

GENERAL INFORMATION

1. 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 precolumn derivatization. The detector is usually an ultraviolet-visible or fluorescence detector depending on the derivatization method used. A recording device (e.g., integrator) is used for transforming the analog signal from the detector and for quantitation. It is preferred that instrumentation be dedicated particularly for amino acid analysis.

General Precautions

Background contamination is always a concern for the analyst in performing amino acid analysis. High purity reagents are necessary (e.g., low purity hydrochloric acid can contribute to glycine contamination). Analytical reagents are changed routinely every few weeks using only high-pressure liquid chromatography (HPLC) grade solvents. Potential microbial contamination and foreign material that might be present in the solvents are reduced by filtering solvents before

use, keeping solvent reservoirs covered, and not placing amino acid analysis instrumentation in direct sunlight.

Laboratory practices can determine the quality of the amino acid analysis. Place the instrumentation in a low traffic area of the laboratory. Keep the laboratory clean. Clean and calibrate pipets according to a maintenance schedule. Keep pipet tips in a covered box; the analysts may not handle pipet tips with their hands. The analysts may wear powder-free latex or equivalent gloves. Limit the number of times a test sample vial is opened and closed because dust can contribute to elevated levels of glycine, serine, and alanine.

A well-maintained instrument is necessary for acceptable amino acid analysis results. If the instrument is used on a routine basis, it is to be checked daily for leaks, detector and lamp stability, and the ability of the column to maintain resolution of the individual amino acids. Clean or replace all instrument filters and other maintenance items on a routine schedule.

Reference Standard Material

Acceptable amino acid standards are commercially available for amino acid analysis and typically consist of an aqueous mixture of amino acids. When determining amino acid composition, protein or peptide standards are analyzed with the test material as a control to demonstrate the integrity of the entire procedure. Highly purified bovine serum albumin has been used as a protein standard for this purpose.

Calibration of Instrumentation

Calibration of amino acid analysis instrumentation typically involves analyzing the amino acid standard, which consists of a mixture of amino acids at a number of concentrations, to determine the response factor and range of analysis for each amino acid. The concentration of each amino acid in the standard is known. In the calibration procedure, the analyst dilutes the amino acid standard to several different analyte levels within the expected linear range of the amino acid analysis technique. Then, replicates at each of the different analyte levels can be analyzed. Peak areas obtained for each amino acid are plotted versus the known concentration for each of the amino acids in the standard dilution. These results will allow the analyst to determine the range of amino acid concentrations where the peak area of a given amino acid is an approximately linear function of the amino acid concentration. It is important that the analyst prepare the samples for amino acid analysis so that they are within the analytical limits (e.g., linear working range) of the technique employed in order to obtain accurate and repeatable results.

Four to six amino acid standard levels are analyzed to determine a response factor for each amino acid. The response factor is calculated as the average peak area or peak height per nmol of amino acid present in the standard. A calibration file consisting of the response factor for each amino acid is prepared and used to calculate the concentration of each amino acid present in the test sample. This calculation involves dividing the peak area corresponding to a given ami-

no 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 μL of *Hydrolysis Solution* per 500 μg of lyophilized protein. Freeze the sample tube in a dry ice-acetone bath, and flame seal in vacuum. Samples are typically hydrolyzed at 110°C for 24 hours in vacuum or inert atmosphere to prevent oxidation. Longer hydrolysis times (e.g., 48 and 72 hours) are investigated if there is a concern that the protein is not completely hydrolyzed.

Vapor Phase Hydrolysis This is one of the most common acid hydrolysis procedures, and it is preferred for microanalysis when only small amounts of the sample are available. Contamination of the sample from the acid reagent is also minimized by using vapor phase hydrolysis. Place vials containing the dried samples in a vessel that contains an appropriate amount of *Hydrolysis Solution*. The *Hydrolysis Solution* does not come in contact with the test sample. Apply an inert atmosphere or vacuum (\leq less than 200 μm of

mercury or 26.7 Pa) to the headspace of the vessel, and heat to about 110°C for a 24-hour hydrolysis time. Acid vapor hydrolyzes the dried sample. Any condensation of the acid in the sample vials is minimized. After hydrolysis, dry the test sample in vacuum to remove any residual acid.

Method 2

Tryptophan oxidation during hydrolysis is decreased by using mercaptoethanesulfonic acid (MESA) as the reducing acid.

Hydrolysis Solution 2.5 mol/L MESA solution.

Vapor Phase Hydrolysis About 1 to 100 μg of the protein/peptide under test is dried in a hydrolysis tube. The hydrolysis tube is placed in a larger tube with about 200 μL of the *Hydrolysis Solution*. The larger tube is sealed in vacuum (about 50 μm of mercury or 6.7 Pa) to vaporize the *Hydrolysis Solution*. The hydrolysis tube is heated to 170°C to 185°C for about 12.5 minutes. After hydrolysis, the hydrolysis tube is dried in vacuum for 15 minutes to remove the residual acid.

Method 3

Tryptophan oxidation during hydrolysis is prevented by using thioglycolic acid (TGA) as the reducing acid.

Hydrolysis Solution A solution containing 7 mol/L hydrochloric acid, 10% of trifluoroacetic acid, 20% of thioglycolic acid, and 1% of phenol.

Vapor Phase Hydrolysis About 10 to 50 μg of the protein/peptide under test is dried in a sample tube. The sample tube is placed in a larger tube with about 200 μL of the *Hydrolysis Solution*. The larger tube is sealed in vacuum (about 50 μm of mercury or 6.7 Pa) to vaporize the TGA. The sample tube is heated to 166°C for about 15 to 30 minutes. After hydrolysis, the sample tube is dried in vacuum for 5 minutes to remove the residual acid. Recovery of tryptophan by this method may be dependent on the amount of sample present.

Method 4

Cysteine-cystine and methionine oxidation is performed with performic acid before the protein hydrolysis.

Oxidation Solution The performic acid is prepared fresh by mixing formic acid and 30 percent hydrogen peroxide (9:1), and incubated at room temperature for 1 hour.

Procedure The protein/peptide sample is dissolved in 20 μL of formic acid, and heated at 50°C for 5 minutes; then 100 μL of the *Oxidation Solution* is added. The oxidation is allowed to proceed for 10 to 30 minutes. In this reaction, cysteine is converted to cysteic acid and methionine is converted to methionine sulfone. The excess reagent is removed from the sample in a vacuum centrifuge. This technique may cause modifications to tyrosine residues in the presence of halides. The oxidized protein can then be acid hydrolyzed using *Method 1* or *Method 2*.

Method 5

Cysteine-cystine oxidation is accomplished during the liquid phase hydrolysis with sodium azide.

Hydrolysis Solution 6 mol/L hydrochloric acid containing 0.2% of phenol, to which is added sodium azide to obtain a final concentration of 0.2% (w/v). The added phenol prevents halogenation of tyrosine.

Liquid Phase Hydrolysis The protein/peptide hydrolysis is conducted at about 110°C for 24 hours. During the hydrolysis, the cysteine-cystine present in the sample is converted to cysteic acid by the sodium azide present in the *Hydrolysis So-*

lution. This technique allows better tyrosine recovery than *Method 4*, but it is not quantitative for methionine. Methionine is converted to a mixture of the parent methionine and its two oxidative products, methionine sulfoxide and methionine sulfone.

Method 6

Cysteine-cystine oxidation is accomplished with dimethyl sulfoxide (DMSO).

Hydrolysis Solution 6 mol/L hydrochloric acid containing 0.1% to 1.0% of phenol, to which DMSO is added to obtain a final concentration of 2% (v/v).

Vapor Phase Hydrolysis The protein/peptide hydrolysis is conducted at about 110°C for 24 hours. During the hydrolysis, the cysteine-cystine present in the sample is converted to cysteic acid by the DMSO present in the *Hydrolysis Solution*. As an approach to limit variability and compensate for partial destruction, it is recommended to evaluate the cysteic acid recovery from oxidative hydrolyses of standard proteins containing 1 to 8 mol of cysteine. The response factors from protein/peptide hydrolysates are typically about 30% lower than those for nonhydrolyzed cysteic acid standards. Because histidine, methionine, tyrosine, and tryptophan are also modified, a complete compositional analysis is not obtained with this technique.

Method 7

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

Reducing Solution Transfer 83.3 μL of pyridine, 16.7 μL of 4-vinylpyridine, 16.7 μL of tributylphosphine, and 83.3 μL of water to a suitable container, and mix.

Procedure Add the protein/peptide (between 1 and 100 μg) to a hydrolysis tube, and place in a larger tube. Transfer the *Reducing Solution* to the large tube, seal in vacuum (about 50 μm of mercury or 6.7 Pa), and incubate at about 100°C for 5 minutes. Then remove the inner hydrolysis tube, and dry it in a vacuum desiccator for 15 minutes to remove residual reagents. The pyridylethylated protein/peptide can then be acid hydrolyzed using previously described procedures. The pyridylethylation reaction is performed simultaneously with a protein standard sample containing 1 to 8 mol of cysteine to improve accuracy in the 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 μL of 100 mmol/L iodoacetamide for every 20 nmol of protein present.] The reaction is stopped by adding excess of 2-mercaptoethanol. The protein/peptide is desalted by collecting the protein/peptide fraction from a reversed-phase HPLC separation. The collected sample can be dried in a vacuum centrifuge before acid hydrolysis. The S-carboxyamidomethyl-cysteine formed will be converted to S-carboxymethylcysteine during acid hydrolysis.

Method 10

Cysteine-cystine is reacted with dithiodiglycolic acid or dithiodipropionic acid to produce a mixed disulfide. [Note: The choice of dithiodiglycolic acid or dithiodipropionic acid depends on the required resolution of the amino acid analysis method.]

Reducing Solution A solution containing 10 mg of dithiodiglycolic acid (or dithiodipropionic acid) per mL of 0.2 mol/L sodium hydroxide.

Procedure Transfer about 20 μg of the test sample to a hydrolysis tube, and add 5 μL of the *Reducing Solution*. Add 10 μL of isopropyl alcohol, and then remove all of the sample liquid by vacuum centrifugation. The sample is then hydrolyzed using *Method 1*. This method has the advantage that other amino acid residues are not derivatized by side reactions, and the sample does not need to be desalted prior to hydrolysis.

Method 11

Asparagine and glutamine are converted to aspartic acid and glutamic acid, respectively, during acid hydrolysis. Asparagine and aspartic acid residues are added and represented by *Asx*, while glutamine and glutamic acid residues are added and represented by *Glx*. Proteins/peptides can be reacted with bis(1,1-trifluoroacetoxy)iodobenzene (BTI) to convert the asparagine and glutamine residues to diaminopropionic acid and diaminobutyric acid residues, respectively, upon acid hydrolysis. These conversions allow the analyst to determine the asparagine and glutamine content of a protein/peptide in the presence of aspartic acid and glutamic acid residues.

Reducing Solutions Prepare and filter three solutions: a

solution of 10 mmol/L trifluoroacetic acid (*Solution A*), a solution of 5 mol/L guanidine hydrochloride and 10 mmol/L trifluoroacetic acid (*Solution B*), and a freshly prepared solution of *N,N*-dimethylformamide containing 36 mg of BTI per mL (*Solution C*).

Procedure In a clean hydrolysis tube, transfer about 200 μg of the test sample, and add 2 mL of *Solution A* or *Solution B* and 2 mL of *Solution C*. Seal the hydrolysis tube in vacuum. Heat the sample at 60°C for 4 hours in the dark. The sample is then dialyzed with water to remove the excess reagents. Extract the dialyzed sample three times with equal volumes of *n*-butyl acetate, and then lyophilize. The protein can then be acid hydrolyzed using previously described procedures. The α,β -diaminopropionic and α,γ -diaminobutyric acid residues do not typically resolve from the lysine residues upon ion-exchange chromatography based on amino acid analysis. Therefore, when using ion-exchange as the mode of amino acid separation, the asparagine and glutamine contents are the quantitative difference in the aspartic acid and glutamic acid content assayed with underivatized and BTI-derivatized acid hydrolysis. [Note: The threonine, methionine, cysteine, tyrosine, and histidine assayed content can be altered by BTI derivatization; a hydrolysis without BTI will have to be performed if the analyst is interested in the composition of these other amino acid residues of the protein/peptide.]

Methodologies of Amino Acid Analysis General Principles

Many amino acid analysis techniques exist, and the choice of any one technique often depends on the sensitivity required from the assay. In general, about one-half of the amino acid analysis techniques employed rely on the separation of the free amino acids by ion-exchange chromatography followed by postcolumn derivatization (e.g., with ninhydrin or *o*-phthalaldehyde). Postcolumn detection techniques can be used with samples that contain small amounts of buffer components, such as salts and urea, and generally require between 5 and 10 μg of protein sample per analysis. The remaining amino acid techniques typically involve precolumn derivatization of the free amino acids (e.g., phenyl isothiocyanate; 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate or *o*-phthalaldehyde; (dimethylamino)azobenzenesulfonyl chloride; 9-fluorenylmethylchloroformate; and, 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole) followed by reversed-phase HPLC. Precolumn derivatization techniques are very sensitive and usually require between 0.5 and 1.0 μg of protein sample per analysis but may be influenced by buffer salts in the samples. Precolumn derivatization techniques may also result in multiple derivatives of a given amino acid, which complicates the result interpretation. Postcolumn derivatization techniques are generally influenced less by performance variation of the assay than precolumn derivatization techniques.

The following *Methods* may be used for quantitative amino acid analysis. Instruments and reagents for these procedures are available commercially. Furthermore, many modifications of these methodologies exist with different reagent preparations, reaction procedures, chromatographic systems, etc. Specific parameters may vary according to the exact equipment and procedure used. Many laboratories will utilize more than one amino acid analysis technique to exploit the advantages offered by each. In each of these *Methods*, the analog signal is visualized by means of a data acquisition sys-

tem, and the peak areas are integrated for quantification purposes.

Method 1—Postcolumn Ninhydrin Detection General Principle

Ion-exchange chromatography with postcolumn ninhydrin detection is one of the most common methods employed for quantitative amino acid analysis. As a rule, a Li-based cation-exchange system is employed for the analysis of the more complex physiological samples, and the faster Na-based cation-exchange system is used for the more simplistic amino acid mixtures obtained with protein hydrolysates (typically containing 17 amino acid components). Separation of the amino acids on an ion-exchange column is accomplished through a combination of changes in pH and cation strength. A temperature gradient is often employed to enhance separation.

When the amino acid reacts with ninhydrin, the reactant has characteristic purple or yellow color. Amino acids, except imino acid, give a purple color, and show the maximum absorption at 570 nm. The imino acids such as proline give a yellow color, and show the maximum absorption at 440 nm. The postcolumn reaction between ninhydrin and amino acid eluted from column is monitored at 440 and 570 nm, and the chromatogram obtained is used for the determination of amino acid composition.

Detection limit is considered to be 10 pmol for most of the amino acid derivatives, but 50 pmol for proline. Response linearity is obtained in the range of 20 to 500 pmol with correlation coefficients exceeding 0.999. To obtain good composition data, samples larger than 1 μg before hydrolysis are best suited for this amino acid analysis of protein/peptide.

Method 2—Postcolumn OPA Fluorometric Detection General Principle

o-Phthalaldehyde (OPA) reacts with primary amines in the presence of thiol compound, to form highly fluorescent isoindole products. This reaction is utilized for the postcolumn derivatization in analysis of amino acids by ion-exchange chromatography. The rule of the separation is the same as *Method 1*. Instruments and reagents for this form of amino acid analysis are available commercially. Many modifications of this methodology exist.

Although OPA does not react with secondary amines (imino acids such as proline) to form fluorescent substances, the oxidation with sodium hypochlorite allows secondary amines to react with OPA. The procedure employs a strongly acidic cation-exchange column for separation of free amino acids followed by postcolumn oxidation with sodium hypochlorite and postcolumn derivatization using OPA and thiol compound such as *N*-acetyl-L-cysteine and 2-mercaptoethanol. The derivatization of primary amino acids are not noticeably affected by the continuous supply of sodium hypochlorite.

Separation of the amino acids on an ion-exchange column is accomplished through a combination of changes in pH and cation strength. After postcolumn derivatization of eluted amino acids with OPA, the reactant passes through the fluorometric detector. Fluorescence intensity of OPA-derivatized amino acids are monitored with an excitation wavelength of 348 nm and an emission wavelength of 450 nm.

Detection limit is considered to be a few tens of picomole level for most of the amino acid derivatives. Response lineari-

ty is obtained in the range of a few picomole level to a few tens of nanomole level. To obtain good compositional data, the starting with greater than 500 ng of sample before hydrolysis is best suited for the amino acid analysis of protein/peptide.

Method 3—Precolumn PITC Derivatization General Principle

Phenylisothiocyanate (PITC) reacts with amino acids to form phenylthiocarbamyl (PTC) derivatives which can be detected with high sensitivity at 245 nm. Therefore, precolumn derivatization of amino acids with PITC followed by a reversed-phase HPLC separation with UV detection is used to analyze the amino acid composition.

After the reagent is removed under vacuum, the derivatized amino acids can be stored dry and frozen for several weeks with no significant degradation. If the solution for injection is kept cold, no noticeable loss in chromatographic response occurs after three days.

Separation of the PTC-amino acids on a reversed-phase HPLC with ODS column is accomplished through a combination of changes in concentrations of acetonitrile and buffer ionic strength. PTC-amino acids eluted from column are monitored at 254 nm.

Detection limit is considered to be 1 pmol for most of the amino acid derivatives. Response linearity is obtained in the range of 20 to 500 pmol with correlation coefficients exceeding 0.999. To obtain good compositional data, samples larger than 500 ng of protein/peptide before hydrolysis is best suited for this amino acid analysis of proteins/peptides.

Method 4—Precolumn AQC Derivatization General Principle

Precolumn derivatization of amino acids with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) followed by reversed-phase HPLC separation with fluorometric detection is used.

6-Aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) reacts with amino acids to form stable, fluorescent unsymmetric urea derivatives (AQC-amino acids) which are readily amenable to analysis by reversed-phase HPLC. Therefore, precolumn derivatization of amino acids with AQC followed by reversed-phase HPLC separation is used to analyze the amino acid composition.

Separation of the AQC-amino acids on ODS column is accomplished through a combination of changes in 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 $\mu\text{mol/L}$ 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).

o-Phthalaldehyde (OPA) in conjunction with a thiol reagent reacts with primary amine groups to form highly fluorescent isoindole products. 2-Mercaptoethanol or 3-mercaptopropionic acid can be used as the thiol. OPA itself does not fluoresce and consequently produces no interfering peaks. In addition, its solubility and stability in aqueous solution, along with the rapid kinetics for the reaction, make it amenable to automated derivatization and analysis using an autosampler to mix the sample with the reagent. However, lack of reactivity with secondary amino acids has been predominant drawback. This method does not detect amino acids that exist as secondary amines (e.g., proline). To compensate for this drawback, this technique may be combined with another technique described in *Method 7* or *Method 8*.

Precolumn derivatization of amino acids with OPA is followed by a reversed-phase HPLC separation. Because of the instability of the OPA-amino acid derivative, HPLC separation and analysis are performed immediately following derivatization. The liquid chromatograph is equipped with a fluorometric detector for the detection of derivatized amino acids. Fluorescence intensity of OPA-derivatized amino acids is monitored with an excitation wavelength of 348 nm and an emission wavelength of 450 nm.

Detection limits as low as 50 fmol via fluorescence have been reported, although the practical limit of analysis remains at 1 pmol.

Method 6—Precolumn DABS-Cl Derivatization General Principle

Precolumn derivatization of amino acids with (dimethylamino)azobenzenesulfonyl chloride (DABS-Cl) followed by reversed-phase HPLC separation with visible light detection is used.

(Dimethylamino)azobenzenesulfonyl chloride (DABS-Cl) is a chromophoric reagent employed for the labeling of amino acids. Amino acids labeled with DABS-Cl (DABS-amino acids) are highly stable and show the maximum absorption at 436 nm.

DABS-amino acids, all 19 naturally occurring amino acids derivatives, can be separated on an ODS column of a reversed-phase HPLC by employing gradient systems consisting of acetonitrile and aqueous buffer mixture. Separated DABS-amino acids eluted from column are detected at 436 nm in the visible region.

This *Method* can analyze the imino acids such as proline together with the amino acids at the same degree of sensitivity, DABS-Cl derivatization method permits the simultaneous quantification of tryptophan residues by previous hydrolysis of the protein/peptide with sulfonic acids such as mercaptoethanesulfonic acid, *p*-toluenesulfonic acid or methanesulfonic acid described under *Method 2* in "Protein Hydrolysis". The other acid-labile residues, asparagine and glutamine, can also be analysed by previous conversion into diaminopropionic acid and diamino butyric acid, respectively,

by treatment of protein/peptide with BTI described under *Method 11* in "Protein Hydrolysis".

The non-proteinogenic amino acid, norleucine cannot be used as internal standard in this method, as this compound is eluted in a chromatographic region crowded with peaks of primary amino acids. Nitrotyrosine can be used as an internal standard, because it is eluted in a clean region.

Detection limit of DABS-amino acid is about 1 pmol. As little as 2 to 5 pmol of an individual DABS-amino acid can be quantitatively analysed with reliability, and only 10 to 30 ng of the dabsylated protein hydrolysate is required for each analysis.

Method 7—Precolumn FMOC-Cl Derivatization General Principle

Precolumn derivatization of amino acids with 9-fluorenylmethyl chloroformate (FMOC-Cl) followed by reversed-phase HPLC separation with fluorometric detection is used.

9-Fluorenylmethyl chloroformate (FMOC-Cl) reacts with both primary and secondary amino acids to form highly fluorescent products. The reaction of FMOC-Cl with amino acid proceeds under mild conditions in aqueous solution and is completed in 30 seconds. The derivatives are stable, only the histidine derivative showing any breakdown. Although FMOC-Cl is fluorescent itself, the reagent excess and fluorescent side-products can be eliminated without loss of FMOC-amino acids.

FMOC-amino acids are separated by a reversed-phase HPLC using ODS column. The separation is carried out by gradient elution varied linearly from a mixture of acetonitrile methanol and acetic acid buffer (10:40:50) to a mixture of acetonitrile and acetic acid buffer (50:50), and 20 amino acid derivatives are separated in 20 minutes. Each derivative eluted from column is monitored by a fluorometric detector set at an excitation wavelength of 260 nm and an emission wavelength of 313 nm.

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

7-fluoro-4-nitrobenzo-2-oxa-1.3-diazole (NBD-F) reacts with both primary and secondary amino acids to form highly fluorescent products. Amino acids are derivatized with NBD-F by heating to 60°C for 5 minutes.

NBD-amino acid derivatives are separated on an ODS column of a reversed-phase HPLC by employing gradient elution system consisting of acetonitrile and aqueous buffer mixture, and 17 amino acid derivatives are separated in 35 minutes. ϵ -Aminocaproic acid can be used as an internal standard, because it is eluted in a clean chromatographic region. Each derivative eluted from column is monitored by a fluorometric detector set at an excitation wavelength of 480 nm and an emission wavelength of 530 nm.

The sensitivity of this method is almost the same as for precolumn OPA derivatization method (*Method 5*), excluding proline to which OPA is not reactive, and might be advantageous for NBD-F against OPA. The detection limit for each amino acid is about 10 fmol. Profile analysis was

achieved for about 1.5 μg of protein hydrolysates in the final precolumn labeling reaction mixture for HPLC.

Data Calculation and Analysis

When determining the amino acid content of a protein/peptide hydrolysate, it should be noted that the acid hydrolysis step destroys tryptophan and cysteine. Serine and threonine are partially destroyed by acid hydrolysis, while isoleucine and valine residues may be only partially cleaved. Methionine can undergo oxidation during acid hydrolysis, and some amino acids (e.g., glycine and serine) are common contaminants. Application of adequate vacuum (≤ 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.

2. 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).

3. 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

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

I. Introduction

1. Fundamental measures to ensure viral safety of JP listed biotechnological/biological products
2. Safety assurance measures described in the Official Monograph and this General Information
3. Items and contents described in this General Information

II. General Matters

1. Purpose
2. Background
3. Unknown risk on the measures taken for ensuring viral safety
4. Applicable range
5. Possible viral contamination to a JP listed biotechnological/biological product (source of virus contamination)
6. Basis for ensuring viral safety
7. Limit of virus test
8. Roles of viral clearance studies

III. Raw material/substrate for drug production

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

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

1. Virus test conducted in advance of purification process
2. Virus test as an acceptance test of an intermediate material, etc.
3. Virus test on a final product

V. Process evaluation on viral clearance

1. Rationale, objective and general items to be concerned with respect to viral clearance process evaluation
2. Selection of virus
3. Design of viral clearance studies
4. Interpretation of viral clearance studies
 - 1) Evaluation of viral clearance factor
 - 2) Calculation of viral clearance index
 - 3) Interpretation of results and items to be concerned at evaluation

VI. Statistics

1. Statistical considerations for assessing virus assays
2. Reproducibility and confidence limit of viral clearance studies

VII. Re-evaluation of viral clearance

VIII. Measurement for viral clearance studies

1. Measurement of virus infective titer
2. Testing by nucleic-acid amplification test (NAT)

IX. Reporting and preservation

X. Others

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, 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 attention 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 safe-

ty?”.

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 exist”, 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 natures, 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 exist” or “unknown viruses, which have special physical/chemical properties that can not be matched to any of the currently known viruses, may exist” 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 thereof 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 con-

taminate 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 virus 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 tolerance, 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 material, 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

Insulin, 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.

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		⊙			
Telfan/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	⊙				

(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 chromatography (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 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 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

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 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

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 production. 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 virus not having such membrane. 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 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
- V₁: Sample volume of the unprocessed material
- T₁: Virus amount (titer) of the unprocessed material
- V₂: Sample volume of the processed material
- T₂: 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 preparation 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 vial 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 quantal or quantitative. Both quantal 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 estimate 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 considered and the system 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 quantal or quantitative. Quantal 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 at viral clearance studies. 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.

4. Capillary Electrophoresis

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

General Principles

Capillary electrophoresis is a physical method of analysis based on the migration, inside a capillary, of charged analytes dissolved in an electrolyte solution, under the influence of a direct-current electric field.

The migration velocity of an analyte under an electric field of intensity E , is determined by the electrophoretic mobility of the analyte and the electro-osmotic mobility of the buffer inside the capillary. The electrophoretic mobility of a solute (μ_{ep}) depends on the characteristics of the solute (electric charge, molecular size and shape) and those of the buffer in which the migration takes place (type and ionic strength of the electrolyte, pH, viscosity and additives). The electrophoretic velocity (v_{ep}) of a solute, assuming a spherical shape, is given by the equation:

$$v_{ep} = \mu_{ep} E = \left(\frac{q}{6\pi\eta r} \right) \left(\frac{V}{L} \right)$$

- q : effective charge of the solute,
- η : viscosity of the electrolyte solution,
- r : Stoke's radius of the solute,
- V : applied voltage,
- L : total length of the capillary.

When an electric field is applied through the capillary filled with buffer, a flow of solvent is generated inside the capillary, called electro-osmotic flow. The velocity of the electro-osmotic flow depends on the electro-osmotic mobility (μ_{eo}) which in turn depends on the charge density on the capillary internal wall and the buffer characteristics. The electro-osmotic velocity (v_{eo}) is given by the equation:

$$v_{eo} = \mu_{eo} E = \left(\frac{\varepsilon\zeta}{\eta} \right) \left(\frac{V}{L} \right)$$

- ε : dielectric constant of the buffer,
- ζ : zeta potential of the capillary surface.

The velocity of the solute (v) is given by:

$$v = v_{ep} + v_{eo}$$

The electrophoretic mobility of the analyte and the electro-osmotic mobility may act in the same direction or in opposite directions, depending on the charge of the solute. In normal capillary electrophoresis, anions will migrate in the opposite

direction to the electro-osmotic flow and their velocities will be smaller than the electro-osmotic velocity. Cations will migrate in the same direction as the electro-osmotic flow and their velocities will be greater than the electro-osmotic velocity. Under conditions in which there is a fast electro-osmotic velocity with respect to the electrophoretic velocity of the solutes, both cations and anions can be separated in the same run.

The time (t) taken by the solute to migrate the distance (l) from the injection end of the capillary to the detection point (capillary effective length) is given by the expression:

$$t = \frac{l}{v_{ep} + v_{eo}} = \frac{l \times L}{(\mu_{ep} + \mu_{eo}) V}$$

In general, uncoated fused-silica capillaries above pH 3 have negative charge due to ionized silanol groups in the inner wall. Consequently, the electro-osmotic flow is from anode to cathode. The electro-osmotic flow must remain constant from run to run if good reproducibility is to be obtained in the migration velocity of the solutes. For some applications, it may be necessary to reduce or suppress the electro-osmotic flow by modifying the inner wall of the capillary or by changing the concentration, composition and/or pH of the buffer solution.

After the introduction of the sample into the capillary, each analyte ion of the sample migrates within the background electrolyte as an independent zone, according to its electrophoretic mobility. Zone dispersion, that is the spreading of each solute band, results from different phenomena. Under ideal conditions the sole contribution to the solute-zone broadening is molecular diffusion of the solute along the capillary (longitudinal diffusion). In this ideal case the efficiency of the zone, expressed as the number of theoretical plates (N), is given by:

$$N = \frac{(\mu_{ep} + \mu_{eo}) \times V \times L}{2 \times D \times L}$$

- D : molecular diffusion coefficient of the solute in the buffer.

In practice, other phenomena such as heat dissipation, sample adsorption onto the capillary wall, mismatched conductivity between sample and buffer, length of the injection plug, detector cell size and unlevelled buffer reservoirs can also significantly contribute to band dispersion.

Separation between two bands (expressed as the resolution, R_s) can be obtained by modifying the electrophoretic mobility of the analytes, the electro-osmotic mobility induced in the capillary and by increasing the efficiency for the band of each analyte, according to the equation:

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

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

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

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

Apparatus

An apparatus for capillary electrophoresis is composed of:—a high-voltage, controllable direct-current power supply, —two buffer reservoirs, held at the same level, containing the prescribed anodic and cathodic solutions,

- two electrode assemblies (the cathode and the anode), immersed in the buffer reservoirs and connected to the power supply,
- a separation capillary (usually made of fused-silica) which, when used with some specific types of detectors, has an optical viewing window aligned with the detector. The ends of the capillary are placed in the buffer reservoirs. The capillary is filled with the solution prescribed in the monograph,
- a suitable injection system,
- a detector able to monitor the amount of substances of interest passing through a segment of the separation capillary at a given time. It is usually based on absorption spectrophotometry (UV and visible) or fluorometry, but conductimetric, amperometric or mass spectrometric detection can be useful for specific applications. Indirect detection is an alternative method used to detect non-UV-absorbing and non-fluorescent compounds,
- a thermostatic system able to maintain a constant temperature inside the capillary is recommended to obtain a good separation reproducibility,
- a recorder and a suitable integrator or a computer.

The definition of the injection process and its automation are critical for precise quantitative analysis. Modes of injection include gravity, pressure or vacuum injection and electrokinetic injection. The amount of each sample component introduced electrokinetically depends on its electrophoretic mobility, leading to possible discrimination using this injection mode.

Use the capillary, the buffer solutions, the preconditioning method, the sample solution and the migration conditions prescribed in the monograph of the considered substance. The employed electrolytic solution is filtered to remove particles and degassed to avoid bubble formation that could interfere with the detection system or interrupt the electrical contact in the capillary during the separation run. A rigorous rinsing procedure should be developed for each analytical method to achieve reproducible migration times of the solutes.

1. Capillary Zone Electrophoresis

Principle

In capillary zone electrophoresis, analytes are separated in a capillary containing only buffer without any anticonvective medium. With this technique, separation takes place because the different components of the sample migrate as discrete bands with different velocities. The velocity of each band depends on the electrophoretic mobility of the solute and the electro-osmotic flow in the capillary (see General Principles). Coated capillaries can be used to increase the separation capacity of those substances adsorbing on fused-silica surfaces.

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

Optimization

Optimization of the separation is a complex process where several separation parameters can play a major role. The

main factors to be considered in the development of separations are instrumental and electrolytic solution parameters.

Instrumental parameters

Voltage: A Joule heating plot is useful in optimizing the applied voltage and column temperature. Separation time is inversely proportional to applied voltage. However, an increase in the voltage used can cause excessive heat production, giving rise to temperature and, as a result thereof, viscosity gradients in the buffer inside the capillary. This effect causes band broadening and decreases resolution.

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

Temperature: The main effect of temperature is observed on buffer viscosity and electrical conductivity, and therefore on migration velocity. In some cases, an increase in capillary temperature can cause a conformational change in proteins, modifying their migration time and the efficiency of the separation.

Capillary: The dimensions of the capillary (length and internal diameter) contribute to analysis time, efficiency of separations and load capacity. Increasing both effective length and total length can decrease the electric fields (working at constant voltage) which increases migration time. For a given buffer and electric field, heat dissipation, and hence sample band-broadening, depend on the internal diameter of the capillary. The latter also affects the detection limit, depending on the sample volume injected and the detection system employed.

Since the adsorption of the sample components on the capillary wall limits efficiency, methods to avoid these interactions should be considered in the development of a separation method. In the specific case of proteins, several strategies have been devised to avoid adsorption on the capillary wall. Some of these strategies (use of extreme pH and adsorption of positively charged buffer additives) only require modification of the buffer composition to prevent protein adsorption. In other strategies, the internal wall of the capillary is coated with a polymer, covalently bonded to the silica, that prevents interaction between the proteins and the negatively charged silica surface. For this purpose, ready-to-use capillaries with coatings consisting of neutral-hydrophilic, cationic and anionic polymers are available.

Electrolytic solution parameters

Buffer type and concentration: Suitable buffers for capillary electrophoresis have an appropriate buffer capacity in the pH range of choice and low mobility to minimize current generation.

Matching buffer-ion mobility to solute mobility, whenever possible, is important for minimizing band distortion. The type of sample solvent used is also important to achieve on-column sample focusing, which increases separation efficiency and improves detection.

An increase in buffer concentration (for a given pH) decreases electro-osmotic flow and solute velocity.

Buffer pH: The pH of the buffer can affect separation by modifying the charge of the analyte or additives, and by changing the electro-osmotic flow. In protein and peptide separation, changing the pH of the buffer from above to below the isoelectric point (pI) changes the net charge of the

solute from negative to positive. An increase in the buffer pH generally increases the electro-osmotic flow.

Organic solvents: Organic modifiers (methanol, acetonitrile, etc.) may be added to the aqueous buffer to increase the solubility of the solute or other additives and/or to affect the degree of ionization of the sample components. The addition of these organic modifiers to the buffer generally causes a decrease in the electro-osmotic flow.

Additives for chiral separations: For the separation of optical isomers, a chiral selector is added to the separation buffer. The most commonly used chiral selectors are cyclodextrins, but crown ethers, polysaccharides and proteins may also be used. Since chiral recognition is governed by the different interactions between the chiral selector and each of the enantiomers, the resolution achieved for the chiral compounds depends largely on the type of chiral selector used. In this regard, for the development of a given separation it may be useful to test cyclodextrins having a different cavity size (α -, β -, or γ -cyclodextrin) or modified cyclodextrins with neutral (methyl, ethyl, hydroxyalkyl, etc.) or ionizable (aminomethyl, carboxymethyl, sulfobutyl ether, etc.) groups. When using modified cyclodextrins, batch-to-batch variations in the degree of substitution of the cyclodextrins must be taken into account since it will influence the selectivity. Other factors controlling the resolution in chiral separations are concentration of chiral selector, composition and pH of the buffer and temperature. The use of organic additives, such as methanol or urea can also modify the resolution achieved.

2. Capillary Gel Electrophoresis

Principle

In capillary gel electrophoresis, separation takes place inside a capillary filled with a gel that acts as a molecular sieve. Molecules with similar charge-to-mass ratios are separated according to molecular size since smaller molecules move more freely through the network of the gel and therefore migrate faster than larger molecules. Different biological macromolecules (for example, proteins and DNA fragments), which often have similar charge-to-mass ratios, can thus be separated according to their molecular mass by capillary gel electrophoresis.

Characteristics of Gels

Two types of gels are used in capillary electrophoresis: permanently coated gels and dynamically coated gels. Permanently coated gels, such as cross-linked polyacrylamide, are prepared inside the capillary by polymerization of the monomers. They are usually bonded to the fused-silica wall and cannot be removed without destroying the capillary. If the gels are used for protein analysis under reducing conditions, the separation buffer usually contains sodium dodecyl sulfate and the samples are denatured by heating a mixture of sodium dodecyl sulfate and 2-mercaptoethanol or dithiothreitol before injection. When non-reducing conditions are used (for example, analysis of an intact antibody), 2-mercaptoethanol and dithiothreitol are not used. Separation in cross-linked gels can be optimized by modifying the separation buffer (as indicated in the free solution capillary electrophoresis section) and controlling the gel porosity during the gel preparation. For cross-linked polyacrylamide gels, the porosity can be modified by changing the concentration of acrylamide and/or the proportion of cross-linker. As a rule, a decrease in the porosity of the gel leads to a decrease

in the mobility of the solutes. Due to the rigidity of these gels, only electrokinetic injection can be used.

Dynamically coated gels are hydrophilic polymers, such as linear polyacrylamide, cellulose derivatives, dextran, etc., which can be dissolved in aqueous separation buffers giving rise to a separation medium that also acts as a molecular sieve. These separation media are easier to prepare than cross-linked polymers. They can be prepared in a vial and filled by pressure in a wall-coated capillary (with no electro-osmotic flow). Replacing the gel before every injection generally improves the separation reproducibility. The porosity of the gels can be increased by using polymers of higher molecular mass (at a given polymer concentration) or by decreasing the polymer concentration (for a given polymer molecular mass). A reduction in the gel porosity leads to a decrease in the mobility of the solute for the same buffer. Since the dissolution of these polymers in the buffer gives low viscosity solutions, both hydrodynamic and electrokinetic injection techniques can be used.

3. Capillary Isoelectric Focusing

Principle

In isoelectric focusing, the molecules migrate under the influence of the electric field, so long as they are charged, in a pH gradient generated by ampholytes having pI values in a wide range (poly-aminocarboxylic acids), dissolved in the separation buffer.

The three basic steps of isoelectric focusing are loading, focusing and mobilization.

Loading step: Two methods may be employed:

- loading in one step: the sample is mixed with ampholytes and introduced into the capillary either by pressure or vacuum;
- sequential loading: a leading buffer, then the ampholytes, then the sample mixed with ampholytes, again ampholytes alone and finally the terminating buffer are introduced into the capillary. The volume of the sample must be small enough not to modify the pH gradient.

Focusing step: When the voltage is applied, ampholytes migrate toward the cathode or the anode, according to their net charge, thus creating a pH gradient from anode (lower pH) to cathode (higher pH). During this step the components to be separated migrate until they reach a pH corresponding to their isoelectric point (pI) and the current drops to very low values.

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

- in the first method, mobilization is accomplished during the focusing step under the effect of the electro-osmotic flow; the electro-osmotic flow must be small enough to allow the focusing of the components;
- in the second method, mobilization is accomplished by applying positive pressure after the focusing step;
- in the third method, mobilization is achieved after the focusing step by adding salts to the cathode reservoir or the anode reservoir (depending on the direction chosen for mobilization) in order to alter the pH in the capillary when the voltage is applied. As the pH is changed, the proteins and ampholytes are mobilized in the direction of the reservoir which contains the added salts and pass the detector.

The separation achieved, expressed as ΔpI , depends on the

pH gradient (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:

Voltage: Capillary isoelectric focusing utilises very high electric fields, 300 V/cm to 1000 V/cm in the focusing step.

Capillary: The electro-osmotic flow must be reduced or suppressed depending on the mobilization strategy (see above). Coated capillaries tend to reduce the electro-osmotic flow.

Solutions: The anode buffer reservoir is filled with a solution with a pH lower than the pI of the most acidic ampholyte and the cathode reservoir is filled with a solution with a pH higher than the pI of the most basic ampholyte. Phosphoric acid for the anode and sodium hydroxide for the cathode are frequently used.

Addition of a polymer, such as methylcellulose, in the ampholyte solution tends to suppress convective forces (if any) and electro-osmotic flow by increasing the viscosity. Commercial ampholytes are available covering many pH ranges and may be mixed if necessary to obtain an expanded pH range. Broad pH ranges are used to estimate the isoelectric point whereas narrower ranges are employed to improve accuracy. Calibration can be done by correlating migration time with isoelectric point for a series of protein markers.

During the focusing step precipitation of proteins at their isoelectric point can be prevented, if necessary, using buffer additives such as glycerol, surfactants, urea or zwitterionic buffers. However, depending on the concentration, urea denatures proteins.

4. Micellar Electrokinetic Chromatography (MEKC)

Principle

In micellar electrokinetic chromatography, separation takes place in an electrolyte solution which contains a surfactant at a concentration above the critical micellar concentration (cmc). The solute molecules are distributed between the aqueous buffer and the pseudo-stationary phase composed of micelles, according to the partition coefficient of the solute. The technique can therefore be considered as a hybrid of electrophoresis and chromatography. It is a technique that can be used for the separation of both neutral and charged solutes, maintaining the efficiency, speed and instrumental suitability of capillary electrophoresis. One of the most widely used surfactants in MEKC is the anionic surfactant sodium dodecyl sulfate, although other surfactants, for example cationic surfactants such as cetyltrimethylammonium salts, are also used.

The separation mechanism is as follows. At neutral and alkaline pH, a strong electro-osmotic flow is generated and moves the separation buffer ions in the direction of the cathode. If sodium dodecyl sulfate is employed as the surfactant, the electrophoretic migration of the anionic micelle is in the opposite direction, towards the anode. As a result, the overall micelle migration velocity is slowed down compared to the bulk flow of the electrolytic solution. In the case of neutral solutes, since the analyte can partition between the

micelle and the aqueous buffer, and has no electrophoretic mobility, the analyte migration velocity will depend only on the partition coefficient between the micelle and the aqueous buffer. In the electropherogram, the peaks corresponding to each uncharged solute are always between that of the electro-osmotic flow marker and that of the micelle (the time elapsed between these two peaks is called the separation window). For electrically charged solutes, the migration velocity depends on both the partition coefficient of the solute between the micelle and the aqueous buffer, and on the electrophoretic mobility of the solute in the absence of micelle.

Since the mechanism in MEKC of neutral and weakly ionized solutes is essentially chromatographic, migration of the solute and resolution can be rationalized in terms of the retention factor of the solute (k'), also referred to as mass distribution ratio (D_m), which is the ratio of the number of moles of solute in the micelle to those in the mobile phase. For a neutral compound, k' is given by:

$$k' = \frac{t_R - t_0}{t_0 \left(1 - \frac{t_R}{t_{mc}}\right)} = K \frac{V_S}{V_M}$$

t_R : migration time of the solute,

t_0 : analysis time of an unretained solute (determined by injecting an electro-osmotic flow marker which does not enter the micelle, for instance methanol),

t_{mc} : micelle migration time (measured by injecting a micelle marker, such as Sudan III, which migrates while continuously associated in the micelle),

K : partition coefficient of the solute,

V_S : volume of the micellar phase,

V_M : volume of the mobile phase.

Likewise, the resolution between two closely-migrating solutes (R_S) is given by:

$$R_S = \frac{\sqrt{N}}{4} \times \frac{\alpha - 1}{\alpha} \times \frac{k'_b}{k'_b + 1} \times \frac{1 - \left(\frac{t_0}{t_{mc}}\right)}{1 + \left(\frac{t_0}{t_{mc}}\right)k'_a}$$

N : number of theoretical plates for one of the solutes,

α : selectivity,

k'_a and k'_b : retention factors for both solutes, respectively ($k'_b > k'_a$).

Similar, but not identical, equations give k' and R_S values for electrically charged solutes.

Optimization

The main parameters to be considered in the development of separations by MEKC are instrumental and electrolytic solution parameters.

Instrumental parameters

Voltage: Separation time is inversely proportional to applied voltage. However, an increase in voltage can cause excessive heat production that gives rise to temperature gradients and viscosity gradients of the buffer in the cross-section of the capillary. This effect can be significant with high conductivity buffers such as those containing micelles. Poor heat dissipation causes band broadening and decreases resolution.

Temperature: Variations in capillary temperature affect the partition coefficient of the solute between the buffer and the micelles, the critical micellar concentration and the

viscosity of the buffer. These parameters contribute to the migration time of the solutes. The use of a good cooling system improves the reproducibility of the migration time for the solutes.

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

Electrolytic solution parameters

Surfactant type and concentration: The type of surfactant, in the same way as the stationary phase in chromatography, affects the resolution since it modifies separation selectivity. Also, the $\log k'$ of a neutral compound increases linearly with the concentration of surfactant in the mobile phase. Since resolution in MEKC reaches a maximum when k' approaches the value of $\sqrt{t_m/t_0}$, modifying the concentration of surfactant in the mobile phase changes the resolution obtained.

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

Organic solvents: To improve MEKC separation of hydrophobic compounds, organic modifiers (methanol, propanol, acetonitrile, etc.) can be added to the electrolytic solution. The addition of these modifiers usually decreases migration time and the selectivity of the separation. Since the addition of organic modifiers affects the critical micellar concentration, a given surfactant concentration can be used only within a certain percentage of organic modifier before the micellization is inhibited or adversely affected, resulting in the absence of micelles and, therefore, in the absence of partition. The dissociation of micelles in the presence of a high content of organic solvent does not always mean that the separation will no longer be possible; in some cases the hydrophobic interaction between the ionic surfactant monomer and the neutral solutes forms solvophobic complexes that can be separated electrophoretically.

Additives for chiral separations: For the separation of enantiomers using MEKC, a chiral selector is included in the micellar system, either covalently bound to the surfactant or added to the micellar separation electrolyte. Micelles that have a moiety with chiral discrimination properties include salts of *N*-dodecanoyl-L-amino acids, bile salts, etc. Chiral resolution can also be achieved using chiral discriminators, such as cyclodextrins, added to the electrolytic solutions which contain micellized achiral surfactants.

Other additives: Several strategies can be carried out to modify selectivity, by adding chemicals to the buffer. The addition of several types of cyclodextrins to the buffer can also be used to reduce the interaction of hydrophobic solutes with the micelle, thus increasing the selectivity for this type of compound.

The addition of substances able to modify solute-micelle interactions by adsorption on the latter, is used to improve the selectivity of the separations in MEKC. These additives

may be a second surfactant (ionic or non-ionic) which gives rise to mixed micelles or metallic cations which dissolve in the micelle and form co-ordination complexes with the solutes.

Quantification

Peak areas must be divided by the corresponding migration time to give the corrected area in order to:

- compensate for the shift in migration time from run to run, thus reducing the variation of the response,
- compensate for the different responses of sample constituents with different migration times.

Where an internal standard is used, verify that no peak of the substance to be examined is masked by that of the internal standard.

Calculations

From the values obtained, calculate the content of the component or components being examined. When prescribed, the percentage content of one or more components of the sample to be examined is calculated by determining the corrected area(s) of the peak(s) as a percentage of the total of the corrected areas of all peaks, excluding those due to solvents or any added reagents (normalization procedure). The use of an automatic integration system (integrator or data acquisition and processing system) is recommended.

System Suitability

In order to check the behavior of the capillary electrophoresis system, system suitability parameters are used. The choice of these parameters depends on the mode of capillary electrophoresis used. They are: retention factor (k') (only for micellar electrokinetic chromatography), apparent number of theoretical plates (N), symmetry factor (A_S) and resolution (R_S). In previous sections, the theoretical expressions for N and R_S have been described, but more practical equations that allow these parameters to be calculated from the electropherograms are given below.

Apparent Number of Theoretical Plates

The apparent number of theoretical plates (N) may be calculated using the expression:

$$N = 5.54 \left(\frac{t_R}{w_h} \right)^2$$

t_R : migration time or distance along the baseline from the point of injection to the perpendicular dropped from the maximum of the peak corresponding to the component,

w_h : width of the peak at half-height.

Resolution

The resolution (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.

5. 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 minimum pyrogenic dose of endotoxin per kg body mass (EU/kg), and M is equal to the maximum dose of product per kg per hour.

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.

6. 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.

- 1) The antimicrobial spectrum
- 2) Action time for killing microorganisms
- 3) Action durability
- 4) Effect of the presence of proteins
- 5) Influence on the human body
- 6) Solubility in water
- 7) Influence on the object to be disinfected
- 8) Odor
- 9) Convenience of use
- 10) Easy disposability
- 11) Influence on the environment at disposal
- 12) 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 μm or smaller is used for the sterilization. However, in this method, a filter with a pore size of 0.45 μm or smaller is permitted to be used.

7. Guideline for Residual Solvents, Residual Solvents Test, and Models for the Test in Monographs

1. Guideline for Residual Solvents

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

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 should not exceed the limits, except for in a special case. Accordingly, pharmaceutical manufacturers should assure the quality of their products by performing the test with the products according to the Residual Solvents Test, to keep the limits recommended in the Guideline.

2. Residual Solvents Test

Method—Perform the test as directed in the Residual Solvents Test under the General Tests, Processes and Apparatus.

International harmonization—The test may also be performed according to the Residual solvents in the EP or the Organic volatile impurities in the USP. Even in this case, Monographs should be described in the JP style.

3. Models for the Test in Monographs

The following are typical examples for the test in Monographs, but these do not necessarily imply that other suitable operating conditions can not be used. It is important to prepare (draft) monographs according to the Guideline for the Preparation of the Japanese Pharmacopoeia.

1) A model for test item, amounts of test sample and reference standard (reference substance), preparation of the sample solution and the standard solution, injection amount for gas chromatography, calculation formula, and preparation of the internal standard solution

Residual solvents (or name of the solvent)—Weigh accurately about 0.200 g of $\triangle\triangle\triangle$ (name of the substance to be tested), add exactly 5 mL of the internal standard solution to dissolve, add water to make exactly 20 mL, and use this solution as the sample solution. If necessary filter or centrifuge. Separately, weigh exactly 0.10 g of $\circ\circ\circ$ reference substance (name of the solvent), put in a vessel containing 50 mL of water, and add water to make exactly 100 mL. Pipet 5 mL of this solution, and add water to make exactly 100 mL. Pipet 2 mL of this solution, add exactly 5 mL of the internal standard solution and 20 mL of water, and use this solution as the standard solution. Perform the test with 1 μ L each of the sample solution and standard solution as directed (in the head-space method) under Gas Chromatography according to the following conditions, and calculate the ratios, Q_T and Q_S , of the peak area of $\triangle\triangle\triangle$ (name of the substance to be tested) to that of the internal standard of each solution, respectively. The amount of $\triangle\triangle\triangle$ should be not more than $\times \times$ ppm.

$$\begin{aligned} & \text{Amount of } \circ\circ\circ \text{ (}\mu\text{g)} \\ & = \text{amount of } \circ\circ\circ \text{ reference substance (}\mu\text{g)} \times \frac{Q_T}{Q_S} \end{aligned}$$

Internal standard solution—A solution of $\triangle\triangle\triangle$ in $\nabla\nabla\nabla$ (name of solvent) (1 in 1000).

2) Models for operating conditions for a head-space sample injection device

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

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

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) Models for operating conditions and system suitability

In the operating conditions, generally, items required for the test such as detector, column, column 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:

Operating conditions—

Detector: Specify in the following manner: “Hydrogen flame-ionization detector”.

Column: Specify the inside diameter, length, material of column, name of stationary phase liquid and thickness of stationary phase in the following manner: “Coat the inside wall of a fused silica tube, 0.3 mm in inside diameter and 30 m in length, to 0.25 μ m thickness with polyethylene glycol 20M.” Describe the inside diameter and length of the column, and thickness or particle size of the stationary phase based on the data obtained for validation of the test method.

Column temperature: Specify in the following manner: “A constant temperature of about \times °C.” or “Maintain at 40°C for 20 minutes, then increase to 240°C at 10°C per minute, and keep at 240°C for 20 minutes.”

Carrier gas: Specify in the following manner: “Helium”.

Flow rate: Specify in the following manner: “Adjust the flow rate so that the retention time of $\blacktriangle\blacktriangle\blacktriangle$ is about \times minutes.” or “35 cm/second”. Describe the flow rate based on the data obtained for validation of the test method.

Time span of measurement: About \times times as long as the

retention time of $\triangle\triangle\triangle$ beginning after the air peak.

System suitability—

Test for required detectability: Specify in the following manner: "Measure exactly \times mL of the standard solution, and add $\square\square\square$ to make exactly \times mL. Confirm that the peak area of $\square\square\square$ obtained from \times μ L of this solution is equivalent to \times to $\times\times\%$ of that of $\square\square\square$ obtained from the standard solution". This item should be specified when, in Purity, the amount of an impurity is controlled by comparing the area of a specific peak from the sample solution with that of the peak of $\times\times\times$ from the standard solution and the system repeatability alone is not sufficient to check the system suitability.

System performance: Specify in the following manner: "Dissolve \times g of $\square\square\square$ and \times g of $\triangle\triangle\triangle$ in \times mL of $\circ\circ$ \circ . When the procedure is run with \times μ L of this solution under the above operating conditions, $\square\square\square$ and $\triangle\triangle\triangle$ are eluted in this order with the resolution between these peaks being not less than \times , and the number of theoretical plates and the symmetry factor of the peak of $\square\square\square$ are not less than $\times\times$ steps and not more than $\times\times\times$, respectively." This item should be specified in all of the test methods. Generally, the order of elution and the resolution, and in case of need (such as when the peak is asymmetrical) the symmetry factor, should be specified. The order of elution and the resolution may be replaced by the resolution and the number of theoretical plates. When no suitable reference substance is available to check separation, the number of theoretical plates and the symmetry factor of the peak of the substance to be tested may be specified.

System repeatability: Specify in the following manner: "When the test is repeated \times times with \times μ L of the solution for the system suitability test under the above operating conditions, the relative standard deviation of the peak areas of $\triangle\triangle\triangle$ is not more than $\times\%$." The system repeatability should be specified in any case, except in a qualitative test.

Examples of operating conditions and system suitability

Test conditions (1)

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 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%.

Test conditions (2)

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 μ m 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 μ m 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 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%.

Test conditions (3)

Operating conditions—

Detector: Hydrogen flame-ionization detector

Column: Coat the inside wall of a fused silica tube, 0.32 mm in inside diameter and 30 m in length, to 0.25 μ m 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%.

8. International Harmonization Implemented in the Japanese Pharmacopoeia Fifteenth 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 Fifteenth Edition (JP 15). 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 15 shows the items as they appear in JP 15. In the Remarks column, notes on any differences between JP 15 and the agreement are shown as occasion demands.

The date on which the agreement has been signed is shown on the top of each table. In the case where the harmonized items have been revised, this is indicated in parenthesis.

Oct. 2004 (Rev. 2)

Harmonized items	JP 15	Remarks
Residue on Ignition/Sulphated Ash Test (Introduction) Procedure	2.44 Residue on Ignition Test (Introduction) Procedure	JP's particular description: Explanation of the description in monograph, etc.

Nov. 2003

Harmonized items	JP 15	Remarks
Specific Surface Area (Introduction) Multi-point measurement Single-point measurement Sample preparation Outgassing Adsorbate Quantity of sample Method 1: The dynamic flow method Method 2: The volumetric method Reference materials Figure 1 Schematic diagram of the dynamic flow method apparatus Figure 2 Schematic diagram of the volumetric method apparatus	3.02 Specific Surface Area (Introduction) Multi-point measurement Single-point measurement Sample preparation Method 1: The dynamic flow method Method 2: The volumetric method Reference materials Fig. 3.02-1 Schematic diagram of the dynamic flow method apparatus Fig. 3.02-2 Schematic diagram of the volumetric method apparatus	JP's particular description: Explanation on this test.

June 2004

Harmonized items	JP 15	Remarks
(Introduction)	3.04 Particle Size Determination (Introduction)	JP's particular description: Explanation on this test.
Optical microscopy	Method 1. Optical microscopy	JP's particular description: Explanation on this test.
Apparatus	Apparatus	
Adjustment	Adjustment	
Illumination	Illumination	
Visual characterization	Visual characterization	JP's particular description: Explanation on the method of particle size measurement.
Photographic characterization	Photographic characterization	
Preparation of the mount	Preparation of the mount	
Crystallinity characterization	Crystallinity characterization	
Limit test of particle size by microscopy	Limit test of particle size by microscopy	
Particle size characterization	Particle size characterization	
Particle shape characterization	Particle shape characterization	
General observations	General observations	
Figure 1 Commonly used measurements of particle size	Fig. 3.04-1 Commonly used measurements of particle size	
Figure 2 Commonly used descriptions of particle shape	Fig. 3.04-2 Commonly used descriptions of particle shape	
Analytical sieving	Method 2. Analytical sieving method	JP's particular description: Explanation on this method.
Principles of analytical sieving	Principles of analytical sieving	
Test sieves	Test sieves	
Test specimen	Test specimen	
Agitation methods	Agitation methods	
Endpoint determination	Endpoint determination	
Sieving methods	Sieving methods	JP's particular description: How to treat the fine powder adherent on back surface of the sieve.
1) Methanical agitation dry sieving method	Methanical agitation Dry sieving method	
2) Air entrainment methods air jet and sonic sifter sieving	Air entrainment methods Air jet and sonic shifter sieving	
Table 1 Size of standard sieve series in range of interest	Table 3.04-1 Size of standard sieve series in range of interest	
Interpretation	Interpretation	

Jan. 2000

Harmonized items	JP 15	Remarks
<p>Bacterial Endotoxins Test</p> <p>(Introduction)</p> <p>Apparatus</p> <p>Preparation of standard endotoxin stock solution</p> <p>Preparation of standard endotoxin solution</p> <p>Preparation of sample solutions</p> <p>Determination of maximum valid dilution</p> <p>Gel-clot technique</p> <p>(1) Preparatory testing</p> <p>(i) Test for confirmation of labeled lysate sensitivity</p> <p>(ii) Test for interfering factors</p> <p>(2) Limit test</p> <p>(i) Procedure</p> <p>(ii) Interpretation</p> <p>(3) Assay</p> <p>(i) Procedure</p> <p>(ii) Calculation and interpretation</p> <p>Photometric techniques</p> <p>(1) Turbidimetric techniques</p> <p>(2) Chromogenic technique</p> <p>(3) Preparatory testing</p> <p>(i) Assurance of criteria for the standard curve</p> <p>(ii) Test for interfering factors</p> <p>(4) Assay</p> <p>(i) Procedure</p> <p>(ii) Calculation</p> <p>(iii) Interpretation</p>	<p>4.01 Bacterial Endotoxins Test</p> <p>(Introduction)</p> <p>Apparatus</p> <p>Preparation of standard endotoxin stock solution</p> <p>Preparation of standard endotoxin solution</p> <p>Preparation of sample solutions</p> <p>Determination of maximum valid dilution</p> <p>Gel-clot technique</p> <p>(1) Preparatory testing</p> <p>(i) Test for confirmation of labeled lysate sensitivity</p> <p>(ii) Test for interfering factors</p> <p>(2) Limit test</p> <p>(i) Procedure</p> <p>(ii) Interpretation</p> <p>(3) Assay</p> <p>(i) Procedure</p> <p>(ii) Calculation and interpretation</p> <p>Photometric techniques</p> <p>(1) Turbidimetric techniques</p> <p>(2) Chromogenic technique</p> <p>(3) Preparatory testing</p> <p>(i) Assurance of criteria for the standard curve</p> <p>(ii) Test for interfering factors</p> <p>(4) Assay</p> <p>(i) Procedure</p> <p>(ii) Calculation</p> <p>(iii) Interpretation</p>	<p>JP's particular description: Endotoxin 10000 Reference Standard and Endotoxin 100 Reference Standard.</p> <p>JP's particular description: Addition of the preparation of sample solutions for containers, and deletion of that for medical devices.</p> <p>JP's particular description: Addition of the concentration unit of sample solutions in the case where the endotoxin limit per equivalent is specified.</p> <p>JP's particular description: The test for assurance of criteria for the standard curve must be carried out for each lot of lysate reagent.</p> <p>JP's particular description: Two conditions which the test must meet are specified. Explanation of the test method where the interfering action is found. Explanation of the usual methods for eliminating the interference.</p> <p>JP's particular description: Addition of the alternative requirement to which solution D must meet for valid test.</p>

Regents, test solutions	Deletion
Amebocyte lysate	Deletion
Lysate TS	Deletion
Water for bacterial endotoxins test (BET)	Deletion

Oct. 2002

Harmonized items	JP 15	Remarks
Sterility (Introduction) Precautions against microbial contamination Culture media and incubation temperatures Fluid thioglycollate medium Soya-bean casein digest medium Sterility Growth promotion test of aerobes, anaerobes and fungi Validation test Membrane filtration Direct inoculation Test for sterility of the product to be examined Membrane filtration Aqueous solutions Soluble solids Oils and oily solutions Ointments and creams Direct inoculation of the culture medium Oily liquids Ointments and creams Catgut and other surgical sutures for veterinary use 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 Table 2.6.1.-1. Strains of the test micro-organisms suitable for use in the Growth Promotion Test and the Validation Test Table 2.6.1.-2. Minimum quantity to be used for each medium	4.06 Sterility Test (Introduction) Media and rinsing fluids Fluid thioglycollate medium Soybean-casein digest medium Sterility of media Growth promotion test Validation test Membrane filtration Direct inoculation Test for sterility of the products to be examined Membrane filtration a) Liquid medicines b) Solid medicines c) Oils and oily solutions d) Ointments and creams Direct inoculation of the culture medium a) Oily liquids b) Ointments and creams Cultivation and observation, Observation and interpretation of results Table 4.06-1. Microorganisms for growth promotion test and the validation test Table 4.06-3. Minimum quantity to be used for each medium	 The same statement appears in the introduction. Note 1 – 5 Note 6 Note 7 The items not included in JP. Note 8 – 9 A part of this is directed in the General Test.

Table 2.6.1.-3. Minimum number of items to be tested	Table 4.06-2. Number of items to be taken from the lot	Note 10 – 11
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- Note: 1) Non-pharmaceutical media: Not to be used.
 2) Water content of agar: Not being specified.
 3) Effective period of media: Unnecessary to be validated.
 4) Effective period of media stored in hermetic containers: Usable for maximum one year.
 5) Medium for sterility test of the products containing a mercurial preservative: Specified.
 6) Periodic growth promotion test for a ready-made powder medium: Specified.
 7) Amount of the rinsing fluid each time in the membrane-filtration method: 100 mL per filter.
 8) Transferring amount from turbid medium to fresh medium: A suitable amount.
 9) Requirements for the retesting in the case when the evidence of microbial growth is found: Specified.
 10) The table of 'Number of items to be taken from the lot': Specified as a part of the General Test.
 11) Number of large-volume products (more than 100 mL) to be taken from the lot: Maximum 10 containers.

Feb. 2004

Harmonized items	JP 15	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	Content uniformity	
Solid dosage forms	Solid dosage forms	
Liquid dosage forms	Liquid dosage forms	
Calculation of acceptance value	Calculation of acceptance value	
Mass variation	Mass variation	JP's particular description: Assuming that the concentration of drug substance is uniform in each lot.
Uncoated or film-coated tablets	Uncoated or film-coated tablets	
Hard capsules	Hard capsules	
Soft capsules	Soft capsules	
Solid dosage forms other than tablets and capsules	Solid dosage forms other than tablets and capsules	
Liquid dosage forms	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	Calculation of acceptance value	
Criteria	Criteria	
Solid and liquid dosage forms	Solid and liquid dosage forms	
Table 1	Table 6.02-1	JP's particular description: Addition of "(divided forms, lyophilized forms)" and "(true solution)".
Application of content uniformity (CU) and mass variation (MV) test for dosage forms	Application of content uniformity (CU) and mass variation (MV) test for dosage forms	
Table 2	Table 6.02-2	The phrases "at time of manufacture" and "For purposes of this Pharmacopoeia" are deleted

June 2004 (Rev.1)

Harmonized items	JP 15	Remarks
Test for Extractable Volume of Parenteral Preparations (Introduction)	6.05 Test for Extractable Volume of Parenteral Preparations (Introduction)	JP's particular description: Explanation of this Test
Single-dose containers	(1) Single-dose containers	
Multi-dose containers	(2) Multi-dose containers	
Cartridges and prefilled syringes	(3) Cartridges and prefilled syringes	
Parenteral infusions	(4) Parenteral infusions	

June 2004 (Rev.1)

Harmonized items	JP 15	Remarks
Particulate Matter in Injectables (Introduction)	6.07 Insoluble Particulate Matter Test for Injections (Introduction)	The phrase "other than gas bubbles" is deleted.
Method 1. Light obscuration particle count test	Method 1. Light obscuration particle count test	JP's particular description: Description of evaluation frequency.
	Apparatus	JP's particular description
	Calibration	JP's particular description
	Manual methods	JP's particular description
	Electronic methods	JP's particular description
	Automated methods	JP's particular description
	Sample volume accuracy	JP's particular description
	Sample flow rate	JP's particular description
	Sensor	JP's particular description
	Particle resolution	JP's particular description
	Particle counting accuracy	JP's particular description
	Threshold accuracy	JP's particular description
	Reagents	JP's particular description
General precautions	General precautions	
Method	Method	
Evaluation	Evaluation	JP's particular description: "equal to or more than 100 mL".
Method 2. Microscopic particle count test	Method 2. Microscopic particle count test	
	Apparatus	
General precautions	General precautions	
Method	Method	
Evaluation	Evaluation	JP's particular description: "equal to or more than 100 mL".
1. Circular diameter graticule	Fig. 6.07-1 Circular diameter graticule	

June 2004

Harmonized items	JP 15	Remarks
Disintegration	6.09 Disintegration	JP's particular description: This test is applied also to Granules and Pills.
Apparatus	Apparatus	
Basket-rack assembly	Basket-rack assembly	JP's particular description: The apparatus may be varied somewhat provided the specifications.
Disk	Disk	
	Auxiliary tube	JP's particular description
Procedure	Procedure	JP's particular description: Making use of water also as the test fluid. Setting each intervals of the immersion. Making a definition of complete disintegration. Setting a procedure for Granules.
	(1) Immediate-release preparation	JP's particular description: Setting a test for Granules and Pills.
	(2) Enteric coated preparations	JP's particular description: Setting a test for enteric coated preparations.
Figure 1 Disintegration apparatus	Fig. 6.09-1 Disintegration apparatus Fig. 6.09-2 Auxiliary tube	JP's particular description.

June 2004

Harmonized items	JP 15	Remarks
Dissolution	6.10 Dissolution Test	JP's particular description: The test also aims at preventing significant bioinequivalence.
Apparatus	Apparatus	
Apparatus 1 (Basket apparatus)	Apparatus for Basket Method (Apparatus 1)	
Apparatus 2 (Paddle apparatus)	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)		
Apparatus 4 (Flow-through cell)	Apparatus for Flow-Through Cell Method (Apparatus 3)	JP's particular description: A pump without the pulsation may also be used.
Procedure	Procedure	
Apparatus 1 or 2	Basket Method or Paddle Method	
Immediate-release dosage forms	Immediate-release dosage forms	
Procedure	Procedure	
Dissolution medium	Dissolution medium	
Time	Time	
Extended-release dosage forms	Extended-release dosage forms	
Procedure	Procedure	
Dissolution medium	Dissolution medium	
Time	Time	

Delayed-release dosage forms Procedure Method A Method B Time	Delayed-release dosage forms Procedure Dissolution medium Time not specified	In the Harmonized text: Alternative usage of Method A or B. JP's particular description. JP's particular description: Time is specified each for the 1st and 2nd fluids.
Apparatus 3 Immediate-release dosage forms Procedure Dissolution medium Time Extended-release dosage forms Procedure Dissolution medium Time Delayed-release dosage forms Procedure Time		
Apparatus 4 Immediate-release dosage forms Procedure Dissolution medium Time Extended-release dosage forms Procedure Dissolution medium Time Delayed-release dosage forms Procedure Time	Flow-Through Cell Method Immediate-release dosage forms Procedure Dissolution medium Time Extended-release dosage forms Procedure Dissolution medium Time	
Interpretation	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	Immediate-release dosage forms Interpretation 1 Interpretation 2	JP's particular description: Setting Interpretation 2.
Extended-release dosage forms	Extended-release dosage forms Interpretation 1 Interpretation 2	JP's particular description: Setting Interpretation 2.
Delayed-release dosage forms	Delayed-release dosage forms	Non-harmonized items: Different dissolution medium. Deletion of disharmonized part on the value Q.

	<p>Interpretation 1</p> <p>Interpretation 2</p> <p>Acceptance Table 6.10-1</p> <p>Acceptance Table 6.10-2</p> <p>Acceptance Table 6.10-3</p> <p>Acceptance Table 6.10-4</p> <p>Fig. 6.10-1 Apparatus 1. Basket stirring element</p> <p>Fig. 6.10-2 Paddle stirring element</p> <p>Fig. 6.10-2 a Alternative sinker</p> <p>Figure 3 Apparatus not specified</p> <p>Fig. 6.10-3 Apparatus 3 (top) large cell tablets and capsules (bottom) tablet holder for the large cell</p> <p>Fig. 6.10-4 Apparatus 3 (top) small cell tablets and capsules (bottom) tablet holder for the small cell</p>	<p>JP's particular description: Setting Interpretation 2.</p> <p>The value Q is specified in the individual monograph.</p>
<p>Acceptance Table 1</p> <p>Acceptance Table 2</p> <p>Acceptance Table 3</p> <p>Acceptance Table 4</p> <p>Figure 1 Apparatus 1. Basket stirring element</p> <p>Figure 2 Paddle stirring element</p> <p>Figure 2 a Alternative sinker</p> <p>Figure 3 Apparatus</p> <p>Figure 4 Apparatus 4 (top) large cell tablets and capsules (bottom) tablet holder for the large cell</p> <p>Figure 5 Apparatus 4 (top) small cell tablets and capsules (bottom) tablet holder for the small cell</p>		

Sep. 2002 (Rev. 1)

Harmonized items	JP 15	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 15	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 15	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	

July 2003 (Rev. 1)

Harmonized items	JP 15	Remarks
Carboxymethylcellulose Calcium	Carmellose Calcium	
Definition	origin	
Identification A	Identification (1)	
Identification B	Identification (2)	
Identification C	Identification (3)	
Identification D	Identification (4)	
Alkalinity	Purity (1) Alkali	
Loss on drying	Loss on drying	
Residue on ignition	Residue on ignition	
Limit of chloride	Purity (2) Chloride	
Limit of sulfate	Purity (3) Sulfate	

Nov. 2003 (Rev. 1)

Harmonized items	JP 15	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) Oxalate	
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 15	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) Oxalate	
Readily carbonisable substances	Purity (5) Readily carbonizable substances	
Aluminium	not specified	
Water	Water	
Sulphated ash	Residue on ignition	
Assay	Assay	

Oct. 2001

Harmonized items	JP 15	Remarks
Croscarmellose Sodium	Definition	
Croscarmellose Sodium	origin	
Identification A	Identification (1)	
B	Identification (2)	
C	Identification (3)	
pH	pH	
Settling volume	Precipitation test	
Degree of substitution	Degree of substitution	
Loss on drying	Residue on ignition	
Loss on drying	Residue on ignition	
Packaging and storage	Containers and storage	

Oct. 2001

Harmonized items	JP 15	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	

Feb. 2003

Harmonized items	JP 15	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 15	Remarks
Saccharin Sodium	Saccharin Sodium Hydrate	
Definition	limits of content	
Identification A, B	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 15	Remarks
Cellacefate	Cellulose Acetate Phthalate	
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 15	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 15	Remarks
Powdered Cellulose	Powdered 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	
Loss on drying	Loss on drying	
Residue on ignition	Residue on ignition	

Feb. 2004 (Rev. 1)

Harmonized items	JP 15	Remarks
Corn Starch	Corn Starch	
Definition	origin	
Identification A	Identification (1)	
Identification B	Identification (2)	
Identification C	Identification (3)	
pH	pH	
Loss on drying	Loss on drying	
Residue on ignition	Residue on ignition	
Limit of iron	Purity (1) Iron	
Limit of oxidizing substances	Purity (2) Oxidizing substances	
Sulfur dioxide determination	Purity (3) Sulfur dioxide	

Feb. 2003 (Rev. 1)

Harmonized items	JP 15	Remarks
Anhydrous Lactose	Anhydrous Lactose	
Definition	origin	
Labeling	labeling of relative quantities of α and β lactose	
Identification	Identification	
Specific rotation	Optical rotation	
Clarity and color of solution	Purity (1) Clarity and color of solution	
Acidity or alkalinity	Purity (2) Acid and alkali	
Protein and light-absorbing impurities	Purity (4) Proteins and light absorbing substances	
Loss on drying	Loss on drying	
Water	Water	
Residue on ignition	Residue on ignition	
Content of alpha and beta anomers	Isomer ratio	

Sep. 2002

Harmonized items	JP 15	Remarks
Lactose Monohydrate	Lactose Hydrate	
Definition	origin	
Identification	Identification	
Specific rotation	Specific rotation	
Clarity and color of solution	Purity (1) Clarity and color of solution	
Acidity or alkalinity	Purity (2) Acid and alkali	
Protein and light-absorbing impurities	Purity (4) Protein and light absorbing substances	
Water	Water	
Residue on ignition	Residue on ignition	

Feb. 2004

Harmonized items	JP 15	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) Acid Purity (4) Related substances Residue on ignition Assay	System suitability is not specified.

Feb. 2004

Harmonized items	JP 15	Remarks
Butyl Parahydroxybenzoate Definition Identification A Appearance of solution Acidity Related substances Sulphated ash Assay	Butyl Parahydroxybenzoate limits of content Identification Purity (1) Clarity and color of solution Purity (2) Acid Purity (4) Related substances Residue on ignition Assay	System suitability is not specified.

Feb. 2004

Harmonized items	JP 15	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) Acid Purity (4) Related substances Residue on ignition Assay	System suitability is not specified.

Feb. 2004

Harmonized items	JP 15	Remarks
Methyl Parahydroxybenzoate Definition Identification A Appearance of solution	Methyl Parahydroxybenzoate limits of content Identification (1) Purity (1) Clarity and color of solution	

Acidity	Purity (2) Acid	System suitability is not specified.
Related substances	Purity (4) Related substances	
Sulphated ash	Residue on ignition	
Assay	Assay	

Oct. 2001

Harmonized items	JP 15	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	

Nov. 2003

Harmonized items	JP 15	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	

July, 2000

Harmonized items	JP 15	Remarks
Benzy Alcohol	Benzy Alcohol	
Definition	limits of the content	
Identification	Identification	
Appearance of solution	Purity (1) Clarity and color of solution	
Refractive index	Refractive index	
Acidity	Purity (2) Acid	
Benzaldehyde and other related substances	Purity (3) Benzaldehyde and other related substances	
Peroxide value	Purity (4) Peroxide value	
Residue on evaporation	Purity (5) Residue on evaporation	
Assay	Assay	

Nov. 2003

Harmonized items	JP 15	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	

Sep. 2002

Harmonized items	JP 15	Remarks
Amino Acid Analysis	General Information Amino Acid Analysis	
Apparatus	Apparatus	
General Precautions	General Precautions	
Reference Standard Material	Reference Standard Material	
Calibration of Instrumentation	Calibration of Instrumentation	
Repeatability	Repeatability	
Sample Preparation	Sample Preparation	
Internal Standards	Internal Standards	

Protein Hydrolysis	Protein Hydrolysis
Method 1	Method 1
Hydrolysis Solution	Hydrolysis Solution
Procedure	Procedure
Method 2	Method 2
Hydrolysis Solution	Hydrolysis Solution
Vapor Phase Hydrolysis	Vapor Phase Hydrolysis
Method 3	Method 3
Hydrolysis Solution	Hydrolysis Solution
Vapor Phase Hydrolysis	Vapor Phase Hydrolysis
Method 4	Method 4
Oxidation Solution	Oxidation Solution
Procedure	Procedure
Method 5	Method 5
Hydrolysis Solution	Hydrolysis Solution
Liquid Phase Hydrolysis	Liquid Phase Hydrolysis
Method 6	Method 6
Hydrolysis Solution	Hydrolysis Solution
Vapor Phase Hydrolysis	Vapor Phase Hydrolysis
Method 7	Method 7
Reducing Solution	Reducing Solution
Procedure	Procedure
Method 8	Method 8
Stock Solutions	Stock Solutions
Reducing Solution	Reducing Solution
Procedure	Procedure
Method 9	Method 9
Stock Solutions	Stock Solutions
Carboxymethylation Solution	Carboxymethylation Solution
Buffer Solution	Buffer Solution
Procedure	Procedure
Method 10	Method 10
Reducing Solution	Reducing Solution
Procedure	Procedure
Method 11	Method 11
Reducing Solutions	Reducing Solutions
Procedure	Procedure
Methodologies of amino acid analysis general principles	Methodologies of Amino Acid Analy- sis General Principles
Method 1-Postcolumn ninhydrin detection general principle	Method 1-Postcolumn Ninhydrin Detection 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 derivatization general principle	Method 5-Precolumn OPA Derivatization 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

Oct. 1999

Harmonized items	JP 15	Remarks
Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)	General Information	
Characteristics of polyacrylamide gels	1. Characteristics of Polyacrylamide Gels	
Denaturing polyacrylamide gel electrophoresis	2. Polyacrylamide Gel Electrophoresis under Denaturing Conditions	
Reducing conditions	1) Reducing conditions	
Non-reducing conditions	2) Non-reducing conditions	
Characteristics of discontinuous buffer system gel electrophoresis	3. Characteristics of Discontinuous Buffer System Gel Electrophoresis	
Preparing vertical discontinuous buffer SDS polyacrylamide gels	4. Preparing Vertical Discontinuous Buffer SDS-Polyacrylamide Gels	
Assembling of the gel moulding cassette	1) Assembling of the gel moulding cassette	
Preparation of the gel	2) Preparation of the gel	
Mounting the gel in the electrophoresis apparatus and electrophoretic separation	3) Mounting the gel in the electrophoresis apparatus and electrophoretic separation	
Detection of protein in gels	5. Detection of Proteins in Gels	
Coomassie staining	1) Coomassie staining	
Silver staining	2) Silver staining	
Drying of stained SDS polyacrylamide gels	6. Drying of Stained SDS-Polyacrylamide Gels	
Molecular-mass determination	7. Molecular-Mass Determination	
Validation of the test	8. Suitability of the Test	
Quantification of impurities	9. Quantification of Impurities	
Reagents, test solutions	Test Solutions	
Blocking solution	Blocking TS	
Coomassie staining solution	Coomassie staining TS	
Destaining solution	Destaining TS	
Developer solution	Developer TS	

Fixing solution	Fixing TS
Silver nitrate reagent	Silver nitrate TS for silver staining
Trichloroacetic acid reagent	Trichloroacetic acid TS for fixing
Table 1 – Preparation of resolving gel	Table 1. Preparation of resolving gel
Table 2 – Preparation of stacking gel	Table 2. Preparation of stacking gel

Sep. 2002

Harmonized items	JP 15	Remarks
Capillary Electrophoresis	General Information	
Apparatus	Capillary Electrophoresis	
Capillary zone electrophoresis	Apparatus	
Optimisation	1. Capillary Zone Electrophoresis	
Instrumental parameters	Optimization	
Voltage	Instrumental parameters	
Polarity	Voltage	
Temperature	Polarity	
Capillary	Temperature	
Electrolytic solution parameters	Capillary	
Buffer type and concentration	Electrolytic solution parameters	
Buffer pH	Buffer type and concentration	
Organic solvents	Buffer pH	
Additives for chiral separations	Organic solvents	
Capillary gel electrophoresis	Additives for chiral separations	
Characteristics of gels	2. Capillary Gel Electrophoresis	
Capillary isoelectric focusing	Characteristics of Gels	
Loading step	3. Capillary Isoelectric Focusing	
loading in one step	Loading step	
sequential loading	loading in one step	
Focusing step	sequential loading	
Mobilisation step	Focusing step	
Optimisation	Mobilization step	
Voltage	Optimization	
Capillary	Voltage	
Solutions	Capillary	
Micellar electrokinetic chromatography (MEKC)	Solutions	
Optimisation	4. Micellar Electrokinetic Chromatography (MEKC)	
Instrumental parameters	Optimization	
Voltage	Instrumental parameters	
Temperature	Voltage	
Capillary	Temperature	
Electrolytic solution parameters	Capillary	
Surfactant type and concentration	Electrolytic solution parameters	
Buffer pH	Surfactant type and concentration	
	Buffer pH	

Organic solvents	Organic solvents	
Additives for chiral separations	Additives for chiral separations	
Other additives	Other additives	
Quantification	Quantification	
Calculations	Calculations	
System Suitability	System Suitability	
Apparent number of theoretical plates	Apparent Number of Theoretical Plates	
Resolution	Resolution	
Symmetry factor	Symmetry Factor	
Signal-to-noise ratio	Signal-to-noise Ratio	

Feb. 2004

Harmonized items	JP 15	Remarks
Tablet Friability	General Information Tablet Friability Test	

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Harmonized items	JP 15	Remarks
Total Protein Assay	General Information Total Protein Assay	
Method 1	Method 1	
Standard Solution	Standard Solution	
Test Solution	Test Solution	
Procedure	Procedure	
Light-scattering	Light-Scattering	
Calculations	Calculations	
Method 2	Method 2	
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	
Standard solutions	Standard Solutions	

Explanatory footnote "Example: the Minimum Requirements for Biological Products and individual monograph of JP" is added.

Test solution	Test Solution	
Blank	Blank	
Coomassie reagent	Coomassie Reagent	
Procedure	Procedure	
Calculations	Calculations	
Method 4	Method 4	
Standard solutions	Standard Solutions	
Test solution	Test Solution	
Blank	Blank	
Reagents	Reagents and Solutions	
BCA Reagent	BCA Reagent	
Copper sulfate reagent	Copper Sulfate Reagent	
Copper-BCA reagent	Copper-BCA Reagent	
Procedure	Procedure	
Calculations	Calculations	
Method 5	Method 5	
Standard solutions	Standard Solutions	
Test solution	Test Solution	
Blank	Blank	
Biuret reagent	Biuret Reagent	
Procedure	Procedure	
Calculations	Calculations	
Interfering substances	Interfering Substances	
Comments	Comments	
Method 6	Method 6	
Standard solutions	Standard Solutions	
Test solution	Test Solution	
Blank	Blank	
Reagents	Reagents and Solutions	
Borate buffer	Borate Buffer	
Stock OPA reagent	Stock OPA Reagent	
OPA reagent	OPA Reagent	
Procedure	Procedure	
Calculations	Calculations	
Method 7	Method 7	
Procedure A	Procedure A	
Procedure B	Procedure B	
Calculations	Calculations	

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Harmonized items	JP 15	Remarks
Isoelectric Focusing	General Information	
Theoretical aspects	Isoelectric Focusing	
Practical aspects	Theoretical Aspects	
	Practical Aspects	

Apparatus Isoelectric Focusing in polyacrylamide gels: detailed procedure Preparation of the gels 1) 7.5 per cent polyacrylamide gel 2) Preparation of the mould Method Variations to the detailed procedure (subject to validation) Validation of iso-electric focusing procedures Specified variation to the general method Point to consider Figure-Mould Reagents Fixing solution for isoelectric focusing in polyacrylamide gel	Apparatus Isoelectric Focusing in Polyacrylamide Gels: Detailed Procedure Preparation of the Gels 7.5% Polyacrylamide gel Preparation of the mould Method Variations to the Detailed Procedure (Subject to Validation) Validation of Iso-Electric Focusing Procedures Specified Variations to the General Method Points to Consider Figure. Mould Reagents and Solutions Fixing solution for isoelectric focusing in polyacrylamide gel Coomassie staining TS Destaining solution	Coomassie staining TS and destaining solution are specified.
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June 2004

Harmonized items	JP 15	Remarks
Powder Flow	General Information Powder Flow	
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
Recommendation for shear cell	4.2 Recommendation for shear cell
Table 1 Flow properties and corresponding angle repose	Table 1 Flow properties and corresponding angle repose
Table 2 Scale of flowability	Table 2 Scale of flowability

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Harmonized items	JP 15	Remarks
Peptide Mapping	General Information	
Purpose and scope	Peptide Mapping Purpose and Scope	
The peptide map	The Peptide Map	
Isolation and purification	Isolation and Purification	
Selective cleavage of peptide bonds	Selective Cleavage of Peptide Bonds	
Pretreatment of sample	Pretreatment of Sample	
Pretreatment of the cleavage agent	Pretreatment of the Cleavage Agent	
Pretreatment of the protein	Pretreatment of the Protein	
Establishment of optimal digestion conditions	Establishment of Optimal Digestion Conditions	
pH	pH	
Temperature	Temperature	
Time	Time	
Amount of cleavage agent	Amount of Cleavage Agent	
Chromatographic separation	Chromatographic Separation	
Chromatographic column	Chromatographic Column	
solvent	Solvent	
Mobile phase	Mobile Phase	
Gradient selection	Gradient Selection	
Isocratic selection	Isocratic Selection	
Other parameters	Other Parameters	
Validation	Validation	
Analysis and identification of peptides	Analysis and Identification of Peptides	
Table 1. Examples of cleavage agents.	Table 1. Examples of Cleavage Agents	
Table 2. Techniques used for the separation of peptides.	Table 2. Techniques Used for the Separation of Peptides	

9. Isoelectric Focusing

This test is harmonized with the European Pharmacopoeia and the U.S. Pharmacopoeia. 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 is estimated by determining the minimum pI difference (ΔpI), which is necessary to separate 2 neighboring bands:

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

D : Diffusion coefficient of the protein

dpH/dx : pH gradient

E : Intensity of the electric field, in volts per centimeter

$-d\mu/dpH$: Variation of the solute mobility with the pH in the region close to the pI

Since D and $-d\mu/dpH$ for a given protein cannot be altered, the separation can be improved by using a narrower pH range and by increasing the intensity of the electric field. Resolution between protein bands on an IEF gel prepared with carrier ampholytes can be quite good. Improvements in resolution may be achieved by using immobilized pH gradients where the buffering species, which are analogous to carrier ampholytes, are copolymerized within the gel matrix. Proteins exhibiting pIs differing by as little as 0.02 pH units may be resolved using a gel prepared with carrier ampholytes while immobilized pH gradients can resolve proteins differing by approximately 0.001 pH units.

Practical Aspects

Special attention must be paid to sample characteristics and/or preparation. Having salt in the sample can be prob-

lematic 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

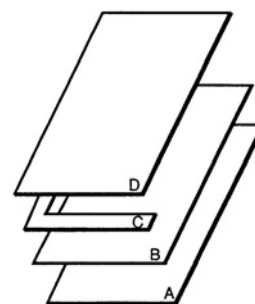


Figure. Mould

volumes with water. Mix carefully and degas the solution.

Preparation of the mould Place the polyester film on the lower glass plate, apply the spacer, place the second glass plate and fit the clamps. Place 7.5% polyacrylamide gel prepared before use on a magnetic stirrer, and add 0.25 volumes of a solution of ammonium persulfate (1 in 10) and 0.25 volumes of *N,N,N',N'*-tetramethylethylenediamine. Immediately fill the space between the glass plates of the mould with the solution.

Method

Dismantle the mould and, making use of the polyester film, transfer the gel onto the cooled support, wetted with a few millilitres of a suitable liquid, taking care to avoid forming air bubbles. Prepare the test solutions and reference solutions as specified in the monograph. Place strips of paper for sample application, about 10 mm × 5 mm in size, on the gel and impregnate each with the prescribed amount of the test and reference solutions. Also apply the prescribed quantity of a solution of proteins with known isoelectric points as pH markers to calibrate the gel. In some protocols the gel has pre-cast slots where a solution of the sample is applied instead of using impregnated paper strips. Cut 2 strips of paper to the length of the gel and impregnate them with the electrolyte solutions: acid for the anode and alkaline for the cathode. The compositions of the anode and cathode solutions are given in the monograph. Apply these paper wicks to each side of the gel several millimetres from the edge. Fit the cover so that the electrodes are in contact with the wicks (respecting the anodic and cathodic poles). Proceed with the isoelectric focusing by applying the electrical parameters described in the monograph. Switch off the current when the migration of the mixture of standard proteins has stabilized. Using forceps, remove the sample application strips and the 2 electrode wicks. Immerse the gel in fixing solution for isoelectric focusing in polyacrylamide gel. Incubate with gentle shaking at room temperature for 30 minutes. Drain off the solution and add 200 mL of destaining solution. Incubate with shaking for 1 hour. Drain the gel, add coomassie staining TS. Incubate for 30 minutes. Destain the gel by passive diffusion with destaining solution until the bands are well visualized against a clear background. Locate the position and intensity of the bands in the electropherogram as prescribed in the monograph.

Variations to the Detailed Procedure (Subject to Validation)

Where reference to the general method on isoelectric focusing is made, variations in methodology or procedure may be made subject to validation. These include:

- the use of commercially available pre-cast gels and of commercial staining and destaining kits,
- the use of immobilized pH gradients,
- the use of rod gels,
- the use of gel cassettes of different dimensions, including ultra-thin (0.2 mm) gels,
- variations in the sample application procedure, including different sample volumes or the use of sample application masks or wicks other than paper,
- the use of alternate running conditions, including variations in the electric field depending on gel dimensions and equipment, and the use of fixed migration times rather than subjective interpretation of band stability,
- the inclusion of a pre-focusing step,
- the use of automated instrumentation,
- the use of agarose gels.

Validation of Iso-Electric Focusing Procedures

Where alternative methods to the detailed procedure are employed they must be validated. The following criteria may be used to validate the separation:

- formation of a stable pH gradient of desired characteristics, assessed for example using colored pH markers of known isoelectric points,
- comparison with the electropherogram provided with the chemical reference substance for the preparation to be examined,
- any other validation criteria as prescribed in the monograph.

Specified Variations to the General Method

Variations to the general method required for the analysis of specific substances may be specified in detail in monographs. These include:

- the addition of urea in the gel (3 mol/L concentration is often satisfactory to keep protein in solution but up to 8 mol/L can be used): some proteins precipitate at their isoelectric point. In this case, urea is included in the gel formulation to keep the protein in solution. If urea is used, only fresh solutions should be used to prevent carbamylation of the protein,
- the use of alternative staining methods,
- the use of gel additives such as non-ionic detergents (e.g. octylglucoside) or zwitterionic detergents (e.g., CHAPS or CHAPSO), and the addition of ampholyte to the sample, to prevent proteins from aggregating or precipitating.

Points to Consider

Samples can be applied to any area on the gel, but to protect the proteins from extreme pH environments samples should not be applied close to either electrode. During method development the analyst can try applying the protein in 3 positions on the gel (i.e. middle and both ends); the pattern of a protein applied at opposite ends of the gel may not be identical.

A phenomenon known as cathodic drift, where the pH gradient decays over time, may occur if a gel is focused too long. Although not well understood, 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).♦

10. Laser Diffraction Measurement of Particle Size

Particle size is one of the important factors concerning the powder characteristics of solid-state drugs and pharmaceutical excipients, and therefore prompt and highly accurate determination method is necessary. The laser diffraction method is considered as a potent candidate, but due to the limit of kind of particles and the range of particle diameter that can be measured, the optical microscopy method should be sometimes applied in parallel. In the pharmaceutical field, the reliable and reproducible particle size determination method is required for quality control of the composition of the formulation, especially, of the pharmaceutical excipients that are difficult to apply the analytical sieving method due to the particle diameter of not larger than 100 μm .

In the Japanese Pharmacopoeia, the optical microscopy (Method 1) for the particles ranging from approximately 1 μm to 100 μm and the analytical sieving method (Method 2) for the particles of not smaller than 75 μm are listed as the powder particle size determination.

This document describes the laser diffraction technique for particle size determination as the method for analyzing the particles having the diameter ranging from approximately 1 μm to 1000 μm by utilizing the diffraction phenomenon against the laser beam (Fraunhofer diffraction). There are methods utilizing the principles such as the Mie scattering other than the Fraunhofer diffraction, which can be also used for particle size determination of the particles in sub-micron range (not larger than 1 μm).

1. Principle of laser diffraction technique

The laser diffraction technique is based upon the beam diffraction phenomenon (Fraunhofer diffraction), in which particle size-dependent intensity patterns exhibit when the powder particles are exposed to the parallel rays, and accurate and reproducible particle size distribution image can be obtained by mathematical analysis of the diffraction pattern. In other words, this apparatus is designed to detect the beams diffracted to each angle by the particles when the laser beam is radiated to the powder sample dispersed in a liquid or gas, in a way that the beam passes over the specimen and, the diffraction pattern is obtained and recorded for data analysis. For the analysis of data, the particles are qualified as spherical form. In this technique, at every particle diameter interval that is provided in the apparatus, distribution of the spherical equivalent diameter in the standard volume is calculated by inverse operation from the optical model to attain the highest coherence to the diffraction intensity pattern.

For the particles in sub-micron region, it is not easy to obtain an accurate particle size distribution due to difficulty in distinction of the particle diameter-dependent scattering pattern because of weakness of the forward light scattering intensity, and it is generally considered that the Fraunhofer diffraction can not be applied for the evaluation of particle diameter in the sub-micron range. Therefore, for the particles in sub-micron region, the method that applies the Mie scattering theory is introduced here. The particle diameter is measured by the analysis of the scattered lights toward back and side that are easy to catch the signals of transmitted and

refracted light. However, since the scattering patterns of the non-spherical particles vary depending on the particle shape, the correct particle diameter can not be obtained even if the accurate physical properties (refraction index or transmittance) of the dispersing medium are given. Therefore, the Mie scattering theory can be effectively applied if the particles are nearly spherical, and for the other cases, evaluation of the data is in general difficult due to strongly be affected by the physical properties or the particle concentration.

2. Apparatus

A typical set-up for a laser diffraction instrument is given in Figure 1. As the light source, the laser beam of a single wavelength is generally used. A beam expander is equipped as the laser beam processing unit H for irradiation to the particle ensemble dispersed. When the sample (particle ensemble F) passes through the laser beam, a part of the laser beam is scattered by the particles, and the diffraction images given by the scattered and transmitted beams are transcribed by the detector J placed at the focal length of the Fourier lens D.

A representative sample is dispersed in a liquid or gas, and the recirculating system consisting of the optical measurement cell, a dispersion bath (usually equipped with the stirrer and ultrasonic elements), a pump and tubing are commonly most used.

(1) Calibration of apparatus

According to the description in ISO standard 13320-1 (1999): Particle size analysis-Laser diffraction methods-Part 1: General principles, apparatus should be calibrated by using the plural standard particles for particle size measurement having a known particle size distribution and over at least one decade of size.

(2) Particle size determination in sub-micron region

In order to extend the measuring range of particle diameter to sub-micron region, apparatuses based on the Mie scattering theory have been developed and used. This type of instrument is designed so that the scattered lights toward back and side are detected and analyzed, but there are large variations in the analytical results depending on the type of instrument. However, this type of apparatus can produce reproducible data even in a sub-micron region, if the apparatus used and the sample tested are restricted under the specific conditions in which the input number for data analysis, e.g., physical properties, are fixed for the apparatus.

(3) Particle size determination of emulsion

In case of particle size determination of emulsion, the method utilizing dynamic light scattering or static light scattering is generally used. Since the emulsion particles transmit the light, the laser diffraction method is not very appropriate to apply. However, it is possible to evaluate emulsion particle diameter when the apparatus has been appropriately calibrated using the standard particles for particle size measurement.

3. Measurement

In case of the laser diffraction method, the particle concentration affects the accuracy of measurement. Therefore, for the sample having the size near the upper measuring limit of particle diameter, the particle concentration should be high enough to attain the static analysis. On the contrary, for the sample having smaller particle diameter, it is necessary for accurate measurement to adjust the particle concentration lower to some extent to prevent the multiple scattering.

(1) Conditions of measurement

·Location of the apparatus: It is important that the appara-

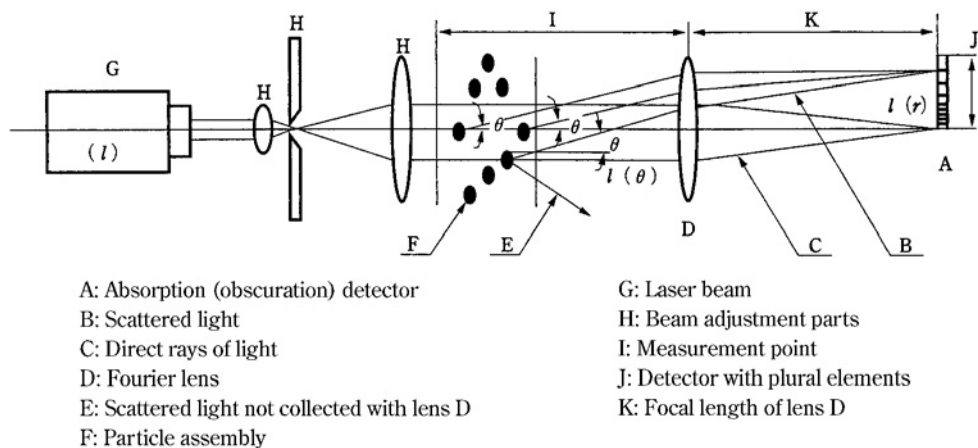


Fig. 1 Construction of the laser diffraction apparatus

tus is not affected by electrical noise and mechanical vibrations. It is also desirable that the apparatus is located under the environment where there is no variation of temperature and humidity to the extent that affects the measurement and is no exposure of direct sun light.

- Blank measurement: After selection of proper alignment of the optical part of the instrument, a blank measurement performed in a pure dispersing medium using the same method as that for measurement of the sample.
- Preliminary observation of the sample: Prior to the measurement, the powder sample should be observed in advance by visual/microscopic inspection to confirm the approximate particle diameter range and particle shape.
- Establishment of the measurement conditions: The time for measurement, the reading time of detector and the number of repetition should be experimentally decided in accordance with required measurement accuracy.
- Measurement of the sample: The sample is measured under the established measurement conditions. Data analysis is performed based on the pulse of dispersed particles from the difference of diffraction intensity between the sample and the blank.

(2) Preparation of the sample

The dry powder sample can be dispersed in a liquid or gas, and the appropriate method is selected depending on the purpose of measurement. There are two types of dispersion method, wet type using a liquid as the dispersion medium and dry type using a gas as the dispersion medium. It is advisable that the powder sample for measurement is divided to an appropriate apparent volume using a sample splitting technique.

The result of the particle size distribution much varies depending on the conditions of preparation of the sample. Therefore, the procedures and conditions of sample preparation should be defined in advance.

(3) Sampling

The amount of the sample necessary for the laser diffraction for powder particle size measurement has decreased due to technical advancement of the apparatus, but the sample for the measurement should represent the whole sample. Therefore, how to sample a small amount for actual measurement from a sample of large volume is an important problem. It is general that a large amount of the sample is divided/reduced to reach the amount necessary for measure-

ment, and the divider or the cone and quartering method, etc. are available for this purpose. The cone and quartering method is used for reduction of a small amount of sample with poor flowability, and is a simple method without using any divider.

For sampling of the powder particles dispersed in a liquid, the liquid is first stirred uniformly and mixed, and it should be confirmed that no precipitate remains at the bottom of the vessel. Then, the amount necessary for measurement is quickly sampled using a pipette, etc. In case of the sample that contains larger particles, it is easy to cause precipitates, and they are precipitated at the bottom after once stopping agitation. Therefore, the sampling of the suspension should be made under agitation.

(4) Pretreatment of the sample for measurement

If the sample contains the particles or agglomerates over the upper limit of measurable particle diameter range of the laser diffraction instrument, they should be removed by sieving in advance, and the mass and percent of such particles or agglomerates removed should be recorded.

(5) Liquids used as the dispersion medium

A variety of liquids is available for the dispersion of the powder samples, and at selection of the dispersion liquids, the followings should be taken into account:

- be compatible with the materials used in the instrument (O-ring, tubing, etc.)
- not dissolve or alter the size of particular materials,
- favour easy and stable dispersion of the particulate materials,
- have suitable viscosity in order to enable recirculation,
- not be hazardous to health and should meet safety requirements.

A low-foaming surfactant and dispersant may be used to facilitate the wetting of the particles, and to stabilize the dispersion. A preliminary check on the dispersion quality can be made by visual/microscopic inspection of the suspension.

(6) Dispersion of the agglomerated particles by ultrasonication

For the powder sample containing fine particles, intense physical power should be given to disperse the agglomerated particles. The ultrasonication is generally used for this purpose. In this case, since the ultrasonic output, irradiation time and the volumes of both the suspension and the vessel

used significantly affect the intensified dispersion effect, the optimum irradiation conditions should be established taking account of these factors. In case that an ultrasonic bath is used for dispersion, kinetic condition of the dispersant and dispersion effect may change depending on the altitude of its surface. Therefore, it is necessary to confirm the resonance point where the ultrasonic wave is intensely irradiated. Further, in case of stronger ultrasonic output or longer irradiation time, the particles for measurement may be destructed. Therefore, the change of the particle size distribution depending on the variation of the irradiation conditions should be checked to determine the appropriate ultrasonic output and irradiation time.

(7) Dispersion gases as the dispersion medium

An appropriate dry type dispersion apparatus using compressed gas is used for dispersion of the powder sample in a gas. Any substance that may affect the measurement, such as oil, water and particulate matter, should not be contaminated in the dispersion medium gas, and such substances should be removed, if necessary, by a filter, etc. In case of the dry dispersion method, it is advisable to measure all the samples reduced. A larger amount of sample diminishes the statistical error at a rough and large particle size area even if the particle size distribution is broad. It is also important that the particles are dispersed uniformly. It can be confirmed by comparing the results obtained by dry and wet dispersion methods. It is desirable that the equivalent results are obtained by dry and wet measurements.

4. Data analysis

Various softwares have been developed to analyze the data utilizing some mathematical methods, and the particle diameter and particle size distribution can be calculated from the data to meet its object. As for the accuracy of measurement, the reproducibility required for each apparatus is attained when measured on the independent triplicate samplings of the same batch.

(1) Calculation of the particle size distribution

The instrument manufacturers may use different algorithms for calculation of the particle size distribution from the intensity of diffraction (or scattering) beams obtained by the laser diffraction instrument, but the principle of estimating particle diameter from the intensity of light diffraction obtained by the detector under the diffraction (or scattering) theory is fundamentally the same. Since this method is based upon the optical model that assumes spherical particle, the sphere equivalent diameter is obtained for the non-spherical particles. The laser diffraction method can not distinguish between the diffraction of a single particle and that of the agglomerate or of the primary particle cluster that constitutes an aggregates or agglomerates.

(2) Weaving of the results

For the samples having particle size distributions, the median diameter or the modal diameter are used as the characteristic diameters, and the particle size distribution is expressed either as the cumulative size distribution or the frequency size distribution. The cumulative size distribution is expressed as a quantitative proportion of the particles smaller than the specified particle diameter among the whole sample. The analytical sieving method is generally used for the particle size distribution determination, and this cumulative distribution is represented as the cumulative undersize distribution. On the other hand, the frequency size distribution is ex-

pressed as the frequency that is shared by the particles that belong to the specified particle diameter fraction among the whole sample.

Mathematical expression of the particle size distribution

The particle size distribution is expressed by a function of either the normal distribution or the lognormal distribution. The lognormal distribution is characterized by distribution pattern that spreads out toward large particle size range, and is generally applied for the samples having a large amount of larger particles and a small amount of smaller particles.

5. Experimental considerations on the measurement

Accurate particle diameter measurement can not be expected for the laser diffraction method in case of the samples having wide range of particle size distribution. Therefore, the setting the measurement range is essential for the measurement of the particle diameter by the laser diffraction method, because the scattering intensity is much influenced by the lower limit diameter ($1\ \mu\text{m}$) and upper limit diameter ($1000\ \mu\text{m}$) of sample particles. It is desirable in this method that the measurement diameter range is confirmed by interpolation using the standard sample having a known particle size distribution considering the accuracy of measurement and the sensitivity of instrument used. For the particles of not larger than $10\ \mu\text{m}$, it is difficult to obtain accurate particle diameter, because the difference of diffraction pulse detected by the detector is small. Particularly, for the particles in sub-micron region, there is almost no difference of the scattered light forward, and it is hard to distinguish them. Further, in addition to the error caused by recognizing the non-spherical particles as spherical particles, decrease of the scattering intensity, which is much bigger than that estimated from the reduction of geometrical particle diameter, occurs as the particle diameter decreases, and securing the accuracy of measurement comes to be difficult. Therefore, the lower limit for measurement of the particle diameter by the laser diffraction technique is approximately $1\ \mu\text{m}$, similar to that by the optical microscopic method. In case of the laser diffraction technique, since the scattering intensity is related to the volume concentration of the particles, and the maximum measurement range of particle diameter should be decided taking account of the relative variation of the scattering intensity. A particle of 10 times larger in diameter has a volume of 1000 times larger. This means that in case of $1\ \mu\text{m}$ for lower limit measurement of particle diameter, the particles of $1000\ \mu\text{m}$ have a volume of 1 billion times larger, and consequently the sample necessary for measurement is 10 billion times larger, similar to the case of volume. Therefore, approximately $1000\ \mu\text{m}$ is appropriate for the maximum particle diameter for measurement.

Example of the standard reference particles

Use the standard reference particles for SAP 10-03, the Specification of Association of Powder Process Industry and Engineering, Japan. Either of MBP1-10 or MBP10-100 is used to meet the particle size measurement range ($1 - 10\ \mu\text{m}$ or $10 - 100\ \mu\text{m}$).

11. Media Fill Test

The media fill test (MFT) is one of the processing validations employed to evaluate the propriety of the aseptic

Table 1. Alert and action levels for large numbers of media filled units

Number of units*1	Number of contaminated units		
	Acceptance levels	Alert levels	Action levels
3,000	0	not applicable	≧ 1
4,750	0	1	≧ 2
6,300	0	1 – 2	≧ 3
7,760	0	1 – 3	≧ 4
9,160	0	1 – 4	≧ 5
10,520	1	2 – 5	≧ 6
11,850	1	2 – 6	≧ 7
13,150	1 – 2	3 – 7	≧ 8
14,440	1 – 2	3 – 8	≧ 9
15,710	1 – 3	4 – 9	≧ 10
16,970	1 – 3	4 – 10	≧ 11

*1 It is not necessary to relate the number of units with lot size of actual products.

Table 2. Upper 95% confidence limit of a Poisson variable for numbers of contaminated units

Observed numbers of contaminated units (k)	Upper 95% confidence limit (U)
0	2.9957
1	4.7439
2	6.2958
3	7.7537
4	9.1537
5	10.5130
6	11.8424
7	13.1481
8	14.4346
9	15.7052
10	16.9622

Using the 95% confidence limit (U) in Table 2, the contamination rate (P) of observed numbers of contaminated units (k) per filled units (n) can be calculated as $P = U/n$ (equation 1). For example:

If 5,000 units were filled and two contaminated units were observed, $n = 5,000$ and $U = 6.30$ ($k = 2$) are substituted in the equation 1; $P = 6.30/5,000 = 0.0013$. The upper 95% confidence limit for the contamination rate would be 0.13%.

processing of pharmaceutical products using sterile media, etc. instead of actual products. Therefore, media fill tests should be conducted with the manipulations normally performed in actual processing, e.g. filling and closing operation, operating environment, processing operation, number of personnel involved, etc., and conducted under processing conditions that include “worst case” conditions. Refer to GMP (1), WHO/GMP for pharmaceutical products (2), and ISO 13408 (3), etc. for necessary information to conduct this test.

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. For production batch sizes exceeding 3,000 units, a minimum of three media fill runs should be conducted on separate days. For production batch sizes of less than 3,000 units, see Table 3.

Table 3. Initial performance qualification: Media fills

Production lot size	Numbers of media fill runs	Alert level and action required	Action level and action required
< 500	A minimum of 10 media fill runs using the maximum lot size of the product	One contaminated unit in any run. Investigate cause.	Two contaminated units in single run, or one each in two runs. Investigate cause and repeat initial qualification media fills.
500 – 2,999	A minimum of 3 media fill runs using the maximum lot size of the product	The same as above	The same as above
≧ 3,000	A minimum of 3 media fill runs using at least 3,000 units	When any of the media fill runs exceeds the alert level shown in Table 1, take the action set out in 2.1.1.	When any of the media fill runs exceeds the action level shown in Table 1, take the action set out in 2.1.1

Table 4. Periodic performance requalification: Media fills

Production lot size	Numbers of media fill runs	Alert level and action required	Action level and action required
< 500	A minimum of 3 media fill runs using the maximum lot size of the product		One contaminated unit in any run. Investigate cause and repeat initial qualification media fill runs.
500 – 2,999	One media fill run using the maximum lot size of the product		One contaminated unit. Investigate cause and repeat initial qualification media fill runs.
≧ 3,000	One media fill run using at least 3,000 units	When the media fill run exceeds the alert level shown in Table 1, take the action set out in 2.1.1.	When the media fill run exceeds the action level shown in Table 1, take the action set out in 2.1.1

1.2 Periodic performance requalification

1) Conduct media fill requalifications periodically on each working shift for the filling line. Employees working in the aseptic processing area should be trained for aseptic processing operations and take part in media fills.

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 resumption of use of the filling lines.

3) In cases of facility and equipment modification (interchanging parts may not require requalification), changes in personnel working in critical aseptic processing (e.g. new crews), anomalies in environmental testing results, or a

product sterility test showing contaminated products, conduct 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

A large number of media filled units is required to detect 0.1% contamination rate. The alert level is more than 0.05% but less than 0.1%, and the action level is more than 0.1% contamination rate at the upper 95% confidence level. Table 1 shows alert and action levels for media filled units. Table 2 is to be used for the calculation of contamination rate of contaminated units found in media filled units. The contamination rate of 0.05% in media fills is the minimum acceptable level, and manufacturers should make efforts to achieve lower contamination rate than this. Table 3 shows the alert and action levels for initial performance qualification of an aseptic processing line, and actions required for each level. Table 4 shows the alert and action levels for requalification of an aseptic processing line, and actions required for each level. The alert and action levels for production batch sizes of less than 3,000 units take semiautomatic or manual operation into consideration.

2.1 Actions required for each level

2.1.1 Initial performance qualification

1) When the result of the media fill run is less than the alert level, the media fill run meets the requirement of the MFT.

2) When the results of any media fill run done with at least three replicate runs reach the alert or the action level, an investigation regarding the cause is required, and initial qualification media fills are to be repeated. When the result of each media fill run is less than the alert level, the initial qualification media fills meet the requirement of the MFT.

2.1.2 Requalification

1) When the result of the media fill run is less than the alert level, the media fill run meets the requirement of the MFT.

2) When the result of the media fill run exceeds the alert level, an investigation regarding the cause is required, and one more media fill run is to be done. If the result is less than the alert level, the media fill run meets the requirement of the MFT.

3) When the result of the media fill run exceeds the action level, a prompt review of all appropriate records relating to aseptic production between the current media fill and the last successful one, and an investigation regarding the cause must be conducted simultaneously. If necessary, appropriate action to sequester stored and/or distributed products should be taken. After investigation regarding the cause, repeat three serial media fill runs. If the results are less than the alert level, the media fill runs meet the requirement of the MFT.

2.2 Parameters which affect sterility

When media fill alert and action levels are exceeded, an investigation should be conducted regarding the cause, taking into consideration the following points:

- 1) Microbial environmental monitoring data
- 2) Particulate monitoring data
- 3) Personnel monitoring data (microbial monitoring data on gloves, gowns, etc. at the end of work)
- 4) Sterilization cycles for media, commodities, equipment, etc.
- 5) Calibration of sterilization equipment

- 6) Storage conditions of sterile commodities
- 7) HEPA filter evaluation (airborne particulate levels, DOP test, velocity measurements, etc.)
- 8) Pre and post filter integrity test data (including filter housing assembly)
- 9) Room 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 lot and catalogue number
- 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) Characterization of the microorganisms from any positive units
- 18) 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 and container configurations.

4.1 Media selection and growth promotion

Soybean-casein digest medium or other suitable media are used. As growth promotion testing microorganisms, strains listed in the Sterility Test and, if necessary, one to two representative microorganisms which are frequently isolated in environmental monitoring should be used. The media inoculated with 10 to 100 viable microorganisms of each strain should show obvious growth when incubated at the predetermined temperature for 5 days.

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 media fill evaluation units should be removed and recorded prior to incubation of media filled units. Incubate at 20–25°C for 1 week, and then at 30–

35°C for 1 week (or at 30 – 35°C for 1 week, and then at 20 – 25°C for 1 week), or at 30 – 35°C for 2 weeks. Observe the media filled units for growth of microorganisms at least once between the third day and seventh day and on the last day of the test period, twice in total. Microorganisms present in contaminated units should be characterized.

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.

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 product contact 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 10 to 100 viable 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

Dry powders are bagged in suitable containers (e.g. double heat-sealed polyethylene bags), and are subjected to radiation 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

Chose 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 used as the blanket 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 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

- 1) Good manufacturing practices for pharmaceutical products (WHO-GMP, 1992)
- 2) ISO 13408-1 (Aseptic processing of health care products: Generals)

12. Microbial Attributes of Nonsterile Pharmaceutical Products

The presence of microbial contaminants in nonsterile pharmaceutical products can reduce or even inactivate the therapeutic activity of the product and has the potential to affect adversely the health of patients. Manufacturers, therefore, should ensure as low as possible a contamination level for finished dosage forms, raw materials and packaging components to maintain appropriate quality, safety and efficacy of nonsterile pharmaceutical products. This chapter provides guidelines for acceptable limits of viable microorganisms (bacteria and fungi) existing in raw materials and nonsterile pharmaceutical products. Testing methods for the counting of total viable microorganisms and methods for the detection and identification of specified microorganisms (*Escherichia coli*, *Salmonella*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, etc.) are given under the “Microbial Limit Test”. 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 and in the interpretation of the testing results.

1. Definitions

- 1.1 Nonsterile pharmaceutical products: Nonsterile drugs shown in monographs of the JP and nonsterile products including intermediate products and finished dosage

forms.

- 1.2 Raw materials: All materials, including raw ingredients and excipients, used for the preparation of drugs, except for water and gases.
- 1.3 Bioburden: Number and type of viable microorganisms existing in nonsterile pharmaceutical products.
- 1.4 Action levels: Established bioburden levels that require immediate follow-up and corrective action if they are exceeded.
- 1.5 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.
- 1.6 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 tests for total viable aerobic count and for the detection of specified microorganisms are not applied to antibiotic or bacteriostatic drugs. However, the tests should be done for microorganisms that are not affected by the drug. The test for total viable aerobic count is not applied to drugs containing viable microorganisms 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 status 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 contamination-controlled environment, 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 nonaqueous or dried products, it is not necessary to do the tests for total viable aerobic counts and for the detection of specified microorganisms, 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:

- a) Dosage forms of non-sterile pharmaceutical products (dosage directions);
- b) Manufacturing processes;
- c) Manufacturing frequency;
- d) Characteristics of raw materials (natural raw material, synthetic compound, etc.);
- e) Batch sizes;
- f) Variations in bioburden estimates (changes in batches, seasonal variations, etc.);
- g) Changes affecting the product bioburden (changes in manufacturing process, supplier of raw materials, lot

number of raw materials, etc.);

h) 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" is applied to a nonsterile 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.

- a) Subject pharmaceutical name (product name);
- b) Frequency of sampling and testing;
- c) Sampling methods (including responsible person, quantity, environment, etc. for sampling);
- d) Transfer methods of the samples to the testing area (including storage condition until the tests);
- e) Treatment of the samples (recovery methods of microbial contaminants);
- f) Enumeration of viable microorganisms (including testing quantity, culture media, growth-supporting test of the media, culturing methods, etc.);
- g) Detection of specified microorganisms (including testing quantity, culture media, growth-supporting test of the media, culturing methods, etc.);
- h) Estimation of the number of and characterization of microbial contaminants;
- i) Establishment of "Microbial contamination limits" (including alert level and action level);
- j) Actions to be taken when the levels exceed "Microbial contamination limits";
- k) Persons responsible for the testing and evaluation, etc.;
- l) Other necessary items

5. Microbial contamination limits for nonsterile pharmaceutical products

By establishing "Microbial contamination limits", 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 city water or purified water used in the processing of active ingredients or nonsterile 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.

The microbial contamination limits for nonsterile 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 contamination limits are given. In this guideline, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Candida albicans* are shown as specified microorganisms, but it is also necessary to test certain pharmaceutical products for other microorganisms (for example, certain species of *Clostridia*, *Pseudomonas*, *Burkholderia*, *Aspergillus*, and *Enterobacter species*) that may have the potential to present a microbial risk to patients. The selection of the specified microorganisms was based on following criteria; indicator for poor hygienic practices, pathogenic potential for route of administration, and survival profile of the microorganism and recoverability in the product. Due to the inherent precision limitations of the enumeration methods, a value exceeding the target limit by not more than 2 times.

6. Microbial contamination limits for herbal drugs

Target limits of microbial contamination for herbal drugs and herbal drug containing preparations are shown in Table 3. Category 1 indicates herbal drugs and their preparations to which boiling water is added before use, and category 2 indicates other herbal drugs and their preparations. In this guideline, enterobacteria and other gram-negative bacteria, *Escherichia coli*, *Salmonella*, and *Staphylococcus aureus* are mentioned as specified microorganisms, but other microorganisms such as certain species of *Bacillus cereus*, *Clostridia*, *Pseudomonas*, *Burkholderia*, *Aspergillus* and *Enterobacter species* are also necessary to be tested depending on the origin of the herbal drug raw materials or the preparation method of the preparations.

Table 1. Microbial enumeration limits for raw materials

Microorganisms	Target limit (cfu/g or cfu/mL)
Total aerobic microbial count (TAMC)	≤ 1000
Total combined yeasts/molds count (TYMC)	≤ 100

Table 2. Microbial enumeration limits for nonsterile finished dosage forms

Route of administration	TAMC (cfu/g or cfu/mL)	TYMC (cfu/g or cfu/mL)	Examples of objectionable microorganisms
Inhalation (liquid)	≤ 20	≤ 20	<i>Staphylococcus aureus</i> <i>Pseudomonas aeruginosa</i>
Inhalation (powder)	≤ 100	≤ 50	<i>Staphylococcus aureus</i> <i>Pseudomonas aeruginosa</i>
Nasal	≤ 100	≤ 50	<i>Staphylococcus aureus</i>

			<i>Pseudomonas aeruginosa</i>
Varginal	≤ 100	≤ 50	<i>Escherichia coli</i> <i>Staphylococcus aureus</i> <i>Candida albicans</i>
Otic or Topical (including transdermal patches)	≤ 100*	≤ 50*	<i>Pseudomonas aeruginosa</i> <i>Staphylococcus aureus</i>
Rectal	≤ 1000	≤ 100	Not specified
Oral (solid)	≤ 1000	≤ 100	<i>Escherichia coli</i>
Oral (liquid)	≤ 100	≤ 50	<i>Escherichia coli</i>

* For transdermal patches, the limits are expressed as cfu per transdermal patch.

Table 3. Microbial enumeration limits for herbal drugs and their preparations

Microorganisms	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.

13. 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 manufac-

Table 1. Air-cleanliness requirements for processing of sterile pharmaceutical products

Air cleanliness Grade*1	Maximum number of airborne particulates per m ³	
	at rest	in operation
	≥ 0.5 μm	≥ 0.5 μm
A (Laminar-airflow zone)	3,530	3,530
B (Non laminar-airflow zone)	3,530	353,000
C	353,000	3,530,000
D	3,530,000	—*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	gloves
			(CFU/24–30 cm ²)*3	
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.

tured, 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.

1) 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.

2) Action levels: Established microbial levels (and type of microorganisms, if appropriate) that require immediate follow-up and corrective action if they are exceeded.

3) 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.

4) Contaminants: Particulates and microorganisms causing contamination by adhering to surfaces or by being incorporated into materials.

5) 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.

6) Contamination control: The planning, establishment of systems and implementation activities performed in order to maintain the required cleanliness of a specified space or surface.

7) 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.

8) 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 re-

quirements 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

a) 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.

b) 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.

c) 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.

d) 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.

e) Table 4 shows recommended limits for environmental 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.

f) 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

a) 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.

b) 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

a) 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.

b) Active microbial sampling methods

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.

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 1) on the characteristics of the filtration-type sampler.

4.2 Measurement methods for microorganisms on surfaces

a) 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.

b) 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.

Media

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	1,000 mL

Sterilize by heating in an autoclave at 121°C for 15 to 20 min. pH after sterilization: 7.2 – 7.6.

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	1,000 mL

Sterilize by heating in an autoclave at 121°C for 15 to 20 min. pH after sterilization: 7.2 – 7.6.

Glucose peptone agar medium or Fluid glucose peptone medium

See Microbial Limit Test. Antibiotic is added if necessary.

Nutrient agar medium or Fluid nutrient medium

Meat extract	3.0 g
Peptone	5.0 g
Agar	15.0 g
Water	1,000 mL

Sterilize by heating in an autoclave at 121°C for 15 to 20 min. pH after sterilization: 6.6 – 7.0.

Potato-dextrose agar medium or Fluid potato-dextrose medium

See Microbial Limit Test. Antibiotic is added if necessary.

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	1,000 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.

Sabouraud dextrose agar medium or Fluid sabouraud dextrose medium

See Microbial Limit Test. Antibiotic is added if necessary.

Soybean-casein digest agar medium or Fluid soybean-casein digest medium

See Microbial Limit Test.

Thioglycolate agar medium or thioglycolate medium I for sterility test

See Sterility Test. The agar concentration of Thioglycolate agar medium is about 1.5%.

*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%.

Rinsing Liquids

Buffered sodium chloride-peptone solution (pH 7.0)

See Microbial Limit Test.

LP liquid

Casein peptone	1.0 g
Soybean lecithin	0.7 g
Polysorbate 80	1.0 – 20.0 g
Water	1,000 mL

Sterilize by heating in an autoclave at 121°C for 15 to 20 min. pH after sterilization: 7.2.

Phosphate buffered solution (pH 7.2)

See Microbial Limit Test.

Ringer's solution, 1/4 concentration

Sodium chloride	2.25 g
Potassium chloride	0.105 g
Calcium chloride dihydrate	0.16 g
Water	1,000 mL

Sterilize by heating in an autoclave at 121°C for 15 to 20 min. pH after sterilization: 7.0.

Thiosulfate-Ringer's solution

Sodium thiosulfate pentahydrate	0.8 g
Ringer's solution, 1/4 concentration	1,000 mL

Sterilize by heating in an autoclave at 121°C for 15 to 20 min. pH after sterilization: 6.6.

14. 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* strain FH or equivalent species or strains) and one of which should be an arginine hydrolyser (i.e., *M. orale* CH 19299 or equivalent species or strains). The mycoplasma strains used for the positive control tests should be obtained from an official or suitably accredited agency, and handled appropriately. Inoculate the culture medium with no more than 100 colony-forming units (CFU).

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 four or more agar plates. After the surfaces of the inoculated plates are dried, one half of the plates should be incubated under aerobic conditions in an atmosphere of air containing 5 to 10 percent carbon dioxide and adequate humidity, and the other half under anaerobic 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 two vessels containing 100 mL of broth medium. One vessel should be incubated under aerobic conditions, and the other under anaerobic conditions.

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.

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 inoculated with aerobic or anaerobic broth cultures should be incubated aerobically or anaerobically, respectively 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 no more than 100 CFU of *M. hyorhinis* DBS 1050 or *M. orale* CH 19299.

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 obtained from an official or suitably accredited agency, and handled appropriately. 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* DBS 1050 or *M. orale* CH 19299. Use an inoculum of no more than 100 CFU 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 super-

nant) to each of two or more culture dishes. Perform the same procedure for the positive (2 types of mycoplasma, such as *M. hyorhina* and *M. orale*) 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. hyorhina* of 100 CFU 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. Add drops of mineral oil or suitable equivalent as needed during the reaction to prevent evaporation.

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. Add drops of mineral oil or suitable equivalent as needed during the reaction to prevent evaporation.

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 (each 1.25 mol)	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	68 μL	77 μL
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
Magnesium chloride hexahydrate	20 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 (diameter 35 mm), 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 less *M. hyorhinis*) 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.

15. Peptide Mapping

This test is harmonized with the European Pharmacopoeia

and the U.S. Pharmacopoeia.

Purpose and Scope

Peptide mapping is an identity test for proteins, especially those obtained by r-DNA technology. It involves the chemical or enzymatic treatment of a protein resulting in the formation of peptide fragments followed by separation and identification of the fragments in a reproducible manner. It is a powerful test that is capable of identifying single amino acid changes resulting from events such as errors in the reading of complementary DNA (cDNA) sequences or point mutations. Peptide mapping is a comparative procedure because the information obtained, compared to a reference standard or reference material similarly treated, confirms the primary structure of the protein, is capable of detecting whether alterations in structure have occurred, and demonstrates process consistency and genetic stability. Each protein presents unique characteristics which must be well understood so that the scientific and analytical approaches permit validated development of a peptide map that provides sufficient specificity.

This chapter provides detailed assistance in the application of peptide mapping and its validation to characterize the desired protein product, to evaluate the stability of the expression construct of cells used for recombinant DNA products and to evaluate the consistency of the overall process, to assess product stability as well as to ensure the identity of the protein product, or to detect the presence of protein variant.

The Peptide Map

Peptide mapping is not a general method, but involves developing specific maps for each unique protein. Although the technology is evolving rapidly, there are certain methods that are generally accepted. Variations of these methods will be indicated, when appropriate, in specific monographs.

A peptide map may be viewed as a fingerprint of a protein and is the end product of several chemical processes that provide a comprehensive understanding of the protein being analyzed. Four major steps are necessary for the development of the procedure: isolation and purification of the protein, if the protein is part of a formulation; selective cleavage of the peptide bonds; chromatographic separation of the peptides; and analysis and identification of the peptides. A test sample is digested and assayed in parallel with a reference standard or a reference material. Complete cleavage of peptide bonds is more likely to occur when enzymes such as endoproteases (e.g., trypsin) are used, instead of chemical cleavage reagents. A map should contain enough peptides to be meaningful. On the other hand, if there are too many fragments, the map might lose its specificity because many proteins will then have the same profiles.

Isolation and Purification

Isolation and purification are necessary for analysis of bulk drugs or dosage forms containing interfering excipients and carrier proteins and, when required, will be specified in the monograph. Quantitative recovery of protein from the dosage form should be validated.

Selective Cleavage of Peptide Bonds

The selection of the approach used for the cleavage of peptide bonds will depend on the protein under test. This selection process involves determination of the type of cleavage to be employed—enzymatic or chemical—and the type of

cleavage agent within the chosen category. Several cleavage agents and their specificity are shown in Table 1. This list is not all-inclusive and will be expanded as other cleavage agents are identified.

Table 1. Examples of Cleavage Agents

Type	Agent	Specificity
Enzymatic	Trypsin (EC 3.4.21.4)	C-terminal side of Arg and Lys
	Chymotrypsin (EC 3.4.21.1)	C-terminal side of hydrophobic residues (e.g., Leu, Met, Ala, aromatics)
	Pepsin (EC 3.4.23.1 & 2)	Nonspecific digest
	Lysyl endopeptidase (Lys-C Endopeptidase) (EC 3.4.21.50)	C-terminal side of Lys
	Glutamyl endopeptidase (from <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	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	Asp and Pro
	BNPS-skatole	Trp

Pretreatment of Sample Depending on the size or the configuration of the protein, different approaches in the pretreatment of samples can be used. For monoclonal antibodies the heavy and light chains will need to be separated before mapping. If trypsin is used as a cleavage agent for proteins with a molecular mass greater than 100,000 Da, lysine residues must be protected by citraconylation or maleylation; otherwise, many peptides will be generated.

Pretreatment of the Cleavage Agent Pretreatment of cleavage agents—especially enzymatic agents—might be necessary for purification purposes to ensure reproducibility of the map. For example, trypsin used as a cleavage agent will have to be treated with tosyl-L-phenylalanine chloromethyl ketone to inactivate chymotrypsin. Other methods, such as purification of trypsin by HPLC or immobilization of enzyme on a gel support, have been successfully used when only a small amount of protein is available.

Pretreatment of the Protein Under certain conditions, it might be necessary to concentrate the sample or to separate the protein from added substances and stabilizers used in formulation of the product, if these interfere with the mapping procedure. Physical procedures used for pretreatment can include ultrafiltration, column chromatography, and lyophilization.

Other pretreatments, such as the addition of chaotropic agents (e.g., urea) can be used to unfold the protein prior to mapping. To allow the enzyme to have full access to cleavage sites and permit some unfolding of the protein, it is often necessary to reduce and alkylate the disulfide bonds prior to digestion.

Digestion with trypsin can introduce ambiguities in the tryptic map due to side reactions occurring during the digestion reaction, such as nonspecific cleavage, deamidation, disulfide isomerization, oxidation of methionine residues, or formation of pyroglutamic groups created from the deamidation of glutamine at the N-terminal side of a peptide. Furthermore, peaks may be produced by autohydrolysis of trypsin. Their intensities depend on the ratio of trypsin to protein. To avoid autohydrolysis, solutions of proteases may be prepared at a pH that is not optimal (e.g., at pH 5 for trypsin), which would mean that the enzyme would not become active until diluted with the digest buffer.

Establishment of Optimal Digestion Conditions Factors that affect the completeness and effectiveness of digestion of proteins are those that could affect any chemical or enzymatic reactions.

pH: The pH of the digestion mixture is empirically determined to ensure the optimization of the performance of the given cleavage agent. For example, when using cyanogen bromide as a cleavage agent, a highly acidic environment (e.g., pH 2, formic acid) is necessary; however, when using trypsin as a cleavage agent, a slightly alkaline environment (pH 8) is optimal. As a general rule, the pH of the reaction milieu should not alter the chemical integrity of the protein during the digestion and should not change during the course of the fragmentation reaction.

Temperature: A temperature between 25°C and 37°C is adequate for most digestions. The temperature used is intended to minimize chemical side reactions. The type of protein under test will dictate the temperature of the reaction milieu, because some proteins are more susceptible to denaturation as the temperature of the reaction increases. For example, digestion of recombinant bovine somatotropin is conducted at 4°C, because at higher temperatures it will precipitate during digestion.

Time: If sufficient sample is available, a time course study is considered in order to determine the optimum time to obtain a reproducible map and avoid incomplete digestion. Time of digestion varies from 2 to 30 hours. The reaction is stopped by the addition of an acid which does not interfere in the tryptic map or by freezing.

Amount of Cleavage Agent: Although excessive amounts of cleavage agent are used to accomplish a reasonably rapid digestion time (i.e., 6 to 20 hours), the amount of cleavage agent is minimized to avoid its contribution to the chromatographic map pattern. A protein to protease ratio between 20:1 and 200:1 is generally used. It is recommended that the cleavage agent can be added in two or more stages to optimize cleavage. Nonetheless, the final reaction volume remains small enough to facilitate the next step in peptide mapping—the separation step. To sort out digestion artifacts that might be interfering with the subsequent analysis, a blank determination is performed, using a digestion control with all the reagents, except the test protein.

Chromatographic Separation

Many techniques are used to separate peptides for

mapping. The selection of a technique depends on the protein being mapped. Techniques that have been successfully used for separation of peptides are shown in Table 2. In this section, a most widely used reverse-phase High Performance Liquid Chromatographic (RP-HPLC) method is described as one of the procedures of chromatographic separation.

Table 2. Techniques Used for the Separation of Peptides

Reverse-Phase High Performance Liquid Chromatography (RP-HPLC)
Ion-Exchange Chromatography (IEC)
Hydrophobic Interaction Chromatography (HIC)
Polyacrylamide Gel Electrophoresis (PAGE), nondenaturing
SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)
Capillary Electrophoresis (CE)
Paper Chromatography-High Voltage (PCHV)
High-Voltage Paper Electrophoresis (HVPE)

The purity of solvents and mobile phases is a critical factor in HPLC separation. HPLC-grade solvents and water that are commercially available, are recommended for RP-HPLC. Dissolved gases present a problem in gradient systems where the solubility of the gas in a solvent may be less in a mixture than in a single solvent. Vacuum degassing and agitation by sonication are often used as useful degassing procedures. When solid particles in the solvents are drawn into the HPLC system, they can damage the sealing of pump valves or clog the top of the chromatographic column. Both pre- and post-pump filtration is also recommended.

Chromatographic Column The selection of a chromatographic column is empirically determined for each protein. Columns with 100 Å or 300 Å pore size with silica support can give optimal separation. For smaller peptides, octylsilane chemically bonded to totally porous silica articles, 3 to 10 μm in diameter (L7) and octadecylsilane chemically bonded to porous silica or ceramic micro-particles, 3 to 10 μm in diameter (L1) column packings are more efficient than the butyl silane chemically bonded to totally porous silica particles, 5 to 10 μm in diameter (L26) packing.

Solvent The most commonly used solvent is water with acetonitrile as the organic modifier to which less than 0.1% trifluoroacetic acid is added. If necessary, add isopropyl alcohol or n-propyl alcohol to solubilize the digest components, provided that the addition does not unduly increase the viscosity of the components.

Mobile Phase Buffered mobile phases containing phosphate are used to provide some flexibility in the selection of pH conditions, since shifts of pH in the 3.0 to 5.0 range enhance the separation of peptides containing acidic residues (e.g., glutamic and aspartic acids). Sodium or potassium phosphates, ammonium acetate, phosphoric acid, and a pH between 2 and 7 (or higher for polymer-based supports) have also been used with acetonitrile gradients. Acetonitrile containing trifluoroacetic acid is used quite often.

Gradient Selection Gradients can be linear, nonlinear, or include step functions. A shallow gradient is recommended in order to separate complex mixtures. Gradients are optimized to provide clear resolution of one or two peaks that will become "marker" peaks for the test.

Isocratic Selection Isocratic HPLC systems using a single

mobile phase are used on the basis of their convenience of use and improved detector responses. Optimal composition of a mobile phase to obtain clear resolution of each peak is sometimes difficult to establish. Mobile phases for which slight changes in component ratios or in pH significantly affect retention times of peaks in peptide maps should not be used in isocratic HPLC systems.

Other Parameters Temperature control of the column is usually necessary to achieve good reproducibility. The flow rates for the mobile phases range from 0.1 to 2.0 mL per minute, and the detection of peptides is performed with a UV detector at 200 to 230 nm. Other methods of detection have been used (e.g., postcolumn derivatization), but they are not as robust or versatile as UV detection.

Validation This section provides an experimental means for measuring the overall performance of the test method. The acceptance criteria for system suitability depend on the identification of critical test parameters that affect data interpretation and acceptance. These critical parameters are also criteria that monitor peptide digestion and peptide analysis. An indicator that the desired digestion endpoint was achieved is by the comparison with a Reference Standard, which is treated exactly as the article under test. The use of a reference standard or reference material in parallel with the protein under test is critical in the development and establishment of system suitability limits. In addition a specimen chromatogram should be included with the Reference Standard or Reference Material for additional comparison purposes. Other indicators may include visual inspection of protein or peptide solubility, the absence of intact protein, or measurement of responses of a digestion-dependent peptide. The critical system suitability parameters for peptide analysis will depend on the particular mode of peptide separation and detection and on the data analysis requirements.

When peptide mapping is used as an identification test, the system suitability requirements for the identified peptides covers selectivity and precision. In this case, as well as when identification of variant protein is done, the identification of the primary structure of the peptide fragments in the peptide map provides both a verification of the known primary structure and the identification of protein variants by comparison with the peptide map of the reference standard/reference material for the specified protein. The use of a digested reference standard or reference material for a given protein in the determination of peptide resolution is the method of choice. For an analysis of a variant protein, a characterized mixture of a variant and a reference standard or reference material can be used, especially if the variant peptide is located in a less-resolved region of the map. The index of pattern consistency can be simply the number of major peptides detected. Peptide pattern consistency can be best defined by the resolution of peptide peaks. Chromatographic parameters—such as peak-to-peak resolution, maximum peak width, peak area, peak tailing factors, and column efficiency—may be used to define peptide resolution. Depending on the protein under test and the method of separation used, single peptide or multiple peptide resolution requirements may be necessary.

The replicate analysis of the digest of the reference standard or reference material for the protein under test yields measures of precision and quantitative recovery. Recovery of the identified peptides is generally ascertained by the use of internal or external peptide standards. The

precision is expressed as the relative standard deviation (RSD). Differences in the recovery and precision of the identified peptides are expected; therefore, the system suitability limits will have to be established for both the recovery and the precision of the identified peptides. These limits are unique for a given protein and will be specified in the individual monograph.

Visual comparison of the relative retention times, the peak responses (the peak area or the peak height), the number of peaks, and the overall elution pattern is completed initially. It is then complemented and supported by mathematical analysis of the peak response ratios and by the chromatographic profile of a 1:1 (v/v) mixture of sample and reference standard or reference material digest. If all peaks in the sample digest and in the reference standard or reference material digest have the same relative retention times and peak 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 de-

velopment of a peptide map for the specified protein.

Analysis and Identification of Peptides

This section gives guidance on the use of peptide mapping during development in support of regulatory applications.

The use of a peptide map as a qualitative tool does not require the complete characterization of the individual peptide peaks. However, validation of peptide mapping in support of regulatory applications requires rigorous characterization of each of the individual peaks in the peptide map. Methods to characterize peaks range from N-terminal sequencing of each peak followed by amino acid analysis to the use of mass spectroscopy (MS).

For characterization purposes, when N-terminal sequencing and amino acids analysis are used, the analytical separation is scaled up. Since scale-up might affect the resolution of peptide peaks, it is necessary, using empirical data, to assure that there is no loss of resolution due to scale-up. Eluates corresponding to specific peptide peaks are collected, vacuum-concentrated, and chromatographed again, if necessary. Amino acid analysis of fragments may be limited by the peptide size. If the N-terminus is blocked, it may need to be cleared before sequencing. C-terminal sequencing of proteins in combination with carboxypeptidase and MALDITOF-MS can also be used for characterization purposes.

The use of MS for characterization of peptide fragments is by direct infusion of isolated peptides or by the use of on-line LC-MS for structure analysis. In general, it includes electrospray and matrix-assisted laser desorption ionization coupled to time-of-flight analyzer (MALDITOF) as well as fast atom bombardment (FAB). Tandem MS has also been used to sequence a modified protein and to determine the type of amino acid modification that has occurred. The comparison of mass spectra of the digests before and after reduction provides a method to assign the disulfide bonds to the various sulfhydryl-containing peptides.

If regions of the primary structure are not clearly demonstrated by the peptide map, it might be necessary to develop a secondary peptide map. The goal of a validated method of characterization of a protein through peptide mapping is to reconcile and account for at least 95% of the theoretical composition of the protein structure.

16. 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.

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.

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.

17. Plastic Containers for Pharmaceutical Products

Various kinds of plastics are used in the manufacture of containers for pharmaceutical products. Such plastics should not alter the efficacy, safety or stability of the pharmaceutical products. In selecting a suitable plastic container, it is desirable to have full information on the manufacturing processes of the plastic container including the substances added. Since each plastic has specific properties and a wide variety of pharmaceutical products may be stored in containers made from it, 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. This judgement should be carried out by verifying that a type sample of the container for the pharmaceutical preparation fulfills the essential requirements, i.e., the design specifications, according to experiments and/or scientific documentation, etc. In addition, the compatibility must be ensured based upon an appropriate quality assurance system.

Furthermore, in introducing a plastic container, it is desirable that proper disposal after use is taken into consideration.

Essential Requirements in Designing Plastic Containers for Pharmaceutical Products

The plastic material 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 alter the efficacy or stability of the pharmaceutical products contained therein. In addition, the possible toxic hazards of 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 the intended usage.

The quality of the pharmaceutical products contained 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, care should be taken that the container is impermeable to the solvent in the case of solvents other than water. The concentration of the pharmaceuticals must not be decreased by more than a certain level due to the absorption 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 possible high temperature or low temperature or cycles thereof 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.

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 criteria for the evaluation, and to clarify the rationales for the selection. The tests should be conducted using samples of the whole or a part of the prototype container. If the container consists of plural parts of different materials, each part should be tested separately. Such materials as laminates, composites, and 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 contacts 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

- 1) preparations contacting blood:
Acute toxicity test, cytotoxicity test, sensitization test and hemolysis test
- 2) preparations contacting skin or mucous membranes:
Cytotoxicity test and sensitization test
- 3) 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:

(A) Selection of Tests

· Guidelines for Basic Biological Tests of Medical Devices and Materials (PAB Notification, YAKU-KI NO.99, June 27, 1995), Principles and selection of tests

· ISO 10993-1: Biological evaluation of medical devices—Evaluation and testing

(B) Acute Toxicity Test

· ASTM F750-82: Standard practice for evaluating material extracts by systemic injection in the mice

· BS5736: Part 3 Method of test for systemic toxicity; assessment of acute toxicity of extracts from medical devices

· USP 24 <88> Biological reactivity tests, in vivo

(C) Cytotoxicity Test

· Guidelines for Basic Biological Tests of Medical Devices and Materials, I. Cytotoxicity Test 10. Cytotoxicity test using extract of medical device or material

· ISO 10993-5: Biological evaluation of medical devices—Tests for cytotoxicity : in vitro methods

· USP 24 <87> Biological reactivity tests, in vitro

(D) Hemolysis Test

· Guidelines for Basic Biological Tests of Medical Devices and Materials, VII. Hemolysis Test

· ISO 10993-4: Biological evaluation of medical devices—Selection of tests for interaction with blood. Annex D

· ASTM F756-82: Standard practice for assessment of hemolytic properties of materials

(E) Sensitization Test

· Guidelines for Basic Biological Tests of Medical Devices and Materials, II. Sensitization Test

· ISO 10993-10: Biological evaluation of medical devices—Tests for irritation and sensitization

Test Results to be Recorded per Production Unit

At the line production phase, it is required to establish the values of acceptable limits on at least the test items mentioned below and to record the test results of each production unit of plastic containers for pharmaceutical products. In addition, it is desirable to clarify the rationales for setting the values of limits. However, these requirements should not be applied to orally administered preparations except liquid ones.

1) Combustion Tests: Residue of ignition, heavy metals. If necessary, the amounts of the specified metals (lead, cadmium, etc.)

2) Extraction Tests: pH, ultraviolet absorption spectra, potassium permanganate-reducing substances, foaming, non-volatile residue

3) Cytotoxicity Test

4) Any other necessary tests for the specific container for aqueous infusions.

18. Powder Flow

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 (♦ ♦).

Four commonly reported methods for testing powder flow are (1) angle of repose, (2) compressibility index of Hausner ratio, (3) flow rate through an orifice, and (4) shear cell. In addition, numerous variations of each of these basic methods are available.

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. 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 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.

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.

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:

(1) 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.

(2) 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.

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

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

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	36 – 40
Passable	41 – 45
Poor	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:

- 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.
- 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 successfully or reproducibly prepared, this method is not appropriate. Determine the angle of repose by measuring the height of the cone of powder and calculating the angle of repose, α , from the following equation:
 $\tan \alpha = \text{height}/0.5 \text{ base}$

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_f , of the powder after tapping the material until no further volume changes occur (refer to the General Test Method, 3.01 Determination of Bulk and Tapped Densities). The compressibility index and the Hausner ratio are calculated as follows:

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

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

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

$$\text{Compressibility Index} = 100 \times \{(\rho_T - \rho_B) / \rho_T\}$$

$$\text{Hausner Ratio} = \rho_T / \rho_B$$

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_f , (3) the bulk density, ρ_B , and (4) the tapped density, ρ_T :

- The diameter of the cylinder used
- The number of times the powder is tapped to achieve the tapped density
- The mass of material used in the test
- Rotation of the sample during tapping

2.3 Recommended Procedure for Compressibility Index and Hausner Ratio

Use a 250-mL volumetric cylinder with a test sample weight of 100 grams. Smaller weights and volumes may be used, but weight of the test sample and volume of the cylinder used 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 grams 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.

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.

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:

- The diameter and shape of the orifice
- The type of container material (metal, glass, plastic)
- 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. 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:

- Diameter of opening >6 times the diameter of the particles
- 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

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.

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. 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

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.*, 72: 163-168(1965).

19. 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: Nonsterile parenterals

Category IC: Oral products made with aqueous bases (including syrup products to be dissolved or suspended before use)

Category II: All the dosage forms listed under Category I made with nonaqueous 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 niger 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 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. niger* 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 10⁸ microorganisms per mL. In the case of *A. niger*, 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 10⁸ per mL. Use these suspensions as the inocula.

Liquid cultures: After culturing each of the four strains except for *A. niger* 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 10⁸ 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.

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.

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

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.

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.

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.

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.

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.

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.

20. 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 amphixenosis infections common between human beings and animals.”.

This General Information describes safety assurance against infection 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 pri-

mary 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) and “Guidance for quality and safety assurance of a drug product, etc. which is derived from human cell/tissue (Attachment 2 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 (sero-

logic 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 sanitarly 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 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.

21. Quality Control of Water for Pharmaceutical Use

Water used for producing pharmaceutical products, cleaning pharmaceutical containers and equipment, and the like is referred to as “Pharmaceutical Water.” To consistently assure the quality of pharmaceutical water, it is important to verify through appropriate validation that water of required quality is supplied, and to maintain that quality by routine control.

Types of Pharmaceutical Water

① Water

The specifications for *Water* are specified in the corresponding monograph of the Japanese Pharmacopoeia. It is required for *Water* to meet the quality standards for drinking water provided by the Japanese Water Supply Law and an additional requirement for ammonium of “no greater than

0.05 mg/L.” In preparing *Water* at various facilities from source water such as well water or industrial water, it is needed to assure compliance with specifications set forth for *Water* in the Japanese Pharmacopoeia. Furthermore, when *Water* is used after storage for a long period of time, microbial proliferation should be prevented.

Water is used as source water for “*Purified Water*” and “*Water for Injection*.” It also is used in the production of intermediates of active pharmaceutical ingredients (APIs) and in pre-washing the equipments used for pharmaceutical purposes.

② Purified Water

The specifications of *Purified Water* are included in “Official Monographs” of the Japanese Pharmacopoeia. *Purified Water* is prepared from *Water* by distillation, ion-exchange treatment, reverse osmosis (RO), ultrafiltration (UF) capable of removing substances having molecular weights of 6,000 and above, or a combination of these methods. Because *Purified Water* contains no components capable of inhibiting microbial growth, appropriate microbiological control is needed. Particularly in the case of ion-exchange treatment, reverse osmosis or ultrafiltration, the appropriate treatment for microbial growth inhibition or periodical sterilization should be conducted.

Purified Water which has been sterilized or treated with chemical agents for the purpose of microbial growth inhibition or maintaining the endotoxin level within an appropriate control range should be appropriately controlled in order to maintain the quality in compliance with standards set forth for the individual purposes of use. *Purified Water* that has been sterilized is referred to as “*Sterilized Purified Water*.”

③ Water for Injection

The specifications of “*Water for Injection*” are described in “Official Monographs” of the Japanese Pharmacopoeia. *Water for Injection* is prepared from *Water* or *Purified Water* by distillation, or from *Purified Water* by reverse osmosis (RO), ultrafiltration (UF) capable of removing substances having molecular weights of 6,000 and above, or a combination of RO and UF. The quality of processing water by RO and/or UF should be maintained consistently at the same level as that prepared by distillation, by the validation through long-term operation of the process and elaborate routine control. Because *Water for Injection* contains no components that inhibit microbial growth, stringent control is needed for microorganisms and endotoxins. The standard for endotoxins requires a level lower than 0.25 EU/mL.

Selection of Pharmaceutical Water

Depending on the purpose of use, pharmaceutical water can be selected from the above-described group of pharmaceutical waters that allow us to assure the quality of the final product without causing any trouble during the production process. Table 1 shows some example criteria for such selection (in the case of charge-in water for the preparation of drug products).

For the production of sterile drug products, for which contamination by microorganisms or endotoxins is not permitted, *Water for Injection* should be used. For ophthalmics and eye ointments, *Water for Injection* or *Purified Water* should be used. For the production of non-sterile drug products, water of a quality higher than that of *Purified Water* should be used, however for a non-sterile drug product that requires

Table 1. Criteria for Selecting Pharmaceutical Water (Charge-In Water)

Classification	Class of Pharmaceutical Water	Application	Remarks
Drug Product	<i>Water for Injection</i>	Injections, Ophthalmics, Eye Ointments	
	<i>Purified Water</i>	Ophthalmics, Eye Ointment	For ophthalmics and eye ointments for which precautions should be taken against microbial contamination, purified water that is subjected to microbiological control by treatment with sterilization, ultrafiltration, or the like, 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, aerosols, and the like for which precautions should be taken against microbial contamination, select and use purified water subjected to appropriate microbiological control.
Active Pharmaceutical Ingredient (API)	<i>Water for Injection</i>	Sterile APIs, and APIs rendered sterile in the formulation process	
	<i>Purified Water</i>	APIs, APIs rendered sterile in the formulation process, and API intermediates	In the production of APIs that are rendered sterile in the formulation process and have no subsequent processes capable of removing endotoxins, Purified Water that is controlled to maintain endotoxins at a low level should be used.
	<i>Water</i>	API Intermediates	

care against microbiological contamination, such as liquids, ointments, suspensions, emulsions, suppositories, and aerosols, water should have a quality appropriately controlled from microbiological viewpoints, considering the possible impacts of preservatives formulated in the dosage form. Water for pre-washing containers or equipment surfaces that come in direct contact with the drug should be of a quality higher than that of *Water*. Water for final rinsing should have the same quality as that of charge-in water.

For the production of non-sterile drug products, the quality of water should be higher than that of *Purified Water*; however for the production of liquids, ointments, suspensions, emulsions, suppositories, and aerosols for which care should be taken against microbial contamination, the quality of water should be controlled appropriately from microbiological viewpoints in consideration of the possible effects of formulated preservatives. Water for pre-washing containers or equipment surfaces that come in direct contact with the product should be of a quality not less than that of *Water*. The water for final rinsing should be the same quality as that of charge-in water.

In selecting pharmaceutical water for Active Pharmaceutical Ingredient (API), the characteristics and formulation

process of the drug products for which the API is used should be considered, so that the quality of the final drug products is maintained at an appropriate level. Water used in the production of sterile API or to clean pharmaceutical containers or equipment surfaces that come in direct contact with the product should have the quality controlled chemically and microbiologically at the level of *Water* or higher, even if the water is to be used at an earlier stage of a synthetic or extraction process. In the final purification process, water should have a quality equal to *Purified Water* or higher. Water used for the final rinsing of containers or equipment surfaces that come in direct contact with the product should have the same quality as that of the charge-in water. Pharmaceutical water used in sterile API should be *Water for Injection*. Similarly, in the case of APIs for drug products, where endotoxin control is required and there are no subsequent processes capable of removing endotoxins, *Water for Injection* or *Purified Water*, for which endotoxins are controlled to an appropriate level, should be used.

Quality Control of Pharmaceutical Water

3.1 Outline

Routine control of pharmaceutical water is conducted on

the premise that the production of water of a required quality has been thoroughly verified by validation studies at an earlier stage of the system that produces the pharmaceutical water (pharmaceutical water system). If these requirements are met, the following control methods are applicable.

For routine control, the control of electrical conductivity and Total Organic Carbon (TOC) is markedly useful. Items to be controlled regularly should be determined according to the intended use of the pharmaceutical water. In addition to the above, control should be made for several chemical substances, viable counts, endotoxins, insoluble foreign particles, and the like. The measurement frequency should be determined in consideration of the stability of the water quality in question.

The following shows microbiological control items and control items for electrical conductivity and Total Organic Carbon (TOC), for which special attention should be paid. Similar considerations should be made of the other control items so that the water quality meets the specifications of pharmaceutical water.

3.2 Sampling

Monitoring should be conducted at a sufficient enough frequency to ensure that the pharmaceutical water system is in control and that water of acceptable quality is continuously produced. Sampling should be made at representative locations in the production and supply systems of the pharmaceutical water, with particular care that the collected sample reflects the pharmaceutical water system. Normally, point-of-use sites are appropriate for the sampling locations. Sampling frequency is established on the basis of data from validation studies of the pharmaceutical water system. In making a sampling plan for pharmaceutical water system, it is important to cover all important points of the system by thoroughly considering the quality characteristics required for the water to be sampled. Particularly in microbiological control, attention should be paid to the difference in conditions among sampling locations.

3.3 Alert and Action Levels

In a pharmaceutical water system, monitoring is done for microorganisms and other quality attributes that assure water of acceptable quality is produced during operation within the designed specifications. Monitoring data thus obtained is compared against an alert level, action level, other control levels, and allowable limits for pharmaceutical waters. This implies the alert level and action level are used for process control, rather than for judging the acceptance.

Definition of Alert Level

An alert level indicates that, when exceeded, a process may have drifted from its normal operating condition. Alert levels constitute a warning and exceeding them does not necessarily require a corrective action. Alert levels are generally established either at a mean + 2 σ on the basis of past trend analyses or at a level of 70% (50% for viable counts) of the action criteria, whichever is smaller.

Definition of Action Level

An action level indicates that, when exceeded, a process has drifted from its normal operating range. Exceeding an action level indicates that corrective action must be taken to bring the process back within its normal operating range.

Alert and action levels should be established within process and product specification tolerances and based on integrated

considerations for technology and product quality. Consequently, exceeding an alert or action level does not necessarily indicate that the product quality has been compromised.

3.4 Microbiological Monitoring

The main purpose of a microbiological monitoring program for pharmaceutical water system is to predict any microbiological quality deterioration of the produced water and to prevent any adverse effects it may have on product quality. Consequently, detecting all of the microorganisms present may not be necessary; however it is required to adopt a monitoring technique capable of detecting a wide range of microorganisms, including slow growing microorganisms.

The following indicates culture-based microbiological monitoring methods for pharmaceutical water systems. In adopting a rapid detection system for microorganisms, it is needed to confirm in advance that the obtained microbial counts are equivalent to or above the data obtained through incubation.

3.4.1 Media and Incubation Conditions

There are many mesophilic bacteria of heterotrophic type that are adaptable to poor nutrient water environments. In many pharmaceutical water systems, heterotrophic bacteria may form bio-films and cause water quality deterioration. It, therefore, is useful to monitor the water quality by use of R2A Agar Medium, which is excellent for growing bacteria of oligotrophic type. On the other hand, in routine microbial monitoring, an approach that identifies the trend in microbiological quality change is widely employed; a standard agar plate is used for counting the total number of viable microorganisms capable of proliferating at 30 – 35°C 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.

3.4.2 Media Growth Promotion Test

In the media growth promotion test with R2A Agar Medium, use the strains listed below or other strains considered to be equivalent to these strains. Prior to the media growth promotion 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, or the like

Dilute the fluid containing the starved strain in purified water with sterile purified water to prepare a fluid containing about 50 – 200 cfu per mL. When pipeting 1 ml of the diluted solution onto the R2A agar medium to be used for incubating at 20 – 25°C or 30 – 35°C for 4 – 7 days, a sufficient number of recovered colonies of the microorganism must be observed in comparison with the inoculated cfus.

In the media growth promotion test with standard agar medium, use the strains listed below or other strains considered to be equivalent to these strains. When pipeting 1 mL of the solution containing the microorganism for incubating at 30 – 35°C for 48 hours, a sufficient number of recovered colonies of the microorganism must be observed in comparison with the inoculated counts.

Staphylococcus aureus: ATCC 6538, NCIMB 9518, CIP 4.83 or NBRC 13276

Pseudomonas aeruginosa: ATCC 9027, NCIMB 8626, CIP 82.118 or NBRC 13275

Table 2. Methods for Assessment of Viable Counts in pharmaceutical Water

Method	Pharmaceutical Water		
	Water	Purified Water in Bulk	Water for Injection in Bulk
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

Colon bacillus (*Escherichia coli*): ATCC 8739, NCIMB 8545, CIP 53.126 or NBRC 3972

3.4.3 Action Levels for Microorganisms in Pharmaceutical Water System

The following action levels are considered appropriate and generally applicable to pharmaceutical water systems. Action levels for *Purified Water* and *Water for Injection* should be those obtained using R2A Agar Medium.

Action Levels for viable counts in various pharmaceutical waters

Water: 100 cfu/mL (according to the criteria in the Water Supply Law)

Purified Water: 100 cfu/mL

Water for Injection: 10 cfu/100 mL

(cfu: colony-forming units)

When actual counts exceed such action levels in validation studies or routine control studies, it is necessary to characterize the isolated microorganisms and to sterilize or disinfect the affected system.

3.5 Physicochemical Monitoring

Physicochemical monitoring of a pharmaceutical water system is generally performed using electrical conductivity and Total Organic Carbon (TOC) as indicators. Monitoring by electrical conductivity can predict probable quantities of the total inorganic salts present. Monitoring by TOC can estimate the total quantity of the organic compounds present. Basically, the tests for electrical conductivity and total organic carbon specified in “General Tests” of the Japanese Pharmacopoeia are applied as these physicochemical monitoring methods. However, tests for monitoring are different in profile from those described in “Monographs” of the Japanese Pharmacopoeia. The following describes the particulars for completing the part that cannot be worked out with the individual descriptions in “General Tests” of the Pharmacopoeia alone. When monitoring is done at a production

facility using electrical conductivity or TOC as an indicator, appropriate alert and action levels, as well as countermeasures against contingency should be established for each indicator.

3.5.1 Monitoring with an Indicator of Electrical Conductivity

Measurement of electrical conductivity for monitoring is usually conducted continuously with an in-line meter having a flow-through type or pipe-insertion type cell. Alternatively, batch testing may be done using a dip type cell with samples taken at point-of-use sites or other appropriate locations of the pharmaceutical water system. The following guidelines for the operation control of a pharmaceutical water system address how to interpret the results of electrical conductivity tests, and how to approve the continuation of operation, depending on whether measurements are made at the standard temperature (20°C) or at temperatures outside the standard.

(1) When monitoring is done at the standard temperature (20°C)

Tests for electrical conductivity of the Japanese Pharmacopoeia commonly require the tester to measure at the standard temperature (20°C), however measurement at a temperature within a range of $20 \pm 5^\circ\text{C}$ may be acceptable on condition that the correct equation is used. When monitoring electrical conductivity at the standard temperature of *Purified Water* and *Water for Injection*, the recommended allowable (action level) conductivity is as follows.

· Action Level 1.0 $\mu\text{S}/\text{cm}$ (20°C)

The above allowable conductivity is established on the supposition of in-line monitoring, so this standard level may be changed in batch testing.

(2) When monitoring is done at temperatures other than the standard temperature

When the quality of water is monitored by the measurement of electrical conductivity at a temperature other than

the standard temperature, the following three-stage approach is applied. The method described below complies with the description in “General Chapter <645> WATER CONDUCTIVITY” of the United States Pharmacopoeia (USP28, 2005), which specifies the establishment of allowable levels of electrical conductivity individually corresponding to different measurement temperatures and pH levels of specimens measured by the three-stage approach (Stage 1 to Stage 3).

Stage 1

1. When a non-temperature-compensated conductivity instrument is used, measure the temperature of the water specimen, in addition to the conductivity.

2. Using Table 3, find the conductivity value corresponding to the measured temperature or a temperature listed in Table 3 just below the measured temperature that can be used to calculate the allowable electrical conductivity at the measured temperature.

3. If the measured conductivity is not greater than the allowable conductivity value, the water specimen is judged as passing the electrical conductivity test. If the conductivity value exceeds the allowable conductivity value, proceed with Stage 2.

Table 3 Stage 1 Allowable Electrical Conductivity Measured at Different Temperatures*

Temperature (°C)	Allowable Conductivity (μS/cm)	Temperature (°C)	Allowable Conductivity (μS/cm)
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

*Applied to the conductivity value measured with a non-temperature-compensated conductivity instrument.

Stage 2

1. Transfer a sufficient amount of water specimen to a suitable container, and stir the specimen. Adjust the temperature ($25 \pm 1^\circ\text{C}$), and while vigorously agitating the specimen, measure the conductivity periodically. If the change in conductivity is not greater than $0.1 \mu\text{S}/\text{cm}$, due to the uptake of atmospheric carbon dioxide, interpret the observed value as the conductivity of the specimen (in equilibrium with the atmosphere).

2. If the observed conductivity value at 25°C (with the specimen in equilibrium with the atmosphere) is not greater than $2.1 \mu\text{S}/\text{cm}$, the specimen is judged as passing the conductivity test. If the observed value is greater than the level, proceed with Stage 3.

Stage 3

1. While maintaining the sample at $25 \pm 1^\circ\text{C}$, perform the conductivity measurement as explained in Step 1 of Stage 2. Add 0.3 mL of saturated potassium chloride solution to 100

mL of the water specimen, and determine the pH to the nearest 0.1 pH.

2. Use Table 4 to find the allowable conductivity value corresponding to the observed pH. If the observed conductivity is lower than the allowable level, the water specimen is judged as passing the electrical conductivity test. If the observed value is greater than the allowable level, or if the pH of the specimen is outside of a range of pH 5.0–7.0, the water specimen is judged as unacceptable.

Table 4 Third Step Allowable Electrical Conductivity at Different pHs*

pH	Allowable Conductivity (μS/cm)	pH	Allowable Conductivity (μS/cm)
5.0	4.7		
5.1	4.1	6.1	2.4
5.2	3.6	6.2	2.5
5.3	3.3	6.3	2.4
5.4	3.0	6.4	2.3
5.5	2.8	6.5	2.2
5.6	2.6	6.6	2.1
5.7	2.5	6.7	2.6
5.8	2.4	6.8	3.1
5.9	2.4	6.9	3.8
6.0	2.4	7.0	4.6

*Applied only to a sample in equilibrium with the air at 25°C .

3.5.2 Monitoring with an Indicator of Total Organic Carbon (TOC)

Drinking Water Standards (Prescribed under the Article 4 of the Japanese Water Supply Law) require that TOC should be “not greater than 5 ppm”. However it is preferable for individual facilities to conduct TOC monitoring on *Water* with alert and action levels separately determined for water-quality control by TOC monitoring.

The Japanese Pharmacopoeia specifies the Test for Total Organic Carbon, and usually, TOC measurement is conducted using an apparatus in compliance with the specifications, however if the apparatus conforms to the apparatus suitability test requirements described in “General Chapter <643> TOTAL ORGANIC CARBON” of the United States Pharmacopoeia (USP28, 2005), otherwise described in “Methods of Analysis 2.2.44. TOTAL ORGANIC CARBON IN WATER FOR PHARMACEUTICAL USE” of the European Pharmacopoeia (EP 5.0, 2005), the apparatus may be used for monitoring a pharmaceutical water supplying system, provided that sufficiently pure water not contaminated with ionic organic substances or organic substances containing nitrogen, sulfur or chlorine atoms is used as source water for 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 a sample solution containing ionic organic substances or organic substances comprised of nitrogen, sulfur, or halogens such as chlorine and the like; therefore, the apparatus should be selected appropriately depending on the purity of phar-

maceutical water to be measured and on the contamination risk in the case of apparatus failure.

3.6 Storage of Water for Injection

In storing *Water for Injection*, the water should be heated during circulation or other action to inhibit microbial proliferation. The maximum storage time allowed should also be appropriately established on the basis of validation studies, with considerations given to risks of contamination and quality deterioration.

22. Rapid Identification of Microorganisms Based on Molecular Biological Method

This chapter describes the methods for the identification or estimation of microorganisms (bacteria and fungi), found in in-process control tests or lot release tests of pharmaceutical products, at the species or genus level based on their DNA sequence homology. The identification of isolates found in the sterility test or aseptic processing can be helpful for investigating the causes of contamination. Furthermore, information on microorganisms found in raw materials used for pharmaceutical products, processing areas of pharmaceutical products, and so on is useful in designing measures to control the microbiological quality of drugs. For the identification of microorganisms, phenotypic analysis is widely used, based on morphological, physiological, and biochemical features and analysis of components. Commercial kits based on differences in phenotype patterns have been used for the identification of microorganisms, but are not always applicable to microorganisms found in raw materials used for pharmaceutical products and in processing areas of pharmaceutical products. In general, the identification of microorganisms based on phenotypic analysis needs special knowledge and judgment is often subjective. It is considered that the evolutionary history of microorganisms (bacteria and fungi) is memorized in their ribosomal RNAs (rRNAs), so that systematic classification and identification of microorganisms in recent years have been based on the analysis of these sequences. This chapter presents a rapid method to identify or estimate microorganisms based on partial sequences of divergent regions of the 16S rRNA gene for bacteria and of the internal transcribed spacer 1 (ITS1) region located between 18S rRNA and 5.8S rRNA for fungi, followed by comparison of the sequences with those in the database. Methods described in this chapter do not take the place of usual other methods for the identification, and can be modified based on the examiner's experience, and on the available equipment or materials. Other gene regions besides those mentioned in this chapter can be used if appropriate.

Apparatuses

(1) DNA sequencer

Various types of sequencers used a gel board or capillary can be used.

(2) DNA amplifier

To amplify target DNA and label amplified (PCR) products with sequencing reagents.

Procedures

The following procedures are described as an example.

1. Preparation of template DNA

It is important to use a pure cultivated bacterium or fungus for identification. In the case of colony samples, colonies are picked up with a sterilized toothpick (in the case of fungi, a small fragment of colony sample is picked up), and suspended in 0.3 mL of DNA releasing solution in a 1.5 mL centrifuge tube. In the case of culture fluid, a 0.5 mL portion of fluid is put in a 1.5 mL centrifuge tube and centrifuged at 10,000 rpm for 10 min. After removal of the supernatant, the pellet is suspended in 0.3 mL of DNA releasing solution, and then heated at 100°C for 10 min. In general, PCR can be run for bacteria and yeasts heated in DNA releasing solution. For fungi, DNA extraction after treatment with a mixer or ultrasonic generator may be necessary before PCR.

2. PCR

Add 2 μL of template DNA in PCR reaction solution. Use 10F/800R primers for bacteria and ITS1F/ITS1R primers for fungi, and then perform 30 amplification cycles at 94°C for 30 sec, 55°C for 60 sec, and 72°C for 60 sec. DNA fragments are amplified about 800 bp in the case of bacteria and about 150 – 470 bp depending on the strain in the case of fungi. Include a negative control (water instead of the test solution) in the PCR.

3. Confirmation of PCR products

Mix 5 μL of PCR product with 1 μL of loading buffer solution, place it in a 1.5 w/v% agarose gel well, and carry out electrophoresis with TAE buffer solution (1-fold concentration). Carry out the electrophoresis together with appropriate DNA size markers. After the electrophoresis, observe PCR products on a trans-illuminator (312 nm) and confirm the presence of a single band of the targeted size. If multiple bands are observed, cut the targeted band out of the gel, and extract DNA by using appropriate commercial DNA extraction kit.

4. Purification of PCR products

Remove unincorporated PCR primers and deoxynucleoside triphosphates (dNTP) from PCR products by using appropriate purification methods.

5. Quantification of purified DNA

When purified DNA is measured by spectrophotometer, calculate 1 $\text{OD}_{260\text{ nm}}$ as 50 $\mu\text{g}/\text{mL}$.

6. Labeling of PCR products with sequencing reagents

Use an appropriate fluorescence-labeled sequencing reagent suitable for the available DNA sequencer or its program and label the PCR products according to the instructions provided with the reagent.

7. Purification of sequencing reagent-labeled PCR products

Transfer the product in 75 μL of diluted ethanol (7 in 10) into a 1.5 mL centrifuge tube, keep in an ice bath for 20 min, and centrifuge at 15,000 rpm for 20 min. After removal of supernatant, add 250 μL of diluted ethanol (7 in 10) to the precipitate and centrifuge at 15,000 rpm for 5 min. Remove the supernatant and dry the precipitate.

8. DNA homology analysis

Place sequencing reagent-labeled PCR products in the DNA sequencer and read the nucleotide sequences of the PCR products. Compare the partial nucleotide sequence with those in the BLAST database.

Judgment

If sequencing data show over 90% identity with a sequence in the database, in general, judgment may be made as follows.

1. In the case of bacteria, compare about 300 nucleotides between positions 50 to 350 in the product obtained with the 10F primer, with the BLAST database. Higher ranked species are judged as identified species or closely related species.
2. In the case of fungi, compare sequencing data for the product obtained with the ITS1F primer, with the BLAST database. Higher ranked species are judged as identified species or closely related species.

Reagents, Test Solutions

(1) 0.5 mol/L Disodium dihydrogen ethylenediamine tetraacetate TS

Dissolve 18.6 g of disodium dihydrogen ethylenediamine tetraacetate dihydrate in water to make 100 mL.

(2) 1 mol/L Tris buffer solution, pH 8.0

Dissolve 24.2 g of 2-amino-2-hydroxymethyl-1,3-propanediol in a suitable amount of water, adjust the pH to 8.0 with 0.2 mol/L hydrochloric acid TS, and add water to make 200 mL.

(3) TE buffer solution

Mix 1.0 mL of 1 mol/L tris buffer solution, pH 8.0 and 0.2 mL of 0.5 mol/L disodium dihydrogen ethylenediamine tetraacetate TS, and add water to make 100 mL.

(4) DNA releasing solution

Divide TE buffer solution containing Triton X-100 (1 in 100) into small amounts and store frozen until use.

(5) PCR reaction solution

10-fold buffer solution*	5 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.

(6) Sequencing reagent

There are many kinds of sequencing methods, such as the dye-primer method for labeling of primer, the dye-terminator method for labeling of dNTP terminator and so on. Use an appropriate sequencing reagent kit for the apparatus and program to be used.

(7) 50-Fold concentrated TAE buffer solution

Dissolve 242 g of 2-amino-2-hydroxymethyl-1,3-propanediol in 57.1 mL of acetic acid (100) and 100 mL of 0.5 mol/L disodium dihydrogen ethylenediamine tetraacetate TS, and add water to make 1000 mL.

(8) 1-Fold concentrated TAE buffer solution

Diluted 50-fold concentrated TAE buffer solution (1 in 50) prepared before use is referred to as 1-fold concentrated TAE buffer solution.

(9) Agarose gel

Mix 1.5 g of agarose, 2.0 mL of 50-fold concentrated TAE buffer solution, 10 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.

(10) Loading buffer solution (6-fold concentrated)

Dissolve 0.25 g of bromophenol blue, 0.25 g of xylene cyanol FF and 1.63 g of disodium dihydrogen ethylenediamine tetraacetate dihydrate in 50 mL of water, and add 30 mL of glycerol and water to make 100 mL.

(11) PCR primers

For	Primer
Bacteria	10F 5'-GTTTGATCCTGGCTCA-3' 800R 5'-TACCAGGGTATCTAATCC-3'
Fungi	ITS1F 5'-GTAACAAGGT(T/C)TCCGT-3' ITS1R 5'-CGTTCTTCATCGATG-3'

23. 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.

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 interpolation in the

presence of suitable molecular-mass standards.

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 or separating (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 forms 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 broad limits 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

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.

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. *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 position at room temperature to allow polymerization to occur.

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.

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 μg to 10 μg of protein per band. Silver staining is the most sensitive method for staining proteins in gels and a band containing 10 ng to 100 ng can be detected. 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.

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.

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

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

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, add to the final rinsing a step of 5 minutes in a diluted solution of concentrated glycerin (1 in 50).

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 front end of the molecular mass marker migrates 80% of the migrating distance of the dye, 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.

Test solutions:

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.

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.

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

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.

Destaining TS A mixture of water, methanol and acetic acid (100) (5:4:1).

Blocking TS To 10 mL of acetic acid (100) add water to make 100 mL.

Trichloroacetic acid TS for fixing Dissolve 10 g of trichloroacetic acid in a mixture of water and methanol (5:4) to make 100 mL.

24. 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

$\text{g}/\text{cm}^3 = 1000 \text{ kg}/\text{m}^3$).

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).

25. 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 produc-

tion 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 vari-

ation 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 high-frequency 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 sterilization 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: Estimation of population of microorganisms on products
- 11) USP <1222> Terminally Sterilized Pharmaceutical Products - Parametric Release

26. Tablet Friability Test

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

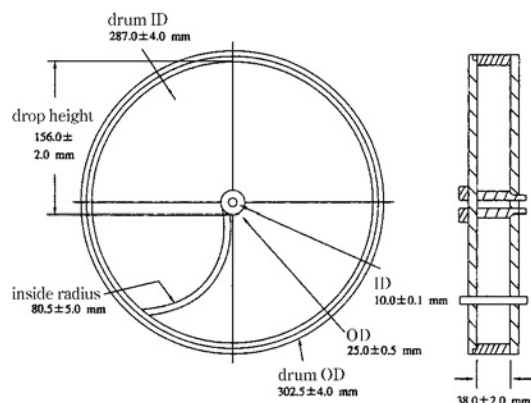
The Tablet Friability Test is a method to determine the friability of compressed uncoated tablets. The test procedure presented in this chapter is generally applicable to most compressed tablets. Measurement of tablets friability supplements other physical strength measurement, such as tablet crushing 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 surface, and not 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 outside diameter of the central shaft 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 weight loss is greater than the targeted value, the test should be repeated twice and the mean of the three tests determined. A maximum mean weight loss from the three samples of not more than 1% 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 bench top 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.

27. 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.

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.

Product: A generic term used to describe raw materials, intermediate products, and finished products, to be sterilized.

Bioburden: Numbers and types of viable microorganisms in a product to be sterilized.

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} .

Integrity test: A non-destructive test which is used to predict the functional performance of a filter instead of the microorganism challenge test.

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.

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.

(i) 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.

(ii) 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.

(i) 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.

(ii) Microwave method

Microorganisms are killed by the heat generated by microwave 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</i>	ATCC 7953, NBRC 13737, JCM 9488
	<i>stearo-thermophilus</i>	ATCC 12980, NBRC 12550, JCM 2501
	<i>Bacillus atrophaeus</i>	ATCC 9372, NBRC 13721
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

(i) 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.

(ii) 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°C for 7 days in the case of *G. stearothermophilus* and at 30 – 35°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.

References

- 1) ISO 11134 Industrial moist heat sterilization
- 2) ISO 11135 Ethylene oxide sterilization
- 3) ISO 11137 Radiation sterilization
- 4) ISO 11138 Biological indicators
- 5) ISO 11140 Chemical indicators
- 6) ISO 11737 Microbiological methods
Part 1: Estimation of population of microorganisms on products

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

Trans parenteral nutrition solutions (TPNs) are nutrient preparations for intravenous injection packed in a large volume. 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: (1) High-Performance Liquid Chromatography using a fluorescence photometric detector (HPLC with fluorescence detection), (2) Inductivity Coupled Plasma-Atomic Emission Spectrometry (ICP-AES method), (3) 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., (1) Quinolinol complexing method, (2) 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.

Preparation of sample solution

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

Preparation of a series of standard solutions for calibration curve

Pipet 1 mL of water for aluminum test exactly, and after adding 10 μ 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).

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 alu-

minum. 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.

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.

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).

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.

The following points should be noted in connection with measuring trace amounts of aluminum in TPN preparations.

- 1) 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.
- 2) Before the measurement, it is necessary to confirm that the characteristic properties of the sample do not affect the formation of the complex.

- 3) 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).

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.

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

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

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.

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.

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

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.

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).

Tetramethylammonium hydroxide TS $(CH_3)_4NOH$

It is a 25% aqueous solution, prepared for aluminum test. Further, it contains not more than 1 ppb of aluminum.

Water for aluminum test Same as the monograph *Purified Water*. Further, it contains not more than 1 ppb of aluminum.

29. Total Protein Assay

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

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 non-ionic 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- μ m porosity or clarification by centrifugation may be performed to reduce the effect of light-scattering, especially if the solution is noticeably turbid.

Calculations Calculate the concentration, C_U , of protein in the test specimen by the formula:

$$C_U = C_S (A_U/A_S),$$

in which C_S is the concentration of the Standard Solution; and A_U and A_S are the corrected absorbances of the Test Solution and the Standard Solution, respectively.

Method 2

This method, commonly referred to as the Lowry assay, is based on the reduction by protein of the 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 substances, a procedure for precipitation of the protein from the test specimen may be used. Where separation of interfering substances from the protein in the test specimen is necessary, proceed as directed below for Interfering Substances prior to preparation of the Test Solution. The effect of interfering substances can be minimized by dilution provided the concentration of the protein under test remains sufficient for accurate measurement. Variations of the Lowry test that are indicated in national regulatory 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 remove interfering substances by precipitation of proteins before testing. This technique also can be used to concentrate proteins from a dilute solution.

Sodium Deoxycholate Reagent Prepare a solution of sodium deoxycholate in water having a concentration of 150 mg in 100 mL.

Trichloroacetic Acid Reagent Prepare a solution of trichloroacetic acid in water having a concentration of 72 g in 100 mL.

Procedure Add 0.1 mL of Sodium Deoxycholate Reagent to 1 mL of a solution of the protein under test. Mix on a vortex mixer, and allow to stand at room temperature for 10 minutes. Add 0.1 mL of Trichloroacetic Acid Reagent, and mix on a vortex mixer. Centrifuge at $3000 \times g$ 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 Test Solution. [Note: Color development reaches a maximum in 20 to 30 minutes during incubation at room temperature, after which there is a gradual loss of color. Most interfering substances cause a lower color yield; however, some detergents cause a slight increase in color. A high salt concentration may cause a precipitate to form. Because different protein species may give different color response intensities, the standard protein and test protein should be the same.]

Method 3

This method, commonly referred to as the Bradford assay, is based on the absorption shift from 470 nm to 595 nm observed when the brilliant blue G-250 dye binds to protein. The Coomassie Brilliant Blue G-250 dye binds most readily to arginyl and lysyl residues in the protein, which can lead to variation in the response of the assay to different proteins.

Standard Solutions Unless otherwise specified in the individual monograph, dissolve the reference standard or the reference material for the protein under test in the buffer used to prepare the Test Solution. Dilute portions of this solution with the same buffer to obtain not fewer than five Standard Solutions having concentrations between 100 μg and 1 mg of protein per mL, the concentrations being evenly spaced.

Test Solution Dissolve a suitable quantity of the protein under test in the appropriate buffer to obtain a solution having a concentration within the range of the concentrations of the Standard Solutions.

Blank Use the buffer used to prepare the Test Solution and the Standard Solutions.

Coomassie Reagent Dissolve 100 mg of brilliant blue G-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 rea-

¹⁾ Example: the Minimum Requirements for Biological Products and individual monograph of JP.♦

²⁾ Dye purity is important in the reagent preparation.

gent. Filter the reagent before use.]

Procedure Add 5 mL of the Coomassie Reagent to 100 μ L of each Standard Solution, the Test Solution, and the Blank, and mix by inversion. Avoid foaming, which will lead to poor reproducibility. Determine the absorbances of the solutions from the Standard Solutions and the Test Solution at 595 nm, with a suitable spectrophotometer, using the Blank to set the instrument to zero.

[Note: Do not use quartz (silica) spectrophotometer cells: the dye binds to this material. Because different protein species may give different color response intensities, the standard protein and test protein should be the same.] There are relatively few interfering substances, but detergents and ampholytes in the test specimen should be avoided. Highly alkaline specimens may interfere with the acidic reagent.

Calculations [Note: The relationship of absorbance to protein concentration is nonlinear; however, if the standard curve concentration range is sufficiently small, it will approach linearity.] Using the linear regression method, plot the absorbances of the solutions from the Standard Solutions versus the protein concentrations, and determine the standard curve best fitting the plotted points. From the standard curve so obtained and the absorbance of the Test Solution, determine the concentration of protein in the Test Solution.

Method 4

This method, commonly referred to as the bicinchoninic acid or BCA assay, is based on reduction of the cupric (Cu^{2+}) ion to cuprous (Cu^+) ion by protein. The bicinchoninic acid reagent is used to detect the cuprous ion. The method has few interfering substances. When interfering substances are present, their effect may be minimized by dilution, provided that the concentration of the protein under test remains sufficient for accurate measurement.

Standard Solutions Unless otherwise specified in the individual monograph, dissolve the reference standard or the reference material for the protein under test in the buffer used to prepare the Test Solution. Dilute portions of this solution with the same buffer to obtain not fewer than five Standard Solutions having concentrations between 10 and 1200 μ g 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 absor-

bances of the solutions from the Standard Solutions and the Test Solution in quartz cells at 562 nm, with a suitable spectrophotometer, using the Blank to set the instrument to zero. After the solutions are cooled to room temperature, the color intensity continues to increase gradually. If substances that will cause interference in the test are present, proceed as directed for Interfering Substances under Method 2. Because different protein species may give different color response intensities, the standard protein and test protein should be the same.

Calculations [Note: The relationship of absorbance to protein concentration is nonlinear; however, if the standard curve concentration range is sufficiently small, it will approach linearity.] Using the linear regression method, plot the absorbances of the solutions from the Standard Solutions versus the protein concentrations, and determine the standard curve best fitting the plotted points. From the standard curve so obtained and the absorbance of the Test Solution, determine the concentration of protein in the Test Solution.

Method 5

This method, commonly referred to as the Biuret assay, is based on the interaction of cupric (Cu^{2+}) ion with protein in an alkaline solution and the resultant development of absorbance at 545 nm.

Standard Solutions Unless otherwise specified in the individual monograph, prepare a solution of Albumin Human for which the protein content has been previously determined by nitrogen analysis (using the nitrogen-to-protein conversion factor of 6.25) or of the reference standard or reference material for the protein under test in sodium chloride solution (9 in 1000). Dilute portions of this solution with sodium chloride solution (9 in 1000) to obtain not fewer than three Standard Solutions having concentrations between 0.5 and 10 mg per mL, the concentrations being evenly spaced. [Note: Low responses may be observed if the sample under test has significantly different level of proline than that of Albumin Human. A different standard protein may be employed in such cases.]

Test Solution Prepare a solution of the test protein in sodium chloride solution (9 in 1000) having a concentration within the range of the concentrations of the Standard Solutions.

Blank Use sodium chloride solution (9 in 1000).

Biuret Reagent Dissolve about 3.46 g of copper (II) sulfate pentahydrate in 10 mL of water, with heating if necessary, and allow to cool (Solution A). Dissolve about 34.6 g of sodium citrate dihydrate and 20.0 g of anhydrous sodium carbonate in 80 mL of water, with heating if necessary, and allow to cool (Solution B). Mix Solutions A and B, and dilute with water to 200 mL. This Biuret Reagent is stable at room temperature for 6 months. Do not use the reagent if it develops turbidity or contains any precipitate.

Procedure To one volume of the Standard Solutions and a solution of the Test Solution add an equal volume of sodium hydroxide solution (6 in 100), and mix. Immediately add a volume of Biuret Reagent equivalent to 0.4 volume of the Test Solution, and mix. Allow to stand at a temperature between 15°C and 25°C for not less than 15 minutes. Within 90 minutes after the addition of the Biuret Reagent, determine the absorbances of the Standard Solutions and the solution from the Test Solution at the wavelength of maximum absorbance at 545 nm, with a suitable spectrophotome-

ter, using the Blank to set the instrument to zero. [Note: Any solution that develops turbidity or a precipitate is not acceptable for calculation of protein concentration.]

Calculations Using the least-squares linear regression method, plot the absorbances of the Standard Solutions versus the protein concentrations, and determine the standard curve best fitting the plotted points, and calculate the correlation coefficient for the line. [Note: Within the given range of the standards, the relationship of absorbance to protein concentration is approximately linear.] A suitable system is one that yields a line having a correlation coefficient of not less than 0.99. From the standard curve and the absorbance of the Test Solution, determine the concentration of protein in the test specimen, making any necessary correction.

Interfering Substances To minimize the effect of interfering substances, the protein can be precipitated from the initial test specimen as follows. Add 0.1 volume of 50% trichloroacetic acid to 1 volume of a solution of the test specimen, withdraw the supernatant layer, and dissolve the precipitate in a small volume of 0.5 mol/L sodium hydroxide TS. Use the solution so obtained to prepare the Test Solution.

Comments This test shows minimal difference between equivalent IgG and albumin samples. Addition of the sodium hydroxide and the Biuret Reagent as a combined reagent, insufficient mixing after the addition of the sodium hydroxide, or an extended time between the addition of the sodium hydroxide solution and the addition of the Biuret Reagent will give IgG samples a higher response than albumin samples. The trichloroacetic acid method used to minimize the effects of interfering substances also can be used to determine the protein content in test specimens at concentrations below 500 μg per mL.

Method 6

This fluorometric method is based on the derivatization of the protein with *o*-phthalaldehyde (OPA), which reacts with the primary amines of the protein (i.e., NH_2 -terminal amino acid and the ϵ -amino group of the lysine residues). The sensitivity of the test can be increased by hydrolyzing the protein before testing. Hydrolysis makes the α -amino group of the constituent amino acids of the protein available for reaction with the OPA reagent. The method requires very small quantities of the protein.

Primary amines, such as tris(hydroxymethyl)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 an amine reacts with OPA can be unstable. The use of automated procedures to standardize this procedure may improve the accuracy and precision of the test.

Standard Solutions Unless otherwise specified in the individual monograph, dissolve the reference standard or the reference material for the protein under test in the buffer used to prepare the Test Solution. Dilute portions of this solution with the same buffer to obtain not fewer than five Standard Solutions having concentrations between 10 and 200 μg of protein per mL, the concentrations being evenly spaced.

Test Solution Dissolve a suitable quantity of the protein under test in the appropriate buffer to obtain a solution having a concentration within the range of the concentrations of the Standard Solutions.

Blank Use the buffer used to prepare the Test Solution and the Standard Solutions.

Reagents and Solutions—

Borate Buffer Dissolve about 61.83 g of boric acid in water, and adjust with potassium hydroxide to a pH of 10.4. Dilute with water to 1000 mL, and mix.

Stock OPA Reagent Dissolve about 120 mg of *o*-phthalaldehyde in 1.5 mL of methanol, add 100 mL of Borate Buffer, and mix. Add 0.6 mL of polyoxyethylene (23) lauryl ether, and mix. This solution is stable at room temperature for at least 3 weeks.

OPA Reagent To 5 mL of Stock OPA Reagent add 15 μL of 2-mercaptoethanol. Prepare at least 30 minutes prior to use. This reagent is stable for one day.

Procedure Adjust each of the Standard Solutions and the Test Solution to a pH between 8.0 and 10.5. Mix 10 μL of the Test Solution and each of the Standard Solutions with 100 μL of OPA Reagent, and allow to stand at room temperature for 15 minutes. Add 3 mL of 0.5 mol/L sodium hydroxide TS, and mix. Using a suitable fluorometer, determine the fluorescent intensities of solutions from the Standard Solutions and the Test Solution at an excitation wavelength of 340 nm and an emission wavelength between 440 and 455 nm. [Note: The fluorescence of an individual specimen is read only once because irradiation decreases the fluorescent intensity.]

Calculations The relationship of fluorescence to protein concentration is linear. Using the linear regression method, plot the fluorescent intensities of the solutions from the Standard Solutions versus the protein concentrations, and determine the standard curve best fitting the plotted points. From the standard curve so obtained and the fluorescent intensity of the Test Solution, determine the concentration of protein in the test specimen.

Method 7

This method is based on nitrogen analysis as a means of protein determination. Interference caused by the presence of other nitrogen-containing substances in the test protein can affect the determination of protein by this method. Nitrogen analysis techniques destroy the protein under test but are not limited to protein presentation in an aqueous environment.

Procedure A Determine the nitrogen content of the protein under test as directed elsewhere in the Pharmacopoeia. Commercial instrumentation is available for the Kjeldahl nitrogen assay.

Procedure B Commercial instrumentation is available for nitrogen analysis. Most nitrogen analysis instruments use pyrolysis (i.e., combustion of the sample in oxygen at temperatures approaching 1000°C), which produces nitric oxide (NO) and other oxides of nitrogen (NO_x) from the nitrogen present in the test protein. Some instruments convert the nitric oxides to nitrogen gas, which is quantified with a thermal conductivity detector. Other instruments mix nitric oxide (NO) with ozone (O_3) to produce excited nitrogen dioxide (NO_2^*), which emits light when it decays and can be quantified with a chemiluminescence detector. A protein reference standard or reference material that is relatively pure and is similar in composition to the test proteins is used to optimize the injection and pyrolysis parameters and to evaluate consistency in the analysis.

Calculations The protein concentration is calculated by dividing the nitrogen content of the sample by the known nitrogen content of the protein. The known nitrogen content of the protein can be determined from the chemical composition of the protein or by comparison with the nitrogen

content of the appropriate reference standard or reference material.

30. 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 words, 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.

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

(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.

(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.

(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.

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.

(1) Accuracy/Trueness

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.

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) Precision

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 oper-

ating 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).

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.

(3) Specificity

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.

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).

(4) Detection limit

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

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.

(5) Quantitation limit

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%.

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.

(6) Linearity

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.

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.

(7) Range

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.

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.

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.

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

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

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.

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

Validation characteristics \ Type of test	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

Terminology used in the validation of analytical procedures

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.

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.

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.

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

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.

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.

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.

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