

GENERAL INFORMATION

G1 Physics and Chemistry

Guideline for Residual Solvents and Models for the Residual Solvents Test

1. Guideline for Residual Solvents

Refer to the Guideline for Residual Solvents in Pharmaceuticals (PAB/ELD Notification No. 307; dated March 30, 1998).

Since the acceptable limits of residual solvents recommended in the Guideline were estimated to keep the safety of patients, the levels of residual solvents in pharmaceuticals must not exceed the limits, except for in a special case. Pharmaceutical manufacturers should assure the quality of their products by establishing their own specification limits or manufacturing process control limits for residual solvents present in their products in consideration of the limits recommended in the Guideline and the observed values in their products, and by performing the test with the products according to the Residual Solvents Test.

2. Residual Solvents Test

Generally, the test is performed by using gas chromatography <2.02> as directed in the Residual Solvents Test <2.46>. If only the class 3 solvents with low toxic potential to man are present in the products, Loss on Drying Test <2.41> can be applied in place of gas chromatography, in which case the limit value of residual solvents is not more than 0.5%.

The test may also be performed according to the EP method (2.4.24 Identification and control of residual solvents) or the USP method (<467> Residual Solvents). Even in this case, description should be in the JP style and the system suitability test should be performed according to the JP rule.

3. Models for the operating conditions and system suitability of gas chromatography for residual solvents test

The following are typical examples of the operating conditions of gas chromatography for residual solvents test, described in the EP and the USP, but these do not necessarily imply that other suitable operating conditions can not be used. In the operating conditions, generally, items required for the test such as detector, column, column temperature, injection port temperature, detector temperature, carrier gas, flow rate, and time span of measurement should be specified, and in the system suitability, items such as test for required detectability, system performance, and system repeatability should be specified. The following are several models for the operating conditions and the system suitability:

3.1. Models for operating conditions for a head-space sample injection device (Models described in the EP and the USP)

(i) Operating conditions (1) for the head-space sample

injection device—

Equilibration temperature for inside vial	A constant temperature of about 80°C
Equilibration time for inside vial	60 minutes
Transfer-line temperature	A constant temperature of about 85°C

Carrier gas	Nitrogen
Pressurisation time	30 seconds
Injection volume of sample	1.0 mL

(ii) Operating conditions (2) for the head-space sample

injection device—

Equilibration temperature for inside vial	A constant temperature of about 105°C
Equilibration time for inside vial	45 minutes
Transfer-line temperature	A constant temperature of about 110°C

Carrier gas	Nitrogen
Pressurisation time	30 seconds
Injection volume of sample	1.0 mL

(iii) Operating conditions (3) for the head-space sample

injection device—

Equilibration temperature for inside vial	A constant temperature of about 80°C
Equilibration time for inside vial	45 minutes
Transfer-line temperature	A constant temperature of about 105°C

Carrier gas	Nitrogen
Pressurisation time	30 seconds
Injection volume of sample	1.0 mL

3.2. Models for operating conditions and system suitability of gas chromatography

(i) Test conditions (1) (A model described under Procedure A in the EP and the USP)

Operating conditions—

Detector: Hydrogen flame-ionization detector.

Column: Coat the inside wall of a fused silica tube, 0.32 mm (or 0.53 mm) in inside diameter and 30 m in length, to 1.8 μ m (or 3 μ m) thickness with 6% cyanopropylphenyl-methyl silicon polymer for gas chromatography. Use a guard column if necessary.

Column temperature: Maintain at 40°C for 20 minutes, then increase to 240°C at 10°C per minute if necessary, and keep at 240°C for 20 minutes.

Injection port temperature: A constant temperature of about 140°C.

Detector temperature: A constant temperature of about 250°C.

Carrier gas: Helium.

Flow rate: 35 cm/second.

Split ratio: 1:5.

System suitability—

System performance: When the procedure is run with the standard solution under the above operating conditions, the

resolution between the peaks is not less than 1.0. (Note: In the case that the number of substances to be tested is two or more.)

System repeatability: When the test is repeated 3 times with the standard solution under the above operating conditions, the relative standard deviation of the peak areas of the substance to be tested is not more than 15%.

(ii) Test conditions (2) (A model described under Procedure B in the EP and the USP)

Operating conditions—

Detector: Hydrogen flame-ionization detector.

Column: Coat the inside wall of a fused silica tube, 0.32 mm (or 0.53 mm) in inside diameter and 30 m in length, to 0.25 μm thickness with polyethylene glycol 20M for gas chromatography. Use a guard column if necessary.

Column temperature: Maintain at 50°C for 20 minutes, then increase to 165°C at 6°C per minute if necessary, and keep at 165°C for 20 minutes.

Injection port temperature: A constant temperature of about 140°C.

Detector temperature: A constant temperature of about 250°C.

Carrier gas: Helium.

Flow rate: 35 cm/second.

Split ratio: 1:5.

System suitability—

System performance: When the procedure is run with the standard solution under the above operating conditions, the resolution between the peaks is not less than 1.0. (Note: In the case that the number of substances to be tested is two or more.)

System repeatability: When the test is repeated 3 times with the standard solution under the above operating conditions, the relative standard deviation of the peak areas of the substance to be tested is not more than 15%.

(iii) Test conditions (3) (A model described in Method I under Other Analytical Procedures in the USP)

Operating conditions—

Detector: Hydrogen flame-ionization detector.

Column: Coat the inside wall of a fused silica tube, 0.53 mm in inside diameter and 30 m in length, to 5 μm thickness with 5% phenyl-methyl silicon polymer for gas chromatography. If necessary use a guard column prepared by coating the inside wall of a fused silica tube, 0.53 mm in inside diameter and 5 m in length, to 5 μm thickness with 5% phenyl-methyl silicon polymer for gas chromatography.

Column temperature: Maintain at 35°C for 5 minutes, then increase to 175°C at 8°C per minute, further increase to 260°C at 35°C per minute if necessary, and keep at 260°C for 16 minutes.

Injection port temperature: A constant temperature of about 70°C.

Detector temperature: A constant temperature of about 260°C.

Carrier gas: Helium.

Flow rate: 35 cm/second.

Split ratio: Splitless.

System suitability—

System performance: When the procedure is run with the standard solution under the above operating conditions, the resolution between the peaks is not less than 1.0. (Note: In the case that the number of substances to be tested is two or more.)

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 areas of the substance to be tested is not more than 15%.

Inductively Coupled Plasma Atomic Emission Spectrometry

Inductively coupled plasma (ICP) atomic emission spectrometry is a method for qualitative and quantitative analysis for the element in the sample specimen, which is nebulized into argon plasma induced by radio frequency power, by measuring the wavelength and the intensity of the emission spectra generated by the target element, atomized and excited in the plasma. Argon gas plasma used in this method is characteristic of higher temperature of 6000 ~ 8000 K and electronic density of 10^{15} cm^{-3} .

When the high energy is externally given to an atom, the orbit transition of the most exterior electron is occurred and attained to the excited state. When the excited state of atom returns to the basic state, the energy obtained by the excitation is radiated as a light. The emitted light has an intrinsic wave number ν and wave length λ , attributed to the individual element. Assuming the *Planck* constant h and the light velocity c , the emitted light energy ΔE is expressed by the following formula.

$$\Delta E = h\nu = hc/\lambda$$

Since there are many of combinations of the orbit transition energy level of the most exterior electron and the emitted light energy, usually, there are plenty of spectral lines emitted from an element, summing up every strong and weak emitted lights. However, the number of emitted lights of being in the ultra-violet and visible region and having suitable detection sensitivity for qualitative and quantitative analysis are limited to a certain number. Since the atomic emission spectra give intrinsic wave number or wave length for the respective element, many of elements contained in a sample specimen can be identified by the spectroscopic analysis of the emitted lights. Furthermore, the quantitative analysis for an element can also be done by measuring the spectral intensity of the emitted light.

The characteristics of ICP emission spectrometry are summarized as follows;

- (i) Microanalysis for plenty of elements is possible.
- (ii) Analytical precision is high, since the stable plasma generation can be kept.
- (iii) Linearity of the calibration curve is ensured in the wide range of 4 ~ 5 digit.
- (iv) Multi-element simultaneous analysis is possible.
- (v) Chemical interferences are almost negligible.

The most serious problem in the ICP emission spectrometry is that the spectral interferences are inevitable to this analysis, and disturb the analysis for the target element. It results from many of emitted spectral lines due to coexisting elements, because the atomization and excitation of elements are done in very high temperature of plasma. Thus it is the key technique to obtain an accurate analytical result how to reduce the spectral interferences and how to correct the in-

terfered spectra.

The present method is superior to analyze inorganic impurities and/or coexisting elements in drug substances and preparations specifically, and also to analyze metal residues in crude drugs and preparations. The qualitative and quantitative analysis of alkali, alkali earth, and heavy metals are possible, and also of many other elements which are required for suitable control for the safety assurance of drug medicines. Furthermore, since the simultaneous analysis for lots of elements are possible, this method can be used for the profile analysis of inorganic impurities such as metal elements for the quality control of drug substances.

In this *General Information*, a spectroscopic detection method of atomic emission spectra (AES) due to metal elements introduced into inductively coupled plasma (ICP), is described as ICP-AES. Separately, since ICP is not only a good excitation source, but also a good ionizing source, inductively coupled plasma-mass spectrometry (ICP-MS) is also possible by using ICP as an ionizing source.

1. Apparatus

1.1. Composition of the apparatus

The apparatus is composed of the excitation source unit, sample introduction unit, the emission unit, the spectrophotometry unit, the detection unit, and the data processing unit.

The excitation source unit is composed of the electric power source circuit, the control circuit to supply and to control electric energy, and the gas supplying part. The sample introduction unit is the part of introducing the sample into the emission unit and composed of a nebulizer and a spray chamber, *etc.*

The emission unit is a part where the target element in the sample is excited and emitted, and it is composed of the torch and the radio frequency induction coil. The torch usually consists of a threefold tube, in which the sample is introduced by the central tube. Argon is used for the gas to form plasma and also to carry the sample. There are two types of observation way of the light emitted, one is the radial viewing, and the other the axial viewing.

The spectroscopy unit is a part separating the emitted light into the individual spectral line and composed of convergent lens system and optical elements such as the diffraction grating, *etc.* There are two types of spectroscopes, one is the wavelength scanning type of monochromator, the other the fixed wavelength type of polychromator. For measuring spectral lines in the vacuum UV region, in which the wavelength is not more than 190 nm, the evacuation of the interior of the spectroscopy or the substitution of air for argon or nitrogen gas are necessary.

Detection unit is a part converting the incident light intensity to the corresponding strength of the electric signal and composed of the detector and the signal processing system. Regarding the detector, either the photomultiplier or the charge transfer device (CTD) such as the charge coupled device, the charge injection device, and the photo diode array *etc.*, is used.

Data processing unit is a part of processing the detected signal and the measurement result and of giving the calibration curve *etc.*, which are given by the displaying apparatus or the printer, *etc.*

1.2. Attachments

1.2.1. Ultrasonic nebulizer

It is an instrument for atomizing the sample solution with an ultrasonic vibrator, dehydrating it by heating and cooling, and introducing it into the emission unit by the carrier gas. By using this attachment, the sample introducing efficiency is remarkably increased.

1.2.2. Hydride generation apparatus

It is an instrument that, after reducing the compounds such as arsenic, selenium, and antimony in the sample solution to the volatile hydrides with sodium tetrahydroborate *etc.*, the vapor/liquid separation is carried out and the gas components only introduced into the emission unit with the carrier gas. In comparison with the usual nebulizer, the sample introducing efficiency is remarkably increased.

2. Pretreatment of sample

Usually, organic samples such as drug substances are ignited or digested by the dry ashing method or the acid digestion method, respectively. Then the sample solution is prepared by dissolving the residues with small amount of nitric acid or hydrochloric acid. Separately, as for the digestion resistant samples such as crude drugs, the microwave digestion apparatus is available to the digestion of those samples in tightly closed vessels.

Where the sample can be dissolved in water or an appropriate solvent, the sample solution can be simply prepared by dilution or dissolution, provided that a correct analysis is assured previously, either by the dry ashing method or the acid digestion method.

2.1. Dilution and/or dissolution method

Take a specified amount of sample in the respective monograph, then dilute by water or an appropriate solvent, or dissolve in water or a specified solvent to prepare the sample solution.

2.2. Dry ashing method

Applying the “*Residue on Ignition Test*”, take a specified amount of sample in the crucible, moisten it with a small amount of sulfuric acid, then heat gently to carbonize it thoroughly. After cooling, moisten the charred residue with a small amount of sulfuric acid, heat gently again, then ignite the residue to ash in the range of 400 ~ 600°C. Dissolve the ash by adding a small amount of nitric acid or hydrochloric acid to prepare the sample solution.

Separately, applying the 3rd Method in the “*Heavy Metals Limit Test*”, ignite the sample to ash without moistening it with sulfuric acid. In this case, since it is digested and incinerated in the open system, be careful to the vaporization loss of low boiling point elements such as mercury.

2.3. Acid digestion method

Take an amount of sample into the beaker or the flask as specified in the monograph, add nitric acid, sulfuric acid, or the mixture of these acids, then digest by heating. If it is necessary, an auxiliary oxidant such as hydrogen peroxide may be available. After the digestion is completed, add a small amount of nitric acid or hydrochloric acid to the residues, dissolve them by heating to prepare the sample solution.

Acid digestion can be done either in the open or in the closed system. Regarding the closed system, the heat digestion at high temperature under high pressure conditions might be applicable by using the pressure tolerable digestion vessel of polytetrafluoroethylene fitted with the outer tube of stainless steel. Where the heat digestion method is used in the

closed system, be careful enough to accidents such as the expansion and the liquid leak.

2.4. Microwave digestion method

Take an amount of sample into the pressure tolerable closed vessel as specified in the monograph, add an appropriate amount of nitric acid, then digest under heating conditions by using the microwave digestion apparatus. Since it is a digestion procedure under high temperature/high pressure conditions, the temperature and the pressure in the closed vessel should be appropriately controlled, in consideration of amounts of the acid and the sample.

The pretreatment method should be selected appropriately, corresponding to the attribute of the sample and the target element. Where the ignition or the digestion is done in the open system, be careful to the vaporization loss of the target element or the contamination from the operating environment. Furthermore, where the residues obtained by the ignition or the digestion are dissolved in acid solution and prepared to the sample solution, if necessary, the filtration with membrane filter (pore size: 1 μm) may be useful.

3. Procedure

3.1. Performance test of the spectroscope

3.1.1. Wavelength calibration

The wavelength calibration should be suitably performed according to the indicated methods and procedures of the apparatus, since there is specific calibration method of the individual instrument. For example, some of calibration methods are available, such as a method of using standard solution containing several elements, spectral lines of mercury lamp, and emission lines of argon gas, *etc.*

3.1.2. Wavelength resolution

The wavelength resolution is usually specified by the half wavelength (nm) of the spectral lines for some representative elements. During the lower and the higher wavelength, following spectral lines of As (193.696 nm), Mn (257.610 nm), Cu (324.754 nm), and Ba (455.403 nm) are usually selected. The specification of the half wavelength for the individual element is responsible to the user, because the characteristics of the instrument and the spectroscope are intrinsic and different each other.

However, in the case of the simultaneous analysis type of instrument fitted with semiconductor detector, this specification is not always required.

3.2. Preparation of sample solution and standard solution

Sample of drug substances, preparations, and crude drugs are pretreated by using anyone of the method described in the above section (2. Pretreatment of sample) and prepared to the sample solution. Usually, the sample solution is prepared to as a dilute nitric acid solution, while hydrochloric acid is also available in place of nitric acid.

Where the reference standard solutions are specified in the Japanese Pharmacopoeia (JP) and/or the Japanese Industrial Standard (JIS), the standard solutions are prepared by diluting those official standards to the definite concentrations with the water for ICP analysis *etc.* When those reference standard solutions are not specified both in JP and JIS, the reference substances certified and provided by the official organization or the academic association *etc.*, are used for the preparation of the standard solutions. Where the reference standard solutions or the reference substances are not given by the above mentioned official or semi-official organizations, either the pure metals or the compounds con-

taining the target element of high purity (not less than 99.99%) can be used for preparing the calibration standards, in which one or more target elements are included. In case of preparing multi-component standard solution, the reagent solutions and the element combinations are required not to form precipitates, and not to give spectral interferences with the analytical line of the target element.

3.3. Optimization of the operating conditions

The operating conditions are usually as follows.

After the plasma is attained to the stable generation by the warm-up driving for 15 ~ 30 minutes, the optimum operating conditions are adjusted for the intended use. Radio frequency power output of 0.8 ~ 1.4 kW, argon gas flow rate for the cooling gas of 10 ~ 18 L/min, for auxiliary gas of 0 ~ 2 L/min, and for carrier gas of 0.5 ~ 2 L/min, sample introduction of 0.5 ~ 2 mL/min are usual operating conditions. Where the radial viewing method is applied, the observation height is adjusted to 10 ~ 25 mm above the induction coil, and the axial viewing is applied, the optical axis is adjusted to obtain the greatest emission intensity. The integration time is set appropriately between 1 and several decades of second, in consideration of the stability of the emission intensity.

As the analytical line of the individual target element, the representative emission lines are shown in Table 1. However, where the concentration of the target element is markedly high, it is recommended either to dilute the sample solution or to select an appropriate another spectral line, based on the expected concentration of the element. Furthermore, when the spectral interferences due to coexisting elements are occurred, another spectral line should be selected so as to be free from the interference.

Where this test method is specified in the respective monograph, the necessary operating conditions such as the analytical line (nm), the radio frequency power unit (kW), argon gas flow rate (L/min) *etc.*, should be specified for the intended use. However, all the specified conditions except for the analytical line are the referring ones, and the optimization of those conditions are expected to be found according to the individual instrument and the observation way *etc.*

3.4. Water and reagents

Water and reagents for this test are as follows.

(i) As for water, use water for ICP analysis described below,

Water for ICP analysis: The electric conductivity should be below 1 $\mu\text{S} \cdot \text{cm}^{-1}$ (25°C). Further it should be confirmed that the contaminated impurities do not interfere with the emission of the target element.

(ii) The quality of reagents should be suitable, as it does not contain the interfering substances of the test.

(iii) As for argon gas, use that specified below,

Argon gas: The purity should be not less than 99.99 vol%, as specified in JIS K 1105. Either the liquefied argon or pressurized argon gas can be used.

3.5. Procedure

After confirmation of the normal action of the instrumental parts usually switched on electrically, the electric source of the instrument body and the surrounding equipments should be switched on. After setting argon gas flow rate to the decided value, switch on the radio frequency source, and generate argon plasma. After confirming the stable plasma is given, the sample solution and the standard solution prepared as specified in the individual monograph, are intro-

Table 1 The representative analytical line (nm) of various metal elements

Al	396.153	Fe	259.940	Os	225.585	Sn	189.980
As	193.759	Hg	184.950	Pb	220.351	Sr	407.771
B	249.773	In	230.606	Pd	340.458	Tl	276.787
Ba	455.404	Ir	224.268	Pt	214.423	V	309.311
Be	313.042	Li	670.784	Rb	780.023	W	207.911
Cd	214.438	Mg	279.553	Rh	233.477	Zn	213.856
Co	228.616	Mn	257.610	Ru	240.272		
Cr	205.552	Mo	202.030	Sb	206.833		
Cu	324.754	Ni	221.647	Se	196.090		

duced into the plasma, and the emission intensity is measured at the indicated analytical line. Where the qualitative test for the confirmation or the identification is performed, the measurement wavelength range of the emission spectra should be sufficiently wide, in which all the specified analytical lines are included.

Where the emission lines in vacuum UV region are measured by using the vacuum type spectroscopy, the air around the optical axis between the emission part and the spectroscopy part should be substituted for argon or nitrogen gas.

4. System suitability

When this test is applied to the limit test or the quantitative test of metal elements *etc.*, it is required to confirm previously whether the performance ability of the apparatus is adequate or not by the system suitability test specified below. However, in the assay test, the test for 4.1. Confirmation of detection and evaluation of linearity, is not required.

4.1. Confirmation of detection and evaluation of linearity

In the limit test of metal elements *etc.*, where the pretreatment method of the sample specimen is specified in the respective monograph, it can be speculated what is the concentration of the specified limit in the sample solution ($\mu\text{g/mL}$). Assuming that the concentration of the target element in the standard solution is 10 ~ 20 times of that of the sample solution, the standard solution of the target element can be prepared, as mentioned in the section 3.2. Next, diluting this standard solution to 1/10 times of the concentration, this is the system suitability test solution.

Under the optimum conditions for an individual instrument, ICP emission spectra of the standard solution and the system suitability test solution of the target element are measured and the followings should be confirmed. Regarding the system suitability test solution, it should be confirmed that the spectra of the target element are clearly observed at the specified wavelength, and the emission intensity is within a specified range (for example, 80 ~ 120%) compared to the theoretical emission intensity, which is calculated by multiplying the concentration of the standard solution by the dilution ratio.

4.2. System repeatability

As for the standard solution of the target element described in the section 4.1, when the emission intensity measurement is repeated 6 times under the optimum conditions for an individual instrument, the relative standard deviation of the emission intensity for the target element should be below a certain specified value (for example, not more than 3.0%).

In case of the assay test, one of the standard solutions for the calibration curve, which are specified in the monograph, can be selected as a test solution of the system repeatability.

5. Interferences and their reduction or correction

Interferences accompanied to ICP-AES are a general expression of the negative effects caused by the coexisting substances or the matrix containing the target element. Various interferences are classified to the spectral interference and the non-spectral one, and in the latter there are interferences such as the physical interference and the ionization interference *etc.* Those interferences can be removed or reduced by the application of a suitable reduction or correction methods.

5.1. Physical interference

When there are differences of physical properties such as the viscosity, the density, and the surface tension *etc.* between the sample solution and the standard solution, the analytical result might be affected by the differences, due to the induction of the difference in the sample nebulization efficiency. It is called the physical interference. In order to remove or reduce this kind of interference, dilute the sample solution until the interference cannot occur, or conform the liquid characteristics of the sample solution to those of the standard solution (*Matrix matching method*). Separately, it is a useful correction method to adopt the internal standard method (*Intensity ratio method*) or the standard addition method as an assay method.

5.2. Ionization interference

The ionization interference indicates the effect that the marked increase of electronic density in plasma may affect the ionization ratio of the target element, where the coexisting element highly contained in the sample solution are easily ionized and release a great number of electrons in the plasma. The reduction or the correction method against the ionization interference effect is essentially the same as that described in the section 5.1 Physical interference. Furthermore, the ionization interference is moderately reduced by the selection of the observation way and the adjustments of the observation height, the radio frequency power output, and the flow rate of carrier gas, *etc.*

5.3. Spectral interference

The spectral interference indicates that analytical results are affected by the overlapping of various emission lines or continuous spectra to the analytical line of the target element. The causes and the correction methods to this interference are shown below.

5.3.1. Interference by the coexisting elements

This interference occurs when the emission line of coexisting element in the sample solution come close to the analytical line of the target element. The degree of the interference depends on the resolution ability of the spectroscopy, the wavelength difference between two emission lines, and the intensity ratio. To avoid this interference another analytical line can also be available. Where an appropriate analytical line cannot be found, it is necessary to do the spectral interference correction.

The inter-element correction is one of the correction methods for the spectral interference. If the effect of coexisting element on the analytical line of the target element are measured in advance as a function of the emission intensity or the concentration by using the known concentration of two-component or multi-component standard solution, the

degree of interference on the emission intensity of the analytical line or the concentration will be speculated by measuring the emission intensity of the coexisting element together with measurement of the target element. Separately, the matrix matching method or the mathematical spectral separation technique, by which the spectra of coexisting element superimposed to the analytical line are separated, can also be used for the correction.

5.3.2. Background interference

The background interference indicates the following effect that analytical results are affected by the increase of background due to the emission lines caused by the element highly contained in the sample. In this case, this effect will be removed by applying the following correction method. Based on the background behavior before and after the analytical line, estimate the background intensity at the position of the analytical line, and obtain an intrinsic emission intensity of the target element by subtraction of this background intensity from the apparent emission intensity.

Furthermore where the pretreatment of organic sample is not complete, the molecular band spectra (NO, OH, NH, CH, etc.) generated by elements of N, O, H, C in the sample solution may happen to affect the intensity of the analytical line. This kind of interference is moderately reduced by the selection of the observation way and the adjustments of the observation height, the radio frequency power output, and the flow rate of carrier gas, etc.

6. Qualitative and quantitative analysis

6.1. Qualitative analysis

6.1.1. Identification of inorganic impurities such as metal elements

In JP, the confirmation test for drug substances is usually specified by applying the method of spectral analysis, by which the structural features of the target molecule can be totally found. Drug substances frequently contain specific elements such as N, S, P, halogens, and metals etc. in their molecule, which are required for their existence in the individual specification. Where the existence is not confirmed by such as spectral analyses, it is required for their confirmation by using chemical reactions. Thus the present method can be applied to their confirmation test, except for nitrogen (N).

When a few of wavelengths and their relative intensity of the target element in the sample solution coincide with those of the standard solution, the existence of the target element is confirmed. In place of the standard solution, the spectra library attached to the individual instrument or the wavelength table provided by the academic society, can also be used for the confirmation.

6.1.2. Profile analysis

If the target elements such as metal catalysts, inorganic elements, and other high toxicity elements such as Pb and As, which should be ordinarily controlled in view of the drug safety, are decided to be controlled, the profile analysis of those inorganic impurities can be performed by the present method as a part of the good manufacturing practice of drug substances.

Although the analytical line of the individual element can be selected referring to Table 1, an appropriate another emission line can also be available, when the spectral interferences are supposed to affect the analytical results. The standard solution of the individual element is adjusted to a

suitable concentration, in consideration of a permitted limit of the respective element, specified separately. However, when the multi-component standard solution is prepared, it should be confirmed beforehand that any trouble such as coprecipitation does not occur.

The confirmation test of the target element should be done according to the section 6.1.1, and the impurity content of each element in the sample specimen is roughly estimated by the ratio of emission intensity of the sample solution and the standard solution at the analytical line (*1 point calibration method*).

6.2. Quantitative analysis

The quantitative evaluation of inorganic impurities in the sample specimen is usually done by measuring the emission intensity during a certain time of integration.

6.2.1. Calibration curve method

Regarding to the target element, not less than 4 kinds of the calibration standard solutions with different concentration are prepared. By using these calibration standards, plot the emission intensity at the analytical line *versus* the concentration to obtain the calibration curve. After measuring the emission intensity of the sample solution, the concentration of the target element is determined by the calibration curve.

6.2.2. Internal standard method

Regarding to the target element, not less than 4 kinds of calibration standard solutions with different concentration, in which a definite concentration of internal standard is contained, are prepared. Usually, yttrium (Y) is used as an internal standard. By using these calibration standards, plot the ratio of emission intensity of the target element and the internal standard *versus* the concentration to obtain the calibration curve. In the preparation of the sample solution, the internal standard element is added to give the same concentration as the calibration standards. After measuring the ratio of the emission intensity of the target element and the internal standard, the concentration of the target element is determined by the calibration curve.

Applying this method to inorganic impurity analysis, it should be confirmed that the internal standard element is not contained in the sample. As for the internal standard element, it is expected that changes of the emission intensity due to the operational conditions and the solution characteristics, are similar to those of the target element, and the selection of emission line is necessary so as not to give the spectral interference to the analytical line.

6.2.3. Standard addition method

Take not less than 4 sample solutions of equivalent volume, add the target element to these solutions to prepare the calibration standard solutions with different concentrations containing zero addition. After measuring the emission intensity of these calibration standards, plot the emission intensity at the analytical line *versus* the concentration. The concentration of the target element is determined to be the concentration, at which the regression line and the abscissa cross each other.

This method can be applied only to the case of analysis with no spectral interference, or where the background and the spectral interference are exactly corrected, and the relation between the emission intensity and the concentration keeps good linearity.

References

- 1) Japanese Industrial Standard: *General Rules for Atomic*

- Emission Spectrometry* (JIS K 0116), Japanese Standard Association (2003).
- 2) US Pharmacopeia 31 (2008), <730> *PLASMA SPECTROCHEMISTRY*.
 - 3) European Pharmacopoeia 6.0 (2008), 2.2.57 *INDUCTIVELY COUPLED PLASMA-ATOMIC EMISSION SPECTROMETRY*.
 - 4) *Data Book on Analytical Chemistry*, Ed. by The Japan Society for Analytical Chemistry, pp. 88 – 90 (2004), Maruzen Press.
 - 5) European Medicines Agency: *Guideline on the specification limits for residues of metal catalysts or metal reagents* (2008).

Near Infrared Spectrometry

Near infrared spectrometry (NIR) is one of spectroscopic methods used to qualitatively and quantitatively evaluate substances from analysis of data obtained by determining their absorption spectrum of light in the near-infrared range.

The near-infrared range lies between the visible light and infrared light, typically of wavelengths (wave numbers) between 750 and 2500 nm ($13333 - 4000 \text{ cm}^{-1}$). The absorption of near-infrared light occurs due to harmonic overtones from normal vibration or combination tones in the infrared range (4000 to 400 cm^{-1}), primarily absorption of O-H, N-H, C-H and S-H that involve hydrogen atoms, in particular. For instance the asymmetrical stretching vibration of N-H occurs in the vicinity of 3400 cm^{-1} , but the absorption due to the first harmonic overtone occurs in the vicinity of 6600 cm^{-1} (wavelength 1515 nm), which is near double 3400 cm^{-1} .

Absorption in the near-infrared range is far weaker than absorption due to normal vibration that occurs in the infrared range. Furthermore, in comparison with visible light, near-infrared light has longer wavelength, which makes it possible for the light to penetrate to a depth of several mm into solid specimens including fine particles. This method is often utilized as a nondestructive analysis, as changes occurring with absorbed light spectrum (transmitted light or reflected light) in this process provide physical and chemical information pertaining to specimens.

Conventional spectrometry, such as calibration curve method, is used as a method for analyzing near-infrared absorption spectrum whenever applicable. Ordinarily, however, chemometrics methods are used for analysis. Chemometrics ordinarily involve quantification of chemical data, as well as numerical and statistical procedures for computerization of information. Chemometrics for near-infrared spectrometry includes various types of multivariate analysis such as multiple regression analysis, to perform qualitative or quantitative evaluation of active substances.

Near-infrared spectrometry is used as a rapid and nondestructive method of analysis that replaces conventional and established analysis methods for water determinations or substance verifications. It is necessary to perform a comparison test to evaluate this method against an existing analysis method, to verify that this method is equivalent to such existing analysis method, before using this analysis method as a quality evaluation test method in routine tests.

Applications of near-infrared spectrometry in the phar-

maceutical field include qualitative or quantitative evaluation of ingredients, additives or water contents of active substances or preparations. Furthermore, near-infrared spectrometry can also be used for evaluation of physical conditions of substances, such as crystal forms, crystallinity, particle diameters. It is also possible to perform spectrometry on samples that are located in a remote location away from equipment main units, without sampling, by using optical fibers. It can therefore be used as an effective means to perform pharmaceutical manufacturing process control online.

1. Equipment

Near-infrared spectrophotometers can either be a distributed near-infrared spectrophotometer or a Fourier transform near-infrared spectrophotometer¹⁾. Interference filter-type near-infrared spectrophotometers that use interference filter in the spectrometry section are also available, however, this type of equipment is hardly used in the field of pharmaceutical quality control.

1.1. Distributed near-infrared spectrophotometer

This equipment is comprised of light source section, sample section, spectrometry section, photometry section, signal processing section, data processing section, display-record-output section. Halogen lamps, tungsten lamps, light emitting diodes and other such devices that can emit high intensity near-infrared light in a stable manner are used in the light source section. The sample section is comprised of a sample cell and a sample holder. Equipment that have an optical fiber section that is comprised of optical fibers and a collimator are equipped with a function for transmitting light to sample section, which is remotely located away from the spectrophotometer main unit. Quartz is ordinarily used as material for optical fibers.

The spectrometry section is intended to extract light of required wavelength, using dispersive devices and is comprised of slits, mirrors and dispersive devices. Potential dispersive devices include prisms, diffraction grating, acousto-optical tunable filters (AOTF), or liquid crystal tunable filters (LCTF).

The photometry section is comprised of detectors and amplifiers. Sensors include semiconductor detectors (silicon, lead sulfide, indium-gallium-arsenic, indium-antimony), as well as photomultiplier tubes. Detecting methods that use semiconductor detectors generally perform detections with single elements, but there are also occasions where array-type detectors that use multiple elements are used. Such detectors are capable of simultaneously detecting multiple wavelengths (wave numbers). The signal processing section separates signals required for measurements from output signals fed by amplifiers and then outputs such isolated signals. The data processing section performs data conversions and spectral analysis, etc. The display-record-output section outputs data, analysis results and data processing results to a printer.

1.2. Fourier transform near-infrared spectrophotometer

The configuration of the equipment is fundamentally same as that of the distributed-type equipment described in Section 1.1, except for the spectrometry section and the signal processing section.

The spectrometry section is comprised of interferometers, sampling signal generators, detectors, amplifiers, A/D conversion devices, etc. Interferometers include Michelson inter-

ferometers, transept interferometers and polarization interferometers. The signal processing section is equipped with functions that are required for spectrometer, as well as a function for translating acquired interference waveform (interferogram) into absorption spectrum by Fourier transformation.

2. Determination

There are three types of measurement methods that are used with near-infrared spectrometry: transmittance method, diffuse reflectance method and transmittance reflectance method. The selection of measurement methods relies on the shape of samples and applications. The transmittance method or diffuse reflectance method is used for solid samples, including fine particles. The transmittance method or transmittance reflectance method is used for liquid samples.

2.1. Transmittance method

The degree of decay for incident light intensity as the light from a light source passes through a sample, is represented as transmittance rate T (%) or absorbance A with the transmittance method. A sample is placed in the light path between a light source and a detector, the arrangement of which is ordinarily same as that of the spectroscopic method.

$$T = 100t$$

$$T = I/I_0 = 10^{-\alpha c l}$$

I_0 : Incident light intensity

I : Transmitted light intensity

α : Absorptivity

c : Solution concentration

l : Layer length (sample thickness)

$$A = -\log t = \log (1/t) = \log (I_0/I) = \alpha c l$$

This method is applied for taking measurements of samples that are liquids and solutions. Quartz glass cells and flow cells are used, with the layer length of 1–5 mm along. Furthermore, this method can also be applied for taking measurements of samples that are solids, including fine particles. It is also known as diffuse transmittance method. Selecting appropriate layer length is critical for this method, since the transmitted light intensity varies depending on grain sizes and surface conditions of samples.

2.2. Diffuse reflectance method

The ratio of the reflection light intensity I_r , emitted from the sample in a wide reflectance range and a control reflection light intensity I_r , emitted from surface of a substance, is expressed as reflectance R (%) with the diffuse reflectance method. The near-infrared light penetrates to a depth of several mm into solid samples, including fine particles. In that process, transmission, refraction, reflection and dispersion are repeated, and diffusion takes place, but a portion of the diffused light is emitted again from the surface of the sample and captured by a detector. The spectrum for the diffuse reflectance absorbance (A_r) can ordinarily be obtained by plotting logarithm of inverse numbers for reflectance ($1/r$) against wavelengths (wave numbers).

$$R = 100r$$

$$r = I_r/I_r$$

I_r : Reflection light intensity of light, diffuse reflected off the sample

I_r : Control reflection light intensity of light emitted from surface of reference substance

$$A_r = \log (1/r) = \log (I_r/I)$$

The intensity of diffuse reflectance spectrum can also be expressed with the Kubelka-Munk (K-M) function. The K-M function is derived, based on the existence of a sample with sufficient thickness, and expressed in terms of light scattering coefficient, which is determined by absorptivity, grain size, shape and fill condition (compression).

This method is applied to solid samples, including fine particles, and requires a diffuse reflector.

2.3. Transmittance reflectance method

The transmittance reflectance method is a combination of the transmittance method and reflectance method. A mirror is used to re-reflect a light that has passed through a sample in order to take a measurement of transmittance reflectance rate, T^* (%). Light path must be twice the thickness of the sample. On the other hand, the light reflected off a mirror and enters into a detector is used as the control light. When this method is applied to suspended samples, however, a metal plate or a ceramic reflector with rough surface that causes diffuse reflectance is used instead of a mirror.

Transmittance reflectance absorbance (A^*) is obtained by the following formula with this method:

$$T^* = 100t^*$$

$$t^* = I/I_T$$

I : Intensity of transmitted and reflected light, in cases where a sample is placed

I_T : Intensity of reflected light, in cases where is no sample

$$A^* = \log (1/t^*)$$

This is a method that is applied to solid samples, including fine particles, as well as liquids and suspended samples. The thickness of a sample must be adjusted when applying this method to a solid sample. Ordinarily adjustment is made by setting absorbance to 0.1–2 (transmittance of 79–1%), which provides the best linearity and S/N ratio of detector. A cell with appropriate layer length, according to the grain size of the fine particle, must be selected when applying the method to a fine particle sample.

3. Factors that affect spectrum

Following items must be considered as factors that can affect spectrum when applying near-infrared spectrometry, particularly when conducting quantitative analysis.

(i) Sample temperature: A significant change (wavelength shift, for example) can occur when the temperature varies by a several degree (°C). Care must be taken, particularly when the sample is a solution or contains water.

(ii) Water or residual solvent: Water or residual solvent contents of a sample, as well as water (humidity) in the environment wherein measurements are taken, can potentially significantly affect absorption band of the near-infrared range.

(iii) Sample thickness: The thickness of a sample is a factor for spectral changes and therefore needs to be controlled at a certain thickness. A sample may be considered to be of adequate thickness for the diffuse reflectance method, however, if the thickness is less than a certain amount, for example, the sample may have to be placed on a support plate with high reflectance to take measurements by the transmittance reflectance method.

(iv) Fill condition of sample: The condition of sample fill can potentially affect spectrum, when taking measure-

ments of samples that are solids or fine particles. Care must be taken when filling samples in a cell, to ensure that a certain amount is filled through a specific procedure.

(v) Optical characteristics of samples: When a sample is physically, chemically or optically uneven, relatively large beam size must be used, multiple samples must be used, measurements must be taken at multiple points on the same sample, or a sample must be pulverized to ensure averaging of the sample. Grain size, fill condition, as well as roughness of surface can also affect fine particle samples.

(vi) Crystal forms: Variations in crystal structures (crystal forms) can also affect spectrum. In cases where multiple crystal forms exist, it is necessary to have consideration for characteristics of samples to be considered and care must be taken to ensure that even standard samples for calibration curve method have diversified distributions similar to that of samples that are subject to analysis.

(vii) Temporal changes in characteristics of samples: Samples can potentially undergo chemical, physical or optical property changes, due to passing of time or storage after sampling, and such changes affect spectrum in a subtle manner. For instance even with identical samples, if elapsed times differ, then their characteristics of near-infrared spectrum can vary significantly. In creating calibration curves, therefore, measurements must be taken offline in a laboratory or online in manufacturing process (or inline) and samples for calibration curves must be prepared with adequate considerations for the passing of time before measurements are taken.

4. Control of equipment performance^{2,3)}

4.1. Accuracy of wavelengths (wave numbers)

The accuracy of wavelengths (wave numbers) of an equipment is derived from the deviation of substances for which peak absorption wavelengths (wave numbers) have been defined, such as polystyrene, mixture of rare earth oxides (dysprosium, holmium and erbium; 1:1:1) or steam, from the figures indicated on the equipment. Tolerance figures in the vicinity of 3 peaks are ordinarily set in the following manner:

- 1200 ± 1 nm (8300 ± 8 cm⁻¹)
- 1600 ± 1 nm (6250 ± 4 cm⁻¹)
- 2000 ± 1.5 nm (5000 ± 4 cm⁻¹)

Since the location of absorption peaks vary, depending on the substance used as reference, absorption peaks of wavelengths (wave numbers) that are closest to the above 3 peaks are selected for suitability evaluations. A mixture of rare earth oxides, for instance, would indicate characteristic absorption peaks at 1261 nm, 1681 nm and 1971 nm.

Absorption peaks at 1155 nm, 1417 nm, 1649 nm, 2352 nm (layer length: 1.0 mm) can be used, when taking measurements with transmittance method that involve the use of dichloromethane as reference. The absorption peak of steam at 7306.7 cm⁻¹ can be used with a Fourier transformation-type spectrophotometer, as its wave number resolution ability is high.

Other substances can also be used as reference, so long as their adequacy for the purpose can be verified.

4.2. Spectroscopic linearity

Appropriate standard plates, such as plate-shaped polymer impregnated with varying concentrations of carbon (carbon-doped polymer standards), can be used to evaluate spectroscopic linearity. In order to verify linearity, however,

standard plates with no less than 4 levels of concentration within the reflectance of 10–90% must be used. When measurements are expected to be taken with absorbance of no less than 1.0, it is necessary to add standard plates with reflectance of either 2% or 5% or both.

In order to plot absorbance (A_{OBS}) of such standard plates at locations in the vicinity of wavelengths 1200 nm, 1600 nm and 2000 nm against absorbance (A_{REF}) assigned to each standard plate, verifications must be made to ensure that the gradient of linearity obtained are ordinarily within the range 1.0 ± 0.05 for each of these wavelengths and 0 ± 0.05 for ordinate intercept.

4.3. Spectrophotometric noise

The spectrophotometric noise of the equipment can be checked using appropriate reflectance standard plates, such as white-colored reflecting ceramic tiles or reflective thermoplastic resin (such as polytetrafluoroethylene).

4.3.1. High flux noise

Spectrophotometric noise is evaluated by using standard plates with high reflectance, such as reflectance of 99%. Standard plates are used to take measurements for both samples and control samples. Generally, the average value obtained from calculation of mean square root (RMS) of noise for each 100 nm segments in the wavelength range of 1200–2200 nm ordinarily must not be more than 0.3×10^{-3} and individual values must not exceed 0.8×10^{-3} .

$$RMS = \{1/N \cdot \Sigma(A_i - A_m)^2\}^{1/2}$$

N : Number of measurement points per segment

A_i : Absorbance at each measurement point of segment

A_m : Average absorbance for segment

4.3.2. Low flux noise

Spectrophotometric noise is evaluated by using standard plates with low reflectance, such as reflectance of 10%, when the amount of light is low. In such cases, light source, optical system, detector and electronic circuit systems all have some impact on noise. Similar to the cases of high flux noise, generally, the average value obtained from calculation of RMS for each 100 nm segments in the wavelength range of 1200–2200 nm ordinarily must not be more than 1.0×10^{-3} and individual values must not exceed 2.0×10^{-3} .

5. Application to qualitative or quantitative analysis

Unlike in the infrared range, mainly harmonic overtones and combinations manifest as spectrum in the near-infrared range. Such absorbance spectrums are often observed as overlay of absorption bands of functional groups and atomic groups. The near-infrared spectrometry, therefore, differs from conventional analysis methods and it is usually necessary to establish analysis methods that correspond to each application, by preparing model analysis methods using methodologies of chemometrics, such as multivariate analysis.

Characteristics of near-infrared absorption spectrum must be emphasized and effects of complexities of spectrums, as well as overlay of absorption bands must be reduced by performing mathematical preprocesses, such as primary or secondary spectral differentiation processes or normalizations, which becomes one of vital procedures in establishing analysis methods that use methodologies of chemometrics. While there are many chemometrics methodologies and mathematical preprocessing methods for data, appropriate combinations must be selected that suit the purposes of

intended analysis.

Evaluation of validity based on analysis parameters is ordinarily required for the analysis validation when establishing a near-infrared analysis method. Selection of parameters that are appropriate for applications must be made for its intended use. Furthermore, following issues must be considered, in conformity with attributes of the near-infrared spectrometry.

(i) Whether or not wavelengths (wave numbers) intended for the particular analysis method, are suitable for evaluation of characteristics of a sample in performing analysis under given conditions.

(ii) Whether or not the method is adequately robust to deal with variables such as handling of samples (for instance fill condition for fine particle samples, etc.) and configuration matrix.

(iii) Whether or not about the same level of accuracy or precision can be obtained, in comparison with the existing and established analysis methods, which are available as standards.

(iv) Sustaining and managing performance of an analysis method, once established, are critical. Continuous and systematic maintenance and inspection work must therefore be implemented. Furthermore, it must be determined whether or not appropriate evaluation procedures are available to deal with change controls or implementation of re-validation on changes made in manufacturing processes or raw materials, as well as changes arising from replacement of major components in equipment.

(v) Whether or not there are appropriate evaluation procedures in place to verify validity of transferring implementation of an analysis, which presupposes the use of a specific equipment, from such originally intended equipment to another equipment (model transfer) for the purpose of sharing the analysis method.

5.1. Qualitative analysis

Qualitative analysis, such as verification of substances, is performed after preparing a reference library that includes inter-lot variations within tolerance range and chemometrics methodologies, such as multivariate analysis, have been established. Minute quality characteristic variations between lots can also be estimated by using this method.

Furthermore, multivariate analysis includes direct analysis methods that consider wavelengths (wave numbers) and absorptions as variables, such as wavelength correlation method, residual sum of squares, range sum of squares, along with factor analysis method, cluster analysis method, discriminant analysis method, as well as SIMCA (soft independent modeling of class analogy).

It is also possible to consider the overall near-infrared absorption spectrum as a single pattern and to identify parameters obtained by applying multivariate analysis methods or characteristic wavelength (wave number) peaks of the sample substance as indices for monitoring, for the purpose of manufacturing process control for active substances or preparations.

5.2. Quantitative analysis

Quantitative analysis uses spectrums of sample groups and analysis values obtained through the existing and established analysis methods, to obtain quantitative models with methodologies of chemometrics. These are used to calculate concentrations of individual ingredients and material values of samples being measured, using conversion formulas.

Chemometrics methodologies for obtaining quantitative models include multiple regression analysis method, main ingredient regression analysis method and PLS (partial least squares) regression analysis method.

In cases where the composition of a sample is simple, concentrations of ingredients in the sample that are subject to analysis can be calculated, by plotting a calibration curve using the absorbance of a specific wavelength (wave number) or the correlating relationship between the parameters and concentration, using samples for preparation of calibration curves with known concentrations (calibration curve method).

6. Reference

- 1) General Rules for Near-infrared Spectrophotometric Analysis, JIS K 0134 (2002), Japanese Industrial Standards
- 2) Near-infrared Spectrophotometry, 2.2.40, European Pharmacopoeia 5.0 (2005)
- 3) Near-infrared Spectrophotometry, <1119>, US Pharmacopoeia 30 (2007)

pH Test for Gastrointestinal Medicine

In this test, medicine for the stomach and bowels, which is said to control stomach acid, is stirred in a fixed amount of the 0.1 mol/L hydrochloric acid for a fixed duration, and the pH value of this solution is obtained. The pH value of a stomach medicine will be based on the dose and the dosage of the medicine (when the dosage varies, a minimum dosage is used) and expressed in the pH value obtained from the test performed by the following procedure.

1. Preparation of Sample

Solid medicine which conforms to the general regulations for medicine (the powdered medicine section) can be used as a sample. When the medicine is in separate packages, the content of 20 or more packages is accurately weighed to calculate the average mass for one dose and mixed evenly to make a sample. For granules and similar types in separate packages, among the solid medicine which does not conform to the general regulations for medicine (the powdered medicine section), the content of 20 or more packages is accurately weighed to calculate the average mass for one dose and is then powdered to make sample. For granules and similar types not in separate packages, among solid medicine which does not conform to the general regulations for medicine (the powdered medicine section), 20 doses or more are powdered to make a sample. For capsules and tablets, 20 doses or more are weighed accurately to calculate the average mass for one dose or average mass and then powdered to make a sample.

Liquid medicine is generously mixed to make a sample.

2. Procedure

Put 50 mL of the 0.1 mol/L hydrochloric acid with the molarity coefficient adjusted to 1.000, or equivalent 0.1 mol/L hydrochloric acid with its volume accurately measured in a 100-mL beaker. Stir this solution with a magnetic stirrer and a magnetic stirrer rotator (35 mm length, 8 mm diameter) at the speed of about 300 revolutions per minute.

While stirring, add the accurately weighed one-dose sample. After 10 minutes, measure the pH value of the solution using the pH Determination. The solution temperature should be maintained at $37 \pm 2^\circ\text{C}$ throughout this operation.

System Suitability

In order to ensure the reliability on the results of drug analyses, it is essential to verify that the test method to be applied to the test, including the method prescribed in the Japanese Pharmacopoeia (JP), can give the results adequate for its intended use using the analytical system in the laboratory in which the test is to be performed, then to carry out system suitability testing for confirming that the analytical system maintains the state suitable for the quality test.

1. Definition and role of system suitability

“System Suitability” is the concept for ensuring that the performance of the analytical system is as suitable for the analysis of the drug as was at the time when the verification of the test method was performed using the system.

Usually, system suitability testing should be carried out at every series of drug analysis. The test procedures and acceptance criteria of system suitability testing must be prescribed in the test methods of drugs. The results of drug analyses are not acceptable unless the requirements of system suitability have been met.

System suitability testing is an integral part of test methods using analytical instruments, and based on the concept that the equipments, electronic data processing systems, analytical operations, samples to be analyzed and operators constitute an integral system that can be evaluated, when the test procedures and acceptance criteria of system suitability testing are prescribed in the test methods.

2. Points to consider in setting system suitability

Parameters of system suitability testing to be prescribed in the test method depend on the intended use and type of analytical method. Since system suitability testing is to be carried out in a routine manner, it is preferable to select the parameters necessary for ensuring that the analytical system maintains the state suitable for the analysis of the drug and to prescribe its test procedure able to carry out easily and rapidly.

For example, in the case of quantitative purity tests using liquid chromatography or gas chromatography, the evaluation of parameters such as “System performance” (to confirm the ability to analyze target substance specifically), “System repeatability” (to confirm that the degree of variation in the analytical results of target substance in replicate injections is within the allowable limit) and “Test for required detectability” (to confirm the linearity of chromatographic response around the specification limit) are usually required.

The followings are supplements to the section of system suitability prescribed in “Liquid Chromatography”.

2.1. System repeatability of HPLC and GC

2.1.1. Allowable limit of system repeatability

It is described in the section of system suitability in “Liquid Chromatography” that “In principle, total number of replicate injections should be 6”, and “The allowable limit of “System repeatability” should be set at an appropriate

level based on the data when suitability of the method for the evaluation of quality of the drug was verified, and the precision necessary for the quality test”.

Based on the above description, an allowable limit of system repeatability for 6 replicate injections should be set in consideration with the following descriptions. However, in the case that the test method prescribed in the JP monograph is used for the test, the allowable limit of system repeatability prescribed in the monograph should be applied.

(i) Assay for drug substance (for drug substance with the content nearby 100%): An adequate allowable limit should be set at the level that the chromatographic system is able to give the precision suitable for the evaluation of variation in the content of active ingredient within and among the batches of drug substance. For example, the allowable limit of “not more than 1.0%” is usually recommended for the drug substances whose width of content specification are not more than 5%, as is in the case of content specification of 98.0 – 102.0% which is often observed in the assay using liquid chromatography.

(ii) Assay for drug products: An adequate allowable limit should be set considering the width of content specification of the drug product and the allowable limit prescribed in the assay of drug substance (when the drug product is analyzed by a method with the same chromatographic conditions as those used for the analysis of drug substance).

(iii) Purity test for related substances: An adequate allowable limit should be set considering the concentration of active ingredients in the solution used for the system suitability testing. In the case that a solution with active ingredient concentration of 0.5 – 1.0% is used for the test of system repeatability, an allowable limit of “not more than 2.0%” is usually recommended.

Recommendations for allowable limits described above should not be applicable to gas chromatography.

2.1.2. Method for decreasing the number of replicate injections without losing the quality of system repeatability testing

It is described in the section of system suitability in “Liquid Chromatography” that “In principle, total number of replicate injections should be 6. However, in the case that a long time is necessary for one analysis, such as the analysis using the gradient method, or the analysis of samples containing late eluting components, it may be acceptable to decrease the number of replicate injections by adopting new allowable limit of “System repeatability” which can guarantee a level of “System repeatability” equivalent to that at 6 replicate injections.”

In consideration of the above description, a method for decreasing the number of replicate injections without losing the quality of system repeatability testing is adopted. One can set the test for system repeatability with reduced number of replicate injections by utilizing this method, if necessary, and can also apply it as an alternative for the method prescribed in a monograph.

The following table shows the allowable limits to be attained in the test at 3 – 5 replicate injections ($n = 3 - 5$) to keep the quality test equivalent to that of test at $n = 6$.

However, it should be kept in mind that since decrease in the number of replicate injections results in increase in the weight of each injection, it becomes more important to perform the test by the experienced operator, and to maintain the equipment in a suitable state.

Table Allowable limits to be attained in the test at 3 – 5 replicate injections ($n = 3 - 5$) to keep the quality of test equivalent to that of test at $n = 6$ *

Allowable limit prescribed in the test of $n = 6$		Allowable limit (RSD)					
		1%	2%	3%	4%	5%	10%
Allowable limit to be attained	$n = 5$	0.88%	1.76%	2.64%	3.52%	4.40%	8.81%
	$n = 4$	0.72%	1.43%	2.15%	2.86%	3.58%	7.16%
	$n = 3$	0.47%	0.95%	1.42%	1.89%	2.37%	4.73%

* The probability for inadequate analytical systems to meet the requirements of system suitability testing, is supposed to be 5%.

3. Points to consider at the change of analytical system (Change control of analytical system)

When the test method and analytical system verified is continuously used for the quality test without any change, it is sufficient to confirm the compliance to the requirements of system suitability at every series of drug analysis.

However, when the test is performed for a long period, a situation in which some changes in the analytical system are inevitable, may occur. These changes don't affect the quality of the product itself, but they affect the scale in the evaluation of product quality. If the change in the analytical system may induce a significant deviation of the scale, it may lead to the acceptance of products with inadequate quality and/or the rejection of products with adequate quality. Thus, at the time of change in the analytical system, it is necessary to check whether the change is appropriate or not, to avoid the deviation of the scale in the evaluation of product quality.

In the case of the change of test method, it is required to perform an adequate validation depending on the extent of the change.

On the other hand, in the case of the change of analytical system in a laboratory, such as renewal of apparatus or column of liquid chromatography, and the change of operator, it is necessary to perform at least system suitability testing using the system after change, and to confirm that the equivalency of the results before and after change.

In the case that equivalent results would not be obtained after change, for example, when a renewal of column of liquid chromatograph may induce a significant change of elution pattern, such as the reversal of elution order between target ingredient of the test and substance for checking resolution, it is required to perform a revalidation of the analytical system for the test using new column, since it is uncertain whether the specificity and/or other validation characteristics necessary for estimating target ingredient is kept or not.

Test for Trace Amounts of Aluminum in Trans Parenteral Nutrition (TPN) Solutions

Trans parenteral nutrition solutions (TPNs) are nutrient preparations for intravenous injection. Since toxic effects to the central nervous system, bone, etc. due to trace amounts

of aluminum have recently been reported in several countries, testing methods for trace amounts of aluminum contaminating TPNs are required for the official standard. The following three analytical methods are available: High-Performance Liquid Chromatography using a fluorescence photometric detector (HPLC with fluorescence detection), Inductivity Coupled Plasma-Atomic Emission Spectrometry (ICP-AES method), Inductivity Coupled Plasma-Mass Spectrometry (ICP-MS method). Detection sensitivity by HPLC with fluorescence detection is about $1 \mu\text{g/L}$ (ppb), while ICP-AES fitted with special apparatus and ICP-MS have higher sensitivity.

Since TPNs are nutrient preparations, they contain many nutrients such as sugars, amino acids, electrolytes, etc., in various compositions. Thus, care is needed in the selection of a suitable analytical method, because these coexisting components may affect the measurement of trace amounts of aluminum.

In view of the general availability of HPLC apparatus, the present general information describes procedures for the determination of trace levels of aluminum in TPNs by means of HPLC with a fluorescence photometric detector, using two kinds of fluorescent chelating agents, i.e., Quinolinol complexing method, Lumogallion complexing method.

1. Quinolinol complexing method

After forming a complex of aluminum ion in the sample solution with quinolinol, the assay for aluminum by HPLC fitted with a fluorescence photometer is performed.

1.1. Preparation of sample solution

Pipet 1 mL of the sample (TPNs) exactly, and after adding $10 \mu\text{L}$ of water for aluminum test, make up the sample solution to 10 mL exactly by adding the mobile phase.

1.2. Preparation of a series of standard solutions for calibration curve

Pipet 1 mL of water for aluminum test exactly, and after adding $10 \mu\text{L}$ each of standard solutions of aluminum (1)–(5), make up the standard solutions for calibration curve to 10 mL (Aluminum concentration: 0, 1.25, 2.5, 5.0, and 10.0 ppb).

1.3. Standard testing method

Pipet 0.1 mL each of the sample solution and standard solutions, and perform the test by HPLC under the following conditions. Calculate the aluminum content in the sample solution using a calibration curve method.

Operating conditions—

Detector: Fluorescence photometer(excitation wavelength: 380 nm, emission wavelength: 520 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 μ m in particle diameter).

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

Mobile phase: A mixture of 8-quinolinol in acetonitrile (3 in 100) and diluted 0.5 mol/L ammonium acetate TS (2 in 5) (1:1).

Flow rate: Adjust the flow rate so that the retention time of aluminum/8-quinolinol complex is about 9 minutes.

System suitability—

The correlation coefficient of the calibration curve, which is prepared using a series of standard solutions, is not less than 0.99.

Furthermore there is an alternative method, in which the chelating agent 8-quinolinol is not included in the mobile phase. In this method also, aluminum is detected as a complex with 8-quinolinol in the sample solution by using HPLC fitted with fluorescence photometer. But it is necessary to form a more stable aluminum/8-quinolinol complex in the sample solution, because the chelating agent is not included in the mobile phase. Further, since the analytical wavelength for the fluorescence detection is different from that in the standard method, excitation WL: 370 nm, emission WL: 504 nm, the detection sensitivity is different. Thus, it is appropriate to obtain the calibration curve between 0 – 25 ppb of aluminum. Other than the above-mentioned differences, the size of column, column temperature, and the mobile phase are also different from those used in the standard method, so suitable analytical conditions should be established for performing precise and reproducible examinations of trace amounts of aluminum in the sample specimen.

2. Lumogallion complexing method

After forming a complex of aluminum ion in the sample specimen with the fluorescent reagent of lumogallion, the solution is examined by HPLC fitted with a fluorescence photometer.

2.1. Preparation of sample solution

Pipet 70 μ L of the sample specimen (TPN) exactly, add 0.15 mL of lumogallion hydrochloric acid TS and 0.6 mL of buffer solution for aluminum test, pH 7.2 exactly, then mix the solution. After this solution has been allowed to stand for 4 hours at 40°C, it can be used for the measurement as a sample solution.

2.2. Preparation of a series of standard solutions for calibration curve

Pipet 1 mL each of standard aluminum solutions (1) – (5) exactly, and add diluted nitric acid for aluminum test (1 in 100) to make exactly 100 mL. Pipet 70 μ L each of these solutions exactly, and add exactly 0.15 mL of lumogallion hydrochloric acid TS and exactly 0.6 mL of buffer solution for aluminum test, pH 7.2 then allow to stand for 4 hours at 40°C to make a series of standard solutions for obtaining the calibration curve (Aluminum: 0, 1.07, 2.13, 4.27, and 8.54 ppb).

2.3. Standard examination method

Take 0.1 mL each of the sample solution and standard aluminum solutions for the calibration curve, and perform HPLC analysis under the following conditions. Calculate the aluminum content in the sample solution by using a calibration

curve method.

Operating conditions—

Detector: Fluorescence photometer(excitation wavelength 505 nm, emission wavelength 574 nm)

Column: A stainless steel column 6.0 mm in inside diameter and 10 cm in length, packed with octylsilanized silica gel for liquid chromatography (5 μ m in particle diameter).

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

Mobile phase: Take 100 mL of 2-propanol, and add a diluted 1 mol/L acetic acid-sodium acetate buffer solution of pH 5.0 (1 in 10) to make 1000 mL.

Flow rate: Adjust the flow rate so that the retention time of aluminum/lumogallion complex is about 5 minutes.

System suitability—

The correlation coefficient of the calibration curve, which is prepared using a series of standard solutions, is not less than 0.99.

3. Notes

(i) Regarding water, solvents, reagents, vessels and other tools used for the examination, select those not contaminated with aluminum. Further, keep the testing environment clean and free from dust in the testing room.

(ii) Before the measurement, it is necessary to confirm that the characteristic properties of the sample do not affect the formation of the complex.

(iii) Reference substances of river water for analysis of trace elements, distributed by the Japan Society for Analytical Chemistry, contain certified amounts of aluminum: JSAC 0301-1 and JSAC 0302 (a known amount of aluminum is artificially added to JSAC 0301-1).

4. Standard Solutions, Reagents and Test Solutions

Other than the standard solutions, reagents and test solutions specified in the Japanese Pharmacopoeia, those described below can be used in this test.

(i) ***N,N*-Bis(2-hydroxyethyl)-2-aminoethane sulfonic acid** $C_6H_{15}NO_5S$ White crystals or powder.

(ii) **Hydrochloric acid for aluminum test** Same as the reagent *Hydrochloric acid*. Further, it contains not more than 1 ppb of aluminum.

(iii) **Lumogallion** [5-Chloro-2-hydroxy-3(2,4-dihydroxyphenylazo)benzenesulfonic acid] $C_{12}H_9ClN_2O_6S$ Red-brown to dark brown powder. Further, it contains not more than 1 ppm of aluminum.

(iv) **Lumogallion hydrochloric acid TS** Dissolve 0.86 g of lumogallion in 300 mL of 2-propanol, and add 350 mL of diluted *Hydrochloric acid for aluminum test* (9 in 50) and *Water for aluminum test* to make 1000 mL exactly.

(v) **Nitric acid for aluminum test** Same as the reagent *Nitric acid*. Further, it contains not more than 1 ppb of aluminum.

(vi) **pH buffer solution for aluminum test, pH 7.2** Dissolve 106.6 g of *N,N*-bis(2-hydroxyethyl)-2-aminoethane sulfonic acid in 800 mL of *Water for aluminum test*, adjust the pH 7.2 by using *Tetramethylammonium hydroxide aqueous solution*, and add *Water for aluminum test* to make 1000 mL.

(vii) **Standard aluminum solution** Pipet a constant volume each of *Water for aluminum test* or the *Standard aluminum stock solution*, dilute and adjust the aluminum concentration to 0, 1.25, 2.5, 5.0, and 10 ppm by using diluted *Nitric acid for aluminum test* (1 in 100), to make

Standard aluminum solutions (1) – (5).

(viii) **Tetramethylammonium hydroxide TS** $(\text{CH}_3)_4\text{NOH}$ It is a 25% aqueous solution, prepared for aluminum test. Further, it contains not more than 1 ppb of aluminum.

(ix) **Water for aluminum test** It contains not more than 1 ppb of aluminum.

Validation of Analytical Procedures

The validation of an analytical procedure is the process of confirming that the analytical procedure employed for a test of pharmaceuticals is suitable for its intended use. In other word, the validation of an analytical procedure requires us to demonstrate scientifically that risks in decision by testing caused by errors from analytical steps are acceptably small. The performance of an analytical procedure is established by various kinds of validation characteristics. The validity of a proposed analytical procedure can be shown by demonstrating experimentally that the validation characteristics of the analytical procedure satisfy the standards set up according to the acceptable limits of testing.

When an analytical procedure is to be newly carried in the Japanese Pharmacopoeia, when a test carried in the Japanese Pharmacopoeia is to be revised, and when the test carried in the Japanese Pharmacopoeia is to be replaced with a new test according to regulations in general notices, analytical procedures employed for these tests should be validated according to this document.

1. Required data for analytical procedures to be carried in the Japanese Pharmacopoeia

1.1. Outline

This section should provide a brief explanation of the principle of a proposed analytical procedure, identify the necessity of the analytical procedure and its advantage compared with other procedures, and summarize the validation. When an analytical procedure is revised, the limitation of the current analytical procedure and the advantage offered by the new analytical procedure should be described.

1.2. Analytical procedure

This section should contain a complete description of the analytical procedure to enable skilled persons to evaluate correctly the analytical procedure and replicate it if necessary. Analytical procedures include all important operating procedures for performing analyses, the preparation of standard samples, reagents and test solutions, precautions, procedures to verify system suitability (e.g. the verification of the separating performance of a chromatographic system), formulas to obtain results, the number of replications and so forth. Any instruments and apparatus that are not stated in the Japanese Pharmacopoeia should be described in detail. The physical, chemical or biological characteristics of any new reference standards should be clarified and their testing methods should be established.

1.3. Data showing the validity of analytical procedures

This section should provide complete data showing the validity of the analytical procedures. This includes the experimental designs to determine the validation characteristics, experimental data, calculation results and results of hypothesis tests.

2. Validation characteristics

The definition of typical validation characteristics to be assessed in validation of analytical procedures and examples of assessing procedures are given below.

The terminology and definitions of the validation characteristics may possibly vary depending upon the fields to which analytical procedures are applied. The terminology and definitions shown in this document are established for the purpose of the Japanese Pharmacopoeia. Typical methods for assessing the validation characteristics are shown in the item of assessment. Various kinds of methods to determine the validation characteristics have been proposed and any methods that are widely accepted will be accepted for the present purpose. However, since values of the validation characteristics may possibly depend upon methods of determination, it is required to present the methods of determining the validation characteristics, the data and calculation methods in sufficient detail.

Although robustness is not listed as a validation characteristic, it should be considered during the development of analytical procedures. Studying the robustness may help to improve analytical procedures and to establish appropriate analytical conditions including precautions.

2.1. Accuracy/Trueness

2.1.1. Definition

The accuracy is a measure of the bias of observed values obtained by an analytical procedure. The accuracy is expressed as the difference between the average value obtained from a large series of observed values and the true value.

2.1.2. Assessment

The estimate of accuracy of an analytical method is expressed as the difference between the total mean of observed values obtained during investigation of the reproducibility and the true value. The theoretical value is used as the true value (e.g., in the case of titration methods, etc.). When there is no theoretical value or it is difficult to obtain a theoretical value even though it exists, a certified value or a consensus value may be used as the true value. When an analytical procedure for a drug product is considered, the observed value of the standard solution of the drug substance may be used as the consensus value.

It may be inferred from specificity data that an analytical procedure is unbiased.

The estimate of accuracy and a 95% confidence interval of the accuracy should be calculated using the standard error based on the reproducibility (intermediate precision). It should be confirmed that the confidence interval includes zero or that the upper or lower confidence limits are within the range of the accuracy required of the analytical procedure.

2.2. Precision

2.2.1. Definition

The precision is a measure of the closeness of agreement between observed values obtained independently from multiple samplings of a homogenous sample and is expressed as the variance, standard deviation or relative standard deviation (coefficient of variation) of observed values.

The precision should be considered at three levels with different repetition conditions; repeatability, intermediate precision and reproducibility.

(i) Repeatability/Intra-assay precision

The repeatability expresses the precision of observed values obtained from multiple samplings of a homogenous

sample over a short time interval within a laboratory, by the same analyst, using the same apparatus and instruments, lots of reagents and so forth (repeatability conditions).

(ii) Intermediate precision

The intermediate precision expresses the precision of observed values obtained from multiple samplings of a homogenous sample by changing a part of or all of the operating conditions including analysts, experimental dates, apparatus and instruments and lots of reagents within a laboratory (intermediate precision condition).

(iii) Reproducibility

The reproducibility expresses the precision of observed values obtained from multiple samplings of a homogenous sample in different laboratories (reproducibility condition).

2.2.2. Assessment

A sufficient volume of a homogenous sample should be prepared before studying the precision. The solution is assumed to be homogenous. When it is difficult to obtain a homogenous sample, the following samples may be used as homogenous samples; e.g., a large amount of drug products or mixture of drug substance and vehicles that are crushed and mixed well until they can be assumed to be homogenous.

Suitable experimental designs such as one-way layout may be employed when more than one level of precision is to be investigated simultaneously. A sufficient number of repetitions, levels of operating conditions and laboratories should be employed. Sources of variations affecting analytical results should be evaluated as thoroughly as possible through the validation.

It is required to show the variance, standard deviation and relative standard deviation (coefficient of variation) of each level of precision. The 90% confidence interval of the variance and corresponding intervals of the standard deviation and relative standard deviation should also be established. The validity of the proposed analytical procedure for its intended use may be confirmed by comparing obtained values with the required values of the analytical procedure. Whether the proposed analytical procedure is acceptable may normally be decided based on the reproducibility.

2.3. Specificity

2.3.1. Definition

The specificity is the ability of an analytical procedure to measure accurately an analyte in the presence of components that may be expected to be present in the sample matrix. The specificity is a measure of discriminating ability. Lack of specificity of an analytical procedure may be compensated by other supporting analytical procedures.

2.3.2. Assessment

It should be confirmed that the proposed analytical procedure can identify an analyte or that it can accurately measure the amount or concentration of an analyte in a sample. The method to confirm the specificity depends very much upon the purpose of the analytical procedure. For example, the specificity may be assessed by comparing analytical results obtained from a sample containing the analyte only with results obtained from samples containing excipients, related substances or degradation products, and including or excluding the analyte. If reference standards of impurities are unavailable, samples that are expected to contain impurities or degradation products may be used (e.g. samples after accelerated or stress tests).

2.4. Detection limit

2.4.1. Definition

The detection limit is the lowest amount or concentration of the analyte in a sample that is detectable, but not necessarily quantifiable.

2.4.2. Assessment

The detection limit should be normally determined so that producer's and consumer's risks are less than 5%. The detection limit may be calculated using the standard deviation of responses of blank samples or samples containing an analyte close to the detection limit and the slope of the calibration curve close to the detection limit. The following equation is an example to determine the detection limit using the standard deviation of responses of blank samples and the slope of the calibration curve.

$$DL = 3.3\sigma/slope$$

DL: detection limit

σ : the standard deviation of responses of blank samples

slope: slope of the calibration curve

The noise level may be used as the standard deviation of responses of blank samples in chromatographic methods. It should be ensured that the detection limit of the analytical procedure is lower than the specified limit for testing.

2.5. Quantitation limit

2.5.1. Definition

The quantitation limit is the lowest amount or concentration of the analyte in a sample that can be determined. The precision expressed as the relative standard deviation of samples containing an analyte at the quantitation limit is usually 10%.

2.5.2. Assessment

The quantitation limit may be calculated using the standard deviation of responses of blank samples or samples containing an analyte close to the quantitation limit and the slope of the calibration curve close to the quantitation limit. The following equation is an example to determine the quantitation limit using the standard deviation of responses of blank samples and the slope of the calibration curve.

$$QL = 10\sigma/slope$$

QL: quantitation limit

σ : the standard deviation of responses of blank samples

slope: slope of the calibration curve

The noise level may be used as the standard deviation of responses of blank samples in chromatographic methods. It should be ensured that the quantitation limit of the analytical procedure is lower than the specified limit for testing.

2.6. Linearity

2.6.1. Definition

The linearity is the ability of an analytical procedure to elicit responses linearly related to the amount or concentration of an analyte in samples. A well-defined mathematical transformation may sometimes be necessary to obtain a linear relationship.

2.6.2. Assessment

Responses are obtained after analyzing samples with various amounts or concentrations of an analyte according to described operating procedures. The linearity may be evaluated in terms of the correlation coefficient, and the slope and y-intercept of the regression line. It may be also helpful for evaluating the linearity to plot residual errors from the

regression line against the amount or concentration and to confirm that there is no particular tendency in the graph. Samples with five different amounts or concentrations of an analyte should be usually investigated.

2.7. Range

2.7.1. Definition

The range for the validation of analytical procedures is the interval between the lower and upper limits of the amount or concentration of an analyte providing sufficient accuracy and precision. The range for the validation of analytical procedures for an analytical procedure with linearity is the interval between the lower and upper limits providing sufficient accuracy, precision and linearity.

2.7.2. Assessment

When the range for the validation of analytical procedures is investigated, 80 to 120% of specified limits of testing should be usually considered. The accuracy, precision and linearity should be evaluated using samples containing the lower and upper limits and in the middle of the range.

3. Categories of tests employing analytical procedures

Tests covered with this document are roughly classified into three categories shown below according to their purposes. The table lists the normally required validation characteristics to be evaluated in the validation of analytical procedures used in these tests. This list should be considered to represent typical validation characteristics. A different approach to validating analytical procedures should be considered depending upon the characteristics of analytical procedures and their intended use.

(i) Type I Identification. Tests for identifying major components in pharmaceuticals according to their characteristics.

(ii) Type II Impurity tests. Tests for determination of impurities in pharmaceuticals.

(iii) Type III Tests for assaying drug substances, active

ingredients, and major components in pharmaceuticals.

(Additives such as stabilizing agents and preservatives are included in major components.) Tests for determining performance of pharmaceuticals, such as dissolution testing.

4. Terminology used in the validation of analytical procedures

(i) Analytical procedure: This document covers analytical procedures applied to identification, and ones that provides responses depending upon the amount or concentration of analytes in samples.

(ii) Laboratory: The laboratory means an experimental room or facility where tests are performed. In this document different laboratories are expected to perform an analytical procedure using different analysts, different experimental apparatus and instruments, different lots of reagents and so forth.

(iii) Number of replications: The number of replications is one that is described in analytical procedures. An observed value is often obtained by more than one measurement in order to achieve good precision of analytical procedures. Analytical procedures including the number of replications should be validated. This is different from repetition in the validation of analytical procedures to obtain accuracy or precision.

(iv) Observed value: The value of a characteristic obtained as the result of performing an analytical procedure.

(v) Consumer's risk: This is the probability that products out of the specification of tests are decided to be accepted after testing. It is usually expressed as β , and is called the probability of type II error or the probability of false negative in impurity tests.

(vi) Producer's risk: This is the probability that products satisfying the specification of tests are decided to be rejected after testing. It is usually expressed as α , and is called the probability of type I error or the probability of false positive in impurity tests.

(vii) Robustness: The robustness is a measure of the capacity to remain unaffected by small but deliberate variations in analytical conditions. The stability of observed values may be studied by changing various analytical conditions within suitable ranges including pH values of solutions, reaction temperature, reaction time or amount of reagents added. When observed values are unstable, the analytical procedure should be improved. Results of studying robustness may be reflected in the developed analytical procedure as precautions or significant digits describing analytical conditions.

(viii) Test: Tests mean various tests described in general tests and official monographs in the Japanese Pharmacopoeia such as impurity tests and assay. They include sampling methods, specification limits and analytical procedures.

Table Lists of validation characteristics required to be evaluated in tests of each type

Type of test Validation characteristics	Type I	Type II		Type III
		Quantitation test	Limit test	
Accuracy/Trueness	—	+	—	+
Precision				
Repeatability	—	+	—	+
Intermediate precision	—	—*	—	—*
Reproducibility	—	+*	—	+*
Specificity**	+	+	+	+
Detection limit	—	—	+	—
Quantitation limit	—	+	—	—
Linearity	—	+	—	+
Range	—	+	—	+

— Usually need not to be evaluated.

+ Usually need to be evaluated.

* Either intermediate precision or reproducibility should be evaluated depending upon circumstances in which analytical procedures or tests are performed. The latter should be normally evaluated in the validation of analytical procedures proposed to be included in the Japanese Pharmacopoeia.

** The lack of the specificity of an analytical procedure may be compensated by other relevant analytical procedures

G2 Solid-state Properties

Laser Diffraction Measurement of Particle Size

This test is harmonized with the European Pharmacopoeia and the U.S. Pharmacopoeia.

The laser light diffraction technique used for the determination of particle-size distribution is based on the analysis of the diffraction pattern produced when particles are exposed to a beam of monochromatic light. Historically, the early laser diffraction instruments only used scattering at small angles. However, the technique has since been broadened to include laser light scattering in a wider range and application of the Mie theory, in addition to the Fraunhofer approximation and anomalous diffraction.

The technique cannot distinguish between scattering by single particles and scattering by clusters of primary particles, i.e. by agglomerates or aggregates. As most particulate samples contain agglomerates or aggregates and as the focus of interest is generally on the size distribution of primary particles, the clusters are usually dispersed into primary particles before measurement.

For non-spherical particles, an equivalent sphere-size distribution is obtained because the technique assumes spherical particles in its optical model. The resulting particle-size distribution may differ from those obtained by methods based on other physical principles (e.g. sedimentation, sieving).

This chapter provides guidance for the measurement of size distributions of particles in different dispersed systems, for example, powders, sprays, aerosols, suspensions, emulsions, and gas bubbles in liquids, through analysis of their angular light-scattering patterns. It does not address specific requirements of particle-size measurement of specific products.

1. Principle

A representative sample, dispersed at an adequate concentration in a suitable liquid or gas, is passed through a beam of monochromatic light, usually a laser. The light scattered by the particles at various angles is measured by a multi-element detector. Numerical values representing the scattering pattern are then recorded for subsequent analysis. These scattering pattern values are then transformed, using an appropriate optical model and mathematical procedure, to yield the proportion of total volume to a discrete number of size classes, forming a volumetric particle-size distribution.

2. Instrument

The instrument is located in an environment where it is not affected by electrical noise, mechanical vibrations, temperature fluctuations, humidity or direct bright light. An example of a set-up of a laser light diffraction instrument is given in Fig. 1. Other equipment may be used.

The instrument comprises a laser light source, beam processing optics, a sample measurement region (or cell), a Fourier lens, and a multi-element detector for measuring the scattered light pattern. A data system is also required for deconvolution of the scattering data into a volumetric size

distribution and associated data analysis and reporting.

The particles can enter the laser beam in 2 positions. In the conventional case the particles enter the parallel beam before the collecting lens and within its working distance. In so-called reversed Fourier optics the particles enter behind the collecting lens and thus, in a converging beam. The advantage of the conventional set-up is that a reasonable path length for the sample is allowed within the working distance of the lens. The second set-up allows only small path lengths but enables measurement of scattered light at larger angles, which is useful when submicron particles are present.

The interaction of the incident light beam and the ensemble of dispersed particles results in a scattering pattern with different light intensities at various angles. The total angular intensity distribution, consisting of both direct and scattered light, is then focused onto a multi-element detector by a lens or a series of lenses. These lenses create a scattering pattern that, within limits, does not depend on the location of the particles in the light beam. Hence, the continuous angular intensity distribution is converted into a discrete spatial intensity distribution on a set of detector elements.

It is assumed that the measured scattering pattern of the particle ensemble is identical to the sum of the patterns from all individual single scattering particles presented in random relative positions. Note that only a limited angular range of scattered light is collected by the lens(es) and, therefore, by the detector.

3. Development of the method

The measurement of particle size by laser diffraction can give reproducible data, even in the sub-micron region, provided the instrument used and the sample tested are carefully controlled to limit variability of the test conditions (e.g. dispersion medium, method of preparation of the sample dispersion).

Traditionally, the measurement of particle size using laser diffraction has been limited to particles in the range of approximately $0.1\ \mu\text{m}$ to 3 mm. Because of recent advances in lens and equipment design, newer instruments are capable of exceeding this range routinely. With the validation report the user demonstrates the applicability of the method for its intended use.

3.1. Sampling

The sampling technique must be adequate to obtain a representative sample of a suitable volume for the particle-size measurement. Sample splitting techniques such as rotating riffler or the cone and quartering method may be applied.

3.2. Evaluation of the dispersion procedure

Inspect the sample to be analyzed, visually or with the aid of a microscope, to estimate its size range and particle shape. The dispersion procedure must be adjusted to the purpose of the measurement. The purpose may be such that it is preferable to deagglomerate clusters into primary particles as far as possible, or it may be desirable to retain clusters as intact as possible. In this sense, the particles of interest may be either primary particles or clusters.

For the development of a method it is highly advisable to check that comminution of the particles does not occur, and conversely, that dispersion of particles or clusters is satisfactory. This can usually be done by changing the dispersing energy and monitoring the change of the particle-size distribution. The measured size distribution must not change sig-

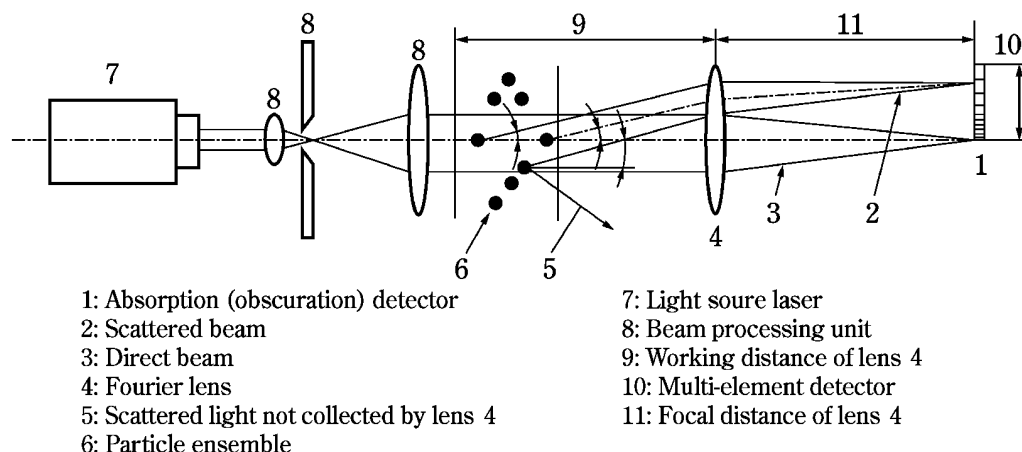


Fig. 1 Example of a set-up of a laser light diffraction instrument

nificantly when the sample is well dispersed and the particles are neither fragile nor soluble. Moreover, if the manufacturing process (e.g. crystallization, milling) of the material has changed, the applicability of the method must be verified (e.g. by microscopic comparison).

Sprays, aerosols and gas bubbles in a liquid should be measured directly, provided that their concentration is adequate, because sampling or dilution generally alters the particle-size distribution.

In other cases (such as emulsions, pastes and powders), representative samples may be dispersed in suitable liquids. Dispersing aids (wetting agents, stabilizers) and/or mechanical forces (e.g. agitation, sonication) are often applied for deagglomeration or deaggregation of clusters and stabilization of the dispersion. For these liquid dispersions, a recirculating system is most commonly used, consisting of an optical measuring cell, a dispersion bath usually equipped with stirrer and ultrasonic elements, a pump, and tubing. Non-recirculating, stirred cells are useful when only small amounts of a sample are available or when special dispersion liquids are used.

Dry powders can also be converted into aerosols through the use of suitable dry powder dispersers, which apply mechanical force for deagglomeration or deaggregation. Generally, the dispersers use the energy of compressed gas or the differential pressure of a vacuum to disperse the particles to an aerosol, which is blown through the measuring zone, usually into the inlet of a vacuum unit that collects the particles. However, for free flowing, coarser particles or granules the effect of gravity may be sufficient to disperse the particles adequately.

If the maximum particle size of the sample exceeds the measuring range of the instrument, the material that is too coarse can be removed by sieving and the mass and percentage of removed material are reported. However, after pre-sieving, note that the sample is no longer representative, unless otherwise proven.

3.3. Optimization of the liquid dispersion

Liquids, surfactants, and dispersing aids used to disperse powders must:

- (i) be transparent at the laser wavelength and practically free from air bubbles or particles;
- (ii) have a refractive index that differs from that of the

test material;

- (iii) be non-solvent of the test material (pure liquid or pre-filtered, saturated solution);
- (iv) not alter the size of the test materials (e.g. by solubility, solubility enhancement, or recrystallization effects);
- (v) favor easily formation and stability of the dispersion;
- (vi) be compatible with the materials used in the instrument (such as O-rings, gaskets, tubing, etc.);
- (vii) possess a suitable viscosity to facilitate recirculation, stirring and filtration.

Surfactants and/or dispersing aids are often used to wet the particles and to stabilize the dispersion. For weak acids and weak bases, buffering of the dispersing medium at low or high pH respectively can assist in identifying a suitable dispersant.

A preliminary check of the dispersion quality can be performed by visual or microscopic inspection. It is also possible to take fractional samples out of a well-mixed stock dispersion. Such stock dispersions are formed by adding a liquid to the sample while mixing it with, for example, a glass rod, a spatula or a vortex mixer. Care must be taken to ensure the transfer of a representative sample and that settling of larger particles does not occur. Therefore a sample paste is prepared or sampling is carried out quickly from a suspension maintained under agitation.

3.4. Optimization of the gas dispersion

For sprays and dry powder dispersions, a compressed gas free from oil, water and particles may be used. To remove such materials from the compressed gas, a dryer with a filter can be used. Any vacuum unit should be located away from the measurement zone, so that its output does not disturb the measurement.

3.5. Determination of the concentration range

In order to produce an acceptable signal-to-noise ratio in the detector, the particle concentration in the dispersion must exceed a minimum level. Likewise, it must be below a maximum level in order to avoid multiple scattering. The concentration range is influenced by the width of the laser beam, the path length of the measurement zone, the optical properties of the particles, and the sensitivity of the detector elements.

In view of the above, measurements must be performed at different particle concentrations to determine the appropri-

ate concentration range for any typical sample of material. (Note: in different instruments, particle concentrations are usually represented by differently scaled and differently named numbers, e.g. obscuration, optical concentration, proportional number of total mass).

3.6. Determination of the measuring time

The time of measurement, the reading time of the detector and the acquisition frequency is determined experimentally in accordance with the required precision. Generally, the time for measurement permits a large number of detector scans or sweeps at short time intervals.

3.7. Selection of an appropriate optical model

Most instruments use either the Fraunhofer or the Mie theory, though other approximation theories are sometimes applied for calculation of the scattering matrix. The choice of the theoretical model depends on the intended application and the different assumptions (size, absorbance, refractive index, roughness, crystal orientation, mixture, etc.) made for the test material. If the refractive index values (real and imaginary parts for the used wavelength) are not exactly known, then the Fraunhofer approximation or the Mie theory with a realistic estimate of the refractive index can be used. The former has the advantages that it is simple and it does not need refractive index values; the latter usually provides less-biased particle-size distributions for small particles. For instance, if the Fraunhofer model is used for samples containing an appreciable amount of small, transparent particles, a significantly larger amount of small particles may be calculated. In order to obtain traceable results, it is essential to document the refractive index values used, since small differences in the values assumed for the real and imaginary part of the complex refractive index may cause significant differences in the resulting particle-size distributions. Small values of the imaginary part of the refractive index (about $0.01 - 0.1i$) are often applied to allow the correction of the absorbance for the surface roughness of the particles. It should be noted, in general, that the optical properties of the substance to be tested, as well as the structure (e.g. shape, surface roughness and porosity) bear upon the final result.

3.8. Validation

Typically, the validity of a procedure may be assessed by the evaluation of its specificity, linearity, range, accuracy, precision and robustness. In particle-size analysis by laser light diffraction, specificity as defined by ICH is not applicable as it is not possible to discriminate different components into a sample, as is neither possible to discriminate between agglomerates from dispersed particles unless properly complemented by microscopic techniques. Exploring a linear relationship between concentration and response, or a mathematical model for interpolation, is not applicable to this procedure. Rather than evaluating linearity, this method requires the definition of a concentration range within which the result of the measurements does not vary significantly. Concentrations below that range produce an error due to a poor signal to noise ratio, while concentrations above that range produce an error due to multiple scattering. The range depends mostly in the instrument hardware. Accuracy should be confirmed through an appropriate instrument qualification and comparison with microscopy, while precision may be assessed by means of a repeatability determination.

The attainable repeatability of the method mainly depends on the characteristics of the material (milled/not milled,

robust/fragile, width of its size distribution, etc.), whereas the required repeatability depends on the purpose of the measurement. Mandatory limits cannot be specified in this chapter, as repeatabilities (different sample preparations) may vary appreciably from one substance to another. However, it is good practice to aim at acceptance criteria for repeatability such as $RSD (\%) \leq 10\%$ [$n = 6$] for any central value of the distribution (e.g. for x_{50}). Values at the sides of the distribution (e.g. x_{10} and x_{90}) are oriented towards less stringent acceptance criteria such as $RSD \leq 15\%$ [$n = 6$]. Below $10 \mu\text{m}$, these values must be doubled. Robustness may be tested during the selection and optimization of the dispersion media and forces. The change of the dispersing energy may be monitored by the change in the particle-size distribution.

4. Measurement

4.1. Precautions

- (i) never look into the direct path of the laser beam or its reflections;
- (ii) earth all instrument components to prevent ignition of solvents or dust explosions;
- (iii) check the instrument set-up (e.g. warm-up, required measuring range and lens, appropriate working distance, position of the detector, no direct bright daylight);
- (iv) in the case of wet dispersions, avoid air bubbles, evaporation of liquid, schlieren or other inhomogeneities in the dispersion; similarly, avoid improper mass-flow from the disperser or turbulent air-flow in the case of dry dispersions; such effects can cause erroneous particle-size distributions.

4.2. Measurement of the light scattering of dispersed sample(s)

After proper alignment of the optical part of the instrument, a blank measurement of the particle-free dispersion medium must be performed using the same method as that used for the measurement of the sample. The background signal must be below an appropriate threshold. The detector data are saved in order to subtract them later from the data obtained with the sample. The sample dispersion is measured according to the developed method.

For each detector element, an average signal is calculated, sometimes together with its standard deviation. The magnitude of the signal from each detector element depends upon the detection area, the light intensity and the quantum efficiency. The co-ordinates (size and position) of the detector elements together with the focal distance of the lens determine the range of scattering angles for each element. Most instruments also measure the intensity of the central (unscattered) laser beam. The ratio of the intensity of a dispersed sample to that in its absence (the blank measurement) indicates the proportion of scattered light and hence the particle concentration.

4.3. Conversion of scattering pattern into particle-size distribution

This deconvolution step is the inverse of the calculation of a scattering pattern for a given particle-size distribution. The assumption of spherical particle shape is particularly important as most algorithms use the mathematical solution for scattering from spherical particles. Furthermore, the measured data always contain some random and systematic errors, which may vitiate the size distributions. Several mathematical procedures have been developed for use in the available instruments. They contain some weighting of devi-

ations between measured and calculated scattering patterns (e.g. least squares), some constraints (e.g. non-negativity for amounts of particles), and/or some smoothing of the size distribution curve.

The algorithms used are specific to each make and model of equipment, and are proprietary. The differences in the algorithms between different instruments may give rise to differences in the calculated particle-size distributions.

4.4. Replicates

The number of replicate measurements (with individual sample preparations) to be performed, depends on the required measurement precision. It is recommended to set this number in a substance-specific method.

5. Reporting of results

The particle-size distribution data are usually reported as cumulative undersize distribution and/or as density distribution by volume. The symbol x is used to denote the particle size, which in turn is defined as the diameter of a volume-equivalent sphere. $Q3(x)$ denotes the volume fraction undersize at the particle size x . In a graphical representation, x is plotted on the abscissa and the dependent variable $Q3$ on the ordinate. Most common characteristic values are calculated from the particle-size distribution by interpolation. The particle sizes at the undersize values of 10%, 50%, and 90% (denoted as x_{10} , x_{50} , and x_{90} respectively) are frequently used. x_{50} is also known as the median particle size. It is recognized that the symbol d is also widely used to designate the particle size, thus the symbol x may be replaced by d .

Moreover, sufficient information must be documented about the sample, the sample preparation, the dispersion conditions, and the cell type. As the results depend on the particular instrument, data analysis program, and optical model used, these details must also be documented.

6. Control of the instrument performance

Use the instrument according to the manufacturer's instructions and carry out the prescribed qualifications at an appropriate frequency, according to the use of the instrument and substances to be tested.

6.1. Calibration

Laser diffraction systems, although assuming idealized properties of the particles, are based on first principles of laser light scattering. Thus, calibration in the strict sense is not required. However, it is still necessary to confirm that the instrument is operating correctly. This can be undertaken using any certified reference material that is acceptable in industrial practice. The entire measurement procedure is examined, including sample collection, sample dispersion, sample transport through the measuring zone, measurement, and the deconvolution procedure. It is essential that the total operational procedure is fully described.

The preferred certified reference materials consist of spherical particles of a known distribution. They must be certified as to the mass-percentage size distribution by an absolute technique, if available, and used in conjunction with an agreed, detailed operation procedure. It is essential that the real and imaginary parts of the complex refractive index of the material are indicated if the Mie theory is applied in data analysis. The representation of the particle-size distribution by volume will equal that of the distribution by mass, provided that the density of the particles is the same for all size fractions.

The response of a laser diffraction instrument is con-

sidered to meet the requirements if the mean value of x_{50} from at least 3 independent measurements does not deviate by more than 3% from the certified range of values of the certified reference material. The mean values for x_{10} and x_{90} must not deviate by more than 5% from the certified range of values. Below 10 μm , these values must be doubled.

Although the use of materials consisting of spherical particles is preferable, non-spherical particles may also be employed. Preferably, these particles have certified or typical values from laser diffraction analysis performed according to an agreed, detailed operating procedure. The use of reference values from methods other than laser diffraction may cause a significant bias. The reason for this bias is that the different principles inherent in the various methods may lead to different sphere-equivalent diameters for the same non-spherical particle.

Although the use of certified reference materials is preferred, other well-defined reference materials may also be employed. They consist of substances of typical composition and particle-size distribution for a specified class of substances. Their particle-size distribution has proven to be stable over time. The results must comply with previously determined data, with the same precision and bias as for the certified reference material.

6.2. Qualification of the system

In addition to the calibration, the performance of the instrument must be qualified at regular time intervals or as frequently as appropriate. This can be undertaken using any suitable reference material as mentioned in the previous paragraph.

The qualification of the system is based on the concept that the equipment, electronics, software and analytical operations constitute an integral system, which can be evaluated as an entity. Thus the entire measurement procedure is examined, including sample collection, sample dispersion, sample transport through the measuring zone, and the measurement and deconvolution procedure. It is essential that the total operational procedure is fully described.

In general, unless otherwise specified in the individual monograph, the response of a laser diffraction instrument is considered to meet the requirements if the x_{50} value does not deviate by more than 10% from the range of values of the reference material. If optionally the values at the sides of the distribution are evaluated (e.g. x_{10} and x_{90}), then these values must not deviate by more than 15% from the certified range of values. Below 10 μm , these values must be doubled.

Note 1: For calibration of the instrument stricter requirements are laid down in 6.1. Calibration.

Note 2: Laser Diffraction Measurement of Particle Size complies with ISO13320-1 (1999) and 9276-1 (1998).

Powder Fineness

This classification is harmonized with the European Pharmacopoeia and the U.S. Pharmacopoeia.

A simple descriptive classification of powder fineness is provided in this chapter. Sieving is most suitable where a majority of the particles are larger than about 75 μm , although it can be used for some powders having smaller particle sizes where the method can be validated. Light

diffraction is also a widely used technique for measuring the size of a wide range of particles.

Where the cumulative distribution has been determined by analytical sieving or by application of other methods, particle size may be characterized in the following manner:

x_{90} : particle size corresponding to 90% of the cumulative undersize distribution

x_{50} : median particle size (ie: 50% of the particles are smaller and 50% of the particles are larger)

x_{10} : particle size corresponding to 10% of the cumulative undersize distribution

It is recognized that the symbol d is also widely used to designate these values. Therefore, the symbols d_{90} , d_{50} , d_{10} may be used.

The following parameters may be defined based on the cumulative distribution.

$Q_r(x)$: cumulative distribution of particles with a dimension less than or equal to x where the subscript r reflects the distribution type

r	Distribution type
0	Number
1	Length
2	Area
3	Volume

Therefore, by definition:

$$Q_r(x) = 0.90 \text{ when } x = x_{90}$$

$$Q_r(x) = 0.50 \text{ when } x = x_{50}$$

$$Q_r(x) = 0.10 \text{ when } x = x_{10}$$

An alternative but less informative method of classifying powder fineness is by use of the descriptive terms in the following table.

Classification of powders by fineness		
Descriptive term	x_{50} (μm)	Cumulative distribution by volume basis, $Q_3(x)$
Coarse	> 355	$Q_3(355) < 0.50$
Moderately fine	180–355	$Q_3(180) < 0.50$ and $Q_3(355) \geq 0.50$
Fine	125–180	$Q_3(125) < 0.50$ and $Q_3(180) \geq 0.50$
Very fine	≤ 125	$Q_3(125) \geq 0.50$

Powder Flow

This test is harmonized with the European Pharmacopoeia and the U.S. Pharmacopoeia.

The widespread use of powders in the pharmaceutical industry has generated a variety of methods for characterizing powder flow. Not surprisingly, scores of references appear in

the pharmaceutical literature, attempting to correlate the various measures of powder flow to manufacturing properties. The development of such a variety of test methods was inevitable; powder behavior is multifaceted and thus complicates the effort to characterize powder flow. The purpose of this chapter is to review the methods for characterizing powder flow that have appeared most frequently in the pharmaceutical literature. In addition, while it is clear that no single and simple test method can adequately characterize the flow properties of pharmaceutical powders, this chapter proposes the standardization of test methods that may be valuable during pharmaceutical development.

Four commonly reported methods for testing powder flow are (1) angle of repose, (2) compressibility index or Hausner ratio, (3) flow rate through an orifice, and (4) shear cell. In addition, numerous variations of each of these basic methods are available. Given the number of test methods and variations, standardizing the test methodology, where possible, would be advantageous.

With this goal in mind, the most frequently used methods are discussed below. Important experimental considerations are identified and recommendations are made regarding standardization of the methods. In general, any method of measuring powder flow should be practical, useful, reproducible, sensitive, and yield meaningful results. It bears repeating that no one simple powder flow method will adequately or completely characterize the wide range of flow properties experienced in the pharmaceutical industry. An appropriate strategy may well be the use of multiple standardized test methods to characterize the various aspects of powder flow as needed by the pharmaceutical scientist.

1. Angle of repose

The angle of repose has been used in several branches of science to characterize the flow properties of solids. Angle of repose is a characteristic related to interparticulate friction, or resistance to movement between particles. Angle of repose test results are reported to be very dependent upon the method used. Experimental difficulties arise due to segregation of material and consolidation or aeration of the powder as the cone is formed. Despite its difficulties, the method continues to be used in the pharmaceutical industry, and a number of examples demonstrating its value in predicting manufacturing problems appear in the literature.

The angle of repose is the constant, three-dimensional angle (relative to the horizontal base) assumed by a cone-like pile of material formed by any of several different methods (described briefly below).

1.1. Basic methods for angle of repose

A variety of angle of repose test methods are reported in the literature. The most common methods for determining the static angle of repose can be classified based on two important experimental variables:

(i) The height of the “funnel” through which the powder passes may be fixed relative to the base, or the height may be varied as the pile forms.

(ii) The base upon which the pile forms may be of fixed diameter or the diameter of the powder cone may be allowed to vary as the pile forms.

1.2. Variations in angle of repose methods

In addition to the above methods, variations of them have been used to some extent.

(i) Drained angle of repose: This is determined by

allowing an excess quantity of material positioned above a fixed diameter base to “drain” from the container. Formation of a cone of powder on the fixed diameter base allows determination of the drained angle of repose.

(ii) Dynamic angle of repose: This is determined by filling a cylinder (with a clear, flat cover on one end) and rotating it at a specified speed. The dynamic angle of repose is the angle (relative to the horizontal) formed by the flowing powder. The internal angle of kinetic friction is defined by the plane separating those particles sliding down the top layer of the powder and those particles that are rotating with the drum (with roughened surface).

1.3. Angle of repose general scale of flowability

While there is some variation in the qualitative description of powder flow using the angle of repose, much of the pharmaceutical literature appears to be consistent with the classification by Carr¹⁾, which is shown in Table 1. There are examples of formulations with an angle of repose in the range of 40 to 50 degrees that manufactured satisfactorily. When the angle of repose exceeds 50 degrees, the flow is rarely acceptable for manufacturing purposes.

Table 1 Flow properties and corresponding angles of repose¹⁾

Flow property		Angle of repose (degrees)
Excellent		25 – 30
Good		31 – 35
Fair	aid not needed	36 – 40
Passable	may hang up	41 – 45
Poor	most agitate, vibrate	46 – 55
Very poor		56 – 65
Very, very poor		> 66

1.4. Experimental considerations for angle of repose

Angle of repose is not an intrinsic property of the powder, that is to say, it is very much dependent upon the method used to form the cone of powder. On this subject, the existing literature raises these important considerations:

(i) The peak of the cone of powder can be distorted by the impact of powder from above. By carefully building the powder cone, the distortion caused by impact can be minimized.

(ii) The nature of the base upon which the powder cone is formed influences the angle of repose. It is recommended that the powder cone be formed on a “common base”, which can be achieved by forming the cone of powder on a layer of powder. This can be done by using a base of fixed diameter with a protruding outer edge to retain a layer of powder upon which the cone is formed.

1.5. Recommended procedure for angle of repose

Form the angle of repose on a fixed base with a retaining lip to retain a layer of powder on the base. The base should be free of vibration. Vary the height of the funnel to carefully build up a symmetrical cone of powder. Care should be taken to prevent vibration as the funnel is moved. The funnel height should be maintained approximately 2 – 4 cm from the top of the powder pile as it is being formed in order to minimize the impact of falling powder on the tip of the cone. If a symmetrical cone of powder cannot be successful-

ly or reproducibly prepared, this method is not appropriate. Determine the angle of repose by measuring the height of the cone of powder and calculating the angle of repose, α , from the following equation:

$$\tan \alpha = \text{height}/(\text{base} \times 0.5)$$

2. Compressibility index and Hausner ratio

In recent years the compressibility index and the closely related Hausner ratio have become the simple, fast and popular methods of predicting powder flow characteristics. The compressibility index has been proposed as an indirect measure of bulk density, size and shape, surface area, moisture content, and cohesiveness of materials because all of these can influence the observed compressibility index. The compressibility index and the Hausner ratio are determined by measuring both the bulk volume and tapped volume of a powder.

2.1. Basic methods for compressibility index and Hausner ratio

While there are some variations in the method of determining the compressibility index and Hausner ratio, the basic procedure is to measure (1) the unsettled apparent volume, V_o , and (2) the final tapped volume, V_t , of the powder after tapping the material until no further volume changes occur. The compressibility index and the Hausner ratio are calculated as follows:

$$\text{Compressibility Index} = (V_o - V_t)/V_o \times 100$$

$$\text{Hausner Ratio} = V_o/V_t$$

Alternatively, the compressibility index and Hausner ratio may be calculated using measured values for bulk density (ρ_{bulk}) and tapped density (ρ_{tapped}) as follows:

$$\text{Compressibility Index} = (\rho_{\text{tapped}} - \rho_{\text{bulk}})/\rho_{\text{tapped}} \times 100$$

$$\text{Hausner Ratio} = \rho_{\text{tapped}}/\rho_{\text{bulk}}$$

In a variation of these methods, the rate of consolidation is sometimes measured rather than, or in addition to, the change in volume that occurs on tapping. For the compressibility index and the Hausner ratio, the generally accepted scale of flowability is given in Table 2.

Table 2 Scale of flowability¹⁾

Compressibility index (%)	Flow character	Hausner ratio
≤ 10	Excellent	1.00 – 1.11
11 – 15	Good	1.12 – 1.18
16 – 20	Fair	1.19 – 1.25
21 – 25	Passable	1.26 – 1.34
26 – 31	Poor	1.35 – 1.45
32 – 37	Very poor	1.46 – 1.59
> 38	Very, very poor	> 1.60

2.2. Experimental considerations for the compressibility index and Hausner ratio

Compressibility index and Hausner ratio are not intrinsic properties of the powder, that is to say, they are dependent upon the methodology used. The existing literature points out several important considerations affecting the determination of the (1) unsettled apparent volume, V_o , (2) the final tapped volume, V_t , (3) the bulk density, ρ_{bulk} , and (4) the tapped density, ρ_{tapped} :

- (i) The diameter of the cylinder used
- (ii) The number of times the powder is tapped to achieve the tapped density
- (iii) The mass of material used in the test
- (iv) Rotation of the sample during tapping

2.3. Recommended procedure for compressibility index and Hausner ratio

Use a 250-mL volumetric cylinder with a test sample weight of 100 g. Smaller weights and volumes may be used, but variations in the method should be described with the results. An average of three determinations is recommended.

3. Flow through an orifice

The flow rate of a material depends upon many factors, some of which are particle-related and some related to the process. Monitoring the rate of flow of material through an orifice has been proposed as a better measure of powder flowability. Of particular significance is the utility of monitoring flow continuously since pulsating flow patterns have been observed even for free flowing materials. Changes in flow rate as the container empties can also be observed. Empirical equations relating flow rate to the diameter of the opening, particle size, and particle density have been determined. However, determining the flow rate through an orifice is useful only with free-flowing materials.

The flow rate through an orifice is generally measured as the mass per time flowing from any of a number of types of containers (cylinders, funnels, hoppers). Measurement of the flow rate can be in discrete increments or continuous.

3.1. Basic methods for flow through an orifice

There are a variety of methods described in the literature. The most common for determining the flow rate through an orifice can be classified based on three important experimental variables:

- (1) The type of container used to contain the powder. Common containers are cylinders, funnels and hoppers from production equipment.
- (2) The size and shape of the orifice used. The orifice diameter and shape are critical factors in determining powder flow rate.
- (3) The method of measuring powder flow rate. Flow rate can be measured continuously using an electronic balance and with some sort of recording device (strip chart recorder, computer). It can also be measured in discrete samples (for example, the time it takes for 100 g of powder to pass through the orifice to the nearest tenth of a second or the amount of powder passing through the orifice in 10 seconds to the nearest tenth of a gram).

3.2. Variations in methods for flow through an orifice

Either mass flow rate or volume flow rate can be determined. Mass flow rate is the easier of the methods, but it biases the results in favor of high-density materials. Since die fill is volumetric, determining volume flow rate may be preferable. A vibrator is occasionally attached to facilitate flow from the container, however, this appears to complicate interpretation of results. A moving orifice device has been proposed to more closely simulate rotary press conditions. The minimum diameter orifice through which powder flows can also be identified.

3.3. General scale of flowability for flow through an orifice

No general scale is available because flow rate is critically dependent on the method used to measure it. Comparison

between published results is difficult.

3.4. Experimental considerations for flow through an orifice

Flow rate through an orifice is not an intrinsic property of the powder. It is very much dependent upon the methodology used. The existing literature points out several important considerations affecting these methods:

- (i) The diameter and shape of the orifice
- (ii) The type of container material (metal, glass, plastic)
- (iii) The diameter and height of the powder bed.

3.5. Recommended procedure for flow through an orifice

Flow rate through an orifice can be used only for materials that have some capacity to flow. It is not useful for cohesive materials. Provided that the height of the powder bed (the 'head' of powder) is much greater than the diameter of the orifice, the flow rate is virtually independent of the powder head. Use a cylinder as the container because the cylinder material should have little effect on flow. This configuration results in flow rate being determined by the movement of powder over powder rather than powder along the wall of the container. Powder flow rate often increases when the height of the powder column is less than two times the diameter of the column. The orifice should be circular and the cylinder should be free of vibration. General guidelines for dimensions of the cylinder are as follows:

- (i) Diameter of opening > 6 times the diameter of the particles
- (ii) Diameter of the cylinder > 2 times the diameter of the opening

Use of a hopper as the container may be appropriate and representative of flow in a production situation. It is not advisable to use a funnel, particularly one with a stem, because flow rate will be determined by the size and length of the stem as well as the friction between the stem and the powder. A truncated cone may be appropriate, but flow will be influenced by the powder-wall friction coefficient, thus, selection of an appropriate construction material is important.

For the opening in the cylinder, use a flat-faced bottom plate with the option to vary orifice diameter to provide maximum flexibility and better ensure a powder-over-powder flow pattern. Rate measurement can be either discrete or continuous. Continuous measurement using an electronic balance can more effectively detect momentary flow rate variations.

4. Shear cell methods

In an effort to put powder flow studies and hopper design on a more fundamental basis, a variety of powder shear testers and methods that permit more thorough and precisely defined assessment of powder flow properties have been developed. Shear cell methodology has been used extensively in the study of pharmaceutical materials. From these methods, a wide variety of parameters can be obtained, including the yield loci representing the shear stress-shear strain relationship, the angle of internal friction, the unconfined yield strength, the tensile strength, and a variety of derived parameters such as the flow factor and other flowability indices. Because of the ability to more precisely control experimental parameters, flow properties can also be determined as a function of consolidation load, time, and other environmental conditions. The methods have been successfully used to determine critical hopper and bin parameters.

4.1. Basic methods for shear cell

One type of shear cell is the cylindrical shear cell which is split horizontally, forming a shear plane between the lower stationary base and the upper movable portion of the shear cell ring. After powder bed consolidation in the shear cell (using a well-defined procedure), the force necessary to shear the powder bed by moving the upper ring is determined. Annular shear cell designs offer some advantages over the cylindrical shear cell design, including the need for less material. A disadvantage, however, is that because of its design, the powder bed is not sheared as uniformly because material on the outside of the annulus is sheared more than material in the inner region. A third type of shear cell (plate-type) consists of a thin sandwich of powder between a lower stationary rough surface and an upper rough surface that is moveable.

All of the shear cell methods have their advantages and disadvantages, but a detailed review is beyond the scope of this chapter. As with the other methods for characterizing powder flow, many variations are described in the literature. A significant advantage of shear cell methodology in general is a greater degree of experimental control. The methodology generally is rather time-consuming and requires significant amounts of material and a well-trained operator.

4.2. Recommendations for shear cell

The many existing shear cell configurations and test methods provide a wealth of data and can be used very effectively to characterize powder flow. They are also helpful in the design of equipment such as hoppers and bins.

Because of the diversity of available equipment and experimental procedures, no specific recommendations regarding methodology are presented in this chapter. It is recommended that the results of powder flow characterization using shear cell methodology include a complete description of equipment and methodology used.

References

- 1) Carr R.L.: Evaluating flow properties of solids. *Chem. Eng.* 1965; 72: 163-168.

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, the Japanese Pharmacopoeia 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 ($1 \text{ g/cm}^3 = 1000 \text{ kg/m}^3$).

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 the Japanese Pharmacopoeia 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 finest 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 powder 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.

The Japanese Pharmacopoeia 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, the Pharmacopoeia 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, the Pharmacopoeia specifies the method of determining the tapped density by measuring the mass of a fixed volume of the tapped powder (constant volume method).

G3 Biotechnological/Biological Products

Amino Acid Analysis

This test is harmonized with the European Pharmacopoeia and the U.S. Pharmacopoeia.

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 pre-column 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 (\leq less than 200 μ m 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 μ L of *Hydrolysis Solution* per 500 μ g 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 (\leq less than 200 μ m 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 μ g of the protein/peptide under test is dried in a hydrolysis tube. The hydrolysis tube is placed in a larger tube with about 200 μ L of the *Hydrolysis Solution*. The larger tube is sealed in vacuum (about 50 μ m 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 hy-

drochloric 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 trypto-

phan 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 pyridylethyl-cysteine recovery. Longer incubation times for the pyridylethylation reaction can cause modifications to the α -amino terminal group and the ϵ -amino group of lysine in the protein.

Method 8

Cysteine-cystine reduction and alkylation is accomplished by a liquid phase pyridylethylation reaction.

Stock Solutions Prepare and filter three solutions: 1 mol/L Tris hydrochloride (pH 8.5) containing 4 mmol/L disodium dihydrogen ethylenediamine tetraacetate (*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 μ L 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 μ g of the test sample to a hydrolysis tube, and add 5 μ L of the *Reducing Solution*. Add 10 μ L 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 diamino-propionic 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 μ g 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 hy-

drolysis 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 μ g 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 μ g 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 μ g 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 the 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.

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 an ODS column is accomplished through a combination of changes in concentrations 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 μ mol/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).

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 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 a predominant drawback. This method does not detect amino acids that exist as secondary amines (e.g., proline). To compensate for this drawback, this technique may be combined with another technique described in *Method 7* or *Method 8*.

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.

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 acid derivatives, can be separated on an ODS column of a reversed-phase HPLC by employing gradient systems consisting of acetonitrile and aqueous buffer mixture. Separated DABS-amino acids eluted from the column are detected at 436 nm in the visible region.

This *Method* can 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.

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 an 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 the column is monitored by a fluorometric detector set at an excitation wavelength of 260 nm and an emission wavelength of 313 nm.

The detection limit is in the low fmol range. A linearity range of 0.1 to 50 $\mu\text{mol/L}$ 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.

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 the column is monitored by a fluorometric detector set at an excitation wavelength of 480 nm and an emission wavelength of 530 nm.

The sensitivity of this method is almost the same as for 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 (≤ 0.0267 kPa) 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 \geq 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.

Basic Requirements for Viral Safety of Biotechnological/Biological Products listed in Japanese Pharmacopoeia

Introduction

The primary role of specification of biotechnological/biological products listed in Japanese Pharmacopoeia (JP) is not only for securing quality control or consistency of the quality but also for assuring their efficacy and safety. In the meantime, the requirements to assure quality and safety of drugs have come to be quite strict recently, and a rigid attitude addressing safety assurance is expected for biotechnological/biological products. The key points for quality and safety assurance of biotechnological/biological products are selection and appropriate evaluation of source material, appropriate evaluation of manufacturing process and maintenance of manufacturing consistency, and control of specific physical properties of the products. Now, how to assure quality and safety of such drugs within a scope of JP has come to be questioned. This General Information describes what sorts of approaches are available to overcome these issues.

It is desired that quality and safety assurance of JP listed products are achieved by state-of-the-art methods and concepts which reflect progress of science and accumulation of experiences. This General Information challenges to show the highest level of current scientific speculation. It is expected that this information will contribute to promotion of scientific understanding of quality and safety assurance of not only JP listed products but also the other biotechnological/biological products and to promotion of active discussion of each Official Monograph in JP.

1. Fundamental measures to ensure viral safety of JP listed biotechnological/biological products

The biotechnological/biological product JP includes the products derived from living tissue and body fluid (urine, blood, etc.) of mammals, etc. Protein drugs derived from cell lines of human or animal origin (e.g., recombinant DNA drug, cell culture drug) are also included. The fundamental measures required for comprehensive viral safety of JP listed

biotechnological/biological products are as follows: 1) acquaintance of possible virus contamination (source of contamination); 2) careful examination of eligibility of raw materials and their sources, e.g. human/animal, and thorough analysis and screening of the sample chosen as a substrate for drug production (e.g., pooled body fluid, cell bank, etc.) to determine any virus contamination and determination of type and nature of the virus, if contaminated; 3) evaluation to determine virus titer and virus-like particles hazardous to human, if exists; 4) selection of production related material (e.g., reagent, immune antibody column) free from infectious or pathogenic virus; 5) performance of virus free test at an appropriate stage of manufacturing including the final product, if necessary; 6) adoption of effective viral clearance method in the manufacturing process to remove/inactivate virus. Combined method sometimes achieves higher level of clearance; 7) development of a deliberate viral clearance scheme; 8) performance of the test to evaluate viral removal and inactivation. It is considered that the stepwise and supplemental adoption of the said measures will contribute to ensure viral safety and its improvement.

2. Safety assurance measures described in the Official Monograph and this General Information

As mentioned in above 1, this General Information describes, in package, points to be concerned with and concrete information on the measures taken for viral safety of JP listed products. Except where any specific caution is provided in Official Monograph of a product in question, Official Monograph provides in general that “Any raw material, substrate for drug production and production related material used for production of drug should be derived from healthy animals and should be shown to be free of latent virus which is infectious or pathogenic to human”, “Cell line and culture method well evaluated in aspects of appropriateness and rationality on viral safety are used for production, and the presence of infectious or pathogenic latent virus to human in process related materials derived from living organisms should be denied”. and “biotechnological/biological drug should be produced through a manufacturing process which is capable of removing infectious or pathogenic virus”, etc., to raise awareness on viral safety and on necessity to conduct test and process evaluation for viral safety.

3. Items and contents described in this General Information

As for viral safety of protein drug derived from cell line of human or animal origin, there is a Notice in Japan entitled “Viral safety evaluation of biotechnology products derived from cell lines of human or animal origin” (Iyakushin No. 329 issued on February 22, 2000 by Director, Evaluation and Licensing Division, Pharmaceutical and Medical Safety Bureau, Ministry of Health and Welfare) to reflect the internationally harmonized ICH Guideline, and as for blood plasma protein fraction preparations, there is a document entitled “Guideline for ensuring viral safety of blood plasma protein fraction preparations”. This General Information for ensuring viral safety of JP listed biotechnological/biological products has been written, referencing the contents of those guidelines, to cover general points and their details to be concerned for ensuring viral safety of not only JP listed biotechnological/biological products but also all products which would be listed in JP in future, i.e., biological products derived from living tissue and body fluid, such as

urine, and protein drugs derived from cell line of human or animal origin (Table 1).

3.1. Purpose

The purpose of this document is to propose the comprehensive concepts of the measures to be taken for ensuring viral safety of biotechnological/biological products derived from living tissue or body fluid of mammals, etc. and of protein drugs derived from cell lines of human or animal origin. That is to say, this document describes the measures and the points of concern on the items, such as ① consideration of the source of virus contamination; ② appropriate evaluation on eligibility at selecting the raw material and on qualification of its source, e.g. human or animal; ③ virus test, and its analysis and evaluation at a stage of cell substrate for drug production; ④ appropriate evaluation to choose product related materials derived from living organisms (e.g. reagent, immune antibody column, etc.); ⑤ conduct of necessary virus test on the product at an appropriate stage of manufacturing; ⑥ development of viral clearance test scheme; ⑦ performance and evaluation of viral clearance test. This document is also purposed to comprehensively describe in details that supplemental and combining adoption of the said measures will contribute to secure viral safety and its improvement.

3.2. Background

One of the most important issues to be cautioned for safety of a biological product, which is directly derived from human or animal, or of a protein drug, which is derived from cell line of human or animal origin (recombinant DNA derived product, cell culture derived product, etc.), is risk of virus contamination. Virus contamination may cause serious situation at clinical use once it occurs. Virus contamination may be from a raw material or from a cell substrate for drug production, or may be from an adventitious factor introduced to the manufacturing process.

JP listed biological drugs or protein drugs derived from cell line have achieved drastic contribution to the medical society, and to date, there has not been any evidence of any safety problem on them caused by virus. But, social requirement of health hazard prevention is strong, and it is now very important to prevent accidental incidence, taking security measures carefully supported by scientific rationality. It is always great concern among the persons involved that under what sort of viewpoint and to what extent we have to pursue for ensuring viral safety of a biotechnological/biological product. Before discussing these issues, two fundamental points have to be reconfirmed. One is that; we have to consider scientific, medical, and social profiles a drug has. In other words, “Medicine is a social asset which is utilized in medical practice paying attention to the risk and benefit from the standpoints of science and society”. It is the destiny and the mission of the medical/pharmaceutical society to realize prompt and stable supply of such a social asset, drug, among the medical work front to bring gospel to the patients.

The other is that; issue of viral safety is independent from safety of the components of a drug per se (narrow sense of safety). It is important to consider that this is the matter of general safety of drug (broad sense of safety). In case of a drug which has been used for a long time in the medical front, such as a JP listed product, its broad sense of safety is considered to have been established epidemiologically, and its usage past records have a great meaning. However,

Table 1 Items described in General Information for Viral Safety Assurance of JP listed Biotechnological/Biological Product

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different from safety of drug per se (its components), taking into account any possibility of virus contamination, we have to say that only the results accumulated can not always assure viral safety of a drug used in future. Accordingly, the basis for securing broad sense of viral safety of JP listed biotechnological/biological products is to pay every atten-

tion to the measures to take for prevention, while evaluating the accumulated results.

Adopting strict regulations and conducting tests at maximum level to the extent theoretically considered may be the ways off assuring safety, but applying such way generally, without sufficient scientific review of the ways and evaluation of usage results, causes excessive requirement of regulation and test not having scientific rationality. As the results, effective and prompt supply of an important drug, already having enough accumulation of experiences, to the medical work front will be hampered, and the drug, a social asset, may not to be utilized effectively. Medicine is a sword used in medical field having double-edge named effectiveness and safety. Effectiveness and safety factors have to be derived as the fruits of leading edge of science, and relatively evaluated on a balance sheet of usefulness. Usefulness evaluation should not be unbalanced in a way that too much emphasis is placed on safety concern without back-up of appropriate scientific rationality. A drug can play an important role as a social asset only when well balanced appropriate scientific usefulness evaluation in addition to social concern of the age are given. In other words, drug is a common asset utilized by society for medication as a fruit of science of the age, and the key point of its utilization lies on a balance of risk and benefit produced from scientific and social evaluation. So, those factors have to be taken into account when target and pursuance levels for ensuring viral safety of a JP listed biotechnological/biological product are reviewed.

And, in general, the risk and benefit of drugs should be considered with the relative comparison to alternative drugs or medical treatment. The usefulness of certain drug should be reviewed finally after the competitive assessment on the risk and benefit on the alternative drugs, relevant drugs and/or alternative medical treatment.

Under such background, the purpose of this article is to describe the scientific and rational measures to be taken for ensuring viral safety of JP listed biotechnological/biological products. Giving scientific and rational measures mean that; appropriate and effective measures, elaborated from the current scientific level, are given to the issues assumable under the current scientific knowledge. In other words, possible contaminant virus is assumed to have the natures of genus, morph, particle size, physical/chemical properties, etc. which are within the range of knowledge of existing virology, and is those assumed to exist in human and animal, tissue and body fluid, which are the source of biotechnological/biological product, reagent, material, additives, etc. Accordingly, viral clearance studies using a detection method which target those viruses have to be designed.

3.3. Unknown risk on the measures taken for ensuring viral safety

There are known and unknown risks.

It is easy to determine a test method and an evaluation standard on the known risk, which exists in the drug per se (pharmaceutical component) or inevitably exists due to a quality threshold, and quantification of such risk is possible. In other words, it is easy to evaluate the known risk on a balance sheet in relation to the benefit, and we can say that valuation even in this respect has been established to some extent.

On the other hand, as for the unknown risk which is inevitable for ensuring viral safety, the subject of the risk can not be defined and quantitative concept is hard to introduce,

and, therefore, taking a counter measure and evaluating its effect are not so easy. Therefore, this is the subject to be challenged calling upon wisdom of the related parties among the society of drug.

Talking about the unknown risk, there are view points that say "It is risky because it is unknown." and "What are the unknowns, and how do we cope with them in ensuring safety?"

The view of "It is risky because it is unknown." is already nothing but a sort of evaluation result, and directly connects to a final decision if it can be used as a drug. Such evaluation/decision has to be made based upon a rational, scientific or social judgment.

For example, in the case that "In a manufacturing process of drug, virus, virus-like particle or retrovirus was detected, but its identification could not be confirmed, and, therefore, its risk can not be denied.", the evaluation of "It is risky because it is unknown." is scientifically rational and reasonable. On the other hand, however, if we reach a decision of "It is risky because it is unknown." due to the reason that "In a manufacturing process of drug, virus, virus-like particle or retrovirus was not detected, but there is a 'concern' that something unknown may exist.", it can not be said that such evaluation is based upon a rational, scientific or social judgment. It goes without saying that the utmost care has to be taken for viral safety, but the substance of 'concern' has to be at least clearly explainable. Otherwise, the 'concern' may result in causing contradiction in the meaningful mission to utilize a social asset, drug, in medical practice.

From scientific view point, we should not be narrow minded by saying "it is risky" because "there is a 'concern' that something unknown may exists", but challenge to clarify the subject of "What is unknown, and how to cope with it for ensuring safety" using wisdom. What is important at the time is to define "what is unknown" based upon current scientific knowledge. Only through this way, is it possible for us to elaborate the measures for ensuring safety.

Once we chase up the substance of unknown risk for viral safety without premises of "what is unknown", "unknown" will be an endless question because it theoretically remains unresolved forever. If this kind of approach is taken, the issue and the measure can not be scientifically connected to each other, which will result in the excessive requirement of regulation and of test to be conducted. Yet, it is unlikely that the measure which has no relation with science will be effective to the subject of "What is unknown is unknown."

For example, "what is unknown" at the "evaluation of a purification process which can completely clear up every virus that contaminated in a manufacturing process" should be the subject of "what sort of existing virus that contaminated is unknown", not on the subject of "what sort of virus that exist in the world is unknown. In the former subject, the premise of the study is based on all the knowledge on viruses including DNA/RNA-virus, virus with/without envelope, particle size, physical/chemical properties, etc. The premise is that the virus contaminated should be within range of existing wisdom and knowledge of virus such as species, type, nature, etc., even though the virus that contaminated is unknown. Under such premise, when evaluation is made on a purification process to decide its capability of clearing a derived virus, which is within the range of existing wisdom and learning, specific viral clearance studies designed to combine a few model viruses with different na-

tures, such as type of nucleic acid, with/without envelope, particle size, physical/chemical properties, etc., would be enough to simulate every sort of the virus already known, and will be a "good measure for ensuring safety".

The issue of "the sort of viruses that exist in the world is unknown" may be a future study item, but it is not an appropriate subject for the viral clearance test. Further, even if the subject of "unknown viruses, which have a particle size smaller than that of currently known viruses, may exists" or "unknown viruses, which have special physical/chemical properties that can not be matched to any of the currently known viruses, may exists" is set up as an armchair theory, any experimental work can not be pursued under the current scientific level, since such virus model is not available. Further, any viral clearance test performed by using the currently available methods and technologies will be meaningless "for ensuring safety", since particle size or natures of such speculated virus are unknown. Likewise, any counter measures can not be taken on the subject of "unknown virus, which can not be detected by currently available screening method, may exist", and conducting any virus detection test at any stage will be useless "for ensuring safety".

The requirement of regulations or tests excessively over scientific rationality will raise human, economical and temporal burden to the pharmaceutical companies, and will adversely affect prompt, effective and economical supply of a drug to the medical front. As drug is a sort of social asset, which has to be scientifically evaluated, how to assure maximization of its safety by means of scientifically rational approach at minimum human, economical and time resources is important.

It is also important to reconfirm that achievement of those issues is on the premise that appropriate measures are taken on the supply source of drugs. For example, in a case of "In a manufacturing process of drug, virus, virus-like particle or retrovirus was not detected, but there is a 'concern' that something unknown may exist.", appropriateness of the test, which resulted in the judgment that "virus, virus-like particle or retrovirus was not detected in a process of drug production", should be a prerequisite premise when judged by science standard at the time. If there is any question on the premise, it is quite natural that the question of "there is a 'concern' that unknown something may exist." will be effective.

3.4. Applicable range

This General Information is on JP listed biological products, derived from living tissue or body fluid, and protein drugs, derived from human or animal cell line, that in Japan. In the case of protein drugs derived from human or animal cell line, the products developed and approved before enforcement of the Notice Iyakushin No. 329 entitled "Viral safety evaluation of biotechnology products derived from cell lines of human or animal origin" should have been treated under the Notice had there been one, and it is inevitable that some products approved after the Notice might not have been sufficiently treated. It is expected that such biodrug will be sufficiently examined to meet such General Information before being listed in JP. On the other hand, blood preparations listed in the biological products standard and covered by "Guideline for securing safety of blood plasma protein fraction preparations against virus", are out of the scope of this General Information. Further, in case of a relatively lower molecular biogenous substance, such as amino acid,

saccharide and glycerin, and of gelatin, which is even classified as infectious or pathogenic polymer, there are cases that viral contamination can not be considered due to its manufacturing or purification process, and that potent viral inactivation/removal procedure that can not be applied to protein, can be used, and, therefore, it is considered reasonable to omit such substances from the subject for application. However, some part of this General Information may be used as reference. Further, a comprehensive assurance measure for viral safety is recommendable on a biotechnological/biological product not listed in JP using this document as a reference so long as it is similar to the biotechnological/biological product JP.

3.5. Possible viral contamination to a JP listed biotechnological/biological product (source of virus contamination)

Promoting awareness of virus contamination to a JP listed biotechnological/biological product (source of virus contamination) and citing countermeasure are important for eradicating any possible virus contamination and raising probability of safety assurance. Many biotechnological/biological products are produced from a "substrate" which is derived from human or animal tissue, body fluid, etc. as an origin/raw material, and in purification or pharmaceutical processing of such products column materials or additives, which are living organism origin, are occasionally used. Accordingly, enough safety measures should be taken against diffusion of the contaminant virus. Further, as mentioned in Notice Iyakushin No. 329, any protein drug derived from cell lines of human or animal origin should be carefully examined with respect to the risk of virus contamination through the cell line, the cell substrate for drug production, and through the manufacturing process applied thereafter.

"Substrate for drug production" is defined as a starting material which is at a stage where it is deemed to be in a position to ensure quality/safety of an active substance. The "substrate for drug production" is sometimes tissue, body fluid, etc. of human or animal per se and pooled material such as urine, and sometimes a material after some treatment. In many cases, it is considered rational that starting point of full-scale test, evaluation and control should be at the stage of "substrate for drug production". The more strict levels of test, evaluation and control achieved at the stage of "substrate for drug production" can more rationalize evaluation and control of the raw material or individual level of upper stream. On the contrary, strict evaluation and control of the raw material or individual level at an upper stream stage can rationalize tests, evaluation or quality control at the stage of "substrate for drug production".

The measures taken for ensuring viral safety on a biotechnological/biological product currently listed in JP can be assumed from the provisions of manufacturing method, specification and test methods of each preparation. However, unitary principles or information with respect to the measures to be taken for ensuring viral safety, totally reviewing the entire process up to the final product rationally and comprehensively, including source/raw material/substrate, purification process, etc. have not been clarified. The most important thing for ensuring viral safety is to take thorough measures to eliminate the risk of virus contamination at any stage of source animal, raw material and substrate. Although not the cases of a biotechnological/biological product, known examples of a virus contamination from

a raw material/substrate for drug production in old times are Hepatitis A Virus (HAV) or Hepatitis C Virus (HCV) contamination in blood protein fraction preparations. It is also well known that Human Immunodeficiency Virus (HIV) infection caused by blood plasma protein fraction preparations occurred in 1980s. The aim of this General Information is to show concrete guidelines for comprehensive viral safety assurance of the JP listed biotechnological/biological products. The pathogenic infectious viruses, currently known to contaminate to raw materials, etc. of drug and have to be cautioned, are HIV, HAV, Hepatitis B Virus (HBV), HCV, Human T-Lymphotropic Virus (HTLV-I/II), Human Parvovirus B19, Cytomegalovirus (CMV), etc. Biotechnological/biological products produced from raw material/cell substrate derived from tissue or body fluid of human or animal origin always have a risk of contamination of pathogenic or other latent virus. Therefore, safety measures should be thoroughly taken. There is also the case that a material, other than the biological component such as raw material/substrate, causes virus contamination. Using enzymatic or monoclonal antibody column or using albumin etc. as a stabilizer, is the example of the case, in which caution has to be taken on risk of virus contamination from the source animal or cell. Further, there is a possibility of contamination from environment or personnel in charge of production or at handling of the product. So, caution has to be taken on these respects as well.

In case of protein drugs derived from cell line of human or animal origin, there may be cases where latent or persistent infectious viruses (e.g., herpesvirus) or endogenous retroviruses exist in the cell. Further, adventitious viruses may be introduced through the routes such as: 1) derivation of a cell line from an infected animal; 2) use of a virus to drive a cell line; 3) use of a contaminated biological reagent (e.g., animal serum components); 4) contamination during cell handling. In the manufacturing process of drug, an adventitious virus may contaminate to the final product through the routes, such as 1) contamination through a reagent of living being origin, such as serum component, which is used for culturing, etc.; 2) use of a virus for introduction of a specific gene expression to code an objective protein; 3) contamination through a reagent used for purification such as monoclonal antibody affinity column; 4) contamination through an additive used for formulation production; 5) contamination at handling of cell and culture media, etc. It is reported that monitoring of cell culture parameter may be helpful for early detection of an adventitious viral contamination.

3.6. Basis for ensuring viral safety

Viral safety of a biotechnological/biological product produced from a raw material/substrate, which derived from tissue, body fluid, cell line, etc. of human or animal origin, can be achieved by supplemental and appropriate adoption of the following plural methods.

- (1) Acquaintance of possible virus contamination (source of contamination).
- (2) Careful examination of eligibility of the raw material and its source, i.e., human or animal, thorough analysis and screening of the sample chosen as the substrate for drug production to determine virus contamination and through examination of the type of virus and its nature, if contaminated.
- (3) Evaluation to determine hazardous properties of the vi-

rus or virus-like particle to human, if exists.

- (4) Choosing a product related material of living organism origin (e.g., reagent, immune anti-body column, etc.) which is free from infectious or pathogenic virus.
- (5) Conduct virus free test at an appropriate stage of manufacturing including the final product, if necessary.
- (6) Adoption of an effective method to remove/inactivate the virus in the manufacturing process for viral clearance. Combined processes sometimes achieve higher level of viral clearance.
- (7) Develop a deliberate viral clearance scheme.
- (8) Conduct test and evaluation to confirm removal/inactivation of the virus.

Manufacturer is responsible for explaining rationality of the way of approach adopted among the comprehensive strategy for viral safety on each product and its manufacturing process. At the time, the approach described in this General Information shall be applicable as far as possible.

3.7. Limit of virus test

Virus test has to be conducted to define existence of virus, but it should be noted that virus test alone can not reach a conclusion of inexistence of virus nor sufficient to secure safety of the product. Examples of a virus not being detected are as follows: 1) Due to statistical reason, there is an inherent quantitative limit, such as detection sensitivity at lower concentration depends upon the sample size. 2) Generally, every virus test has a detection limit, and any negative result of a virus test can not completely deny existence of a virus. 3) A virus test applied is not always appropriate in terms of specificity or sensitivity for detection of a virus which exists in the tissue or body fluid of human or animal origin.

Virus testing method is improved as science and technology progress, and it is important to apply scientifically the most advanced technology at the time of testing so that it can be possible to raise the assurance level of virus detection. It should be noted, however, that the limit as mentioned above can not always be completely overcome. Further, risk of virus contamination in a manufacturing process can not be completely denied, and, therefore, it is necessary to elaborate the countermeasure taken these effects into account.

Reliable assurance of viral free final product can not be obtained only by negative test results on the raw material/substrate for drug production or on the product in general, it is also necessary to demonstrate inactivation/removal capability of the purification process.

3.8. Roles of viral clearance studies

Under the premises as mentioned in the preceding clause that there is a limit of a virus test, that there is a possibility of existence of latent virus in a raw material/substrate for drug production and that there is a risk of entry of a non-endogenous virus in a manufacturing process, one of the important measures for viral safety is how to remove or inactivate the virus, which exists in a raw material, etc. and can not be detected, or the virus, which is contingently contaminated in a manufacturing process. The purpose of viral clearance study is to experimentally evaluate the viral removal/inactivation capability of a step that mounted in a manufacturing process. So, it is necessary to conduct an experimental scale spike test using an appropriate virus that is selected by taking account the properties, such as particle size, shape, with or without envelope, type of nucleic acid (DNA type, RNA type), heat and chemical treatment toler-

ance, etc., with an aim to determine removal/inactivation capability of the virus that can not be detected in a raw material or contingently contaminated.

As mentioned above, the role of viral clearance study is to speculate removal/inactivation capability of a process through a model test, and it contributes to give scientific basis to assure that a biotechnological/biological product of human or animal origin has reached an acceptable level in aspect of viral safety.

At a viral clearance study, it is necessary to adopt an appropriate approach method which is definitive and rational and can assure viral safety of a final product, taking into consideration the source and the properties of the raw material/substrate as well as the manufacturing process.

4. Raw material/substrate for drug production

4.1. Issues relating to animal species and its region as a source of raw material/substrate for drug production and countermeasures to be taken thereto

For manufacturing JP listed biotechnological/biological products, which require measures for viral safety, a raw material/substrate derived mainly from human, bovine, swine or equine is used, and it is obvious that such human and animal has to be healthy nature. A wild animal should be avoided, and it is recommended to use animals derived from a colony controlled by an appropriate SPF (Specific Pathogen-Free) condition and bred under a well deigned hygienic control, including appropriate control for prevention of microbial contamination and contamination monitoring system. If a meat standard for food is available, an animal meeting this standard has to be used. The type of virus to be concerned about depend on animal species, but it may be possible to narrow down the virus for investigation by means of examining the hygiene control, applicability of a meat standard for food, etc. On the other hand, even with the animals of the same species, a different approach may be necessary depending upon the region where the specimen for a raw material/substrate is taken. For example, in case of obtaining raw material/substrate from blood or other specific region, it is necessary to be aware of the risk level, virus multiplication risk, etc. which may specifically exists depending upon its region. Such approach may be different from those applied to body waste such as urine, milk, etc. as a source of raw material/substrate. Further, caution has to be taken on transmissible spongiform encephalopathy (TSE) when pituitary gland, etc. is used as a raw material. This report does not include detailed explanation on TSE, but recommendations are to use raw material derived from 1) animals originated in the countries (area) where incidence of TSE has not been reported; 2) animals not infected by TSE; or 3) species of animal which has not been reported on TSE. It is recommended to discuss the matters concerned with TSE with the regulatory authority if there is any unclear point.

Followings are the raw material/substrate used for manufacturing biotechnological/biological products in Japan.

(1) Biological products derived from human

Blood plasma, placenta, urine, etc. derived from human are used as the sources of raw material of biotechnological/biological product. As for these raw materials, there are 2 cases: 1) Appropriateness can be confirmed by interview or by examination of the individual who supplies each raw ma-

terial, and 2) Such sufficient interview or examination of the individual can not be made due to type of raw material. In case that sufficient examination of individual level is not possible, it is necessary to perform test to deny virus contamination at an appropriate manufacturing stage, for example, the stage to decide it as a substrate for drug production.

(2) Biological products derived from animal besides human

Heparin, gonadotropin, etc. are manufactured from blood plasma or from various organs of bovine, swine and equine.

(3) Protein drug derived from cell line of human or animal origin

In the case of protein drugs derived from cell line of human or animal origin, a cell line of human or animal is the raw material per se, and the substrate for drug production is a cell bank prepared from cloned cell line (master cell bank or working cell bank). Examination at cell bank level is considered enough for viral safety qualification, but it goes without saying that the more appropriate and rational qualification evaluation test of cell bank can be realized when more information is available on the virus of the source animal or on prehistory of driving the cell line, the base of cell bank.

4.2. Qualification evaluation test on human or animal as a source of raw material/substrate for drug production

(1) Biological products derived from human

Body fluid etc. obtained from healthy human must be used for biological products production. Further, in case that interview or examination of the individual, who supplies the raw material, can be possible and is necessary, interview under an appropriate protocol and a serologic test well evaluated in aspects of specificity, sensitivity and accuracy have to be performed, so that only the raw material, which is denied latent HBV, HCV and HIV, will be used. In addition to the above, it is necessary to test for gene of HBV, HCV and HIV by a nucleic amplification test (NAT) well evaluated in aspects of specificity, sensitivity and accuracy.

In case of the raw material (e.g., urine), which can not be tested over the general medical examination of the individual who supplies the material, or of the raw material which is irrational to conduct individual test, the pooled raw material, as the substrate for drug production, has to be conducted at least to deny existence of HBV, HCV and HIV, using a method well evaluated in aspects of specificity, sensitivity and accuracy, such as the antigen test or NAT.

(2) Biological products derived from animal besides human

The animal used for manufacturing biological product has to be under appropriate health control, and has to be confirmed of its health by various tests. Further, it is necessary that the population, to which the animal belongs, has been under an appropriate breeding condition, and that no abnormal individual has been observed in the population. Further, it is necessary to demonstrate information or scientific basis which can deny known causes infection or disease to human, or to deny such animal inherent latent virus by serologic test or by nucleic amplification test (NAT). The infectious virus that is known to be common between human and animal, and known to cause infection in each animal are tentatively listed in Table 2. It is necessary that the table is completed under careful examination, and denial of all of them, by means of tests on individual animal, tissue, body fluid, etc. as a raw material, or on pooled raw material (as a direct substrate for drug production), is not always necessary. Table 2

can be used as reference information, in addition to the other information, such as; source of animal, health condition, health and breeding control, conformity to the meat standard for food, etc., to elaborate to which virus what kind of test has to be performed, and for which virus it is not always necessary to test for, etc. It is important to clarify and record the basis of choosing the virus and the test conducted thereof.

(3) Protein drug derived from cell line of human or animal origin

It is important to conduct thorough investigation on latent endogenous and non-endogenous virus contamination in a master cell bank (MCB), which is the cell substrate for drug production, in accordance with the Notice Iyakushin No. 329 entitled "Viral safety evaluation of biotechnology products derived from cell lines of human or animal origin". Further, it is necessary to conduct an appropriate adventitious virus test (e.g., *in vitro* and *in vivo* test) and a latent endogenous virus test on the cell at the limit of *in vitro* cell age (CAL) for drug production. Each WCB as a starting cell substrate for drug production should be tested for adventitious virus either by direct testing or by analysis of cells at the CAL, initiated from the WCB. When appropriate non-endogenous virus tests have been performed on the MCB and cells cultured up to or beyond the CAL have been derived from the WCB and used for testing for the presence of adventitious viruses, similar tests need not be performed on the initial WCB.

5. Points of concern with respect to manufacturing and virus testing

To ensure viral safety of a biological product derived from tissue, body fluid etc. of human or animal origin, it is necessary to exclude any possibility of virus contamination from a raw material, such as tissue and body fluid, or a substrate, paying attention to the source of virus contamination as mentioned in above 3.5, and to adopt appropriate manufacturing conditions and technologies in addition to enhancement of manufacturing environment, so that virus contamination in the course of process and handling and from operators, facilities and environment can be minimized.

In addition to the above, effective virus test and viral inactivation/removal technology, which are reflected by rapid progress of science, have to be introduced. Adoption of two or more steps with different principles is recommended for virus inactivation/removal process. Further, it is important to minimize any possible virus derivation by using a reagent, which quality is equivalent to that of a drug. Examples of virus inactivation/removal processes are ① heating (It is reported that almost viruses are inactivated by heating at 55 – 60°C for 30 minutes with exceptions of hepatitis virus, etc. and that dry heating at 60°C for 10 – 24 hours is effective in case of the products of blood or urine origin.), ② treatment with organic solvent/surfactant (S/D treatment), ③ membrane filtration (15 – 50 nm), ④ acid treatment, ⑤ irradiation (γ -irradiation, etc.), ⑥ treatment with column chromatograph (e.g. affinity chromatography, ion-exchange chromatography), ⑦ fractionation (e.g. organic solvent or ammonium sulfate fractionation), ⑧ extraction.

5.1. Virus test conducted in advance of purification process

(1) Biological products derived from human

In many cases, samples for virus test before purification

Table 2 Infectious viruses known to be common between human and animal and known to cause infection to each animal

	bovine	swine	sheep	goat	equine
Cowpox virus	⊙				
Paravaccinia virus	⊙	⊙	⊙	⊙	
Murray valley encephalitis virus	⊙	⊙			
Louping ill virus	⊙	⊙	⊙	⊙	
Wesselsbron virus			⊙		
Foot-and-mouth disease virus	⊙	⊙			
Japanese encephalitis virus		⊙			
Vesicular stomatitis virus		⊙			
Bovine papular stomatitis virus	⊙				
Orf virus			⊙		
Borna disease virus			⊙		⊙
Rabies virus	⊙	⊙	⊙	⊙	⊙
Influenza virus		⊙			
Hepatitis E virus		⊙			
Encephalomyocarditis virus	⊙	⊙			
Rotavirus	⊙				
Eastern equine encephalitis virus					⊙
Western equine encephalitis virus					⊙
Venezuelan equine encephalitis virus					⊙
Morbillivirus					⊙
Hendra virus					⊙
Nipah virus		⊙			
Transmissible gastroenteritis virus		⊙			
Porcine respiratory coronavirus		⊙			
Porcine epidemic diarrhea virus		⊙			
Hemagglutinating encephalomyelitis virus		⊙			
Porcine respiratory and reproductive syndrome virus		⊙			
Hog cholera virus		⊙			

Parainfluenza virus Type 3		⊙			
Talfan/Teschen disease virus		⊙			
Reovirus		⊙			
Endogenous retrovirus		⊙			
Porcine adenovirus		⊙			
Porcine circovirus		⊙			
Porcine parvovirus		⊙			
Porcine poxvirus		⊙			
Porcine cytomegalovirus		⊙			
Pseudorabies virus		⊙			
Russian spring-summer encephalitis virus			⊙	⊙	
Rift Valley fever virus			⊙	⊙	
Crimean-Congo hemorrhagic fever virus (Nairovirus)	⊙		⊙	⊙	
Torovirus	⊙				

process are body fluid or tissue of individual collected as a raw material, or its pooled material or extraction as a substrate. As mentioned in 4.2 (1), it is necessary to deny latent HBV, HCV and HIV by the test evaluated enough in aspects of specificity, sensitivity and accuracy. Even in a case that a non-purified bulk before purification process is produced from a substrate, it is not always necessary to conduct virus test again at the stage before purification, so long as the presence of any latent virus can be denied at the stage of substrate by an appropriate virus test, with cases where the non-purified bulk is made from the substrate by adding any reagent etc. of living organisms origin are an exception.

(2) Biological products derived from animal besides human

Similar to (1) above, samples for virus test before purification process are, in many cases, body fluid or tissue of individual collected as a raw material, or its pooled material or extraction as a substrate. In these cases, it is necessary to have a data, which can deny latent virus of probable cause of human infection or disease as mentioned in the above 4.2 (2), or to have a result of serologic test or nucleic amplification test (NAT) evaluated enough in aspects of specificity, sensitivity and accuracy. The concept, which is applied to a case that non-purified bulk before purification process is produced from substrate, is the same as those provided in the above 4.2 (1).

(3) Protein drug derived from cell line of human or animal origin

Generally, substrate in this case is cell bank, and the sample for testing before purification process is a harvested cell after cell culturing or unprocessed bulk which consists of single or pooled complex culture broth. The unprocessed bulk may be sometimes culture broth without cell. Denial of latent virus, which is determined by virus test at a MCB or WCB level, does not always deny latent virus in unprocessed

bulk after culturing. Further, it is noted that the viral test at the CAL is meaningful as a validation but can not guarantee definite assurance of latent virus denial, since the test is generally performed only once. In case of using a serum or a component of blood origin in a culture medium, definite denial of latent virus at the level of unprocessed bulk can not be assured so long as the viral test has not been conducted on each lot at the CAL, since lot renewal can be a variable factor on viral contamination.

A representative sample of the unprocessed bulk, removed from the production reactor prior to further processing, represents one of the most suitable levels at which the possibility of adventitious virus contamination can be determined with a high probability of detection. Appropriate testing for viruses should be performed at the unprocessed bulk level unless virus testing is made more sensitive by initial partial processing (e.g., unprocessed bulk may be toxic in test cell cultures, whereas partially processed bulk may not be toxic). In certain instances it may be more appropriate to test a mixture consisting of both intact and disrupted cells and their cell culture supernatants removed from the production reactor prior to further processing.

In case of unprocessed bulk, it is required to conduct virus test on at least 3 lots obtained from pilot scale or commercial scale production. It is recommended that manufacturers develop programs for the ongoing assessment of adventitious viruses in production batches. The scope, extent and frequency of virus testing on the unprocessed bulk should be determined by taking several points into consideration including the nature of the cell lines used to produce the desired products, the results and extent of virus tests performed during the qualification of the cell lines, the cultivation method, raw material sources and results of viral clearance studies. Screening *in vitro* tests, using one or several cell lines, are generally employed to test unprocessed bulk. If appropriate, a NAT test or other suitable methods may be used.

Generally, harvest material in which adventitious virus has been detected should not be used to manufacture the product. If any adventitious viruses are detected at this level, the process should be carefully checked to determine the cause of the contamination, and appropriate actions taken.

5.2. Virus test as an acceptance test of an intermediate material, etc.

When a biological product is manufactured from tissue, body fluid etc. of human or animal origin, there are cases that an intermediate material, partially processed as a raw material or substrate by outside manufacturer, is purchased and used for manufacturing. In such case, if any test to meet this General Information has been conducted by such outside manufacturer, it is necessary for the manufacturer of the biological product, who purchased the intermediate material, to examine what sort of virus test has to be conducted as acceptance tests, and to keep record on the basis of rationality including the details of the test conducted.

On the other hand, if no test to meet this General Information has been conducted by such outside manufacturer of the raw material, all necessary virus free test has to be conducted to meet this General Information on the intermediate material regarding it as the direct substrate for drug production.

5.3. Virus test on a final product

Virus tests to be conducted on a final product (or on a

product to reach the final product) has to be defined under comprehensive consideration of the type of raw material or substrate, the result of virus test conducted on raw material/substrate, the result of evaluation on viral removal/inactivation process, any possibility of virus contamination in the manufacturing process, etc. Comprehensive viral safety assurance can only be achieved by appropriate selection of the raw material/substrate, an appropriate virus test conducted on the raw material/substrate/intermediate material, the virus test conducted at an appropriate stage of manufacturing, an appropriate viral clearance test, etc. However, there are cases of having specific backgrounds, such as 1) use of the raw material derived from unspecified individual human, 2) possible existence of virus at window period, 3) specific detection limit of virus test, etc. and in these cases, virus contamination to the final product may occur if there is any deficiency on the manufacturing process (e.g., damage of membrane filter) or any mix-up of the raw materials, etc. To avoid such accidental virus contamination, it may be recommended to conduct nucleic amplification test (NAT) on the final product focusing on the most risky virus among those that may possibly to exist in the raw material.

6. Process evaluation on viral clearance

6.1. Rationale, objective and general items to be concerned with respect to viral clearance process evaluation

Evaluation of a viral inactivation/removal process is important for ensuring safety of a biological product derived from tissue or body fluid of human or animal origin. Conducting evaluation on viral clearance is to assure, even to some extent, elimination of the virus, which may exist in a raw material, etc. or may be derived to the process due to unexpected situation. Viral clearance studies should be made by a carefully designed appropriate method, and has to be rationally evaluated.

The objective of viral clearance studies is to assess process step(s) that can be considered to be effective in inactivating/removing viruses and to estimate quantitatively the overall level of virus reduction obtained by the process. This should be achieved by the deliberate addition ("spiking") of significant amounts of a virus at different manufacturing/purification steps and demonstrating its removal or inactivation during the subsequent steps. It is not necessary to evaluate or characterize every step of a manufacturing process if adequate clearance is demonstrated by the use of fewer steps. It should be borne in mind that other steps in the process may have an indirect effect on the viral inactivation/removal achieved. Manufacturers should explain and justify the approach used in studies for evaluating viral clearance.

The reduction of virus infectivity may be achieved by removal of virus particles or by inactivation of viral infectivity. For each production step assessed, the possible mechanism of loss of viral infectivity should be described with regard to whether it is due to inactivation or removal. For inactivation steps, the study should be planned in such a way that samples are taken at different times and an inactivation curve constructed.

6.2. Selection of virus

To obtain broad range of information of viral inactivation/removal, it is desirable that a model virus used for viral clearance studies should be chosen from the viruses with broad range of characteristics in aspects of DNA/RNA, with or without envelope, particle size, significant resistance to

Table 3 Example of viruses which have been used for viral clearance studies

Virus	Family	Genus	Natural host	Genome	Env	Size (nm)	Shape	Resistance
Vesicular Stomatitis Virus	Rhabdo	Vesiculovirus	Equine Bovine	RNA	yes	70 × 150	Bullet	Low
Parainfluenza Virus	Paramyxo	Type 1,3 Respirovirus Type 2,4 Rubulavirus	Various	RNA	yes	100 – 200 +	Pleo-Spher	Low
MuLV	Retro	Type C oncovirus	Mouse	RNA	yes	80 – 110	Spherical	Low
Sindbis Virus	Toga	Alphavirus	Human	RNA	yes	60 – 70	Spherical	Low
BVDV	Flavi	Pestivirus	Bovine	RNA	yes	50 – 70	Pleo-Spher	Low
Pseudorabies Virus	Herpes	Varicellovirus	Swine	DNA	yes	120 – 200	Spherical	Med
Poliovirus Sabin Type 1	Picornia	Enterovirus	Human	RNA	no	25 – 30	Icosahedral	Med
Encephalomyocarditis Virus	Picornia	Cardiovirus	Mouse	RNA	no	25 – 30	Icosahedral	Med
Reovirus Type 3	Reo	Orthoreovirus	Various kind	RNA	no	60 – 80	Spherical	Med
SV 40	Papova	Polyomavirus	Monkey	DNA	no	40 – 50	Icosahedral	Very high
Parvovirus: canine, porcine	Parvo	Parvovirus	Canine Porcine	DNA	no	18 – 24	Icosahedral	Very high

physical/chemical treatment, etc. and it is necessary to combine about 3 model viruses to cover these characteristics.

At choice of a model virus, there are also the ways to choose a virus closely related to or having the same characteristics of the virus known to exist in the raw material. In such case, it is in principle recommendable to choose a virus which demonstrates a higher resistance to inactivation/removal treatment if two or more candidate viruses are available for choice. Further, a virus which can grow at a high titer is desirable for choice, although this may not always be possible. In addition to the above, choosing a virus, which will provide effective and reliable assay result at each step, is necessary, since sample condition to be tested at each step of a production process may influence the detection sensitivity. Consideration should also be given to health hazard which may pose to the personnel performing the clearance studies.

For the other items taken for consideration at choice of virus, the Notice, Iyakushin No. 329 can be used as a reference. Examples of the virus which have been used for viral clearance studies are shown in Table 3 which was derived from Iyakushin No. 329. However, the Notice, Iyakushin No. 329, is on viral safety of a product derived cell line of human or animal origin, and a more appropriate model virus has to be chosen taking into account the origin/raw material of biological products.

6.3. Design of viral clearance studies

The purpose of viral clearance studies is to quantitatively evaluate removal or inactivation capability of a process, in which a virus is intentionally spiked to a specific step of a manufacturing process.

Following are the precautions to be taken at planning viral clearance studies.

(1) Care should be taken in preparing the high-titer virus to avoid aggregation which may enhance physical removal and decrease inactivation thus distorting the correlation with

actual production.

(2) Virus detection method gives great influence to viral clearance factor. Accordingly, it is advisable to gain detection sensitivity of the methods available in advance, and use a method with a detection sensitivity as high as possible. Quantitative infectivity assays should have adequate sensitivity and reproducibility in each manufacturing process, and should be performed with sufficient replicates to ensure adequate statistical validity of the result. Quantitative assays not associated with infectivity may be used if justified. Appropriate virus controls should be included in all infectivity assays to ensure the sensitivity of the method. Also, the statistics of sampling virus when at low concentrations (for example, number of virus is 1-1000/L) should be considered.

(3) Viral clearance studies are performed in a miniature size system that simulates the actual production process of the biotechnological/biological product used by the manufacturer. It is inappropriate to introduce any virus into a production facility because of GMP constraints. Therefore, viral clearance studies should be conducted in a separate laboratory equipped for virological work and performed by staff with virological expertise in conjunction with production personnel involved in designing and preparing a scaled-down version of the purification process. The viral clearance studies should be performed under the basic concept of GLP.

(4) Each factor on a viral clearance study of a process, which is performed in miniature size, should reflect that of actual manufacturing as far as possible, and its rationality should be clarified. In case of chromatograph process, length of column bed, linear velocity, ratio of bed volume per velocity (in other words, contact time), buffer, type of column packing, pH, temperature, protein concentration, salt concentration and concentration of the objective product are all correspondent to those of the actual produc-

tion. Further, similarity of elution profile should be achieved. For the other process, similar concept should be applied. If there is any factor which can not reflect the actual production, its effect to the result should be examined.

(5) It is desirable that two or more inactivation/removal processes of different principles are selected and examined.

(6) As for the process which is expected to inactivate/remove virus, each step should be evaluated in aspect of clearance capability, and carefully determined if it is the stage of inactivation, removal or their combination for designing the test. Generally, in viral clearance test, a virus is spiked in each step which is the object of the test, and after passing through the process in question, the reduction level of infectivity is evaluated. But, in some case, it is accepted that a high potential virus is spiked at a step of the process, and virus concentration of each succeeding step is carefully monitored. When removal of virus is made by separation or fractionation, it is desirable to investigate how the virus is separated or fractionated (mass balance).

(7) For assessment of viral inactivation, unprocessed crude material or intermediate material should be spiked with infectious virus and the reduction factor calculated. It should be recognized that virus inactivation is not a simple, first order reaction and is usually more complex, with a fast "phase 1" and a slow "phase 2". The study should, therefore, be planned in such a way that samples are taken at different times and an inactivation curve constructed. It is recommended that studies for inactivation include at least one time point less than the minimum exposure time and greater than zero, in addition to the minimum exposure time. The reproducible clearance should be demonstrated in at least two independent studies. When there is a possibility that the virus is a human pathogen, it is very important that the effective inactivation process is designed and additional data are obtained. The initial virus load should be determined from the virus which can be detected in the spiked starting material. If this is not possible, the initial virus load may be calculated from the titer of the spiking virus preparation. Where inactivation is too rapid to plot an inactivation curve using process conditions, appropriate controls should be performed to demonstrate that infectivity is indeed lost by inactivation.

(8) If antibody against virus exists in an unprocessed material, caution should be taken at clearance studies, since it may affect the behavior of virus at viral removal or inactivation process.

(9) Virus spiked in unprocessed material should be sufficient enough to evaluate viral removal or inactivation capability of the process. However, the virus "spike" to be added to the unprocessed material should be as small as possible in comparison with the sample volume of the unprocessed material so as not to cause characteristic change of the material by addition of the virus nor to cause behavioral change of the protein in the material.

(10) It is desirable that the virus in the sample is subject for quantitative determination without applying ultracentrifuge, dialysis, storage, etc. as far as possible. However, there may be a case that any handling before quantitative test, such as remove procedure of inhibitor or toxic substance, storage for a period to realize test at a time, etc., is inevitable. If any manipulation, such as dilution, concentration, filtration, dialysis, storage, etc., is applied for preparation of the sample for testing, a parallel control test, which

passes through a similar manipulation, should be conducted to assess infectivity variance at the manipulation.

(11) Buffers and product (desired protein or other component contained therein) should be evaluated independently for toxicity or interference in assays used to determine the virus titer, as these components may adversely affect the indicator cells. If the solutions are toxic to the indicator cells, dilution, adjustment of the pH, or dialysis of the buffer containing spiked virus might be necessary. If the product itself has anti-viral activity, the clearance study may need to be performed without the product in a "mock" run, although omitting the product or substituting a similar protein that does not have anti-viral activity could affect the behaviour of the virus in some production steps.

(12) Many purification schemes use the same or similar buffers or columns, repetitively. The effects of this approach should be taken into account when analyzing the data. The effectiveness of virus elimination by a particular process may vary with the stage in manufacture at which it is used.

(13) Overall reduction factors may be underestimated where production conditions or buffers are too cytotoxic or virucidal and should be discussed on a case-by-case basis. Overall reduction factors may also be overestimated due to inherent limitations or inadequate design of viral clearance studies.

(14) It has to be noted that clearance capability of viral removal/inactivation process may vary depending upon the type of virus. The viral removal/inactivation process, which displays viral clearance by specific principle or mechanism, may be quite effective to the virus, which meets such mechanism of action, but not effective to the other type of viruses. For example, S/D treatment is generally effective to the virus with lipid membrane, but not effective to the non-enveloped virus. Further, some virus is resistant to the general heating process (55 – 60°C, 30 minutes). When clearance is expected for such virus, introduction of a further severe condition or process, which has different principle or mechanism, is necessary. Virus removal by membrane filtration, which is different from S/D or heat treatment in aspect of principle, is effective to a broad range of virus that can not pass through the membrane. Affinity chromatography process, which specifically absorbs the objective protein, can thoroughly wash out the materials other than the objective protein including virus etc. and is generally effective for viral removal. Separation/fractionation of a virus from an objective protein is sometimes very difficult, but there are not so rare that ion exchange chromatography, ethanol fractionation, etc. is effective for clearance of a virus which can not be sufficiently inactivated or removed by the other process.

(15) Effective clearance may be achieved by any of the following: multiple inactivation steps, multiple complementary separation steps, or combinations of inactivation and separation steps. Separation methods may be dependent on the extremely specific physico-chemical properties of a virus which influence its interaction with gel matrices and precipitation properties. However, despite these potential variables, effective removal can be obtained by a combination of complementary separation steps or combinations of inactivation and separation steps. Well designed separation steps, such as chromatographic procedures, filtration steps and extractions, can be also effective virus removal steps provided that they are performed under appropriately controlled conditions.

(16) An effective virus removal step should give reproducible reduction of virus load shown by at least two independent studies.

(17) Over time and after repeated use, the ability of chromatography columns and other devices used in the purification scheme to clear virus may vary. Some estimate of the stability of the viral clearance after several uses may provide support for repeated use of such columns.

(18) The Notice, Iyakushin No. 329, would be used as a reference when viral clearance studies on biological products are designed.

6.4. Interpretation of viral clearance studies

6.4.1. Evaluation on viral clearance factor

Viral clearance factor is a logarithm of reduction ratio of viral amount (infectious titer) between each step applied for viral clearance of a manufacturing process. Total viral clearance factor throughout the process is sum of the viral clearance factor of each step appropriately evaluated.

Whether each and total viral clearance factor obtained are acceptable or should not be evaluated in aspects of every virus that can be realistically anticipated to derive into the raw material or the manufacturing process, and its rationality should be recorded.

In case that existence of any viral particle is recognized in a substrate for drug production, e.g., a substrate of rodent origin for biodrug production, it is important not only to demonstrate removal or inactivation of such virus, but also to demonstrate that the purification process has enough capability over the required level to assure safety of the final product at an appropriate level. The virus amount removed or inactivated in a manufacturing process should be compared with the virus amount assumed to exist in the substrate etc. used for manufacturing drug, and for this purpose, it is necessary to obtain the virus amount in the raw materials/substrate, etc. Such figure can be obtained by measuring infectious titer or by the other method such as transmission electron microscope (TEM). For evaluation of overall process, a virus amount, far larger than that assumed to exist in the amount of the raw materials/substrate which is equivalent to single administration of the final product, has to be removed. It is quite rare that existence of virus can be assumed in a substrate for drug production, with the exception of the substrate of rodent origin, and such suspicious raw material/substrate should not be used for manufacturing drug with a special exceptional case that the drug in question is not available from the other process and is clinically indispensable, and that the information including infectious properties of the virus particle assumed to exist has been clarified.

6.4.2. Calculation of viral clearance index

Viral clearance factor, “*R*”, for viral removal/inactivation process can be calculated by the following formula.

$$R = \log[(V_1 \times T_1)/(V_2 \times T_2)]$$

In which

R: Logarithm of reduction ratio

*V*₁: Sample volume of the unprocessed material

*T*₁: Virus amount (titer) of the unprocessed material

*V*₂: Sample volume of the processed material

*T*₂: Virus amount (titer) of the processed material

At the calculation of viral clearance factor, it is recommendable to use the virus titer detected in the sample prepa-

ration of the unprocessed material after addition of virus, not the viral titer added to the sample preparation wherever possible. If this is not possible, loaded virus amount is calculated from virus titer of the solution used for spike.

6.4.3. Interpretation of results and items to be concerned at evaluation

At the interpretation and the evaluation of the data on effectiveness of viral inactivation/removal process, there are various factors to be comprehensively taken into account, such as ① appropriateness of the virus used for the test, ② design of the viral clearance studies, ③ virus reduction ratio shown in logarithm, ④ time dependence of inactivation, ⑤ factors/items which give influence to the inactivation/removal process, ⑥ sensitivity limit of virus assay method, ⑦ possible effect of the inactivation/removal process which is specific to certain class of viruses.

Additional items to be concerned at appropriate interpretation and evaluation of the viral clearance data are as follows:

(1) Behavior of virus used to the test

At interpretation of the viral clearance results, it is necessary to recognize that clearance mechanism may differ depending upon the virus used for the test. Virus used for a test is generally produced in tissue culture, but behavior of the virus prepared in the tissue culture may be different from that of the native virus. Examples are possible differences of purity and degree of aggregation between the native and the cultured viruses. Further, change of surface properties of a virus, e.g., addition of a sucrose chain which is ascribed to specific nature of a separation process, may give effect to the separation. These matters should be also considered at interpretation of the results.

(2) Design of test

Viral clearance test should have been designed taking into account variation factors of the manufacturing process and scaling down, but there still remain some variance from actual production scale. It is necessary to consider such variance at the interpretation of the data and limitation of the test.

(3) Acceptability of viral reduction data

Total viral clearance factor is expressed as a sum of logarithm of reduction ratio obtained at each step. The summation of the reduction factor of multiple steps, particularly of steps with little reduction (e.g., below 1 log₁₀), may overestimate viral removal/inactivation capability of the overall process. Therefore, virus titer of the order of 1 log₁₀ or less has to be ignored unless justified. Further, viral clearance factor achieved by repeated use of the same or similar method should be ignored for calculation unless justified.

(4) Time dependence of inactivation

Inactivation of virus infectivity frequently shows biphasic curve, which consists of a rapid initial phase and subsequent slow phase. It is possible that a virus not inactivated in a step may be more resistant to the subsequent step. For example, if an inactivated virus forms coagulation, it may be resistant to any chemical treatment and heating.

(5) Evaluation of viral reduction ratio shown logarithm

Viral clearance factor shown in logarithm of reduction ratio of virus titer can demonstrate drastic reduction of residual infectious virus, but there is a limit that infectious titer can never be reduced to zero. For example, reduction in infectivity of a preparation containing 8 log₁₀ infectious unit per mL by a factor of 8 log₁₀ leaves zero log₁₀ per mL or one

infectious unit per mL, taking into account the detection limit of assay.

(6) Variable factor of manufacturing process

Minor variance of a variation factor of a manufacturing process, e.g., contact time of a spiked sample to a buffer or a column, will sometimes give influence to viral removal or inactivation effect. In such case, it may be necessary to investigate to what extent such variance of the factor has given influence to the process concerned in aspect of viral inactivation.

(7) Existence of anti-viral antiserum

Anti-viral antiserum that exists in the sample preparation used for a test may affect sensitivity of distribution or inactivation of a virus, which may result in not only defusing the virus titer but complicating interpretation of the test result. So, existence of anti-viral antiserum is one of the important variable factors.

(8) Introduction of a new process for removal/inactivation

Viral clearance is an important factor for securing safety of drug. In case that an achievement level of infective clearance of a process is considered insufficient, a process which is characterized by inactivation/removal mechanism to meet the purpose or an inactivation/removal process which can mutually complement to the existence process has to be introduced.

(9) Limit of viral clearance studies

Viral clearance studies are useful for contributing to the assurance that an acceptable level of safety in the final product is achieved but do not by themselves establish safety. However, a number of factors in the design and execution of viral clearance studies may lead to an incorrect estimate of the ability of the process to remove virus infectivity, as described above.

7. Statistics

The viral clearance studies should include the use of statistical analysis of the data to evaluate the results. The study results should be statistically valid to support the conclusions reached.

7.1. Statistical considerations for assessing virus assays

Virus titrations suffer the problems of variation common to all biological assay systems. Assessment of the accuracy of the virus titrations and reduction factors derived from them and the validity of the assays should be performed to define the reliability of a study. The objective of statistical evaluation is to establish that the study has been carried out to an acceptable level of virological competence.

Assay

1. Assay methods may be either semiquantitative or quantitative. Both semiquantitative and quantitative assays are amenable to statistical evaluation.

2. Variation can arise within an assay as a result of dilution errors, statistical effects and differences within the assay system which are either unknown or difficult to control. These effects are likely to be greater when different assay runs are compared (between-assay variation) than when results within a single assay run are compared (within-assay variation).

3. The 95% confidence limits for results of within-assay variation normally should be on the order of $\pm 0.5 \log_{10}$ of the mean. Within-assay variation can be assessed by standard textbook methods. Between-assay variation can be monitored by the inclusion of a reference preparation, the esti-

mate of whose potency should be within approximately 0.5 \log_{10} of the mean estimate established in the laboratory for the assay to be acceptable. Assays with lower precision may be acceptable with appropriate justification.

7.2. Reproducibility and confidence limit of viral clearance studies

An effective virus inactivation/removal step should give reproducible reduction of virus load shown by at least two independent studies. The 95% confidence limits for the reduction factor observed should be calculated wherever possible in studies of viral clearance. If the 95% confidence limits for the viral assays of the starting material are $\pm s$, and for the viral assays of the material after the step are $\pm a$, the 95% confidence limits for the reduction factor are $\pm \sqrt{s^2 + a^2}$.

8. Re-evaluation of viral clearance

Whenever significant changes in the production or purification process are made, the effect of that change, both direct and indirect, on viral clearance should be re-evaluated as needed. Changes in process steps may also change the extent of viral clearance.

9. Measurement for viral clearance studies

9.1. Measurement of virus infective titer

Assay methods may be either semiquantitative or quantitative. Semiquantitative methods include infectivity assays in animals or in cultured cell infections dose (CCID) assays, in which the animal or cell culture is scored as either infected or not. Infectivity titers are then measured by the proportion of animals or culture infected. In quantitative methods, the infectivity measured varies continuously with the virus input. Quantitative methods include plaque assays where each plaque counted corresponds to a single infectious unit. Both quantal and quantitative assays are amenable to statistical evaluation.

9.2. Testing by nucleic-acid-amplification test (NAT)

NAT can detect individual or pooled raw material/cell substrate or virus genome at a high sensitivity even in a stage that serum test on each virus is negative. Further, it can detect HBV or HCV gene, which can not be measured in culture system. Window period can be drastically shortened at the test on HBV, HCV and HIV, and the method is expected to contribute as an effective measure for ensuring viral safety. However, depending upon a choice of primer, there may be a case that not all the subtype of objective virus can be detected by this method, and, therefore, it is recommendable to evaluate, in advance, if subtype of a broad range can be detected.

NAT will be an effective evaluation method for virus removal capability for viral clearance. However, in case of viral inactivation process, viral inactivation obtained by this method may be underrated, since there is a case that inactivated virus still shows positive on nucleic acid. Further, at introduction of NAT, cautions should be taken on rationality of detection sensitivity, choice of a standard which is used as run-control, quality assurance and maintenance of a reagent used for primer, interpretation of positive and negative results, etc.

10. Reporting and preservation

All the items relating to virus test and viral clearance studies should be reported and preserved.

11. Others

The Notice, Iyakushin No. 329, should be used as a reference at virus test and viral clearance studies.

Conclusion

As mentioned at the Introduction, assurance of quality/safety etc. of JP listed drugs should be achieved by state-of-the-art methods and concepts reflecting the progress of science and accumulation of experiences.

The basis for ensuring viral safety of JP listed biotechnological/biological products is detailed in this General Information. What is discussed here is that an almost equal level of measures are required for both development of new drugs and for existing products as well, which means that similar level of concerns should be paid on both existing and new products in aspect of viral safety. This document is intended to introduce a basic concept that quality and safety assurance of JP listed product should be based upon the most advanced methods and concepts. This document has been written to cover all conceivable cases, which can be applied to all biotechnological/biological products. Therefore, there may be cases that it is not so rational to pursue virus tests and viral clearance studies in accordance with this document on each product, which has been used for a long time without any safety issue. So, it will be necessary to elaborate the most rational ways under a case-by-case principle taking into due consideration source, origin, type, manufacturing process, characteristics, usages at clinical stage, accumulation of the past usage record, etc. relating to such biotechnological/biological products.

Capillary Electrophoresis

This test is harmonized with the European Pharmacopoeia and the U.S. Pharmacopoeia.

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 electroosmotic 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 electroosmotic flow. The velocity of the electroosmotic flow depends on the electroosmotic mobility

(μ_{eo}) which in turn depends on the charge density on the capillary internal wall and the buffer characteristics. The electroosmotic 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 electroosmotic 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 electroosmotic flow and their velocities will be smaller than the electroosmotic velocity. Cations will migrate in the same direction as the electroosmotic flow and their velocities will be greater than the electroosmotic velocity. Under conditions in which there is a fast electroosmotic 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 electroosmotic flow is from anode to cathode. The electroosmotic 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 electroosmotic 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 electroosmotic mobility induced

in the capillary and by increasing the efficiency for the band of each analyte, according to the equation:

$$R_s = \frac{\sqrt{N}(\mu_{\text{epb}} - \mu_{\text{epa}})}{4(\bar{\mu}_{\text{ep}} + \mu_{\text{eo}})}$$

μ_{epa} and μ_{epb} : electrophoretic mobilities of the two analytes separated,

$\bar{\mu}_{\text{ep}}$: mean electrophoretic mobility of the two analytes

$$\bar{\mu}_{\text{ep}} = \frac{1}{2}(\mu_{\text{epb}} + \mu_{\text{epa}}).$$

Apparatus

An apparatus for capillary electrophoresis is composed of:

- (1) a high-voltage, controllable direct-current power supply,
- (2) two buffer reservoirs, held at the same level, containing the prescribed anodic and cathodic solutions,
- (3) two electrode assemblies (the cathode and the anode), immersed in the buffer reservoirs and connected to the power supply,
- (4) 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,
- (5) a suitable injection system,
- (6) 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,
- (7) a thermostatic system able to maintain a constant temperature inside the capillary is recommended to obtain a good separation reproducibility,
- (8) 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

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

electroosmotic flow in the capillary (see General Principles). Coated capillaries can be used to increase the separation capacity of those substances adsorbing on fused-silica surfaces.

Using this mode of capillary electrophoresis, the analysis of both small ($M_r < 2000$) and large molecules ($2000 < M_r < 100,000$) can be accomplished. Due to the high efficiency achieved in capillary zone electrophoresis, separation of molecules having only minute differences in their charge-to-mass ratio can be effected. This separation mode also allows the separation of chiral compounds by addition of chiral selectors to the separation buffer.

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

(1) *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.

(2) *Polarity*: Electrode polarity can be normal (anode at the inlet and cathode at the outlet) and the electroosmotic flow will move toward the cathode. If the electrode polarity is reversed, the electroosmotic flow is away from the outlet and only charged analytes with electroosmotic mobilities greater than the electroosmotic flow will pass to the outlet.

(3) *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.

(4) *Capillary*: The dimensions of the capillary (length and internal diameter) contribute to analysis time, efficiency of separations and load capacity. Increasing total length can decrease the electric fields (working at constant voltage), and increasing both effective length and total length increase 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

(1) *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 electroosmotic flow and solute velocity.

(2) *Buffer pH*: The pH of the buffer can affect separation by modifying the charge of the analyte or additives, and by changing the electroosmotic 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 electroosmotic flow.

(3) *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 electroosmotic flow.

(4) *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

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 capillary zone electrophoresis section) and controlling the gel porosity during the gel preparation. For cross-linked polyacrylamide gels, the porosity can be modified by changing the concentration of acrylamide and/or the proportion of cross-linker. As a rule, a decrease in the porosity of the gel leads to a decrease in the mobility of the solutes. Due to the rigidity of these gels, only electrokinetic injection can be used.

Dynamically coated gels are hydrophilic polymers, such as linear polyacrylamide, cellulose derivatives, dextran, etc., which can be dissolved in aqueous separation buffers giving rise to a separation medium that also acts as a molecular sieve. These separation media are easier to prepare than cross-linked polymers. They can be prepared in a vial and filled by pressure in a wall-coated capillary (with no electroosmotic 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

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.

(1) Loading step: Two methods may be employed:

- (i) loading in one step: the sample is mixed with ampholytes and introduced into the capillary either by pressure or vacuum;
- (ii) 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.

(2) 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.

(3) Mobilization step: If mobilization is required for detection, use one of the following methods. Three methods are available:

- (i) in the first method, mobilization is accomplished

during the focusing step under the effect of the electroosmotic flow; the electroosmotic flow must be small enough to allow the focusing of the components;

- (ii) in the second method, mobilization is accomplished by applying positive pressure after the focusing step;
- (iii) 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 (dpH/dx), the number of ampholytes having different pI values, the molecular diffusion coefficient (D), the intensity of the electric field (E) and the variation of the electrophoretic mobility of the analyte with the pH ($-d\mu/dpH$):

$$\Delta pI = 3 \sqrt{\frac{D(dpH/dx)}{E(-d\mu/dpH)}}$$

Optimization

The main parameters to be considered in the development of separations are:

- (1) Voltage: Capillary isoelectric focusing utilises very high electric fields, 300 V/cm to 1000 V/cm in the focusing step.
- (2) Capillary: The electroosmotic flow must be reduced or suppressed depending on the mobilization strategy (see above). Coated capillaries tend to reduce the electroosmotic flow.
- (3) 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 electroosmotic 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)

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 tech-

nique 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 electroosmotic 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 electroosmotic 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 electroosmotic 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

(1) *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.

(2) *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.

(3) *Capillary*: As in capillary zone electrophoresis, the dimensions of the capillary (length and internal diameter) contribute to analysis time and efficiency of separations. Increasing both effective length and total length can decrease the electric fields (working at constant voltage), increase migration time and improve the separation efficiency. The internal diameter controls heat dissipation (for a given buffer and electric field) and consequently the sample band broadening.

Electrolytic solution parameters

(1) *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.

(2) *Buffer pH*: Although pH does not modify the partition coefficient of non-ionized solutes, it can modify the electroosmotic flow in uncoated capillaries. A decrease in the buffer pH decreases the electroosmotic flow and therefore increases the resolution of the neutral solutes in MEKC, resulting in a longer analysis time.

(3) *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.

(4) *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.

(5) *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:

- (1) compensate for the shift in migration time from run to run, thus reducing the variation of the response,
- (2) 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 (R_S) 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.

Isoelectric Focusing

This test is harmonized with the European Pharmacopoeia and the U.S. Pharmacopeia. The parts of the text that are not harmonized are marked with symbols (♦ ♦).

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 R is estimated by determining the minimum pI difference (ΔpI), which is necessary to separate 2 neighboring bands:

$$R: \Delta pI = 3 \sqrt{\frac{D(dpH/dx)}{E(-d\mu/dpH)}}$$

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.

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

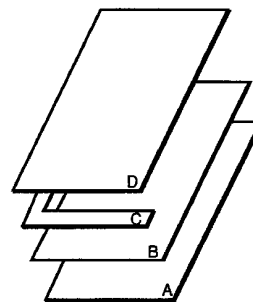


Figure. Mould

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:

- (1) the use of commercially available pre-cast gels and of commercial staining and destaining kits,
- (2) the use of immobilized pH gradients,
- (3) the use of rod gels,
- (4) the use of gel cassettes of different dimensions, including ultra-thin (0.2 mm) gels,
- (5) variations in the sample application procedure, including different sample volumes or the use of sample application masks or wicks other than paper,
- (6) 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,
- (7) the inclusion of a pre-focusing step,
 - (8) the use of automated instrumentation,
 - (9) 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:

- (1) formation of a stable pH gradient of desired characteristics, assessed for example using colored pH markers of known isoelectric points,
- (2) comparison with the electropherogram provided with the chemical reference substance for the preparation to be examined,
- (3) 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:

- (1) 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,
- (2) the use of alternative staining methods,
- (3) the use of gel additives such as non-ionic detergents (e.g. octylglucoside) or zwitterionic detergents (e.g., 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) or 3-[(3-Cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate (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, electroendosmosis 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).♦

Mass Spectrometry of Peptides and Proteins

Mass spectrometry (MS) is based on the ionization of molecules and separation of the electrically charged ions according to the m/z . The results are expressed as a mass spectrum with m/z values of the ions on the x-axis and signal intensity of the ions on the y-axis. The mass of the molecule calculated from the m/z values is expressed in unified atomic mass units (u) or daltons (Da). Tandem mass spectrometry (MS/MS) is based on the selection of the precursor ion in the first stage analyzer, fragmentation of the precursor ion and measurement of the product ions in the second stage mass analyzer. This technique provides useful information for structural analysis of the molecule. Information obtained in MS is qualitative and is sometimes used for qualification. MS and MS/MS are useful for measuring masses of peptides and proteins and for confirming amino acid sequences and post-translational modifications. Both methods are therefore used for identification of pharmaceutical peptides and proteins.

1. Instrument

A mass spectrometer is composed of an ion source, an analyzer, an ion detector, and a data system (Figure 1). A peptide or protein sample introduced into the ion source is ionized by soft-ionization methods, such as matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI). The charged and gas-phased ions are sorted according to the m/z under a vacuum in the analyzer, which may be a quadrupole, time-of-flight, ion trap or Fourier transform ion cyclotron resonance analyzer. The ion flux collected in the detector is converted to an electric signal. Then the signal is recorded as a mass spectrum. MS/MS is carried out by using two mass spectrometers connected in series, an ion-trap mass spectrometer and Fourier transform ion cyclotron resonance mass spectrometer. The precursor ions are generally fragmented by collision-induced dissociation, post-source decay, electron capture dissociation, etc.

2. Analytical mode

2.1. MS

There are two useful modes for MS:

(1) Full scan mode

The signals of the entire ion are acquired over the chosen range of m/z . This mode provides information on the masses of the molecule of interest and different species.

(2) Selected ion monitoring

The signals of the ion at chosen m/z are acquired. This mode is useful for the sensitive measurement of the chosen molecule.

2.2. MS/MS

There are four essential modes for MS/MS:

(1) Product ion analysis

The signals of all the product ions produced from the precursor at chosen m/z are acquired. This mode provides structural information on the analytes and various co-existing species.

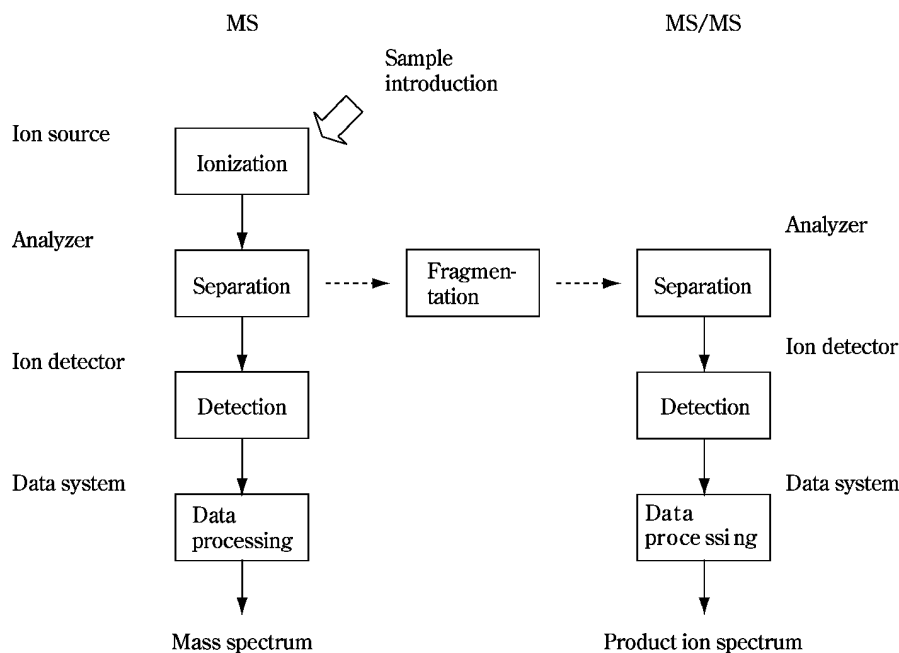


Fig. 1 Concept of MS and MS/MS

(2) Precursor ion scan mode

The precursor that yields the product ion at chosen m/z are monitored. This mode is used for sorting the molecules containing a component of interest.

(3) Constant neutral loss scan mode

The precursor that loses the fragment at chosen m/z are monitored. This mode is useful to sort the molecules containing a component of interest.

(4) Selected reaction monitoring

The product ions at chosen m/z that are produced from the precursor at chosen m/z are monitored. This mode allows for sensitive and selective measurement and is used for quantification of a molecule in a complex mixture.

3. Analytical procedure

3.1. MS

System suitability is tested by measuring the mass of a test sample specified in the monograph. The system performance should be confirmed based on the difference between the calculated mass and observed mass of the test sample. If the detectability and system performance do not meet the criteria, the system should be optimized by adjustment of the ion source, analyzer and detector, as well as by calibration using appropriate molecules. MS is performed according to the sample preparation and operating conditions indicated in the monograph. The general procedure is described as follows.

(1) Matrix-assisted laser desorption/ionization (MALDI)

A desalted peptide or protein sample is dissolved in an appropriate solvent, e.g., an aqueous solution of trifluoroacetic acid. A suitable matrix, such as α -cyano-4-hydroxycinnamic acid, 2,5-dihydroxybenzoic acid, or sinapic acid, is dissolved in an aqueous solution containing acetonitrile and trifluoroacetic acid. A mixture of sample solution and matrix solution is deposited on a sample plate and dried. The sample on the plate is set in the ion source, and ionized by a laser beam at suitable intensity.

(2) Electrospray ionization (ESI)

A desalted peptide or protein sample is dissolved in a suitable solvent, such as an aqueous solution containing acetic acid and methanol or acetonitrile. The sample solution is sprayed through a capillary and held at a potential of several kilovolts. The sample is introduced by using a syringe or HPLC.

3.2. MS/MS

System suitability is tested by MS/MS of the test sample specified in the monograph. The detectability and system performance should be confirmed based on the detection of the product ions specified in the monograph. The sample is ionized in the same way as for MS, and the chosen precursor is fragmented by the suitable conditions specified in the monograph. The signals are recorded as a mass spectrum. A peptide containing disulfide bonds is generally reduced by dithiothreitol, 2-mercaptoethanol and tris (2-carboxyethyl) phosphine. The reduced peptides are derivatized with moniodoacetic acid, iodoacetamide, and 4-biarylpyridine.

4. Identification test

4.1. Mass of the molecule

The monoisotopic mass of the peptide is preferably acquired. If the monoisotopic peak is not detectable, the average mass is calculated using the weighed average of isotopic masses. Deconvolution is effective for calculating the average mass of multiply-charged proteins. The mass should meet the criteria specified in the monograph.

4.2. Amino acid sequence

After measuring the mass of the sample peptide, the presence of the specified product ions that arise from the selected precursor is confirmed according to the conditions indicated in the monograph. Digestion of sample proteins with a suitable enzyme followed by MS/MS is sometimes effective for sequencing of the high-molecular weight proteins which provide insufficient product ions. Details of the diges-

tion procedure are provided in the section on peptide mapping.

5. Glossary

Ion-trap (IT)

Ion-trap refers to the quadrupole ion trap mass analyzer in a restricted sense. Ions stored in the analyzer by applying radio frequency voltage to ring electrodes are separated by subsequent ejection of the ions from the analyzer by varying the voltage on the ring electrodes. This allows multiple stage MS in which a selected ion is repeatedly trapped, fragmented and ejected.

Electrospray ionization (ESI)

The sample in solution is sprayed through a capillary and held at high-voltage at atmospheric pressure. The sample is ionized by a formation of charged liquid droplets. High-molecular weight proteins are detected as multiply-charged ions. The analyzer can be connected with HPLC.

Quadrupole (Q)

The analyzer is composed of four parallel electrodes which have a hyperboloidal or cylindrical cross-section. The ions transmitted to the analyzer are separated by varying the potential of direct and radio frequency components applied to the rods so that the filter for sorting the m/z values of ions is changed.

Collision-induced dissociation (CID)

When an ion collides with a neutral atom or molecule (He, Ar, N_2 and so on), some of the translational energy of the collision is converted into internal energy, thereby causing dissociation. The terms low-energy CID and high-energy CID refer to those CIDs for which the translational energy of the precursor ions is lower than 1000 eV and higher than 1000 eV, respectively.

Electron capture dissociation (ECD)

Multiply-charged positive ions interact with low energy electrons producing charge-reduced radical ions, which readily dissociate. This method is primarily used for MS/MS in FT-ICR MS or IT MS.

Time-of-flight (TOF)

The ionized sample is accelerated at high-voltage and separated based on the time required for an ion to travel to the detector. There are two types of analyzer, a linear type in which ions travel linearly from the ion source to the detector, and a reflectron type where ions are inverted by a reflectron. The latter type allows high-resolution measurement by correction of the variation in the initial energy of ions.

Fourier transform ion cyclotron resonance (FT-ICR)

The analyzer is based on the principle that the cyclotron frequency of the ions in a magnetic field is inversely proportional to its m/z value. Ions are excited to a larger radius orbit using radio frequency energy and their image current is detected on receiver plates. The resulting data are devolved by applying a Fourier transform to give a mass spectrum.

Post-source decay (PSD)

Metastable ion decay occurs by excess internal energy and collision with residual gas during ion acceleration out of the MALDI ion source and prior to reaching the detector. This method is used for MS/MS by using MALDI-TOF MS with a reflectron mode.

Matrix-assisted laser desorption/ionization (MALDI)

The sample, which is mixed with a suitable matrix and deposited on a target plate, is ionized by irradiation with nanosecond laser pulses. Proteins, carbohydrates, oligonucleotides, and lipids can be ionized without any dissociation. Singly-charged ions are mainly detected.

Mycoplasma Testing for Cell Substrates used for the Production of Biotechnological/Biological Products

This document describes the currently available methods of mycoplasma testing that should be performed for cell substrates that are used in the manufacture of biotechnological/biological products.

Methods suggested for detection of mycoplasma are, A. culture method, B. indicator cell culture method, and C. polymerase chain reaction (PCR) method.

Mycoplasma testing should be performed on the master cell bank (MCB) and the working cell bank (WCB), as well as on the cell cultures used during the manufacturing process of the product. For the assessment of these cells, mycoplasma testing should be performed using both methods A and B. Method B, however, does not detect only DNA derived from mycoplasma. Therefore, if a positive result is obtained only from method B, method C can be used to determine whether mycoplasma is actually present. When method C is used, it is necessary to demonstrate the rationale for determining a negative result. In such a case, the sensitivity and specificity of the method, the appropriateness of the sample preparation, and the suitability of the selection of the test method, including selection of reagents, reaction conditions and primers should be taken into account.

Prior to mycoplasma testing, the sample should be tested to detect the presence of any factors inhibiting the growth of mycoplasma. If such growth-inhibiting factors are detected they should be neutralized or eliminated by an appropriate method, such as centrifugation or cell passage.

If the test will be performed within 24 hours of obtaining the sample, the sample should be stored at a temperature between 2°C and 8°C. If more than 24 hours will elapse before the test is performed, the sample should be stored at -60°C or lower.

If mycoplasma is detected, additional testing to identify the species may be helpful in determining the source of contamination.

A. Culture Method

1. Culture Medium

Both agar plates and broth are used. Each lot of agar and broth medium should be free of antibiotics except for penicillin. Refer to the Minimum Requirements for Biological Products regarding selection of the culture media. Other culture media may be used if they fulfill the requirements described in the following section 2.

2. Suitability of Culture Medium

Each lot of medium should be examined for mycoplasma growth-promoting properties. To demonstrate the capacity of the media to detect known mycoplasma, each test should

include control cultures of at least two known species or strains of mycoplasma, one of which should be a dextrose fermenter (i.e., *M. pneumoniae* ATCC15531 or equivalent species or strains) and one of which should be an arginine hydrolyser (i.e., *M. orale* ATCC23714 or equivalent species or strains). The mycoplasma strains used for the positive control tests should be those with a low number of passages obtained from an official or suitably accredited agency, and handled appropriately. Inoculate the culture medium with 100 colony-forming units (CFU) or 100 color-changing units (CCU) or less.

3. Culture and Observation

1) Inoculate no less than 0.2 mL of test sample (cell suspension) in evenly distributed amounts over the surface of each of two or more agar plates. After the surfaces of the inoculated plates are dried, the plates should be incubated under microaerophilic conditions in an atmosphere of nitrogen containing 5 to 10 percent carbon dioxide and adequate humidity at $36 \pm 1^\circ\text{C}$ for no less than 14 days.

2) Inoculate no less than 10 mL of the test sample (cell suspension) into each of one or more vessels containing 100 mL of broth medium, and incubate at $36 \pm 1^\circ\text{C}$.

If the culture medium for the sample cells contains any growth-inhibiting factors, such as antibiotics, these factors must be removed. A method such as centrifugation is recommended for this purpose. Refer to the Validation tests for growth-inhibiting factors described in the Minimum Requirements for Biological Products for the detection of growth-inhibiting factors.

3) Subculture 0.2 mL of broth culture from each vessel on the 3rd, 7th, and 14th days of incubation onto two or more agar plates. The plates should be inoculated microaerophilically at $36 \pm 1^\circ\text{C}$ for no less than 14 days.

4) Examination of all plates for mycoplasma colonies should be done microscopically on the 7th and 14th day at 100 times magnification or greater.

B. Indicator Cell Culture Method

Using Vero cell culture substrate, pretest the suitability of the method using an inoculum of 100 CFU or 100 CCU or less of *M. hyorhinis* (ATCC29052, ATCC17981 or equivalent species or strains) and *M. orale* (ATCC23714 or equivalent species or strains).

An equivalent indicator cell substrate and suitable mycoplasma strains may be acceptable if data demonstrate at least equal sensitivity for the detection of known mycoplasma contaminants. The mycoplasma strains should be those with a low number of passages obtained from an official or suitably accredited agency, and handled appropriately, and the unit of inoculation should be determined before use. The cell substrate used should be obtained from a qualified cell bank and certified to be mycoplasma free. The acquired cells should be carefully cultured and propagated, and sufficient volumes of seed stock should be prepared with the proper precautions to avoid mycoplasma contamination. The stock should be tested for mycoplasma contamination using at least one of the methods described in this document, then frozen for storage. For each test a new container from the stock should be thawed and used within 6 passages.

Indicator cell cultures should be grown on cover slips submerged in culture dishes or equivalent containers for one day. Inoculate no less than 1 mL of the test sample (cell culture supernatant) into two or more of the culture dishes.

The test should include a negative (non-infected) control and two positive mycoplasma controls, such as *M. hyorhinis* (ATCC29052, ATCC17981 or equivalent species or strains) and *M. orale* (ATCC23714 or equivalent species or strains). Use an inoculum of 100 CFU or 100 CCU or less for the positive controls.

Incubate the cell cultures for 3 to 6 days at $36 \pm 1^\circ\text{C}$ in an atmosphere of air containing 5 percent carbon dioxide.

Examine the cell cultures after fixation for the presence of mycoplasma by epifluorescence microscopy (400 to 600 times magnification or greater) using a DNA-binding fluorochrome, such as bisbenzimidazole or an equivalent stain. Compare the microscopical appearance of the test cultures with that of the negative and positive controls.

Procedure

1) Aseptically place a sterilized glass cover slip into each cell culture dish (35 mm diameter).

2) Prepare Vero cell suspension in Eagle's minimum essential medium containing 10 percent bovine calf serum at a concentration of 1×10^4 cells per 1 mL. The bovine calf serum should be tested and confirmed to be free from mycoplasma prior to use.

3) Inoculate aliquots of 2 mL of the Vero cell suspension into each culture dish. Ensure that the cover slips are completely submerged, and not floating on the surface of the culture medium. Incubate the cultures at $36 \pm 1^\circ\text{C}$ in an atmosphere of air containing 5 percent carbon dioxide for one day, so that the cells are attached to the glass cover slip.

4) Replace 2 mL of the culture medium with fresh medium, then add 0.5 mL of the test sample (cell culture supernatant) to each of two or more culture dishes. Perform the same procedure for the positive (2 types of mycoplasma, such as *M. hyorhinis* (ATCC29052, ATCC17981 or equivalent species or strains) and *M. orale* (ATCC23714 or equivalent species or strains) and negative controls.

5) Incubate the cultures for 3 to 6 days at $36 \pm 1^\circ\text{C}$ in an atmosphere of air containing 5 percent carbon dioxide.

6) Remove the culture medium from the culture dishes, and add 2 mL of a mixture of acetic acid (100) and methanol (1:3) (fixative) to each dish; then, allow them to stand for 5 minutes.

7) Remove the fixative from each dish, then add the same amount of fixative again, and leave the dishes to stand for 10 minutes.

8) Remove the fixative and then completely air-dry all the dishes.

9) Add 2 mL of bisbenzamide fluorochrome staining solution to each culture dish. Cover the dishes and let them stand at room temperature for 30 minutes.

10) Aspirate the staining solution and rinse each dish with 2 mL of distilled water 3 times. Take out the glass cover slips and dry them.

11) Mount each cover slip with a drop of a mounting fluid. Blot off surplus mounting fluid from the edges of the cover slips.

12) Examine by epifluorescence microscopy at 400 to 600 times magnification or greater.

13) Compare the microscopic appearance of the test sample with that of the negative and positive controls.

14) The test result is judged to be positive if there are more than 5 cells per 1000 (0.5%) that have minute fluorescent spots that appear to surround, but are outside, the cell nucleus.

C. Polymerase Chain Reaction (PCR) Detection Method

The PCR method is a highly specific method that enables the detection of trace amounts of mycoplasma DNA, and has come to be widely used in recent years as a means of detecting mycoplasma contamination. However, the sensitivity and specificity depend on the procedure employed, and a positive result from PCR does not always indicate the presence of viable mycoplasma.

The PCR method is based on amplifying DNA extracted from the cell culture with specific primers so that the presence of the target DNA is detected. A two-step PCR (nested PCR) is recommended in order to increase sensitivity and specificity. The tests should include both a positive control (such as *M. hyorhinis* (ATCC29052, ATCC17981 or equivalent species or strains) of 100 CFU or 100 CCU or less) and a negative control.

Mycoplasma DNA from the sample of cells or cell cultures is amplified using primers which should be able to amplify some common conserved mycoplasma DNA sequence. The amplification should be performed using an appropriate heat-resistant DNA polymerase, and suitable conditions. The amplified DNA can be identified after agarose gel electrophoresis, followed by ethidium bromide staining and UV irradiation of the gel.

For this method, it is important to use primers that are specific to mycoplasma by choosing base sequences that are well-conserved for a wide range of mycoplasma species, for example, the spacer region between the 16S-23S ribosome genes.

It is recommended that a two-step PCR using nested primers should be performed to increase the sensitivity and specificity, if the one-step PCR is negative.

The primers to be selected for the second stage of a two-step PCR are nested primers from the inner portion of the sequence. The outer and inner primers should have proven effectiveness and specificity as described in publications or be validated experimentally.

It is possible to increase the accuracy of the detection of mycoplasma DNA by performing PCR tests after cultivation of mycoplasma that may be present in samples using Vero cells.

The following is an example of a two-step PCR procedure. The reagents and reaction conditions in this example are not exclusive. If the suitability of other reagents and conditions is verified, they may be used. If another procedure is used, the procedure should be justified and documented in detail, and the information provided should include the sensitivity and specificity of the method.

Example Procedure**1. Preparation of template**

1) Place 600 μL of the test cell suspension (if necessary, subcultured with Vero cells) in a tube and dissolve the cells with 0.1% SDS or an equivalent. Add an equal volume (600 μL) of TE (10 mmol/L tris-hydrochloric acid (pH 8.0), 1 mmol/L EDTA) buffer-saturated phenol, and mix.

2) Centrifuge at $15,000 \text{ min}^{-1}$ for 5 minutes at room temperature.

3) Transfer 400 μL of the supernatant to another tube, and add 10 μL of 3 mol/L sodium acetate.

4) Add 1 mL (2.5 volumes) of ethanol (95) and stir thoroughly. Ice the mixture for 15 minutes, then centrifuge at $15,000 \text{ min}^{-1}$ for 10 minutes at 4°C .

5) Discard the supernatant and rinse the precipitate once

or twice with 200 to 300 μL of 80% ethanol. Remove the rinse solution using a pipette. Centrifuge at $15,000 \text{ min}^{-1}$ for 10 minutes at 4°C , then remove the supernatant thoroughly and dry up the precipitate.

6) Dissolve the precipitate in 40 μL of distilled water.

2. Perform the same procedure for the positive and negative controls**3. First stage of a two-step PCR**

1) Make a mixture of the heat-resistant DNA polymerase, dNTP solution, outer primer, and reaction buffer solution (including Mg ions), and place 90 μL in each tube.

2) Add 10 μL of the template prepared as above to each tube containing the first stage PCR solution (90 μL).

3) Perform the DNA amplification by repeating 30 cycles of denaturation at 94°C for 30 seconds, annealing at an appropriate temperature for the primer (55°C for the primer in this example), and elongation at 72°C for 2 minutes.

4. Second stage of a two-step PCR

1) Make a mixture of the heat-resistant DNA polymerase, dNTP solution, inner primer, and reaction buffer solution (including Mg ions), and place 99 μL in each tube.

2) Add 1 μL of the first stage PCR product from each tube to a tube containing the second stage PCR solution (99 μL).

3) Perform the DNA amplification by repeating 30 cycles of denaturation at 94°C for 30 seconds, annealing at an appropriate temperature for the primer (55°C for the primer in this example), and elongation at 72°C for 2 minutes.

5. Agarose gel electrophoresis

1) Mix 10 μL of each of the first stage and second stage PCR products with 2 μL of an appropriate dye as a migration marker, and perform 1% agarose gel electrophoresis.

2) Stain the gel with ethidium bromide and take a photograph under UV irradiation.

3) The test is judged to be positive if a DNA band is detected.

[An Example of Primer]

For mycoplasma detection

Outer primer

F1 : 5'-ACACCATGGGAG(C/T)TGGTAAT-3'

R1 : 5'-CTTC(A/T)TCGACTT(C/T)CAGACCCAAGG-CAT-3'

Inner primer

F2 : 5'-GTG(G/C)GG(A/C)TGGATCACCTCCT-3'

R2 : 5'-GCATCCACCA(A/T)A(A/T)AC(C/T)CTT-3'

() indicates a mixture.

[PCR reaction solution]

	[First stage]	[Second stage]
dNTP solution (1.25 mmol/L)	16 μL	16 μL
Primer (10 pmol/ μL)	F1 2 μL	F2 2 μL
Primer (10 pmol/ μL)	R1 2 μL	R2 2 μL
Heat-resistant DNA polymerase (1 U/ μL)	2 μL	2 μL
Reaction buffer solution		
25 mmol/L magnesium chloride hexahydrate	8 μL	8 μL
10-fold buffer solution*	10 μL	10 μL
Sterile distilled water	50 μL	59 μL

*Composition of 10-fold buffer solution
2-amino-2-hydroxymethyl-1,3-

propanediol-hydrochloric acid (pH 8.4)	100 mmol/L
Potassium chloride	500 mmol/L
Gelatin	0.1 g/L

[Method of cultivating mycoplasma within Vero cells]

- 1) Use at least two cell culture dishes for each of the test sample, positive control and negative control.
- 2) Into each cell culture dish (35 mm diameter), inoculate 2 mL of the Vero cell suspension (1×10^4 cells per 1 mL) in Eagle's minimum essential medium containing 10 percent bovine calf serum (tested in advance using the PCR method to verify that it does not contain any detectable mycoplasma DNA). Incubate the cultures at $36 \pm 1^\circ\text{C}$ in an atmosphere of air containing 5 percent carbon dioxide for one day.
- 3) Replace the culture media with fresh media, and add 0.5 mL of the test sample (cell culture supernatant) to each of two or more Vero cell culture dishes. Perform the same procedure for the positive (such as 100 CFU or 100 CCU or less of *M. hyorhinis* (ATCC29052, ATCC17981 or equivalent species or strains)) and negative controls.
- 4) Incubate the Vero cell culture dishes for the test sample, positive and negative controls for 3 to 6 days at $36 \pm 1^\circ\text{C}$ in an atmosphere of air containing 5 percent carbon dioxide.

Peptide Mapping

This test is harmonized with the European Pharmacopoeia and the U.S. Pharmacopoeia.

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, to evaluate the consistency of the overall process and to assess product stability as well as to ensure the identity of the protein product, or to detect the presence of protein variant.

1. 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.

2. 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.

3. 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.

3.1. 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, too many peptides will be generated.

3.2. 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.

3.3. 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

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 <i>S. aureus</i> 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
Chemical	Clostripain (EC 3.4.22.8)	C-terminal side of Arg
	Cyanogen bromide	C-terminal side of Met
	2-Nitro-5-thio-cyanobenzoic acid	N-terminal side of Cys
	<i>o</i> -Iodosobenzoic acid	C-terminal side of Trp and Tyr
	Dilute acid BNPS-skatole	Asp and Pro Trp

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.

3.4. 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.

(i) **pH:** The pH of the digestion mixture is empirically determined to ensure the optimal 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.

(ii) **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 somatotropin is conducted at 4°C, because at higher temperatures it will precipitate during digestion.

(iii) **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.

(iv) **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.

4. 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.

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.

4.1. 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.

4.2. Solvent

The most commonly used solvent is water with acetonitrile as the organic modifier to which less than 0.1% trifluoroa-

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), nondenaturing
SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)
Capillary Electrophoresis (CE)
Paper Chromatography-High Voltage (PCHV)
High-Voltage Paper Electrophoresis (HVPE)

cetic 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.

4.3. 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.

4.4. 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.

4.5. 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.

4.6. 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.

4.7. 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 or reference material, 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.

5. 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 digestion

and MALDI-TOF 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 MALDI-TOF MS analyzer 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.

Qualification of Animals as Origin of Animal-derived Medicinal Products provided in the General Notices of Japanese Pharmacopoeia and Other Standards

Introduction

The Official Gazette issued on March 29, 2002 announced that General Notices of the Japanese Pharmacopoeia and other standards were amended to add a provision that "When a drug product or a drug substance which is used to manufacture a drug product, is manufactured from a raw material of animal origin, the animal in question should be in principle a healthy subject, if not otherwise provided."

The Notice *Iyaku-hatsu* No. 0329001, which was issued on the same date, provided that "Healthy subject herein provided is the animal which does not cause any disease or any infection to human being at an appropriate production process and use of the drug product, and as for the oral or external drug for example, the animal, as its raw material of animal origin, should be confirmed at this stage to meet the Food Standard. It has to be noted that this standard of healthy subject has to be revised timely taking into account the up-to-date information with respect to the amphi xenosis infections common between human beings and animals."

This General Information describes safety assurance against infection associated with the use of drugs, which are manufactured from raw materials of animal origin, to follow up the Notice as mentioned above.

1. Basic concept

When drugs derived from raw materials of animal origin including human are used, it is important to take into account any possibility that communicable disease agents such as virus may cause infectious disease or any possible hazards to patients. In such case, it goes without saying that the primary subject that has to be considered is the absence of any infectious agents such as virus in the raw materials of animal origin including human as the source of the drug. More important points are whether the drugs derived from such raw

materials are free of such infectious agents and whether there is any possibility of transmission of infectious agents when the drugs are administered to patient. The eligibility of animals including human, as the source of raw materials of drugs, in other words "the subject which is free from any disease or transmission of infectious agents that is infectious to human being at an appropriate production process and use of the drug product" is that "The drug should be entirely free from any risk of infections by means of whole procedures which include evaluation of appropriateness of the animals including human as the source of their raw materials, establishment of appropriate production processes and their appropriate control, and strict adherence to the clinical indications of the final product."

2. Animals including human as the source of raw materials of drugs

What is the most clear and appropriate preventive measures against infection to human being due to administration of drugs which are derived from animals including human is to assure the absence of any infectious agents such as virus in its raw materials or an appropriate critical raw material by each of the followings: (1) the use of raw materials of healthy animal origin, which are proved to be free from communicable disease agents to human, or (2) the use of appropriate critical raw materials (e.g., cell substrate, blood plasma, pooled urine after some treatments) for drug production, which are proved to be free from communicable disease agents after certain appropriate processing on raw materials of animal origin.

As for raw materials of drugs of human origin, cell, tissue, blood, placenta, urine, etc. are used. Whenever it is sufficient and possible each donor, as the origin of such raw materials, should be asked his (her) health condition and undergoes his (her) medical examination at this stage, so that the appropriateness as a donor can be confirmed from the standpoint of safety concerning communicable disease agents such as virus.

For example, "Basic concept on handling and use of a drug product, etc. which is derived from cell/tissue" (Attachment 1 of the Notice Iyaku-Hatsu No. 1314 dated December 26, 2000) issued by the Director-General of the Medicinal Safety Bureau, Ministry of Health and Welfare, states that since the cell/tissue supplied by a human donor comes to be applied to patients without processing through any sufficient inactivation or removal of communicable disease agents, the selection and qualification criteria on such donor has to be established. These criteria are to be composed with the respect to the check items on the case history and the physical conditions as well as the test items on the various transmission of infectious agents through cell/tissue, and that the appropriateness of these criteria has to be clarified. Hepatitis Type-B (HBV), Hepatitis Type-C (HCV), Human Immune Deficiency Viral infections (HIV), Adult T-Cell Leukemia and Parvovirus B19 Infections should be denied through the interview to the donor and the tests (serologic test, nucleic-acid amplification test, etc.). Further, if necessary, Cytomegalovirus infection and EB Virus infection should be denied by tests. "Infections caused by bacteria such as *Treponema pallidum*, *Chlamydia*, *Gonococci*, *Tubercule bacillus*, etc.", "septicemia and its suspicious case", "vicious tumor", "serious metabolic or endocrine-related disorders", "collagenosis and haematological disorder",

"hepatic disease" and "dementia (transmissible spongiform encephalopathies and its suspicious case)" should be checked on the case history or by the interview, etc. and the experience of being transfused or/and transplanted should be checked to confirm eligibility as a donor. The most appropriate check items and test methods then available are to be used, which need to be reconsidered at appropriate timing taking into account the updated knowledge and the progress of the science and the technologies. At screening of a donor, reexaminations has to be made at appropriate timing using the eligible check items and the test methods taking into account the window period (Initial period after infection, in which antibody against bacteria, fungi or virus is not detected.)

In the case of plasma derivatives produced from the donated blood in Japan, the donor should be checked by means of self-assessed report about health conditions, and a serologic check and a nucleic acid amplification test (NAT) on mini pooled plasma should be performed at the stage of donated blood. Further, the plasma material (i.e., critical raw material) for fractionation should be stored 4 months in minimum so that the arrangement could be taken based on the information available after collection of the blood and the blood infusion to exclude the possibility of using any critical raw material which might cause infection to patients.

On the other hand, as for the materials such as urine which are taken from the unspecified number of the donors and come to be critical raw materials for drug production after some treatments, it is unrealistic and not practical to conduct the tests of virus infection, etc. on the individual donor. Consequently, appropriate tests such as virus test has to be performed on such critical raw materials for drug production.

In the case of the animals besides human, the wild ones should be excluded. Only the animals, which are raised under well sanitarily controlled conditions taken to prevent bacterial contamination or under the effective bacterial pollution monitoring systems, have to be used, and it is recommended that the animals from a colony appropriately controlled under specific pathogen-free (SPF) environment are to be used as far as possible. Further, for the animals regulated under the Food Standard, only the animals that met this standard should be used. It should be confirmed by appropriate tests that the animals were free from pathogen, if necessary.

The concrete measures to avoid transmittance or spread of infectivity of prion, which is considered to be the pathogen of transmissible spongiform encephalopathies (TSEs), as far as possible are the followings: ① avoidance of use of animals, which are raised in the areas where high incidence or high risk of TSEs (Scrapie in sheep and goat, bovine spongiform encephalopathies (BSE) in cattle, chronic wasting disease (CWD) in deer, new type of Creutzfeldt-Jacob-Disease (CJD) in human, etc.) is reported, and humans, who have stayed long time (more than 6 months) in such areas, as raw materials or related substances of drugs; ② avoidance of use of any substances that are derived from the individual infected with scrapie, BSE, CJD, etc.; ③ avoidance of using a material derived from organ, tissue and cell, etc. of high risk of TSEs; and ④ taking appropriate measures basing on the information collected, which includes incidence of TSEs, the results of epidemiological investigation and the experimental research on prion, and incidence of tardive infection on

donors after collecting raw materials, etc.

3. Human or animal cells which are used as critical raw materials for drug production

Cell substrates derived from humans or animals are used for drug production. In such case, it is desirable that the humans or the animals, which are the origins of the cell substrates, are healthy subjects. However, it is considered practical that viral safety of the drugs derived from the cell substrates are evaluated on the cells, which are so called critical raw materials for production of such drugs. In such case, the safety should be confirmed through the test and analysis on established cell bank thoroughly with respect to virus etc., as far as possible. The items and the methods of the tests that have been followed in this case are described in detail in the Notice of Japanese version on the internationally accepted ICH Guideline entitled "Viral safety evaluation of biotechnology products derived from cell lines of human or animal origin" (Iyakushin No. 329 issued on February 22, 2000 by Director, Evaluation and Licensing Division, Pharmaceutical and Medical Safety Bureau, Ministry of Health and Welfare). In the meantime, it is important how to handle the cell in case that any virus has been detected under the cell level tests. This Notice describes how to cope with this situation as follows: "It is recognised that some cell lines used for the manufacture of product will contain endogenous retroviruses, other viruses or viral sequences. In such circumstances, the action plan recommended for manufacturer is described in Section V (Rationale and action plan for viral clearance studies and virus tests on purified bulk) of the Notice. The acceptability of cell lines containing viruses other than endogenous retroviruses will be considered on an individual basis by the regulatory authorities, by taking into account a risk/benefit analysis based on the benefit of the product and its intended clinical use, the nature of the contaminating viruses, their potential for infecting humans or for causing disease in humans, the purification process for the product (e.g., viral clearance evaluation data), and the extent of the virus tests conducted on the purified bulk." For example, it is well known that Type A-, R- and C-endogenous particles like retrovirus are observed in the cells of the rodents used most often for drug production. It is also known that they are not infectious to human and is not dangerous, and CHO cells are generally used for drug production. The established cell lines (e.g., NAMALWA Cell, BALL-1 Cell, etc.) derived from cancer patients are sometimes used, but through the thorough virus tests, etc., their safety are confirmed. The established cell lines are assumed to be safer than the primary cultured cells which are hard to conduct the thorough virus test.

4. Establishment and control of appropriate production process and adherence to the clinical indication of final product for safety assurance

Safety assurance against potential infections at only the level of animals that are source of raw materials of drugs is limited. Further, "health of animal" can not be defined univocally, and the various factors have to be taken into account. The final goal of this subject is to protect human from any infectious disease caused by drugs. Achieving this goal, the establishment and control of appropriate production processes of each drug and the adherence to the clinical indications of the final product are important.

As mentioned above, the rodent cells used most often for

the production of the drugs are known to have endogenous retrovirus-like particles sometimes. The reason why such cells can be used for the production of the drugs is that multiple measures are applied for safety in the purification stages which include appropriate inactivation or removal processes. There are cases in which the production procedure involves intentional use of a virus or a microorganism. In this case, relevant measures capable of removing or inactivating of such virus or microorganism are appropriately incorporated in the purification process, so that the risk of infection to human can be fully denied and its safety can be assured when it is used as a drug. Further, even in the case that it is difficult to clarify the risk of contamination of the infectious agents or that the raw material are contaminated by viruses etc., the raw material in question may be used for the production of drugs so long as appropriate inactivation or removal processes are introduced, their effectiveness can be confirmed and the safety can be assured by appropriate control of the manufacturing processes under GMP, etc.

5. Conclusion

The qualification of animals including human, as the source of raw materials of drugs, in other words "the subject which does not cause any infectious diseases to human being at an appropriate production process and use of the drug product" is that "the drug has to be entirely free from any risk of infections by means of whole procedures which include evaluation of appropriateness of the animal including human as the source of their raw materials, establishment of appropriate production processes and their appropriate control, and strict adherence to the clinical indication of the final product."

To cope with this subject, the advanced scientific measures, which actually reflect the updated knowledge and progress of the science and the technology about infectious diseases in human and infection of animal origin, have to be taken into account timely.

SDS-Polyacrylamide Gel Electrophoresis

This test is harmonized with the European Pharmacopoeia and the U.S. Pharmacopoeia.

The SDS-Polyacrylamide Gel Electrophoresis is used for the characterization of proteins in biotechnological and biological products and for control of purity and quantitative determinations.

This technique is a suitable analytical method with which to identify and to assess the homogeneity of proteins in biotechnological and biological products. The method is also routinely used for the estimation of protein subunit molecular masses and for determining the subunit compositions of purified proteins.

Ready-to-use gels and reagents are widely available on the market and can be used instead of those described in this text, provided that they give equivalent results and that they meet the validity requirements given below under Validation of the Test.

1. Characteristics of Polyacrylamide Gels

The sieving properties of polyacrylamide gels are afforded

by the three-dimensional network of fibers and pores which is formed as the bifunctional bisacrylamide cross-links adjacent polyacrylamide chains. Polymerization is catalyzed by a free radical-generating system composed of ammonium persulfate and *N,N,N',N'*-tetramethylethylenediamine (TEMED).

As the acrylamide concentration of a gel increases, its effective pore size decreases. The effective pore size of a gel is operationally defined by its sieving properties; that is, by the resistance it imparts to the migration of macromolecules. There are limits on the acrylamide concentration that can be used. At high acrylamide concentrations, gels break much more easily and are difficult to handle. As the pore size of a gel decreases, the migration rate of a protein through the gel decreases. By adjusting the pore size of a gel, through manipulating the acrylamide concentration, the resolution of the method can be optimized for a given protein product. Thus, the physical characteristics of a given gel are determined by the relative concentrations of acrylamide and bisacrylamide, used in its preparation.

In addition to the composition of the gel, the state of the protein is an important determinant of the electrophoretic mobility. In the case of proteins, the electrophoretic mobility is dependent on the pK values of the charged groups and the size of the molecule. It is also influenced by the type, concentration and pH of the buffer, the temperature and the field strength, as well as by the nature of the support material.

2. Polyacrylamide Gel Electrophoresis under Denaturing Conditions

The method cited as an example is limited to the analysis of monomeric polypeptides with a mass range of 14,000 to 100,000 daltons. It is possible to extend this mass range by various techniques (e.g., by using gradient gels, particular buffer systems, etc.), but those techniques are not discussed in this chapter.

Analysis by electrophoresis on sodium dodecyl sulfate (SDS) polyacrylamide gel (SDS-Polyacrylamide Gel Electrophoresis) under denaturing conditions is the most common mode of electrophoresis used in assessing the quality of proteins in biotechnological and biological products, and will be the focus of the example described here. Typically, analytical electrophoresis of proteins is carried out in polyacrylamide gels under conditions that ensure dissociation of the proteins into their individual polypeptide subunits and that minimize aggregation. Most commonly, the strongly anionic detergent SDS is used in combination with heat to dissociate the proteins before they are loaded on the gel. The denatured polypeptides bind to SDS, become negatively charged and exhibit a consistent charge-to-mass ratio regardless of protein type. Because the amount of SDS bound is almost always proportional to the molecular mass of the polypeptide and is independent of its amino acid sequence, SDS-polypeptide complexes migrate through polyacrylamide gels with mobilities that are dependent on the size of the polypeptides.

The electrophoretic mobilities of the resultant SDS-polypeptide complexes all assume the same functional relationship to their molecular masses. Migration of SDS-complexes occurs toward the anode in a predictable manner, with low-molecular-mass complexes migrating faster than larger ones. The molecular mass of a protein can therefore

be estimated from its relative mobility calibrated in SDS-Polyacrylamide Gel Electrophoresis and the occurrence of a single band in such a gel is a criterion of purity.

However, modifications to the polypeptide backbone, such as N- or O-linked glycosylation, have a significant impact on the apparent molecular mass of a protein, since SDS does not bind to a carbohydrate moiety in a manner similar to a polypeptide. Thus, a consistent charge-to-mass ratio is not maintained. The apparent molecular masses of proteins that have undergone post-translational modifications do not truly reflect the masses of the polypeptides.

2.1. Reducing conditions

Polypeptide subunits and three-dimensional structure of proteins are often fixed, at least in part, by the presence of disulfide bonds. A goal of SDS-Polyacrylamide Gel Electrophoresis under reducing conditions is to disrupt this structure by reducing the disulfide bonds. Complete denaturation and dissociation of proteins by treatment with 2-mercaptoethanol or dithiothreitol (DTT) will result in unfolding of the polypeptide backbone and subsequent complexation with SDS. Under these conditions, the molecular masses of the polypeptide subunits can be calculated by linear regression in the presence of suitable molecular-mass standards.

2.2. Non-reducing conditions

For some analyses, complete dissociation of the protein of interest into subunit peptides is not desirable. In the absence of treatment with reducing agents such as 2-mercaptoethanol or DTT, disulfide covalent bonds remain intact, preserving the oligomeric form of the protein. Oligomeric SDS-protein complexes migrate more slowly than their SDS-polypeptide subunits. In addition, non-reduced proteins may not be completely saturated with SDS and, hence, may not bind the detergent in the expected mass ratio. This makes molecular-mass determinations of these molecules by SDS-Polyacrylamide Gel Electrophoresis less straightforward than analyses of fully denatured polypeptides, since it is necessary that both standards and unknown proteins be in similar configurations for valid comparisons. However, the staining of a single band in such a gel is a criterion of purity.

3. Characteristics of Discontinuous Buffer System Gel Electrophoresis

The most widely used electrophoretic method for the analysis of complex mixtures of proteins involves the use of a discontinuous buffer system consisting of two contiguous, but distinct gels: a resolving (lower) gel and a stacking (upper) gel. The two gels are cast with different porosities, pH, and ionic strengths. In addition, different mobile ions are used in the gel and electrode buffers. The buffer discontinuity acts to concentrate large-volume samples in the stacking gel, resulting in improved resolution. When power is applied, a voltage drop develops across the sample solution which drives the proteins into the stacking gel. Glycinate ions from the electrode buffer follow the proteins into the stacking gel. A moving boundary region is rapidly formed with the highly mobile chloride ions in the front and the relatively slow glycinate ions in the rear. A localized high-voltage gradient is formed between the leading and trailing ion fronts, causing the SDS-protein complex to form into a very thin zone (called the stack) and to migrate between the chloride and glycinate phases. Regardless of the height of the applied sample solution in the wells, all SDS-protein complexes condense within a very narrow range and enter the resolving

gel as a well-defined, thin zone of high protein density. The large-pore stacking gel does not retard the migration of most proteins and serves mainly as an anticonvective medium. At the interface of the stacking and resolving gels, the proteins experience a sharp increase in retardation due to the smaller pore size of the resolving gel. Once the proteins are in the resolving gel, their mobility continues to be slowed down by the molecular sieving effect of the matrix. The glycinate ions overtake the proteins, which then move in a space of uniform pH formed by the tris(hydroxymethyl)aminomethane and glycine. Molecular sieving causes the SDS-polypeptide complexes to separate on the basis of their molecular masses.

4. Preparing Vertical Discontinuous Buffer SDS-Polyacrylamide Gels

4.1. Assembling of the gel moulding cassette

Clean the two glass plates (size: e.g. 10 cm × 8 cm), the sample comb made of polytetrafluoroethylene, the two spacers and the silicone rubber tubing (diameter, e.g. 0.6 mm × 35 cm) with mild detergent and rinse extensively with water. Dry all the items with a paper towel or tissue. Lubricate the spacers and the silicone rubber tubing with non-silicone grease. Apply the spacers along each of the two short sides of the glass plate 2 mm away from the edges and 2 mm away from the long side corresponding to the bottom of the gel. Begin to lay the silicone rubber tubing on the glass plate by using one spacer as a guide. Carefully twist the silicone rubber tubing at the bottom of the spacer and follow the long side of the glass plate. While holding the silicone rubber tubing with one finger along the long side again twist the tubing and lay it on the second short side of the glass plate, using the spacer as a guide. Place the second glass plate in perfect alignment and hold the mould together by hand pressure. Apply two clamps on each of the two short sides of the mould. Carefully apply four clamps on the longer side of the gel mould, thus forming the bottom of the gel mould. Verify that the silicone rubber tubing is running along the edge of the glass plates and has not been extruded while placing the clamps.

4.2. Preparation of the gel

In a discontinuous buffer SDS polyacrylamide gel, it is recommended to pour the resolving gel, let the gel set, and then pour the stacking gel, since the compositions of the two gels in acrylamide-bisacrylamide, buffer and pH are different.

4.2.1. Preparation of the resolving gel

In a conical flask, prepare the appropriate volume of solution containing the desired concentration of acrylamide for the resolving gel, using the values given in Table 1. Mix the components in the order shown. Where appropriate, before adding the ammonium persulfate solution and the tetramethylethylenediamine (TEMED), filter the solution if necessary under vacuum through a cellulose acetate membrane (pore size: 0.45 µm); keep the solution under vacuum by swirling the filtration unit until no more bubbles are formed in the solution. Add appropriate amounts of ammonium persulfate solution and TEMED as indicated in Table 1, swirl and pour immediately into the gap between the two glass plates of the mould. Leave sufficient space for the stacking gel (the length of the teeth of the sample comb plus 1 cm). Using a pipette, carefully overlay the solution with water-saturated isobutanol. Leave the gel in a vertical posi-

tion at room temperature to allow polymerization to occur.

4.2.2. Preparation of the stacking gel

After polymerization is complete (about 30 minutes), pour off the isobutanol and wash the top of the gel several times with water to remove the isobutanol overlay and any unpolymerized acrylamide. Drain as much fluid as possible from the top of the gel, and then remove any remaining water with the edge of a paper towel.

In a conical flask, prepare the appropriate volume of solution containing the desired concentration of acrylamide, using the values given in Table 2. Mix the components in the order shown. Where appropriate, before adding the ammonium persulfate solution and the TEMED, filter the solution if necessary under vacuum through a cellulose acetate membrane (pore size: 0.45 µm); keep the solution under vacuum by swirling the filtration unit until no more bubbles are formed in the solution. Add appropriate amounts of ammonium persulfate solution and TEMED as indicated in Table 2, swirl and pour immediately into the gap between the two glass plates of the mould directly onto the surface of the polymerized resolving gel. Immediately insert a clean sample comb into the stacking gel solution, taking care to avoid trapping air bubbles. Add more stacking gel solution to fill completely the spaces of the sample comb. Leave the gel in a vertical position and allow to polymerize at room temperature.

4.3. Mounting the gel in the electrophoresis apparatus and electrophoretic separation

After polymerization is complete (about 30 minutes), remove the sample comb carefully. Rinse the wells immediately with water or with the running buffer for SDS-Polyacrylamide Gel Electrophoresis to remove any unpolymerized acrylamide. If necessary, straighten the teeth of the sample comb of the stacking gel with a blunt hypodermic needle attached to a syringe. Remove the clamps on one short side, carefully pull out the silicone rubber tubing and replace the clamps. Proceed similarly on the other short side. Remove the silicone rubber tubing from the bottom part of the gel. Mount the gel in the electrophoresis apparatus. Add the electrophoresis buffers to the top and bottom reservoirs. Remove any bubbles that become trapped at the bottom of the gel between the glass plates. This is best done with a bent hypodermic needle attached to a syringe. Never pre-run the gel before loading solutions, such as samples, since this will destroy the discontinuity of the buffer systems. Before loading solutions, such as samples, carefully rinse the stacking gel wells with the running buffer for SDS-Polyacrylamide Gel Electrophoresis. Prepare the test and reference solutions in the recommended sample buffer and treat as specified in the individual monograph. Apply the appropriate volume of each solution to the stacking gel wells. Start the electrophoresis using suitable operating conditions for the electrophoresis equipment to be used. There are commercially available gels of different surface area and thickness that are appropriate for various types of electrophoresis equipment. Electrophoresis running time and current/voltage may need to be altered depending on the type of apparatus used, in order to achieve optimum separation. Check that the dye front is moving into the resolving gel. When the dye is reaching the bottom of the gel, stop the electrophoresis. Remove the gel assembly from the apparatus and separate the glass plates. Remove the spacers, cut off and discard the stacking gel and immediately proceed with staining.

5. Detection of Proteins in Gels

Coomassie staining is the most common protein staining method, with a detection level of the order of $1\ \mu\text{g}$ to $10\ \mu\text{g}$ of protein per band. Silver staining is the most sensitive method for staining proteins in gels and a band containing 10 ng to 100 ng can be detected. All of the steps in gel staining are done at room temperature with gentle shaking in any convenient container. Gloves must be worn when staining gels, since fingerprints will stain.

5.1. Coomassie staining

Immerse the gel in a large excess of Coomassie staining TS and allow to stand for at least 1 hour. Remove the staining solution.

Destain the gel with a large excess of destaining TS. Change the destaining solution several times, until the stained protein bands are clearly distinguishable on a clear background. The more thoroughly the gel is destained, the smaller is the amount of protein that can be detected by the method. Destaining can be speeded up by including 2 to 3 g of anion-exchange resin or a small sponge in the destaining TS.

NOTE: the acid-alcohol solutions used in this procedure do not completely fix proteins in the gel. This can lead to losses of some low-molecular-mass proteins during the staining and destaining of the gel. Permanent fixation is obtainable by allowing the gel to stand in trichloroacetic acid TS for fixing for 1 hour before it is immersed in Coomassie staining TS.

5.2. Silver staining

Immerse the gel in a large excess of fixing TS and allow to stand for 1 hour. Remove the fixing solution, add fresh fixing solution and incubate either for at least 1 hour or overnight, if convenient. Discard the fixing solution and wash the gel in water for 1 hour. Soak the gel for 15 minutes in a 1 vol% glutaraldehyde solution. Wash the gel twice for 15 minutes in water. Soak the gel in fresh silver nitrate TS for silver staining for 15 minutes, in darkness. Wash the gel three times for 5 minutes in water. Immerse the gel for about 1 minute in developer TS until satisfactory staining has been obtained. Stop the development by incubation in blocking TS for 15 minutes. Rinse the gel with water.

6. Drying of Stained SDS-Polyacrylamide Gels

Depending on the staining method used, gels are pretreated in a slightly different way. For Coomassie staining, after the destaining step, allow the gel to stand in a diluted solution of concentrated glycerin (1 in 10) for at least 2 hours (overnight incubation is possible). For silver staining, after the final rinse, allow the gel to stand in a diluted solution of concentrated glycerin (1 in 50) for 5 minutes.

Immerse two sheets of porous cellulose film in water and incubate for 5 to 10 minutes. Place one of the sheets on a drying frame. Carefully lift the gel and place it on the cellulose film. Remove any trapped air bubbles and pour 2 to 3 mL of water around the edges of the gel. Place the second sheet on top and remove any trapped air bubbles. Complete the assembly of the drying frame. Place in an oven or leave at room temperature until dry.

7. Molecular-Mass Determination

Molecular masses of proteins are determined by comparison of their mobilities with those of several marker proteins of known molecular mass. Mixtures of proteins with precisely known molecular masses blended for uniform staining are

commercially available for calibrating gels. They are obtainable in various molecular mass ranges. Concentrated stock solutions of proteins of known molecular mass are diluted in the appropriate sample buffer and loaded on the same gel as the protein sample to be studied.

Immediately after the gel has been run, the position of the bromophenol blue tracking dye is marked to identify the leading edge of the electrophoretic ion front. This can be done by cutting notches in the edges of the gel or by inserting a needle soaked in India ink into the gel at the dye front. After staining, measure the migration distances of each protein band (markers and unknowns) from the top of the resolving gel. Divide the migration distance of each protein by the distance traveled by the tracking dye. The normalized migration distances so obtained are called the relative mobilities of the proteins (relative to the dye front) and conventionally denoted as R_f . Construct a plot of the logarithm of the relative molecular masses (M_r) of the protein standards as a function of the R_f values. Note that the graphs are slightly sigmoid. Unknown molecular masses can be estimated by linear regression analysis or interpolation from the curves of $\log M_r$ against R_f as long as the values obtained for the unknown samples are positioned along the linear part of the graph.

8. Suitability of the Test (Validation)

The test is not valid unless the proteins of the molecular mass marker are distributed along 80% of the length of the gel and over the required separation range (e.g., the range covering the product and its dimer or the product and its related impurities) the separation obtained for the relevant protein bands shows a linear relationship between the logarithm of the molecular mass and the R_f as described in 7. Additional requirements with respect to the solution under test may be specified in individual monographs.

9. Quantification of Impurities

Where the impurity limit is specified in the individual monograph, a reference solution corresponding to that level of impurity should be prepared by diluting the test solution. For example, where the limit is 5%, a reference solution would be a 1:20 dilution of the test solution. No impurity (any band other than the main band) in the electrophoretogram obtained with the test solution may be more intense than the main band obtained with the reference solution.

Under validated conditions, impurities may be quantified by normalization to the main band, using an integrating densitometer. In this case, the responses must be validated for linearity.

10. Test solutions:

(i) 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.

(ii) Developer TS: Dissolve 2 g of citric acid monohydrate in water to make 100 mL. To 2.5 mL of this solution add 0.27 mL of formaldehyde solution and water to make 500 mL.

(iii) Fixing TS: To 250 mL of methanol add 0.27 mL of formaldehyde solution and water to make 500 mL.

(iv) Silver nitrate TS for silver staining: To 40 mL of sodium hydroxide TS add 3 mL of ammonia solution (28), then add dropwise 8 mL of a solution of silver nitrate (1 in 5) while stirring, and add water to make 200 mL.

Table 1 Preparation of resolving gel

Solution components	Component volumes (mL) per gel mould volume of							
	5 mL	10 mL	15 mL	20 mL	25 mL	30 mL	40 mL	50 mL
6% Acrylamide								
Water	2.6	5.3	7.9	10.6	13.2	15.9	21.2	26.5
Acrylamide solution ⁽¹⁾	1.0	2.0	3.0	4.0	5.0	6.0	8.0	10.0
1.5 mol/L Tris solution (pH 8.8) ⁽²⁾	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
100 g/L SDS ⁽³⁾	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
100 g/L APS ⁽⁴⁾	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED ⁽⁵⁾	0.004	0.008	0.012	0.016	0.02	0.024	0.032	0.04
8% Acrylamide								
Water	2.3	4.6	6.9	9.3	11.5	13.9	18.5	23.2
Acrylamide solution ⁽¹⁾	1.3	2.7	4.0	5.3	6.7	8.0	10.7	13.3
1.5 mol/L Tris solution (pH 8.8) ⁽²⁾	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
100 g/L SDS ⁽³⁾	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
100 g/L APS ⁽⁴⁾	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED ⁽⁵⁾	0.003	0.006	0.009	0.012	0.015	0.018	0.024	0.03
10% Acrylamide								
Water	1.9	4.0	5.9	7.9	9.9	11.9	15.9	19.8
Acrylamide solution ⁽¹⁾	1.7	3.3	5.0	6.7	8.3	10.0	13.3	16.7
1.5 mol/L Tris solution (pH 8.8) ⁽²⁾	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
100 g/L SDS ⁽³⁾	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
100 g/L APS ⁽⁴⁾	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED ⁽⁵⁾	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02
12% Acrylamide								
Water	1.6	3.3	4.9	6.6	8.2	9.9	13.2	16.5
Acrylamide solution ⁽¹⁾	2.0	4.0	6.0	8.0	10.0	12.0	16.0	20.0
1.5 mol/L Tris solution (pH 8.8) ⁽²⁾	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
100 g/L SDS ⁽³⁾	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
100 g/L APS ⁽⁴⁾	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED ⁽⁵⁾	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02
14% Acrylamide								
Water	1.4	2.7	3.9	5.3	6.6	8.0	10.6	13.8
Acrylamide solution ⁽¹⁾	2.3	4.6	7.0	9.3	11.6	13.9	18.6	23.2
1.5 mol/L Tris solution (pH 8.8) ⁽²⁾	1.2	2.5	3.6	5.0	6.3	7.5	10.0	12.5
100 g/L SDS ⁽³⁾	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
100 g/L APS ⁽⁴⁾	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED ⁽⁵⁾	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02
15% Acrylamide								
Water	1.1	2.3	3.4	4.6	5.7	6.9	9.2	11.5
Acrylamide solution ⁽¹⁾	2.5	5.0	7.5	10.0	12.5	15.0	20.0	25.0
1.5 mol/L Tris solution (pH 8.8) ⁽²⁾	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
100 g/L SDS ⁽³⁾	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
100 g/L APS ⁽⁴⁾	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED ⁽⁵⁾	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02

(1) Acrylamide solution: 30% acrylamide/bisacrylamide (29:1) solution

(2) 1.5 mol/L Tris solution (pH 8.8): 1.5 mol/L tris-hydrochloride buffer solution, pH 8.8

(3) 100 g/L SDS: 100 g/L solution of sodium dodecyl sulfate

(4) 100 g/L APS: 100 g/L solution of ammonium persulfate. Ammonium persulfate provides the free radicals that drive polymerization of acrylamide and bisacrylamide. Since ammonium persulfate solution decomposes slowly, fresh solutions must be prepared before use.

(5) TEMED: *N,N,N',N'*-tetramethylethylenediamine

(v) Destaining TS: A mixture of water, methanol and acetic acid (100) (5:4:1).

(vi) Blocking TS: To 10 mL of acetic acid (100) add water to make 100 mL.

(vii) Trichloroacetic acid TS for fixing: Dissolve 10 g of

trichloroacetic acid in a mixture of water and methanol (5:4) to make 100 mL.

Table 2 Preparation of stacking gel

Solution components	Component volumes (mL) per gel mould volume of							
	1 mL	2 mL	3 mL	4 mL	5 mL	6 mL	8 mL	10 mL
Water	0.68	1.4	2.1	2.7	3.4	4.1	5.5	6.8
Acrylamide solution ⁽¹⁾	0.17	0.33	0.5	0.67	0.83	1.0	1.3	1.7
1.0 mol/L Tris solution (pH 6.8) ⁽²⁾	0.13	0.25	0.38	0.5	0.63	0.75	1.0	1.25
100 g/L SDS ⁽³⁾	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
100 g/L APS ⁽⁴⁾	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
TEMED ⁽⁵⁾	0.001	0.002	0.003	0.004	0.005	0.006	0.008	0.01

(1) Acrylamide solution: 30% acrylamide/bisacrylamide (29:1) solution

(2) 1.0 mol/L Tris solution (pH 6.8): 1 mol/L tris-hydrochloride buffer solution, pH 6.8

(3) 100 g/L SDS: 100 g/L solution of sodium dodecyl sulfate

(4) 100 g/L APS: 100 g/L solution of ammonium persulfate. Ammonium persulfate provides the free radicals that drive polymerization of acrylamide and bisacrylamide. Since ammonium persulfate solution decomposes slowly, fresh solutions must be prepared before use.

(5) TEMED: *N,N,N',N'*-tetramethylethylenediamine

Total Protein Assay

This test is harmonized with the European Pharmacopoeia and the U.S. Pharmacopeia. The parts of the text that are not harmonized are marked with symbols (♦ ♦).

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 (UV method)

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 (Lowry method)

This method, commonly referred to as the Lowry assay, is based on the reduction by protein of the phosphomolybdic tungstic 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 sub-

stances, 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 documents¹⁾ 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 re-

move 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 $3000 \times g$ 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 the 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 (Bradford method)

This method, commonly referred to as the Bradford assay, is based on the absorption shift from 470 nm to 595 nm observed when Coomassie brilliant blue G-250 binds to protein. The Coomassie brilliant blue G-250 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 Coomassie brilliant blue G-250²⁾ 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 (Bicinchoninic acid method)

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.

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 (Biuret method)

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 ver-

sus 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 (Fluorometric method)

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)amino-methane 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 (Nitrogen method)

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 mate-

rial.

- ♦1) Example: Minimum Requirements for Biological Products and individual monograph in JP.♦
- 2) Purity of the reagent is important.

G4 Microorganisms

Decision of Limit for Bacterial Endotoxins

The endotoxin limit for injections is to be decided as follows:

$$\text{Endotoxin limit} = K/M$$

where K is a threshold pyrogenic dose of endotoxin per kg body mass (EU/kg), and M is equal to the maximum bolus dose of product per kg body mass. When the product is to be injected at frequent intervals or infused continuously, M is the maximum total dose administered in a single hour period.

M is expressed in mL/kg for products to be administered by volume, in mg/kg or mEq/kg for products to be administered by mass, and in Unit/kg for products to be administered by biological units. Depending on the administration route, values for K are set as in the following table.

Intended route of administration	K (EU/kg)
Intravenous	5.0
Intravenous, for radiopharmaceuticals	2.5
Intraspinal	0.2

Notes:

- 1) For products to be administered by mass or by units, the endotoxin limit should be decided based on the labeled amount of the principal drug.
- 2) Sixty kg should be used as the average body mass of an adult when calculating the maximum adult dose per kg.
- 3) The pediatric dose per kg body mass should be used when this is higher than the adult dose.
- 4) The K values for the intravenous route are applicable to drugs to be administered by any route other than those shown in the table.

Disinfection and Sterilization Methods

Disinfection and Sterilization Methods are applied to kill microorganisms in processing equipment/utensils and areas used for drug manufacturing, as well as to perform microbiological tests specified in the monographs, and so differ from "Terminal Sterilization" and "Filtration Method" described in "Terminal Sterilization and Sterilization Indicators". The killing effect on microorganisms or the

estimated level of sterility assurance is greatly variable, so the conditions for disinfection and sterilization treatment must be chosen appropriately for each application. Generally, the following methods are to be used singly or in combination after appropriate optimization of operation procedures and conditions, in accordance with the kind and the degree of the contaminating microorganisms and the nature of the item to which the methods are applied.

The validation of sterilization in accordance with Terminal Sterilization and Sterilization Indicators is required when the methods are applied to the manufacturing processes of drug products.

1. Disinfection methods

These methods are used to reduce the number of living microorganisms, but do not always remove or kill all microorganisms present. Generally, disinfection is classified into chemical disinfection with the use of chemical drugs (disinfectants) and physical disinfection with the use of moist heat, ultraviolet light, and other agents.

1.1. Chemical disinfection

Microorganisms are killed with chemical drugs. The killing effect and mechanisms of a chemical drug differ depending on the type, applied concentration, action temperature, and action time of the chemical drug used, the degree of contamination on the object to be disinfected, and the species and state (e.g., vegetative bacteria or spore bacteria) of microorganisms.

Therefore, in applying the method, full consideration is required of the sterility and permissible storage period of the prepared chemical drug, the possibility of resistance of microorganisms at the site of application, and the effect of residual chemical drug on the product. In selecting a suitable chemical drug, the following items should be considered in relation to the intended use.

- (i) The antimicrobial spectrum
- (ii) Action time for killing microorganisms
- (iii) Action durability
- (iv) Effect of the presence of proteins
- (v) Influence on the human body
- (vi) Solubility in water
- (vii) Influence on the object to be disinfected
- (viii) Odor
- (ix) Convenience of use
- (x) Easy disposability
- (xi) Influence on the environment at disposal
- (xii) Frequency of occurrence of resistance

1.2. Physical disinfection

Microorganisms are killed without a chemical drug.

(i) Steam flow method

Microorganisms are killed by direct application of steam. This method is used for a product which may be denatured by the moist heat method. As a rule, the product is kept in flowing steam at 100°C for 30 – 60 minutes.

(ii) Boiling method

Microorganisms are killed by putting the object in boiling water. This method is used for a product which may be denatured by the moist heat method. As a rule, the product is put in boiling water for 15 minutes or more.

(iii) Intermittent method

Microorganisms are killed by heating for 30 – 60 minutes repeatedly, three to five times, once a day in water at 80 – 100°C or in steam. This method is used for a product which

may be denatured by the moist heat method. There is another method called the low temperature intermittent method with repeated heating at 60 – 80°C. During the intermission periods between heating or warming, a suitable temperature for the growth of microorganisms of 20°C or higher, must be maintained.

(iv) Ultraviolet method

As a rule, microorganisms are killed by irradiation with ultraviolet rays at a wavelength of around 254 nm. This method is used for products which are resistant to ultraviolet rays, such as smooth-surfaced articles, facilities, and equipment, or water and air. This method does not suffer from the occurrence of resistance, which is observed in chemical disinfection, and shows a killing effect on bacteria, fungi, and viruses. It must be taken into consideration that direct ultraviolet irradiation of the human body can injure the eyes and skin.

2. Sterilization methods

2.1. Heating methods

In these methods, the heating time before the temperature or pressure reaches the prescribed value differs according to the properties of the product, the size of the container, and the conditions. The duration of heating in conducting these methods is counted from the time when all the parts of the product have reached the prescribed temperature.

(i) Moist heat method

Microorganisms are killed in saturated steam at a suitable temperature and pressure. This method is generally used for heat-stable substances, such as glass, porcelain, metal, rubber, plastics, paper, and fiber, as well as heat-stable liquids, such as water, culture media, reagents, test solutions, liquid samples, etc. As a rule, one of the following conditions is used.

- 115 – 118°C for 30 minutes
- 121 – 124°C for 15 minutes
- 126 – 129°C for 10 minutes

(ii) Dry-heat method

Microorganisms are killed in dry-heated air. This method is generally used for heat-stable substances, such as glass, porcelain, and metal, as well as heat-stable products, such as mineral oils, fats and oils, powder samples, etc. This method is generally conducted in the way of direct heating by gas or electricity or circulating heated air. As a rule, one of the following conditions is used.

- 160 – 170°C for 120 minutes
- 170 – 180°C for 60 minutes
- 180 – 190°C for 30 minutes

2.2. Irradiation methods

(i) Radiation method

Microorganisms are killed by gamma-rays emitted from a radioisotope or electron beam and bremsstrahlung (X-ray) generated from an electron accelerator. This method is generally used for radiation-resistant substances such as glass, porcelain, metal, rubber, plastics, fiber, etc. The dose is decided according to the material properties, and the degree of contamination of the product to be sterilized. Special consideration is necessary of the possibility of qualitative change of the product after the application of the method.

(ii) Microwave method

Microorganisms are killed by the heat generated by direct microwave irradiation. This method is generally used for microwave-resistant products such as water, culture media,

test solutions, etc. As a rule, microwave radiation with a wavelength of around 2450 ± 50 MHz is used.

2.3. Gas methods

Microorganisms are killed by a sterilizing gas. Suitable gases for killing microorganisms include ethylene oxide gas, formaldehyde gas, hydrogen peroxide gas, chlorine dioxide gas, etc. Temperature, humidity, the concentration of gas, and the exposure time differ in accordance with the species of gas used. As sterilizing gases are generally toxic to humans, full consideration is required of the environmental control for the use of gases and the concentration of residual gas. In some of the gas methods, it may be difficult to measure or estimate quantitatively the killing of microorganisms.

2.4. Filtration method

Microorganisms are removed by filtration with a suitable filtering device. This method is generally used for gas, water, or culture media and test solutions containing a substance that is water-soluble and unstable to heat. As a rule, a filter having a pore size of $0.22 \mu\text{m}$ or smaller is used for the sterilization. However, in this method, a filter with a pore size of $0.45 \mu\text{m}$ or smaller is permitted to be used.

Media Fill Test (Process Simulation)

The media fill test is one of the process validations employed to evaluate the propriety of the aseptic processing of pharmaceutical products using sterile media, etc. instead of actual products. Therefore, media fill should be conducted under conditions that simulate routine manufacturing procedures, e.g. filling and sealing, operating environment, processing operation, number of personnel involved, etc., and include permissible worst case conditions. Process simulation can be applied to the other aseptic manufacturing processes in addition to aseptic manufacturing processes for finished drug products such as “filling” and “sealing”.

1. Frequency of media fills

1.1. Initial performance qualification

Initial performance qualification should be conducted for each new facility, item of equipment, filling line, and container design (except for multiple sizes of the same container design), etc. As referring to Table 1, a sufficient number of units should be used to simulate aseptic manufacturing process. A minimum of three consecutive separate successful runs should be performed on each separate day.

1.2. Periodic performance requalification

1) As referring to Table 2, a sufficient number of units should be used to simulate aseptic manufacturing process. Media fill run should be conducted at least on semi-annual base for each shift and processing line. All personnel working in the critical processing area should be trained about aseptic processing and participate in a media fill run at least once a year.

2) When filling lines have not been used for over six months, conduct appropriate numbers of media fill runs in the same way as for the initial performance qualification prior to the use of the filling lines.

3) In cases of facility and equipment modification (interchanging parts may not require requalification), major changes in personnel working in critical aseptic processing, anomalies in environmental monitoring results, or a product sterility test showing contaminated products, conduct ap-

Table 1 Initial performance qualification

Minimum number of simulations	Number of units filled per simulation	Contaminated units in any of the three simulations	Action
3	< 5000	≥ 1	Investigation, corrective measures, restart validation
3	5000 – 10000	1	Investigation, consideration of repeat of one media fill
		> 1	Investigation, corrective measures, restart validation
3	> 10000	1	Investigation
		> 1	Investigation, corrective measures, restart validation

Table 2 Periodic performance requalification

Minimum number of simulations	Number of units filled per simulation	Contaminated units	Action
Every half year	< 5000	1	Investigation, revalidation
	5000 – 10000	1	Investigation, consideration of repeat media fill
		> 1	Investigation, corrective measures, revalidation
	> 10000	1	Investigation
		> 1	Investigation, corrective measures, revalidation

appropriate numbers of media fill runs in the same way as for the initial performance qualification prior to the scheduled media fills.

2. Acceptance criteria of media fills

Both in initial performance qualification and periodic performance qualification, the target should be zero growth regardless of number of units filled per simulation. Where contaminated units are found, action shown in Tables 1 and 2 should be taken.

2.1. Investigation of positive units

Where contaminated units are found in media fill, an investigation should be conducted regarding the cause, taking into consideration the following points:

- 1) Microbial monitoring data

- 2) Particulate monitoring data
- 3) Personnel monitoring data (microbial monitoring data on gloves, gowns, etc. at the end of work)
- 4) Sterilization cycle data for media, commodities, equipment, etc.
- 5) Calibration data of sterilization equipment
- 6) Storage conditions of sterile commodities
- 7) HEPA filter evaluation (integrity tests, velocity, etc.)
- 8) Pre and post filter integrity test data (including filter housing assembly)
- 9) Air flow patterns and pressures
- 10) Unusual events that occurred during the media fill run
- 11) Characterization of contaminants
- 12) Hygienic control and training programs
- 13) Gowning procedures and training programs
- 14) Aseptic processing technique and training programs
- 15) Operator's health status (especially coughing, sneezing, etc., due to respiratory diseases)
- 16) Other factors that affect sterility

3. Data guidance for media fills

Each media fill run should be fully documented and the following information recorded:

- 1) Data and time of media fill
- 2) Identification of filling room and filling line used
- 3) Container/closure type and size
- 4) Volume filled per container
- 5) Filling speed
- 6) Filter type and integrity test result (in case of filtration)
- 7) Type of media filled
- 8) Number of units filled
- 9) Number of units not incubated and reason
- 10) Number of units incubated
- 11) Number of units positive
- 12) Incubation time and temperature
- 13) Procedures used to simulate any step of a normal production fill (e.g., mock lyophilization or substitution of vial headspace gas)
- 14) Microbiological monitoring data obtained during the media fill set-up and run
- 15) List of personnel who took part in the media fill
- 16) Growth promotion results of the media (in case of powder fill, an antimicrobial activity test for the powder is necessary)
- 17) Identification and characterization of the microorganisms from any positive units
- 18) Product(s) covered by the media fill
- 19) Investigation of runs with a positive unit or failed runs
- 20) Management review

4. Media fill procedures

Methods to validate aseptic processing of liquid, powder and freeze-dried products are described. Basically, it is possible to apply media fill procedures for liquid products to other dosage forms.

4.1. Media selection and growth promotion

Soybean-casein digest medium or other suitable media are used. When strains listed in the Microbial Limit Test <4.05> and, if necessary, one or two representative microorganisms which are frequently isolated in environmental monitoring are inoculated under the specified conditions, each strain should show obvious growth.

4.2. Sterile medium preparation

The medium is sterilized according to the pre-validated method.

4.3. Incubation and inspection of media filled units

Leaking or damaged units should be removed and recorded prior to incubation of media filled units. Units should be incubated at 20 – 35°C for not less than 14 days. The use of another temperature range should be justified. If two temperatures are used for incubation, the units are typically incubated for at least 7 days at each temperature starting with the lower temperature. Established temperature should be kept within $\pm 2.5^\circ\text{C}$. Observe the media filled units for growth of microorganisms on the last day of the incubation. Microorganisms in contaminated units should be followed to identification and characterization. For the identification of contaminants, “Rapid Identification of Microorganisms Based on Molecular Biological Method” shown in General Information or appropriate commercial kit for identification of microorganisms may be applicable.

A. Liquid products

Media fill procedure

Media fill should include normal facility/equipment operations and clean-up routines. Containers, closures, parts of the filling machine, trays, etc. are washed and sterilized according to the standard operating procedures. Media fills should be conducted under processing conditions that include “worst case” conditions, e.g., correction of line stoppage, repair or replacement of filling needles/tubes, replacement of on-line filters, permitted interventions, duration and size of run, number of personnel involved, etc.

It is not necessary to put all worst-case scenarios in a single media fill run, but all worst-case scenarios should be evaluated intentionally. While advanced processing lines are highly automated, often operated at relatively high speeds, and are designed to limit operator intervention, there are processing lines showing frequent human interventions. Although the most accurate simulation model would be the full batch size and duration because it most closely simulates the actual production run, other appropriate models can be justified.

A predetermined volume of medium is filled into sterilized containers at a predetermined filling speed and the containers are sealed. The media are contacted with all interior surfaces in the containers by an appropriate method, and then incubated at the predetermined temperature.

B. Powder products

B.1 Powder selection and antimicrobial activity test

Actual products or placebo powder are used. In general, lactose monohydrate, D-mannitol, polyethylene glycol 6,000, carboxymethyl cellulose salts or media powder, etc. are used as placebo powders. Prior to employing any of the powders, evaluate whether the powder has antimicrobial activity. Media powders are dissolved in water and other powders in liquid medium, and the solutions are inoculated with less than 100 CFU microorganisms of each kind, shown in 4.1, for the growth promotion test. If obvious growth appears in the medium incubated at the predetermined temperature for 5 days, the powder has no antimicrobial activity and is available for the media fill test.

B.2 Sterilization of powders

Powders are bagged in suitable containers (e.g. double heat-sealed polyethylene bags), and are subjected to radia-

tion sterilization.

B.3 Sterility of filling powders

The powders must pass the Sterility Test. However, if the sterilization is fully validated, sterility testing of the powders can be omitted.

B.4 Media fill procedures

Choose a suitable procedure from among the following procedures.

1) Fill sterilized liquid media into containers by suitable methods, and then fill actual products or sterilized placebo powder with the powder filling machine. If sterilized powder media are used as a placebo powder, fill sterilized water instead of sterilized liquid media.

2) Distribute liquid media into containers, and then sterilize them in an autoclave. Remove the containers to the filling area, and then fill actual products or sterilized placebo powder into the containers with the powder filling machine.

3) Fill actual products or sterilized placebo powder into containers with the powder filling machine, and then fill sterilized liquid media into the containers by appropriate methods. If sterilized powder media are used as a placebo powder, fill sterilized water instead of sterilized liquid media.

C. Lyophilized products

In the case of lyophilized products, it may be impossible to conduct a media fill run in the same way as used for actual processing of lyophilized products. The process of freezing and lyophilization of the solution may kill contaminant organisms and change the characteristics of the media too. The use of inert gas as a blanket gas may inhibit the growth of aerobic bacteria and fungi. Therefore, in general, the actual freezing and lyophilization process should be avoided and air is used as the blanket gas. For products manufactured under an anaerobic atmosphere, process simulation should be performed with the use of anaerobic growth media and the inert gas such as nitrogen gas.

Media fill procedures

Use the following method or other methods considered to be equivalent to these methods.

1) After filling of the media into containers by the filling machine, cap the containers loosely and collect them in pre-sterilized trays.

2) After placing the trays in the lyophilizer, close the chamber door, and conduct lyophilization according to the procedures for production operation. Hold them without freezing and boiling-over under weak vacuum for the predetermined time.

3) After the vacuum process, break the vacuum, and seal the stoppers.

4) Contact the media with all product contact surfaces in the containers by appropriate methods, and then cultivate them at the predetermined temperature.

References

ISO 13408-1 (2008): Aseptic processing of health care products: General requirements.

Microbial Attributes of Non-sterile Pharmaceutical Products

This chapter is harmonized with the European Pharmacopoeia and the U.S. Pharmacopeia. The parts of the text that are not harmonized are marked with symbols (♦ ♦).

The presence of certain micro-organisms in non-sterile preparations may have the potential to reduce or even inactivate the therapeutic activity of the product and has a potential to adversely affect the health of the patient. Manufacturers have therefore to ensure a low bioburden of finished dosage forms by implementing current guidelines on Good Manufacturing Practice during the manufacture, storage and distribution of pharmaceutical preparations. ♦This chapter provides guidelines for acceptable limits of viable micro-organisms (bacteria and fungi) existing in raw materials and non-sterile pharmaceutical products. ♦ Microbial examination of non-sterile products is performed according to the methods given in the Microbial Limit Test <4.05> on Microbiological Examination of Non-sterile Products: I. *Microbial Enumeration Tests* and II. *Tests for Specified Micro-organisms*. ♦When these tests are carried out, a microbial control program must be established as an important part of the quality management system of the product. Personnel responsible for conducting the tests should have specialized training in microbiology, biosafety measures and in the interpretation of the testing results. ♦

♦1. Definitions

- (i) Non-sterile pharmaceutical products: Non-sterile drugs shown in monographs of the JP and non-sterile finished dosage forms.
- (ii) Raw materials: All materials, including raw ingredients and excipients, used for the preparation of drugs, except for water and gases.
- (iii) Bioburden: Number and type of viable micro-organisms existing in non-sterile pharmaceutical products.
- (iv) Action levels: Established bioburden levels that require immediate follow-up and corrective action if they are exceeded.
- (v) Alert levels: Established bioburden levels that give early warning of a potential drift from normal bioburden level, but which are not necessary grounds for definitive corrective action, though they may require follow-up investigation.
- (vi) Quality management system: The procedures, operation methods and organizational structure of a manufacturer (including responsibilities, authorities and relationships between these) needed to implement quality management.

2. Scope

In general, the test for total viable aerobic count is not applied to drugs containing viable micro-organisms as an active ingredient.

3. Sampling plan and frequency of testing

3.1. Sampling methods

Microbial contaminants are usually not uniformly distributed throughout the batches of non-sterile pharmaceutical products or raw materials. A biased sampling plan, therefore, cannot be used to estimate the real bioburden in the product. A sampling plan which can properly reflect the sta-

tus of the product batch should be established on the basis of the bioburden data obtained by retrospective validation and/or concurrent validation. In general, a mixture of samples randomly taken from at least different three portions, almost the same amount for each portion, is used for the tests of the product. When the sampling is difficult in a clean area, special care is required during sampling to avoid introducing microbial contamination into the product or affecting the nature of the product bioburden. If it is confirmed that the product bioburden is stable for a certain period, as in the case of non-aqueous or dried products, it is not necessary to do the tests, immediately after the sampling.

3.2. Testing frequency

The frequency of the tests should be established on the basis of a variety of factors unless otherwise specified. These factors include:

- (i) Dosage forms of non-sterile pharmaceutical products (usage);
- (ii) Manufacturing processes;
- (iii) Manufacturing frequency;
- (iv) Characteristics of raw materials (natural raw material, synthetic compound, etc.);
- (v) Batch sizes;
- (vi) Variations in bioburden estimates (changes in batches, seasonal variations, etc.);
- (vii) Changes affecting the product bioburden (changes in manufacturing process, supplier of raw materials, batch number of raw materials, etc.);
- (viii) Others.

In general, the tests may be performed at a high frequency during the initial production of a drug to get information on the microbiological attributes of the product or raw materials used for the production. However, this frequency may be reduced as bioburden data are accumulated through retrospective validation and/or concurrent validation. For example, the tests may be performed at a frequency based on time (e.g., weekly, monthly or seasonally), or on alternate batches.

4. Microbial control program

When the "Microbial Limit Test <4.05>" is applied to a non-sterile pharmaceutical product, the methods for the recovery, cultivation and estimation of the bioburden from the product must be validated and a "Microbial control program" covering the items listed below must be prepared.

- (i) Subject pharmaceutical name (product name);
- (ii) Frequency of sampling and testing;
- (iii) Sampling methods (including responsible person, quantity, environment, etc. for sampling);
- (iv) Transfer methods of the samples to the testing area (including storage condition until the tests);
- (v) Treatment of the samples (recovery methods of microbial contaminants);
- (vi) Enumeration of viable micro-organisms (including testing quantity, culture media, growth-supporting test of the media, culturing methods, etc.);
- (vii) Detection of specified micro-organisms (including testing quantity, culture media, growth-supporting test of the media, culturing methods, etc.);
- (viii) Estimation of the number of and characterization of microbial contaminants;
- (ix) Establishment of "Microbial acceptance criteria" (including alert level and action level);

(x) Actions to be taken when the levels exceed “Microbial acceptance criteria”;

(xi) Persons responsible for the testing and evaluation, etc.;

(xii) Other necessary items. ♦

5. Microbial acceptance criteria for non-sterile pharmaceutical products

By establishing “Microbial acceptance criteria” for non-sterile pharmaceutical products based upon the total aerobic microbial count (TAMC) and the total combined yeasts/moulds count (TYMC), ♦it is possible to evaluate at the initial processing stage of the product whether the microbiological quality of the raw materials is adequate or not. Furthermore, it is then possible to implement appropriate corrective action as needed to maintain or improve the microbiological quality of the product. ♦ The target limits of microbial levels for raw materials (synthetic compounds and minerals) are shown in Table 1.

♦In general, synthetic compounds have low bioburden levels due to the high temperatures, organic solvents, etc., used in their manufacturing processes. Raw materials originated from plants and animals in general have higher bioburdens than synthetic compounds.

The microbial quality of the water used in the processing

of active ingredients or non-sterile pharmaceuticals may have a direct effect on the quality of the finished dosage form. This means it is necessary to keep the level of microbial contaminants in the water as low as possible. ♦

Acceptance criteria for microbiological quality for non-sterile finished dosage forms are shown in Table 2. ♦These microbial limits are based primarily on the type of dosage form, water activity, and so on. For oral liquids and pharmaceutical products having a high water activity, in general, low microbial acceptance criteria are given. ♦

Table 2 includes a list of specified micro-organisms for which acceptance criteria are set. The list is not necessarily

Table 1 Acceptance criteria for microbiological quality of non-sterile substances for pharmaceutical use

	Total Aerobic Microbial Count (CFU/g or CFU/mL)	Total Combined Yeasts/Moulds Count (CFU/g or CFU/mL)
Substances for pharmaceutical use	10 ³	10 ²

Table 2 Acceptance criteria for microbiological quality of non-sterile dosage forms

Route of administration	Total Aerobic Microbial Count (CFU/g or CFU/mL)	Total Combined Yeasts/Moulds Count (CFU/g or CFU/mL)	Specified Micro-organism
Non-aqueous preparations for oral use	10 ³	10 ²	Absence of <i>Escherichia coli</i> (1 g or 1 mL)
Aqueous preparations for oral use	10 ²	10 ¹	Absence of <i>Escherichia coli</i> (1 g or 1 mL)
Rectal use	10 ³	10 ²	—
Oromucosal use Gingival use Cutaneous use Nasal use Auricular use	10 ²	10 ¹	Absence of <i>Staphylococcus aureus</i> (1 g or 1 mL) Absence of <i>Pseudomonas aeruginosa</i> (1 g or 1 mL)
Vaginal use	10 ²	10 ¹	Absence of <i>Pseudomonas aeruginosa</i> (1 g or 1 mL) Absence of <i>Staphylococcus aureus</i> (1 g or 1 mL) Absence of <i>Candida albicans</i> (1 g or 1 mL)
Transdermal patches (limits for one patch including adhesive layer and backing)	10 ²	10 ¹	Absence of <i>Staphylococcus aureus</i> (1 patch) Absence of <i>Pseudomonas aeruginosa</i> (1 patch)
Inhalation use (more rigorous requirements apply to liquid preparations for nebulization)	10 ²	10 ¹	Absence of <i>Staphylococcus aureus</i> (1 g or 1 mL) Absence of <i>Pseudomonas aeruginosa</i> (1 g or 1 mL) Absence of bile-tolerant gram-negative bacteria (1g or 1 mL)

♦**Table 3** Acceptance criteria for crude drugs and crude drug preparations

Micro-organisms	Category 1 (CFU/g or CFU/mL)	Category 2 (CFU/g or CFU/mL)
Aerobic bacteria	10 ⁷	10 ⁵
Molds and yeasts	10 ⁴	10 ³
Enterobacteria and other gram-negative bacteria	※	10 ³
<i>Escherichia coli</i>	10 ²	Not detected
<i>Salmonella</i>	Not detected	Not detected
<i>Staphylococcus aureus</i>	※	※

※ : The limits are not specified. ♦

exhaustive and for a given preparation it may be necessary to test for other micro-organisms depending on the nature of the starting materials and the manufacturing process.

If it has been shown that none of the prescribed tests will allow valid enumeration of micro-organisms at the level prescribed, a validated method with a limit of detection as close as possible to the indicated acceptance criterion is used.

In addition to the micro-organisms listed in Table 2, the significance of other micro-organisms recovered should be evaluated in terms of:

- (i) the use of the product: hazard varies according to the route of administration (eye, nose, respiratory tract);
- (ii) the nature of the product: does the product support growth, does it have adequate antimicrobial preservation?
- (iii) the method of application;
- (iv) the intended recipient: risk may differ for neonates, infants, the debilitated;
- (v) use of immunosuppressive agents, corticosteroids;
- (vi) presence of disease, wounds, organ damage.

Where warranted, a risk-based assessment of the relevant factors is conducted by personnel with specialized training in microbiology and the interpretation of microbiological data.

For raw materials, the assessment takes account of processing to which the product is subjected, the current technology of testing and the availability of materials of the desired quality. Acceptance criteria are based on individual results or on the average of replicate counts when replicate counts are performed (e.g. direct plating methods).

When an acceptance criterion for microbiological quality is prescribed it is interpreted as follows:

- 10¹ CFU: maximum acceptable count = 20,
- 10² CFU: maximum acceptable count = 200,
- 10³ CFU: maximum acceptable count = 2000, and so forth.

♦6. Acceptance criteria for crude drugs

Target limits of microbial contamination for crude drugs and crude drug preparations are shown in Table 3. Category 1 includes crude drugs and crude drug preparations which are used for extraction by boiling water or to which boiling water is added before use. Category 2 includes crude drugs which are taken directly without extraction process and

directly consumed crude drug preparations containing powdered crude drugs. In this guideline, enterobacteria and other gram-negative bacteria, *Escherichia coli*, *Salmonella*, and *Staphylococcus aureus* are mentioned as specified micro-organisms, but other micro-organisms such as certain species of *Bacillus cereus*, *Clostridia*, *Pseudomonas*, *Burkholderia*, *Asperigillus* and *Enterobacter* species are also necessary to be tested depending on the origin of raw materials for crude drugs or the preparation method of crude drug preparations. ♦

Microbiological Evaluation of Processing Areas for Sterile Pharmaceutical Products

This chapter describes the methods for the control and evaluation of microbial contamination in areas used for the processing of sterile pharmaceutical products. Such processing areas are classified into critical areas and clean areas according to the required levels of air-cleanliness. A critical area is a defined space in which the airborne particulate and microorganism levels are controlled to meet grade A. The cleanliness requirements for such a space extend to the surfaces of the facilities and equipment which form or are located within the space, as well as to the supplied raw materials, chemicals, water, etc. Environmental conditions, such as temperature, humidity, and air pressure, are also controlled in this space when required. A clean area is a controlled space such that the levels of contaminants (particulates and microorganisms) in air, gases and liquids are maintained within specified limits, which are less stringent than those of grade A. When sterile pharmaceutical products are manufactured, the environment, facilities/equipment, and personnel should be routinely monitored to ensure appropriate microbiological control in the processing areas. The detection of microorganisms should be performed under normal operational conditions, using an appropriate sampling device, according to an environmental control program established previously. The sampling, cultivation, counting, and evaluation methods for airborne microorganisms, as well as those found on surfaces, should also be chosen appropriately, depending on the monitoring purpose, monitoring items, and microorganisms being detected. Sampling devices, measurement methods, media, culture conditions, frequency of monitoring, and recommended limits for environmental microorganisms shown in this chapter are for information only, and are not requirements.

1. Definitions

For the purposes of this chapter, the following definitions apply.

(i) Processing areas: Areas in which actions such as cultivation, extraction/purification, weighing of raw materials, washing and drying of containers and stoppers, preparation of solutions, filling, sealing and packaging are performed, including the gowning area.

(ii) Action levels: Established microbial levels (and type of microorganisms, if appropriate) that require immediate follow-up and corrective action if they are exceeded.

(iii) Alert levels: Established microbial levels (and type

of microorganisms if appropriate) that give early warning of a potential drift from normal operating conditions, but which are not necessarily grounds for definitive corrective action, though they may require follow-up investigation.

(iv) Contaminants: Particulates and microorganisms causing contamination by adhering to surfaces or by being incorporated into materials.

(v) Cleanliness: A quantity which indicates the condition of cleanliness of a monitored item, expressed as mass or number of contaminants contained in a certain volume or area.

(vi) Contamination control: The planning, establishment of systems and implementation activities performed in order to maintain the required cleanliness of a specified space or surface.

(vii) Shift: Scheduled period of work or production, usually less than 12 hours in length, during which operations are conducted by a single defined group of workers.

(viii) Characterization of contaminants: Procedures for classifying contaminants so that they can be differentiated. In routine control, classification to the genus level is sufficient; as required, identification to the species level is performed.

2. Air-cleanliness of processing areas for sterile pharmaceutical products

Airborne particulates in areas used for the processing of pharmaceutical products may act physically as a source of insoluble particles in the products, and biologically as a carrier of microorganisms. So, it is necessary to control strictly the number of particles in the air. The air-cleanliness criteria are shown in Table 1.

2.1. Terminally sterilized products

Solutions should generally be prepared in a grade C environment. Solution preparation may be permitted in a grade D environment if additional measures are taken to minimize contamination. For parenterals, filling should be done in a grade A workstation in a grade B or C environment. The requirements during the preparation and filling of other sterile products are generally similar to those for parenterals.

2.2. Sterile products prepared aseptically after filtration

The handling of starting materials and the preparation of solutions should be done in a grade C environment. These activities may be permitted in a grade D environment if additional measures are taken to minimize contamination, such as the use of closed vessels prior to filtration. After sterile

filtration, the product must be handled and filled into containers under grade A aseptic conditions.

2.3. Sterile products prepared aseptically from sterile starting materials

The handling of starting materials and all further processing should be done under grade A conditions.

3. Microbiological environmental monitoring program

Environmental monitoring is especially important in sterility assurance for sterile pharmaceutical products that are manufactured by aseptic processing. The major purpose of environmental monitoring is to predict potential deterioration of the processing environment before it occurs, and to produce high-quality, sterile pharmaceutical products under appropriate contamination control.

3.1. Monitoring of environmental microorganisms

(i) An environmental control program document is prepared for each area used for the processing of sterile pharmaceutical products. The procedures in the document include: 1) items to be monitored, 2) type of microorganisms to be monitored, 3) frequency of monitoring, 4) methods of monitoring, 5) alert and action levels, and 6) actions to be taken when specified levels are exceeded.

(ii) The aseptic processing areas and other processing areas maintained under controlled conditions are monitored on a routine basis. The critical processing areas where sterile products are in contact with environmental air are monitored during every operational shift. The items to be monitored include the air, floor, walls, equipment surfaces, and the gowns and gloves of the personnel. Table 2 shows suggested frequencies of the environmental monitoring.

(iii) The sampling devices used for monitoring environmental microorganisms, as well as the methods and culture media, should be suitable to detect microorganisms that may be present (aerobic bacteria, anaerobic bacteria, molds, yeast, etc.). The cultivation conditions, such as incubation temperature and time, are selected to be appropriate for the specific growth requirements of microorganisms to be detected. Table 3 shows the culture media and cultivation conditions that are generally used in testing for environmental microorganisms.

(iv) The number of microorganisms in the samples is estimated by using the Membrane Filtration, Pour Plating, Spread Plating, or Serial Dilution (Most Probable Number) Methods described in the Microbial Limit Test.

(v) Table 4 shows recommended limits for environmental

Table 1 Air-cleanliness requirements for processing of sterile pharmaceutical products

Air cleanliness	Maximum number of airborne particulates per m ³	
	at rest	in operation
	≥ 0.5 μm	≥ 0.5 μm
Grade* ¹		
A (Laminar-airflow zone)	3530	3530
B (Non laminar-airflow zone)	3530	353000
C	353000	3530000
D	3530000	—* ²

*1 The maximum permitted number of particles in the “in operation” condition corresponds to the standards described under USP <1116> as follows.

Grade A: Class 100 (M3.5); Grade B: Class 10,000 (M5.5); Grade C: Class 100,000 (M6.5);
Grade D: no corresponding standard.

*2 The limit for this area will depend on the nature of the operation carried out there.

Table 2 Suggested frequency of environmental monitoring

Processing area	Frequency of monitoring
Critical area (Grade A)	Each shift
Clean area adjacent to critical area (Grade B)	Each shift
Other clean areas (Grade C, D)	
Potential product/container contact areas	Twice a week
Non-product/container contact areas	Once a week

Table 3 Media and culture conditions

Microorganisms to be detected	Media* ¹	Culture conditions
Aerobes	Soybean-casein digest agar (or fluid) medium Brain-heart infusion agar (or fluid) medium Nutrient agar (or fluid) medium	30 – 35°C* ² More than 5 days* ³
Yeast and fungi	Soybean-casein digest agar (or fluid) medium Sabouraud dextrose agar (or fluid) medium Potato-dextrose agar (or fluid) medium Glucose peptone agar (or fluid) medium	20 – 25°C* ² More than 5 days* ³
Anaerobes* ⁴	Soybean-casein digest agar medium Fluid cooked meat medium Reinforced clostridial agar (or fluid) medium Thioglycolate medium I (or thioglycolate agar medium) for sterility test	30 – 35°C More than 5 days* ³

*1 If necessary, antibiotics may be added to media in an appropriate concentration (see Microbial Limit Test). If the existence of disinfectants that may interfere with the test on the surface of the specimen is suspected, add a substance to inactivate them.

*2 When soybean-casein digest agar medium is used for the detection of aerobes, yeast and fungi, incubation at 25 to 30°C for more than 5 days is acceptable.

*3 If a reliable count is obtained in a shorter incubation time than 5 days, this may be adopted.

*4 Generally, anaerobes are not targets for the monitoring. For the detection of anaerobes, agar medium is incubated in an appropriate anaerobic jar.

Table 4 Recommended limits for environmental microorganisms*¹

Grade	Airborne microorganisms* ² (CFU/m ³)	Minimum air sample (m ³)	CFU on a surface	
			instruments/facilities (CFU/24–30 cm ²)* ³	gloves
A	< 1	0.5	< 1	< 1
B	10	0.5	5	5
C	100	0.2	25	—
D	200	0.2	50	—

*1 Maximum acceptable average numbers of microorganisms under each condition.

*2 These values are by using a slit sampler or equivalent.

*3 Viable microbe cell number per contact plate (5.4 – 6.2 cm in diameter). When swabbing is used in sampling, the number of microorganisms is calculated per 25 cm². For gloves, usually, put their all fingers on the plate.

microorganisms. The alert and action levels may be adjusted if necessary after sufficient data have been accumulated. The most important point in environmental monitoring is to confirm that an acceptable value of each monitoring item is maintained consistently.

(vi) Microorganisms isolated are characterized if necessary. In addition, analysis of hourly or daily variation of airborne particulate numbers will provide data to assist in the control of the cleanliness of processing areas.

3.2. Evaluation of environmental monitoring data

(i) The data from the environmental monitoring are evaluated on a routine basis for each area and location. The

source of any discrepancy should be investigated immediately and the investigation should be documented in a report. After corrective action has been taken, follow-up monitoring should be done to demonstrate that the affected area is once again within specification.

(ii) The report is reviewed and approved by personnel responsible for quality control and distributed to all key personnel associated with the aseptic processing operation.

4. Sampling devices and measuring methodology

Various types of sampling devices and measurement methods are available for the sampling and measurement of

microorganisms in the air and on surfaces, and appropriate samplers and measuring methodology are selected according to the purpose of monitoring and the items to be monitored.

4.1. Evaluation of airborne microorganisms

4.1.1. Settle plates

Petri dishes of a specified diameter containing a suitable culture medium are placed at the measurement location and the cover is removed there. The plates are exposed for a given time and the microorganisms deposited from the air onto the agar surface are enumerated after incubation. This method is not effective for quantitative monitoring of total airborne microorganisms because it does not detect microorganisms that do not settle onto the surface of the culture media, and the settling velocity of aggregates of microorganisms is affected by air currents and disturbances in airflow. Although the results obtained by the settle plate method are only qualitative or semi-quantitative, this method is suitable for long-term evaluation of possible contamination of products or devices by airborne microorganisms.

4.1.2. Active microbial sampling methods

4.1.2.1. Measuring methods

Methods in which a fixed volume of air is aspirated include filtration-type sampling devices and impact-type sampling devices. With the filter-type sampling devices the desired volume of air can be collected by appropriately changing the air intake rate or the filter size. However, care must be taken to ensure that sterility is maintained while the filter is placed in and removed from the holder. When air sampling devices are used in critical areas, care must be taken to avoid disturbance of the airflow around the products. There are two types of filters; wet-type used gelatin filters and dry-type used membrane filters. With the dry-type filters, static electricity effects can make it impossible to collect quantitatively microorganisms on the filter. When an impact-type sampling device is used, the following points are important: 1) The speed at which the collected air strikes the culture medium surface must be sufficient to capture the microorganisms, but must not have an adverse effect on the collected microorganisms. 2) A sufficient volume of air must be sampled so that even extremely low levels of microbiological contaminants are detected, but the procedure must not cause a significant change in the physical or chemical properties of the culture medium. 3) When the device is used in critical areas, care must be exercised to ensure that the processing of the sterile pharmaceutical products is not adversely affected by the air disturbance.

4.1.2.2. Sampling devices

The most commonly used samplers are as follows: Slit sampler, Andersen sampler, pinhole sampler, centrifugal sampler and filtration-type sampler. Each sampler has specific characteristics. The slit sampler is a device to trap microorganisms in a known volume of air passed through a standardized slit. The air is impacted on a slowly revolving Petri dish containing a nutrient agar. The rotation rate of the Petri dish and the distance from the slit to the agar surface are adjustable and it is possible to estimate the number of microorganisms in the air passed through the device for a period of up to 1 hr. The Andersen sampler consists of a perforated cover and several pieces of Petri dishes containing a nutrient agar, and a known volume of air passed through the perforated cover impacts on the agar medium in the Petri dishes. The sampler is suitable for the determination of the distribution of size ranges of microorganism particulates in

the air. The pinhole sampler resembles the slit of the slit sampler, but has pinholes in place of the slit. A known volume of air passed through several pinholes impacts on agar medium in a slowly revolving Petri dish. The centrifugal sampler consists of a propeller that pulls a known volume of air into the device and then propels the air outward to impact on a tangentially placed nutrient agar strip. The sampler is portable and can be used anywhere, but the sampling volume of air is limited.

See above the Measuring methods <4.1.2.1> on the characteristics of the filtration-type sampler.

4.2. Measurement methods for microorganisms on surfaces

4.2.1. Contact plates

Use a contact plate with an appropriate contact surface. The culture medium surface should be brought into contact with the sampling site for several seconds by applying uniform pressure without circular or linear movement. After contact and removal, the plates are covered and, as soon as possible, incubated using appropriate culture conditions. After a contact plate has been used, the site to which the plate was applied must be wiped aseptically to remove any adherent culture medium.

4.2.2. Swabs

A piece of sterilized gauze, absorbent cotton, cotton swab, or other suitable material premoistened with an appropriate rinse fluid is stroked in closely parallel sweeps or slowly rotated over the defined sampling area. After sampling, the swab is agitated in a specified amount of an appropriate sterilized rinse fluid, and the rinse fluid is assayed for viable organisms.

5. Test methods for collection performance of a sampling device for airborne microorganisms

The testing of the collection performance of sampling devices for airborne microorganisms is performed in accordance with JIS K 3836 (Testing methods for collection efficiency of airborne microbe samplers) or ISO 14698 - 1 (Cleanrooms and associated controlled environments. Biocontamination control. General principles).

6. Growth-promotion test of media and confirmation of antimicrobial substances

This test and confirmation are performed according to "Effectiveness of culture media and confirmation of antimicrobial substances" in the Microbial Limit Test <4.05>.

7. Media

(i) Soybean-casein digest agar medium or Fluid soybean-casein digest medium

See Microbial Limit Test.

(ii) Sabouraud dextrose agar medium or Fluid sabouraud dextrose medium

See Microbial Limit Test. Antibiotic is added if necessary.

(iii) Potato-dextrose agar medium or Fluid potato-dextrose medium

See Microbial Limit Test. Antibiotic is added if necessary.

(iv) Glucose peptone agar medium or Fluid glucose peptone medium

Glucose	20.0 g
Yeast extract	2.0 g

Magnesium sulfate heptahydrate	0.5 g
Peptone	5.0 g
Potassium dihydrogen phosphate	1.0 g
Agar	15.0 g
Water	1000 mL

Mix all the components, and sterilize by heating in an autoclave at 121°C for 15 to 20 minutes. pH after sterilization: 5.6 – 5.8. Just prior to use, add 0.10 g of benzylpenicillin potassium and 0.10 g of tetracyclin per liter of medium as sterile solutions or, alternatively, add 50 mg of chloramphenicol per liter of medium. Antibiotics are added, as appropriate.

(v) Thioglycolate agar medium or thioglycolate medium I for sterility test

See Sterility Test. The agar concentration of Thioglycolate agar medium is about 1.5%.

(vi) Brain-heart infusion agar medium or Fluid brain-heart infusion medium

Bovine brain extract powder*¹

An amount equivalent to 200 g of calf brain

Bovine heart extract powder*²

An amount equivalent to 250 g of the material

Peptone	10.0 g
Glucose	2.0 g
Sodium chloride	5.0 g

Disodium hydrogenphosphate dodecahydrate 2.5 g

Agar 15.0 g

Water 1000 mL

Sterilize by heating in an autoclave at 121°C for 15 to 20 min. pH after sterilization: 7.2 – 7.6.

(vii) Nutrient agar medium or Fluid nutrient medium

Meat extract 3.0 g

Peptone 5.0 g

Agar 15.0 g

Water 1000 mL

Sterilize by heating in an autoclave at 121°C for 15 to 20 min. pH after sterilization: 6.6 – 7.0.

(viii) Fluid cooked meat medium

Bovine heart extract powder*²

An amount equivalent to 450 g of the material

Peptone 20.0 g

Glucose 2.0 g

Sodium chloride 5.0 g

Water 1000 mL

Sterilize by heating in an autoclave at 121°C for 15 to 20 min. pH after sterilization: 7.2 – 7.6.

(ix) Reinforced clostridial agar medium or Fluid reinforced clostridial medium

Meat extract 10.0 g

Peptone 10.0 g

Yeast extract 3.0 g

Soluble starch 1.0 g

Glucose 5.0 g

L-Cystein hydrochloride monohydrate 0.5 g

Sodium chloride 5.0 g

Sodium acetate trihydrate 3.0 g

Agar 15.0 g

Water 1000 mL

For fluid medium, add 0.5 g of agar. Sterilize by heating in an autoclave at 121°C for 15 to 20 min. pH after sterilization: 6.7 – 6.9.

*1 **Bovine brain extract powder** Dried extract of bovine fresh brain. A yellow-brown powder having a characteristic odor.

Loss on drying: not more than 5%.

*2 **Bovine heart extract powder** Dried extract of bovine fresh heart. A yellow-brown powder having a characteristic odor.

Loss on drying: not more than 5%.

8. Rinsing Liquids

(i) Phosphate buffer (pH 7.2)

See Microbial Limit Test.

(ii) Buffered sodium chloride-peptone solution (pH 7.0)

See Microbial Limit Test.

(iii) Ringer's solution, 1/4 concentration

Sodium chloride 2.25 g

Potassium chloride 0.105 g

Calcium chloride dihydrate 0.16 g

Water 1000 mL

Sterilize by heating in an autoclave at 121°C for 15 to 20 min. pH after sterilization: 7.0.

(iv) Thiosulfate-Ringer's solution

Sodium thiosulfate pentahydrate 0.8 g

Ringer's solution, 1/4 concentration 1000 mL

Sterilize by heating in an autoclave at 121°C for 15 to 20 min. pH after sterilization: 6.6.

(v) LP liquid

Casein peptone 1.0 g

Soybean lecithin 0.7 g

Polysorbate 80 1.0 – 20.0 g

Water 1000 mL

Sterilize by heating in an autoclave at 121°C for 15 to 20 min. pH after sterilization: 7.2.

Preservatives-Effectiveness Tests

The purpose of the Preservatives-Effectiveness Tests is to assess microbiologically the preservative efficacy, either due to the action of product components themselves or any added preservative(s), for multi-dose containers. The efficacy of the preservatives is assessed by direct inoculation and mixing of the test strains in the product, and titration of survival of the test strains with time.

Preservatives must not be used solely to comply with GMP for drugs or to reduce viable aerobic counts. In addition, preservatives themselves are toxic substances. Therefore, preservatives must not be added to products in amounts which might jeopardize the safety of human beings, and consideration must be given to minimizing the amounts of preservative used. These tests are commonly used to verify that products maintain their preservative effectiveness at the design phase of formulation or in the case of periodic monitoring. Although these tests are not performed for lot

release testing, the efficacy of the preservative present in the product packaged in the final containers should be verified throughout the entire dating period.

1. Products and their Categories

The products have been divided into two categories for these tests. Category I products are those made with aqueous bases or vehicles, and Category II products are those made with nonaqueous bases or vehicles. Oil-in-water emulsions are considered Category I products, and water-in-oil emulsions Category II products. Category I is further divided into three subtypes depending on the dosage forms.

Category IA: Injections and other sterile parenterals

Category IB: Non-sterile parenterals

Category IC: Oral products in liquid forms (including syrup products to be dissolved or suspended before use)

Category II: All the dosage forms listed under Category I made with non-aqueous bases or vehicles.

2. Test Microorganisms and Culture Media

The following strains or those considered to be equivalent are used as the test microorganisms.

Escherichia coli ATCC 8739, NBRC 3972

Pseudomonas aeruginosa ATCC 9027, NBRC 13275

Staphylococcus aureus ATCC 6538, NBRC 13276

Candida albicans ATCC 10231, NBRC 1594, JCM 2085

Aspergillus brasiliensis ATCC 16404, NBRC 9455

These test microorganisms are representative of those that might be found in the environment in which the product is manufactured, used or stored, and they are also recognized as opportunistic pathogens. In addition to these strains designated as test microorganisms, it is further recommended to use strains that might contaminate the product and grow on or in it, depending on its characteristics. For the test microorganisms received from coordinated collections of microorganisms, one passage is defined as the transfer of microorganisms from an established culture to fresh medium, and microorganisms subjected to not more than five passages should be used for the tests. Single-strain challenges rather than mixed cultures should be used. The test strains can be harvested by growth on solid agar or liquid media.

Cultures on agar plate media: Inoculate each of the five test strains on the surface of agar plates or agar slants. For growth of bacteria, use Soybean-Casein Digest Agar Medium, and for yeasts and moulds, use Sabouraud Glucose Agar, Glucose-Peptone Agar or Potato Dextrose Agar Medium. Incubate bacterial cultures at 30°C to 35°C for 18 to 24 hours, the culture of *C. albicans* at 20°C to 25°C for 40 to 48 hours and the culture of *A. brasiliensis* at 20°C to 25°C for one week or until good sporulation is obtained. Harvest these cultured cells aseptically using a platinum loop, etc. Suspend the collected cells in sterile physiological saline or in 0.1% Peptone Water and adjust the viable cell count to about 10^8 microorganisms per mL. In the case of *A. brasiliensis*, suspend the cultured cells in sterile physiological saline or 0.1% Peptone Water containing 0.05 w/v% of polysorbate 80 and adjust the spore count to about 10^8 per mL. Use these suspensions as the inocula.

Liquid cultures: After culturing each of the four strains except for *A. brasiliensis* in a suitable medium, remove the medium by centrifugation. Wash the cells in sterile physiological saline or 0.1% Peptone Water and resuspend them in the same solution with the viable cell or spore count of the inoculum adjusted to about 10^8 per mL.

When strains other than the five listed above are cultured, select a culture medium suitable for growth of the strain concerned. The cell suspension may also be prepared by a method suitable for that strain. Use the inoculum suspensions within 24 hours after they have been prepared from the cultivations on agar plate media or in liquid media. Store the inoculum suspensions in a refrigerator if it is not possible to inoculate them into the test specimens within 2 hours. Titrate the viable cell count of the inocula immediately before use, and then calculate the theoretical viable cell count per mL (g) of the product present just after inoculation.

3. Test Procedure

3.1. Category I products

Inject each of the cell suspensions aseptically into five containers containing the product and mix uniformly. When it is difficult to inject the cell suspension into the container aseptically or the volume of the product in each container is too small to be tested, transfer aseptically a sufficient volume of the product into each of alternative sterile containers, and mix the inoculum. When the product is not sterile, incubate additional containers containing the uninoculated product as controls and calculate their viable cell counts (the viable counts of bacteria and those of yeasts and moulds). A sterile syringe, spatula or glass rod may be used to mix the cell suspension uniformly in the product. The volume of the suspension mixed in the product must not exceed 1/100 of the volume of the product. Generally, the cell suspension is inoculated and mixed so that the concentration of viable cells is 10^5 to 10^6 cells per mL or per gram of the product. Incubate these inoculated containers at 20°C to 25°C with protection from light, and calculate the viable cell count of 1 mL or 1 g of the product taken at 0, 14 and 28 days subsequent to inoculation. Record any marked changes (e.g., changes in color or the development of a bad odor) when observed in the mixed samples during this time. Such changes should be considered when assessing the preservative efficacy of the product concerned. Express sequential changes in the viable counts as percentages, with the count at the start of the test taken as 100. Titration of the viable cell counts is based, in principle, on the Pour Plate Methods in "Microbial Limit Tests". In this case, confirm whether any antimicrobial substance is present in the test specimen. If a confirmed antimicrobial substance needs to be eliminated, incorporate an effective inactivator of the substance in the buffer solution or liquid medium to be used for dilution of the test specimen, as well as in the agar plate count medium. However, it is necessary to confirm that the inactivator has no effect on the growth of the microorganisms. When the occurrence of the preservative or the product itself affects titration of the viable cell count and there is no suitable inactivator available, calculate the viable cell counts by the Membrane Filtration Method in "Microbial Limit Tests".

3.2. Category II products

The procedures are the same as those described for Category I products, but special procedures and considerations are required for both uniform dispersion of the test microorganism in the product and titration of viable cell counts in the samples.

For semisolid ointment bases, heat the sample to 45°C to 50°C until it becomes oily, add the cell suspension and disperse the inoculum uniformly with a sterile glass rod or spatula. Surfactants may also be added to achieve uniform

dispersion, but it is necessary to confirm that the surfactant added has no effect on survival or growth of the test microorganisms and that it does not potentiate the preservative efficacy of the product. For titration of the viable cell count, a surfactant or emulsifier may be added to disperse the product uniformly in the buffer solution or liquid medium. Sorbitan monooleate, polysorbate 80 or lecithin may be added to improve miscibility between the liquid medium and semisolid ointments or oils in which test microorganisms were inoculated. These agents serve to inactivate or neutralize many of the most commonly used preservatives.

Table 1 Interpretation criteria by product category

Product category	Microorganisms	Interpretation criteria	
		After 14 days	After 28 days
Category IA	Bacteria	0.1% of inoculum count or less	Same or less than level after 14 days
	Yeasts/moulds	Same or less than inoculum count	Same or less than inoculum count
Category IB	Bacteria	1% of inoculum count or less	Same or less than level after 14 days
	Yeasts/moulds	Same or less than inoculum count	Same or less than inoculum count
Category IC	Bacteria	10% of inoculum count or less	Same or less than level after 14 days
	Yeasts/moulds	Same or less than inoculum count	Same or less than inoculum count
Category II	Bacteria	Same or less than inoculum count	Same or less than inoculum count
	Yeasts/moulds	Same or less than inoculum count	Same or less than inoculum count

4. Interpretation

Interpret the preservative efficacy of the product according to Table 1. When the results described in Table 1 are obtained, the product examined is considered to be effectively preserved. There is a strong possibility of massive microbial contamination having occurred when microorganisms other than the inoculated ones are found in the sterile product to be examined, and caution is required in the test procedures and/or the control of the manufacturing process of the product. When the contamination level in a nonsterile product to be examined exceeds the microbial enumeration limit specified in "Microbial Attributes of Nonsterile Pharmaceutical Products" in General Information, caution is also required in the test procedures and/or the control of the manufacturing process of the product.

5. Culture Media

Culture media and buffer solution used for Preservatives-

Effectiveness Tests are described below. Other media may be used if they have similar nutritive ingredients and selective and growth-promoting properties for the microorganisms to be tested.

- (i) Soybean Casein Digest Agar Medium
- | | |
|-----------------|---------|
| Casein peptone | 15.0 g |
| Soybean peptone | 5.0 g |
| Sodium chloride | 5.0 g |
| Agar | 15.0 g |
| Water | 1000 mL |

Mix all of the components and sterilize at 121°C for 15 – 20 minutes in an autoclave. pH after sterilization: 7.1 – 7.3.

- (ii) Sabouraud Glucose Agar Medium
- | | |
|------------------------------------|---------|
| Peptone (animal tissue and casein) | 10.0 g |
| Glucose | 40.0 g |
| Agar | 15.0 g |
| Water | 1000 mL |

Mix all of the components and sterilize at 121°C for 15 – 20 minutes in an autoclave. pH after sterilization: 5.4 – 5.8.

- (iii) Glucose Peptone (GP) Agar Medium
- | | |
|--------------------------------|---------|
| Glucose | 20.0 g |
| Yeast extract | 2.0 g |
| Magnesium sulfate heptahydrate | 0.5 g |
| Peptone | 5.0 g |
| Monobasic potassium phosphate | 1.0 g |
| Agar | 15.0 g |
| Water | 1000 mL |

Mix all of the components and sterilize at 121°C for 15 – 20 minutes in an autoclave. pH after sterilization: 5.6 – 5.8.

- (iv) Potato Dextrose Agar Medium
- | | |
|----------------|---------|
| Potato extract | 4.0 g |
| Glucose | 20.0 g |
| Agar | 15.0 g |
| Water | 1000 mL |

Mix all of the components and sterilize at 121°C for 15 – 20 minutes in an autoclave. pH after sterilization: 5.4 – 5.8.

- (v) 0.1% Peptone Water
- | | |
|-----------------|---------|
| Peptone | 1.0 g |
| Sodium chloride | 8.0 g |
| Water | 1000 mL |

Mix all of the components and sterilize at 121°C for 15 – 20 minutes in an autoclave. pH after sterilization: 7.2 – 7.4.

Rapid Counting of Microbes using Fluorescent Staining

This chapter provides rapid methods using fluorescence staining for the quantitative estimation of viable microorganisms. Incubation on an agar medium has been widely used for quantitative estimation of viable microorganisms, but a number of environmental microorganisms of interest are not easy to grow in culture under usual conditions, thus new microbial detection methods based on fluorescence or luminescence have been developed. In the fluorescence staining method, microorganisms are stained with fluorescent dye, and can easily be detected and counted with various sorts of apparatus, such as a fluorescence microscope or flow cytometer. Methods are available to detect total

microorganisms, including both dead and viable cells, or to detect only cells with a specified bioactivity by choosing the dye reagent appropriately. Nucleic acid staining reagents, which bind with DNA or RNA, detect all cells containing nucleic acids, whether they are live or dead. This technique is the most fundamental for the fluorescence staining method. On the other hand, fluorescent vital staining methods target the respiratory activity of the microorganism and the activity of esterase, which is present universally in microorganisms. In the microcolony method, microcolonies in the early stage of colony formation are counted. The CFDA-DAPI double staining method and the microcolony method are described below. These methods can give higher counts than the other techniques, because these rapid and accurate techniques provide quantitative estimation of viable microorganisms based on a very specific definition of viability, which may be different from that implicit in other methods. The procedures of these methods described here may be changed as experience with the methods is accumulated. Therefore, other reagents, instruments and apparatus than those described here may also be used if there is a valid reason for so doing.

1. CFDA-DAPI double staining method

Fluorescein diacetate (FDA) reagents are generally used for the detection of microorganisms possessing esterase activity. These reagents are hydrolyzed by intracellular esterase, and the hydrolyzed dye exhibits green fluorescence under blue excitation light (about 490 nm). Modified FDAs such as carboxyfluorescein diacetate (CFDA) are used because of the low stainability of gram-negative bacteria with FDA. The principle of the CFDA-DAPI double staining method, which also employs a nucleic acid staining reagent, 4',6-diamidino-2-phenylindole (DAPI), is as follows. The nonpolar CFDA penetrates into the cells and is hydrolyzed to fluorescent carboxyfluorescein by intracellular esterase. The carboxyfluorescein is accumulated in the living cells due to its polarity, and therefore green fluorescence due to carboxyfluorescein occurs when cells possessing esterase activity are illuminated with blue excitation light. No fluorescent carboxyfluorescein is produced with dead cells, since they are unable to hydrolyze CFDA. On the other hand, DAPI binds preferentially to the adenine and thymine of DNA after penetration into both viable and dead microorganisms, and consequently all of the organisms containing DNA exhibit blue fluorescence under ultraviolet excitation light. Therefore, this double staining method enables to count specifically only live microorganisms possessing esterase activity under blue excitation light, and also to determine the total microbial count (viable and dead microorganisms) under ultraviolet excitation light.

1.1. Apparatus

1.1.1. Fluorescence microscope or fluorescence observation apparatus

Various types of apparatus for counting fluorescence-stained microorganisms are available. Appropriate filters are provided, depending on the fluorescent dye reagents used. A fluorescence microscope, laser microscope, flow cytometer, and various other types of apparatus may be used for fluorescence observation.

1.2. Instruments

- (i) Filtering equipment (funnels, suction flasks, suction pumps)
- (ii) Membrane filters (poresize: 0.2 μm); A suitable filter

that can trap particles on the surface can be used such as polycarbonate filter, alumina filter, etc.

- (iii) Glass slide
- (iv) Cover glass
- (v) Ocular micrometer for counting (with 10 \times 10 grids)

1.3. Procedure

An example of the procedure using fluorescence microscope is described below.

1.3.1. Preparation of samples

Prepare samples by ensuring that microbes are dispersed evenly in the liquid (water or buffer solution).

1.3.2. Filtration

Set a membrane filter (poresize: 0.2 μm) on the funnel of the filtering equipment. Filter an appropriate amount of sample to trap microbes in the sample on the filter.

1.3.3. Staining

Pour sufficient amount of buffer solution for CFDA staining, mixed to provide final concentration of 150 $\mu\text{g/mL}$ of CFDA and 1 $\mu\text{g/mL}$ of DAPI, into the funnel of the filtering equipment and allow staining in room temperature for 3 minutes, then filter the liquid by suction. Pour in sufficient amount of aseptic water, suction filter and remove excess fluorescent reagent left on the filter. Thoroughly dry the filter.

1.3.4. Slide preparation

Put one drop of immersion oil for fluorescence microscope on the glass slide. Place the air dried filter over it, with the filtering side on the top. Then put one drop of immersion oil for fluorescence microscope on the surface of the filter, place a cover glass. Put another drop of immersion oil for fluorescence microscope on the cover glass when using an oil immersion objective lens.

1.3.5. Counting

Observe and count under fluorescence microscope, with 1000 magnification. In case of CFDA-DAPI double staining method, count the microorganisms (with esterase activity) exhibiting green fluorescence under the blue excitation light first to avoid color fading by the ultraviolet light, then count the microorganisms (with DNA) exhibiting blue fluorescence under the ultraviolet excitation light in the same microscopic field. Count the organisms exhibiting fluorescence on more than 20 randomly selected fields among 100 grids observed through an ocular micrometer of the microscope, and calculate the total number of organisms using the following formula. The area of the microscopic field should be previously determined with the ocular and objective micrometers. The amount of the sample to be filtered must be adjusted so that the cell number per field is between 10 and 100. It might be necessary to reprepare the sample in certain instances. (In such case that the average count number is not more than 2 organisms per field, or where more than 5 fields are found which have no organism per field, it is assumed that the microorganism count is below the detection limit.)

Number of microbes (cells/mL)

$$= \{(\text{average number of microbes per visual field}) \times (\text{area of filtration})\} / \{(\text{amount of sample filtered}) \times (\text{area of one microscopic field})\}$$

1.4. Reagents and test solutions

- (i) Aseptic water: Filter water through a membrane filter with 0.2 μm pore size to remove particles, then sterilize it by heating in an autoclave at 121°C for 15 minutes. Water for injection may be used.

(ii) CFDA solution, 10 mg/mL: Dissolve 50 mg of CFDA in dimethylsulfoxide to prepare a 5 mL solution. Store at -20°C in light shielded condition.

(iii) Buffer solution for CFDA staining: Dissolve 5 g of sodium chloride with 0.5 mL of 0.1 mol/L disodium dihydrogen ethylenediamine tetraacetate TS and diluted disodium hydrogen phosphate TS (1 in 3) to prepare 100 mL of solution. Add sodium dihydrogen phosphate dihydrate solution (1 in 64) to adjust the pH level to 8.5. Filter the solution through a membrane filter with a pore size of $0.2\ \mu\text{m}$.

(iv) DAPI solution, 10 μg /mL: Dissolve 10 mg of DAPI in 100 mL of aseptic water. Dilute this solution 10 times with aseptic water and filter through a membrane filter with a pore size of $0.2\ \mu\text{m}$. Store at 4°C in light shielded condition.

(v) Immersion oil for fluorescence microscope

2. Microcolony method

Microcolonies, which are in early stages of colony formation, are fluorescently stained, then observed and counted under fluorescence microscope or other suitable systems. This method enables to count the number of proliferative microorganisms, with short incubation time. In this method, the organisms are trapped on a membrane filter, the filter is incubated on a medium for a short time, and the microcolonies are counted. By this method, even colonies which are undetectable with the naked eye can be identified, so viable organisms can be counted rapidly and with high precision. Various nucleic acid staining reagents can be used for staining of microcolonies.

2.1. Apparatus

2.1.1. Fluorescence microscope or fluorescence observation apparatus

Various types of apparatus for counting fluorescence-stained microorganisms are available. Appropriate filters are provided, depending on the fluorescence dye reagents used. A fluorescence microscope, laser microscope and various other types of apparatus may be used for fluorescence observation.

2.2. Instruments

(i) Filtering equipment (funnels, suction flasks, suction pumps)

(ii) Membrane filters (pore size: $0.2\ \mu\text{m}$); A suitable filter that can trap particles on the surface can be used such as polycarbonate filter, alumina filter, etc.

(iii) Glass slide

(iv) Cover glass

(v) Filter paper (No. 2)

(vi) Ocular micrometer for counting (with 10×10 grids)

2.3. Procedure

An example of the procedure using a fluorescence microscope is described below.

2.3.1. Preparation of samples

Prepare samples by ensuring that microbes are dispersed evenly in the liquid (water or buffer solution).

2.3.2. Filtration

Set a membrane filter (pore size: $0.2\ \mu\text{m}$) on the funnel of the filtering equipment. Filter an appropriate amount of sample to trap microbes in the sample on the filter.

2.3.3. Incubation

Remove the filter from the filtering equipment and place it with filtering side facing up on a culture medium avoiding formation of airbubbles between the filter and the medium. Incubate at a suitable temperature for appropriate hours in a

dark place. It should be noted that the appropriate incubation conditions (such as medium, incubation temperature and/or incubation time) are different, depending on the sample.

2.3.4. Fixation

Soak filter paper with an appropriate amount of neutral buffered formaldehyde test solution, then place the filter that has been removed from the culture medium on top with filtering side up, and allow to remain at room temperature for more than 30 minutes to fix the microcolonies.

2.3.5. Staining

Soak filter paper with an appropriate amount of staining solution (such as $1\ \mu\text{g}/\text{mL}$ of DAPI, 2% polyoxyethylenesorbitan monolaurate), then place the filter on top with filtering side up, and then leave at room temperature, light shielded for 10 minutes to stain microcolonies. Wash the filter by placing it with the filtering side facing up on top of a filter paper soaked with aseptic water for 1 minute. Thoroughly air dry the filter.

2.3.6. Slide preparation

Put one drop of immersion oil for fluorescence microscope on the slide glass. Place an air dried filter over it, with the filtering side on the top. Then, put one drop of immersion oil for fluorescence microscope on top, place a cover glass.

2.3.7. Counting

Count the organisms exhibiting fluorescence on more than 20 randomly selected fields among the 100 grids observed through an ocular micrometer of the microscope with 400 or 200 magnification, and calculate the total number of organisms using the following formula. The area of the microscopic field should be previously determined with the ocular and objective micrometers. In such case that the average count number is not more than 2 microcolonies per field, or where more than 5 fields are found which have no microcolony per field, it is assumed that the microorganism count is below the detection limit.

Number of microcolonies (cells/mL)

$$= \{(\text{average number of microcolonies per visual field}) \times (\text{area of filtration})\} / \{(\text{amount of sample filtered}) \times (\text{area of one microscopic field})\}$$

2.4. Reagents and test solutions

(i) Aseptic water: Filter water through a membrane filter with $0.2\ \mu\text{m}$ pore size to remove particles, and sterilize it by heating in an autoclave at 121°C for 15 minutes. Water for injection may be used.

(ii) Staining solution: Dissolve 10 mg of DAPI in 100 mL of aseptic water. Dilute the solution 10 times with aseptic water and filter through a membrane filter with pore size of $0.2\ \mu\text{m}$. Store at 4°C in light shielded condition. Dissolve polyoxyethylene sorbitan monolaurate to the final concentration of 2%, when using.

(iii) Neutral buffered formaldehyde solution (4w/v% formaldehyde solution; neutrally buffered).

(iv) Immersion oil for fluorescence microscope

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.

1. Apparatuses

(i) DNA sequencer

Various types of sequencers used a gel board or capillary can be used.

(ii) DNA amplifier

To amplify target DNA and label amplified (PCR) products with sequencing reagents.

2. Procedures

The following procedures are described as an example.

2.1. 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 from culture fluid is better because some of colony samples can disturb PCR reaction.

2.2. PCR

Add 2 μ L of template DNA in PCR reaction solution. Use 10F/800R primers (or 800F/1500R primers in the case to analyze also a latter part of 16S rRNA) 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.

2.3. Confirmation of PCR products

Mix 5 μ L of PCR product with 1 μ L 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.

2.4. Purification of PCR products

Remove unincorporated PCR primers and deoxynucleoside triphosphates (dNTP) from PCR products by using appropriate purification methods.

2.5. Quantification of purified DNA

When purified DNA is measured by spectrophotometer, calculate 1 OD_{260 nm} as 50 μ g/mL.

2.6. 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.

2.7. Purification of sequencing reagent-labeled PCR products

Transfer the product in 75 μ L 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 μ L 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.

2.8. 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.

3. Judgment

If sequencing data show over 90% identity with a sequence in the database, in general, judgment may be made as follows.

- (i) In the case of bacteria, compare the nucleotides in the product obtained with the 10F primer (the 800F primer when 800F/1500R primers are used) with the BLAST database. Higher ranked species are judged as identified species or closely related species.
- (ii) 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.

4. Reagents, Test Solutions

(i) 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.

(ii) 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.

(iii) 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.

(iv) DNA releasing solution: Divide TE buffer solution containing 1 vol% of polyoxyethylene (10) octylphenyl ether into small amounts and store frozen until use.

(v) PCR reaction solution

10-fold buffer solution*	5 μ L
dNTP mixture**	4 μ L
10 μ mol/L Sense primer	1 μ L
10 μ mol/L Anti-sense primer	1 μ L
Heat-resistant DNA polymerase (1 U/ μ L)	1 μ L
Water	36 μ L

* 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.

(vi) 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.

(vii) 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.

(viii) 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.

(ix) Agarose gel: Mix 1.5 g of agarose, 2.0 mL of 50-fold concentrated TAE buffer solution, 10 μ L 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.

(x) 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.

(xi) PCR primers

For	Primer
Bacteria	10F 5'-GTTTGATCCTGGCTCA-3'
	800R 5'-TACCAGGGTATCTAATCC-3'
	800F 5'-GGATTAGATACCCTGGTA-3'
	1500R 5'-TACCTTGTTACGACTT-3'
Fungi	ITS1F 5'-GTAACAAGGT(T/C)TCCGT-3'
	ITS1R 5'-CGTTCTTCATCGATG-3'

(xii) Polyoxyethylene(10)octylphenyl ether: A pale yellow, viscous liquid.

Sterility Assurance for Terminally Sterilized Pharmaceutical Products

As indicated in the "Terminal Sterilization and Sterilization Indicators", the pharmaceuticals to which terminal sterilization can be applied, generally must be sterilized so that a sterility assurance level of 10^{-6} or less is obtained. The sterility assurance level of 10^{-6} or less can be proven by using a sterilization process validation based on physical and microbiological methods, but cannot be proven by sterility tests of the sterilized products. This chapter deals with the necessary requirements for the appropriate management of the important control points of the sterilization process for the parametric release of products, without performing sterility tests on products which have been subjected to terminal sterilization (in the case of radiation sterilization, called dosimetric release). Parametric release is a method that can be applied in cases where the sterilization system is clearly defined, important control points are clearly specified, and the sterilization system process can be validated by microbiological methods using appropriate biological indicators.

1. Definitions

The definitions of the terminology used in this chapter are provided below.

1.1. Terminal sterilization

A process whereby a product is sterilized in its final container or packaging, and which permits the measurement and evaluation of quantifiable microbial lethality.

1.2. Validation

A documented procedure for obtaining, recording and interpreting the results needed to show that a process will consistently yield a product complying with predetermined specifications.

1.3. Periodic re-validation

Validation that is regularly performed to reconfirm that a process is consistently yielding a product complying with predetermined specifications. It should confirm that variables and the acceptable ranges are permissible to yield a product consistently of the required quality.

1.4. Facility/equipment qualification

This is to provide evidence that the manufacturing facilities/equipment, measuring equipment, and manufacturing environment control facilities, etc. have been properly selected, correctly installed, and are operated in conformity with the specifications at the time of installation and during operation.

1.5. Operation qualification

This is to provide evidence to confirm physically, chemically and microbiologically that equipment, operated in accordance with its operational instructions, operates as specified and affords a product meeting the specifications.

1.6. Support system for sterilization process

This refers to the facility/equipment that is associated with the sterilization devices, such as the preconditioning and aeration for ethylene oxide sterilization, the steam supply equipment for moist heat sterilization, and the loading devices for radiation sterilization.

1.7. Quality system

The procedures, resources and organizational structure of a manufacturer (responsibilities, authorities and relationships between these) required to implement quality management.

1.8. Change control system

A system designed to evaluate all of the changes that may affect the quality of the pharmaceutical product, in order to ensure that the process is continuously controlled.

1.9. F_0 value

Assume a value of 10°C for the Z value defined as the number of degrees of temperature required for a 10-fold change in the D value. The F_0 value indicates the time (minutes) required to give the equivalent lethality at T_b of the sterilization heat obtained by integrating the lethality rate (L) over an entire heating cycle.

$$L = \log^{-1} \frac{T_0 - T_b}{Z} = 10^{\frac{T_0 - T_b}{Z}}$$

T_0 = Temperature inside the chamber or inside the product to be sterilized

T_b = Reference temperature (121°C)

$$F_0 = \int_{t_0}^{t_1} L dt$$

$t_1 - t_0$ = Processing time (minutes)

1.10. Control device

A general term for the devices and measurement equipment, including the equipment for controlling, measuring and recording the physical parameters that can be measured (temperature, humidity, pressure, time, radiation dose, etc.).

1.11. Parametric release

A release procedure based on an evaluation of the production records and critical parameters of the sterilization process (temperature, humidity, pressure, time, radiation dose, etc.) based on the results of validation, in lieu of release based on testing results of the final product.

2. Sterilization Validation

2.1. Subject of the Implementation

A manufacturer of sterile pharmaceuticals (hereafter, "manufacturer") must establish a quality system, implement product sterilization validation for the categories below as a general rule, and continuously control the sterilization process based on the results of the sterilization validation.

a) Sterilization process

b) Sterilization process support system

2.2. Documenting Sterilization Validation Procedure

2.2.1. The manufacturer must prepare a "Sterilization Validation Procedure" defining the items listed below regarding the procedures for managing the sterilization

process.

a) Details related to the range of duties of the persons responsible for the validation, as well as the extent of their authority

b) Details related to the implementation period for the sterilization validation

c) Details related to the creation, modification, and approval of the sterilization validation plan documents

d) Details related to the reporting, evaluation, and approval of the sterilization validation implementation results

e) Details related to the storage of documentation concerning the sterilization validation

f) Other required matters

2.2.2. The sterilization validation procedure must list the names of the enactors, the date of enactment, and when there are revisions, must also list the revisers, date of revisions, revised sections and reasons for the revisions.

2.2.3. The manufacturer must properly store and maintain the sterilization validation procedure after clarifying the procedures related to alterations and deletions of the contents of the sterilization validation procedure.

2.3. Persons Responsible for the Validation

The manufacturer must assign persons to be responsible for the sterilization validation. The responsible parties must perform each of the duties listed below according to the sterilization validation procedure.

2.3.1. For products that are to be produced according to the sterilization validation procedure, a written sterilization validation implementation plan must be prepared. The implementation plan will specify the following points based on a consideration of the implementation details of the sterilization validation.

a) Subject pharmaceutical name (product name)

b) Purpose of the applicable sterilization validation

c) Expected results

d) Verification methods (including inspection results and evaluation methods)

e) Period of verification implementation

f) Names of persons performing the sterilization validation (persons-in-charge)

g) Names of the persons who created the plan, creation date, and in the event of revisions, the names of the revisers, date of the revisions, revised sections, and reasons for revision.

h) Technical requirements for the applicable sterilization validation

i) Other required matters for the implementation of the applicable sterilization validation

2.3.2. The following sterilization validation is implemented according to the plan defining the items above.

a) When the manufacturing license and additional (modification) licenses for product production are obtained, implementation items for the sterilization validation to be executed

1 Product qualification

2 Facility/equipment qualification

1) Installation qualification

2) Operation qualification

3 Performance qualification

1) Physical performance qualification

2) Microbiological performance qualification

b) Sterilization validation to be executed until it is time to renew the manufacturing license

- 1 Re-validation when there are changes
 - 2 Periodic re-validation (The items implemented, etc. must be determined based on a consideration of relevant factors such as the sterilization method.)
- 2.3.3.** Evaluate the results of the sterilization validation and verify that sterility is assured.
- 2.3.4.** Make a written report of the results of the sterilization validation to the manufacturer's authorized person.
- 2.3.5.** Perform the day-to-day management of the sterilization process.

3. Microorganism Control Program

When parametric release is adopted, it is important to control the bioburden in the raw materials of the product, the containers and stoppers, and in the product before sterilization. The bioburden is measured with a previously specified method and frequency, and when required, surveys of the characteristics of the isolated microorganisms are made to investigate their resistance to the applicable sterilization method. Refer to the "Microbiological Evaluation of Process Areas for Sterile Pharmaceutical Products" regarding the method for evaluating the environmental microorganisms in the processing areas of pharmaceutical products.

4. Sterilization Indicators

Biological indicators (BI), chemical indicators (CI), and dosimeters are among the means used to monitor a sterilization process and as indices of sterility (refer to Terminal Sterilization and Sterilization Indicators). When using sterilization indicators it is important to consider environmental and human safety, and to take all necessary precautions. The BI used for sterilization validation and daily process control must be defined in the specification, and recorded in writing. When BI are used for daily process control it must be verified that the loading pattern on the form, product, or simulated product has a resistance equal to or greater than that used for the microbiological performance qualification.

5. Establishment of a Change Control System

Changes which have a large effect on the sterile quality, such as changes in sterilization equipment, loading pattern, and sterilization conditions, correspond to changes of the parametric release conditions for the relevant pharmaceutical product. A change control system must be defined in the sterilization validation procedure; and when there are changes in the causes of variation that have been previously specified, there must be an investigation of the causes of variation and of acceptable conditions to verify that the pharmaceutical product is guaranteed always to conform to the quality standards. Furthermore, before modifications are made to a sterilization process that has been validated, it is mandatory to obtain approval for the implementation of the modifications in question from the appropriate authorized person.

6. Release Procedure

A release procedure must be created to clarify the conditions required for shipment based on parametric release of terminally sterilized products. The following points must be evaluated and recorded when a product is released.

- Depending on the sterilization method, some of these items may be omitted or modified.
- a) Batch record
 - b) Microorganism evaluation data of production environment

- c) Bioburden data for the raw materials and product before sterilization
- d) Data related to the sterilization indicators
- e) Data on the maintenance management of the sterilization process and sterilization process support systems
- f) Data on the management of sterilization parameters
- g) Data on the calibrations of measurement equipment
- h) Re-validation data
- i) Other

7. Critical Control Points

The important control points for each sterilization method are presented.

7.1. Moist heat sterilization

Moist heat sterilization is a method for killing microorganisms in which saturated water vapor is generated or introduced into a sterilization chamber at the appropriate temperature and pressure, and the chamber is then heated for a certain period of time. It is roughly classified into saturated vapor sterilization, in which the target microorganisms are directly exposed to the saturated vapor, and unsaturated vapor sterilization, in which the fluid inside a container, such as an ampule, is subjected to moist heat energy or highfrequency energy from the outside.

7.1.1. Important control points

A process control procedure must be created, specifying the process parameters that affect the sterile quality of the pharmaceutical product, and the permissible range of variation for each parameter. The important control points for the moist heat sterilization are indicated below.

- a) Heating history (usually indicated by F_0 value)
- b) Temperature
- c) Pressure
- d) Time
- e) Product loading format/loading density
- f) Other necessary matters

7.1.2. Utilities

The utilities and control devices required for moist heat sterilization determine the quality and precision.

- a) Quality of the vapor used
- b) Quality of the air introduced into the sterilization chamber to restore pressure, etc.
- c) Quality of the water used for cooling
- d) Precision of the temperature control devices
- e) Precision of the pressure control devices
- f) Precision of the time control devices
- g) Other

7.2. Ethylene oxide gas sterilization

Ethylene oxide gas allows sterilization at low temperatures, so there is typically little injury to the substance being sterilized; however, since the gas is toxic it must be handled with extreme caution. The sterilization process consists of preconditioning, a sterilization cycle, and aeration. The preconditioning is performed before the sterilization cycle to process the product so that temperature and relative humidity in the room or container are within the range in the specifications. The sterilization cycle indicates the stage at which the actual sterilization is performed, and consists of removal of the air, conditioning (when used), injection of the sterilization gas, maintenance of the sterilization conditions, removal of the sterilization gas, and replacement of the air. The aeration is the process of eliminating the residual ethylene oxide gas from the product, either inside the sterili-

zation chamber or in a separate location.

7.2.1. Important control points

The important control points for the ethylene oxide gas sterilization are indicated below.

7.2.1.1. Preconditioning (when performed)

- a) Time, temperature, humidity
- b) Product loading pattern/loading density
- c) Sterilization loading temperature and/or humidity
- d) Time from the end of preconditioning until the start of the sterilization
- e) Other necessary matters

7.2.1.2. Conditioning

- a) If pressure reduction is performed, the pressure achieved and required time
- b) Reduced pressure maintenance period
- c) Time, temperature, pressure, humidity
- d) Sterilization loading temperature and humidity
- e) Other necessary matters

7.2.1.3. Sterilization cycle

- a) Pressure increase, injection time, and final pressure for the injection of the sterilization gas
- b) Concentration of the ethylene oxide gas (it is desirable to analyze directly the gas concentration inside the sterilization chamber, but the following alternatives are acceptable if direct analysis is difficult)
 - i) Mass of gas used
 - ii) Volume of gas used
 - iii) Conversion calculation using the initial low pressure level and the gas injection pressure
- c) Temperature within the sterilization chamber
- d) Temperature of the loaded products to be sterilized
- e) Effect time (exposure time)
- f) Product loading pattern/loading density
- g) BI placement points and cultivation results
- h) Other necessary matters

7.2.1.4. Aeration

- a) Time, temperature
- b) Loaded sterilized substance temperature
- c) Pressure variation in the sterilization chamber and/or the aeration room
- d) Rate of change of the air or other gases in the aeration room
- e) Other necessary matters

7.2.2. Utilities

The utilities and control devices required for ethylene oxide sterilization determine the quality and precision.

- a) Quality of the ethylene oxide gas
- b) Quality of the injected vapor or water
- c) Quality of the replacement air after the completion of sterilization
- d) Quality of the BI
- e) Precision of the temperature control devices
- f) Precision of the pressure control devices
- g) Precision of the humidity control devices
- h) Precision of the time control devices
- i) Other

7.3. Irradiation Sterilization

Irradiation sterilization refers to methods of killing microorganisms through exposure to ionizing radiation. The types of ionizing radiation used are gamma-rays (γ -rays) emitted from a radioisotope such as ^{60}Co or ^{137}Cs , or electron beams and bremsstrahlung (X-ray) generated from an electron accelerator. In the case of γ -rays, the cells are killed

by secondarily generated electrons, while in the case of the electron beam, the cells are killed by the electrons generated directly from the electron accelerator. For this reason, the processing time for electron beam sterilization is generally shorter than that for γ -ray sterilization; but, since the penetration of the γ -rays is better than that of the electron beam, there must be appropriate consideration of the density and thickness of the substance being sterilized when choosing between these methods. For an irradiation sterilization process, the control procedures primarily make use of dosimeters and measure the absorbed dose in the substance being sterilized. This is called dosimetric release.

7.3.1. Important control points

The important control points for the irradiation sterilization are indicated below.

7.3.1.1. γ -ray radiation

- a) Irradiation time (timer setting or conveyor speed)
- b) Absorbed dose
- c) Product loading pattern
- d) Other necessary matters

7.3.1.2. Electron beam and X-ray radiation

- a) Electron beam characteristics (average electron beam current, electron energy, scan width)
- b) Conveyor speed
- c) Absorbed dose
- d) Product loading pattern
- e) Other necessary matters

7.3.2. Utilities

A traceable calibration, performed according to national standards, must be performed for the radiation devices and dose measurement systems. This calibration must be performed as specified in a written plan in order to verify that the equipment is kept within the required range of accuracy.

7.3.2.1. Required calibration items for gamma-radiation equipment

- a) Cycle time or conveyor speed
- b) Weighing device
- c) Dose measurement system
- d) Other

7.3.2.2. Required calibration items for electron-beam and X-ray radiation equipment

- a) Electron beam characteristics
- b) Conveyor speed
- c) Weighing device
- d) Dose measurement system
- e) Other

References

- 1) Validation Standards, PAB Notification No.158, Ministry of Health and Welfare 1995
- 2) Sterilization Validation Standards, PMSB/IGD Notification No.1, Ministry of Health and Welfare 1997
- 3) Quality Assurance Standards for Medical Devices, PAB Notification No.1128, Ministry of Health and Welfare 1994
- 4) ISO 9000 series, International Standards for Quality Assurance
- 5) ISO 11134 Industrial moist heat sterilization
- 6) ISO 11135 Ethylene oxide sterilization
- 7) ISO 11137 Radiation sterilization
- 8) ISO 11138 Biological indicators
- 9) ISO 11140 Chemical indicators
- 10) ISO 11737-1 Microbiological Methods Part 1: Estima-

- tion of population of microorganisms on products
- 11) USP <1222> Terminally Sterilized Pharmaceutical Products - Parametric Release

Terminal Sterilization and Sterilization Indicators

Sterilization is a process whereby the killing or removal of all forms of viable microorganisms in substances is accomplished. It is achieved by terminal sterilization or a filtration method. For substances to which terminal sterilization can be applied, an appropriate sterilization method should be selected in accordance with the properties of the product, including the packaging, after full consideration of the advantages and disadvantages of each sterilization method, from among the heat method, irradiation method and gas method. After installation of the sterilizer (including design and development of the sterilization process), validation is required to confirm that the sterilization process is properly performing its designed function, under conditions of loading and unloading of the product, on the basis of sufficient scientific evidence. After the process has been validated and the sterilization of the product commenced, the process must be controlled correctly, and qualification tests of the equipment and procedures must be performed regularly.

The bioburden per product, prior to terminal sterilization, must be evaluated periodically or on the basis of batches. Refer to the ISO standard (ISO 11737-1) relevant to bioburden estimation. For a substance to which terminal sterilization can be applied, generally use sterilization conditions such that a sterility assurance level of less than 10^{-6} can be obtained. The propriety of the sterilization should be judged by employing an appropriate sterilization process control, with the use of a suitable sterilization indicator, and if necessary, based on the result of the sterility test. The filtration procedure is used for the sterilization of a liquid product, to which terminal sterilization can not be applied. Concerning the disinfection and/or sterilization necessary for processing equipment and areas of pharmaceutical products, and performing microbiological tests specified in the monographs, see Disinfection and Sterilization Methods.

1. Definitions

The definitions of the terms used in this text are as follows.

(i) Terminal sterilization: A process whereby a product is sterilized in its final container or packaging, and which permits the measurement and evaluation of quantifiable microbial lethality.

(ii) Product: A generic term used to describe raw materials, intermediate products, and finished products, to be sterilized.

(iii) Bioburden: Numbers and types of viable microorganisms in a product to be sterilized.

(iv) Sterility assurance level (SAL): Probability of a viable microorganism being present in a product unit after exposure to the proper sterilization process, expressed as 10^{-n} .

(v) Integrity test: A non-destructive test which is used to predict the functional performance of a filter instead of the microorganism challenge test.

(vi) D value: The value which shows the exposure time

(decimal reduction time) or absorbed dose (decimal reduction dose) required to cause a 1-logarithm or 90% reduction in the population of test microorganisms under stated exposure conditions.

(vii) Sterilization indicator: Indicators used to monitor the sterilization process, or as an index of sterility, including biological indicators (BI), chemical indicators (CI), dosimeters and the like.

2. Sterilization

2.1. Heat Method

In the heat method, microorganisms are killed by heating.

2.1.1. Moist heat method

Microorganisms are killed in saturated steam under pressure. In this method, factors which may affect the sterilization include temperature, steam pressure and exposure time. Therefore, in routine sterilization process control, it is required to monitor continuously the temperature, steam pressure and exposure time, and they should be included in the specifications of the sterilizer.

2.1.2. Dry-heat method

Microorganisms are killed in dry heated air. This method is usually conducted in a batch-type dry heat sterilizer or a tunnel-type dry heat sterilizer. In this method, factors which may affect the sterilization include temperature and exposure time. Therefore, in routine sterilization process control, it is required to monitor continuously the temperature and exposure time, and they should be included in the specifications of the sterilizer.

2.2. Irradiation method

Microorganisms are directly killed by ionizing radiation, or by the heat generated by microwave radiation.

2.2.1. Radiation method

Ionizing radiations which may be used are gamma (γ) rays emitted from a radioisotope such as cobalt 60, an electron beam and bremsstrahlung (X rays) generated from an electron accelerator. Although any procedure can be applied to thermally unstable products with no radioactivity residue, it is necessary to consider the possibility of material degradation. Although a 25 kGy dose is traditionally used as a sterilization dose, there are some ways to calculate the dose as follows: the bioburden of the substance to be sterilized is measured and the sterilization dose is calculated based on the mean bioburden and the standard resistance distribution (Method 1 in ISO 11137), the dose is calculated from the fraction positive information from a sterility test in which representative product samples are exposed to a substerilizing dose (Method 2 in ISO 11137), or the dose is calculated based on the bioburden and D value of the most resistant microorganisms (Log method) (see 5.3.). In the case of the radiation sterilization procedure, factors which may affect the sterilization include dose (absorbed dose) and exposure time. Therefore, in γ ray sterilization process control, it is required to determine the dose (the absorbed dose) at appropriate intervals and to monitor continuously the exposure time in terms of the operating parameters (the conveyer speed, the cycle time). The dose control mechanism should be included in the specifications of the sterilizer. In the case of electron beam or bremsstrahlung irradiation, it is required to monitor the acceleration voltage, the beam current and beam scanning width besides the above-mentioned items.

2.2.2. Microwave method

Microorganisms are killed by the heat generated by micro-

wave radiation, usually at the frequency of 2450 ± 50 MHz. This method is applied to liquids or water-rich products in sealed containers. Since a glass or plastic container may be destroyed or deformed due to the rise of the inner pressure, the containers must be certified to be able to withstand the heat and the inner pressure generated during microwave sterilization. Leakage of electromagnetic radiation must be at a sufficiently low level to cause no harm to humans and no interference with radio communications and the like. In this method, factors which may affect the sterilization include temperature, processing time and microwave output power. Therefore, in routine sterilization process control, it is required to monitor continuously the temperature, time and the microwave output power, and they should be included in the specifications of the sterilizer.

2.3. Gas method

Ethylene oxide (EO) is widely used as a sterilization gas. Since EO gas has an explosive nature, a 10–30% mixture with carbon dioxide is commonly used. Also, as EO gas is a strong alkylating agent, it can not be applied to the products which are likely to react with or absorb it. Furthermore, because EO gas is toxic, the residual concentration of EO gas and other secondarily generated toxic gases in products sterilized with EO gas must be reduced to less than the safe levels thereof by means of aeration and the like before the product is shipped. In this method, factors which may affect the sterilization include temperature, gas concentration (pressure), humidity and exposure time. Therefore, in routine sterilization process control, it is required to monitor continuously the temperature, gas concentration (pressure), humidity and exposure time, and they should be included in the specifications of the sterilizer.

3. Filtration method

Microorganisms are removed by using a sterilizing filter made of an appropriate material. However, this method is not intended for microorganisms smaller than bacteria. Generally, a sterilizing filter challenged with more than 10^7 microorganisms of a strain of *Brevundimonas diminuta* (ATCC 19146, NBRC 14213, JCM 2428), cultured under the appropriate conditions, per square centimeter of effective filter area should provide a sterile effluent. In this method, factors which may affect the sterilization include pressure, flow rate, filter unit characteristics and the like. In routine filtration process control, it is required to perform integrity tests of the sterilizing filter after each filtration process (also prior to the filtration process, if necessary).

4. Sterilization Indicators

4.1. Biological indicator (BI)

A BI is prepared from specific microorganisms resistant to the specified sterilization process and is used to develop and/or validate a sterilization process. The dry type BI is classified into two kinds. In one, bacterial spores are added to a carrier such as filter paper, glass or plastic and then the carriers are dried and packaged. In the other, bacterial spores are added to representative units of the product to be sterilized or to simulated products. Packaging materials of the BI should show good heat penetration in dry heat sterilization and good gas or steam penetration in ethylene oxide and moist heat sterilizations. It should be confirmed that any carrier does not affect the D value of the spores. In the case of a liquid product, the spores may be suspended in the same solution as the product or in a solution showing an equivalent

effect in the sterilization of biological indicator. However, when the spores are suspended in liquid, it is necessary to ensure that the resistance characteristics of the spores are not affected due to germination.

Typical examples of biological indicator

Sterilization	Representative microorganisms*	Strain name
Moist heat method	<i>Geobacillus stearothermophilus</i>	ATCC 7953, NBRC 13737, JCM 9488 ATCC 12980, NBRC 12550, JCM 2501
Dry heat method	<i>Bacillus atrophaeus</i>	ATCC 9372, NBRC 13721
Gas method	<i>Bacillus atrophaeus</i>	ATCC 9372, NBRC 13721

* In addition to these microorganisms, other microorganisms with the greatest resistance to the sterilization procedure concerned, found in the bioburden, can be used as the biological indicator.

4.1.1. D value of BI

Methods for determination of the D value include the survival curve method and the fraction negative method (Stumbo, Murphy & Cochran procedure, Limited Spearman-Kärber procedure and the like). In using marketed BIs, it is usually unnecessary to determine the D value before use if the D value indicated on the label has been determined by a standardized biological indicator evaluation resistometer (BIER) under strictly prescribed conditions in accordance with ISO 11138-1. It is acceptable that the D value indicated on the label shows a scattering of not more than ± 30 seconds.

4.1.2. Setting up procedure of BI

4.1.2.1. In the case of dry materials

A Dry type BI is placed at predetermined cold spots in the product to be sterilized or a suitable product showing an equivalent effect in the sterilization. The BIs are usually primary packaged in the same way as the product, including a secondary packaging, if applicable.

4.1.2.2. In the case of wet materials

Spores are suspended as the BI in the same solution as the product or in an appropriate similar solution, and should be placed at cold spots in the sterilizer.

4.1.3. Culture conditions of BI

Soybean casein digest medium is generally used. General culture conditions are at $55 - 60^\circ\text{C}$ for 7 days in the case of *G. stearothermophilus* and at $30 - 35^\circ\text{C}$ for 7 days in the case of *B. atrophaeus*.

4.2. Chemical indicator (CI)

CI is an indicator which shows a color change of a substance applied to a paper slip, etc. as a result of physical and/or chemical change due to exposure to heat, gas or radiation. The CI can be classified into three types. The first is employed to identify whether or not sterilization has already been implemented, the second is employed to control the sterilization process (for example, its color changes after sterilization for a sufficient time), and the third is the Bowie

& Dick type used to evaluate the effectiveness of air removal during the pre-vacuum phase of the pre-vacuum sterilization cycle.

4.3. Dosimeter

In the radiation (γ -ray) method, the sterilization effect depends on the absorbed radiation dose, so the sterilization process control is mainly performed by measuring the dose. A dosimeter is installed at a position corresponding to the minimum dose region of an exposed container or a position where the dose is in a known relation to that in the above region. Measurement should be done for each radiation batch. If there are many containers in the same batch, dosimeters should be employed so that more than one dosimeter is always installed at the effective radiation section of the irradiation chamber. It should be noted that dosimeters may be affected by environmental conditions (temperature, humidity, ultraviolet light, time until reading, etc.) before and during irradiation. Practical dosimeters for γ -ray and bremsstrahlung sterilization include the dyed polymethylmethacrylate dosimeter, clear polymethylmethacrylate dosimeter, ceric-cerous sulfate dosimeter, alanine-EPR dosimeter and the like. A dosimeter for gamma radiation can not generally be used for sterilization process control with an electron beam of less than 3 MeV energy. Dosimeters for electron beam sterilization include the cellulose acetate dosimeter, radiochromic film dosimeter and the like. A practical dosimeter must be calibrated against an appropriate national or international standard dosimetry system.

5. Determination of sterilization conditions using microorganism as an indicator

Taking account of the characteristics upon the sterilization concerned, bioburden, etc. of a product to be sterilized, chose a suitable method from the followings and determine the conditions.

5.1. Half-cycle method

In this method, a sterilization time of twice as long as that required to inactivate all of 10^6 counts of BI placed in the product is used, regardless of bioburden count in the product being sterilized or the resistance of the objective microorganisms to the sterilization.

5.2. Overkill method

In this method, a sterilization condition giving a sterility assurance level of not more than 10^{-6} counts is used, regardless of bioburden count in the product being sterilized or the resistance of the objective microorganisms to the sterilization. Generally, a sterilization condition providing 12 logarithmic reduction (12D) of a known count of BI of more than 1.0 D value is used.

5.3. Combination of BI and bioburden

Generally, a count of mean bioburden added three times of its standard deviation obtained by an extensive bioburden estimation is considered as the maximum bioburden count, and the sterilization time (or radiation dose) is calculated with the bioburden count based on an objective sterility assurance level. When this procedure is used, it is required to determine the resistance of the bioburden to the sterilization as well as the bioburden count in the product being sterilized. If a more resistant microorganism than the BI spore is found in the bioburden estimation, it should be used as the BI.

$$\text{Sterilization time (or radiation dose)} = D \times \log \frac{N_0}{N}$$

D: D value of the BI

N: Sterility assurance level

*N*₀: Maximum bioburden count in the product

5.4. Absolute bioburden method

The sterilization conditions are determined by employing the D value of the most resistant microorganism found in the product or environment by the resistant estimation and being based on the bioburden count in the product. Generally, a count of mean bioburden added three times of its standard deviation obtained by an extensive bioburden estimation is employed as the bioburden count. When this procedure is used, it is required to make frequent counting and resistance determination of microorganisms in daily bioburden estimation.

6. References

- (i) ISO 11134 Industrial moist heat sterilization
- (ii) ISO 11135 Ethylene oxide sterilization
- (iii) ISO 11137 Radiation sterilization
- (iv) ISO 11138 Biological indicators
- (v) ISO 11140 Chemical indicators
- (vi) ISO 11737 Microbiological methods
Part 1: Estimation of population of microorganisms on products

G5 Crude Drugs

Aristolochic Acid

Aristolochic acid, which occurs in plants of *Aristolochiaceae*, is suspected to cause renal damage. It is also reported to be oncogenic (see References).

Aristolochic acid toxicity will not be a problem if crude drugs of the origin and parts designated in the JP are used, but there may be differences in crude drug nomenclature between different countries, and it is known that crude drug preparations not meeting the specifications of the JP are circulating in some countries. Consequently, when crude drugs or their preparations are used, it is important that the materials should not include any plant containing aristolochic acid.

Since Supplement I to JP14, the test for aristolochic acid I was added to the Purity under Asiasarum Root, which consists of the rhizome and root. Because the aerial part of the plant may contain aristolochic acid and may have been improperly contaminated in Asiasarum Root.

It is considered that Akebia Stem, Sinomenium Stem and Saussurea Root do not contain aristolochic acid, unless plants of origin other than that designated in the JP are used. However, contamination of aristolochic acid might occur, as mentioned above. In this case, the test described in the Purity under Asiasarum Root is useful for checking the presence of aristolochic acid.

References:

Drug & Medical Device Safety Information (No.161) (July, 2000).

New England Journal of Medicine (June 8, 2000).

Mutation Research 515, 63 – 72 (2002).

Purity Tests on Crude Drugs Using Genetic Information

The first step in the quality assurance of natural products is the use of raw materials from the right part of the right origin (the right source). Therefore, it is clearly stated in Article 4 of the General Rules For Crude Drugs that the source of a crude drug is the approval or rejection criteria. There are various methods for differentiating the sources of crude drugs, such as morphological methods, organoleptic tests, and chemical methods, and appropriate methods for each are described in the individual monographs. Morphological methods, organoleptic tests, and chemical methods are discrimination methods for species that are based on the phenotypic characteristics of the crude drugs. On the other hand, together with recent progress in molecular biology techniques and the accumulation of genetic information on plants, differentiating methods of crude drugs based on genotypes have been established. Unlike morphological and other methods that are based on phenotypic characteristics, the genotypic methods are not affected by environmental factors. Also, the methods have several advantages, such as specialized expertise and skill for classification are not needed, and objective results are easily obtained.

The evolution of living organisms is accomplished by genetic mutation, and differences among the nucleotide sequences of genes of closely related species reflect the strain relationships between the species. Based on this theory, in recent years methods that classify species phylogenetically using the nucleotide sequence of rDNA that codes for ribosomal RNA (rRNA) on the nuclear genome have been adopted. In the same way, the sequence of this rDNA is also most often used in the classification of higher plants based on the genotype. In particular, it is very easy to classify closely related species using the intergenic transcriber space (ITS) region of the rDNA region, since by comparison with the coded gene region nucleotide substitution is more often undertaken. Furthermore, since the genes on the nuclear genome originate from the parents' genom, there is an advantage that interspecies hybrids can be detected. Higher plants also have mitochondrial genes and chloroplastic genes. Although the genes on these genomes are also often used for classification, interspecies hybrids cannot be confirmed because the genes are normally uniparental inheritance.

The two methods presented here have been developed based on the reported identification methods of *Atractylodes Lancea* Rhizome and *Atractylodes Rhizome*^{1,2)} utilizing the gene sequence of the ITS of rDNA. Inter-laboratory validation study for purity test of *Atractylodes Rhizome* targeted for *Atractylodes Lancea* Rhizome have been completed. The plant sources for *Atractylodes Lancea* Rhizome stipulated in the individual monographs are *Atractylodes lancea* De Candolle or *A. chinensis* Koidzumi (*Compositae*), while those for *Atractylodes Rhizome* are *A. japonica* Koidzumi ex Kitamura or *A. ovata* De Candolle (*Compositae*). The approval or rejection of the source of *Atractylodes Lancea* Rhizome is, in principle, determined by the description of the crude drug, including microscopy, while that of *Atractylodes Rhizome* is determined by the description of the crude drug, including microscopy, together with color reac-

tion, which is an identification test. In the above scientific paper, it was shown that these 4 plant species can be clearly classified by comparing the nucleotide sequences of the ITS mentioned above, and that the species can be easily classified without performing sequence analysis by performing PCR using a species specific primer set or by using a restriction enzyme which recognizes species specific sequence.

In validation studies, the simplicity of the test is given maximum consideration. We examined methods that observe PCR amplification bands using species specific primer sets (Mutant Allele Specific Amplification: Method 1) and that observe DNA fragments produced by restriction enzyme treatment of the PCR products, which are prepared using a primer set common to each plant source (PCR-Restriction Fragment Length Polymorphism: Method 2), and do not involve nucleotide sequence analyses. In these methods based on PCR, an extremely small amount of template DNA is amplified to billions to hundred-billions times. Therefore, when using them as identification tests for powdered crude drugs, the target DNA fragment can be observed even if the vast majority of the crude drug for analysis is not appropriate plant species and there is only a minute amount of powder from a crude drug derived from a suitable plant. (Consequently, in identification tests, either a cut or a whole crude drug must be used, as long as one is careful to avoid contamination by powder originating from other crude drugs.) On the other hand, when used as a purity test, the form of the crude drug is irrelevant as long as the gene amplification is performed properly and the target gene is not polymorphic, so if DNA fragments of an inappropriate plant are confirmed in the purity test, regardless of the form of the crude drug, it becomes clear there is contamination by an inappropriate crude drug.

The methods shown here are reference information and at the present stage results obtained using the methods do not affect the approval or rejection of the crude drug in each monograph. Furthermore, by performing the sequence analysis outlined in the previous paper, it goes without saying that more accurate decision concerning the source species can be made.

1. DNA Amplification Equipment

DNA amplification equipment is used to amplify the DNA which is extracted from a crude drug and then purified. Since there are slight differences in the methods of temperature control, and so on depending on the equipment used, there may be differences in the intensity, etc. of the PCR amplification bands even if PCR is carried out under the stipulated conditions. Therefore, when judging results based solely on the presence or absence of PCR amplification bands as in 3. Methods 1, confirm that only proper amplification bands are obtained when performing PCR using DNA obtained from samples confirmed beforehand to be the source species. If proper amplification bands are not obtained, the PCR temperature conditions should be slightly adjusted. This equipment can be used for the restriction enzyme treatment in 4. Method 2.

2. General precautions

Crude drugs are different from fresh plants in that they are dried products and a certain amount of time has passed since they were harvested. Therefore, in many cases the DNA has undergone fragmentation. Furthermore, various substances that can block or interfere with the PCR reaction

may be present in the plant. For these reasons, the extraction and purification of template DNA is the process that should receive the greatest amount of attention. In the case of *Atractylodes* crude drugs, the periderm should be removed using a clean scalpel or other clean instrument before pulverizing the sample because very often there are inhibitory substances present in the periderm.

3. Method 1 (Mutant Allele Specific Amplification Method)

Generally, this method is referred to as Mutant Allele Specific Amplification (MASA) or Amplification Refractory Mutation System (ARMS), and it provides nucleotide sequence information of sample-derived template DNA, based upon the presence or absence of DNA amplification in PCR using a species specific primer set.

3.1. Procedure

The following is an example procedure.

3.1.1. Preparation of template DNA

There are various methods with which to extract and purify DNA from the samples. It is recommended that commercially available DNA extraction kits be used when considering their advantages of not using any noxious reagents and not requiring any complicated purification procedures. In this case, attention should be paid to the final amount (concentration) of DNA obtained, and the initial amount of initial sample and the volume of liquid to elute the DNA need to be controlled. When extraction and purification are performed using silica gel membrane type kits stipulated in notifications³⁾ related to inspection methods of the foods produced by recombinant DNA techniques, it is appropriate to use 200 mg of sample, 1 mL of AP1 buffer solution, 2 μ L of RNase A, and 325 μ L of AP2 buffer solution. Also, the most important things are that the supernatant loaded on the first column is clear and that there is no need to load 1 mL unreasonably. Furthermore, 50 μ L is an appropriate volume used in the final elution of the DNA, and normally the initial eluate is used as the DNA sample stock solution.

3.1.2. Confirmation of purity of DNA in DNA sample stock solution and assay of DNA

The purity of the DNA in the stock solution can be confirmed by the $OD_{260\text{ nm}}/OD_{280\text{ nm}}$ ratio using a spectrophotometer. A ratio of 1.5 indicates that the DNA has been adequately purified. The amount of DNA is calculated using $1\text{ }OD_{260\text{ nm}} = 50\text{ }\mu\text{g/mL}$. The measurement mentioned above is performed using appropriately diluted DNA sample stock solution. Based on the results obtained, dilute with water to the concentration needed for the subsequent PCR reactions, dispense the solution into micro tubes as the sample DNA solution, and if necessary store frozen at not over -20°C . The dispensed DNA sample is used immediately after thawing and any remaining solution should be discarded and not refrozen. If the concentration of the DNA sample stock solution does not reach the concentration stipulated in PCR, it is used as a DNA sample solution.

3.1.3. PCR

When a commercially available PCR enzyme mentioned in the above notification⁴⁾ is used, it is appropriate that 25 μ L of reaction mixture consisting of 2.5 μ L of the PCR buffer solution containing magnesium, dNTP (0.2 mmol/L), 5' and 3' primer (0.4 μ mol/L), Taq DNA polymerase (1.25 units), and 5 μ L of 10 ng/ μ L sample DNA solution (50 ng of DNA) is prepared on ice. Among them, the PCR buffer solution

and dNTP are provided as adjuncts to the enzyme. When conducting purity tests on *Atractylodes Lancea* Rhizome in *Atractylodes* Rhizome, the primer sets used are C and D (C is positive with *A. lancea*, D is positive with *A. chinensis*) as described in the paper mentioned above (*J. Nat. Med.* 60, 149 – 156, 2006), however, when a combination of primer A and B is used, it is possible to confirm the source species of each of the respective specimens. In order to confirm that the DNA has been extracted correctly, the reaction solution containing the positive control primer (Pf and Pr) as shown below should be prepared. In addition, the negative control solutions which are not containing DNA sample or either of the primer sets should be prepared and simultaneously conduct PCR.

Pf: 5'-CAT TGT CGA AGC CTG CAC AGC A-3'

Pr: 5'-CGA TGC GTG AGC CGA GAT ATC C-3'

The PCR reaction is performed under the following conditions: starting the reaction at 95°C for 10 minutes, 30 cycles of 0.5 minutes at 95°C and 0.75 minutes at 68°C (69°C only when using the primer set C), terminate reaction at 72°C for 7 minutes, and store at 4°C . The resulting reaction mixture is used for the following process as PCR amplification reaction solution.

3.1.4. Agarose gel electrophoresis and detection of PCR products

After completion of the PCR reaction, mix 5 μ L of the PCR amplification reaction solution with an appropriate volume of gel loading buffer solution, add the mixture to the wells of 2 w/v% agarose gel, and then perform electrophoresis using 1-fold TAE buffer solution (refer to General Information, Rapid Identification of Microorganisms Based on Molecular Biological Method). Run in parallel an appropriate DNA molecular mass standard. Electrophoresis is terminated when the bromophenol blue dye in the gel loading buffer has advanced to a point corresponding to 1/2 to 2/3 the length of the gel.

Stain the gel after electrophoresis when not using gel stained in advance with ethidium bromide. Place the gel that has undergone electrophoresis and staining in a gel image analyzer, irradiate with ultraviolet light (312 nm), and detected its electrophoresis pattern. Compare this to the DNA molecular mass standard and determine the absence or presence of the target amplification band.

3.2. Judgement

Confirm at first that a 305 bp band is found with the reaction solution to which the positive control primer set has been added, and confirm there are no bands in a solution with no primer sets and a solution with no sample DNA solution. Next, if a 226 bp band is confirmed when the primer set C is added or if a 200 bp band is confirmed when the primer set D is added, the sample is judged to be *Atractylodes Lancea* Rhizome (in the case of cut crude drug, contamination of *Atractylodes Lancea* Rhizome is observed) and it is rejected. The sample is judged not to be *Atractylodes Lancea* Rhizome (in the case of cut crude drug, there is no contamination of *Atractylodes Lancea* Rhizome) and the purity test is acceptable if a 305 bp band is confirmed with the positive control primer set, bands are not observed in reaction solution without primer and reaction solution without DNA sample solution, and a 226 bp band is not observed with the primer set C and a 200 bp band is not observed with the primer set D. If a band is not observed with

the positive control primer, it is to be concluded that the DNA extraction failed and the procedure should be started over again from the DNA extraction step. If bands are confirmed in reaction solutions without primer sets or without DNA sample solution, it should be assumed that there was an error in the PCR procedure and therefore the procedure should be repeated again from the step 3.1.3. PCR.

4. Method 2 (PCR-Restriction Fragment Length Polymorphism)

Generally, this method is referred to as PCR-Restriction Fragment Length Polymorphism (RFLP), and it provides nucleotide sequence information of sample-derived template DNA, based upon the DNA fragment pattern produced by restriction enzyme treatment of the PCR products, which are amplified by using a primer set common to the DNA sequence of the objective plant.

The test is performed with 25 samples randomly taken from a lot, and each sample is designated with a number from 1 to 25. Differentiation of the sources is performed by individual PCR-RFLP measurement of the samples, and decision of the acceptability of the purity is dependent on how many nonconforming samples are present in the first 20 samples, taken in numerical order, for which judgement is possible as described below.

4.1. Procedure

The following is an example procedure.

4.1.1. Preparation of template DNA

There are various methods with which to extract and purify DNA from the samples. It is recommended that commercially available DNA extraction kits be used, when considering their advantages of not using noxious reagents and not requiring complicated purification procedures. Recently, PCR reagents that inhibit the effect of PCR enzyme-inhibiting substances present in samples have become commercially available, and by using these reagents, it is possible to prepare the template DNA from the sample simply by incubating the sample with the DNA extraction reagent. Here, a recommended DNA preparing procedure using such PCR reagents is described for the convenience of experimenters.

Cut 20 mg of the sample into small pieces with a clean knife, add 400 μ L of the DNA extraction reagent, and incubate at 55°C overnight (16–18 hours). Then heat at 95°C for 5 minutes to inactivate the enzyme in the reagent. Centrifuge to precipitate the sample, and use 50 μ L of the supernatant liquid as the template DNA solution. The DNA solution prepared in this method can not be used for concentration measurement based on OD_{260 nm}, because it contains many foreign substances affecting OD₂₆₀ value from the sample.

The composition of the DNA extraction reagent is as follows:

2-Amino-2-hydroxymethyl-1,3-propanediol-hydrochloric acid (pH 8.0)	20 mmol/L
Ethylenediamine tetraacetate	5 mmol/L
Sodium chloride	400 mmol/L
Sodium dodecyl sulfate	0.3%
Proteinase K	200 μ g/mL

4.1.2. PCR

In the method using the PCR enzyme and PCR reagent as described²⁾, the reaction mixture is prepared on an ice bath in a total volume of 20 μ L of a solution containing 10.0 μ L of 2-fold concentrated PCR reagent, 5'- and 3'-primers (0.5 μ mol/L), Taq DNA polymerase (0.5 units) and 0.5 μ L of

template DNA solution.

The PCR reaction is performed under the following conditions: 95°C for 10 minutes, 40 cycles of 95°C for 0.5 minute, 65°C for 0.25 minute, and 72°C for 0.25 minute and 72°C for 7 minutes. Store the solution at 4°C, and use this solution as the PCR amplified solution. A negative control (containing water instead of the template DNA solution) must be included in the procedure.

The sequence of each primer is as follows:

5'-primer: 5'-GGC ACA ACA CGT GCC AAG GAA AA-3'

3'-primer: 5'-CGA TGC GTG AGC CGA GAT ATC C-3'

4.1.3. Restriction enzyme treatment

The treatment is performed on individual reaction solutions using two enzymes, *Fau* I and *Msp* I. In the case of *Fau* I, to an appropriate amount of the reaction solution, composed of a reaction buffer containing 1.0 unit of enzyme, add 3.0 μ L of PCR products while cooling in an ice bath to make 15.0 μ L. In the case of *Msp* I, to an appropriate amount of the reaction solution, composed of a reaction buffer containing 20.0 units of enzyme, add 3.0 μ L of PCR products while cooling in an ice bath to make 15.0 μ L. Incubate these solutions at the temperature recommended by the manufacturer for 2 hours, and then inactivate the enzyme by heating at 72°C for 10 minutes. The negative control of the PCR reaction is also treated in the same manner.

4.1.4. Agarose gel electrophoresis and detection of DNA fragments

After the restriction enzyme treatment, mix the total amount of the reaction solution and an appropriate amount of the gel loading buffer solution, place it in a 4 w/v% agarose gel well, and carry out electrophoresis with 1-fold concentrated TAE buffer solution (see "Rapid Identification of Microorganisms Based on Molecular Biological Methods" under General Information). Carry out the electrophoresis together with appropriate DNA molecular mass standard. Stop the electrophoresis when the bromophenol blue included in the loading buffer solution has moved about 2 cm from the well. The 4 w/v% agarose gel is sticky, difficult to prepare and hard to handle, so that it is better to use a commercially available precast gel.

After the electrophoresis, stain the gel, if it is not already stained, with ethidium bromide, and observe the gel on an illuminating device under ultraviolet light (312 nm) to confirm the electrophoretic pattern.

4.2. Judgement

4.2.1. Judgment of each sample

Confirm that no band is obtained with the negative control of the PCR, other than the primer dimer (about 40 bp) band. A sample treated with *Fau* I, showing bands of about 80 bp and 60 bp, or that treated with *Msp* I, showing bands of about 90 bp and 50 bp, is judged as *Atractylodes Lancea* Rhizome. A sample not showing any band other than a band at about 140 bp and the primer dimer band is judged as *Atractylodes Rhizome*. If a sample does not show any band other than the primer dimer band, it is considered that PCR products were not obtained, and judgement is impossible for the sample.

4.2.2. Judgment of the purity

Judgment of the purity is based on the result of the judgment of each sample. If there is no sample that is judged as *Atractylodes Lancea* Rhizome among 20 samples taken in

order of the numbering, excluding any sample for which judgement is impossible, the lot is acceptable for purity. When there is one sample that is judged as *Atractylodes Lancea* Rhizome among the 20 samples, perform the same test with 25 newly taken samples from the lot, and if there is no sample that is judged as *Atractylodes Lancea* Rhizome, the lot is acceptable for purity. When there is a sample that is judged as *Atractylodes Lancea* Rhizome in the second test, or there is more than one sample that is judged as *Atractylodes Lancea* Rhizome in the first test, the lot is not acceptable for purity.

5. Reference

- 1) Y. Guo, *et al.*, *J. Nat. Med.* 60, 149 – 156 (2006).
- 2) K. Kondo, *et al.*, *J. Jpn. Bot.* 84, 356 – 359 (2009).
- 3) Notification No. 110, Director of Food Health Department, March 2001; Partial Amendment: Notification No. 0629002, 2.2.1.2, Director of Food Safety Department, June 2006.
- 4) Notification No. 0629002, 2.1.3.1.1, Director of Food Safety Department, June 2006.

On the Scientific Names of Crude Drugs Listed in the JP

The notation system of the scientific names for the original plants and animals of crude drugs listed in JP is not necessary the same as the taxonomic system used in the literature. The reason for this is that the JP is not an academic text, but an ordinance. The relationship between the scientific names used in the JP and those generally used taxonomically is indicated in the following table, to avoid misunderstanding by JP users owing to differences in the notation system.

Scientific names used in the JP and the corresponding taxonomic names

Crude Drug	Scientific names used in the JP = Scientific names being used taxonomically (Combined notation, Standard form for author or authors)	Family
	Scientific names that are different from those written in JP but identical to them taxonomically or being regarded as identical, and typical sub-classified groups belonging to their species. The names marked with “*” are those being written together in JP.	
Acacia アラビアゴム	<i>Acacia senegal</i> Willdenow = <i>Acacia senegal</i> (L.) Willd.	Leguminosae
	Other species of the same genus	
Achyranthes Root ゴシツ	<i>Achyranthes fauriei</i> Leveille et Vaniot = <i>Achyranthes fauriei</i> H. Lev. & Vaniot	Amaranthaceae
	<i>Achyranthes bidentata</i> Blume	
Agar カンテン	<i>Gelidium elegans</i> Kuetzing	Gelidiaceae
	Other species of the same genus	
	Other red algae	
Akebia Stem モクツウ	<i>Akebia quinata</i> Decaisne = <i>Akebia quinata</i> (Thunb. ex Houtt.) Decne.	Lardizabalaceae
	<i>Akebia trifoliata</i> Koidzumi = <i>Akebia trifoliata</i> (Thunb.) Koidz.	
Alisma Rhizome タクシャ	<i>Alisma orientale</i> Juzepczuk = <i>Alisma orientale</i> (Sam.) Juz.	Alismataceae
	<i>Alisma plantago-aquatica</i> L. var. <i>orientale</i> Sam.	

Aloe アロエ	<i>Aloe ferox</i> Miller = <i>Aloe ferox</i> Mill.	<i>Liliaceae</i>
	Hybrid between <i>Aloe ferox</i> Miller and <i>Aloe africana</i> Miller <i>Aloe africana</i> Miller = <i>Aloe africana</i> Mill.	
	Hybrid between <i>Aloe ferox</i> Miller and <i>Aloe spicata</i> Baker	
Alpinia Officinarum Rhizome リョウキョウ	<i>Alpinia officinarum</i> Hance	<i>Zingiberaceae</i>
Amomum Seed シュクシャ	<i>Amomum xanthioides</i> Wallich = <i>Amomum xanthioides</i> Wall.	<i>Zingiberaceae</i>
	<i>Amomum villosum</i> Lour. var. <i>xanthioides</i> (Wall.) T. L. Wu & Senjen	
Anemarrhena Rhizome チモ	<i>Anemarrhena asphodeloides</i> Bunge	<i>Liliaceae</i>
Angelica Dahurica Root ビャクシ	<i>Angelica dahurica</i> Benth. et Hooker filius ex Franchet et Savatier = <i>Angelica dahurica</i> (Hoffm.) Benth. & Hook. f. ex Franch. & Sav.	<i>Umbelliferae</i>
Apricot Kernel キョウニン	<i>Prunus armeniaca</i> Linné = <i>Prunus armeniaca</i> L.	<i>Rosaceae</i>
	<i>Prunus armeniaca</i> Linné var. <i>ansu</i> Maximowicz = <i>Prunus armeniaca</i> L. var. <i>ansu</i> Maxim.	
	<i>Prunus sibirica</i> Linné = <i>Prunus sibirica</i> L.	
Aralia Rhizome ドクカツ	<i>Aralia cordata</i> Thunberg = <i>Aralia cordata</i> Thunb.	<i>Araliaceae</i>
Areca ビンロウジ	<i>Areca catechu</i> Linné = <i>Areca catechu</i> L.	<i>Palmae</i>
Artemisia Capillaris Flower インチンコウ	<i>Artemisia capillaris</i> Thunberg = <i>Artemisia capillaris</i> Thunb.	<i>Compositae</i>
Asiasarum Root サイシン	<i>Asiasarum sieboldii</i> F. Maekawa = <i>Asiasarum sieboldii</i> (Miq.) F. Maek.	<i>Aristolochiaceae</i>
	<i>Asarum sieboldii</i> Miq.	
	<i>Asarum sieboldii</i> Miq. var. <i>seoulense</i> Nakai	
	<i>Asiasarum heterotropoides</i> F. Maekawa var. <i>mandshuricum</i> F. Maekawa = <i>Asiasarum heterotropoides</i> (F. Schmidt) F. Maek. var. <i>mandshuricum</i> (Maxim.) F. Maek.	
	<i>Asarum heterotropoides</i> F. Schmidt var. <i>mandshuricum</i> (Maxim.) Kitag.	
Asparagus Tuber テンモンドウ	<i>Asparagus cochinchinensis</i> Merrill = <i>Asparagus cochinchinensis</i> (Lour.) Merr.	<i>Liliaceae</i>
Astragalus Root オウギ	<i>Astragalus membranaceus</i> Bunge = <i>Astragalus membranaceus</i> (Fisch.) Bunge	<i>Leguminosae</i>
	<i>Astragalus mongholicus</i> Bunge	
	<i>Astragalus membranaceus</i> (Fisch.) Bunge var. <i>mongholicus</i> (Bunge) Hsiao	

Atractylodes Lancea Rhizome ソウジュツ	<i>Atractylodes lancea</i> De Candolle = <i>Atractylodes lancea</i> (Thunb.) DC.	Compositae
	<i>Atractylodes chinensis</i> Koidzumi = <i>Atractylodes chinensis</i> (DC.) Koidz.	
	Hybrid between above species	
Atractylodes Rhizome ジャクジュツ	<i>Atractylodes japonica</i> Koidzumi ex Kitamura = <i>Atractylodes japonica</i> Koidz. ex Kitam.	Compositae
	<i>Atractylodes macrocephala</i> Koidzumi = <i>Atractylodes macrocephala</i> Koidz.	
	* <i>Atractylodes ovata</i> De Candolle = <i>Atractylodes ovata</i> (Thunb.) DC.	
Bear Bile ユウタン	<i>Ursus arctos</i> Linné = <i>Ursus arctos</i> L.	Ursidae
	Other animals of the related genus	
Bearberry Leaf ウワウルシ	<i>Arctostaphylos uva-ursi</i> Sprengel = <i>Arctostaphylos uva-ursi</i> (L.) Spreng.	Ericaceae
Belladonna Root ベラドンナコン	<i>Atropa belladonna</i> Linné = <i>Atropa belladonna</i> L.	Solanaceae
Benincasa Seed トウガシ	<i>Benincasa cerifera</i> Savi	Cucurbitaceae
	<i>Benincasa hispida</i> (Thunb.) Cogn.	
	<i>Benincasa cerifera</i> Savi forma <i>emarginata</i> K. Kimura et Sugiyama = <i>Benincasa cerifera</i> Savi f. <i>emarginata</i> K. Kimura & Sugiyama	
Benzoin アンソッコウ	<i>Styrax benzoin</i> Dryander = <i>Styrax benzoin</i> Dryand.	Styracaceae
	Other species of the same genus	
Bitter Cardamon ヤクチ	<i>Alpinia oxyphylla</i> Miquel = <i>Alpinia oxyphylla</i> Miq.	Zingiberaceae
Bitter Orange Peel トウヒ	<i>Citrus aurantium</i> Linné = <i>Citrus aurantium</i> L.	Rutaceae
	<i>Citrus aurantium</i> Linné var. <i>daidai</i> Makino = <i>Citrus aurantium</i> L. var. <i>daidai</i> Makino	
	<i>Citrus aurantium</i> L. 'Daidai'	
Brown Rice コウベイ	<i>Oryza sativa</i> Linné = <i>Oryza sativa</i> L.	Gramineae
Bupleurum Root サイコ	<i>Bupleurum falcatum</i> Linné = <i>Bupleurum falcatum</i> L.	Umbelliferae
	<i>Bupleurum chinense</i> DC. <i>Bupleurum scorzonnerifolium</i> Willd.	
Burdock Fruit ゴボウシ	<i>Arctium lappa</i> Linné = <i>Arctium lappa</i> L.	Compositae
Calumba コロンボ	<i>Jateorhiza columba</i> Miers	Menispermaceae
Capsicum トウガラシ	<i>Capsicum annuum</i> Linné = <i>Capsicum annuum</i> L.	Solanaceae

Cardamon ショウブク	<i>Elettaria cardamomum</i> Maton	<i>Zingiberaceae</i>
Cassia Seed ケツメイシ	<i>Cassia obtusifolia</i> Linné = <i>Cassia obtusifolia</i> L.	<i>Leguminosae</i>
	<i>Cassia tora</i> Linné = <i>Cassia tora</i> L.	
Catalpa Fruit キササゲ	<i>Catalpa ovata</i> G. Don	<i>Bignoniaceae</i>
	<i>Catalpa bungei</i> C. A. Meyer = <i>Catalpa bungei</i> C. A. Mey.	
Chrysanthemum Flower キクカ	<i>Chrysanthemum morifolium</i> Ramatuelle = <i>Chrysanthemum morifolium</i> Ramat.	<i>Compositae</i>
	<i>Chrysanthemum indicum</i> Linné = <i>Chrysanthemum indicum</i> L.	
Cimicifuga Rhizome ショウマ	<i>Cimicifuga simplex</i> Turczaninow = <i>Cimicifuga simplex</i> (DC.) Turcz.	<i>Ranunculaceae</i>
	<i>Cimicifuga dahurica</i> Maximowicz = <i>Cimicifuga dahurica</i> (Turcz.) Maxim.	
	<i>Cimicifuga heracleifolia</i> Komarov = <i>Cimicifuga heracleifolia</i> Kom.	
	<i>Cimicifuga foetida</i> Linné = <i>Cimicifuga foetida</i> L.	
Cinnamon Bark ケイヒ	<i>Cinnamomum cassia</i> Blume	<i>Lauraceae</i>
Cinnamon Oil ケイヒ油	<i>Cinnamomum cassia</i> Blume	<i>Lauraceae</i>
	<i>Cinnamomum zeylanicum</i> Nees	
Citrus Unshiu Peel チンピ	<i>Citrus unshiu</i> Marcowicz = <i>Citrus unshiu</i> (Swingle) Marcow.	<i>Rutaceae</i>
	<i>Citrus reticulata</i> Blanco ‘Unshiu’	
	<i>Citrus reticulata</i> Blanco	
Clematis Root イレイセン	<i>Clematis chinensis</i> Osbeck	<i>Ranunculaceae</i>
	<i>Clematis mandshurica</i> Ruprecht = <i>Clematis mandshurica</i> Rupr.	
	<i>Clematis hexapetala</i> Pallas = <i>Clematis hexapetala</i> Pall.	
Clove チョウジ Clove oil チョウジ油	<i>Syzygium aromaticum</i> Merrill et Perry = <i>Syzygium aromaticum</i> (L.) Merr. & M. L. Perry	<i>Myrtaceae</i>
	* <i>Eugenia caryophyllata</i> Thunberg = <i>Eugenia caryophyllata</i> Thunb. <i>Eugenia caryophyllus</i> (Spreng.) Bullock & S. G. Harrison	
Cnidium Monnieri Fruit ジャシヨウシ	<i>Cnidium monnieri</i> Cusson = <i>Cnidium monnieri</i> (L.) Cusson	<i>Umbelliferae</i>
Cnidium Rhizome センキュウ	<i>Cnidium officinale</i> Makino	<i>Umbelliferae</i>
Coix Seed ヨクイニン	<i>Coix lacryma-jobi</i> Linné var. <i>mayuen</i> Stapf = <i>Coix lacryma-jobi</i> L. var. <i>mayuen</i> (Rom. Caill.) Stapf	<i>Gramineae</i>

Condurango コンズランゴ	<i>Marsdenia cundurango</i> Reichenbach filius = <i>Marsdenia cundurango</i> Rchb. f.	<i>Asclepiadaceae</i>
Coptis Rhizome オウレン	<i>Coptis japonica</i> Makino = <i>Coptis japonica</i> (Thunb.) Makino	<i>Ranunculaceae</i>
	<i>Coptis japonica</i> (Thunb.) Makino var. <i>dissecta</i> (Yatabe) Nakai <i>Coptis japonica</i> (Thunb.) Makino var. <i>japonica</i> <i>Coptis japonica</i> (Thunb.) Makino var. <i>major</i> (Miq.) Satake	
	<i>Coptis chinensis</i> Franchet = <i>Coptis chinensis</i> Franch.	
	<i>Coptis deltoidea</i> C. Y. Cheng et Hsiao	
	<i>Coptis teeta</i> Wallich = <i>Coptis teeta</i> Wall.	
Cornus Fruit サンシュユ	<i>Cornus officinalis</i> Siebold et Zuccarini = <i>Cornus officinalis</i> Siebold & Zucc.	<i>Cornaceae</i>
Corydalis Tuber エンゴサク	<i>Corydalis turtchaninovii</i> Besser forma <i>yanhusuo</i> Y. H. Chou et C. C. Hsu = <i>Corydalis turtchaninovii</i> Besser f. <i>yanhusuo</i> (W. T. Wang) Y. H. Chou & C. C. Hsu	<i>Papaveraceae</i>
	<i>Corydalis yanhusuo</i> W. T. Wang	
Crataegus Fruit サンザシ	<i>Crataegus cuneata</i> Siebold et Zuccarini = <i>Crataegus cuneata</i> Siebold & Zucc.	<i>Rosaceae</i>
	<i>Crataegus pinnatifida</i> Bunge var. <i>major</i> N. E. Brown = <i>Crataegus pinnatifida</i> Bunge var. <i>major</i> N. E. Br.	
Cyperus Rhizome コウブシ	<i>Cyperus rotundus</i> Linné = <i>Cyperus rotundus</i> L.	<i>Cyperaceae</i>
Digenea マクリ	<i>Digenea simplex</i> C. Agardh = <i>Digenea simplex</i> (Wulfen) C. Agardh	<i>Rhodomelaceae</i>
Dioscorea Rhizome サンヤク	<i>Dioscorea japonica</i> Thunberg = <i>Dioscorea japonica</i> Thunb.	<i>Dioscoreaceae</i>
	<i>Dioscorea batatas</i> Decaisne = <i>Dioscorea batatas</i> Decne.	
	<i>Dioscorea opposita</i> Thunb.	
Dolichos Seed ヘンズ	<i>Dolichos lablab</i> Linné = <i>Dolichos lablab</i> L.	<i>Leguminosae</i>
Eleutherococcus Senticosus Rhizome シゴカ	<i>Eleutherococcus senticosus</i> Maximowicz = <i>Eleutherococcus senticosus</i> (Rupr. & Maxim.) Maxim.	<i>Araliaceae</i>
	* <i>Acanthopanax senticosus</i> Harms = <i>Acanthopanax senticosus</i> (Rupr. & Maxim.) Harms	
Ephedra Herb マオウ	<i>Ephedra sinica</i> Stapf	<i>Ephedraceae</i>
	<i>Ephedra intermedia</i> Schrenk et C. A. Meyer = <i>Ephedra intermedia</i> Schrenk & C. A. Mey.	
	<i>Ephedra equisetina</i> Bunge	

Epimedium Herb インヨウカク	<i>Epimedium pubescens</i> Maximowicz = <i>Epimedium pubescens</i> Maxim.	Berberidaceae
	<i>Epimedium brevicornu</i> Maximowicz = <i>Epimedium brevicornu</i> Maxim.	
	<i>Epimedium wushanense</i> T. S. Ying	
	<i>Epimedium sagittatum</i> Maximowicz = <i>Epimedium sagittatum</i> (Siebold & Zucc.) Maxim.	
	<i>Epimedium koreanum</i> Nakai	
	<i>Epimedium grandiflorum</i> Morren var. <i>thunbergianum</i> Nakai = <i>Epimedium grandiflorum</i> Morr. var. <i>thunbergianum</i> (Miq.) Nakai	
	<i>Epimedium sempervirens</i> Nakai	
Eucommia Bark トチュウ	<i>Eucommia ulmoides</i> Oliver = <i>Eucommia ulmoides</i> Oliv.	Eucommiaceae
Euodia Fruit ゴシユユ	<i>Euodia ruticarpa</i> Hooker filius et Thomson = <i>Euodia ruticarpa</i> (A. Juss.) Hook. f. & Thomson	Rutaceae
	* <i>Evodia rutaecarpa</i> Benth = <i>Evodia rutaecarpa</i> (A. Juss.) Benth. <i>Tetradium ruticarpum</i> (A. Juss.) Hartley	
	<i>Euodia officinalis</i> Dode	
	* <i>Evodia officinalis</i> Dode <i>Evodia rutaecarpa</i> (A. Juss.) Benth. var. <i>officinalis</i> (Dode) Huang	
	<i>Euodia bodinieri</i> Dode	
	* <i>Evodia bodinieri</i> Dode <i>Evodia rutaecarpa</i> (A. Juss.) Benth. var. <i>bodinieri</i> (Dode) Huang	
Fennel ウイキョウ	<i>Foeniculum vulgare</i> Miller = <i>Foeniculum vulgare</i> Mill.	Umbelliferae
Fennel Oil ウイキョウ油	<i>Foeniculum vulgare</i> Miller = <i>Foeniculum vulgare</i> Mill.	Umbelliferae
	<i>Illicium verum</i> Hooker filius = <i>Illicium verum</i> Hook. f.	Illiciaceae
Forsythia Fruit レンギョウ	<i>Forsythia suspensa</i> Vahl = <i>Forsythia suspensa</i> (Thunb.) Vahl	Oleaceae
	<i>Forsythia viridissima</i> Lindley = <i>Forsythia viridissima</i> Lindl.	
Fritillaria Bulb バイモ	<i>Fritillaria verticillata</i> Willdenow var. <i>thunbergii</i> Baker = <i>Fritillaria verticillata</i> Willd. var. <i>thunbergii</i> (Miq.) Baker	Liliaceae
	<i>Fritillaria thunbergii</i> Miq.	
Gambir アセンヤク	<i>Uncaria gambir</i> Roxburgh = <i>Uncaria gambir</i> (Hunter) Roxb.	Rubiaceae
Gardenia Fruit サンシシ	<i>Gardenia jasminoides</i> Ellis	Rubiaceae
	<i>Gardenia jasminoides</i> Ellis f. <i>longicarpa</i> Z. W. Xie & Okada	
Gastrodia Tuber テンマ	<i>Gastrodia elata</i> Blume	Orchidaceae
Gentian ゲンチアナ	<i>Gentiana lutea</i> Linné = <i>Gentiana lutea</i> L.	Gentianaceae

Geranium Herb ゲンノショウコ	<i>Geranium thunbergii</i> Siebold et Zuccarini = <i>Geranium thunbergii</i> Siebold & Zucc.	<i>Geraniaceae</i>
Ginger ショウキョウ	<i>Zingiber officinale</i> Roscoe	<i>Zingiberaceae</i>
Ginseng ニンジン	<i>Panax ginseng</i> C. A. Meyer = <i>Panax ginseng</i> C. A. Mey. * <i>Panax schinseng</i> Nees	<i>Araliaceae</i>
Glehnia Root and Rhizome ハマボウフウ	<i>Glehnia littoralis</i> Fr. Schmidt ex Miquel = <i>Glehnia littoralis</i> F. Schmidt ex Miq.	<i>Umbelliferae</i>
Glycyrrhiza カンゾウ	<i>Glycyrrhiza uralensis</i> Fischer = <i>Glycyrrhiza uralensis</i> Fisch. <i>Glycyrrhiza glabra</i> Linné = <i>Glycyrrhiza glabra</i> L.	<i>Leguminosae</i>
Hemp Fruit マシニン	<i>Cannabis sativa</i> Linné = <i>Cannabis sativa</i> L.	<i>Moracea</i>
Honey ハチミツ	<i>Apis mellifera</i> Linné = <i>Apis mellifera</i> L. <i>Apis cerana</i> Fabricius	<i>Apidae</i>
Houttuynia Herb ジュウヤク	<i>Houttuynia cordata</i> Thunberg = <i>Houttuynia cordata</i> Thunb.	<i>Saururaceae</i>
Immature Orange キジツ	<i>Citrus aurantium</i> Linné var. <i>daidai</i> Makino = <i>Citrus aurantium</i> L. var. <i>daidai</i> Makino <i>Citrus aurantium</i> L. 'Daidai' <i>Citrus natsuda</i> Hayata <i>Citrus aurantium</i> Linné = <i>Citrus aurantium</i> L. <i>Citrus aurantium</i> L. subsp. <i>hassaku</i> (Tanaka) Hiroe = <i>Citrus hassaku</i> hort. ex Tanaka	<i>Rutaceae</i>
Imperata Rhizome ボウコン	<i>Imperata cylindrica</i> Beauvois = <i>Imperata cylindrica</i> (L.) P. Beauv. <i>Imperata cylindrica</i> (L.) P. Beauv. var. <i>major</i> (Nees) C. E. Hubb.	<i>Gramineae</i>
Ipecac トコン	<i>Cephaelis ipecacuanha</i> A. Richard = <i>Cephaelis ipecacuanha</i> (Brot.) A. Rich. <i>Cephaelis acuminata</i> Karsten = <i>Cephaelis acuminata</i> H. Karst.	<i>Rubiaceae</i>
Japanese Angelica Root トウキ	<i>Angelica acutiloba</i> Kitagawa = <i>Angelica acutiloba</i> (Siebold & Zucc.) Kitag. <i>Angelica acutiloba</i> Kitagawa var. <i>sugiyamae</i> Hikino = <i>Angelica acutiloba</i> (Siebold & Zucc.) Kitag. var. <i>sugiyamae</i> Hikino	<i>Umbelliferae</i>

Japanese Gentian リュウタン	<i>Gentiana scabra</i> Bunge	<i>Gentianaceae</i>
	<i>Gentiana scabra</i> Bunge var. <i>buergeri</i> (Miq.) Maxim.	
	<i>Gentiana manshurica</i> Kitagawa = <i>Gentiana manshurica</i> Kitag.	
	<i>Gentiana triflora</i> Pallas = <i>Gentiana triflora</i> Pall.	
	<i>Gentiana triflora</i> Pall. var. <i>japonica</i> Hara	
Japanese Valerian カノコソウ	<i>Valeriana fauriei</i> Briquet = <i>Valeriana fauriei</i> Briq.	<i>Valerianaceae</i>
	<i>Valeriana fauriei</i> Briq. f. <i>yezoensis</i> Hara	
Jujube Seed サンソウニン	<i>Zizyphus jujuba</i> Miller var. <i>spinosa</i> Hu ex H. F. Chou = <i>Zizyphus jujuba</i> Mill. var. <i>spinosa</i> (Bunge) Hu ex H. F. Chou	<i>Rhamnaceae</i>
Jujube タイソウ	<i>Zizyphus jujuba</i> Miller var. <i>inermis</i> Rehder = <i>Zizyphus jujuba</i> Mill. var. <i>inermis</i> (Bunge) Rehder	<i>Rhamnaceae</i>
Koi コウイ	<i>Zea mays</i> Linné = <i>Zea mays</i> L.	<i>Gramineae</i>
	<i>Manihot esculenta</i> Crantz	<i>Euphorbiaceae</i>
	<i>Solanum tuberosum</i> Linné = <i>Solanum tuberosum</i> L.	<i>Solanaceae</i>
	<i>Ipomoea batatas</i> Poirét = <i>Ipomoea batatas</i> (L.) Poir	<i>Convolvulaceae</i>
	<i>Ipomoea batatas</i> (L.) Lam.	
	<i>Oryza sativa</i> Linné = <i>Oryza sativa</i> L.	<i>Gramineae</i>
Leonurus Herb ヤクモソウ	<i>Leonurus japonicus</i> Houttuyn = <i>Leonurus japonicus</i> Houtt.	<i>Labiatae</i>
	<i>Leonurus sibiricus</i> Linné = <i>Leonurus sibiricus</i> L.	
Lilium Bulb ジャクゴウ	<i>Lilium lancifolium</i> Thunberg = <i>Lilium lancifolium</i> Thunb.	<i>Liliaceae</i>
	<i>Lilium brownii</i> F. E. Brown var. <i>colchesteri</i> Wilosn = <i>Lilium brownii</i> F. E. Br. var. <i>colchesteri</i> (Van Houtte) E. H. Wilson ex Elwes	
	<i>Lilium brownii</i> F. E. Brown var. <i>viridulum</i> Baker	
	<i>Lilium brownii</i> F. E. Brown = <i>Lilium brownii</i> F. E. Br.	
	<i>Lilium pumilum</i> De Candolle = <i>Lilium pumilum</i> DC.	
Lindera Root ウヤク	<i>Lindera strychnifolia</i> Fernandez-Villar = <i>Lindera strychnifolia</i> (Siebold & Zucc.) Fern.-Vill.	<i>Lauraceae</i>
	<i>Lindera aggregata</i> (Sims) Kosterm.	
Lithospermum Root シコン	<i>Lithospermum erythrorhizon</i> Siebold et Zuccarini = <i>Lithospermum erythrorhizon</i> Siebold & Zucc.	<i>Boraginaceae</i>

Longan Aril リュウガンニク	<i>Euphoria longana</i> Lamarck = <i>Euphoria longana</i> Lam.	<i>Sapindaceae</i>
	<i>Dimocarpus longan</i> Lour.	
Lonicera Leaf and Stem ニンドウ	<i>Lonicera japonica</i> Thunberg = <i>Lonicera japonica</i> Thunb.	<i>Caprifoliaceae</i>
Loquat Leaf ビワヨウ	<i>Eriobotrya japonica</i> Lindley = <i>Eriobotrya japonica</i> (Thunb.) Lindl.	<i>Rosaceae</i>
Lycium Bark ジコッピ	<i>Lycium chinense</i> Miller = <i>Lycium chinense</i> Mill.	<i>Solanaceae</i>
	<i>Lycium barbarum</i> Linné = <i>Lycium barbarum</i> L.	
Lycium Fruit クコシ	<i>Lycium chinense</i> Miller = <i>Lycium chinense</i> Mill.	<i>Solanaceae</i>
	<i>Lycium barbarum</i> Linné = <i>Lycium barbarum</i> L.	
Magnolia Bark コウボク	<i>Magnolia obovata</i> Thunberg = <i>Magnolia obovata</i> Thunb.	<i>Magnoliaceae</i>
	* <i>Magnolia hypoleuca</i> Siebold et Zuccarini = <i>Magnolia hypoleuca</i> Siebold & Zucc.	
	<i>Magnolia officinalis</i> Rehder et Wilson = <i>Magnolia officinalis</i> Rehder & E. H. Wilson	
	<i>Magnolia officinalis</i> Rehder et Wilson var. <i>biloba</i> Rehder et Wilson = <i>Magnolia officinalis</i> Rehder & E. H. Wilson var. <i>biloba</i> Rehder & E. H. Wilson	
Magnolia Flower シンイ	<i>Magnolia salicifolia</i> Maximowicz = <i>Magnolia salicifolia</i> (Siebold & Zucc.) Maxim.	<i>Magnoliaceae</i>
	<i>Magnolia kobus</i> De Candolle = <i>Magnolia kobus</i> DC.	
	<i>Magnolia biondii</i> Pampanini = <i>Magnolia biondii</i> Pamp.	
	<i>Magnolia sprengeri</i> Pampanini = <i>Magnolia sprengeri</i> Pamp.	
	<i>Magnolia heptapeta</i> Dandy = <i>Magnolia heptapeta</i> (Buchoz) Dandy	
	* <i>Magnolia denudata</i> Desrousseaux = <i>Magnolia denudata</i> Desr.	
Mallotus Bark アカメガシワ	<i>Mallotus japonicus</i> Mueller Argoviensis = <i>Mallotus japonicus</i> (Thunb.) Müll. Arg.	<i>Euphorbiaceae</i>
Mentha Herb ハッカ Mentha Oil ハッカ油	<i>Mentha arvensis</i> Linné var. <i>piperascens</i> Malinvaud = <i>Mentha arvensis</i> L. var. <i>piperascens</i> Malinv.	<i>Labiatae</i>
	<i>Mentha haplocalyx</i> Briq.	
	Hybrid originated from <i>Mentha arvensis</i> L. var. <i>piperascens</i> Malinv. as the mother species	
Moutan Bark ボタンピ	<i>Paeonia suffruticosa</i> Andrews	<i>Paeoniaceae</i>
	* <i>Paeonia moutan</i> Sims	

Mulberry Bark ソウハクヒ	<i>Morus alba</i> Linné = <i>Morus alba</i> L.	<i>Moraceae</i>
Nelumbo Seed レンニク	<i>Nelumbo nucifera</i> Gaertner = <i>Nelumbo nucifera</i> Gaertn.	<i>Nymphaeaceae</i>
Notopterygium キョウカツ	<i>Notopterygium incisum</i> Ting ex H. T. Chang	<i>Umbelliferae</i>
	<i>Notopterygium forbesii</i> Boissieu	
Nuphar Rhizome センコツ	<i>Nuphar japonicum</i> De Candolle = <i>Nuphar japonicum</i> DC.	<i>Nymphaeaceae</i>
Nutmeg ニクズク	<i>Myristica fragrans</i> Houttuyn = <i>Myristica fragrans</i> Houtt.	<i>Myristicaceae</i>
Nux Vomica ホミカ	<i>Strychnos nux-vomica</i> Linné = <i>Strychnos nux-vomica</i> L.	<i>Loganiaceae</i>
Ophiopogon Tuber バクモンドウ	<i>Ophiopogon japonicus</i> Ker-Gawler = <i>Ophiopogon japonicus</i> (L. f.) Ker Gawl.	<i>Liliaceae</i>
Oriental Bezoar ゴオウ	<i>Bos taurus</i> Linné var. <i>domesticus</i> Gmelin = <i>Bos taurus</i> L. var. <i>domesticus</i> Gmelin	<i>Bovidae</i>
Oyster Shell ボレイ	<i>Ostrea gigas</i> Thunberg = <i>Ostrea gigas</i> Thunb.	<i>Ostreidae</i>
Panax Japonicus Rhizome チクセツニンジン	<i>Panax japonicus</i> C. A. Meyer = <i>Panax japonicus</i> C. A. Mey.	<i>Araliaceae</i>
Peach Kernel トウニン	<i>Prunus persica</i> Batsch = <i>Prunus persica</i> (L.) Batsch	<i>Rosaceae</i>
	<i>Prunus persica</i> Batsch var. <i> davidiana</i> Maximowicz = <i>Prunus persica</i> (L.) Batsch var. <i> davidiana</i> (Carrière) Maxim.	
	<i>Prunus davidiana</i> (Carrière) Franch.	
Peony Root シャクヤク	<i>Paeonia lactiflora</i> Pallas = <i>Paeonia lactiflora</i> Pall.	<i>Paeoniaceae</i>
Perilla Herb ソヨウ	<i>Perilla frutescens</i> Britton var. <i>acuta</i> Kudo = <i>Perilla frutescens</i> (L.) Britton var. <i>acuta</i> (Thunb.) Kudo	<i>Labiatae</i>
	<i>Perilla frutescens</i> Britton var. <i>crispa</i> Decaisne = <i>Perilla frutescens</i> (L.) Britton var. <i>crispa</i> (Thunb.) Decne.	
Peucedanum Root ゼンコ	<i>Peucedanum praeruptorum</i> Dunn	<i>Umbelliferae</i>
	<i>Angelica decursiva</i> Franchet et Savatier = <i>Angelica decursiva</i> (Miq.) Franch. & Sav.	
	* <i>Peucedanum decursivum</i> Maximowicz = <i>Peucedanum decursivum</i> (Miq.) Maxim.	
Pharbitis Seed ケンゴシ	<i>Pharbitis nil</i> Choisy = <i>Pharbitis nil</i> (L.) Choisy	<i>Convolvulaceae</i>
Phellodendron Bark オウバク	<i>Phellodendron amurense</i> Ruprecht = <i>Phellodendron amurense</i> Rupr.	<i>Rutaceae</i>
	<i>Phellodendron amurense</i> Rupr. var. <i>sachalinense</i> F. Schmidt <i>Phellodendron amurense</i> Rupr. var. <i>japonicum</i> (Maxim.) Ohwi <i>Phellodendron amurense</i> Rupr. var. <i>lavalleyi</i> (Dode) Sprangue	
	<i>Phellodendron chinense</i> Schneider = <i>Phellodendron chinense</i> C. K. Schneid.	

Picrasma Wood ニガキ	<i>Picrasma quassioides</i> Bennet = <i>Picrasma quassioides</i> (D. Don) Benn.	<i>Simaroubaceae</i>
Pinellia Tuber ハング	<i>Pinellia ternata</i> Breitenbach = <i>Pinellia ternata</i> (Thunb.) Breitenb.	<i>Araceae</i>
Plantago Herb シャゼンソウ	<i>Plantago asiatica</i> Linné = <i>Plantago asiatica</i> L.	<i>Plantaginaceae</i>
Plantago Seed シャゼンシ	<i>Plantago asiatica</i> Linné = <i>Plantago asiatica</i> L.	<i>Plantaginaceae</i>
Platycodon Root キキョウ	<i>Platycodon grandiflorum</i> A. De Candolle = <i>Platycodon grandiflorum</i> (Jacq.) A. DC.	<i>Campanulaceae</i>
Pogostemon Herb カッコウ	<i>Pogostemon cablin</i> Benthham = <i>Pogostemon cablin</i> (Blanco) Benth.	<i>Labiatae</i>
Polygala Root オンジ	<i>Polygala tenuifolia</i> Willdenow = <i>Polygala tenuifolia</i> Willd.	<i>Polygalaceae</i>
Polygonatum Rhizome オウセイ	<i>Polygonatum falcatum</i> A. Gray	<i>Liliaceae</i>
	<i>Polygonatum sibiricum</i> Redouté	
	<i>Polygonatum kingianum</i> Collett et Hemsley = <i>Polygonatum kingianum</i> Collett & Hemsl.	
	<i>Polygonatum cyrtonema</i> Hua	
Polygonum Root カシュウ	<i>Polygonum multiflorum</i> Thunberg = <i>Polygonum multiflorum</i> Thunb.	<i>Polygonaceae</i>
Polyporus Sclerotium チョレイ	<i>Polyporus umbellatus</i> Fries = <i>Polyporus umbellatus</i> (Pers.) Fries	<i>Polyporaceae</i>
Poria Sclerotium ブクリョウ	<i>Wolfiporia cocos</i> Ryvarden et Gilbertson = <i>Wolfiporia cocos</i> (Schw.) Ryv. & Gilbn.	<i>Polyporaceae</i>
	* <i>Poria cocos</i> Wolf = <i>Poria cocos</i> (Schw.) Wolf	
Processed Aconite Root ブシ	<i>Aconitum carmichaeli</i> Debeaux	<i>Ranunculaceae</i>
	<i>Aconitum japonicum</i> Thunberg = <i>Aconitum japonicum</i> Thunb.	
Processed Ginger カンキョウ	<i>Zingiber officinale</i> Roscoe	<i>Zingiberaceae</i>
Prunella Spike カゴソウ	<i>Prunella vulgaris</i> Linné var. <i>lilacina</i> Nakai = <i>Prunella vulgaris</i> L. var. <i>lilacina</i> Nakai	<i>Labiatae</i>
Pueraria Root カッコン	<i>Pueraria lobata</i> Ohwi = <i>Pueraria lobata</i> (Willd.) Ohwi	<i>Leguminosae</i>
Quercus Bark ボクソク	<i>Quercus acutissima</i> Carruthers = <i>Quercus acutissima</i> Carruth.	<i>Fagaceae</i>
	<i>Quercus serrata</i> Murray	
	<i>Quercus mongolica</i> Fischer ex Ledebour var. <i>crispula</i> Ohashi = <i>Quercus mongolica</i> Fisch. ex Ledeb. var. <i>crispula</i> (Blume) Ohashi	
	<i>Quercus variabilis</i> Blume	
Red Ginseng コウジン	<i>Panax ginseng</i> C. A. Meyer = <i>Panax ginseng</i> C. A. Mey.	<i>Araliaceae</i>
	* <i>Panax schinseng</i> Nees	

Rehmannia Root ジオウ	<i>Rehmannia glutinosa</i> Liboschitz var. <i>purpurea</i> Makino = <i>Rehmannia glutinosa</i> Libosch. var. <i>purpurea</i> Makino	<i>Scrophulariaceae</i>
	<i>Rehmannia glutinosa</i> Liboschitz = <i>Rehmannia glutinosa</i> Libosch.	
Rhubarb ダイオウ	<i>Rheum palmatum</i> Linné = <i>Rheum palmatum</i> L.	<i>Polygonaceae</i>
	<i>Rheum tanguticum</i> Maximowicz = <i>Rheum tanguticum</i> Maxim.	
	<i>Rheum officinale</i> Baillon = <i>Rheum officinale</i> Baill.	
	<i>Rheum coreanum</i> Nakai	
	Hybrid between above species	
Rose Fruit エイジツ	<i>Rosa multiflora</i> Thunberg = <i>Rosa multiflora</i> Thunb.	<i>Rosaceae</i>
Rosin ロジン	Several plants of <i>Pinus</i> genus	<i>Pinaceae</i>
Royal Jelly ローヤルゼリー	<i>Apis mellifera</i> Linné = <i>Apis mellifera</i> L.	<i>Apidae</i>
	<i>Apis cerana</i> Fabricius	
Safflower コウカ	<i>Carthamus tinctorius</i> Linné = <i>Carthamus tinctorius</i> L.	<i>Compositae</i>
Saffron サフラン	<i>Crocus sativus</i> Linné = <i>Crocus sativus</i> L.	<i>Iridaceae</i>
Saposhnikovia Root and Rhizome ポウフウ	<i>Saposhnikovia divaricata</i> Schischkin = <i>Saposhnikovia divaricata</i> (Turcz.) Schischk.	<i>Umbelliferae</i>
Sappan Wood ソボク	<i>Caesalpinia sappan</i> Linné = <i>Caesalpinia sappan</i> L.	<i>Leguminosae</i>
Saussurea Root モッコウ	<i>Saussurea lappa</i> Clarke = <i>Saussurea lappa</i> (Decne.) C. B. Clarke	<i>Compositae</i>
	<i>Aucklandia lappa</i> Decne.	
Schisandra Fruit ゴミシ	<i>Schisandra chinensis</i> Baillon = <i>Schisandra chinensis</i> (Turcz.) Baill.	<i>Schisandraceae</i>
Schizonepeta Spike ケイガイ	<i>Schizonepeta tenuifolia</i> Briquet = <i>Schizonepeta tenuifolia</i> Briq.	<i>Labiatae</i>
Scopolia Rhizome ロートコン	<i>Scopolia japonica</i> Maximowicz = <i>Scopolia japonica</i> Maxim.	<i>Solanaceae</i>
	<i>Scopolia carniolica</i> Jacquin = <i>Scopolia carniolica</i> Jacq.	
	<i>Scopolia parviflora</i> Nakai = <i>Scopolia parviflora</i> (Dunn) Nakai	
Scutellaria Root オウゴン	<i>Scutellaria baicalensis</i> Georgi	<i>Labiatae</i>

Senega セネガ	<i>Polygala senega</i> Linné = <i>Polygala senega</i> L.	<i>Polygalaceae</i>
	<i>Polygala senega</i> Linné var. <i>latifolia</i> Torrey et Gray = <i>Polygala senega</i> L. var. <i>latifolia</i> Torr. & A. Gray	
Senna Leaf センナ	<i>Cassia angustifolia</i> Vahl	<i>Leguminosae</i>
	<i>Cassia acutifolia</i> Delile	
Sesame ゴマ	<i>Sesamum indicum</i> Linné = <i>Sesamum indicum</i> L.	<i>Pedaliaceae</i>
Sinomenium Stem and Rhizome ボウイ	<i>Sinomenium acutum</i> Rehder et Wilson = <i>Sinomenium acutum</i> (Thunb.) Rehder & E. H. Wilson	<i>Menispermaceae</i>
Smilax Rhizome サンキライ	<i>Smilax glabra</i> Roxburgh = <i>Smilax glabra</i> Roxb.	<i>Liliaceae</i>
Sophora Root クジン	<i>Sophora flavescens</i> Aiton	<i>Leguminosae</i>
Sweet Hydrangea Leaf アマチャ	<i>Hydrangea macrophylla</i> Seringe var. <i>thunbergii</i> Makino = <i>Hydrangea macrophylla</i> (Thunb.) Ser. var. <i>thunbergii</i> (Siebold) Makino	<i>Saxifragaceae</i>
Swertia Herb センブリ	<i>Swertia japonica</i> Makino = <i>Swertia japonica</i> (Shult.) Makino	<i>Gentianaceae</i>
Toad Venom センソ	<i>Bufo bufo gargarizans</i> Cantor	<i>Bufonidae</i>
	<i>Bufo melanostictus</i> Schneider	
Tragacanth トラガント	<i>Astragalus gummifer</i> Labillardière = <i>Astragalus gummifer</i> Labill.	<i>Leguminosae</i>
Tribulus Fruit シツリシ	<i>Tribulus terrestris</i> Linné = <i>Tribulus terrestris</i> L.	<i>Zygophyllaceae</i>
Trichosanthes Root カロコン	<i>Trichosanthes kirilowii</i> Maximowicz = <i>Trichosanthes kirilowii</i> Maxim.	<i>Cucurbitaceae</i>
	<i>Trichosanthes kirilowii</i> Maximowicz var. <i>japonica</i> Kitamura = <i>Trichosanthes kirilowii</i> Maxim. var. <i>japonica</i> (Miq.) Kitam.	
	<i>Trichosanthes bracteata</i> Voigt = <i>Trichosanthes bracteata</i> (Lam.) Voigt	
Turmeric ウコン	<i>Curcuma longa</i> Linné = <i>Curcuma longa</i> L.	<i>Zingiberaceae</i>
Uncaria Hook チョウトウコウ	<i>Uncaria rhynchophylla</i> Miquel = <i>Uncaria rhynchophylla</i> (Miq.) Miq.	<i>Rubiaceae</i>
	<i>Uncaria sinensis</i> Haviland = <i>Uncaria sinensis</i> (Oliv.) Havil.	
	<i>Uncaria macrophylla</i> Wallich = <i>Uncaria macrophylla</i> Wall.	
Zanthoxylum Fruit サンショウ	<i>Zanthoxylum piperitum</i> De Candolle = <i>Zanthoxylum piperitum</i> (L.) DC.	<i>Rutaceae</i>
	<i>Zanthoxylum piperitum</i> (L.) DC. f. <i>inerme</i> Makino	
Zedoary ガジュツ	<i>Curcuma zedoaria</i> Roscoe	<i>Zingiberaceae</i>

When “Other species of the same genus” is included as its original plants the scientific name is not written in Monograph, however, it is written in this table.

Reference

Terabayashi S. *et al.*: Pharmaceutical and Medical Device Regulatory Science, 41(5), 407 – 418 (2010).

G6 Drug Formulation

Tablet Friability Test

This test is harmonized with the European Pharmacopoeia and the U.S. Pharmacopeia.

The Tablet Friability Test is a method to determine the friability of compressed uncoated tablets. The test procedure presented in this chapter is generally applicable to most compressed tablets. Measurement of tablets friability supplements other physical strength measurement, such as tablet crushing strength.

Use a drum, with an internal diameter between 283 and 291 mm and a depth between 36 and 40 mm, of transparent synthetic polymer with polished internal surfaces, and subject to minimum static build-up (see figure for a typical apparatus). One side of the drum is removable. The tablets are tumbled at each turn of the drum by a curved projection with an inside radius between 75.5 and 85.5 mm that extends from the middle of the drum to the outer wall. The outer diameter of the central ring is between 24.5 and 25.5 mm. The drum is attached to the horizontal axis of a device that rotates at 25 ± 1 rpm. Thus, at each turn the tablets roll or slide and fall onto the drum wall or onto each other.

For tablets with a unit mass equal to or less than 650 mg, take a sample of whole tablets n corresponding as near as possible to 6.5 g. For tablets with a unit mass of more than 650 mg, take a sample of 10 whole tablets. The tablets should be carefully dedusted prior to testing. Accurately weigh the tablet sample, and place the tablets in the drum. Rotate the drum 100 times, and remove the tablets. Remove any loose dust from the tablets as before, and accurately weigh.

Generally, the test is run once. If obviously cracked, cleaved, or broken tablets are present in the tablet sample after tumbling, the sample fails the test. If the results are difficult to interpret or if the mass loss is greater than the targeted value, the test should be repeated twice and the mean of the three tests determined. A maximum mean mass loss from the three samples of not more than 1.0% is considered acceptable for most products.

If tablet size or shape causes irregular tumbling, adjust the

drum base so that the base forms an angle of about 10° with the horizontal and the tablets no longer bind together when lying next to each other, which prevents them from falling freely.

Effervescent tablets and chewable tablets may have different specifications as far as friability is concerned. In the case of hygroscopic tablets, an appropriate humidity-controlled environment is required for testing.

Drums with dual scooping projections, or apparatus with more than one drum, for the running of multiple samples at one time, are also permitted.

G7 Containers and Package

Plastic Containers for Pharmaceutical Products

Various kinds of plastics are used as the materials for manufacturing containers for pharmaceutical products. Such plastics should not have the properties to deteriorate the efficacy, safety or stability of the pharmaceutical products to be packed in the container. In selecting a suitable plastic container, it is desirable to have sufficient information on the manufacturing processes of the plastic container including that on the substances added. Since each plastic has specific properties and a wide variety of pharmaceutical products may be packed in plastic containers, the compatibility of plastic containers with pharmaceutical products should be judged for each combination of container and the specific pharmaceutical product to be contained therein. Such judgement should be performed through a verification that the container for the pharmaceutical preparation can comply with the essential requirements for the container, i.e., the design specifications, based on the data from the experiments on the prototype products of the container and/or information from scientific documentation, etc. In addition, such compatibility must be ensured based upon an appropriate quality assurance system.

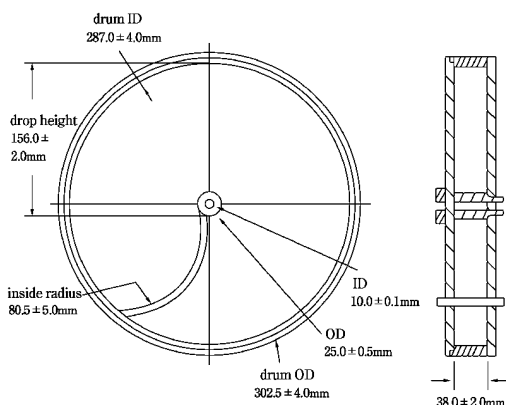
Furthermore, in introducing a plastic container, it is desirable that proper disposal method after use is taken into consideration.

1. Essential Requirements in Designing Plastic Containers for Pharmaceutical Products

The plastic material used for the container should be of high quality. Therefore, recycled plastic materials, which are of unknown constitution, must not be used.

The leachables or migrants from the container should not deteriorate the efficacy or stability of the pharmaceutical products contained therein. In addition, the possible toxic hazards due to the leachables or migrants should not exceed a given level. Furthermore, the amounts of leachable or migratable chemical substances, such as monomers and additives, from the containers to the pharmaceutical products contained therein must be sufficiently small from the viewpoint of safety.

The container should have a certain level of physical properties such as hardness, flexibility, shock resistance, tensile strength, tear strength, bending strength, heat resistance



and the like, in accordance with its intended usage.

The quality of the pharmaceutical products packed in the container must not deteriorate during storage. For example, in the case of pharmaceutical products which are unstable to light, the container should provide a sufficient level of light shielding. In the case of pharmaceutical products which are easily oxidized, the container material should not allow the permeation of oxygen. In the case of aqueous pharmaceutical products and pharmaceutical products that must be kept dry, the container material should not allow the permeation of water vapor. In addition, it is necessary to pay attention to the permeability of solvents other than water through the container. The concentration of the pharmaceuticals must not be decreased by more than a certain level due to the adsorption of the pharmaceuticals on the surface of the container, the migration of the pharmaceuticals into the inside of the material of the container, or the loss of pharmaceuticals through the container. Also, the pharmaceutical products contained therein must not be degraded by an interaction with the material of the container.

The container should not be deformed, should not deteriorate and should not be degraded by the pharmaceutical products contained therein. Unacceptable loss of function of the container should not result from a possible high temperature or low temperature or their repetitions encountered during storage or transportation.

The container should be of a required level of transparency, when it is necessary to examine foreign insoluble matter and/or turbidity of the pharmaceutical products by visual observation.

In the case of pharmaceutical products which must be sterilized, it is required to satisfy the above-mentioned essential requirements of the container after the sterilization, if there is a possibility that the quality of the container may change after the sterilization. There should not be any residue or generation of new toxic substances of more than certain risk level after the sterilization. In addition, the container should not have any inappropriate structure and/or material that might result in any bacterial contamination of the pharmaceutical products contained therein during storage and transportation after sterilization.

2. Toxicity Evaluation of Container at Design Phase

For design verification, the toxicity of the container should be evaluated. For the toxicity evaluation, it is desirable to select appropriate test methods and acceptance criteria for the evaluation, and to explain the rationale for the selection clearly. The tests should be conducted using samples of the whole or a part of the prototype products of the container. If the container consists of plural parts of different materials, each part should be tested separately. Such materials as laminates, composites, and the like are regarded as a single material. To test containers made of such materials, it is recommended to expose the inner surface of the container, which is in contact with the pharmaceutical products contained therein, to the extraction media used in the tests as far as possible.

The tests required for the toxicity evaluation of the container are different depending upon the tissue to which the pharmaceutical products contained therein are to be applied.

The following tests are required for containers for

- (i) preparations in contact with blood:

Acute toxicity test, cytotoxicity test, sensitization

test and hemolysis test

- (ii) preparations in contact with skin or mucous membranes:
Cytotoxicity test and sensitization test
- (iii) liquid orally administered preparations:
Cytotoxicity test

It is recommended to conduct the tests in accordance with the latest versions of the standard test methods on medical devices and materials published in Japan and other countries.

Those standard test methods are listed for information:

2.1. Exemplification of Standard Tests

2.1.1. Selection of Tests

(i) Guidelines for Basic Biological Tests of Medical Devices and Materials (PAB Notification, YAKU-KI NO.99, June 27, 1995), Principles and selection of tests

(ii) ISO 10993-1: Biological evaluation of medical devices—Evaluation and testing

2.1.2. Acute Toxicity Test

(i) ASTM F750-82: Standard practice for evaluating material extracts by systemic injection in the mice

(ii) BS5736: Part 3 Method of test for systemic toxicity; assessment of acute toxicity of extracts from medical devices

(iii) USP 24 <88> Biological reactivity tests, in vivo

2.1.3. Cytotoxicity Test

(i) Guidelines for Basic Biological Tests of Medical Devices and Materials, I. Cytotoxicity Test 10. Cytotoxicity test using extract of medical device or material

(ii) ISO 10993-5: Biological evaluation of medical devices—Tests for cytotoxicity : in vitro methods

(iii) USP 24 <87> Biological reactivity tests, in vitro

2.1.4. Hemolysis Test

(i) Guidelines for Basic Biological Tests of Medical Devices and Materials, VII. Hemolysis Test

(ii) ISO 10993-4: Biological evaluation of medical devices—Selection of tests for interaction with blood. Annex D

(iii) ASTM F756-82: Standard practice for assessment of hemolytic properties of materials

2.1.5. Sensitization Test

(i) Guidelines for Basic Biological Tests of Medical Devices and Materials, II. Sensitization Test

(ii) ISO 10993-10: Biological evaluation of medical devices—Tests for irritation and sensitization

3. Test Results to be Recorded per Production Unit

At the commercial production phase, it is required to establish acceptance criteria on at least the test items listed below and to record the test results of each production unit of plastic containers for pharmaceutical products. In addition, it is desirable to explain the rationale for setting the acceptance criteria clearly. However, these requirements should not be applied to orally administered preparations except for liquid preparations.

(i) Combustion Tests: Residue of ignition, heavy metals. If necessary, the amounts of specified metals (lead, cadmium, etc.)

(ii) Extraction Tests: pH, ultraviolet absorption spectra, potassium permanganate-reducing substances, foaming, non-volatile residue

(iii) Cytotoxicity Test

(iv) Any other tests necessary for the specific container for aqueous infusions.

G8 Water

Quality Control of Water for Pharmaceutical Use

Water used for manufacturing pharmaceutical products and for cleaning their containers and equipments used in the manufacture of the products is referred to as “pharmaceutical water”. For assuring the quality of pharmaceutical water consistently, it is important to verify through appropriate process validation of water processing system that water with the quality suitable for its intended use is produced and supplied, and to keep the quality of produced water through routine works for controlling the water processing system.

1. Types of Pharmaceutical Water

1.1. Water

The specification for “Water” is prescribed in the Japanese Pharmacopoeia (JP) monograph. It is required for *Water* to meet the Quality Standards for Drinking Water provided under the Article 4 of the Japanese Water Supply Law. In the case that *Water* is produced at individual facilities using well water or industrial water as source water, it is necessary for produced water to meet the Quality Standards for Drinking Water and an additional requirement for ammonium of “not more than 0.05 mg/L”. Furthermore, when *Water* is to be used after storing for a period of time, it is necessary to prevent microbial proliferation.

Water is used as source water for *Purified Water* and *Water for Injection*. It is also used for manufacturing intermediates of active pharmaceutical ingredients (APIs), and for pre-washing of the equipments used in the manufacture of pharmaceutical products.

1.2. Purified Water

The specifications for “*Purified Water*” and “*Purified Water in Containers*” are prescribed in the JP monographs. *Purified Water* is prepared by distillation, ion-exchange, reverse osmosis (RO), ultrafiltration (UF) capable of removing substances with molecular masses of not less than approximately 6000, or a combination of these processes from *Water*, after applying some adequate pretreatments if necessary. For the production of *Purified Water*, appropriate control of microorganisms is required. Particularly, in the case that *Purified Water* is prepared by ion-exchange, RO or UF, it is necessary to apply the treatments adequate for preventing microbial proliferation, or to sanitize the system periodically.

When *Purified Water* is treated with chemical agents for sterilizing, preventing microbial proliferation, or maintaining the endotoxin level within an appropriate control range, a specification suitable for the intended use of treated water should be established individually, and a process control for keeping the quality of treated water in compliance with the specification thus established should be performed.

“*Purified Water in Containers*” is prepared from *Purified Water* by introducing it in a tight container.

1.3. Sterile Purified Water

The specification for “*Sterile Purified Water in Containers*” (its alternative name is *Sterile Purified Water*) is

prescribed in the JP monograph.

Sterile Purified Water in Container is prepared from *Purified Water* by 1) introducing it into a hermetic container, sealing up the container, then sterilizing the product, or 2) making it sterile by using a suitable method, introducing the sterilized water into a sterile hermetic container by applying aseptic manipulation, then sealing up the container.

Plastic containers for aqueous injections may be used in place of hermetic containers.

1.4. Water for Injection

The specifications for “*Water for Injection*” and “*Sterile Water for Injection in Containers*” are prescribed in the JP monographs. *Water for Injection* is prepared by distillation or reverse osmosis and/or ultrafiltration (RO/UF), either from *Water* after applying some adequate pretreatments such as ion exchange, RO, etc., or from *Purified Water*.

In the case of water processing systems based on distillation, it is necessary to take care for avoiding contamination of produced water by the impurities accompanied with the entrain.

In the case of water processing system based on RO/UF, it is required to provide water with equivalent quality to that prepared by distillation consistently, based on substantial process validation through long-term operation and elaborate routine control of the system. It is essential to ensure consistent production of water suitable for *Water for Injection* by the entire water processing system including pretreatment facilities, in any systems based on RO/UF. For the water supplied to the system, it is also required to keep the quality suitable as source water through adequate validation and routine control on the water.

For the water processing system based on RO/UF, routine control should be performed by analyzing water specimens, monitoring some quality attributes using in-line apparatus and checking the volume of water passed through the system. In addition, it is recommended to carry out periodical appearance observation and air-leak test on the membranes being currently used. It is also recommended to establish protocols for keeping the performance of membrane modules within appropriate control ranges and for estimating the timing to exchange the modules, through diagnosis on the degree of deterioration based on the results of tensile strength test on the used membrane modules, and visual observation on those modules whether any leakages of membranes have occurred or not, and to what extent they have occurred. Furthermore, it is desirable to establish the frequency of membrane exchange considering with its actual condition of use.

In the case that *Water for Injection* is stored in the water processing system temporarily, a stringent control for microorganisms and endotoxins should be taken. An acceptable criterion of lower than 0.25 EU/mL for endotoxins is specified in the JP monograph of *Water for Injection*.

“*Sterile Water for Injection in Container*” is prepared from *Water for Injection* by 1) introducing it into a hermetic container, sealing up the container, then sterilizing the product, or 2) making it sterile by using a suitable method, introducing the sterilized water into a sterile hermetic container by applying aseptic manipulation, then sealing up the container.

Plastic containers for aqueous injections may be used in place of hermetic containers.

2. Reverse Osmosis and/or Ultrafiltration (RO/UF)

RO/UF are the methods for refining water by using membrane modules based on either reverse osmosis or ultrafiltration, or the modules combining them, and used as the alternative methods for distillation in the production of *Purified Water* or *Water for Injection*.

When *Water for Injection* is produced by RO/UF, a water processing system equipped with pretreatment facilities, facilities for producing *Water for Injection* and facilities for supplying *Water for Injection* is usually used. The pretreatment facilities are used to remove solid particles, dissolved salts and colloids in source water, and placed before the facilities for producing *Water for Injection* so as to reduce the load on the facilities for producing *Water for Injection*. They consist of apparatus properly selected from aggregation apparatus, precipitation-separation apparatus, filtration apparatus, chlorine sterilization apparatus, oxidation-reduction apparatus, residual chlorine-removing apparatus, precise filtration apparatus, reverse osmosis apparatus, ultrafiltration apparatus, ion exchange apparatus, etc., depending on the quality of source water. The facilities for producing *Water for Injection* consist of apparatus for supplying pretreated water, sterilization apparatus with ultraviolet rays, heat exchange apparatus, membrane modules, apparatus for cleaning and sterilizing the facilities, etc. The facilities for supplying *Water for Injection* consist of a reservoir tank for storing *Water for Injection* in the facilities temporarily, pipe lines, heat exchange apparatus, a pump for circulating *Water for Injection* in the facilities, pressure control apparatus, etc.

In the case that *Water for Injection* is stored in the water processing system temporarily, it should usually be circulated in a loop consisting of a reservoir tank and pipe lines at a temperature not lower than 80°C for preventing microbial proliferation.

When *Purified Water* is produced by RO/UF, basic composition of water processing system is almost the same as that for *Water for Injection* described above.

When RO/UF is utilized for preparing pharmaceutical water, it is necessary to select the most appropriate combination of membrane modules in consideration of the quality of source water and the quality of produced water required for its intended use. When the ultrafiltration membrane is used to prepare *Purified Water* or *Water for Injection*, membrane modules capable of removing microorganisms and substances with molecular masses not less than approximately 6000 should be used.

3. Selection of Pharmaceutical Water

Depending on the intended use of pharmaceutical water, the water suitable for assuring the quality of final products without causing any trouble during their manufacturing processes, should be selected from the above 4 types (1.1. ~ 1.4.) of pharmaceutical water specified in the JP. Table 1 exemplifies a protocol for such selection (in the case of pharmaceutical water used for the manufacture of drug products).

Sterile Purified Water in Containers or *Water for Injection* (or *Sterile Water for Injection in Containers*) may be used in place of *Purified Water* or *Purified Water in Containers*.

3.1. Drug Products

For the manufacture of sterile drug products, for which

contamination with microorganisms or endotoxins is not permissible, *Water for Injection* (or *Sterile Water for Injection in Containers*) should be used. For the manufacture of ophthalmics and eye ointments, *Purified Water* (or *Purified Water in Containers*) can also be used.

For the manufacture of non-sterile drug products, water with a quality not lower than that of *Purified Water* (or *Purified Water in Containers*) should be used. However, out of the non-sterile drug products such as liquids, ointments, suspensions, emulsions, suppositories and aerosols, for those which require care against microbiological contamination, *Purified Water* (or *Purified Water in Containers*) adequately controlled from microbiological viewpoints should be used in consideration of the possible impacts of preservatives formulated in the drug products. For the manufacture of products containing crude drugs, it is recommended to select adequate type of water considering with viable counts of the crude drugs used for manufacturing the product and microbial limit required for the product.

Water used for pre-washing of containers or equipment surfaces that comes in direct contact with the drug products should have the quality not lower than that of *Water*. Water used for final rinsing should have an equivalent quality to that of water used for manufacturing drug products.

3.2. Active Pharmaceutical Ingredient (API)

Water used for manufacturing active pharmaceutical ingredient (API) should be selected in consideration of the characteristics of drug product for which the API is to be used, and its manufacturing process, so that the quality of the final drug product is assured.

Water used for manufacturing API or for cleaning containers or equipment surfaces that comes in direct contact with the raw materials or API intermediates, should have the quality not lower than that of *Water* adequately controlled from the chemical and microbiological viewpoints, even if the water is used at an earlier stage of synthetic or extraction process in the manufacture of API. Water used in the final purification process should have the quality equal to or higher than that of *Purified Water* (or *Purified Water in Containers*). Water used for final rinsing of containers or equipment surfaces that comes in direct contact with the APIs should have an equivalent quality to that of water used for manufacturing the APIs.

For manufacturing sterile API, *Sterile Purified Water in Containers* or *Water for Injection* (or *Sterile Water for Injection in Containers*) should be used. Similarly, for manufacturing APIs used for drug products where endotoxin control is required and there are no subsequent processes capable of removing endotoxins, *Water for Injection* (or *Sterile Water for Injection in Containers*), or *Purified Water* (or *Purified Water in Containers*) for which endotoxins are controlled at a low level, should be used.

4. Quality Control of Pharmaceutical Water

4.1. Outline

Verification that water with the quality required for its intended use has been produced by the pharmaceutical water processing system through substantial validation studies at an earlier stage of its operation, is the prerequisite for conducting quality control on pharmaceutical water in a routine and periodical manner. If this prerequisite is fulfilled, the following methods are applicable for quality control of pharmaceutical water.

Table 1 An Exemplified Protocol for Selecting Pharmaceutical Water
(Water Used in the Manufacture of Drug Products or APIs)

Classification	Class of Pharmaceutical Water	Application	Remarks
Drug Product	<i>Water for Injection</i> or <i>Sterile Water for Injection in Containers</i>	Injections, Ophthalmics, Eye Ointments	
	<i>Purified Water</i> or <i>Purified Water in Containers</i>	Ophthalmics, Eye Ointment	For ophthalmics and eye ointments for which precautions should be taken against microbial contamination, <i>Purified Water</i> or <i>Purified Water in Containers</i> kept its viable counts at low levels through sterilization, UF filtration, etc. should be used.
		Aerosols, Liquids and Solutions, Extracts, Elixirs, Capsules, Granules, Pills, Suspensions and Emulsions, Suppositories, Powders, Spirits, Tablets, Syrups, Infusions and Decoctions, Plasters, Tinctures, Troches, Ointments, Cataplasms, Aromatic Waters, Liniments, Lemonades, Fluidextracts, Lotions, and Pharmaceutical preparations of percutaneous absorption type	For liquids, solutions, ointments, suspensions, emulsions, suppositories, aerosols and so on for which precautions should be taken against microbial contamination, <i>Purified Water</i> or <i>Purified Water in Containers</i> adequately controlled from microbiological viewpoints should be used.
Active Pharmaceutical Ingredient (API)	<i>Water for Injection</i> or <i>Sterile Water for Injection in Containers</i>	Sterile APIs, and APIs rendered sterile in the formulation process	
	<i>Purified Water</i> or <i>Purified Water in Containers</i>	APIs, APIs rendered sterile in the formulation process, and API intermediates	In the manufacture of APIs to be rendered sterile in the formulation process and have no subsequent processes capable of removing endotoxins, <i>Purified Water</i> or <i>Purified Water in Containers</i> controlled endotoxins at a low level should be used.
	<i>Water</i>	API Intermediates	

For routine control, it is very useful to control quality of produced water based on the monitoring of electrical conductivity (conductivity) and total organic carbon (TOC). In addition, items to be monitored periodically, such as some specified impurities, viable counts, endotoxins, insoluble particulate matters, etc., should be determined according to the intended use of pharmaceutical water. The frequency of measurement should be determined considering with the variation in the quality of water to be monitored.

The following are points to consider in controlling the quality of produced water from microbiological and physicochemical (conductivity and TOC) viewpoints. It is necessary to monitor other items if necessary, and to confirm that they meet the specifications established individually.

4.2. Sampling

Monitoring should be conducted at an adequate frequency to ensure that the pharmaceutical water processing system is well-controlled and that water with acceptable quality is con-

tinuously produced and supplied. Specimens should be collected at the representative locations in the facilities for producing and supplying water, with particular care so that collected specimens reflect the operating condition of the pharmaceutical water processing system. An adequate protocol for the control of microorganisms at the sampling site should be established considering with the situation around the site.

Sampling frequency should be established based on the data from validation studies on the system. For microbiological monitoring, it is adequate to use the water specimens for the test within 2 hours after sampling. In the case that it is not possible to test within 2 hours, the specimens should be kept at 2 – 8°C and be used for the test within 12 hours.

4.3. Alert and Action Levels

In producing pharmaceutical water using a water processing system, microbiological and physicochemical monitoring is usually carried out to assure that water with required qual-

Table 2 Methods for Assessment of Viable Counts in Pharmaceutical Water

Method	Pharmaceutical Water		
	<i>Water</i>	<i>Purified Water</i>	<i>Water for Injection</i>
Measurement Method	Pour Plate Method or Membrane Filtration	Pour Plate Method or Membrane Filtration	Membrane Filtration
Minimum Sample Size	1.0 mL	1.0 mL	100 mL
Media	Standard Agar Medium	R2A Agar Medium, Standard Agar Medium	R2A Agar Medium, Standard Agar Medium
Incubation Period	Standard Agar Medium: 48 – 72 hours (or longer)	R2A Agar Medium: 4 – 7 days (or longer) Standard Agar Medium: 48 – 72 hours (or longer)	R2A Agar Medium: 4 – 7 days (or longer) Standard Agar Medium: 48 – 72 hours (or longer)
Incubation Temperature	Standard Agar Medium: 30 – 35°C	R2A Agar Medium: 20 – 25°C or 30 – 35°C Standard Agar Medium: 30 – 35°C	R2A Agar Medium: 20 – 25°C or 30 – 35°C Standard Agar Medium: 30 – 35°C

ity is being continuously produced when the system is operating as it designed. The operating condition of the system can be estimated by the comparison of monitoring data thus obtained against the alert level, action level, other levels for controlling the system, and acceptance criteria specified for the water required for its intended use, and also by the trend analysis of monitoring data through plotting them in a control chart. In this manner, the alert level and action level are used for controlling the process of water production, and not used for judging pass/fail of produced water.

4.3.1. Definition of Alert Level

“Alert level” indicates that, when exceeded it, the system is threatening to deviate from its normal operating range. Alert levels are used for giving a warning, and exceeding them does not necessarily require a corrective action. Alert level is generally established either at a mean + 2 σ on the basis of past trend analysis, or at a level of 70% (50% for viable counts) of action level, whichever is smaller.

4.3.2. Definition of Action Level

“Action level” indicates that, when exceeded it, the system has deviated from its normal operating range. Exceeding it indicates that corrective action must be taken to bring the system back within its normal operating range.

Alert and action levels should be established within the specified acceptance criteria of the water required for its intended use in consideration of available technologies and the quality required for the water. Consequently, exceeding an alert or action level does not necessarily indicate that the quality of produced water has become inadequate for its intended use.

4.4. Microbiological Monitoring

The main purpose of microbiological monitoring program for pharmaceutical water processing system is to foresee any microbiological quality deterioration of the produced water, and to prevent any adverse effects on the quality of pharmaceutical products. Consequently, detecting all of the microorganisms present in the water to be monitored may not be necessary. However it is required to adopt a monitoring technique able to detect a wide range of microorganisms, including slow growing microorganisms.

The followings indicate incubation-based microbiological monitoring techniques for pharmaceutical water processing systems. To adopt a rapid microorganism detection technique, it is necessary to confirm in advance that the microbial counts obtained by such techniques are equivalent to those obtained by the incubation-based monitoring techniques.

4.4.1. Media and Incubation Conditions

There are many mesophilic bacteria of heterotrophic type that are adapted to poor nutrient water environments. Heterotrophic bacteria may form bio-films in many pharmaceutical water processing systems, and to cause quality deterioration of the produced water. Therefore, it is useful to monitor microbiological quality of water by using the R2A Agar Medium, which is excellent for growing bacteria of oligotrophic type. On the other hand, in routine microbiological monitoring, an approach by using the Standard Agar Medium, prescribed in the Quality Standards for Drinking Water provided under the Article 4 of the Japanese Water Supply Law, is widely employed. In this approach, the trend in microbiological change of water processing system is estimated from the number of viable microorganisms capable of proliferating at 30 – 35°C in the Standard Agar Medium in a comparatively short period of time.

Table 2 shows examples of measurement methods, minimum sample sizes, media, and incubation periods for estimating viable counts.

The media shown in Table 2 are as follows.

- (i) Standard Agar Medium

Casein peptone	5.0 g
Yeast extract	2.5 g
Glucose	1.0 g
Agar	15.0 g
Water	1000 mL

Mix all the ingredients, and sterilize by heating in an autoclave at 121°C for 15 – 20 minutes. pH after sterilization: 6.9 – 7.1.
- (ii) R2A Agar Medium

Peptone (casein and animal tissue)	0.5 g
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Casamino acid	0.5 g
Yeast extract	0.5 g
Sodium pyruvate	0.3 g
Glucose	0.5 g
Magnesium sulfate heptahydrate	0.05 g
Soluble starch	0.5 g
Dipotassium hydrogen phosphate	0.3 g
Agar	15.0 g
Water	1000 mL

Mix all the ingredients, and sterilize by heating in an autoclave at 121°C for 15 – 20 minutes. pH after sterilization: 7.1 – 7.3.

The following reagents should be used for preparing the R2A Agar Medium.

(i) Casamino acid Prepared for microbial test, by the acid hydrolysis of casein.

Loss on drying <2.41>: Not more than 8% (0.5 g, 105°C, constant mass)

Residue on ignition <2.44>: Not more than 55% (0.5 g)

Nitrogen content <1.08>: Not less than 7% (105°C, constant mass, after drying)

(ii) Sodium pyruvate $\text{CH}_3\text{COCOONa}$ White to pale yellow crystalline powder. Freely soluble in water, and slightly soluble in ethanol (99.5) and in acetone.

Identification (1) Determine the infrared absorption spectrum as directed in the potassium bromide disk method under *Infrared Spectrophotometry* <2.25>: it exhibits absorption maxima at the wave numbers around 1710 cm^{-1} , 1630 cm^{-1} , 1410 cm^{-1} , 1360 cm^{-1} , 1190 cm^{-1} , 1020 cm^{-1} , 980 cm^{-1} , 830 cm^{-1} , 750 cm^{-1} , 630 cm^{-1} and 430 cm^{-1} .

(2) A solution (1 in 20) responds to the *Qualitative Tests* <1.09> for sodium salt (1).

Content: Not less than 97.0%. *Assay*—Weigh accurately about 0.4 g of sodium pyruvate and dissolve it in 200 mL of water. Transfer 20 mL of this solution into an iodine bottle, and cool to 10°C or lower. Add 40 mL of 0.05 mol/L iodine VS and 20 mL of sodium hydroxide solution (17 in 100), then allow to stand in a dark place for 2 hours, and add 15 mL of diluted sulfuric acid (1 in 6). Titrate <2.50> with 0.1 mol/L sodium thiosulfate VS (indicator: starch TS). Perform a blank determination in the same manner, and make any necessary correction.

$$\begin{aligned} \text{Each mL of 0.05 mol/L iodine VS} \\ = 1.834 \text{ mg of } \text{C}_3\text{H}_3\text{NaO}_3 \end{aligned}$$

4.4.2. Media Growth Promotion Test

In the media growth promotion test with the R2A Agar Medium, use the strains listed below or other strains considered equivalent to these strains. Prior to the test, inoculate these strains into *Sterile Purified Water* and incubate at 20 – 25°C for 3 days.

Methylobacterium extorquens: NBRC 15911

Pseudomonas fluorescens: NBRC 15842, ATCC 17386, etc.

Dilute the fluid containing the strain starved in *Purified Water* with *Sterile Purified Water* to prepare a fluid containing about 50 – 200 CFU/mL of viable counts. When pipetting 1 mL of the diluted fluid onto the R2A Agar Medium and incubating at 20 – 25°C for 4 – 7 days, sufficient proliferation of the inoculated strain must be observed.

In the media growth promotion test with the Standard Agar Medium, use the strains listed below or other strains

considered equivalent to these strains. Prepare the fluid containing the strain according to the procedure prescribed in the *Microbial Limit Test* <4.05>. When pipetting 1 mL of the fluid onto the Standard Agar Medium and incubating at 30 – 35°C for 48 hours, sufficient proliferation of the inoculated strain must be observed.

Staphylococcus aureus: ATCC 6538, NCIMB 9518, CIP 4.83 or NBRC 13276

Pseudomonas aeruginosa: ATCC 9027, NCIMB 8626, CIP 82.118 or NBRC 13275

Colon bacillus (*Escherichia coli*): ATCC 8739, NCIMB 8545, CIP 53.126 or NBRC 3972

4.4.3. Action Levels for Microorganisms for Pharmaceutical Water Processing System

The following action levels are considered appropriate and generally applicable to pharmaceutical water processing systems.

Action Levels for viable counts in various types of pharmaceutical water

Water: 100 CFU/mL* (Acceptance criterion prescribed in the Quality Standards for Drinking Water provided under the Article 4 of the Water Supply Law)

Purified Water: 100 CFU/mL**

Water for Injection: 10 CFU/100 mL**

(*Viable counts obtained using the Standard Agar Medium, **Viable counts obtained using the R2A Agar Medium)

Although the action level for *Purified Water* shown above is set at the same level as that for *Water*, in near future, an adequate action level would be set for *Purified Water* in consideration of the current level of technologies. Therefore, for the time being, it is recommended for each facility to perform a higher level of microbiological control of water processing system based on the action level established individually.

When actual counts in validation studies or routine control exceed the above action levels, it is necessary to isolate and identify the microorganisms present in the water, and to sanitize or disinfect the affected system.

4.5. Physicochemical Monitoring

Physicochemical monitoring of a pharmaceutical water processing system is usually performed using conductivity and TOC as the indicators for water quality. By monitoring conductivity, total amounts of inorganic salts present in the water can be estimated, and by monitoring TOC, total amount of organic compounds present in the water can be estimated. Normally, the *Conductivity Measurements* <2.51> and the *Test for Total Organic Carbon* <2.59> specified in the General Tests, Processes and Apparatus of the JP should be applied to these physicochemical monitoring. However, since tests for monitoring are performed in the situations different from those for judging pass/fail to the acceptance criteria prescribed in the monographs, supplements necessary to cover the situations to which the JP general tests cannot be applied, are described below.

To adopt the monitoring using conductivity and TOC as the indicators for inorganic and organic impurities at individual facility, appropriate alert and action levels, and countermeasures against unexpected apparatus failures should be established for each indicator.

4.5.1. Monitoring of Conductivity as the Indicator for Inorganic Impurities

Measurement of conductivity for monitoring is usually

conducted continuously using an in-line apparatus with a flow-through type or pipe-insertion type cell. Alternatively, offline batch testing may be performed using a dip type cell with water specimens taken at point-of-use sites or other appropriate locations of the pharmaceutical water processing system. For the operation control of a pharmaceutical water processing system, guides for judging whether it is adequate to continue the operation of the system or not, based on the results from monitoring of conductivity, are shown below, both for the cases of monitoring at the standard temperature (20°C) by applying *Conductivity Measurements <2.51>* of the JP and monitoring at temperatures other than 20°C by applying <645> *WATER CONDUCTIVITY* of the United States Pharmacopeia (USP) with some modifications.

4.5.1.1. Monitoring of Conductivity by applying the *Conductivity Measurements <2.51>* of the JP

The *Conductivity Measurements <2.51>* of the JP principally requires to measure the conductivity at the standard temperature (20°C). However, measurement at a temperature within a range of 15–30°C may also be acceptable, when the results are corrected using the equation prescribed in the *Conductivity Measurements <2.51>*. In this case, the recommended allowable conductivity (action level) for *Purified Water* and *Water for Injection* is as follows.

· Action Level $1.0 \mu\text{S} \cdot \text{cm}^{-1}$ (20°C)

Since the above allowable conductivity is established for in-line monitoring, an alternative action level may be used for the monitoring based on offline batch testing.

4.5.1.2. Monitoring of Conductivity by applying the <645> *WATER CONDUCTIVITY* of the USP with some modifications

Usually, it is somewhat difficult to control the temperature exactly in in-line conductivity monitoring. Therefore, the following approach can be applied for the monitoring at temperatures other than the standard temperature (20°C) of the JP. This approach is based on the Stages 1 and 2 of the three-stage approach described in “<645> *WATER CONDUCTIVITY*” of the USP.

Stage 1 (In-line Measurement)

- (i) Determine the temperature and the conductivity of the water specimens using a non-temperature-compensated conductivity reading.
- (ii) From the Table 3, find the temperature value equal to or just lower than the measured temperature. Adopt the corresponding conductivity value on this table as the allowable conductivity at the measured temperature.
- (iii) If the observed conductivity is not greater than the allowable conductivity adopted above, the water tested meets the requirement for monitoring conductivity. If the observed conductivity exceeds the allowable conductivity, proceed with Stage 2.

Stage 2 (Off-line Measurement)

- (i) Measure the conductivity of the water specimen, by transferring it into a container and agitating it vigorously in order to attain equilibrium between the water specimen and the atmosphere on absorbing/desorbing carbon dioxide.
- (ii) Transfer a sufficient amount of water to be tested into a suitable container, and stir the water specimen. Adjust the temperature to $25 \pm 1^\circ\text{C}$, and begin agitating the water specimen vigorously, while

Table 3 Stage 1 Allowable Conductivity for Different Temperatures*

Temperature (°C)	Allowable Conductivity ($\mu\text{S} \cdot \text{cm}^{-1}$)	Temperature (°C)	Allowable Conductivity ($\mu\text{S} \cdot \text{cm}^{-1}$)
0	0.6		
5	0.8	55	2.1
10	0.9	60	2.2
15	1.0	65	2.4
20	1.1	70	2.5
25	1.3	75	2.7
30	1.4	80	2.7
35	1.5	85	2.7
40	1.7	90	2.7
45	1.8	95	2.9
50	1.9	100	3.1

* Applicable only to non-temperature-compensated conductivity measurements.

observing the conductivity periodically. When the change in conductivity, due to the uptake of atmospheric carbon dioxide, becomes not greater than $0.1 \mu\text{S} \cdot \text{cm}^{-1}$ per 5 minutes, adopt the observed value as the conductivity (25°C) of the water specimen.

- (iii) If the conductivity of the water specimen at 25°C obtained above is not greater than $2.1 \mu\text{S} \cdot \text{cm}^{-1}$, the water tested meets the requirement for monitoring conductivity. If the observed value exceeds $2.1 \mu\text{S} \cdot \text{cm}^{-1}$, it should be judged that the water tested does not meet the requirement for monitoring conductivity.

4.5.2. Monitoring of TOC as the Indicator for Organic Impurities

The acceptance criterion of TOC is specified as “not greater than 0.50 mg/L (500 ppb)” in the monographs of *Purified Water* and *Water for Injection*. However it is recommended for each facility preparing pharmaceutical water to conduct operation control of pharmaceutical water processing system through TOC monitoring on produced water based on its own alert and action levels for TOC determined individually. The followings are the recommended action levels for TOC.

· Action Level: ≤ 300 ppb (in-line)
 ≤ 400 ppb (off-line)

The Quality Standards for Drinking Water provided under the Article 4 of the Japanese Water Supply Law require that TOC should be “not greater than 3 mg/L (3 ppm)”. Taking the above recommended action levels into consideration, it is also recommended for each facility to conduct quality control of source water through TOC monitoring based on its own alert and action levels for TOC determined individually.

The JP specifies the *Test for Total Organic Carbon <2.59>*, and normally, TOC measurement should be conducted using an apparatus which meets the requirements described in the JP method. However, if a TOC apparatus conforms to the apparatus suitability test requirements described in “<643> *TOTAL ORGANIC CARBON*” of the USP, or those described in the “*Methods of Analysis 2.2.44. TOTAL*

ORGANIC CARBON IN WATER FOR PHARMACEUTICAL USE" of the European Pharmacopoeia (EP), the apparatus can be used for the monitoring of pharmaceutical water processing system, if sufficiently pure water not contaminated with ionic organic substances, or organic substances having nitrogen, sulfur, phosphorus or halogen atoms in their chemical structures, is used as the source water supplied to the system.

A TOC apparatus, characterized by calculating the amount of organic carbon from the difference in conductivity before and after the decomposition of organic substances without separating carbon dioxide from the sample solution, may be influenced negatively or positively, when applied to the water specimens containing ionic organic substances, or organic substances having nitrogen, sulfur, phosphorus or halogen atoms in their chemical structures. Therefore, the apparatus used for TOC monitoring should be selected appropriately in consideration of the purity of the water to be monitored and the contamination risk in the case of apparatus failure.

4.6. Storage of Water for Injection

In storing *Water for Injection* temporarily, adequate measures able to prevent microbial proliferation stringently, such as circulating it in a loop at a high temperature must be taken, and an appropriate storage time should also be established based on the validation studies, in consideration of the risks of contamination and quality deterioration.

5. Points to Consider for Assuring the Quality of Pharmaceutical Water in Containers

There are some specific points to consider for assuring the quality of pharmaceutical water in containers (*Purified Water in Containers*, *Sterile Purified Water in Containers* and *Sterile Water for Injection in Containers*), which are available as commercially products.

5.1. Methods for Preparing Sterile Pharmaceutical Water in Containers and Their Sterilization Validation

The following 2 different preparation methods are described in the monographs of *Sterile Purified Water in Containers* and *Sterile Water for Injection in Containers*.

- (i) Introduce *Purified Water* (or *Water for Injection*) into a hermetic container, seal up the container, then sterilize the product.
- (ii) Make *Purified Water* (or *Water for Injection*) sterile by using a suitable method, introduce the sterilized water into a sterile hermetic container by applying aseptic manipulation, then seal up the container.

For assuring the sterility of pharmaceutical water products, only the validation of final sterilization process is required in the case of preparation method (i), whereas validations of all the processes are indispensable in the case of preparation method (ii), since the latter is based on the idea to assure the sterility of pharmaceutical water products by "aseptically" introducing *Purified Water* (or *Water for Injection*) treated in advance with filtration sterilization, etc. into a sterile hermetic container, and sealing it up.

5.2. Deterioration of Water Quality during the Storage in Containers

5.2.1. Conductivity (as the indicator for inorganic impurities)

The conductivity of pharmaceutical water in containers may increase to some higher levels due to the absorption of carbon dioxide from the atmosphere at the time of its prepa-

ration and that passed through plastic layer of the containers during storage, and also due to ionic substances released from the containers, even if the conductivity of *Purified Water* or *Water for Injection* used for its production is maintained at the level not more than $1.0 \mu\text{S} \cdot \text{cm}^{-1}$. Particularly in the cases of pharmaceutical water products packed in small scale glass containers, it is necessary to pay attention to the change of conductivity during storage.

5.2.2. Potassium Permanganate-reducing Substances or Total Organic Carbon (TOC) (as the indicator for organic impurities)

JP specifies the classical test of potassium permanganate-reducing substances in the monographs of *Purified Water in Containers*, *Sterile Purified Water in Containers* and *Sterile Water for Injection in Containers* for controlling organic impurities in pharmaceutical water in containers. It forms a remarkable contrast to the specifications of *Purified Water* and *Water for Injection*, in which JP requires to control organic impurities in pharmaceutical water in bulk based on the test of TOC (acceptance criterion: not more than 0.5 mg/L (500 ppb)). This is because that it is considered difficult to establish the specification of pharmaceutical water in containers for organic impurities based on the test of TOC from the facts that there were many cases of remarkable increases in TOC values after storage of water in containers. Particularly in the cases of pharmaceutical water products packed in small scale plastic containers, it is necessary to pay attention to the increase of materials released from containers during storage.

The test of potassium permanganate-reducing substances is retained in the specifications of pharmaceutical water in containers, not as the most suitable method for the test of organic impurities present in the water in containers, but as a counter measure for performing the test of the water in containers with the same test method despite of the material (glass, polyethylene or polypropylene, etc) and the size (0.5 – 2000 mL) of the containers, and the duration of storage. Therefore, it is recommended to adopt the test of TOC as the alternative for the test of potassium permanganate-reducing substances, and to perform quality control of pharmaceutical water in containers based on TOC measurements under the responsibility of each manufacturer, if possible.

In such cases, it is recommended to adopt the following values as the levels preferable to attain.

For products containing not more than 10 mL of water:

TOC not greater than 1500 ppb

For products containing more than 10 mL of water:

TOC not greater than 1000 ppb

As for the pharmaceutical water packed in the plastic containers made of polyethylene, polypropylene, etc., in addition to the concern for the release of materials such as monomer, oligomers, plasticizers, etc. from plastics, it is necessary to pay attention to the storage environment of the products to avoid the contaminations with low molecular volatile organics such as ethanol, or low molecular air pollutants such as nitrogen oxides, since these plastics have the properties of permeating various gases.

5.2.3. Microbial Limit (Total Aerobic Viable Counts)

For *Purified Water in Containers*, it is not required to assure the sterility, but it is necessary to produce it by using sanitary or aseptic processes in order to meet the acceptance criterion of " 10^2 CFU/mL" for total aerobic viable counts

throughout the period of their storage. It is also necessary to take special care against microbial contamination during its circulation. In addition, it is recommended to use them as soon as possible after opening their seals.

The acceptance criterion of “ 10^2 CFU/mL” for total aerobic viable counts of *Purified Water in Containers* is at the same level as the action level for viable counts in the production of *Purified Water* (in bulk). However, different from the case of microbiological monitoring of *Purified Water*, Soybean-Casein Digest Agar Medium is used for the test of total aerobic viable counts of *Purified Water in Containers* to detect microorganisms contaminated from the surroundings during its storage and circulation.

5.3. Points to consider in the case that commercially available products of pharmaceutical water in containers are used for the manufacture of pharmaceutical products

It is allowable to use commercially available products of pharmaceutical water in containers (*Purified Water in Containers*, *Sterile Purified Water in Containers* or *Sterile Water for Injection in Containers*) for the manufacture of pharmaceutical products and products for clinical trial, and for the tests of pharmaceutical products. In such cases, it is necessary to consider the following points.

- (i) When such products are used for manufacturing pharmaceutical products, it is recommended to use them soon after confirming their compliances to the requirements of JP monograph from the test results at the time of its receipt or those offered from the supplier of the products.
- (ii) In the case that such products are used for manufacturing pharmaceutical products, it is necessary to validate the process in which the water was used as a part of process validation of pharmaceutical products. In the case that they are used for manufacturing products for clinical trial, it is necessary to confirm that the water doesn't give any adverse effects on the quality of the products.
- (iii) The products of sterile pharmaceutical water in containers should be used only once after opening their seals, and it must be avoided to use them again after storage.
- (iv) It is recommended to prepare a standard operation practice (SOP) adequate for its intended use, considering that the contamination and quality deterioration of the water due to human and laboratory environmental origins might go on rapidly immediately after opening the product seal.

Water to be used in the Tests of Drugs

The water to be used in the tests of drugs is defined as “the water suitable for performing the relevant test” in the paragraph 20 under General Notices of the JP. Therefore, it is necessary to confirm that the water to be used in a test of a drug is suitable for the purpose of the relevant test before its use.

Unless otherwise specified in the individual test method, Purified Water, Purified Water in Containers or the water produced by an appropriate process, such as ion exchange or

ultrafiltration, may be used for these purposes. Water produced for these purposes at other individual facilities may also be used.

The water for tests specified in General Tests in the JP is as follows:

- Water for ammonium limit test: <1.02> Ammonium Limit Test (Standard Ammonium Solution)
- Water for bacterial endotoxins test: <4.01> Bacterial Endotoxins Test
- Water for particulate matter test (for injections): <6.07> Insoluble Particulate Matter Test for Injections
- Water for particulate matter test (for ophthalmic solutions): <6.08> Insoluble Particulate Matter Test for Ophthalmic Solutions
- Water for particulate matter test (for plastic containers): <7.02> Test Methods for Plastic Containers

The water for tests specified in General Information in the JP is as follows:

- Water for aluminum test: Test for Trace Amounts of Aluminum in Trans Parenteral Nutrition (TPN) Solutions
- Water for ICP analysis: Inductively Coupled Plasma Emission Spectral Analysis

The term “water” described in the text concerning tests of drugs means “the water to be used in the tests of drugs” as defined in the paragraph 20 under General Notices.

G9 Others

International Harmonization Implemented in the Japanese Pharmacopoeia Sixteenth Edition

Items for which harmonization has been agreed among the European Pharmacopoeia, the United States Pharmacopoeia and the Japanese Pharmacopoeia are implemented in the Japanese Pharmacopoeia Sixteenth Edition (JP 16). 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 16 shows the items as they appear in JP 16. In the Remarks column, notes on any differences between JP 16 and the agreement are shown as occasion demands.

The date on which the agreement has been signed is shown on the top pf each table. In the case where the harmonized items have been revised and/or corrected, this is indicated in parenthesis.

Aug. 2005 (Rev. 2)

Harmonized items	JP 16	Remarks
Residue on Ignition/Sulphated Ash Test (Introduction)	2.44 Residue on Ignition Test (Introduction)	JP's particular description: Explanation on this test. Explanation of the description in monograph, etc.
Procedure	1. Procedure	

Oct. 2007

Harmonized items	JP 16	Remarks
Characterisation of Crystalline and Partially Crystalline Solids by X-ray Powder Diffraction (XRPD) (Introduction)	2.58 X-Ray Powder Diffraction Method (Introduction)	JP's particular description: Explanation on this method.
Principle	1. Principle	
Instrument	2. Instrument	“ <i>Specimen mounting</i> ” is not stipulated.
Instrument set-up	2.1 Instrument setup	
X-ray radiation	2.2 X-ray radiation	
Radiation protection	2.3 Radiation protection	
Specimen preparation and mounting	3. Specimen preparation and mounting	
Specimen preparation	3.1 Specimen preparation	
Specimen mounting		
Effect of specimen displacement		
Effect of specimen thickness and transparency		
Control of the instrument performance	4. Control of the instrument performance	
Qualitative phase analysis (Identification of phases)	5. Qualitative phase analysis (Identification of phases)	
Quantitative phase analysis	6. Quantitative phase analysis	
Polymorphic samples	6.1 Polymorphic samples	
Methods using a standard	6.2 Methods using a standard	
Estimate of the amorphous and crystalline fractions	7. Estimate of the amorphous and crystalline fractions	
Single crystal structure	8. Single crystal structure	
Figure 1 Diffraction of X-rays by a crystal according to Bragg's law	Fig. 1 Diffraction of X-rays by a crystal according to Bragg's law	
Figure 2 X-ray powder diffraction patterns collected for 5 different solid phases of a substance (the intensities are normalized)	Fig. 2 X-ray powder diffraction patterns collected for 5 different solid phases of a substance (the intensities are normalized)	

Figure 3 Geometric arrangement of the Bragg-Brentano para-focusing geometry	Fig. 3 Geometric arrangement of the Bragg-Brentano para-focusing geometry	
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June 2009 (Rev. 1 – Corr. 1)

Harmonized items	JP 16	Remarks
Bulk Density and Tapped Density of Powders	3.01 Determination of Bulk and Tapped Densities	JP's particular description: Explanation on this method.
	(Introduction)	
Bulk density	1. Bulk density	
Method 1: Measurement in a graduated cylinder	1.1. Method 1: Measurement in a Graduated Cylinder	
Procedure	1.1.1. Procedure	
Method 2: Measurement in a volumeter	1.2. Method 2: Measurement in a Volumeter	
Apparatus	1.2.1. Apparatus	
Procedure	1.2.2. Procedure	
Method 3: Measurement in a vessel	1.3. Method 3: Measurement in a Vessel	
Apparatus	1.3.1. Apparatus	
Procedure	1.3.2. Procedure	
Tapped density	2. Tapped density	
Method 1	2.1. Method 1	
Apparatus	2.1.1. Apparatus	
Procedure	2.1.2. Procedure	
Method 2	2.2. Method 2	
Procedure	2.2.1. Procedure	
Method 3	2.3. Method 3	
Procedure	2.3.1. Procedure	
Measures of powder compressibility	3. Measures of powder compressibility	
Figure 1 Volumeter	Fig. 1 Volumeter	
Figure 2 Measuring vessel (left) and cap (right)	Fig. 2 Measuring vessel (left) and cup (right)	
Figure 3	Fig. 3 Tapping apparatus	

Nov. 2003

Harmonized items	JP 16	Remarks
Specific Surface Area	3.02 Specific Surface Area by Gas Adsorption	JP's particular description: Explanation on this test.
(Introduction)	(Introduction)	
	1. Measurements	
Multi-point measurement	1.1. Multi-point measurement	
Single-point measurement	1.2. Single-point measurement	

Sample preparation Outgassing Adsorbate Quantity of sample	2. Sample preparation	
Method 1: The dynamic flow method	3. Methods	
Method 2: The volumetric method	3.1. Method 1: The dynamic flow method	
Reference materials	3.2. Method 2: The volumetric method	
Figure 1 Schematic diagram of the dynamic flow method apparatus	4. Reference materials	
Figure 2 Schematic diagram of the volumetric method apparatus	Fig. 1 Schematic diagram of the dynamic flow method apparatus	
	Fig. 2 Schematic diagram of the volumetric method apparatus	

May 2007

Harmonized items	JP 16	Remarks
Gas Pycnometric Density of Solids	3.03 Powder Particle Density Determination	
(Introduction)	(Introduction)	JP's particular description: Objects to be measured by this method.
Apparatus	1. Apparatus	
	2. Calibration of apparatus	The range of temperature during the measurement is described in the Procedure.
Method	3. Procedure	
Expression of the results		
Figure 1 Schematic diagram of a gas pycnometer	Fig. 1 Schematic diagram of a gas pycnometer	

June 2004 (Method 1)/May 2007 (Rev. 1) (Method 2)

Harmonized items	JP 16	Remarks
	3.04 Particle Size Determination	
	(Introduction)	JP's particular description: Explanation on this test.
Optical microscopy	1. Method 1. Optical microscopy	JP's particular description: Explanation on this test.
Apparatus	1.1. Apparatus	
Adjustment	1.1.1. Adjustment	
Illumination	1.1.1.1. Illumination	
Visual characterization	1.1.1.2. Visual characterization	JP's particular description: Explanation on the method of particle size measurement.
Photographic characterization	1.1.1.3. Photographic characterization	
Preparation of the mount	1.2. Preparation of the mount	
	1.3. Characterization	
Crystallinity characterization	1.3.1. Crystallinity characterization	

Limit test of particle size by microscopy	1.3.2. Limit test of particle size by microscopy	JP's particular description: Explanation on this method.
Particle size characterization	1.3.3. Particle size characterization	
Particle shape characterization	1.3.4. Particle shape characterization	
General observations	1.3.5. General observations	
Figure 1 Commonly used measurements of particle size	Fig. 1 Commonly used measurements of particle size	
Figure 2 Commonly used descriptions of particle shape	Fig. 2 Commonly used descriptions of particle shape	
Analytical sieving	2. Method 2. Analytical sieving method	
Principles of analytical sieving	Principles of analytical sieving	
Test sieves	2.1. Procedure	
Test specimen	2.1.1. Test sieves	
Agitation methods	2.1.2. Test specimen	
Endpoint determination	2.1.3. Agitation methods	
Sieving methods	2.1.4. Endpoint determination	
1) Methanical agitation dry sieving method	2.2. Sieving methods	
2) Air entrainment methods air jet and sonic sifter sieving	2.2.1. Methanical agitation (Dry sieving method)	
Interpretation	2.2.2. Air entrainment methods Air jet and sonic shifter sieving	
Table 1 Size of standard sieve series in range of interest	2.3. Interpretation	
	Table 1 Size of standard sieve series in range of interest	

Oct. 2009 (Rev. 1 – Corr. 1)

Harmonized items	JP 16	Remarks
Bacterial Endotoxins Test	4.01 Bacterial Endotoxins Test	JP's particular description: Deletion of the preparation of sample solutions for medical devices.
(Introduction)	(Introduction)	
Apparatus	1. Apparatus	
Preparation of standard endotoxin stock solution	2. Preparation of solutions	
Preparation of standard endotoxin solution	2.1. Standard endotoxin stock solution	
Preparation of sample solutions	2.2. Standard endotoxin solution	
Determination of maximum valid dilution	2.3. Sample solutions	
Gel-clot technique	3. Determination of maximum valid dilution	
(1) Preparatory testing	4. Gel-clot techniques	
(i) Test for confirmation of labeled lysate sensitivity	4.1. Preparatory testing	
(ii) Test for interfering factors	4.1.1. Test for confirmation of labeled lysate reagent sensitivity	
(2) Limit test	4.1.2. Test for interfering factors	
(i) Procedure	4.2. Limit test	
	4.2.1. Procedure	

(ii) Interpretation	4.2.2. Interpretation	Prescribed in <9.41> Reagents, Test Solutions
(3) Assay	4.3. Quantitative test	
(i) Procedure	4.3.1. Procedure	
(ii) Calculation and interpretation	4.3.2. Calculation and interpretation	
Photometric techniques	5. Photometric quantitative techniques	
(1) Turbidimetric techniques	5.1. Turbidimetric technique	
(2) Chromogenic technique	5.2. Chromogenic technique	
(3) Preparatory testing	5.3. Preparatory testing	
(i) Assurance of criteria for the standard curve	5.3.1. Test for assurance of criteria for the standard curve	
(ii) Test for interfering factors	5.3.2. Test for interfering factors	
(4) Assay	5.4. Quantitative test	
(i) Procedure	5.4.1. Procedure	
(ii) Calculation	5.4.2. Calculation of endotoxin concentration	
(iii) Interpretation	5.4.3. Interpretation	
Reagents, test solutions		
Amoebocyte lysate		
Lysate TS		
Water for bacterial endotoxins test (BET)		
Table 1	Table 1	
Table 2	Table 2	
Table 3	Table 3	
Table 4	Table 4	

June 2009 (Rev. 1 – Corr. 1) (I. Microbial Enumeration Tests)/Jun. 2008 (Rev. 1) (II. Test for specified Micro-organisms)

Harmonized items	JP 16	Remarks
Microbiological Examination of Non-Sterile Products: Microbial Enumeration Tests	4.05 Microbiological Examination of Non-Sterile Products I. Microbiological Examination of Non-Sterile Products: Microbial Enumeration Tests (Introduction)	
1 Introduction		
2 General procedures	1. General Procedures	
3 Enumeration methods	2. Enumeration Methods	
4 Growth promotion test, suitability of the counting method and negative controls	3. Growth Promotion Test, Suitability of the Counting Method and Negative Controls	
4-1 General considerations		
4-2 Preparation of test strains	3.1. Preparation of test strains	
4-3 Negative control	3.2. Negative control	
4-4 Growth promotion of the media	3.3. Growth promotion of the media	
4-5 Suitability of the counting method in the presence of product	3.4. Suitability of the counting method in the presence of product	

4-6 Results and interpretation 5 Testing of products 5-1 Amount used for the test 5-2 Examination of the product 5-3 Interpretation of the results Table 1 Preparation and use of test micro-organisms Table 2 Common neutralising agents for interfering substances Table 3 Most-probable-number values of micro-organisms Microbiological Examination of Non-Sterile Products: Tests for Specified Micro-organisms 1 Introduction 2 General procedures 3 Growth promoting and inhibitory properties of the media, suitability of the test and negative controls 3-1 Preparation of test strains 3-2 Negative control 3-3 Growth promotion and inhibitory properties of the media 3-4 Suitability of the test method 4 Testing of products 4-1 Bile-tolerant gram-negative bacteria 4-2 <i>Escherichia coli</i> 4-3 <i>Salmonella</i> 4-4 <i>Pseudomonas aeruginosa</i> 4-5 <i>Staphylococcus aureus</i> 4-6 <i>Clostridia</i> 4-7 <i>Candida albicans</i> 5 Recommended solutions and culture media Table II-1 Growth promoting, inhibitory and indicative properties of media Table II-2 Interpretation of results	3.5. Results and interpretation 4. Testing of Products 4.1. Amount used for the test 4.2. Examination of the product 4.3. Interpretation of the results Table 1-1 Preparation and use of test micro-organisms Table 1-2 Common neutralising agents /Method for interfering substances Table 1-3 Most-probable-number values of micro-organisms II. Microbiological Examination of Non-Sterile Products: Tests for Specified Micro-organisms (Introduction) 1. General Procedures 2. Growth Promoting and Inhibitory Properties of the Media, Suitability of the Test and Negative Controls 2.1. Preparation of test strains 2.2. Negative control 2.3. Growth promotion and inhibitory properties of the media 2.4. Suitability of the test method 3. Testing of Products 3.1. Bile-tolerant gram-negative bacteria 3.2. <i>Escherichia coli</i> 3.3. <i>Salmonella</i> 3.4. <i>Pseudomonas aeruginosa</i> 3.5. <i>Staphylococcus aureus</i> 3.6. <i>Clostridia</i> 3.7. <i>Candida albicans</i> 4. Recommended Solutions and Culture Media Table II-1 Growth promoting, inhibitory and indicative properties of media Table II-2 Interpretation of results	
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June 2009 (Rev. 1 – Corr. 3)

Harmonized items	JP 16	Remarks
Sterility (Introduction) Precautions against microbial contamination Culture media and incubation temperatures	4.06 Sterility Test (Introduction) 1. Precautions against microbial contamination 2. Culture media and incubation temperatures	

Media for the test may be prepared as described below, or equivalent commercial media may be used provided that they comply with the growth promotion test.		
Fluid thioglycollate medium	(i) Fluid thioglycollate medium	
Soya-bean casein digest medium	(ii) Soya-bean casein digest medium	
The media used comply with the following tests, carried out before or in parallel with the test on the product to be examined	3. Suitability of the culture medium	
Sterility	3.1. Sterility	
Growth promotion test of aerobes, anaerobes and fungi	3.2. Growth promotion test of aerobes, anaerobes and fungi	
Method suitability test	4. Method suitability test	
Membrane filtration	(i) Membrane filtration	
Direct inoculation	(ii) Direct inoculation	
Test for sterility of the product to be examined	5. Test for sterility of the product to be examined	
The test may be carried out using the technique of membrane filtration or by direct inoculation of the culture media with the product to be examined		
Membrane filtration	5.1. Membrane filtration	Note 7
Aqueous solutions	(i) Aqueous solutions	
Soluble solids	(ii) Soluble solids	
Oils and oily solutions	(iii) Oils and oily solutions	
Ointments and creams	(iv) Ointments and creams	
Direct inoculation of the culture medium	5.2. Direct inoculation of the culture medium	
Oily liquids	(i) Oily liquids	
Ointments and creams	(ii) Ointments and creams	
Catgut and other surgical sutures for veterinary use		The items not included in JP.
Observation and interpretation of results	6. Observation and interpretation of results	
Application of the test to parenteral preparations, ophthalmic and other non-injectable preparations required to comply with the test for sterility	7. Application of the test to parenteral preparations, ophthalmic and other non-injectable preparations required to comply with the test for sterility	
Minimum number of items to be tested	8. Minimum number of items to be tested	
Table 1. Strains of the test micro-organisms suitable for use in the growth promotion test and the method suitability test	Table 1. Strains of the test micro-organisms suitable for use in the growth promotion test and the method suitability test	
Table 2. Minimum quantity to be used for each medium	Table 2. Minimum quantity to be used for each medium	
Table 3. Minimum number of items to be tested	Table 3. Minimum number of items to be tested	Non-harmonized item: The large volume preparation is specified as not less than 100 mL labeled.

Feb. 2004

Harmonized items	JP 16	Remarks
Uniformity of Dosage Units (Introduction)	6.02 Uniformity of Dosage Units (Introduction)	JP's particular description: Additional explanation on Liquids. Additional explanation for the part not containing drug substance.
Content uniformity	1. Content uniformity	
Solid dosage forms	(i) Solid dosage forms	
Liquid dosage forms	(ii) Liquid dosage forms	
Calculation of acceptance value	1.1. Calculation of acceptance value	
Mass variation	2. Mass variation	JP's particular description: Assuming that the concentration of drug substance is uniform in each lot.
Uncoated or film-coated tablets	(i) Uncoated or film-coated tablets	
Hard capsules	(ii) Hard capsules	
Soft capsules	(iii) Soft capsules	
Solid dosage forms other than tablets and capsules	(iv) Solid dosage forms other than tablets and capsules	
Liquid dosage forms	(v) Liquid dosage forms	The phrase "in conditions of normal use. If necessary, compute the equivalent volume after determining the density." is deleted.
Calculation of acceptance value	2.1. Calculation of acceptance value	
Criteria	3. Criteria	
Solid and liquid dosage forms	(i) Solid and liquid dosage forms	
Table 1 Application of content uniformity (CU) and mass variation (MV) test for dosage forms	Table 1 Application of content uniformity (CU) and mass variation (MV) test for dosage forms	JP's particular description: Addition of "(divided forms, lyophilized forms)" and "(true solution)".
Table 2	Table 2	The phrases "at time of manufacture" and "For purposes of this Pharmacopoeia" are deleted

June 2004 (Rev.1)

Harmonized items	JP 16	Remarks
Test for Extractable Volume of Parenteral Preparations (Introduction)	6.05 Test for Extractable Volume of Parenteral Preparations (Introduction)	JP's particular description: Explanation on this Test
Single-dose containers	1. Single-dose containers	
Multi-dose containers	2. Multi-dose containers	
Cartridges and prefilled syringes	3. Cartridges and pre-filled syringes	
Parenteral infusions	4. Parenteral infusions	

June 2004 (Rev.1)

Harmonized items	JP 16	Remarks
Particulate Matter in Injectables (Introduction)	6.07 Insoluble Particulate Matter Test for Injections (Introduction)	

Method 1.	1. Method 1. Light obscuration particle count test	
Light obscuration particle count test	1.1. Apparatus	JP's particular description: Description of evaluation frequency.
	1.1.1. Calibration	JP's particular description
	1.1.1.1. Manual method	JP's particular description
	1.1.1.2. Electronic method	JP's particular description
	1.1.1.3. Automated method	JP's particular description
	1.1.2. Sample volume accuracy	JP's particular description
	1.1.3. Sample flow rate	JP's particular description
	1.1.4. Sensor	JP's particular description
	1.1.4.1. Sensor resolution (Particle size resolution of apparatus)	JP's particular description
	1.1.4.2. Particle counting accuracy	JP's particular description
	1.1.4.3. Threshold accuracy	JP's particular description
General precautions	1.2. General precautions	
Method	1.3. Method	
Evaluation	1.4. Evaluation	JP's particular description: "equal to or more than 100 mL".
Method 2.	2. Method 2. Microscopic particle count test	
Microscopic particle count test	2.1. Apparatus	
	2.2. General precautions	
General precautions	2.3. Method	
Method	2.4. Evaluation	JP's particular description: "equal to or more than 100 mL".
Evaluation	3. Reagents	JP's particular description
1. Circular diameter graticule	Fig. 1 Circular diameter graticule	

Oct. 2007 (Rev.1)

Harmonized items	JP 16	Remarks
Disintegration	6.09 Disintegration Test	JP's particular description: This test is applied to Granules, Dry Syrups and Pills.
Apparatus	1. Apparatus	
Basket-rack assembly	(i) Basket-rack assembly	JP's particular description: The apparatus may be varied somewhat provided the specifications.
Disk	(ii) Disk	
	(iii) Auxiliary tube	JP's particular description
Procedure	2. Procedure	
	2.1. Immediate-release preparations	JP's particular description: Setting a test for Granules, Dry Syrups and Pills. Allowing use of water as the test fluid. Setting each intervals of the immersion. Making a definition of complete disintegration.

	2.2. Enteric coated preparations	Setting a procedure for Granules. JP's particular description: Setting a test for enteric coated preparations.
Figure 1 Disintegration apparatus	Fig. 1 Disintegration apparatus Fig. 2 Auxiliary tube	JP's particular description.

Nov. 2008 (Rev. 2)

Harmonized items	JP 16	Remarks
Dissolution	6.10 Dissolution Test	JP's particular description: The test also aims at preventing significant bioinequivalence.
Apparatus	1. Apparatus	
Apparatus 1 (Basket apparatus)	1.1. Apparatus for Basket Method (Apparatus 1)	
Apparatus 2 (Paddle apparatus)	1.2. Apparatus for Paddle Method (Apparatus 2)	JP's particular description: The sinker is allowed to use in case only when specified in the monograph.
Apparatus 3 (Reciprocating cylinder)	not specified	
Apparatus 4 (Flow-through cell)	1.3. Apparatus for Flow-Through Cell Method (Apparatus 3)	
Apparatus suitability	2. Apparatus suitability	
Procedure	3. Procedure	
Apparatus 1 or 2	3.1. Basket Method or Paddle Method	
Immediate-release dosage forms	3.1.1. Immediate-release dosage forms	
Procedure	(i) Procedure	
Dissolution medium	(ii) Dissolution medium	
Time	(iii) Time	
Extended-release dosage forms	3.1.2. Extended-release dosage forms	
Procedure	(i) Procedure	
Dissolution medium	(ii) Dissolution medium	
Time	(iii) Time	
Delayed-release dosage forms	3.1.3. Delayed-release dosage forms	
Procedure	(i) Procedure	In the Harmonized text: Alternative usage of Method A or B.
Method A		
Method B		
Time	(ii) Dissolution medium (iii) Time	JP's particular description. JP's particular description: Time is specified each for the 1st and 2nd fluids.
Apparatus 3	not specified	
Immediate-release dosage forms		
Procedure		
Dissolution medium		
Time		

Extended-release dosage forms		
Procedure		
Dissolution medium		
Time		
Delayed-release dosage forms		
Procedure		
Time		
Apparatus 4	3.2. Flow-Through Cell Method	
Immediate-release dosage forms	3.2.1. Immediate-release dosage forms	
Procedure	(i) Procedure	
Dissolution medium	(ii) Dissolution medium	
Time	(iii) Time	
Extended-release dosage forms	3.2.2. Extended-release dosage forms	
Procedure	(i) Procedure	
Dissolution medium	(ii) Dissolution medium	
Time	(iii) Time	
Delayed-release dosage forms		
Procedure		
Time		
Interpretation	4. Interpretation	JP's particular description: Follow Interpretation 1 when the value Q is specified in the individual monograph, otherwise follow Interpretation 2.
Immediate-release dosage forms	4.1. Immediate-release dosage forms	JP's particular description: Setting Interpretation 2.
	4.1.1. Interpretation 1	
	4.1.2. Interpretation 2	
Extended-release dosage forms	4.2. Extended-release dosage forms	JP's particular description: Setting Interpretation 2.
	4.2.1. Interpretation 1	
	4.2.2. Interpretation 2	
Delayed-release dosage forms	4.3. Delayed-release dosage forms	Non-harmonized items: Different dissolution medium. Deletion of disharmonized part on the value Q.
	4.3.1. Interpretation 1	JP's particular description: Setting Interpretation 2.
	4.3.2. Interpretation 2	The value Q is specified in the individual monograph.
Acceptance Table 1	Acceptance Table 1	
Acceptance Table 2	Acceptance Table 2	
Acceptance Table 3	Acceptance Table 3	
Acceptance Table 4	Acceptance Table 4	
Figure 1 Apparatus 1, Basket stirring element	Fig. 1 Apparatus 1, Basket stirring element	
Figure 2 Paddle stirring element	Fig. 2 Apparatus 2, Paddle stirring element	
Figure 2a Alternative sinker	Fig. 2a Alternative sinker	

Figure 3 Apparatus 3	not specified	
Figure 4 Apparatus 4 (top) large cell tablets and capsules (bottom) tablet holder for the large cell	Fig. 3 Apparatus 3 (top) large cell for tablets and capsules (bottom) tablet holder for the large cell	
Figure 5 Apparatus 4 (top) small cell tablets and capsules (bottom) tablet holder for the small cell	Fig. 4 Apparatus 3 (top) small cell for tablets and capsules (bottom) tablet holder for the small cell	

Sep. 2002 (Rev. 1)

Harmonized items	JP 16	Remarks
Ethanol	Ethanol	
Identification A	not specified as Identification	Setting Specific gravity as specification.
Identification B	Identification	
Relative density	Specific gravity	Setting Specific gravity at 15°C.
Appearance	Purity (1) Clarity and color of solution	
Acidity or alkalinity	Purity (2) Acid or alkali	
Volatile impurities	Purity (3) Volatile impurities	
Absorbance	Purity (4) Other impurities	
Residue on evaporation	Purity (5) Residue on evaporation	
Storage	Containers and storage	

Sep. 2002 (Rev. 1)

Harmonized items	JP 16	Remarks
Ethanol, Anhydrous	Anhydrous Ethanol	
Definition	limits of content	
Identification A	not specified as Identification	Setting Specific gravity as specification.
Identification B	Identification	
Relative density	Specific gravity	Setting Specific gravity at 15°C.
Appearance	Purity (1) Clarity and color of solution	
Acidity or alkalinity	Purity (2) Acid or alkali	
Volatile impurities	Purity (3) Volatile impurities	
Absorbance	Purity (4) Other impurities	
Residue on evaporation	Purity (5) Residue on evaporation	
Storage	Containers and storage	

Nov. 2003 (Rev. 2)

Harmonized items	JP 16	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
Sulphates	Purity (3) Sulfates
Phosphates	Purity (4) Phosphates
Bromides	Purity (5) Bromides
Iodides	Purity (6) Iodides
Ferrocyanides	Purity (7) Ferrocyanides
Iron	Purity (9) Iron
Barium	Purity (10) Barium
Magnesium and alkaline-earth metals	Purity (11) Magnesium and alkaline-earth materials
Aluminium	not specified
Nitrites	not specified
Potassium	not specified
Loss on drying	Loss on drying
Assay	Assay

Nov. 2008

Harmonized items	JP 16	Remarks
Carmellose	Carmellose	
Definition	Definition	
Identification (1)	Identification (1)	
Identification (2)	Identification (2)	
Purity (1) Chloride	Purity (1) Chloride	
Purity (2) Sulfate	Purity (2) Sulfate	
Loss on drying	Loss on drying	
Residue on ignition	Residue on ignition	

July 2003 (Rev. 1)

Harmonized items	JP 16	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) Alkalinity	
Loss on drying	Loss on drying	
Residue on ignition	Residue on ignition	
Limit of chloride	Purity (2) Chloride	
Limit of sulfate	Purity (3) Sulfate	

Oct. 2001

Harmonized items	JP 16	Remarks
Croscarmellose Sodium	Croscarmellose Sodium	
Definition	origin	
Identification A	Identification (1)	
Identification B	Identification (2)	
Identification C	Identification (3)	
pH	pH	
Settling volume	Precipitation test	
Degree of substitution	Degree of substitution	
Loss on drying	Loss on drying	
Residue on ignition	Residue on ignition	
Packaging and storage	Containers and storage	

Nov. 2003 (Rev. 1)

Harmonized items	JP 16	Remarks
Citric Acid, Anhydrous	Anhydrous Citric Acid	
Definition	limits of the content	
Appearance of solution	Purity (1) Clarity and color of solution	
Sulphates	Purity (2) Sulfates	
Oxalic acid	Purity (3) Oxalic acid	
Readily carbonisable substances	Purity (5) Readily carbonizable substances	
Aluminium	not specified	
Water	Water	
Sulphated ash	Residue on ignition	
Assay	Assay	

Nov. 2003 (Rev. 1)

Harmonized items	JP 16	Remarks
Citric Acid Monohydrate	Citric Acid Hydrate	
Definition	limits of the content	
Appearance of solution	Purity (1) Clarity and color of solution	
Sulphates	Purity (2) Sulfates	
Oxalic acid	Purity (3) Oxalic acid	
Readily carbonisable substances	Purity (5) Readily carbonizable substances	
Aluminium	not specified	
Water	Water	
Sulphated ash	Residue on ignition	
Assay	Assay	

Feb. 2003

Harmonized items	JP 16	Remarks
Saccharin	Saccharin	
Definition	limits of content	
Limit of benzoate and salicylate	Purity (3) Benzoate and salicylate	
Readily carbonisable substances	Purity (5) Readily carbonizable substances	
Loss on drying	Loss on drying	
Residue on ignition	Residue on ignition	
Assay	Assay	
Packaging and storage	Containers and storage	

Feb. 2004 (Rev. 1)

Harmonized items	JP 16	Remarks
Saccharin Sodium	Saccharin Sodium Hydrate	
Definition	limits of content	
Identification B, C	Identification (2)	
Acidity or alkalinity	Purity (2) Acid or alkali	
Limit of benzoate and salicylate	Purity (4) Benzoate and salicylate	
Readily carbonisable substances	Purity (6) Readily carbonizable substances	
Water	Water	
Assay	Assay	

Oct. 2001

Harmonized items	JP 16	Remarks
Cellacefate	Collacefate	
Definition	content of the acetyl and carboxybenzoyl groups	
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 group	
Content of acetyl	Assay (2) Acetyl group	
Packaging and storage	Containers and storage	

May 2005 (Rev. 1)

Harmonized items	JP 16	Remarks
Microcrystalline Cellulose	Microcrystalline Cellulose	
Definition	origin	
Identification A	Identification (1)	
Identification B	Identification (3)	
pH	pH	

Water-soluble substances	Purity (2) Water-soluble substances	
Ether-soluble substances	Purity(3) Diethyl ether-soluble substances	
Conductivity	Conductivity	
Loss on drying	Loss on drying	
Residue on ignition	Residue on ignition	
Bulk density	Bulk density	

May 2005 (Rev. 1)

Harmonized items	JP 16	Remarks
Powdered Cellulose	Powdered Cellulose	
Definition	origin	
Labeling	Labeling of mean degree of polymerization	
Identification A	Identification (1)	
Identification B	Identification (3)	
pH	pH	
Water-soluble substances	Purity (2) Water-soluble substances	
Ether-soluble substances	Purity (3) Diethyl ether-soluble substances	
Loss on drying	Loss on drying	
Residue on ignition	Residue on ignition	

June 2008 (Rev. 1)

Harmonized items	JP 16	Remarks
Talc	Talc	
Definition	origin, content of magnesium	
Identification	Identification	
Acidity and alkalinity	Purity (1) Acidity or alkalinity	
Aluminium	Purity (5) Aluminum	
Calcium	Purity (7) Calcium	
Iron	Purity (4) Iron	
Lead	Purity (6) Lead	
Magnesium	Assay	
Loss on ignition	Loss on ignition	

Oct. 2007 (Rev. 1)

Harmonized items	JP 16	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	

Oct. 2006

Harmonized items	JP 16	Remarks
Rice Starch	Rice Starch	
Definition	Origin	
Identification A	Identification (1)	
Identification B	Identification (2)	
Identification C	Identification (3)	
pH	pH	
Iron	Purity (1) Iron	
Loss on drying	Loss on drying	
Sulphated ash	Residue on ignition	
Oxidising substances	Purity (2) Oxidizing substances	
Sulphur dioxide	Purity (3) Sulfur dioxide	

Oct. 2007 (Rev. 2)

Harmonized items	JP 16	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	
Limit of sulfur dioxide	Purity (3) Sulfur dioxide	

Oct. 2007 (Rev. 1)

Harmonized items	JP 16	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	

May 2005 (Rev.1)

Harmonized items	JP 16	Remarks
Sodium Starch Glycolate	Sodium Starch Glycolate	
Definition	origin, limit of sodium	
Identification A	Identification (1)	
Identification B	Identification (3)	
pH	pH	
Loss on drying	Loss on drying	
Limit of iron	Purity (2) Iron	
Limit of sodium chloride	Purity (4) Sodium chloride	
Limit of sodium glycolate	Purity (3) Sodium glycolate	
Assay	Assay	

June 2008 (Rev. 3)

Harmonized items	JP 16	Remarks
Anhydrous Lactose	Anhydrous Lactose	
Definition	origin	
Clarity and color of solution	Purity (1) Clarity and color of solution	
Specific rotation	Optical rotation	
Acidity or alkalinity	Purity (2) Acidity or alkalinity	
Loss on drying	Loss on drying	
Residue on ignition	Residue on ignition	
Water	Water	
Protein and light-absorbing impurities	Purity (4) Proteins and light absorbing substances	
Content of alpha and beta anomers	Isomer ratio	

June 2008 (Rev. 2)

Harmonized items	JP 16	Remarks
Lactose Monohydrate	Lactose Hydrate	
Definition	origin	
Clarity and color of solution	Purity (1) Clarity and color of solution	
Identification	Identification	
Specific rotation	Optical rotation	
Acidity or alkalinity	Purity (2) Acid and alkali	
Residue on ignition	Residue on ignition	
Water	Water	

Protein and light-absorbing impurities	Purity (4) Protein and light absorbing substances	
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Feb. 2004

Harmonized items	JP 16	Remarks
Ethyl Parahydroxybenzoate Definition Identification A Appearance of solution Acidity Related substances Sulphated ash Assay	Ethyl Parahydroxybenzoate limits of content Identification (1) Purity (1) Clarity and color of solution Purity (2) Acidity Purity (4) Related substances Residue on ignition Assay	System suitability is not specified.

Feb. 2004

Harmonized items	JP 16	Remarks
Butyl Parahydroxybenzoate Definition Identification A Appearance of solution Acidity Related substances Sulphated ash Assay	Butyl Parahydroxybenzoate limits of content Identification (1) Purity (1) Clarity and color of solution Purity (2) Acidity Purity (4) Related substances Residue on ignition Assay	System suitability is not specified.

Feb. 2004

Harmonized items	JP 16	Remarks
Propyl Parahydroxybenzoate Definition Identification A Appearance of solution Acidity Related substances Sulphated ash Assay	Propyl Parahydroxybenzoate limits of content Identification (1) Purity (1) Clarity and color of solution Purity (2) Acidity Purity (4) Related substances Residue on ignition Assay	System suitability is not specified.

Feb. 2004

Harmonized items	JP 16	Remarks
Methyl Parahydroxybenzoate Definition Identification A Appearance of solution Acidity Related substances	Methyl Parahydroxybenzoate limits of content Identification (1) Purity (1) Clarity and color of solution Purity (2) Acidity Purity (4) Related substances	System suitability is not specified.

Sulphated ash Assay	Residue on ignition Assay	
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Nov. 2003

Harmonized items	JP 16	Remarks
Hydroxypropyl Methylcellulose	Hypromellose	
Definition	limits of content of methoxy group and hydroxypropoxy group	
Labeling	labeling of viscosity	
Identification (1)	Identification (1)	
Identification (2)	Identification (2)	
Identification (3)	Identification (3)	
Identification (4)	Identification (4)	
Identification (5)	Identification (5)	
Viscosity	Viscosity	
Method 1	Method I	
Method 2	Method II	
pH	pH	
Heavy metals	Purity Heavy metals	
Loss on drying	Loss on drying	
Residue on ignition	Residue on ignition	
Assay	Assay	

June 2006

Harmonized items	JP 16	Remarks
Hypromellose Phthalate	Hypromellose Phthalate	
Definition	origin, limit of carboxybenzoyl	
Packaging and storage	Containers and storage	
Viscosity	Viscosity	
Water	Water	
Residue on ignition	Residue on ignition	
Chloride	Purity (1) Chloride	
Limit of free phthalic acid	Purity (3) Phthalic acid	
Phthalyl content	Assay	

June 2008 (Rev. 2)

Harmonized items	JP 16	Remarks
Benzyl Alcohol	Benzyl Alcohol	
Definition	limits of the content	
Refractive index	Refractive index	
Acidity	Purity (2) Acidity	
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	
Nov. 2005 (Rev. 1)		
Harmonized items	JP 16	Remarks
Methylcellulose	Methylcellulose	
Definition	limits of content of methoxy group	
Labeling	labeling of viscosity	
Identification (1)	Identification (1)	
Identification (2)	Identification (2)	
Identification (3)	Identification (3)	
Identification (4)	Identification (4)	
Identification (5)	Identification (5)	
Viscosity	Viscosity	
Method 1	Method I	
Method 2	Method II	
pH	pH	
Heavy metals	Purity Heavy metals	
Loss on drying	Loss on drying	
Residue on ignition	Residue on ignition	
Assay	Assay	

Nov. 2005

Harmonized items	JP 16	Remarks
Anhydrous Dibasic Calcium Phosphate	Anhydrous Dibasic Calcium Phosphate	
Definition	limit of content	
Identification (1)	Identification (1)	
Identification (2)	Identification (2)	
Acidinsoluble substances	Purity (1) Acid-insoluble substance	
Chloride	Purity (2) Chloride	
Sulfate	Purity (3) Sulfate	
Carbonate	Purity (4) Carbonate	
Barium	Purity (6) Barium	
Loss on ignition	Loss on ignition	
Assay	Assay	

Nov. 2005

Harmonized items	JP 16	Remarks
Dibasic Calcium Phosphate	Dibasic Calcium Phosphate Hydrate	
Definition	limit of content	
Identification (1)	Identification (1)	
Identification (2)	Identification (2)	
Acidinsoluble substances	Purity (1) Acid-insoluble substances	
Chloride	Purity (2) Chloride	
Sulfate	Purity (3) Sulfate	

Carbonate	Purity (4) Carbonate	
Barium	Purity (6) Barium	
Loss on ignition	Loss on ignition	
Assay	Assay	

Nov. 2008

Harmonized items	JP 16	Remarks
Particle-size Analysis by Laser Light Diffraction	General Information	
Laser Diffraction Measurement of Particle Size	Laser Diffraction Measurement of Particle Size	
Introduction	Introduction	
Principle	1. Principle	
Instrument	2. Instrument	
Development of the method	3. Development of the method	
Sampling	3.1. Sampling	
Evaluation of the dispersion procedure	3.2. Evaluation of the dispersion procedure	
Optimisation of the liquid dispersion	3.3. Optimization of the liquid dispersion	
Optimisation of the gas dispersion	3.4. Optimization of the gas dispersion	
Determination of the concentration range	3.5. Determination of the concentration range	
Determination of the measuring time	3.6. Determination of the measuring time	
Selection of an appropriate optical model	3.7. Selection of an appropriate optical model	
Validation	3.8. Validation	
Measurement	4. Measurement	
Precautions	4.1. Precautions	
Measurement of the light scattering of dispersed sample(s)	4.2. Measurement of the light scattering of dispersed sample(s)	
Conversion of scattering pattern into particle-size distribution	4.3. Conversion of scattering pattern into particle-size distribution	
Replicates	4.4. Replicates	
Reporting of results	5. Reporting of results	
Control of the instrument performance	6. Control of the instrument performance	
Calibration	6.1. Calibration	
Qualification of the system	6.2. Qualification of the system	
Figure 1 Example of a set-up of laser light diffraction instrument	Fig. 1 Example of a set-up of laser light diffraction instrument	
<i>Note</i>	<i>Note 1</i> <i>Note 2</i>	The first sentence of the harmonized text is described as Note 2.

May 2007

Harmonized items	JP 16	Remarks
Powder Fineness	General Information Powder Fineness	

June 2004

Harmonized items	JP 16	Remarks
Powder Flow	General Information Powder Flow	
(Introduction)	(Introduction)	
Angel of repose	1. Angel of repose	
Basic methods for angel of repose	1.1 Basic methods for angel of repose	
Variations in angel of repose methods	1.2 Variations in angel of repose methods	
Angel of repose general scale of flowability	1.3 Angel of repose general scale of flowability	
Experimental considerations for angle of repose	1.4 Experimental considerations for angle of repose	
Recommended procedure for angle of repose	1.5 Recommended procedure for angle of repose	
Compressibility index and Hausner ratio	2. Compressibility index and Hausner ratio	
Basic methods for compressibility index and Hausner ratio	2.1 Basic methods for compressibility index and Hausner ratio	
Experimental considerations for the compressibility index and Hausner ratio	2.2 Experimental considerations for the compressibility index and Hausner ratio	
Recommended procedure for compressibility index and Hausner ratio	2.3 Recommended procedure for compressibility index and Hausner ratio	
Flow through an orifice	3. Flow through an orifice	
Basic methods for flow through an orifice	3.1 Basic methods for flow through an orifice	
Variations in methods for flow through an orifice	3.2 Variations in methods for flow through an orifice	
General scale of flowability for flow through an orifice	3.3 General scale of flowability for flow through an orifice	
Experimental considerations for flow through an orifice	3.4 Experimental considerations for flow through an orifice	
Recommended procedure for flow through an orifice	3.5 Recommended procedure for flow through an orifice	
Shear cell methods	4. Shear cell methods	
Basic methods for shear cell	4.1 Basic methods for shear cell	
Recommendations for shear cell	4.2 Recommendations for shear cell	
Table 1 Flow properties and corresponding angle of repose	Table 1 Flow properties and corresponding angle of repose	
Table 2 Scale of flowability	Table 2 Scale of flowability	

Sep. 2002

Harmonized items	JP 16	Remarks
Amino Acid Analysis	General Information	
Apparatus	Amino Acid Analysis	
General precautions	Apparatus	
Reference standard material	General precautions	
Calibration of instrumentation	Reference standard material	
Repeatability	Calibration of instrumentation	
Sample preparation	Repeatability	
Internal standards	Sample preparation	
Protein hydrolysis	Internal standards	
Method 1	Protein hydrolysis	
Hydrolysis solution	Method 1	
Procedure	Hydrolysis solution	
Method 2	Procedure	
Hydrolysis solution	Method 2	
Vapor phase hydrolysis	Hydrolysis solution	
Method 3	Vapor phase hydrolysis	
Hydrolysis solution	Method 3	
Vapor phase hydrolysis	Hydrolysis solution	
Method 4	Vapor phase hydrolysis	
Oxidation solution	Method 4	
Procedure	Oxidation solution	
Method 5	Procedure	
Hydrolysis solution	Method 5	
Liquid phase hydrolysis	Hydrolysis solution	
Method 6	Liquid phase hydrolysis	
Hydrolysis solution	Method 6	
Vapor phase hydrolysis	Hydrolysis solution	
Method 7	Vapor phase hydrolysis	
Reducing solution	Method 7	
Procedure	Reducing solution	
Method 8	Procedure	
Stock solutions	Method 8	
Reducing solution	Stock solutions	
Procedure	Reducing solution	
Method 9	Procedure	
Stock solutions	Method 9	
Carboxymethylation solution	Stock solutions	
Buffer solution	Carboxymethylation solution	
Procedure	Buffer solution	
Method 10	Procedure	
Reducing solution	Method 10	
Procedure	Reducing solution	
	Procedure	

Method 11	Method 11
Reducing solutions	Reducing solutions
Procedure	Procedure
Methodologies of amino acid analysis general principles	Methodologies of amino acid analysis general principles
Method 1-Postcolumn ninhydrin detection general principle	Method 1-Postcolumn ninhydrin de- tection general principle
Method 2-Postcolumn OPA fluoro- metric detection general principle	Method 2-Postcolumn OPA fluoro- metric detection general principle
Method 3-Precolumn PITC derivati- zation general principle	Method 3-Precolumn PITC derivati- zation general principle
Method 4-Precolumn AQC derivati- zation general principle	Method 4-Precolumn AQC derivati- zation general principle
Method 5-Precolumn OPA derivati- zation general principle	Method 5-Precolumn OPA derivati- zation general principle
Method 6-Precolumn DABS-Cl derivatization general principle	Method 6-Precolumn DABS-Cl derivatization general principle
Method 7-Precolumn FMOC-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

June 2010 (Corr. 2)

Harmonized items	JP 16	Remarks
Capillary Electrophoresis	General Information	
Apparatus	Apparatus	
Capillary zone electrophoresis	1. Capillary Zone Electrophoresis	
Optimisation	Optimization	
Instrumental parameters	Instrumental parameters	
Voltage	(1) Voltage	
Polarity	(2) Polarity	
Temperature	(3) Temperature	
Capillary	(4) Capillary	
Electrolytic solution parameters	Electrolytic solution parameters	
Buffer type and concentration	(1) Buffer type and concentration	
Buffer pH	(2) Buffer pH	
Organic solvents	(3) Organic solvents	
Additives for chiral separations	(4) Additives for chiral separations	
Capillary gel electrophoresis	2. Capillary Gel Electrophoresis	
Characteristics of gels	Characteristics of Gels	
Capillary isoelectric focusing	3. Capillary Isoelectric Focusing	
Loading step	(1) Loading step	
loading in one step	(i) loading in one step	

sequential loading	(ii) sequential loading	
Focusing step	(2) Focusing step	
Mobilisation step	(3) Mobilization step	
Optimisation	Optimization	
Voltage	(1) Voltage	
Capillary	(2) Capillary	
Solutions	(3) Solutions	
Micellar electrokinetic chromatography (MEKC)	4. Micellar Electrokinetic Chromatography (MEKC)	
Optimisation	Optimization	
Instrumental parameters	Instrumental parameters	
Voltage	(1) Voltage	
Temperature	(2) Temperature	
Capillary	(3) Capillary	
Electrolytic solution parameters	Electrolytic solution parameters	
Surfactant type and concentration	(1) Surfactant type and concentration	
Buffer pH	(2) Buffer pH	
Organic solvents	(3) Organic solvents	
Additives for chiral separations	(4) Additives for chiral separations	
Other additives	(5) 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	

Sep. 2002

Harmonized items	JP 16	Remarks
Isoelectric Focusing	General Information	
	Isoelectric Focusing	
	General principle	
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	
Reagents	Reagents and Solutions	
Fixing solution for isoelectric focusing in polyacrylamide gel	Fixing solution for isoelectric focusing in polyacrylamide gel	JP's particular description
	Coomassie staining TS	JP's particular description
	Destaining solution	
Figure-Mould	Figure. Mould	

Sep. 2002

Harmonized items	JP 16	Remarks
Peptide Mapping	General Information	
Purpose and scope	Peptide Mapping	
The peptide map	Purpose and scope	
Isolation and purification	1. The peptide map	
Selective cleavage of peptide bonds	2. Isolation and purification	
Pretreatment of sample	3. Selective cleavage of peptide bonds	
Pretreatment of the cleavage agent	3.1. Pretreatment of sample	
	3.2. Pretreatment of the cleavage agent	
Pretreatment of the protein	3.3. Pretreatment of the protein	
Establishment of optimal digestion conditions	3.4. Establishment of optimal digestion conditions	
pH	(i) pH	
Temperature	(ii) Temperature	
Time	(iii) Time	
Amount of cleavage agent	(iv) Amount of cleavage agent	
Chromatographic separation	4. Chromatographic separation	
Chromatographic column	4.1. Chromatographic column	
solvent	4.2. Solvent	
Mobile phase	4.3. Mobile phase	
Gradient selection	4.4. Gradient selection	
Isocratic selection	4.5. Isocratic selection	
Other parameters	4.6. Other parameters	
Validation	4.7. Validation	
Analysis and identification of peptides	5. 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	

Oct. 1999

Harmonized items	JP 16	Remarks
Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)	General Information 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	2.1. Reducing conditions	
Non-reducing conditions	2.2. 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	4.1. Assembling of the gel moulding cassette	
Preparation of the gel	4.2. Preparation of the gel	
Mounting the gel in the electrophoresis apparatus and electrophoretic separation	4.3. Mounting the gel in the electrophoresis apparatus and electrophoretic separation	
Detection of protein in gels	5. Detection of Proteins in Gels	
Coomassie staining	5.1. Coomassie staining	
Silver staining	5.2. 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 (Validation)	
Quantification of impurities	9. Quantification of Impurities	
Reagents, test solutions	10. 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	

Sep. 2002

Harmonized items	JP 16	Remarks
Total Protein Assay	General Information Total Protein Assay	
Method 1	Method 1 (UV method)	
Standard solution	Standard Solution	

Test solution	Test Solution	Explanatory footnote “Example: the Minimum Requirements for Biological Products and individual monograph of JP” is added.
Procedure	Procedure	
Light-scattering	Light-Scattering	
Calculations	Calculations	
Method 2	Method 2 (Lowry method)	
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 reagent	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 (Bradford method)	
Standard solutions	Standard Solutions	
Test solution	Test Solution	
Blank	Blank	
Coomassie reagent	Coomassie Reagent	
Procedure	Procedure	
Calculations	Calculations	
Method 4	Method 4 (Bicinchonic acid method)	
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 (Biuret method)	
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 (Fluorometric method)	
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 (Nitrogen method)	
Procedure A	Procedure A	
Procedure B	Procedure B	
Calculations	Calculations	

Nov. 2005

Harmonized items	JP 16	Remarks
Microbiological Examination of Non-Sterile Products: Acceptance criteria for pharmaceutical preparations and substances for pharmaceutical use Table 2. Acceptance criteria for microbiological quality of non-sterile substances for pharmaceutical use Table 1. Acceptance criteria for microbiological quality of non-sterile dosage forms	General Information Microbial Attributes of Non-Sterile Pharmaceutical Products 5. Microbial acceptance criteria for non-sterile pharmaceutical products Table 1. Acceptance criteria for microbiological quality of non-sterile substances for pharmaceutical use Table 2. Acceptance criteria for microbiological quality of non-sterile dosage forms Table 3. Acceptance criteria for crude drugs and crude drug preparations	JP's particular description: 1. Definitions 2. Scope 3. Sampling plan and frequency of testing 4. Microbial control program JP's particular description: Explanation of the microbial acceptance criteria JP's particular description: 6. Acceptance criteria for crude drugs and crude drug preparations JP's particular description

Feb. 2004

Harmonized items	JP 16	Remarks
Tablet Friability	General Information Tablet Friability Test	

Atomic Weight Table (2010)

In 1961 it was decided that the atomic weights of the elements would be based on values relative to the mass number of 12 (no fractions) for carbon (^{12}C). Ever since, there has been a marked improvement in the quality and quantity of data on the nuclide masses and isotope ratios of the elements using physical methods such as mass spectrometry. The Commission on Isotope Abundances and Atomic Weights (CIAAW) of the International Union of Pure and Applied Chemistry (IUPAC) collected and examined newly measured data and publishes a new atomic weight table every two years (in the odd years). Based on this table, in April of each year the Atomic Weight Subcommittee of the Chemical Society of Japan also publishes an atomic weight table. The numbers of the following Atomic Weight Table (2010) is based on the numbers published by the IUPAC in 2007¹⁾. For a more detailed explanation, the user is referred to a report²⁾ and a review³⁾ published by the CIAAW.

The atomic weight values of each of the elements shown in the atomic weight tables are, as stated in the preface to the table, for elements that originate on Earth and are present in substances that exist naturally. Atomic weights are, with the exception of single nuclide elements (elements consisting of one stable nuclide), not natural constants like the speed of light, but rather change depending on a variety of factors, such as the method of treatment or the origin of the substance containing that element. This is because the atomic weight is dependent on the relative frequency (isotope ratio) of the stable nuclides comprising each of the respective elements. Due to advancements in measurement techniques, the isotopic frequencies of each of the elements are not necessarily constant, and fluctuate due to a variety of processes that occur on the Earth. We have come to learn that this is reflected in the atomic weights. The result of this is that differences have arisen in the accuracy of the atomic weights between elements. The figures in parentheses that follow the atomic weight values in the atomic weight tables represent the uncertainty with respect to the last digit in the atomic weight. For example, in the case of hydrogen, 1.00794(7) means 1.00794 ± 0.00007 .

The atomic weight of a single nuclide element is the most accurate and the precision is also high. This is because it is not necessary to consider the isotope ratio since single nuclide elements do not possess a multiple number of stable isotopes. The atomic weights of such elements are determined based on the mass⁴⁾ of each nuclide determined by physical techniques, taking into consideration the uncertainty with constant criteria.

Among the elements, the majority of samples gathered on Earth exhibit a constant isotope composition, however, some specific samples have isotope compositions that are different to these. These kinds of elements are indicated by a “g”, which means the value in the atomic weight table cannot be used as is, depending on the sample, as the atomic weight of these elements. In relation to this, oxygen for example exists in a number of forms on Earth, such as in air, salt water, fresh water, and in rocks, and because the isotope compositions fluctuate among these substances, oxygen is not an element for which only one value can be used. Thus, an “r” is attached to an element for which a precise atomic weight cannot be given, no matter how much progress is made in techniques for measuring the isotope composition. On the other hand, it is also possible, depending on the element, to use an isotope that has undergone artificial fractionation as a reagent. Typical elements that are representative include hydrogen, lithium, boron, and uranium. This type of element is identified by an “m”, and particularly in cases where the atomic weight is a problem, it is necessary to be careful by referring to the label of the reagent.

- 1) IUPAC Inorganic Chemistry Division, CIAAW: Standard Atomic Weights Revised. *Chem. Int.*, **29**, 18 (2007).
- 2) IUPAC Inorganic Chemistry Division, CIAAW: Atomic Weights of the Elements 2007. *Pure Appl. Chem.*, **81**, 2131 (2009).
- 3) J. R. De Laeter *et al.*: Atomic Weights of the Elements: Review 2000. *Pure Appl. Chem.*, **75**, 683 (2003).
- 4) G. Audi *et al.*: The A_{ME} 2003 Atomic Mass Evaluation (II). Tables, graphs and references. *Nucl. Phys. A*, **729**, 337 (2003).

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(Scaled to $A_r(^{12}\text{C}) = 12$, where ^{12}C is a neutral atom in its nuclear and electronic ground state)

The atomic weights of many elements are not invariant but depend on the origin and treatment of the material. The standard values of $A_r(\text{E})$ and the uncertainties (in parentheses, following the last significant figure to which they are attributed) apply to elements of natural terrestrial origin. The footnotes to this table elaborate the types of variation which may occur for individual elements and which may be larger than the listed uncertainties of values of $A_r(\text{E})$. Names of elements with atomic number 112 to 118 are provisional.

Name	Symbol	Atomic Number	Atomic Weight	Footnotes
Hydrogen	H	1	1.00794(7)	g m r
Helium	He	2	4.002602(2)	g r
Lithium	Li	3	[6.941(2)] ¹	g m r
Beryllium	Be	4	9.012182(3)	
Boron	B	5	10.811(7)	g m r
Carbon	C	6	12.0107(8)	g r
Nitrogen	N	7	14.0067(2)	g r
Oxygen	O	8	15.9994(3)	g r
Fluorine	F	9	18.9984032(5)	
Neon	Ne	10	20.1797(6)	g m
Sodium	Na	11	22.98976928(2)	
Magnesium	Mg	12	24.3050(6)	
Aluminium	Al	13	26.9815386(8)	
Silicon	Si	14	28.0855(3)	r
Phosphorus	P	15	30.973762(2)	
Sulfur	S	16	32.065(5)	g r
Chlorine	Cl	17	35.453(2)	g m r
Argon	Ar	18	39.948(1)	g r
Potassium	K	19	39.0983(1)	
Calcium	Ca	20	40.078(4)	g
Scandium	Sc	21	44.955912(6)	
Titanium	Ti	22	47.867(1)	
Vanadium	V	23	50.9415(1)	
Chromium	Cr	24	51.9961(6)	
Manganese	Mn	25	54.938045(5)	
Iron	Fe	26	55.845(2)	
Cobalt	Co	27	58.933195(5)	
Nickel	Ni	28	58.6934(4)	r
Copper	Cu	29	63.546(3)	r
Zinc	Zn	30	65.38(2)	r
Gallium	Ga	31	69.723(1)	
Germanium	Ge	32	72.64(1)	
Arsenic	As	33	74.92160(2)	
Selenium	Se	34	78.96(3)	r
Bromine	Br	35	79.904(1)	
Krypton	Kr	36	83.798(2)	g m
Rubidium	Rb	37	85.4678(3)	g
Strontium	Sr	38	87.62(1)	g r
Yttrium	Y	39	88.90585(2)	
Zirconium	Zr	40	91.224(2)	g
Niobium	Nb	41	92.90638(2)	
Molybdenum	Mo	42	95.96(2)	g r
Technetium*	Tc	43		
Ruthenium	Ru	44	101.07(2)	g
Rhodium	Rh	45	102.90550(2)	

Name	Symbol	Atomic Number	Atomic Weight	Footnotes
Palladium	Pd	46	106.42(1)	g
Silver	Ag	47	107.8682(2)	g
Cadmium	Cd	48	112.411(8)	g
Indium	In	49	114.818(3)	
Tin	Sn	50	118.710(7)	g
Antimony	Sb	51	121.760(1)	g
Tellurium	Te	52	127.60(3)	g
Iodine	I	53	126.90447(3)	
Xenon	Xe	54	131.293(6)	g m
Caesium(Cesium)	Cs	55	132.9054519(2)	
Barium	Ba	56	137.327(7)	
Lanthanum	La	57	138.90547(7)	g
Cerium	Ce	58	140.116(1)	g
Praseodymium	Pr	59	140.90765(2)	
Neodymium	Nd	60	144.242(3)	g
Promethium*	Pm	61		
Samarium	Sm	62	150.36(2)	g
Europium	Eu	63	151.964(1)	g
Gadolinium	Gd	64	157.25(3)	g
Terbium	Tb	65	158.92535(2)	
Dysprosium	Dy	66	162.500(1)	g
Holmium	Ho	67	164.93032(2)	
Erbium	Er	68	167.259(3)	g
Thulium	Tm	69	168.93421(2)	
Ytterbium	Yb	70	173.054(5)	g
Lutetium	Lu	71	174.9668(1)	g
Hafnium	Hf	72	178.49(2)	
Tantalum	Ta	73	180.94788(2)	
Tungsten	W	74	183.84(1)	
Rhenium	Re	75	186.207(1)	
Osmium	Os	76	190.23(3)	g
Iridium	Ir	77	192.217(3)	
Platinum	Pt	78	195.084(9)	
Gold	Au	79	196.966569(4)	
Mercury	Hg	80	200.59(2)	
Thallium	Tl	81	204.3833(2)	
Lead	Pb	82	207.2(1)	g r
Bismuth*	Bi	83	208.98040(1)	
Polonium*	Po	84		
Astatine*	At	85		
Radon*	Rn	86		
Francium*	Fr	87		
Radium*	Ra	88		
Actinium*	Ac	89		
Thorium*	Th	90	232.03806(2)	g
Protactinium*	Pa	91	231.03588(2)	
Uranium*	U	92	238.02891(3)	g m
Neptunium*	Np	93		
Plutonium*	Pu	94		
Americium*	Am	95		
Curium*	Cm	96		
Berkelium*	Bk	97		
Californium*	Cf	98		
Einsteinium*	Es	99		
Fermium*	Fm	100		
Mendelevium*	Md	101		
Nobelium*	No	102		
Lawrencium*	Lr	103		
Rutherfordium*	Rf	104		
Dubnium*	Db	105		
Seaborgium*	Sg	106		
Bohrium*	Bh	107		
Hassium*	Hs	108		
Meitnerium*	Mt	109		
Darmstadtium*	Ds	110		

Name	Symbol	Atomic Number	Atomic Weight	Footnotes
Roentgenium	Rg	111		
Copernicium*	Cn	112		
Ununtrium*	Uut	113		
Ununquadium*	Uuq	114		
Ununpentium*	Uup	115		
Ununhexium*	Uuh	116		
Ununoctium*	Uuo	118		

* : Element has no stable isotopes.

† : Commercially available Li materials have atomic weights that range between 6.939 and 6.996 ; if a more accurate value is required, it must be determined for the specific material.

g : Geological specimens are known in which the element has an isotopic composition outside the limits for normal material. The difference between the atomic weight of the element in such specimens and that given in the table may exceed the stated uncertainty.

m : Modified isotopic compositions may be found in commercially available material because it has been subjected to an undisclosed or inadvertent isotopic fractionation. Substantial deviations in atomic weight of the element from that given in the table can occur.

r : Range in isotopic composition of normal terrestrial material prevents a more precise $A_r(E)$ being given ; the tabulated $A_r(E)$ value should be applicable to any normal material.

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