[Appendix 6]

General Information
(Related to Article 2 Subparagraph 6)

General Information

General Information

Analytical Method for Alkylsulphonic Acid Esters
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This chapter is a method that measures the alkyl methansulfonate esters, alkyl ethylsulfonate esters, alkyl propylsulfonate esters, or methane sulfonyl chloride, which are potential impurities in the production process of pharmaceutical products using chromatography mass spectrometry (GC-MS).

1. Analytical method for alkyl (methyl, ethyl, and isopropyl) methanesulfonates in methanesulfonic acid`

The analytical method for alkyl (methyl, ethyl, and isopropyl) methanesulfonates in methanesulfonic acid has been validated in the range of 0.5 to 100 ppm. Adjust the concentration of the test solution appropriately and apply it if it deviates from the validated range due to reasons such as high concentration in the initial synthesis step.

Apparatus

Gas chromatography-mass spectrometer

Procedure **1)** ***Preparation of standard solution*** Accurately weigh about 50 mg of methyl methanesulfonate, ethyl methanesulfonate, and isopropyl methanesulfonate reference standard, respectively, and add the internal standard solution to make exactly 50 mL and use it as the standard stock solution. Add the internal standard solution to 74 μL of the standard stock solution to prepare exactly 10 mL, take exactly 100 μL of this solution again, and add the internal standard solution to it to prepare exactly 10 mL and use it as Standard Solution (1). Add the internal standard solution to 3 mL of Standard Solution (1) to make exactly 10 mL and use it as Standard Solution (2).

Internal standard solution: The solution of methylene chloride of butyl methanesulfonate (7 in 100,000,000).

2) Preparation of the test solution

Weigh accurately about 0.74 g of this drug and add exactly 10 mL of water and 10 mL of internal standard solution, shake it slowly to mix, and perform extraction. Separate the organic layer and transfer it to a vial containing sodium sulfate anhydrous, shake it to mix, and then take the supernatant and filter it using a membrane filter. Discard the first filtrate and use the subsequent filtrate as the test solution.

3) ***Apparatus operating conditions***

Detector: Mass spectrometer

Column: A fused silica column, about 0.25 mm in internal diameter and about 15 m in length, coated with 100% methyl polysiloxane for gas chromatography-Mass spectrometer to 1 μm in thickness, or an equivalent. Column temperature: Inject the sample at 55 ºC, maintain the temperature for 1 minute, and then increase it to 135 ºC at a rate of 10 ºC per minute.

Flow rate: 1 mL/min

Carrier gas: Helium

Injection mode: Pulsed splitless (250 kPa, 0.25 min)

Injection vol.: 2 μL

Inlet temp.: 240 ºC

Detector temp.:

Transfer line temp.: 280 ℃

Ion source: 230 ℃

Analyzer: 150 ℃

Measurement mode: SIM

Ions:

Ingredient name	Ions (m/z)
Methyl methanesulfonate	80
Ethyl methanesulfonate	79
Isopropyl methanesulfonate	123
Butyl methanesulfonate	56

System suitability:

Test for required detectability: The signal-to-noise ratio of each peak of methyl methanesulfonate, ethyl methanesulfonate, and isopropyl methanesulfonate obtained from Standard Solution (2) is NLT 10.

System performance: The resolution between the peaks of ethyl methanesulfonate and isopropyl methanesulfonate obtained from Standard Solution (1) is NLT 3.

4) ***Testing method***

Test according to the operating conditions described above, and calculate the peak area ratio, Q_T and Q_S, of each drug substance in the test solution and Standard Solution (1) to the peak area of the internal standard.

$$\text{Amount (ppm) of each alkyl (methyl, ethyl, and isopropyl) methanesulfonate} = (Q_T / Q_S) \times (M_S / M_T) \times C \times 0.148$$

Q_S: Peak area ratio of each drug substance to the peak area of the internal standard in Standard Solution (1)

Q_T: Peak area ratio of each drug substance to the peak area of the internal standard in the test solution

M_S: Amount of the reference standard for each drug substance (mg)

M_T: Amount of the sample (mg)

C: Purity of the reference standard for each drug substance (%)

0.148: Dilution factor

2. Analytical method for methanesulfonyl chloride in methanesulfonic acid

The analytical method for methanesulfonyl chloride in methanesulfonic acid has been validated in the range of 0.05 to 50 ppm.

Apparatus Gas chromatography-mass spectrometer

Preparation **1)** ***Preparation of standard solution*** Accurately weigh about 50 mg of methanesulfonyl chloride RS, add methylene chloride to make exactly 10 mL and use it as the standard stock solution. Take exactly 1 mL of the standard stock solution and add methylene chloride to make exactly 10 mL. Then, take exactly 300 μL of this solution dilute, add methylene chloride to make exactly 10 mL, and use it as the Standard Solution (1). Take exactly 500 μL of Standard Solution (1) and 100 μL of the internal standard solution and add methylene chloride to make exactly 15 mL, and use it as the Standard Solution (2). Take exactly 25 μL of the Standard Solution (1) and 100 μL of the internal standard solution, add methylene chloride to prepare exactly 15 mL, and use it as the Standard Solution (3).

Internal standard solution: The solution of methylene

chloride of butyl methanesulfonate (7 in 100,000).

2) *Preparation of the test solution*

Weigh accurately about 7.4 g of this drug and add 5 mL of water. Mix it slowly. After cooling, add exactly 5 mL of methylene chloride and 100 μL of the internal standard solution to the solution. Shake well to mix. Separate the organic layer, transfer it to a vial containing sodium sulfate anhydrous, and shake. Extraction is repeated twice with 5 mL of methylene chloride each time. Collect the organic layer and filter it through a membrane filter. Discard the first filtrate and use the subsequent filtrate as the test solution.

3) *Apparatus operating conditions*

Detector: Mass spectrometer

Column: A fused silica column, about 0.25 mm in internal diameter and about 15 m in length, coated with 100% methyl polysiloxane for gas chromatography-Mass spectrometer to 1 μm in thickness, or equivalent.

Column temperature: Inject the sample at 40 ℃, maintain the temperature for 4 minutes, and then increase it to 200 ℃ at a rate of 40 ℃ per minute. At the end of the analysis, raise the temperature of the column to 270 ℃ and maintain it for 8 minutes.

Flow rate: 1 mL/min

Carrier gas: Helium

Injection mode: Pulsed splitless (60 kPa, 0.1 min)

Injection vol.: 5 μL

Inlet temp.: 240 ℃

Detector temp.:

Transfer line temp.: 280 ℃

Ion source: 230 ℃

Analyzer: 150 ℃

Mode: SIM

Ions:

Drug substance	Ions (m/z)
Methanesulfonyl chloride	79
Butyl methanesulfonate	56

System suitability:

Test for required detectability: The signal-to-noise ratio of the peak of methanesulfonyl chloride obtained from Standard Solution (3) is NLT 10.

System Performance: The resolution between the peaks of methanesulfonyl chloride and butyl methanesulfonate obtained from Standard Solution (2) is NLT 5.

4) *Analytical procedures*

Test according to the operating conditions described above to calculate the peak area ratio, Q_T and Q_S, of methanesulfonyl chloride in the test solution and standard solution (2) to the peak area of the internal standard.

$$\text{Amount (ppm) of methanesulfonyl chloride} = (Q_T / Q_S) \times (M_S / M_T) \times C \times 1.5$$

Q_S: Peak area ratio of methanesulfonyl chloride to the peak area of the internal standard in Standard Solution (2)

Q_T: Peak area ratio of methanesulfonyl chloride to the peak area of the internal standard in the test solution

M_S: Amount of the reference standard for methanesulfonyl chloride (mg)

M_T: Amount of the sample (mg)

C: Purity of the methanesulfonyl chloride reference standard (%)

1.5: Dilution factor

3. Analytical method for alkyl (methyl, ethyl, and isopropyl) methanesulfonates in drug substances

The analytical method for alkyl (methyl, ethyl, and isopropyl) methane sulfonates in drug substances has been validated in the range of 0.2 to 5 ppm using betahistine mesylate. When applying other drug substances, especially if the concentration deviates from the validated range due to high concentration in the initial synthesis step, etc., adjust the concentration of the test solution appropriately and check its effectiveness.

Apparatus Gas chromatography-mass spectrometer

Procedure **1) *Preparation of standard solution*** Weigh accurately about 25 mg of methyl methanesulfonate, ethyl methanesulfonate, and isopropyl methanesulfonate reference standard, respectively, and add toluene to prepare exactly 5 mL. Take exactly 50 μL of this solution, add the internal standard solution to make exactly 25 mL and use it as Standard Solution (1). Take exactly 20 μL of Standard Solution (1), and add the internal standard solution to make exactly 20 mL. Take exactly 0.5 mL of this solution and 0.5 mL of the derivatization reagent, transfer them into a headspace vial, and immediately seal the vial tightly with a PTFE-coated silicone membrane and an aluminum cap. Use this as Standard Solution (2). Take exactly 500 μL of Standard Solution (1), and add the internal standard solution to prepare exactly 20mL. Take exactly 0.5 mL of this solution and 0.5 mL of the derivatization solution, transfer them into a headspace vial, and immediately seal the vial tightly with a PTFE-coated silicone membrane and an aluminum cap. Use this as Standard Solution (3).

Internal standard solution: A mixture (4:1) solution of the acetonitrile of butyl methanesulfonate and water (1 in 5,000,000)

Derivatization solution: Weigh accurately 30 mg of sodium thiosulfate anhydrous and 60 g of sodium iodide and dissolve them in water to make exactly 50 mL.

※ Precipitation may be observed in the derivatization reaction, but it does not affect the quantification.

2) *Preparation of the test solution*

Weigh accurately about 25 mg of this drug and put it into a headspace vial. Add exactly 0.5 mL of the internal standard solution and 0.5 mL of the derivatization solution to the vial and immediately seal it tightly with a PTFE-coated silicone septum and an aluminum cap. Use it as the blank test solution.

Blank test solution: Take 0.5 mL of the derivatization solution and 0.5 mL of the internal standard solution and put them into a headspace vial. Immediately seal the vial tightly with a PTFE-coated silicone septum and an aluminum cap, and use it as the blank test solution.

3) *Apparatus operating conditions*

Detector: Mass spectrometer

Column: A fused silica column, about 0.25 mm in internal diameter and about 30 m in length, coated with cyanopolysiloxane for gas chromatography-Mass spectrometer to 1 μm in thickness, or equivalent.

Column temperature: Inject the sample at 40 ℃, maintain

the temperature for 1 minute, then increase it to 130 ℃ at a rate of 10 ℃ per minute. At the end of the analysis, raise the temperature of the column to 240 ℃ and maintain it for 7 minutes.

Carrier gas: Helium
Flow rate: 0.5 mL/min
Split ratio: 1:20

Conditions for the sample injection apparatus for the headspace

Equilibrium temp.: 60 ℃
Equilibrium time: 30 minutes
Transfer line temp.: 120 ℃

Injection vol.: 1 mL
Inlet temp.: 220 ℃
Detector temp.:

Transfer line temp.: 280 ℃
Ion source: 250 ℃
Analyzer: 200 ℃

Mode: SIM
Ions:

Drug substance	Quantitation (m/z)	Qualification (m/z)
Methyl iodide*	142	127
Ethyl iodide*	156	127
Isopropyl iodide*	170	127
Butyl iodide*	184	127

*Derivatization products

System suitability:

Test for required detectability: The signal-to-noise ratio of each peak of alkyl iodides obtained from Standard Solution (2) is NLT 10.

System performance: The resolution between the peaks of ethyl iodide and isopropyl iodide obtained from Standard Solution (3) is NLT 1.5.

4) *Testing method*

Test according to the operating conditions described above, and calculate the peak area ratio, Q_T and Q_S, of each drug substance in the test solution and standard solution (3) to the peak area of the internal standard.

Amount (ppm) of each alkyl (methyl, ethyl, and isopropyl) methanesulfonate = $(Q_T / Q_S) \times (M_S / M_T) \times C \times 0.05$

Q_S: Peak area ratio of each drug substance to the peak area of the internal standard in Standard Solution (3)
Q_T: Peak area ratio of each drug substance to the peak area of the internal standard in the test solution
M_S: Amount of the reference standard for each drug substance (mg)
M_T: Amount of the sample (mg)
C: Purity of the reference standard for each drug substance (%)
0.05: Dilution factor

4. Analytical method for alkyl (methyl, ethyl, and isopropyl) methanesulfonates in drug substances

The analytical method for alkyl (methyl, ethyl, and isopropyl) methane sulfonates in drug substances has been validated in the range of 0.2 to 5 ppm. When applied to different drug substances, especially when the concentration of alkyl toluenesulfonates deviates from the validated range, adjust the concentration of the test solution appropriately and check its effectiveness.

Apparatus Gas chromatography-mass spectrometer

Procedure **1) *Preparation of standard solution*** Weigh accurately about 25 mg of methyl toluenesulfonate, ethyl toluenesulfonate, isopropyl toluenesulfonate standard, respectively, and add toluene to make exactly 5 mL. Take exactly 50 μL of this solution, add the internal standard solution to make exactly 25 mL and use it as Standard Solution (1). Take exactly 40 μL of Standard Solution (1), add the internal standard solution to make exactly 20 mL. Take exactly 0.5 mL of this solution and 0.5 mL of the derivatization solution, transfer them into a headspace vial, and immediately seal the vial tightly with a PTFE-coated silicone membrane and an aluminum cap. Use it as Standard Solution (2). Take exactly 500 μL of Standard Solution (1), and add the internal standard solution to prepare exactly 20mL. Take exactly 0.5 mL of this solution and 0.50 mL of the derivatization solution, transfer them into a headspace vial, and immediately seal the vial tightly with a PTFE-coated silicone membrane and an aluminum cap. Use it as Standard Solution (3).

Internal standard solution: A mixture (4:1) solution of the acetonitrile of butyl methanesulfonate and water (1 in 5,000,000)

Derivatization solution: Weigh accurately 30 mg of sodium thiosulfate anhydrous and 60 g of sodium iodide and dissolve them in water to make exactly 50 mL.

※ Precipitation may be observed in the derivatization reaction, but it does not affect the quantification.

2) *Preparation of the test solution* Weigh accurately about 25 mg of this drug and put it into a headspace vial. Add exactly 0.5 mL of the internal standard solution and 0.5 mL of the derivatization solution to the vial and immediately seal it tightly with a PTFE-coated silicone septum and an aluminum cap. Use it as the test solution.

Blank test solution: Take 0.5 mL of the derivatization solution and 0.5 mL of the internal standard solution and put them into a headspace vial. Immediately seal the vial tightly with a PTFE-coated silicone septum and an aluminum cap, and use it as the blank test solution.

3) *Apparatus operating conditions*

Detector: Mass spectrometer

Column: A fused silica column, about 0.25 mm in internal diameter and about 30 m in length, coated with cyanopolysiloxane for gas chromatography-Mass spectrometer to 1 μm in thickness, or an equivalent.

Column temperature: Inject the sample at 40 ℃, maintain the temperature for 1 minute, then increase it to 130 ℃ at a rate of 10 ℃ per minute. At the end of the analysis, raise the temperature of the column to 240 ℃ and maintain it for 7 minutes.

Carrier gas: Helium
Flow rate: 0.5 mL/min
Split ratio: 1:20

Conditions for the sample injection apparatus for the headspace

Equilibrium temp.: 60 ℃
Equilibrium time: 30 minutes
Transfer line temp.: 120 ℃

Injection vol.: 1 mL
Inlet temp.: 220 °C
Detector temp.:
Transfer line temp.: 280 °C
Ion source: 250 °C
Analyzer: 200 °C
Mode: SIM
Ions:

Drug substance	Quantitation (m/z)	Qualification ion (m/z)
Methyl iodide*	142	127
Ethyl iodide*	156	127
Isopropyl iodide*	170	127
Butyl iodide*	184	127

*Derivatization products

System suitability:

Test for required detectability: The signal-to-noise ratio of each peak of alkyl iodides obtained from Standard Solution (2) is NLT 10.

System performance: The resolution between the peaks of ethyl iodide and isopropyl iodide obtained from Standard Solution (3) is NLT 1.5.

4) *Testing method*

Test according to the operating conditions described above, and calculate the peak area ratio, Q_T and Q_S, of each drug substance in the test solution and Standard Solution (3) to the peak area of the internal standard.

$$\text{Amount of each alkyl (methyl, ethyl, and isopropyl) toluenesulfonate (ppm)} = (Q_T / Q_S) \times (M_S / M_T) \times C \times 0.05$$

Q_S: Peak area ratio of each drug substance to the peak area of the internal standard in Standard Solution (3)
Q_T: Peak area ratio of each drug substance to the peak area of the internal standard in the test solution
M_S: Amount of the reference standard for each drug substance (mg)
M_T: Amount of the sample (mg)
C: Purity of the reference standard for each drug substance (%)
0.05: Dilution factor

5. Analytical method for alkyl (methyl, ethyl, and isopropyl) benzene sulfonates in drug substances

The analytical method for alkyl (methyl, ethyl, and isopropyl) benzene sulfonates in drug substances was evaluated in the range of 2.5 to 40 ppm using amlodipine besylate. When applied to different drug substances, especially when the concentration of alkyl benzene sulfonates deviates from the effectiveness evaluation range, adjust the concentration of the test solution and the standard solution appropriately and validate it.

※ In the case of clopidogrel besylate, the use of this test method is not suitable as methyl benzene sulfonate is observed as an artificial degradation product during the gas chromatography.

Apparatus Gas chromatography-mass spectrometer

Procedure 1) *Preparation of standard solution* Weigh accurately about 25 mg of each methyl benzenesulfonate, ethyl benzenesulfonate, isopropyl benzenesulfonate reference standard, and add toluene to prepare exactly 5 mL. Take exactly 50 μL of this solution, add the internal standard solution to make exactly 25 mL and use it as Standard Solution (1). Take exactly 40 μL of Standard Solution (1), and add the internal standard solution to it to prepare exactly 20mL. Take exactly 0.5 mL of this solution and 0.5 mL of the derivatization solution. Transfer them into a headspace vial and immediately seal the vial tightly with a PTFE-coated silicone membrane and an aluminum cap. Use this as Standard Solution (2). Take exactly 500 μL of Standard Solution (1), and add the internal standard solution to it to prepare exactly 20mL. Take exactly 0.5 mL of this solution and 0.5 mL of the derivatization solution. Transfer them into a headspace vial and immediately seal the vial tightly with a PTFE-coated silicone membrane and an aluminum cap. Use this as Standard Solution (3).

Internal standard solution: A mixture (4:1) solution of the acetonitrile of butyl methanesulfonate and water (1 in 5,000,000)

Derivatization solution: Weigh accurately 30 mg of sodium thiosulfate anhydrous and 60 g of sodium iodide and dissolve them in water to make exactly 50 mL.

※ Precipitation may be observed in the derivatization reaction, but it does not affect the quantification.

2) *Preparation of the test solution* Weigh accurately about 25 mg of this drug and put it into a headspace vial. Add exactly 0.5 mL of the internal standard solution and 0.5 mL of the derivatization solution to the vial and immediately seal it tightly with a PTFE-coated silicone septum and an aluminum cap. Use it as the test solution.

Blank test solution: Take 0.5 mL of the derivatization solution and 0.5 mL of the internal standard solution and put them into a headspace vial. Immediately seal the vial tightly with a PTFE-coated silicone septum and an aluminum cap, and use it as the blank test solution.

3) *Apparatus operating conditions*

Detector: Mass spectrometer

Column: A fused silica column, about 0.25 mm in internal diameter and about 30 m in length, coated with cyanopolysiloxane for gas chromatography-Mass spectrometer to 1 μm in thickness, or an equivalent.

Column temperature: Inject the sample at 40 °C, maintain the temperature for 1 minute, and then increase it to 130 °C at a rate of 10 °C per. At the end of the analysis, raise the temperature of the column to 240 °C and maintain it for 7 minutes.

Carrier gas: Helium
Flow rate: 0.5 mL/min
Split ratio: 1:20

Conditions for the sample injection apparatus for the headspace
Equilibrium temp.: 60 °C
Equilibrium time: 30 minutes
Transfer line temp.: 120 °C
Injection vol.: 1 mL
Inlet temp.: 220 °C
Detector temp.:
Transfer line temp.: 280 °C
Ion source: 250 °C
Analyzer: 200 °C
Mode: SIM

Ions:

Drug substance	Quantitation (m/z)	Qualification ion (m/z)
Methyl iodide*	142	127
Ethyl iodide*	156	127
Isopropyl iodide*	170	127
Butyl iodide*	184	127

*Derivatization products

System suitability:

Test for required detectability: The signal-to-noise ratio of each peak of alkyl iodides obtained from Standard Solution (2) is NLT 10.

System performance: The resolution between the peaks of ethyl iodide and isopropyl iodide obtained from Standard Solution (3) is NLT 1.5.

4) *Testing method*

Test according to the operating conditions described above, and calculate the peak area ratio, Q_T and Q_S, of each drug substance in the test solution and standard solution (3) to the peak area of the internal standard.

Amount of each alkyl (methyl, ethyl, and isopropyl) benzenesulfonate (ppm) = $(Q_T / Q_S) \times (M_S / M_T) \times C \times 0.05$

Q_S: Peak area ratio of each drug substance to the peak area of the internal standard in Standard Solution (3)
Q_T: Peak area ratio of each drug substance to the peak area of the internal standard in the test solution
M_S: Amount of the reference standard for each drug substance (mg)
M_T: Amount of the sample (mg)
C: Purity of the reference standard for each drug substance (%)
0.05: Dilution factor

Analytical Method for Quality Analysis of Recombinant Monoclonal Antibody Drugs
재조합 단클론항체의약품 품질분석 시험법

This general information introduces the analytical procedures for murine, chimeric, humanized IgG isotype monoclonal antibodies, and subtypes (e.g., IgG1 and IgG2). Subtypes show differences in amino acid sequence and in the number of disulfide bonds, and in some cases, they require subtype-specific analysis. This general information applies to monoclonal antibodies for therapeutic and prophylactic use and for in vivo diagnostic purposes. It does not apply to monoclonal antibodies designated by regulatory agencies for use as reagents in the manufacture of medicinal products.

Polyclonal antibodies naturally occurring in serum or plasma are part of the immune system and can bind to many different targets. In contrast, therapeutic monoclonal antibodies for human use are derived from monoclonal cells, and are preparations of an immunoglobulin (or a fragment of an immunoglobulin) that have the specificity to bind only to specific targets. Therapeutic monoclonal antibodies generally bind to and neutralize water-soluble antigens, and their mechanism of action (MOA) often involves blocking ligands from binding to their cognate receptor. Alternatively, for other types of therapeutic monoclonal antibodies, they can recognize and bind to cell bound antigens and in these cases may also engage the immune system through Fc (crystallizable fragment) mediated effector functions as part of their MOA. Generally, monoclonal antibodies that have a single defined binding specificity are monospecific, whereas bispecific monoclonal antibodies that can bind to two different targets (antigens) can also be produced through genetic recombination. IgG-type monoclonal antibodies have a molecular weight of approximately 150 kD, and each molecule consists of two heavy and light polypeptide chains that have a molecular weight of approximately 50 and 25 kD, respectively. An antibody is a Y-shaped structure joined by disulfide bonds, where each arm of the Y is called a Fab domain, and the stem of the Y is called an Fc domain. In addition, monoclonal antibodies are glycoproteins that have a glycosylation site in the Fc portion located on each of the heavy chains and have possible additional glycosylation sites in the Fab domain, depending on the molecule.

The specificity of a monoclonal antibody is based on its antigen binding site, which is located in the Fab domain of the molecule. The Fc domain contains receptor binding sites that are associated with antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity.

Monoclonal antibodies are produced through a system based on a master cell bank and a working cell bank, and the produced antibodies are recovered and purified to remove impurities related to the manufacturing process to acceptable standards.

Currently licensed monoclonal antibody therapeutics include those involved in the activation of effector cells, cell necrosis, cross-linked induced apoptosis, antagonism against several targets, and agonist antibodies.

This general information includes purity assessment by size-exclusion chromatography (SE-HPLC, SEC), capillary electrophoresis, and analysis of oligosaccharides, and it provides validated procedures for each of them.

Although this mainly focuses on IgG-type monoclonal antibodies, the principles of the tests included can also be applied to other related molecules, such as IgM or other isotype antibodies, antibody fragments, and Fc-fusion proteins. Furthermore, when chemicals are bound to an antibody, such tests can also be performed on the antibodies before modification.

Alternative methods and/or procedures may be used if they provide advantages in terms of accuracy, sensitivity, precision, selectivity, automation and suitability for data digitization, or in other special circumstances. Such alternative procedures and methods shall be validated and compared using existing test methods through appropriate validation and must be shown to give equivalent or better results.

1. Size-exclusion chromatography

Although size exclusion chromatography (SEC) is robust for measuring monomers and high-molecular-weight species (HMWS, aggregates), the quantification of low-molecular-weight species (LMWS, fragments) can varies greatly depending on the mAb studied. For quantification of LMWS, better results can be obtained using capillary electrophoresis sodium dodecyl sulfate (CE-SDS).

Mobile phase: Add 10.5 g of potassium (mono)hydrogen phosphate, 19.1 g of potassium dihydrogen phosphate, and 18.6 g of potassium chloride to water. Adjust the pH to 6.2 ± 0.1. Pass

through a membrane filter of ≤ 0.45-µm or smaller pore size.

System suitability solution: Prepare a 10 mg/mL solution by adding 200 µL of mobile phase to one vial of a certified monoclonal IgG system compatibility standard and redissolving. The prepared *system suitability* solution should be used within 24 h after reconstitution and stored at 2 - 8°C if not used immediately.

System suitability blank test solution: Use the mobile phase.

Test solution: Prepare the sample by diluting it to 10 mg/mL in *Mobile phase*.

Blank test solution: Prepare the blank test solution by diluting the formulation buffer in the mobile phase at the equivalent ratio to the sample.

Chromatographic system

- Mode: LC
- Detector: UV 280 nm
- Column: 7.8 mm × 30 cm; 5 μm L59 (a column for protein segregation in the range of 5-7000 kDa, hydrophilically coated silica or composite material filler with a diameter of 1.5 to 10 μm).
- Temperatures
  Column: Ambient
  Autosampler: Maintain at 2–8°C.
- Flow rate: 0.5 mL/min
- Injecting volume: 20 μL
- Run time: 30 min.

System suitability

- The chromatogram of the system suitability solution should be consistent with the typical chromatogram as illustrated in the certificate for the certified monoclonal IgG system suitability reference standard.
  [Note: The approximate retention time for polymer, dimer, monomer, and LMWS is 12, 15, 18, and 22 min, respectively.]
- The chromatographic profiles of the system suitability standard solution, which was injected before and after bracket injection of the test solution, should be consistent with each other, and each should match the typical chromatogram illustrated in the certificate for the approved monoclonal IgG system suitability reference standard.
- The percent peak area of the HMWS in the system suitability solution must be 0.4–0.67%.
- The percent peak area of the main peak in the system suitability solution must be 99.1–99.6%.
- The percent peak area of the LMWS in the system suitability solution must be NMT 0.2%.

Analysis: The system suitability solution should be injected at least once before and after the injection (bracketing) of the test solution. Injection of blank test solution should be included as the final injection before the final System suitability solution injection.

Calculation: Using a suitable electronic integrator or computer system, analyze the bracketed injected System suitability solution. Exclude from integration any peaks that are present in the blank test solution. Protein-related peaks that elute before the main peak are classified as HMWS; those that elute after the main peak are classified as LMWS. Report the percentage of peak areas for the HMWS, main peak, and LMWS.

2. CAPILLARY SDS ELECTROPHORESIS (REDUCED AND NONREDUCED)

Capillary sodium dodecyl sulfate (CE-SDS) electrophoresis is a highly sensitive analytical method used for the quantitative estimation of product-derived impurities such as non-glycosylated molecules, half antibodies, and antibody fragments, and thus is also useful as a stability-indicating assay. After denaturing the sample with SDS, full antibody analysis is possible under nonreduction conditions, and the analysis of light and heavy chains (LC and HC, respectively) is possible under reduction conditions.
[Note: There may be differences in sample preparation depending on the stability of each antibody. If 15 minutes of incubation at 70°C leads to fragmentation or cleavage of disulfide bonds of a particular antibody, adjust the incubation time accordingly.]

SDS sample buffer solution: Prepare a solution containing 100 mM tris-hydrochloride (tris-HCl), with a pH of 8.95±0.05, and 1% (w/w) SDS.

SDS gel buffer solution: Buffer solution at a pH of 8.0 containing 0.2% (w/w) SDS and an appropriate gel

Alkylation solution: Prepare immediately before use with 250 mM iodoacetamide in water.

System suitability solution: Dissolve 1 vial of certified monoclonal IgG system suitability reference standard in 0.5 mL of water for injection (WFI) to a final concentration of 4 mg/mL.

Internal standard solution: Prepare 5 mg/mL of the internal standard solution by dissolving a 10 kDa internal reference standard in water containing 0.5% (w/w) SDS and 0.2% (w/v) sodium azide.

Non-reduction system suitability solution: Mix 25 µL of the system suitability solution with 4 µL of the internal standard solution. Mix 66 µL of SDS sample buffer solution with 5 µL of the alkylation solution. Mix the two solutions thoroughly, and heat up the mixture at 70°C for 15 min. Allow it to cool for 3 min at room temperature, centrifuge at 300 × g for 1 min, and transfer 100 µL into autosampler vials.

Reduction system suitability solution: Mix 25 µL of the system suitability solution with 66 µL of sample buffer solution. Add 4 µL of internal standard solution and 5 µL of β-mercaptoethanol. Mix thoroughly, and heat up the mixture at 70°C for 15 min. Allow to cool for 3 min at room temperature, centrifuge at 300 × g for 1 min, and transfer 100 µL into autosampler vials.
[Note: In some cases, DTT (dithiothreitol) can be used as an alternative reducing agent instead of β-mercaptoethanol. The amount used should be optimized in such cases.]

Blank test solution: Mix 45 µL of water with 50 µL of SDS sample buffer solution. Add 4 μL of the internal standard solution and 5 μL of neat β-mercaptoethanol for the reducing conditions or 5 μL of the alkylation solution for the nonreducing conditions. to this solution. Mix thoroughly, and heat the mixture at 70°C for 15 min. Allow it to cool for 3 min at room temperature, centrifuge at 300 × g for 1 min, and transfer 100 µL into autosampler vials.

Nonreduced test solution: Dilute 100 µg of the sample to be tested with 50–95 µL of SDS sample buffer solution to a final volume of 95 µL. Add 4 µL of the internal standard solution and 5 µL of the alkylation solution. Mix thoroughly, and heat the mixture at 70°C for 15 min. Allow it to cool for 3 min at room temperature, centrifuge at 300 × g for 1 min, and transfer 100 µL into autosampler vial.

Reduced test solution: Dilute 100 µg of the sample to be tested with 50–95 µL of SDS sample buffer solution to a final volume of 95 µL. Add 4 µL of the internal standard solution and 5 µL of *β*-mercaptoethanol. Mix thoroughly, and heat the mixture at 70°C for 15 min. Allow to cool for 3 min at room temperature, centrifuge at 300 × g for 1 min, and transfer 100 µL into autosampler vial.

Instrumental conditions

- Mode: CE-SDS
- Detector: UV or diode array 220 nm
- Capillary: Fused-silica capillary (i.d. 50 µm) cut to a total length of 30 cm so that the effective length is 20 cm^3. The following preconditioning and prefilling steps are required between each run.
- Temperature
  Sample storage: Approximately 25°C
  Capillary: Approximately25°C
- Preconditioning of the capillary: Rinse for 3 min at 70 psi with 0.1 N sodium hydroxide followed by 0.1 N hydrochloric acid for 1 min at 70 psi and water for 1 min at 70 psi.
- Prefilling of capillary: Rinse for 10 min at 70 psi with SDS gel buffer solution.
- Sample injection: Electrokinetic injection, 5.0 kV reversed polarity for 20s
- Voltage segregation
  Non-reduction conditions: 40 min at 15 kV, reversed polarity.
  Reduction conditions: 40 min at 15 kV, reversed polarity.

System suitability requirements

(1) ***Reduction conditions***

- The electropherogram of the reducing system suitability solution should be consistent with the typical electropherogram as illustrated in the certificate for certified monoclonal IgG system suitability reference standard.
- The main peaks of the heavy chain and the nonglycosylated heavy chain should be clearly identifiable. The resolution between the nonglycosylated heavy chain and the intact heavy chain is NLT 1.2.
- Calculate the ratio of nonglycosylation of the heavy chain by dividing the nonglycosylated heavy chain peak area by the sum of all heavy chain peaks and multiplying by 100. The ratio of nonglycosylated of the total heavy chain in the reduction system suitability solution should be within 0.75–1.34%.

(2) ***Non-reduction conditions***

- The electropherogram of the non-reduction system suitability solution should be consistent with the typical electropherogram as illustrated in certificate for USP monoclonal IgG system suitability reference standard.
- The main peak should be clearly identifiable. The resolution between the IgG main peak and Fragment 1 (F1) is NLT1.3.
- Calculate the relative amount of the main peak by dividing the time-corrected peak area of the IgG main peak by the sum of the time-corrected peak areas of all peaks, excluding internal standard peaks and all system peaks, and multiply by 100. The relative amount of the main IgG peak of the system suitability solution should be within the limits of 61.4–86.4%.
- The relative standard deviation for the migration of the internal standard in the bracketing blank test solution should be NMT 2%. No other peaks should be detected after the internal standard is detected.

Analysis:

Analyze the blank test solution, system suitability solution, and the test solution.

[Note: Depending on the instrument requirements, the electric field applied across the capillary and the conditions for the sample injection can be adjusted to achieve system suitability.]

Apply a field strength of 500 V/cm (reversed polarity) for 40 min by using the SDS gel buffer solution as the electrolyte in both buffer solution reservoirs. Electrophorese the test sample, using a 20-s reversed polarity and electrokinetic injection at 5.0 kV, into the anodic end of the capillary, and record the electropherograms at 220 nm. Rinse for 3 min at 70 psi with 0.1 N sodium hydroxide followed by 0.1 N hydrochloric acid for 1 min at 70 psi. and water for 1 min at 70 psi. Repeat injection at least twice. Calculate the time-corrected peak areas of all peaks that migrate after the internal standard peak.

Reduction conditions: Calculate the relative amount of the test solution peak by dividing the sum of the time-corrected peak areas of the heavy chain, non-glycosylated heavy chain, and light chain by the sum of all time-corrected peak areas that appear after the internal standard peak and multiply by 100.

Non-reduction conditions: Calculate the relative amount of the test solution peak by dividing the sum of the time-corrected peak area of the main IgG peak by the sum of all time-corrected peak areas that appear after the internal standard peak and multiply by 100.

3. Oligosaccharide analysis- Analysis of N-linked oligosaccharide of monoclonal antibodies

The inclusion of oligosaccharide analysis in the specifications for monoclonal antibody drug substances should be defined on a product-specific basis. Identification or quantitative analysis of oligosaccharide profiles can be performed using one or more of the following assay methods, and other validated assays with differences in the method of segregation, labelling, detection, etc. may also be used.

[Note: For monoclonal antibodies with N-linked oligosaccharides present in both Fc and Fab, the domains must be separated through additional test solution pretreatment (e.g., enzyme treatment such as papain) to confirm the glycan structures binding site. At this time, additional test solution preparation steps require verification.]

[Note: Numerical acceptance criteria may be defined for individual glycan structures. If applicable, acceptance criteria must be derived from product-specific information taking into consideration a suitable range of historical batch data.]

A. Method A: Capillary electrophoresis with laser-induced fluorescence detection

Run buffer solution: Prepare 100 mM triethanolamine (TEA) and 10% glycerol at pH 7.5 by dissolving 1.492 g of TEA and 10 g of glycerol in 80 mL of water. Adjust with 1 N hydrochloric acid to a pH of 7.5, and dilute with water to a final

volume of 100 mL.

Sample buffer solution: Dilute 1 mL of run buffer solution with 9 mL of water.

Enzyme reaction buffer solution: Prepare 50 mM sodium phosphate, pH 7.5.

APTS labeling reagent: Dissolve 5 mg of 8-aminopyrene-1,3,6-trisulfonate (APTS) in 48 µL of 15% acetic acid, and mix well.

Test solution: Add 2 µL of peptide N-glycosidase F (PNGase F) (5 units/mL) to 50 µg of the sample, and adjust with enzyme reaction buffer solution to a 50 µL volume.
[Note: Since the unit definition for PNGase F varies depending on the commercial sources, It is necessary to experimentally determine the appropriate quantity of PNGase F required for the complete release of N-linked oligosaccharides in the sample.]
Incubate it at 37°C for 18 h. Separate released oligosaccharides by centrifugation using a 10,000 molecular-weight-cut-off (MWCO) centrifugal filter. Dry the released oligosaccharides in a vacuum centrifugal. Add 2 µL of APTS labeling reagent and 2 µL of 1 M sodium cyanoborohydride to the dried oligosaccharides. Incubate at 55°C for 90 min. Add 46 µL of water to quench the reaction and mixture. Add 5 µL of the APTS-labeled sample to 95 µL of the sample buffer solution.

System suitability solution: Resuspend the glycan standards (10 µg) in 50 µL of water, and mix on a vortex mixer. Add 5 µL of glycan standard (1 µg) to a microcentrifuge tube. Dry the glycan standard using a centrifugal vacuum evaporator at ambient temperature. Add 2 µL of sodium cyanoborohydride and 2 µL of the APTS labeling reagent to each standard. Mix the standards on a vortex mixer, and centrifuge briefly to settle the liquid before incubation at 55°C for 90 min. Quench the reaction by adding 46 µL of water to each standard. Standards can be stored at −20°C for up to 5 weeks.

Instrumental conditions

- Mode: CE
- Detector: Laser-induced fluorescence detector (488-nm excitation wavelength; 520-nm emission wavelength)
- Capillary: 50-µm inner diameter bare fused-silica capillary with 50-cm total length and 40-cm segregation length
- Sample injection: 10-s hydrodynamic injection
- Segregation voltage: 22 kV for 50 min
- Samples: Test solution and system suitability solution

Analysis: Integrate peaks in the resultant electropherogram, report the relative time-corrected percentage areas of major glycan structure peaks. Compare the peaks of the test solution and the system suitability solution.

B. Method B: Liquid chromatography with fluorescence detection

Enzyme reaction buffer solution: 100 mM sodium phosphate, pH 7.5.

Labeling buffer solution: Prepare 8% (w/v) sodium acetate trihydrate and 4% (w/v) boric acid solutions in methanol by dissolving 800 mg of sodium acetate trihydrate and 400 mg of boric acid in 10 mL of methanol.

Labeling reagent solution: Prepare a 100 mg/mL stock solution of anthranilic acid (2-AA) dissolved in labeling buffer solution. Mix with an equal volume of 1 M sodium cyanoborohydride in methanol for a final Labelling reagent solution containing 50 mg/mL of 2-AA and 0.5 M sodium cyanoborohydride.
[Note: Keep the labelling reagent protected from light.]

Solution A: 50 mM ammonium formate, pH 4.4.
Solution B: Acetonitrile
Mobile phase:

Time (min)	Solution A (%)	Solution B (%)
0.0	32.5	67.5
48.0	42.5	57.5
49.0	100	0
53.0	100	0
54.0	32.5	67.5
60.0	32.5	67.5

Test solution preparation: Transfer 80 µg of the sample to a microcentrifuge tube Add 3 µL of PNGase F (2.5 units/mL).
[Note: The unit definition for PNGase F may differ among commercial sources. It may be necessary to experimentally determine the appropriate quantity of PNGase F required to achieve the complete release of the N-linked oligosaccharides in the sample.]
A dd14 µL of enzyme reaction buffer solution. Add water to make 70 µL of the total volume, and incubate at 37°C for 18 h. Add 70 µL of the labeling reagent solution, and incubate at 70°C for 2 h in a fume hood. Allow to cool, and add 60 μL of 50% methanol to each sample. Mix, then centrifuge. Remove 140 µL of the supernatant, and transfer to a microcentrifuge tube. Add 500 µL of acetonitrile to the supernatant-containing tube. Load the sample onto a hydrophilic-interaction solid-phase extraction cartridge to remove excess labeling reagent. Wash the cartridge with 95% acetonitrile (approximately 10 mL). Elute the labeled glycans from the cartridge with 0.5 mL of 20% acetonitrile.

Dilute the eluate 1:1 with acetonitrile.

- Mode: LC
- Detector: Fluorescence (360-nm excitation wavelength; 420-nm emission wavelength)
- Column: 4.6 mm × 15 cm; 3 μm packing L68
- Column temperature: Maintain at 30°C.
- Flow rate: 0.8 mL/min
- Injecting volume: 50 uL

Analysis: Integrate peaks in the resulting chromatogram, and calculate the relative percentage peak areas of the major glycan structures. Compare the peaks of the test solution and the system suitability solution.

4. Oligosaccharide Analysis—Sialic Acid Analysis
[Note: The range of the standard curve or the mass of the sample may require modification depending on the sialic acid content of the monoclonal antibodies.]

Solution A: Prepare 100 mM sodium hydroxide by diluting 10.4 mL of a 50% (w/w) sodium hydroxide solution in 2 L of water. [Note: Use high-quality water of high resistivity (18 MΩ-cm or better) that contains as little dissolved oxygen as possible.]

Solution B: Prepare a solution containing 100 mM sodium hydroxide and 1 M sodium acetate by adding 82.0 g of sodium acetate to 800 mL of water. Add 5.2 mL of 50% (w/w) sodium hydroxide, and dilute with water to a final volume of 1000 mL.

Mobile phase:

Time (min)	Solution A (%)	Solution B (%)
0.0	93	7
10.0	70	30
11.0	70	30
12.0	93	7

15.0	93	7

Sodium acetate buffer solution: 20 mM sodium acetate solution, pH 5.2.

Standard stock solution: Prepare 0.2 mM solutions of USP N-Acetylneuraminic Acid RS (NeuAc) and USP N-Glycolylneuraminic Acid RS (NeuGc) in 20 mM sodium acetate buffer solution, pH 5.2. Dilute an appropriate volume of this solution with 20 mM sodium acetate buffer solution pH 5.2, to obtain a 0.02 mM standard stock solution.

Internal standard stock solution: Prepare a 0.1 mM solution of 3-deoxy-D-glycero-D-galacto-2-nonulosonic acid (KDN) in 20 mM sodium acetate buffer solution, adjusted to a pH of 5.2.

Standard solutions: Prepare as indicated in the Table below.

Standard	Concentration (μM) of reference material	Volume (μL) of Standard stock solution	Volume (μL) of 20 mM Sodium acetate buffer solution, pH 5.2	Volume (μL) of Internal Standard stock solution
1	10	250	235	15
2	5	125	360	15
3	4	100	385	15
4	2	50	435	15
5	1	25	460	15
6	0.4	10	475	15

System suitability solution: Prepare 3 μM solutions of NeuAc, NeuGc, and KDN in 20 mM sodium acetate buffer solution, adjusted to a pH of 5.2. Store the system suitability solution at −70°C.

Test solution: Pipette a volume of the sample equivalent to 0.5 mg into a microcentrifuge tube. Dilute with 20 mM sodium acetate buffer solution, pH 5.2, to bring the total volume 475 μL. Confirm the pH using a test strip, and add 10 μL of 10 milliunit/μL of neuraminidase. Incubate for 5 h at 37°C. Add 15 μL of internal standard stock solution. Mix with a vortex mixer, and transfer the sample to an autosampler vial.
[Note: A slight adjustment in the sample preparation may be necessary depending on the test sample and the quality of the enzyme. Adjust the incubation time accordingly.]

Chromatographic system

- Mode: LC
- Detector: Integrated amperometric detector with gold electrode
- Column: 4 mm × 25 cm; 10 μm L46
- Flow rate: 1 mL/min
- Injection volume: 50 μL
- Use the following waveform for the electrochemical detector.

Time (s)	Potential (V)	Integration
0.00	+0.10	-
0.20	+0.10	Begin
0.40	+0.10	End
0.41	-2.00	-
0.42	-2.00	-
0.43	+0.60	-
0.44	-0.10	-
0.50	-0.10	-

Analysis: Analyze the standard solutions, system suitability solutions, and the test solution. Integrate the NeuAc, NeuGc, and *Internal standard stock solution* peaks in each chromatogram. For the *Standard solutions*, evaluate the NeuAc and NeuGc peak areas relative to the peak area of the internal standard as follows.

$$\text{NeuAc peak area}(\%) = \frac{\text{NeuAc peak}}{\text{Peak area of internal standard}} \times 100$$

$$\text{NeuGc peak area}(\%) = \frac{\text{NeuGc peak area}}{\text{peak area of internal standard}} \times 100$$

Draw a standard curve (calibration curve) between the concentration of the standard solution and the peak area percentage of NeuAc and NeuGc.. Calculate the concentration of NeuAc and NeuGc in the *test solution* from the standard curve using the area percentage of the NeuAc and NeuGc peaks in the test solution. Divide the determined NeuAc and NeuGc concentrations by the test solution concentration to calculate the molar ratio (i.e., numbers of NeuAc and NeuGc per molecule of sample).

5. Glossary

- 2-AA: Anthranilic acid
- APTS: 8-Aminopyrene-1,3,6-trisulfonate
- CE-SDS: Capillary Electrophoresis-Sodium dodecyl sulfate
- DTT: Dithiothreitol
- Fab: Fragment antigen-binding
- F1: Fragment 1
- GlcNAc: N-Acetylglucosamine
- HC: Heavy chain
- HMWS: High molecular weight species
- KDN: 3-deoxy-D-glycero-D-galacto-2-nonulosonic acid
- LC: Light chain
- LMWS: Low molecular weight species
- MWCO: Molecular-weight-cut-off
- NeuAC: Sialic acid, N-AcetylNeuraminic aicd
- NeuGC: N-glycolylneuraminic acid
- SE-HPLC, SEC: Size-exclusion chromatography
- TEA: Triethanolamine
- USP: United States Pharmacopoeia

Assessment and Control of Metal (Elemental) Impurities in Drug Products
완제의약품 중 금속(원소)불순물 평가 및 관리

1. Introduction

Elemental impurities pharmaceuticals, including metal, metalloid, and non-metal elements, may originate from several sources; they may be residual catalysts that were added intentionally in synthesis of drug substances or may be present as impurities, e.g., through interactions with processing equipment or container/closure systems or by being present in components (drug substances and excipients, etc.) of the drug product. Because elemental impurities do not provide any therapeutic benefit to the patient, their levels in the drug product should be controlled within acceptable limits, except when they are specified in monographs.

The permitted daily exposures (PDEs) of elemental impurities are established to protect the health of all patients based on the evaluation of the toxic data of elemental impurities. More strict limits are not needed, if the elemental impurities in drug products do not exceed the PDEs. In some cases, lower levels of elemental impurities may be warranted when levels below toxicity thresholds have been shown to have an impact on other quality attributes of the drug product (e.g., element catalyzed degradation of drug substances). Additionally, pharmaceutical quality considerations may warrant other limits for elements with high PDEs.

The assessment and control of elemental impurities in finished pharmaceuticals are conducted using a risk-based management approach.

2. Scope

This guideline applies to finished pharmaceuticals. It encompasses finished pharmaceuticals containing refined proteins and polypeptides (including proteins and polypeptides produced from recombinant or non-recombinant origins), their derivatives, and products of which they are components (e.g., conjugates) are within the scope of this guideline, as are drug products containing synthetically produced polypeptides, polynucleotides, and oligosaccharides.

However, this guideline does not apply to crude drugs, radiopharmaceuticals, vaccines, cell metabolites, DNA products, allergenic extracts, cells, whole blood, cellular blood components or blood derivatives including plasma and plasma derivatives, dialysate solutions not intended for systemic circulation, and elements that are intentionally included in the drug product for therapeutic benefit. Furthermore, this guideline does not apply to drug products based on genes (gene therapy), cells (cell therapy) and tissue (tissue engineering), which are known as advanced therapy medicinal products.

3. The PDEs of Elemental Impurities for Oral, Parenteral and Inhalation Routes of Administration and Element Classification

The PDEs of elemental impurities established for preparations for oral, parenteral and inhalation routes of administration are shown in Table 1. If the PDEs for the other administration routes are necessary, generally consider the oral PDE as a starting point in the establishment, and assess if the elemental impurity is expected to have local effects when administered by the intended route of administration.

Parenteral drug products with a maximum daily volume up to 2 liters calculate permissible concentrations from PDEs using this volume. For products whose daily volumes, as specified by labeling and/or established by clinical practice, may exceed 2 liters (e.g., saline, dextrose, total parenteral nutrition, solutions for irrigation), a 2-liter volume may be used to calculate permissible concentrations from PDEs.

Table 1. PDEs for Elemental Impurities

Element	Class	Oral PDE (μg/day)	Parenteral PDE (μg/day)	Inhalation PDE (μg/day)
Cd	1	5	2	3
Pb	1	5	5	5
As	1	15	15	2
Hg	1	30	3	1
Co	2A	50	5	3
V	2A	100	10	1
Ni	2A	200	20	6
TI	2B	8	8	8
Au	2B	300	300	3
Pd	2B	100	10	1
Ir	2B	100	10	1
Os	2B	100	10	1
Rh	2B	100	10	1
Ru	2B	100	10	1
Se	2B	150	80	130
Ag	2B	150	15	7
Pt	2B	100	10	1
Li	3	550	250	25
Sb	3	1200	90	20
Ba	3	1400	700	300
Mo	3	3000	1500	10
Cu	3	3000	300	30
Sn	3	6000	600	60
Cr	3	11000	1100	3

As shown in Table 1, elemental impurities are divided into three classes based on their toxicity (PDE) and likelihood of being present in the finished pharmaceutical product. The likelihood of occurrence is derived from several factors including: probability of use in manufacturing processes, probability of being a co-isolated impurity with other elemental impurities in raw materials used in manufacturing processes, and the observed natural abundance and environmental distribution of the element. The classification scheme is intended to focus the risk assessment on those elements that are the most toxic but also have a reasonable probability of inclusion in the drug product (see Table 2). The elemental impurity classes are:

Class 1: The elements, As, Cd, Hg, and Pb, are human toxicants with limited or no use in pharmaceutical manufacturing. Their presence in drug products typically comes from commonly used materials (e.g., mined excipients). Because of their unique nature, these four elements require evaluation during the risk assessment, across all potential sources of elemental impurities and routes of administration. The outcome of the risk assessment will determine those components that may require additional controls which may in some cases include testing for Class 1 elements. The assessment results identify components requiring additional controls, including testing for Class 1 elements, if deemed necessary.

Class 2: Elements in this class are generally considered as route-dependent human toxicants. Class 2 elements are further divided in sub-classes 2A and 2B based on their relative likelihood of occurrence in the drug product.

- Class 2A: Elements like Co, Ni, and V have relatively high probability of occurrence in the drug product and thus require risk assessment across all potential sources of elemental impurities and routes of administration (as indicated).
- Class 2B: Elements like Ag, Au, Ir, Os, Pd, Pt, Rh, Ru, Se, and Tl have a reduced probability of occurrence in the drug product related to their low abundance and low potential to be co-isolated with other materials. As a result, they may be excluded from the risk assessment unless they are intentionally added during the manufacture of drug substances, excipients or other components of the drug product.

Class 3: The elements in this class have relatively low

toxicities by the oral route of administration (high PDEs, generally > 500 μg/day) but may require consideration in the risk assessment for inhalation and parenteral routes. For oral routes of administration, unless these elements are intentionally added, they do not need to be considered during the risk assessment. For parenteral and inhalation products a risk assessment for potential inclusion of these elemental impurities should be conducted unless the route-specific PDE is above 500 μg/day. The elements in this class include: Ba, Cr, Cu, Li, Mo, Sb, and Sn.

4. Risk Assessment and Control of Elemental Impurities

In developing controls for elemental impurities in drug products, the principles of quality risk management should be considered. The risk assessment should be based on scientific knowledge and principles. The risk assessment would be focused on assessing the levels of elemental impurities in a drug product in relation to the PDEs. Information for this risk assessment includes but is not limited to: measured data of drug products and components, measured data and the risk assessment result supplied by drug substance and/or excipient manufacturers, and/or data available in published literature. The risk assessment and control approaches should be documented in an appropriate manner. The level of effort and formality of the risk assessment should be proportional to the level of risk. It is neither always appropriate nor always necessary to use a formal risk management process (using recognized tools and/or formal procedures, e.g., standard operating procedures.)

The use of informal risk management processes (using empirical tools and/or internal procedures) may also be considered acceptable.

A. General Principles

The risk assessment process involves three steps:

1) Identify known and potential sources of elemental impurities that may find their way into the drug product.

2) Evaluate the presence of specific elemental impurities by comparing observed or predicted levels with established PDEs.

3) Summarize and document the risk assessment. Identify if controls built into the process are sufficient or identify additional controls to be considered to limit elemental impurities in the drug product.

In many cases, the steps are considered simultaneously. The outcome of the risk assessment may be the result of iterations to develop a final approach to ensure the potential elemental impurities do not exceed the PDE certainly.

B. Potential Sources of Elemental Impurities

In considering the manufacturing process of a drug product, potential sources of elemental impurities.

- Residual impurities derived from metals intentionally added during the manufacturing of drug substances, excipients, or components of other drug products. The risk assessment of drug substances should deal with the potential for adulteration of metal impurities in drug products.
- Elemental impurities that are not intentionally added and are potentially present in the drug substance, water or excipients used in the preparation of the drug product.
- Elemental impurities that are potentially introduced into the drug substance and/or drug product from manufacturing equipment.
- Elemental impurities that may leach into the drug substance and drug product from container closure systems.

Each source may contribute elemental impurities to the drug and during the risk assessment, the potential contributions from each of these sources should be considered to determine the overall contribution of elemental impurities to the drug product.

C. Identification of Potential Elemental Impurities

***Potential elemental impurities derived from intentionally added catalysts and inorganic reagents*:** If any element listed in Table 2 is intentionally added, it should be considered in the risk assessment. For this category, the identity of the potential impurities is known and techniques for controlling elemental impurities are easily characterized and defined.

***Potential elemental impurities that may be present in drug substances and/or excipients*:** While not intentionally added, some elemental impurities may be present in some drug substances and/or excipients. The possibility for inclusion of these elements in the drug product should be reflected in the risk assessment.

For orally administered drugs, the risk assessment should evaluate the potential inclusion of Class 1 and Class 2A elemental impurities in the drug product. For parenteral and inhalation routes of administration, the risk assessment should evaluate the possibility for inclusion of the Class 1, Class 2A and Class 3 elemental impurities as shown in Table 2.

Potential elemental impurities derived from manufacturing equipment: The contribution of elemental impurities from this source may be limited and the subset of elemental impurities that should be considered in the risk assessment will depend on the manufacturing equipment used in the production of the drug product. Applying of process knowledge, selecting of equipment, conducting equipment qualification, and implementing GMP controls can minimize the contribution of elemental impurities from manufacturing equipment. Assess the specific elemental impurities of concern should be assessed based on knowledge of the composition of the components of the manufacturing equipment that come in contact with components of the drug product. The risk assessment of this source of elemental impurities is one that can potentially be utilized for many drug products using similar process trains and processes.

In general, the processes used to prepare a given drug substance are considerably more aggressive than processes used in preparing the drug product when assessed relative to the potential to leach or remove elemental impurities from manufacturing equipment. Contributions of elemental impurities from drug product processing equipment would be expected to be lower than contributions observed for the drug substance. However, when this is not the case based on process knowledge or understanding, the potential for incorporation of elemental impurities from the drug product manufacturing equipment in the risk assessment (e.g., hot melt extrusion) should be considered.

***Elemental impurities leached from container closure systems*:** Identify potential elemental impurities introduced from container closure systems based on scientific understanding of interactions between a specific drug product type and its packaging. When a review of the materials of construction demonstrates that the container closure system does not contain elemental impurities, no additional risk assessment needs to be performed. It is recognized that the probability of elemental leaching into solid dosage forms is minimal and does not require further consideration in the risk assessment. For liquid and semi-solid dosage forms there is a higher probability that elemental impurities could leach from the container closure system during the shelf-life of the drug product. Studies to understand potential

leachable from the container closure system (after washing, sterilization, irradiation, etc.) should be performed. This source of elemental impurities is typically addressed during evaluation of the container closure system for the drug product.

- Factors that should be considered (for liquid and semi-solid dosage forms) include, but are not limited to: hydrophilicity/hydrophobicity, ionic content, pH, temperature (low vs room temperature and processing conditions), contact surface area, container/component composition, terminal sterilization, packaging process, component sterilization, and duration of storage.

Table 2 provides recommendations for inclusion of elemental impurities in the risk assessment. This table can be applied to all sources of elemental impurities in the drug product.

D. Evaluation

Upon the completion of the potential elemental impurity identification process,, there are two possible outcomes:

1) The risk assessment process does not identify any potential elemental impurities. The conclusion of the risk assessment and supporting information and data should be documented.

2) The risk assessment process identifies one or more potential elemental impurities. For any elemental impurities identified in the process, the risk assessment should consider if there are multiple sources of the identified elemental impurity or impurities and document the conclusion of the assessment and supporting information.

The risk assessment can be facilitated with information about the potential elemental impurities provided by suppliers of drug substances, excipients, container closure systems, and manufacturing equipment. The data that support this risk assessment can come from a number of sources that include, but are not limited to: prior knowledge, published literature, data generated from similar processes, supplier information or data, testing of the components of the drug product, and testing of the drug product.

During the risk assessment, various factors influencing the quantity of the potential impurity in the drug product should be considered in the risk assessment. These include but are not limited to: efficiency of removal of elemental impurities during further processing; natural abundance of elements (especially important for the categories of elements which are not intentionally added); prior knowledge of elemental impurity concentration ranges, and the composition of the drug product.

E. Summary of Risk Assessment Process

The risk assessment process involves evaluating all information obtained during the process to identify significant elemental impurities that may be present or observed in the drug product.

The summary should consider the significance of the observed or predicted level of the elemental impurity relative to the PDE of the elemental impurity. As a measure of the significance of the observed elemental impurity level, a control threshold is defined as a level that is 30% of the established PDE in the drug product. This threshold may be used to determine the need for additional controls.

If the total elemental impurity level from all sources in the drug product is expected to be consistently less than 30% of the PDE, then additional controls are not required, provided the applicant has appropriately assessed the data and demonstrated adequate controls on elemental impurities.

Table 2. Elements to be Considered in the Risk Assessment

Element	Class	If intentionally added	If not intentionally added		
		(all routes)	Oral	Parenteral	Inhalation
Cd	1	necessary	necessary	necessary	necessary
Pb	1	necessary	necessary	necessary	necessary
As	1	necessary	necessary	necessary	necessary
Hg	1	necessary	necessary	necessary	necessary
Co	2A	necessary	necessary	necessary	necessary
V	2A	necessary	necessary	necessary	necessary
Ni	2A	necessary	necessary	necessary	necessary
TI	2B	necessary	unnecessary	unnecessary	unnecessary
Au	2B	necessary	unnecessary	unnecessary	unnecessary
Pd	2B	necessary	unnecessary	unnecessary	unnecessary
Ir	2B	necessary	unnecessary	unnecessary	unnecessary
Os	2B	necessary	unnecessary	unnecessary	unnecessary
Rh	2B	necessary	unnecessary	unnecessary	unnecessary
Ru	2B	necessary	unnecessary	unnecessary	unnecessary
Se	2B	necessary	unnecessary	unnecessary	unnecessary
Ag	2B	necessary	unnecessary	unnecessary	unnecessary
Pt	2B	necessary	unnecessary	unnecessary	unnecessary
Li	3	necessary	unnecessary	necessary	necessary
Sb	3	necessary	unnecessary	necessary	necessary
Ba	3	necessary	unnecessary	unnecessary	necessary
Mo	3	necessary	unnecessary	unnecessary	necessary
Cu	3	necessary	unnecessary	necessary	necessary
Sn	3	necessary	unnecessary	unnecessary	necessary

Element	Class	If intentionally added	If not intentionally added		
		(all routes)	Oral	Parenteral	Inhalation
Cr	3	necessary	unnecessary	unnecessary	necessary

If the risk assessment fails to demonstrate that an elemental impurity level is consistently less than the control threshold, controls should be established to ensure that the elemental impurity level does not exceed the PDE in the drug product.

Consideration should be given to the variability of elemental impurity levels, including variability from analytical methods, specific sources, and within the drug product. Data from three representative production scale lots or six pilot scale lots can establish the level and variability of elemental impurities. For components with inherent variability, additional data may be necessary.

The variability of the level of an elemental impurity should be factored into the application of the control threshold to drug products. Sources of variability may include:

- Variability of the analytical method;
- Variability of the elemental impurity level in the specific sources;
- Variability of the elemental impurity level in the drug product.

The level and variability of an elemental impurity can be established by providing the data from three (3) representative production scale lots or six (6) representative pilot scale lots of the component or components or drug product. For some components that have inherent variability (e.g., mined excipients), additional data may be needed to apply the control threshold. There are many acceptable approaches to summarizing and documenting the risk assessment that may include: tables, written summaries of considerations and conclusions of the assessment. The summary should identify the elemental impurities, their sources, and necessary controls and acceptance criteria.

5. Control of Elemental Impurities

Controlling elemental impurities is one part of the overall control strategy for a drug product that assures that elemental impurities do not exceed the PDEs. If the level of an elemental impurity may exceed the control threshold, additional measures should be implemented to prevent exceeding the PDE. Approaches that can be pursued include but are not limited to:

- Modification of the steps in the manufacturing process that result in the reduction of elemental impurities below the control threshold through specific or non-specific purification steps;
- Implementation of in-process or upstream controls, designed to limit the concentration of the elemental impurity below the control threshold in the drug product;
- Establishment of specification limits for excipients or raw materials (e.g., synthetic intermediates);
- Establishment of specification limits for the drug substance;
- Establishment of specification limits for the drug product;
- Selection of appropriate container closure systems.

6. Converting between PDEs and Concentration Limits

The PDEs, reported in micrograms per day (μg/day) give the maximum permitted quantity of each element that may be contained in the maximum daily intake of a drug product. Because the PDE reflects only total exposure from the drug product, it is useful to convert the PDE into concentrations as a tool in evaluating elemental impurities in drug products or their components. The options listed below describe some acceptable approaches to establishing concentrations of elemental impurities in drug products or components that would assure that the drug product does not exceed the PDEs. Any of these options may be selectable as long as the resulting permitted concentrations assure that the drug product does not exceed the PDEs. Options for establishing concentrations ensuring PDE compliance include:

Option 1: Common permitted concentration limits of elements across drug product components for drug products with daily intakes of not more than 10 grams:

This option is not intended to imply that all elements are present at the same concentration, but rather provides a simplified approach to the calculations.

The option assumes the daily intake (amount) of the drug product is 10 grams or less, and that elemental impurities identified in the risk assessment (the target elements) are present in all components of the drug product. Using Equation 1 below, and a daily intake of 10 grams of drug product, this option calculates a common permissible target elemental concentration for each component in the drug product. This approach, for each target element, allows determination of a fixed common maximum concentration in micrograms per gram in each component.

$$Concentration(\mu g/g) = \frac{PDE(\mu g/day)}{daily\ amount\ of\ drug\ product(g/day)} \quad (1)$$

The permitted concentrations for Option 1 are provided in Table 3.

Table 3. 1Permitted Concentrations of Elemental Impurities for Option 1

Element	Class	Oral Concentration (μg/g)	Parenteral Concentration (μg/g)	Inhalation Concentration (μg/g)
Cd	1	0.5	0.2	0.3
Pb	1	0.5	0.5	0.5
As	1	1.5	1.5	0.2
Hg	1	3	0.3	0.1
Co	2A	5	0.5	0.3
V	2A	10	1	0.1
Ni	2A	20	2	0.6
TI	2B	0.8	0.8	0.8
Au	2B	30	30	0.3
Pd	2B	10	1	0.1
Ir	2B	10	1	0.1
Os	2B	10	1	0.1
Rh	2B	10	1	0.1
Ru	2B	10	1	0.1
Se	2B	15	8	13
Ag	2B	15	1.5	0.7
Pt	2B	10	1	0.1
Li	3	55	25	2.5
Sb	3	120	9	2
Ba	3	140	70	30
Mo	3	300	150	1
Cu	3	300	30	3
Sn	3	600	60	6
Cr	3	1100	110	0.3

If all the components in a drug product do not exceed the concentrations specified in Option 1 for all target elements identified in the risk assessment, then all these components may be used in any proportion in the drug product. If the permissible concentrations in Table 3 are not applied, Options 2a, 2b, or 3 should be followed.

Option 2a: Common permitted concentration limits across drug product components for a drug product with a specified daily intake:

This option is similar to Option 1, except that the drug daily intake is not assumed to be 10 grams. The common permitted concentration of each element is determined using Equation 1 and the actual maximum daily intake. This approach, for each target element, allows determination of a fixed common maximum concentration in micrograms per gram in each component based on the actual daily intake provided. If all components in a drug product do not exceed the Option 2a concentrations for all target elements identified in the risk assessment, then all these components may be used in any proportion in the drug product.

Option 2b: Permitted concentration limits of elements in individual components of a drug product with a specified daily intake:

This option requires additional information that may be assembled regarding the potential for specific elemental impurities to be present in specific drug product components. Permitted concentrations based on the distribution of elements in the components (e.g., higher concentrations in components with the presence of an element in question) may be set. For each element identified as potentially present in the components of the drug product, the maximum expected mass of the elemental impurity in the final drug product can be calculated by multiplying the mass of each component material times the permitted concentration established in each material and summing over all components in the drug product, as described in Equation 2. The total mass of the elemental impurity in the drug product should comply with the PDEs given in Table 1, unless justified according to other relevant sections of this chapter. If the risk assessment has determined that a specific element is not a potential impurity in a specific component, there is no need to establish a quantitative result for that element in that component. This approach allows that the maximum permitted concentration of an element in certain components of the drug product be higher than the limits set in Option 1 or Option 2a, but this should be compensated by lower allowable concentrations in the other components of the drug product. Equation 2 may be used to demonstrate that component-specific limits for each element in each component of a drug product assure that the PDE will be met.

$$PDE = (\mu g/\text{day}) \geq \sum_{k=1}^{N} C_k \cdot M_k \qquad (2)$$

k = an index for each of N components in the drug product
C_k = permitted concentration of the elemental impurity in component k (μg/g)
M_k = mass of component k in the maximum daily intake of the drug product (g)

Option 3: Finished Product Analysis:

The concentration of each element may be measured in the final drug product. Equation 1 may be used with the maximum total daily dose of the drug product to calculate a maximum permitted concentration of the elemental impurity.

7. Speciation and Other Considerations

Chemical speciation refers to the distribution of metals among various chemical species, including isotopic composition, electronic or oxidation state, and complex or molecular structure. When the toxicities of different species of the same element are known, the PDE has been established using the toxicity information on the species expected to be in the drug product.

When elemental impurity measurements are used in the risk assessment, total elemental impurity levels in drug products may be used to assess compliance with the PDEs. The identification of speciation is not particularly expected, however such information could be used to justify lower or higher levels when the identified species is more or less toxic, respectively, than the species used for the calculation of the PDEs.

When total elemental impurity levels in components are used in the risk assessment, providing information on release of an elemental impurity from the component in which it is found. However, such information could be used to justify levels higher than those based on the total elemental impurity content of the drug product.

8. Analytical Procedures

The determination of elemental impurities should be conducted using appropriate procedures suitable for their intended purposes. Unless otherwise justified, individual and specific testing methods should be employed for each elemental impurity identified during the risk assessment. Pharmacopoeia procedures or suitable alternative procedures for determining levels of elemental impurities should be used.

9. Lifecycle Management

If changes to the drug product or components have the potential to change the elemental impurity content of the drug product, the risk assessment, including established controls for elemental impurities, should be re-evaluated. Such changes may encompass synthetic routes, excipient suppliers, raw materials, processes, equipment, container closure systems or facilities. All changes should adhere to the internal change management process and, if required, comply with relevant regional regulatory requirements.

Biological Test Design and Data Preprocessing Method
생물학적 시험 설계 및 자료 전처리법

A biological test data preparation encompasses group methods to prepare the data by appropriate application of the selected statistical analysis method, in the case of implementing the biological test by applying an appropriate statistical analysis (hereinafter referred to as "statistical analysis") on the measured or observed values from the biological test. A biological analysis is various tests generally used for the determine the potency of biological products. To implement a biological analysis, an experimental plan is prepared according to the design of experiment, and the data require rigorous statistical analysis. Therefore, this chapter focuses on the preparation of test data to increase the efficiency and accuracy for the test purpose in terms of statistical analysis and interpretation. In particular, in order to have the prerequisites for statistical analysis, it includes the necessary processes for designing a test based on statistical techniques and processing the test results derived therefrom.

The requirements for ensuring statistical adequacy in the implementation of a biological test can be summarized as follows. First, the sample population should be selected by using an appropriate test design method. Second, a specific probability distribution model should be used as a basic assumption in statistical analysis for data analysis. Otherwise, it is necessary to apply a data transformation for analysis. Third, if any outliers are in the test data, it is necessary to apply an appropriate statistical analysis to remove them. Lastly, Lastly, biological tests may frequently result in missing data, e.g. loss of test animals, dropout of subjects and failure of the experiment. In this case, appropriate measures for any missing data (hereafter, "missing value") might be required.

1. Test Design

The allocation of the different treatments to different experimental units (animals, tubes, etc.) should be made by some strictly random process. Any other choice of experimental conditions that is not deliberately allowed for in the experimental design should also be made randomly. Examples are the choice of positions for cages in a laboratory and the order in which treatments are administered. In particular, a group of animals receiving the same dose of any preparation should not be treated together (at the same time or in the same position) unless there is strong evidence that the relevant source of variation (for example, between times, or between positions) is negligible. Random allocations may be obtained from computers by using the built-in randomization function. The analyst must check whether a different series of numbers is produced every time the function is started. The preparations allocated to each experimental unit should be as independent as possible. Within each experimental group, the dilutions allocated to each treatment are not normally divisions of the same dose, but should be prepared individually. Without this precaution, the variability inherent in the preparation will not be fully represented in the experimental error variance and the result will be an under-estimation of the residual error leading to; an unjustified increase in the stringency of the test for the analysis of variance; an under-estimation of the true confidence limits for the test, which are calculated from the residual error.

The allocation of experimental units (animals, tubes, etc.) to different treatments may be made in various ways.

1) Completely Randomized Design

If the totality of experimental units appears to be reasonably homogeneous with no indication that variability in response will be smaller within certain recognizable sub-groups, the allocation of the units to the different treatments should be made randomly. If units in sub-groups such as physical positions or experimental days are likely to be more homogeneous than the totality of the units, the precision of the assay may be increased by introducing one or more restrictions into the design. A careful distribution of the units over these restrictions permits irrelevant sources of variation to be eliminated. By appropriately randomize the experimental units based on the additional restrictions, such as the date of the experiment, inappropriate sources of variability are eliminated. For example, when 6 treatments (3 reference standards of S1, S2 and S3, and 3 samples of T1, T2 and T3) are tested four times, the experiment is performed in sequence or positioning by randomization as follows.

	Sequence 1	**Sequence 2**	**Sequence 3**	**Sequence 4**	**Sequence 5**	**Sequence 6**
Run 1	T3	S1	S2	T2	T1	S3
Run 2	S1	T3	T2	S3	T1	S2
Run 3	T1	T3	S1	S3	T2	S2
Run 4	S1	S2	T3	S3	T1	T2

2) Randomized Block Design

In this design it is possible to segregate an identifiable source of variation, such as the sensitivity variation between litters of experimental animals or the variation between Petri dishes in a diffusion microbiological assay. The design requires that every treatment be applied an equal number of times in every block (litter or Petri dish) and is suitable only when the block is large enough to accommodate all treatments once. It is also possible to use a randomized design with repetitions. The treatments should be allocated randomly within each block. For example, when 4 doses of a reference standard (S1, S2, S3, S4) and a sample (T1, T2, T3, T4) are tested in 5 petri dishes (5 blocks), respectively, the test is performed following arrangement.

	Position 1	**Position 2**	**Position 3**	**Position 4**	**Position 5**	**Position 6**	**Position 7**	**Position 8**
Block 1	S4	T1	T2	S2	S1	T3	S3	T4
Block 2	T3	S3	T4	S4	T1	T2	S2	S1
Block 3	T2	S2	S1	T3	S3	T4	S4	T1
Block 4	T4	S4	T1	T2	S2	S1	T3	S3
Block 5	S1	T3	S3	T4	S4	T1	T2	S2

3) Latin Square Design

This design is appropriate when the response may be affected by two different sources of variation each of which can assume k different levels or positions. For example, in a plate assay of an antibiotic the treatments may be arranged in a $k \times k$ array on a large plate, each treatment occurring once in each row and each column. For example, when 6 treatments (3 reference standards of S1, S2 and S3, and 3 samples of T1, T2 and T3) are tested in 2 plates ($k = 6$), the treatments are assigned to each plate by randomization as follows. The design is suitable when the number of rows, the number of columns and the number of treatments is equal. Variations due to differences in response among the k rows and among the k columns may be segregated, thus reducing the error.

Plate 1						
	Row 1	**Row 2**	**Row 3**	**Row 4**	**Row 5**	**Row 6**
Column 1	T1	T2	S3	T3	S2	S1
Column 2	S3	S2	S1	T2	T1	T3
Column 3	S1	T1	S2	S3	T3	T2
Column 4	T2	T3	T1	S1	S3	S2
Column 5	S2	S1	T3	T1	T2	S3
Column 6	T3	S3	T2	S2	S1	T1

Plate 2						
	Row 1	**Row 2**	**Row 3**	**Row 4**	**Row 5**	**Row 6**
Column 1	T2	S1	S3	T3	T1	S2
Column 2	S3	S2	T1	S1	T2	T3
Column 3	S1	T1	T2	S2	T3	S3
Column 4	T1	T3	S1	S3	S2	T2
Column 5	T3	S3	S2	T2	S1	T1
Column 6	S2	T2	T3	T1	S3	S1

4) Crossover Design

This design is useful when the experiment can be sub-divided into blocks but it is possible to apply only 2 treatments to each block. For example, if 2 doses of a reference standard (T1 and T2) and of a sample (S1 and S2) are tested, this is known as a twin cross-over test, the test is performed following arrangement. According to this design, Units that received one preparation in the first part of the test receive the other preparation on the second occasion, and units receiving small doses in one part of the test receive large doses in the other. Between 2 time points, the reference standard and the sample also cross-over each other.

Group of units	Time point 1	Time point 2
Group 1	S1	T2
Group 2	S2	T1
Group 3	T1	S2
Group 4	T2	S1

2. Preprocessing of Test Data

1) Data Transformation

In the biological analysis, it is essential to meet the necessary conditions for statistical analysis of test results. Otherwise, the appropriate data transformation should be used on response values. The method of data conversion is as follows:

(1) Log Transformation

Natural logarithms or common logarithms can be used. Log transformation is useful when the uniformity of distribution is not satisfactory. In addition, When the data has a right-skewed distribution with a high positive (+) skewness, log transformation can improve the normality of the data distribution.

$$\text{Log transformation: } \eta = ln\, y \text{ or } log_{10}\, y$$

(2) Square Root Transformation

It is a useful data conversion method when the values obtained from counting have a Poisson distribution. It also improves the normality of data distribution when the data have a left-skewed distribution with negative (-) skewness caused by over log transformation.

$$\text{Square root transformation: } \eta = \sqrt{y}$$

(3) Square Transformation

The square transformation may be a useful method when the dose is proportional to the area rather than the diameter of the clear zone, for example, in Microbial Assays for Antibiotics. In addition, When the data has a left-skewed distribution with a high negative (-) skewness, square transformation can improve the normality of the data distribution.

$$\text{Square transformation: } \eta = y^2$$

Meanwhile, if the dose-response relationship obtained by counting experimental units are responsive to each treatment group, rather than measured values of each experimental unit, the dose-response relationship is linearized using various methods, such as Probit transformation, Logit transformation, Gompit transformation, etc. It is called quantal dose-response relationship. It is applicable to binominal distribution of data with values measured by only two nominal scales. For example, it applies when the efficacy of drug in a group of experimental units treated with a specific dosage is determined only by a "Yes" or "No". Similarly, it applies when the response to a specific dosage is determined as either “survival” or “death”.

(4) Probit Transformation

When the number of experimental units is n in each treatment groups, and the number of units is r, the corresponding ratio, p $(= r/n)$ is regarded as cumulative probability function (Φ) of the standard normal distribution (A normal distribution represented by z-scores with a average of 0 and a standard deviation of 1). The z-score corresponding to this ratio (p), denoted as $\Phi^{-1}(p)$, is referred to as the "normit." The value of normit plus 5 is called Probit. Even if 5 is not added, it is also called Probit transformation. Regardless of adding 5, the result of potency calculation remains same.

$$\text{Probit Transformation: } \eta = \Phi^{-1}(p)$$

(5) Logit Transformation

Quantal dose-response relationship can be explained by using a method to use a value of logit function corresponding to the antilog of logistic distribution. Where, logit transformation $logit(p)$ is $ln\{p/(1-p)\}$.

$$\text{Logit transformation: } \eta = logit(p) = ln\{p/(1-p)\}$$

(6) Gompit Transformation

Probit and logit transformations are the data transformation methods to be used when the log values $ln(dose)$ of dose schematized to responses are symmetry. If such schematization does not show symmetry, Gompit transformation is used. Transformation of responses by the Gompit function is expressed as $ln\{-ln(1-p)\}$. It is also called cloglog (complementary log-log transformation).

$$\text{Gompit transformation: } \eta = gompit(p) = ln\{-ln(1-p)\}$$

A method of data transformation is selected as appropriate method which can linearize the given data according to types of regression models and types of measurements or observations in monographs.

2) Detection of Outliers

It is justified to exclude the outliers from analysis if any response as a measured value does not meet the requirements of analysis for any issue during the analysis process or is deviated significantly from the statistical standard of general distribution compared to other measured values. However, random elimination or maintenance of outliers could be a cause of severe

Systematic error. Therefore, it is not correct to remove specific outliers simply based on their relative sizes, rather than based on the proper cause determination or practical analysis experience. The measurement estimated as an outlier can be detected and removed by using one of the following statistical methods.

(1) Dixon's Q-Test

It is a method to detect outliers based an assumption that the measurements of the equal response would be obtained in the group of experimental animals where the equal dose is used. In this test, when n measurements from a single group are arranged in ascending order ($y_1, y_2, \cdots, y_n$, i.e., y_1 = minimum; y_n = maximum), for both extremes, or one extreme's outlier candidate, the ratio ($Q_{calc} = G/R$) of the gap ($G = y_n - y_{n-1}$ or $G = y_2 - y_1$) between the outlier candidate and the nearest measurement to the range (range, $R = y_n - y_1$) of all measurements is compared with the critical value ($Q_{critical}$) suggested by Dixon to determine the outliers. In other words, if it is $Q_{calc} > Q_{critical}$, it is determined as an outliers. The equation to calculate the ratio between the range and gap, Q_{calc}, is applied as follows depending on the number of observations, i.e., sample size (or type of calculation).

Sample size (n) and type of calculation	Candidate outlier is the minimum (y_1)	Candidate outlier is the maximum (y_n)
3 - 7 (type=10)	$Q_{10} = (y_2 - y_1)/(y_n - y_1)$	$Q_{10} = (y_n - y_{n-1})/(y_n - y_1)$
8 - 10 (type=11)	$Q_{11} = (y_2 - y_1)/(y_{n-1} - y_1)$	$Q_{11} = (y_n - y_{n-1})/(y_n - y_2)$
(type=12)	$Q_{12} = (y_2 - y_1)/(y_{n-2} - y_1)$	$Q_{12} = (y_n - y_{n-1})/(y_n - y_3)$
(type=20)	$Q_{20} = (y_3 - y_1)/(y_n - y_1)$	$Q_{20} = (y_n - y_{n-2})/(y_n - y_1)$
11 - 13 (type=21)	$Q_{21} = (y_3 - y_1)/(y_{n-1} - y_1)$	$Q_{21} = (y_n - y_{n-2})/(y_n - y_2)$
> 14 (type=22)	$Q_{22} = (y_3 - y_1)/(y_{n-2} - y_1)$	$Q_{22} = (y_n - y_{n-2})/(y_n - y_3)$

(2) Grubbs' Test

It is a method to detect outliers based on an assumption that the measurements of the equal response would be obtained in the group of experimental animals where the equal dose is used, or to detect outliers in the residual values when the obtained residual value from the linear or non-linear regression model follows a specific distribution. In this test, when n measurements from a single group are arranged in ascending order ($y_1, y_2, \cdots, y_n$, i.e., y_1 = minimum; y_n = maximum), for both extremes, or one extreme's outlier candidate, G_{calc} ($= (y_n - \overline{y})/s_y$ or $= (\overline{y} - y_1)/s_y$) obtained from the average ($\overline{y}$) and standard deviation (s_y) of overall data is compared with the critical value ($Q_{critical}$) suggested by Grubbs to determine the outliers. In other words, if it is $G_{calc} > G_{critical}$, it is determined as an outlier. In other words, upon $G_{calc} > G_{critical}$, it is determined as an outlier. This method is called calculation type 10 (type=10). For the calculation type 11 (type=11), G_{calc} ($= (y_n - y_1)/s_y$) is obtained by dividing the range of measurements by standard deviation, which can remove the outliers at both extremes one by one. Sometimes, U_{calc} is used instead of G_{calc}, which refers to the ratio ($U_{calc} = S^2_{y,(2\sim n)}/S^2_{y,(n)}$ or $U_{calc} = S^2_{y,(1\sim n-1)}/S^2_{y,(n)}$) of standard deviation excluding candidate outliers ($S^2_{y,(2\sim n)}$ or $S^2_{y,(1\sim n-1)}$) by standard variance ($S^2_{y,(n)}$) including candidate outliers. For the calculation type 20 (type=20), two values at both extremes are defined as candidate outliers to calculate and determine U_{calc}.

Type of calculation	Candidate outlier is the minimum (y_1)	Candidate outlier is the maximum (y_n)
type=10	$G_{10} = (\overline{y} - y_1)/s_y$ $U_{10} = S^2_{y,(2\sim n)}/S^2_{y,(n)}$	$G_{10} = (y_n - \overline{y})/s_y$ $U_{10} = S^2_{y,(1\sim n-1)}/S^2_{y,(n)}$
type=11	$G_{11} = (y_n - y_1)/s_y$ $U_{11} = S^2_{y,(2\sim n-1)}/S^2_{y,(n)}$	
type=20	$G_{20} = (\overline{y} - y_2)/s_y$ $U_{20} = S^2_{y,(3\sim n)}/S^2_{y,(n)}$	$G_{20} = (y_{n-1} - \overline{y})/s_y$ $U_{20} = S^2_{y,(1\sim n-2)}/S^2_{y,(n)}$

(3) Huber's Test

It is a method to detect outliers on the basis of Median Absolute Deviation (MAD), which is calculated based on the median of measurements. In this test, the median (y_{median}) of n measurements ($y_1, y_2, \cdots, y_n$) from a single group, the absolute of difference between the individual measurements and that median ($u_i = | y_i - y_{median} |$), and the median ($(u_{median})$ of those values are obtained, and multiplied by consistency constant (c) according to probability distribution, to obtain the MAD ($c \times u_{median}$). The consistency constant is usually calculated as $c = 1/Q(0.75)$, where $Q(0.75)$ refers to 75% percentile in a specific probability distribution. In the case of assumption of normal distribution $Q(0.75) = \Phi^{-1}(0.75)$ and the consistency constant is 1.482602. Among the measurements (y_i), y_i corresponding to $| y_i - y_{median} | \geqq c \times u_{median} \times k$ determines outliers. In this case, k have the value of 3 (high), 2.5 (moderate), or 2 (low) depending on the prudence of determination.

3) Missing Value Method

During the implementation of bioassay, an accident totally unconnected with the applied treatments may lead to the loss of one or more responses. For example, one of experimental animals in a group may die, or one of samples may be lost during the experimental operation. If it is considered that the accident is in no way connected with the composition of the preparation administered, the exact calculations can still be performed but the formulae are necessarily more complicated and can only be given within the framework of general linear models However, there exists an approximate method which keeps the simplicity of the balanced design by replacing the missing response by a calculated value. The loss of information is taken into account by diminishing the degrees of freedom for the total sum of squares and for the residual error by the number of missing values and using one of the formulae below for the missing values. It should be borne in mind that this is only an approximate method, and that the exact method is to be preferred. If more than one observation is missing, the same formulae can be used. The procedure is to make a rough guess at all the missing values except one, and to use the proper formula for this one, using all

the remaining values including the rough guesses. Fill in the calculated value. Continue by similarly calculating a value for the first rough guess. After calculating all the missing values in this way the whole cycle is repeated from the beginning, each calculation using the most recent guessed or calculated value for every response to which the formula is being applied. This continues until 2 consecutive cycles give the same values. Provided that the number of values replaced is small relative to the total number of observations in the full experiment (say less than 5 per cent), the approximation implied in this replacement and reduction of degrees of freedom by the number of missing values so replaced is usually fairly satisfactory. The analysis should be interpreted with great care however, especially if there is a preponderance of missing values in one treatment or block, and a biometrician should be consulted if any unusual features are encountered. Replacing missing values in a test without replication is a particularly delicate operation.

(1) Imputation of Missing Values in Completely Randomized Design

Missing values can be imputed with the values (y') obtained by the following equation. It can be imputed by the arithmetic average (T'/k) of other response to the same treatment.

$$y' = \frac{T'}{k}$$

(2) Imputation of Missing Values in Randomized Block Design

Missing values can be imputed with the values (y') obtained by the following equation. Where, B' refers to the sum of response values in the block including missing values; T' refers to the sum of all treatments; G' refers to the sum of all responses recorded in analysis; n refers to the number of observations of the block including missing values; and k refers to the number of corresponding treatments.

$$y' = \frac{nB' + kT' - G'}{(n-1)(k-1)}$$

(3) Imputation of Missing Values in Latin Square Design

Missing values can be imputed with the values (y') obtained by the following equation. Where, B' and C' refer to the sum of response values in the rows and columns including missing values, respectively. In this case, $k = n$.

$$y' = \frac{k(B' + C' + T') - 2G'}{(k-1)(k-2)}$$

(4) Imputation of Missing Values by MICE Method

The Multivariate Imputation via Chained Equation (MICE) method is one of the multiple imputation. The MICE considers the uncertainty in the missing values rather than single imputation, such as the average value. In the MICE method, the probability of missing value solely depends on its observed values; the missing values are assumed to be estimated by using their observed values; and imputation is carried out on a variable basis by specifying an imputation model for each variable. For example, assuming to use k variables, if there is any missing value on the first variable (x_1), the repression equation for the first variable is obtained from other variables ($x_2, x_3, \cdots, x_k$) to obtain an estimate, and then the missing value is imputed by that estimate. If there is any missing value on the second variable (x_2), the repression equation for the second variable is obtained from other variables ($x_1, x_3, \cdots, x_k$) to obtain an estimate, and then the missing value is imputed by that estimate. Basically, linear regression is used for estimation of consecutive missing values, while logistic regression is used for categorical missing values. Once a cycle of calculation is completed, a number of data sets is created. In these data sets, only imputed missing values are different from each other. In general, it is recommended sorting these data sets to create a model and combining their results. 2 main variables are usually used in biological test methods, where Predictive Mean Matching (pmm) is used for consecutive variables; Logistic Regression (logreg) is used for binomial variables. The MICE method creates a number of pseudo-complete dataset by imputing the missing values with appropriate values obtained using an appropriate regression model depending on the type of data variables; analyzes each pseudo-complete dataset; and combines the individual analysis results to draw a comprehensive conclusion.

Capillary Electrophoresis
모세관 전기영동법

1. Principle

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

The migration velocity of an analyte under an electric field (E) is determined by the electrophoretic mobility of the analyte and the electro-osmotic mobility of the buffer solution inside the capillary. The electrophoretic mobility (μ_{ep}) depends on the characteristics of the analyte (electric charge and molecular size and shape) and those of the buffer solution (type, ionic strength, pH, viscosity, and additives of the electrolyte solution). The electrophoretic velocity (v_{ep}) of a solute, assumed to have a spherical shape, is calculated using the following equation:

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

q: Effective charge of the solute ion
η: Viscosity of the electrolyte solution
r: Stoke's radius of the solute ion
V: Voltage
L: Total length of the capillary

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

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

ε: Dielectric constant of the buffer solution
ζ: Zeta potential on the internal wall of the capillary

The velocity (v) of the analyte is calculated using the following equation:

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

The electrophoretic mobility of the analyte and the electro-osmotic mobility may act in the same direction or in the opposite direction, depending on the charge of the analyte. In a normal capillary electrophoresis, anions migrate in the opposite direction to the electro-osmotic flow and their velocities are slower than the electro-osmotic velocity. Cations migrate in the same direction as the electro-osmotic flow, and their velocities are faster than the electro-osmotic velocity. Under a condition in which the electro-osmotic velocity is faster than the electrophoretic velocity of the solute, both cations and anions can be separated in the same run.

The time (t) taken by the analyte to migrate the distance from the sample injection port of the capillary to the detection port (effective length of the capillary, ı) can be calculated using the following equation:

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

In general, above pH 3, fused-silica capillaries with an uncoated internal surface exhibit negative charge due to ionization of the silanol group present in the internal wall. As a result, an electro-osmotic flow from the anode to the cathode is generated. It is necessary to keep the electro-osmotic flow constant to achieve high reproducibility in the migration velocity of the solute. For some applications, it may be necessary to suppress the electro-osmotic flow by modifying the internal 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, ions of each analyte of the sample migrate to independent zones within the given electrolyte according to their electrophoretic mobility. Zone broadening, that is, the diffusion of each solute band, results from various reasons. Under ideal conditions, the only phenomenon contributing to the solute-zone broadening is molecular diffusion of the solute inside the capillary (longitudinal diffusion). In the ideal case, the segregation efficiency of the zone is expressed as the number of theoretical plates (N), which is calculated using the following equation:

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

D: Molecular diffusion coefficient of the solute in the buffer solution.

In general practice, other phenomena, such as heat dissipation, sample adsorption onto the capillary wall, mismatched conductivity between the sample and the buffer solution, length of the injection plug, detector cell size, and unleveled electrophoresis buffer reservoirs, can also play a significant role in band diffusion.

Segregation between two bands (expressed as the resolution, R_S) can be achieved by modifying the electrophoretic mobility of the analytes and the electro-osmotic mobility induced in the capillary, and by increasing the segregation efficiency for the zone of each analyte, according to the following 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

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

2. Apparatus

The device for capillary electrophoresis is composed of the following:

(1) An adjustable high-voltage, direct-current power supply

(2) Two electrophoresis buffer reservoirs, held at the same level, which contain specified anodic and cathodic solutions

(3) A pair of electrodes (cathode and the anode), immersed in the electrophoresis buffer reservoirs and connected to the power supply

(4) A capillary for segregation (usually made of fused-silica), which, when used with some specific types of detectors, has an optical viewing window aligned to the detector. Both ends of the capillary are placed in the buffer solution reservoirs. The capillary is packed with solutions specified in the monograph,

(5) A suitable sample injection system

(6) A detector for monitoring the amount of the substance of interest that passes through the detection port of the capillary at a given time. It is usually based on ultraviolet and visible absorption spectrophotometry or fluorometry, but conductimetric, amperometric, or mass spectrometric detection methods can also be useful for specific applications. Indirect detection methods are used to detect non UV-absorbing or non-fluorescent compounds.

(7) A thermostatic system for maintaining a constant temperature inside the capillary is recommended to achieve segregation with high reproducibility.

(8) A recorder and a suitable integrator or a computer

The injection procedure and its automation are critical for accurate quantitative analysis. Injection methods include hydrodynamic, pressure or vacuum, and the electrokinetic method. The amount of each analyte introduced electrokinetically depends on its respective electrophoretic mobility, which determines whether or not to choose this type of injection method.

For the analysis, use the capillary, the buffer solutions, the preconditioning method for the capillary, the sample solution, and the migration conditions specified in the Monograph of the substance of interest. To prevent interference with the detection or interruption of the electrical contact in the capillary during the segregation process, filter the electrolytic solution to be used to remove particles, and degas it to avoid bubble formation. A rigorous capillary-rinsing procedure should be implemented for each analytical method to obtain reproducible measurements of the migration time for the solutes.

3. Capillary Zone Electrophoresis

A. Principle

In capillary zone electrophoresis, analytes are separated in a capillary containing only a buffer solution that does not include any anti-convective medium. In this method, segregation takes place because the analytes 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. Coated capillaries can be used to increase the segregation capacity of the substances that are prone to adhere to the fused-silica surfaces. Using this mode of capillary electrophoresis, both small (Mr < 2000) and large molecules (2000 < Mr < 100000) can be analyzed. Due to its high

segregation efficiency, capillary zone electrophoresis can be used for the segregation of molecules having only a slight difference in their charge to mass ratios. Using this segregation method, optical isomer compounds can also be separated with the addition of chiral selectors to the buffer solution for segregation.

B. Optimization of segregation

Optimization of segregation is a complex process in which several segregation parameters play critical roles. When it comes to segregation, optimization of the segregation conditions can be complicated. The main factors to be considered in the development of this segregation method are instrumental and electrolytic solution parameters.

Instrumental parameters

(1) Voltage The Joule heating plot is useful for the optimization of the applied voltage and the capillary temperature. The segregation time is inversely proportional to the applied voltage. However, an increase in the voltage produces excessive heat, which elevates the temperature inside the capillary, leading to the generation of a viscosity gradient in the buffer solution inside the capillary. This effect results in band broadening, and thus low resolution.

(2) Polarity Electrode polarity is normal voltage (anode at the sample injection port and cathode at the discharged solution outlet) and the electro-osmotic flow move toward the cathode. If the electrode polarity is reversed, the electro-osmotic flow moves from the discharged solution outlet to the injection port direction, and only charged analytes with electrophoretic mobilities faster than the electro-osmotic mobility pass the detection port.

(3) Temperature Temperature mainly affects the viscosity and electrical conductivity of the buffer solution, which, in turn, influence the migration velocity. In some cases, an increase in capillary temperature can induce a conformational change in proteins, modifying their migration times or efficiency of segregation.

(4) Capillary The dimensions of the capillary (length and internal diameter) affect the analysis time, the efficiency of segregation, and the sample load capacity. By increasing both the effective length and the total length of the capillary, the intensity of the electric field (working at constant voltage) can be lowered, which results in a longer migration time. For a given buffer solution and electric field condition, the heat dissipation rate varies depending on the internal diameter of the capillary. Therefore, the degree of sample band-diffusion is affected by the internal diameter of the capillary. Sample band diffusion also have an impact on the detection limit to varying degrees, depending on the sample volume injected and the detection system employed.

Since the adsorption of analytes to be detected on the capillary wall lowers the efficiency, methods to avoid such adsorption should be considered during the establishment of the segregation method. For protein samples, in particular, several strategies have been devised to prevent sample adsorption on the capillary wall. Some of these strategies simply require to modify the composition of buffer solution (use of extreme pH or adsorption of positively charged buffer additives on the internal wall) to avoid protein adsorption. Also, the internal wall of the capillary can be coated with a polymer, covalently bonded to the silica, so that interactions between proteins and the negatively charged silica surface are prevented. For this purpose, ready-to-use capillaries coated with neutral-hydrophilic, cationic, or anionic polymers are available.

Parameters related to electrolytic solutions

(1) Type and concentration of buffer solution Suitable buffer solutions for capillary electrophoresis should have an appropriate buffering capacity with the desired pH range and low mobility to minimize current generation. By matching the mobility of ions in the buffer solution with that of the solute, band distortion can be minimized. The type of solvent used for the sample is also important to achieve zone focusing of the sample inside the capillary, which can increase the efficiency of segregation and improve the sensitivity of detection. An increase in the concentration of a buffer solution under a given pH decreases the electro-osmotic flow and the migration velocity of the solute.

(2) pH of buffer solution The pH of the buffer solution can change the charge of the analyte or additives and influence the electro-osmotic flow, thereby affecting segregation. In the segregation of proteins and peptides, changing the pH of the buffer solution from above to below the isoelectric point (pI) of the sample results in converting the net charge of the solute from negative to positive. An increase in the pH of a buffer solution generally speeds up the electro-osmotic flow.

(3) Organic solvents Organic solvents (methanol, acetonitrile, etc.) may be added to an aqueous buffer solution to increase the solubility of the solute or other additives and/or to change the degree of ionization of the solute components. In general, the addition of such organic solvents to a buffer solution causes a reduction in the electro-osmotic flow.

(4) Additives for the segregation of optical isomers For the segregation of optical isomers, a chiral selector is added to the buffer solution for segregation. The most commonly used chiral selectors are dextrins, but crown ethers, polysaccharides, and proteins may also be used. The recognition of optical isomers is governed by the different interactions between the chiral selector and each optical isomer, so the resolution achieved for chiral compounds depends largely on the type of chiral selectors used. In this regard, to achieve a desired segregation, it may be useful to test cyclodextrins with different cavity sizes (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, it is necessary to take into account batch-to-batch variations in the degree of substitution for cyclodextrins, as this may influence the efficiency of chiral segregation. Other factors affecting the resolution in the chiral segregation include the concentration of the chiral selector, the composition and pH of the buffer solution, and the temperature during analysis. The use of organic additives, such as methanol and urea, can also influence the resolution achieved.

4. Capillary Gel Electrophoresis

A. Principle

In capillary gel electrophoresis, segregation takes place inside a capillary filled with a gel acting as a molecular sieve. Molecules with similar charge to mass ratios are separated according to their molecular size, since smaller molecules move more freely through the gel network than larger molecules, meaning that they migrate faster. Biological macromolecules with similar charge-to-mass ratios (e.g., proteins and DNA fragments) can thus be separated according to their molecular weight by capillary gel electrophoresis.

B. 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. These gels are typically bonded to the fused silica wall, making them non-removable without damaging the capillary. When used for protein analysis under reducing conditions, the segregation buffer solutions usually contain sodium dodecyl sulfate. Before injection, the sample is denatured by heating with a mixture of sodium dodecyl sulfate and 2-mercaptoethanol or dithiothreitol. In an analysis under non-reducing conditions (e.g., analysis of intact antibodies), 2-mercaptoethanol and dithiothreitol are not used. Segregation in cross-linked gels can be optimized through modifying the segregation buffer solution (as described in the section on capillary zone electrophoresis) or by controlling the porosity of the gel at the time of gel preparation. For cross-linked polyacrylamide gels, the porosity of the gel can be modified by changing the concentration of acrylamide and/or the proportion of the cross -linking agent. 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 the gels, only the electrokinetic method can be used for sample injection.

Dynamically coated gels are made of hydrophilic polymers, such as linear polyacrylamide, cellulose derivatives, and dextran, which can be dissolved in aqueous segregation buffer solutions, serving as a segregation medium that also acts as a molecular sieve. These segregation media are easier to prepare compared to cross-linked polymers. They can be prepared in a vial and filled in an inner wall-coated capillary by applying pressure to prevent the generation of electro-osmotic flow. In general, the reproducibility of segregation can be enhanced by replacing the gel before every injection of the solute. The porosity of the gel can be increased by using polymers of higher molecular weight (at a given polymer concentration) or by decreasing the concentration of the polymer (for a given molecular weight of the polymer). A reduction in the porosity of the gel leads to a decrease in the mobility of the solute for the same buffer solution. Since the dissolution of these polymers in the buffer solution still gives low viscosity, both hydrodynamic and electrokinetic injection techniques can be used for sample injection.

5. Capillary Isoelectric Focusing

A. Principle

In isoelectric focusing, the molecules in the sample 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 (polyaminocarboxylic acids) that is dissolved in the segregation buffer solution. There are three basic steps of isoelectric focusing; loading, focusing, and mobilization

(1) Loading step: One of two methods may be used

(i) *Loading in one step* The sample is mixed with ampholytes and introduced into the capillary either by applying pressure or using a vacuum.

(ii) *Sequential loading* Solutions are injected sequentially into the capillary in the order of the leading buffer solution, ampholytes, sample mixed with ampholytes, ampholytes alone, and finally the terminating buffer solution. It is necessary to keep the volume of the sample small so as not to disrupt the pH gradient.

(2) Focusing step: When the voltage is applied, ampholytes migrate toward the cathode or the anode according to their net charges, thus creating a pH gradient from the anode (lower pH) to the cathode (higher pH). The molecules separated in this step migrate until they reach a pH corresponding to their isoelectric point (pI), at which the current drops significantly.

(3) Mobilization step: If mobilization is required for detection, one of the following methods can be used:

(i) In the first method, mobilization of molecules is accomplished during the focusing step under the influence of the electro-osmotic flow. In this case, it is necessary to keep the electro-osmotic flow low enough to allow the focusing of the molecules.

(ii) In the second method, mobilization is accomplished by applying pressure after the focusing step is completed.

(iii) In the third method, mobilization is achieved after the focusing step by adding salts to the cathode or anode reservoir (depending on the direction of mobilization chosen) and applying voltage so as to alter the pH in the capillary, thereby causing migration of the molecules. According to the change in pH, the proteins and ampholytes are mobilized in the direction of the reservoir containing the added salts and pass the detector. The segregation achieved can be expressed as ΔpI, depending 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 change in electrophoretic mobility of the analyte under the pH (-dμ/dpH).

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

B. Optimization

The main parameters determining the segregation conditions are as follows:

(1) Voltage Capillary isoelectric focusing utilizes very high electric fields, 300 to 1000 V/cm, in the focusing step.

(2) Capillary It is necessary to reduce the electro-osmotic flow to zero or to suppress it to the minimum, depending on the method used to mobilize the analyte to the detection port (see above). Inner-coated capillaries tend to reduce the electro-osmotic flow.

(3) Solutions The anode buffer solution reservoir is filled with a solution with a pH lower than the pI of the most acidic ampholyte, while the cathode reservoir is filled with a solution with a pH higher than the pI of the most basic ampholyte. Most typically, phosphoric acid is used for the anode and sodium hydroxide is used for the cathode.

By adding a polymer, such as methylcellulose, to the ampholyte solution, viscosity can be increased, thus suppressing convective forces or the electro-osmotic flow. Ampholytes covering wide pH ranges are commercially available, and these can be mixed if even broader pH ranges are required. Broad pH ranges are used to estimate the isoelectric point, whereas narrow pH ranges are used to improve accuracy of analysis. Calibration of the pH can be done by correlating the migration time with the isoelectric points of a series of protein markers.

By using buffer additives, such as glycerol, surfactants, urea, and zwitterionic buffers, precipitation of proteins at their isoelectric points, which may occur during the focusing step, can be prevented. However, urea may denature proteins in a concentration-dependent manner.

6. Micellar Electrokinetic Chromatography (MEKC)

A. Principle

In micellar electrokinetic chromatography, segregation 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 pseudo-stationary phase made up of micelles and the aqueous buffer solution based on the partition coefficient of the solute. As such, the technique can be considered a hybrid of electrophoresis

and chromatography. MECK is an electrophoresis method that can be used for the segregation of both neutral and charged solutes, while 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 (SDS), although other surfactants like cationic surfactants, including cetyltrimethylammonium salts, are also used.

The segregation mechanism in MECK is as follows: Under neutral and alkaline pH conditions, a strong electro-osmotic flow is generated, which moves the ions in the segregation buffer solution toward the direction of the cathode. If SDS is used as the surfactant, the electrophoretic migration of the anionic micelle is in the opposite direction, towards the anode. As a result, the overall migration velocity of the micelle is slowed down compared to the bulk flow of the electrolytic solution. With neutral solutes, since partition of the analyte takes place between the micelle and the aqueous buffer solution and there is no electrophoretic mobility, the migration velocity of the analyte depends only on the partition coefficient between the micelle and the aqueous buffer solution. In the electropherogram, the peaks corresponding to each uncharged solute are always present between the peak of the electro-osmotic flow marker and that of the micelle (the time elapsed between these two peaks is called the segregation window). For electrically charged solutes, their migration velocity depends both on the partition coefficient of the solute between the micelle and the aqueous buffer solution, and on the electrophoretic mobility of the solute in the absence of the micelle.

Since the principle of segregation in MEKC for neutral or weakly ionized solutes essentially lies in chromatography, migration of the solute and resolution can be rationalized in terms of the retention factor of the solute (k'), that is, the mass distribution ratio (Dm), which is the ratio of the number of moles of the solute in the micelle to those in the mobile phase.

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

t_R: Migration time of the solute
t_0: Migration time of an unretained solute (an electro-osmotic flow marker which does not enter the micelle, for instance, the migration time of methanol)
t_{mc}: Migration time of the micelle (migration time of a micelle marker, such as Sudan III, which co-migrates with the micelle while being continuously associated in the micelle)
K: Partition coefficient of the analyte
V_S: Volume of the micellar phase
V_M: Volume of the mobile phase

In the same way, the resolution between two closely migrating solutes (RS) can be calculated using the following equation:

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

N: Number of theoretical plates for one component of the solutes
a: Selectivity
k'_a, k'_b: Retention factors for each solute ($k'b > k'a$)

Similarly, k' and RS values for electrically charged analytes can be calculated by the same equation.

B. Optimization

The main parameters to be considered in determining the segregation conditions for MEKC are as follows:

Instrumental parameters

(1) Voltage The segregation time is inversely proportional to the voltage applied. However, an increase in voltage produces excessive heat, which gives rise to a temperature gradient and a viscosity gradient in the cross-section of the capillary. This phenomenon is highly likely to be observed when high conductivity buffer solutions, such as those containing micelles, are used. Poor heat dissipation causes band broadening, and thus low resolution.

(2) Temperature Variations in capillary temperature affect the partition coefficient of the solute between the buffer solution and the micelle, the critical micellar concentration, and the viscosity of the buffer solution. These parameters influence the migration time of the solutes. The use of a proper cooling system can help improve the reproducibility of the migration time for the solutes.

(3) Capillary As in the case of capillary zone electrophoresis, the dimensions of the capillary (length and internal diameter) affect the analysis time and the efficiency of segregation. By increasing both the effective length and the total length, the electric field (working at constant voltage) can be weakened, thus increasing the migration time and enhancing the efficiency of segregation. The internal diameter plays a role in heat dissipation (for a given buffer solution and electric field condition), thereby influencing the band width of the analyte.

Parameters related to electrolytic solutions

(1) Type and concentration of the surfactant The type of surfactant affects resolution since it modifies the selectivity of segregation in the same manner as the stationary phase does in chromatography. Also, as the concentration of the surfactant increases in the mobile phase, the log k' value of a neutral compound increases linearly.

Since the resolution in MEKC reaches a maximum as k′ approaches the value of $\sqrt{t_m/t_0}$, if the concentration of the surfactant in the mobile phase changes, the resolution obtained is also changed.

(2) pH of the buffer solution Although pH does not change the partition coefficient of non-ionized solutes, it can change the electro-osmotic flow inside the uncoated capillaries. In MEKC, a decrease in the pH of the buffer solution results in a reduction in the electro-osmotic flow, which, in turn, increases the resolution of the neutral solutes, giving rise to a longer analysis time.

(3) Organic solvents To enhance the segregation of hydrophobic compounds in MEKC, organic modifiers, such as methanol, propanol, and acetonitrile, may be added to the electrolytic solution. The addition of these modifiers usually decreases the migration time and the selectivity of segregation. Considering that the addition of organic modifiers affects the critical micellar concentration, a given concentration of the surfactant can be used only within a certain concentration range of an organic modifier before reaching the extent to which the micellization is inhibited or adversely affected, resulting in loss of micelles and, therefore, loss of partition. The dissociation of micelles in high concentrations of organic solvents does not always mean that the segregation will no longer be possible, In some cases, due to the hydrophobic interaction between the ionic surfactant monomer and the neutral solutes, solvophilic

complexes that are electrophoretically separable may be formed.

(4) Additives for the segregation of optical isomers For the segregation of optical isomers using MEKC, a chiral selector is included in the micellar system, either by covalent binding it to the surfactant or by adding it to the micellar segregation electrolyte. Examples of micelles that have a moiety with chiral recognition capabilities include salts of N-dodecanoyl-L-amino acid salts, bile salts, etc. Segregation of optical isomers can also be achieved by adding a chiral selector, such as cyclodextrins, to the electrolytic solutions containing micellized achiral surfactants.

(5) Other additives There are several other methods for modifying selectivity, by adding chemicals to the buffer solution. By adding several types of cyclodextrins to the buffer solution, the interaction between hydrophobic solutes and micelles can also be reduced, thereby increasing selectivity.

Alternatively, by adding a substance that adheres to the micelle, solute-micelle interactions can be controlled, thereby improving the selectivity of segregation in MEKC. These additives include different types of ionic or non-ionic surfactants which form mixed micelles or metallic cations which dissolve in the micelle and form co-ordination complexes with the solutes.

7. Quantification

Peak areas must be divided by the corresponding migration time to calculate the corrected area for the following reasons:

(1) To reduce the variation in the response by correcting the variation of the migration time from run to run

(2) To correct the different responses of sample components with different migration times

In cases where an internal standard is used, it is necessary to ensure that no peak of the substance to be examined overlaps with that of the internal standard.

A. Calculations

From the values obtained, calculate the content of the component of interest. For test samples under regulation, the content % of one or more components of the sample to be examined is calculated by determining the area % of the peak of interest with respect to the total area of all peaks after corrections are applied, excluding the peaks of solvents or additives. The use of an automatic integration system (integrator or data entry and processing system) is recommended.

8. System suitability

To determine the suitability of the capillary electrophoresis system, system suitability parameters are used. The choice of these parameters depends on the mode of capillary electrophoresis used. System suitability parameters include the mass distribution ratio (K', for micellar electrokinetic chromatography only), the number of theoretical plates (N), the symmetry factor (AS), and the resolution (RS). The theoretical explanations for N and RS are provided above, but more practical equations that allow these parameters to be calculated from the electropherograms are given below.

Number of theoretical plates

The number of theoretical plates (N) can be calculated using the following equation:

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

t_R: Migration time or distance along the baseline from the point of injection of the solute to the point perpendicularly dropped from the apex of the peak of the component of interest

W_h: Half the width of the peak

Resolution

The resolution (RS) between peaks of two component with similar heights can be calculated using the following equation:

$$R_s = \left(\frac{1.18(t_{R2} - t_{R1})}{W_{h1} + W_{h2}}\right) t_{R2} > t_{R1}$$

t_{R1}, t_{R2}: Migration times or distances along each baseline from the point of injection of the solute to each point perpendicularly dropped from the apexes of two adjacent peaks

W_{h1}, W_{h2}: Half the widths of each peak

When appropriate, the resolution may be calculated by measuring the height of the valley (*H*v) between two partly separated peaks and the height of the smaller peak (*H*p) and then calculating the peak-to-valley ratio.

$$p/v = \frac{H_P}{H_V}$$

Symmetry factor

The symmetry factor (As), which shows the extent of symmetry of a peak, can be calculated using the following equation:

$$A_s = \frac{W_{0.05}}{2d}$$

$W_{0.05}$: Width of the peak at 1/20 of the peak height

d: Distance between the point perpendicularly dropped from the apex of the peak and the ascending point of the peak at 1/20 of the peak height.

Tests for repeatability of the area (standard deviation of areas or of the area-to migration time-ratio) and for repeatability of the migration time (standard deviation of the migration time) should be included in the suitability parameters. The suitability of the capillary washing procedure can be evaluated through testing repeatability of the migration time. An alternative practice to compensate for poor repeatability of the migration time is to use the relative migration time with respect to an internal standard.

A test for determining the signal-to-noise (S/N) ratio for a standard sample (or for determining the limit of quantification) may also be useful.

Signal-to-noise (S/N) ratio

The limit of detection and the limit of quantification correspond to the S/N ratios of 3 or greater and 10 or greater, respectively. The S/N ratio is calculated using the following equation:

$$\mathrm{S/N} = \frac{2H}{h}$$

H: Height of the peak corresponding to the component of interest in the electropherogram obtained with the prescribed reference solution; the distance from the apex of the peak to the extrapolated baseline of the signal observed over a distance equal to twenty times the half the width of the peak is measured.

h: Width of the background in an electropherogram obtained after injection of a blank test solution, observed over a

distance equal to twenty times half the width of the peak in the electropherogram obtained with the prescribed standard solution and, if possible, situated equally around the places where each peak would be found.

Classification of Antibiotic Substances and Antibiotic Drugs
항생물질의 계와 류 분류

I. Glycopeptides Antibiotics

1. Vancomycin Antibiotic Drugs

1) Vancomycin is a substance prepared by culturing *Streptomyces orientalis* or an equivalent substance prepared through other methods.

2) Drugs in this class include vancomycin, vancomycin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of vancomycin ($C_{66}H_{75}Cl_2N_9O_{24}$) in mass (potency).

4) 1.025 mg of vancomycin hydrochloride reference standard ($C_{66}H_{75}Cl_2N_9O_{24}\cdot HCl$) is equivalent to a potency of 1 mg.

2. Teicoplanin Antibiotic Drugs

1) Teicoplanin is a mixture of teicoplanin A_3, teicoplanin $A_{2\text{-}1}$, teicoplanin $A_{2\text{-}2}$, teicoplanin $A_{2\text{-}3}$, teicoplanin $A_{2\text{-}4}$, teicoplanin $A_{2\text{-}5}$, and the related substances that are prepared by culturing *Actinoplanes teichomyceticus*.

2) Drugs in this class include teicoplanin, teicoplanin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of a mixture of teicoplanin A_3 ($C_{72}H_{68}Cl_2N_8O_{28}$) accounting for ≤15 %, A_2 groups [teicoplanin $A_{2\text{-}1}$ ($C_{88}H_{95}Cl_2N_9O_{33}$), teicoplanin $A_{2\text{-}2}$ ($C_{88}H_{97}Cl_2N_9O_{33}$), teicoplanin $A_{2\text{-}3}$ ($C_{88}H_{97}Cl_2N_9O_{33}$), teicoplanin $A_{2\text{-}4}$ ($C_{89}H_{99}Cl_2N_9O_{33}$), and teicoplanin $A_{2\text{-}5}$ (C_{89} $H_{99}Cl_2N_9O_{33}$)] accounting for ≥ 80%, and the related substances accounting for ≤ 5% in mass (potency).

4) 1 mg of teicoplanin reference standard is equivalent to a potency of 1 mg.

II. Rifamycin Antibiotics

1. Rifamycin SV Antibiotic Drugs

1) Rifamycin SV is a substance prepared by culturing *Streptomyces mediterranei* or an equivalent substance prepared through other methods.

2) Drugs in this class include rifamycin SV, rifamycin SV salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of rifamycin SV ($C_{37}H_{47}NO_{12}$) in unit.

4) 1.127 μg of rifamycin SV reference standard ($C_{37}H_{47}NO_{12}$) is equivalent to 1 unit (potency).

2. Rifabutin Antibiotic Drugs

1) Rifabutin is an antibiotic substance synthesized from rifabutin S. It is also known as (1) 6,9-dihydro-5,17,19,21-tetrahydroxy-8,9-(2-spiro-(*N*-isobutyl-4-piperidyl)-2,5-dihydro-1*H*-imidazo]-23-methoxy-2,4,12,16,18,20,22-heptamethyl-6-oxo-2,7-(epoxypentadeca-[1,11,13]-trienimino)-naphtho-[2,1-b]-furan-1,11-(2*H*)-dione-21-acetate and (2) 4-deoxo-3,4-[2-spiro-(*N*-isobutyl-4-piperidyl)-2,5-dihydro-1*H*-imidazo]-rifamycin S.

2) Drugs in this class include rifabutin, rifabutin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of rifabutin ($C_{46}H_{64}N_4O_{11}$) in mass (potency).

4) 1 mg of rifabutin reference standard ($C_{46}H_{64}N_4O_{11}$) is equivalent to a potency of 1 mg.

3. Rifaximin Antibiotic Drugs

1) Rifaximin is a chemical derivative of rifamycin SV, which is prepared by culturing *Streptomyces mediterranei*. It is also known as 4-deoxy-4-methylpyrido-(1',2'-1,2)-imidazo-(5, 4 C)-rifamycin SV.

2) Drugs in this class include rifaximin and preparations containing it.

3) The potency of drugs in this class is expressed as the amount of rifaximin ($C_{43}H_{51}N_3O_{11}$) in mass (potency).

4) 1.008 mg of rifaximin reference standard ($C_{43}H_{51}N_3O_{11}$) is equivalent to a potency of 1 mg.

4. Rifampicin Antibiotic Drugs

1) Rifampicin is a chemical derivative of rifamycin SV, which is prepared by culturing *Streptomyces mediterranei*. It is also known as 3-[(4-methyl-1-piperazinylimino)]methyl rifamycin SV.

2) Drugs in this class include rifampicin, rifampicin salts, and preparations containing them.

3) The potency of antibiotic drugs in this class is expressed as the amount of rifampicin ($C_{43}H_{58}N_4O_{12}$) in mass (potency).

4) 1 mg of rifampicin reference standard ($C_{43}H_{58}N_4O_{12}$) corresponds to a potency of 1 mg.

III. Lincomycins Antibiotics

1. Lincomycin Antibiotic Drugs

1) Lincomycin is a substance prepared by culturing *Streptomyces lincolnensis var. lincolnensis* or an equivalent substance prepared through other methods.

2) Drugs in this class include lincomycin, lincomycin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of lincomycin ($C_{18}H_{34}N_2O_6S$) in mass (potency).

4) 1.112 mg of lincomycin hydrochloride reference standard ($C_{18}H_{34}N_2O_6S.HCl.H_2O$) is equivalent to a potency of 1 mg.

2. Clindamycin Antibiotic Drugs

1) Clindamycin is a chemical derivative of lincomycin. It is also known as (7*S*)-7-chloro-7-deoxylincomycin.

2) Drugs in this class include clindamycin, salts or ester derivatives of clindamycin, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of clindamycin ($C_{18}H_{33}ClN_2O_5S$) in mass (potency).

4) 1.128 mg of clindamycin hydrochloride reference standard ($C_{18}H_{33}ClN_2O_5S.HCl.H_2O$) is equivalent to a potency of 1 mg, and 1.252 mg of clindamycin phosphate reference standard ($C_{18}H_{34}ClN_2O_8PS$) is equivalent to a potency of 1 mg.

IV. Macrolide Antibiotics

1. Dirithromycin Antibiotic Drugs

1) Dirithromycin is a macrolide with a lactone ring composed of 14 elements. It is an erythromysylamine oxazine derivative formed by condensation of the hydroxyl group at the 11th position of erythromycin, in which the ketone at the 9th position of erythromycin is converted to an amine, with 2-(2-methoxyethoxy) acetaldehyde. It is also known as 9-(*S*)-9-deoxy-11-deoxy-9,11-[imino[(1*R*)-2-(2- methoxyethoxy) ethylidene] oxy] erythromycin.

2) Drugs in this class include dirithromycin, its isomer

epidilithromycin, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of dirithromycin ($C_{42}H_{78}N_2O_{14}$) in mass (potency).

4) 1 mg of dirithromycin reference standard ($C_{42}H_{78}N_2O_{14}$) in its anhydrous form is equivalnet to a potency of 1 mg.

2. Rokitamycin Antibiotic Drugs

1) Rokitamycin is 3″-*O*-propionylleucomycin A_5. It is prepared by propionylating the 3″ hydroxyl group of leucomycin A_5, which is prepared by culturing a variant of *Streptomyces Kitasatoensis.*

2) Drugs in this class include rokitamycin, rokitamycin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of rokitamycin ($C_{42}H_{69}NO_{15}$) in mass (potency).

4) 1 mg of rokitamycin reference standard ($C_{42}H_{69}NO_{15}$) is equivalnet to a potency of 1 mg.

3. Roxithromycin Antibiotic Drugs

1) Roxithromycin is a 9-[O-(2-methylethoxy) methyloxime] derivative of erythromycin.

2) Drugs in this class include roxithromycin and preparations containing it.

3) The potency of drugs in this class is expressed as the amount of roxithromycin ($C_{41}H_{76}N_2O_{15}$) in mass (potency).

4. Midecamycin Antibiotic Drugs

1) Midecamycin is a substance prepared by culturing *Streptomyces mycarofaciens* or an equivalent substance prepared through other methods.

2) Drugs in this class include midecamycin, midecamycin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of midecamycin ($C_{41}H_{67}NO_{15}$) in mass (potency).

4) 1 mg of midecamycin reference standard ($C_{41}H_{67}NO_{15}$) is equivalent to a potency of 1 mg.

5. Spiramycin Antibiotic Drugs

1) Spiramycin is a mixture of spiramycin I, spiramycin II, and spiramycin III, which are prepared by culturing *Streptomyces ambofaciens* or an equivalent substance prepared through other methods.

2) Drugs in this class include spiramycin, spiramycin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of spiramycin I ($C_{43}H_{74}N_2O_{14}$) in mass (potency).

4) 1 mg of spiramycin reference standard ($C_{43}H_{74}N_2O_{14}$) is equivalnet to 3200 units (potency).

6. Acetylspiramycin Antibiotic Drugs

1) Acetylspiramycin is an acetyl derivative of spiramycin.

2) Drugs in this class include acetylspiramycin and preparations containing it.

3) The potency of drugs in this class is expressed as the amount of acetylspiramycin II ($C_{47}H_{78}N_2O_{16}$) in mass (potency).

4) 0.8122 mg of acetylspiramycin reference standard ($C_{47}H_{78}N_2O_{16}$) is equivalent to a potency of 1 mg.

7. Azithromycin Antibiotic Drugs

1) Azithromycin is a derivative of antibiotics prepared by culturing *Streptomyces erythreus*. It is also known as 9-deoxo-9a-methyl-9a-aza-9a-homoerythromycin A.

2) Drugs in this class include azithromycin and preparations containing it.

3) The potency of drugs in this class is expressed as the amount of azithromycin ($C_{38}H_{72}N_2O_{12}$) in mass (potency).

4) 1.049 mg of azithromycin reference standard ($C_{38}H_{72}N_2O_{12}.2H_2O$) is equivalent to a potency of 1 mg.

8. Erythromycin Antibiotic Drugs

1) Erythromycin is a substance prepared by culturing *Streptomyces erythreus* or an equivalent substance prepared through other methods.

2) Drugs in this class include erythromycin, salts or derivatives of erythromycin, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of erythromycin ($C_{37}H_{67}NO_{13}$) in mass (potency).

4) 1.053 mg of erythromycin reference standard ($C_{37}H_{67}NO_{13}.2H_2O$) is equivalent to a potency of 1 mg.

9. Josamycin Antibiotic Drugs

1) Josamycin is a substance prepared by culturing *Streptomyces narbonensis var. josamyceticus* or an equivalent substance prepared through other methods.

2) Drugs in this class include josamycin, salts or derivatives of josamycin, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of josamycin ($C_{42}H_{69}NO_{15}$) in mass (potency).

4) 1 mg of josamycin reference standard ($C_{42}H_{69}NO_{15}$) is equivalent to a potency of 1 mg, and 1.068 mg of josamycin propionate reference standard ($C_{45}H_{73}NO_{16}$) is equivalent to a potency of 1 mg.

10. Midecamycin Acetate Antibiotic Drugs

1) Midecamycin acetate is an acetic acid ester of midecamycin.

2) Drugs in this class include midecamycin acetate and preparations containing it.

3) The potency of drugs in this class is expressed as the amount of midecamycin acetate ($C_{45}H_{71}NO_{17}$) in mass (potency).

4) 1 mg of midecamycin acetate reference standard ($C_{45}H_{71}NO_{17}$) is equivalent to a potency of 1 mg.

11. Clarithromycin Antibiotic Drugs

1) Clarithromycin is prepared by methylating the hydroxyl group of erythromycin at the 6th position. It is known as a 6-O-methylerythromycin.

2) Drugs in this class include clarithromycin, clarithromycin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of clarithromycin ($C_{38}H_{69}NO_{13}$) in mass (potency).

4) 1 mg of anhydrous clarithromycin reference standard ($C_{38}H_{69}NO_{13}$) is equivalent to a potency of 1 mg.

12. Kitasamycin Antibiotic Drugs

1) Kitasamycin is a mixture of leucomycin A_1, leucomycin A_3, leucomycin A_4, leucomycin A_5, leucomycin A_6, leucomycin A_7, leucomycin A_8, leucomycin A_9, leucomycin A_{13}, etc., which are prepared by culturing *Streptomyces kitasatoensis*

2) Drugs in this class include kitasamycin, salts or derivatives of kitasamycin, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of leucomycin A_5($C_{39}H_{65}NO_{14}$) in mass (potency).

4) 0.530 mg of kitasamycin reference standard ($C_{39}H_{65}NO_{14}$) is equivalent to a potency of 1 mg.

13. Telithromycin Antibiotic Drugs

1) Telithromycin is a semi-synthetic derivative of erythromycin A, which has a ketone group instead of an L-cladinose group at the 3rd position. It is also known as 11,12-

dideoxy-3-de[(2,6-dideoxy-3-C-methyl- 3-*O*-methyl-alpha-*L*-ribo-hexopyranosyl)oxy]-6-*O*-methyl-oxo-12,11-[oxycarbonyl[[4-[4-(3-pyridinyl)- 1*H*-imidazol-1-yl]butyl]iminol]-erythromycin.

2) Drugs in this class include telithromycin and preparations containing it.

3) The potency of drugs in this class is expressed as the amount of telithromycin ($C_{43}H_{65}N_5O_{10}$) in mass (potency).

4) 1 mg of telithromycin reference standard ($C_{43}H_{65}N_5O_{10}$) is equivalent to a potency of 1 mg.

V. Monobactam Antibiotics

1. Aztreonam Antibiotic Drugs

1) Aztreonam is an antibiotic substance prepared by synthesis. It is also known as [2-[2α,3β(*Z*)]]-2-[[[1-(2-amino-4-thiazolyl)-2-[(2-methyl-4-oxo-1-sulfo-3-azetidinyl) amino]-2-oxoethylidene]amino]oxy]-2-methyl-propionic acid.

2) Drugs in this class include aztreonam, aztreonam salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of aztreonam ($C_{13}H_{17}N_5O_8S_2$) in mass (potency).

4) 1 mg of Aztreonam reference standard ($C_{13}H_{17}N_5O_8S_2$) is equivalent to a potency of 1 mg.

2. Carumonam Antibiotic Drugs

1) Carumonam is a substance prepared by culturing *Pseudomonas acidophilla* G-6302, or prepared from antibiotic derivatives or through other methods. It is also known as (+)-(*Z*)-[[[1-(2-amino-4-thiazolyl)-2-[[(2*S*,3*S*)-2-(carbamoyloxymethyl)-4-oxo-1-sulfo-3-azetidinyl]amino]-2-oxoethylidene]amino]oxy]acetic acid.

2) Drugs in this class include carumonam, carumonam salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of carumonam ($C_{12}H_{14}N_6O_{10}S_2$) in mass (potency).

4) 1.094 mg of carumonam sodium reference standard ($C_{12}H_{12}N_6Na_2O_{10}S_2$) is equivalent to a potency of 1 mg.

VI. Bleomycin Antibiotics

1. Bleomycin Antibiotic Drugs

1) Bleomycin is a mixture of bleomycin acid, bleomycin A_1, bleomycin demethyl A_2, bleomycin A_2, bleomycin A_2'-a, bleomycin A_2'-b, bleomycin A_5, bleomycin B_1', bleomycin B_2, bleomycin B_4, etc., which are prepared by culturing *Streptomyces verticillus*.

2) Drugs in this class include bleomycin, bleomycin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of bleomycin $A_2(X^- = C1^-: C_{55}H_{84}ClN_{17}O_{21}S_3)$ in mass (potency).

4) 1.03 mg of bleomycin hydrochloride reference standard ($C_{55}H_{84}ClN_{17}O_{21}S_3.HCl$) is equivalent to a potency of 1 mg.

VII. Cephem Antibiotics

1. Cefadroxil Antibiotic Drugs

1) Cefadroxil is a derivative of antibiotics prepared by culturing *Cephalosporium acremonium* or *Cephalosporium salmosynnematum*. It is also known as (1) (6*R*,7*R*)-7-[(*R*)-2-amino-2-(*p*-hydroxyphenyl) acetylamino]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid and (2) 7-(D-α-amino-*p*- hydroxyphenylacetylamino)-3-methyl-3-cephem-4-carboxylic acid.

2) Drugs in this class include cefadroxil, cefadroxil salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of cefadroxil ($C_{16}H_{17}N_3O_5S$) in mass (potency).

4) 1.050 mg of cefadroxil reference standard ($C_{16}H_{17}N_3O_5S.H_2O$) is equivalent to a potency of 1 mg.

2. Cefamandole Antibiotic Drugs

1) Cefamandole is a derivative of antibiotics prepared by culturing *Cephalosporium acremonium* or *Cephalosporium salmosynnematum*. It is also known as (1) (6*R*,7*R*)-7-*D*-mandelamido-3-[[(1-methyl-1-*H* tetrazol-5-yl)thio]ethyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid and (2) 7-*D*-mandelamido-3[[(1-methyl-1*H*-tetrazol-5-yl)thio]methyl]-3-cephem-4-carboxylic acid.

2) Drugs in this class include cefamandole, cefamandole salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of cefamandole ($C_{18}H_{18}N_6O_5S_2$) in mass (potency).

4) 1.013 mg of cefamandole reference standard ($C_{18}H_{17}LiN_6O_5S_2$) is equivalent to a potency of 1 mg, and 1.108 mg of cefamandole nafate reference standard ($C_{19}H_{17}N_6NaO_6S_2$) is equivalent to a potency of 1 mg.

3. Cefacetrile Antibiotic Drugs

1) Cefacetrile is a derivative of antibiotics prepared by culturing *Cephalosporium acremonium* or *Cephalosporium salmosynnematum*. It is also known as (1) (6*R*,7*R*)-3-acetoxymethyl-7-cyanoacetylamino-8-oxo-5-thia -1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid and (2) 3-acetoxymethyl-7-cyanoacetylamino-3-cephem-4-carboxylic acid.

2) Drugs in this class include cefacetrile, cefacetrile salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of cefacetrile ($C_{13}H_{13}N_3O_6S$) in mass (potency).

4) 1.065 mg of cefacetrile sodium reference standard ($C_{13}H_{12}N_3NaO_6S$) is equivalent to a potency of 1 mg.

4. Cefazedone Antibiotic Drugs

1) Cefazedone is a derivative of antibiotics prepared by culturing *Cephalosporium acremonium or Cephalosporium salmosynnematum*. It is also known as (1) (6*R*,7*R*)-7-[[(3,5-dichloro-4-oxo-1(4*H*)-pyridine denyl)acetyl]amino]-3-[[(5-methyl-1,3,4-thiadiazol-2-yl)-thio]methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0] oct-2-ene-2-carboxylic acid and (2) 3-(5-methyl-1,3,4-thiadiazolyl-2-mercaptomethyl)-7-(3,5-dichloro-4-pyridone-1-ylacetamido)-3-cephem-4-carboxylic acid.

2) Drugs in this class include cefazedone, cefazedone salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of cefazedone ($C_{18}H_{15}C_{l2}N_5O_5S_3$) in mass (potency).

4) 1.040 mg of cefazedone sodium reference standard ($C_{18}H_{15}C_{l2}N_5O_5S_3$) is equivalent to a potency of 1 mg.

5. Cefazolin Antibiotic Drugs

1) Cefazolin is a derivative of antibiotics prepared by culturing *Cephalosporium acremonium* or *Cephalosporium salmosynnematum*. It is also known as (1) (6*R*,7*R*)-3-[[(5-methyl-1,3,4-thiadiazole-2- yl)thio]methyl]-8-oxo-7-[(1*H*-tetrazol-1-ylacetyl)amino]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid and (2) 7-(1-(1*H*)-tetrazolylacetylamino)-3-[2-(5-methyl-1,3,4-thiadiazolyl)thiomethyl]-Δ^3-cephem-4-carboxylic acid.

2) Drugs in this class include cefazolin, cefazolin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of cefazolin ($C_{14}H_{14}N_8O_4S_3$) in mass (potency).

4) 1 mg of cefazolin reference standard ($C_{14}H_{14}N_8O_4S_3$) is equivalent to a potency of 1 mg.

6. Cefaclor Antibiotic Drugs

1) Cefaclor is a derivative of antibiotics prepared by culturing *Cephalosporium acremonium or Cephalosporium salmosynnematum*. It is also known as (1) (6*R*,7*R*)-7-[[(2*R*)-aminophenylacetyl]amino]-3-chloro-8- oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid and (2) 3-chloro-7-*D*-(2-phenylglycinamido)-3-cephem-4-carboxylic acid.

2) Drugs in this class include cefaclor, cefaclor salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of cefaclor ($C_{15}H_{14}ClN_3O_4S$) in mass (potency).

4) 1.049 mg of cefaclor reference standard ($C_{15}H_{14}ClN_3O_4S.H_2O$) is equivalent to a potency of 1 mg.

7. Cefatrizine Antibiotic Drugs

1) Cefatrizine is a derivative of antibiotics prepared by culturing *Cephalosporium acremonium* or *Cephalosporium salmosynnematum*. It is also known as (1) (6*R*,7*R*)-7-[[(2*R*)-amino-(4-hydroxyphenyl)acetyl]amino]-8-oxo-3-[(1*H*-1,2,3-triazol-4-ylthio)methyl]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid and (2) 7-[D-α-amino-α-(4-hydroxyphenyl)acetamido]-3-(1,2,3-triazol-4(5)-ylthiomethyl) -3-cephem-4-carboxylic acid.

2) Drugs in this class include cefatrizine, adducts of cefatrizine, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of cefatrizine ($C_{18}H_{18}N_6O_5S_2$) in mass (potency).

4) 1.165 mg of cefatrizine propylene glycol reference standard ($C_{18}H_{18}N_6O_5S_2.C_3H_8O_2$) is equivalent to a potency of 1 mg.

8. Cefapirin Antibiotic Drugs

1) Cefapirin is a derivative of antibiotics prepared by culturing *Cephalosporium acremonium* or *Cephalosporium salmosynnematum*. It is also known as (1) (6*R*,7*R*)-3-[(acetyloxy)methyl]-8-oxo-7-[[(4- Pyridinylthio)acetyl]amino]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid and (2) 7-[α-(4-pyridylthio)acetamido] cephalosporanic acid.

2) Drugs in this class include cefapirin, cefapirin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of cefapirin ($C_{17}H_{17}N_3O_6S_2$) in mass (potency).

4) 1.052 mg of cefapirin sodium reference standard ($C_{17}H_{16}N_3NaO_6S_2$) is equivalent to a potency of 1 mg.

9. Cefalexin Antibiotic Drugs

1) Cefalexin is a derivative of antibiotics prepared by culturing *Cephalosporium acremonium* or *Cephalosporium salmosynnematum*. It is also known as (1) (6*R*,7*R*)-7-[[(2*R*)-aminophenylacetyl]amino]-3-methyl-8- oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid and (2) 7-(D-2-amino-2-phenylacetamido)-3-methyl-Δ^3-cephem-4-carboxylic acid.

2) Drugs in this class include cefalexin, cefalexin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of cefalexin ($C_{16}H_{17}N_3O_4S$) in mass (potency).

4) 1.071 mg of cefalexin reference standard ($C_{16}H_{17}N_3O_4S.H_2O$) is equivalent to a potency of 1 mg.

10. Cefalotin Antibiotic Drugs

1) Cefalotin is a derivative of antibiotics prepared by culturing *Cephalosporium acremonium* or *Cephalosporium salmosynnematum*. It is also known as (1) (6*R*,7*R*)-3-[(acetyloxy)methyl]-8-oxo-7-[(2-thienylacetyl)amino]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid and (2) 7-(2-thienylacetamido)cephalosporanic acid.

2) Drugs in this class include cefalotin, cefalotin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of cefalotin ($C_{16}H_{16}N_2O_6S_2$) in mass (potency).

4) 1.055 mg of cefalotin sodium reference standard ($C_{16}H_{15}N_2NaO_6S_2$) dried at 60 °C for 3 hours under a reduced pressure of ≤ 0.7 kPa is equivalent to a potency of 1 mg.

11. Cefetamet Pivoxil Antibiotic Drugs

1) Cefetamet pivoxil is a pivaloyloxymethyl derivative of an antibiotic derivative (cefetamet) prepared by culturing *Cephalosporium acremonium or Cephalosporium salmosynnematum.* It is also known as (1) (6*R*,7*R*)-7-[[(2*Z*) -(2-amino-4-thiazolyl)(methoxyimino)acetyl]amino]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylo acid pivaloyloxymethyl ester and (2) (6*R*,7*R*)-7-[2-(2-amino-4-thiazolyl)glyoxylamido]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid pivaloyloxymethyl ester.

2) Drugs in this class include cefetamet pivoxil, cefetamet pivoxil salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of cefetamet ($C_{14}H_{15}N_5O_5S_2$) in mass (potency).

4) 1.379 mg of cefetamet pivoxil hydrochloride reference standard ($C_{20}H_{25}N_5O_7S_2 \cdot HCl$) is equivalent to a potency of 1 mg.

12. Cefepime Antibiotic Drugs

1) Cefepime is a derivative of antibiotics prepared by culturing *Cephalosporium acremonium* or *Cephalosporium salmosynnematum*. It is also known as (1) 1-[[(6*R*,7*R*)-7-[[(2*Z*)-(2-amino-4-thiazolyl)(methoxyimino)acetyl]amino]-2-carboxy-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-3-yl]methyl]-1-methylpyrrolidiinium and (2) 7-[(Z)-2-(2-aminothiazol-4-yl)-2-methoxyiminoacetamido]-3-(1-methylpyrrolidio)methyl-3-cephem-4-carboxylic acid.

2) Drugs in this class include cefepime, cefepime salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of cefepime ($C_{19}H_{24}N_6O_5S_2$) in mass (potency).

4) 1.152 mg of cefepime hydrochloride reference standard ($C_{19}H_{24}N_6O_5S_2.2HCl.H_2O$) in an anhydrous form is equivalent to a potency of 1 mg.

13. Cefonicid Antibiotic Drugs

1) Cefonicid is a derivative of antibiotics prepared by culturing *Cephalosporium acremonium* or *Cephalosporium salmosynnematum.* It is also known as (1) (6*R*,7*R*)-7-[[(2*R*)-hydroxyphenylacetyl]amino]-8-oxo-3[[[1-(sulfomethyl)-1*H*-tetrazol-5-yl]thio]methyl]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid and (2) (6*R*,7*R*)-7-[(*R*)-mandelamido]-8-oxo-3[[[1-(sulfomethyl)-1*H*-tetrazol-5-yl]thio]methyl]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid.

2) Drugs in this class include cefonicid, cefonicid salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of cefonicid ($C_{18}H_{18}N_6O_8S_3$) in mass (potency).

4) 1.081 mg of cefonicid sodium reference standard ($C_{18}H_{16}N_6Na_2O_8S_3$) is equivalent to a potency of 1 mg.

14. Cefodizime Antibiotic Drugs

1) Cefodizime is a derivative of antibiotics prepared by culturing *Cephalosporium acremonium* or *Cephalosporium salmosynnematum*. It is also known as (1) (6*R*,7*R*)-7-[[(2*Z*)-(2-amino-4-thiazolyl) (methoxyimino)acetyl]amino]-3-[[[5-(carboxymethyl)-4-methyl-2-thiazolyl]thio]methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0] oct-2-ene-2-carboxylic acid and (2) (6*R*,7*R*)-7-[2-(2-amino-4-thiazolyl)glyoxylamido]-3-[[[5-(carboxymethyl)-4-methyl-2-thiazolyl]thio]methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid 7^2(Z)-(*O*-methyloxime).

2) Drugs in this class include cefodizime, cefodizime salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of cefodizime ($C_{20}H_{20}N_6O_7S_4$) in mass (potency).

4) 1.075 mg of cefodizime sodium reference standard ($C_{20}H_{18}N_6Na_2O_7S_4$) is equivalent to a potency of 1 mg.

15. Ceforanide Antibiotic Drugs

1) Ceforanide is an antibiotic derivative prepared by culturing *Cephalosporium acremonium* or *Cephalosporium salmosynnematum*. It is also known as (1) (6*R*,7*R*)-7-[[[2-(aminomethyl)phenyl]acetyl]amino]-3-[[[1-(carboxymethyl)-1*H*-tetrazol-5-yl]thio]methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid and (2) (6*R*,7*R*)-7-[2-(α-amino-*O*-tolyl)acetamido]-3-[[[1-(carboxymethyl)-1*H*-tetrazol-5-yl]thio]methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid.

2) Drugs in this class include ceforanide, ceforanide salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of ceforanide ($C_{20}H_{21}N_7O_6S_2$) in mass.

4) 1 mg of ceforanide reference standard ($C_{20}H_{21}N_7O_6S_2$) is equivalent s to a potency of 1 mg.

16. Cefotaxime Antibiotic Drugs

1) Cefotaxime is a derivative of antibiotics prepared by culturing *Cephalosporium acremonium* or *Cephalosporium salmosymematum*. It is also known as (1) (6*R*,7*R*)-3-[(acetyloxy)methyl]-7-[[(2*Z*)-(2-amino-4-thiazolyl)(methoxyimino)-acetyl]amino]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid and (2) (6*R*,7*R*)-7-[2-(2-amino-4-thiazolyl)glyoxylamido]-3-(hydroxymethyl)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid 7^2-(Z)-(*O*-methyloxime) acetic acid.

2) Drugs in this class include cefotaxime, cefotaxime salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of cefotaxime ($C_{16}H_{17}N_5O_7S_2$) in mass (potency).

4) 1.048 mg of cefotaxime sodium reference standard ($C_{16}H_{16}N_5NaO_7S_2$) is equivalent to a potency of 1 mg.

17. Cefotetan Antibiotic Drugs

1) Cefotetan is a derivative of antibiotics prepared by culturing *Streptomyces oganonensis*. It is also known as (1) (6*R*,7*S*)-7-[[[4-(2-amino-1-carboxy-2-oxoethylidene)-1,3-dithietane-2-yl]carbonyl]amino]-7-methoxy-3-[[(1-methyl-1*H*-tetrazol-5-yl)thio]methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid and (2) (6*R*,7*S*)-7-[4-(carbamoylcarboxymethylene)-1,3-dithietane-2-carboxamido]-7-methoxy-3-[[(1-methyl-1*H*-tetrazol-5-yl)thio]methyl]-8-oxo-5-thia-1-azabicyclo [4.2.0]oct-2-ene-2-carboxylic acid.

2) Drugs in this class include cefotetan, cefotetan salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of cefotetan ($C_{17}H_{17}N_7O_8S_4$) in mass (potency).

4) 1 mg of cefotetan reference standard ($C_{17}H_{17}N_7O_8S_4$) is equivalent to a potency of 1 mg.

18. Cefotiam Antibiotic Drugs

1) Cefotiam is a derivative of antibiotics prepared by culturing *Cephalosporium acremonium* or *Cephalosporium salmosynnematum*. It is also known as (1) (6*R*,7*R*)-7-[[(2-amino-4-thiazolyl)acetyl]amino]-3-[[[1-[2-(dimethylamino)ethyl]-1*H*-tetrazol-5-yl]thio]methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid and (2) 7β-[2-(aminothiazol-4-yl)acetamido]-3-[[[1-(2-dimethylaminoethyl)-1*H*-tetrazol-5 -yl]thio]methyl]ceph-3-ene-4-carboxylic acid.

2) Drugs in this class include cefotiam, cefotiam salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of cefotiam ($C_{18}H_{23}N_9O_4S_3$) in mass (potency).

4) 1.139 mg of cefotiam hydrochloride reference standard ($C_{18}H_{23}N_9O_4S_3.2HCl$) is equivalent to a potency of 1 mg, and 1.463 mg of cefotiam hexetil hydrochloride reference standard ($C_{27}H_{37}N_9O_7S_3.$ 2HCl) is equivalent to a potency of 1 mg.

19. Cefoperazone Antibiotic Drugs

1) Cefoperazone is a derivative of antibiotics prepared by culturing *Cephalosporium acremonium* or *Cephalosporium salmosynnematum*. It is also known as (1) (6*R*,7*R*)-7-[[(2*R*)-[[(4-ethyl-2,3-dioxo-1-piperazinyl)carbonyl]amino](4-hydroxyphenyl)acetyl]amino]-3-[[(1-methyl-1*H*-tetrazol-5-yl)thio]methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid and (2) 7-[*D*-(-)-α-(4-ethyl-2,3-dioxo-1-piperazine carboxamido)-α-(4-hydroxyphenyl)acetamido]-3-[[(1-methyl-1*H*-tetrazol-5-yl)thio]methyl]-3 -cephem-4-carboxylic acid.

2) Drugs in this class include cefoperazone, cefoperazone salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of cefoperazone ($C_{25}H_{27}N_9O_8S_2$) in mass (potency).

4) 1.056 mg of cefoperazone reference standard ($C_{25}H_{27}N_9O_8S_2.2H_2O$) is equivalent to a potency of 1 mg.

20. Cefoxitin Antibiotic Drugs

1) Cefoxitin is a derivative of antibiotics prepared by culturing *Streptomyces lactamdurans*. It is also known as (1) (6R,7S)-3-[[(aminocarbonyl)oxy]methyl]-7-methoxy-8-oxo-7-[(2-thienylacetyl)amino]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid and (2) 3-carbamoyloxymethyl-7α- methoxy-7-[2-(2-thienyl)acetamido]-3-cephem-4-carboxylic acid.

2) Drugs in this class include cefoxitin, cefoxitin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of cefoxitin ($C_{16}H_{17}N_3O_7S_2$) in mass (potency).

4) 1 mg of cefoxitin reference standard ($C_{16}H_{17}N_3O_7S_2$) is equivalent to a potency of 1 mg.

21. Cefuroxime Antibiotic Drugs

1) Cefuroxime is a derivative of antibiotics prepared by culturing *Cephalosporium acremonium or Cephalosporium salmosynnematum*. It is also known as (1) (6*R*,7*R*)-3-[[(aminocarbonyl)oxy]methyl]-7-[[(2Z)-2-furanyl(methoxyimino)acetyl]amino]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid and (2) (6*R*,7*R*)-3-carbamoyloxymethyl-7-[2-(2-furyl)-2-(methoxyimino)acetamido]ceph-3-ene-4-carboxylic acid.

2) Drugs in this class include cefuroxime, cefuroxime salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of cefuroxime ($C_{16}H_{16}N_4O_8S$) in mass (potency).

4) 1.052 mg of cefuroxime sodium reference standard ($C_{16}H_{15}N_4NaO_8S$) is equivalent to a potency of 1 mg, and 1.203 mg of cefuroxime axetil reference standard ($C_{20}H_{22}N_4O_{10}S$) is equivalent to a potency of 1 mg.

22. Cefdinir Antibiotic Drugs

1) Cefdinir is a derivative of antibiotics prepared by culturing *Cephalosporium acremonium* or *Cephalosporium salmosynnematum*. It is also known as (1) (6*R*,7*R*)-7-[[(2*Z*)-(2-amino-4-thiazolyl)(hydroxyimino)acetyl]amino]-3-ethenyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid and (2) *syn*-7-[2 -(2-amino-4-thiazolyl)-2-hydroxyiminoacetamido]-3-vinyl-3-cephem-4-carboxylic acid.

2) Drugs in this class include cefdinir, cefdinir salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of cefdinir ($C_{14}H_{13}N_5O_5S_22$) in mass (potency).

4) 1 mg of cefdinir reference standard ($C_{14}H_{13}N_5O_5S_2$) is equivalent to a potency of 1 mg.

23. Cefditoren Pivoxil Antibiotic Drugs

1) Cefditoren pivoxil is a pivaloyloxymethyl derivative of a derivative of an antibiotic substance (cefditoren), which is prepared by culturing *Cephalosporium acremonium* or *Cephalosporium salmosynnematum.* It is also known as (1) (6*R*,7*R*)-7-[[(2*Z*) (2-amino-4-thiazolyl)(methoxyimino)acetyl]amino]-3-[(1*Z*)-2-(4-methyl-5-thiazolyl)ethenyl]-8-oxo-5- thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid pivaloyloxymethyl ester and (2) (+)-(6*R*,7*R*)-7-[2-(2-amino) -4-thiazolyl)glyoxylamido]-3-[(*Z*)-2-(4-methyl-5-thiazolyl)vinyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid 7^2-(Z)-(O-methyloxime) pivaloyloxymethyl ester.

2) Drugs in this class include cefditoren pivoxil, cefditoren pivoxil salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of cefditoren ($C_{19}H_{18}N_6O_5S_3$) in mass (potency).

4) 1.225 mg of cefditoren pivoxil reference standard ($C_{25}H_{28}N_6O_7S_3$) is equivalent to a potency of 1 mg.

24. Cefradine Antibiotic Drugs

1) Cefradine is a derivative of antibiotics prepared by culturing *Cephalosporium acremonium* or *Cephalosporium salmosynnematum*. It is also known as (1) (6*R*,7*R*)-7-[[(2*R*)-amino-1,4-cyclohexadiene-1-ylacetylamino]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid and (2) 7-(D-2-amino-2-(1,4-cyclohexadienyl)acetamido]-3-methyl-3-cephem-4-carboxylic acid.

2) Drugs in this class include cefradine, cefradine salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of cefradine ($C_{16}H_{19}N_3O_4S$) in mass (potency).

4) 1.111 mg of cefradine reference standard ($C_{16}H_{19}N_3O_4S.2H_2O$) is equivalent to a potency of 1 mg.

25. Cefroxadine Antibiotic Drugs

1) Cefroxadine is a derivative of antibiotics prepared by culturing *Cephalosporium acremonium* or *Cephalosporium salmosynnematum*. It is also known as (1) (6*R*,7*R*)-7-[[(2*R*)-amino-(1,4-cyclohexadiene-1-ylacetyl]amino]-3-methoxy-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid and (2) 7-[D-2-amino-2-(1,4-cyclohexadienyl)acetamide]-3-methoxy-3-cephem-4-carboxylic acid.

2) Drugs in this class include cefroxadine, cefroxadine salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of cefroxadine ($C_{16}H_{19}N_3O_5S$) in mass (potency).

4)1.099 mg of cefroxadine reference standard ($C_{16}H_{19}N_3O_5S.2H_2O$) is equivalent to a potency of 1 mg.

26. Cefmenoxime Antibiotic Drugs

1) Cefmenoxime is a derivative of antibiotics prepared by culturing *Cephalosporium acremonium* or *Cephalosporium salmosynnematum*. It is also known as (1) (6*R*,7*R*)-7-[[(2*Z*)-(2-amino-4-thiazolyl)(methoxyimino)acetyl]amino]-3-[[(1-methyl-1*H*-tetrazol-5-yl)thio]methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid and (2) (6*R*,7*R*)-7-[2-(2-aminothiazolyl)glyoxylamido]-3-[[(1-methyl-1*H*-tetrazol-5-yl)thio]methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid 7^2-(*Z*)-(*O*-methyloxime).

2) Drugs in this class include cefmenoxime, cefmenoxime salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of cefmenoxime ($C_{16}H_{17}N_9O_5S_3$) in mass (potency).

4) 1.036 mg of cefmenoxime hydrochloride reference standard ($C_{16}H_{17}N_9O_5S_3.1/2HCl$) is equivalent to a potency of 1 mg.

27. Cefmetazole Antibiotic Drugs

1) Cefmetazole is a derivative of antibiotics prepared by culturing *Streptomyces jumonjinensis, Cephalosporium acremonium,* or *Cephalosporium salmosymematum.* It is also known as (1) (6*R*,7*S*)-7-[[[(cyanomethyl)thio]acetyl]amino]-7-methoxy-3-[[(1-methyl-1*H*-tetrazol-5-yl)thio]methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid and (2) 7β-[(cyanomethylthio)acetamido]-7α-methoxy-3-[(1-methyl-1H-tetrazol-5-yl)thiomethyl]-3-cephem-4-carboxylic acid.

2) Drugs in this class include cefmetazole, cefmetazole salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of cefmetazole ($C_{15}H_{17}N_7O_5S_3$) in mass (potency).

4) 1 mg of cefmetazole reference standard ($C_{15}H_{17}N_7O_5S_3$) is equivalent to a potency of 1 mg.

28. Cefminox Antibiotic Drugs

1) Cefminox is a derivative of antibiotics prepared by culturing *Streptomyces chartseusis, Cephalosporium acremonium* or *Cephalosporium salmosynnematum,* or through other methods. It is also known as (1) (6*R*,7*S*)-7-[[[[(2*S*)-2-amino-2-carboxyethyl]thio]acetyl]amino]-7-methoxy-3-[[(1-methyl-1*H*-tetrazol-5-yl-)thio]methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid and (2) 7β-(2-D-amino-2-carboxyethylthioacetamido)-7α-methoxy-3-[[(1-methyl-1*H*-tetrazol-5-yl)thio]methyl]-3-cephem-4-carboxylic acid.

2) Drugs in this class include cefminox, cefminox salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of cefminox ($C_{16}H_{21}N_7O_7S_3$) in mass (potency).

4) 1.285 mg of cefminox sodium reference standard ($C_{16}H_{20}N_7NaO_7S_3.7H_2O$) is equivalent to a potency of 1 mg.

29. Cefbuperazone Antibiotic Drugs

1) Cefbuperazone is a derivative of antibiotics prepared by culturing *Cephalosporium acremonium* or *Cephalosporium salmosynnematum*, or through other methods. It is also known as (1) (6*R*,7*S*)-7-[[(2*R*,3*S*)-2-[[(4-ethyl-2,3-dioxo-1-piperazinyl)carbonyl]amino]-3-hydroxy-1-oxobutyl]amino]-7-

methoxy-3-[[(1-methyl-1*H*-tetrazol-5-yl)thio]methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid and (2) 7β-[D-α-(4-ethyl-2,3-dioxo-1-piperazinecarboxamido)-β-(S)-hydroxybutanamido]-7α-methoxy-3-[5-(1-methyl-1,2,3,4-tetrazolyl)thiomethyl]-Δ^3-cephem-4-carboxylic acid.

2) Drugs in this class include cefbuperazone, cefbuperazone salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of cefbuperazone ($C_{22}H_{29}N_9O_9S_2$) in mass (potency).

4) 1.057 mg of cefbuperazone reference standard ($C_{22}H_{29}N_9O_9S_2.2H_2O$) is equivalent to a potency of 1 mg.

30. Cefsulodin Antibiotic Drugs

1) Cefsulodin is a derivative of antibiotics prepared by culturing *Cephalosporium acremonium* or *Cephalosporium salmosynnematum*, or through other methods. It is also known as (1) 4-(aminocarbonyl)-1-[[(6*R*,7*S*)-2- carboxy-8-oxo-7-[[(2*R*)-phenylsulfoacetyl]amino]-5-thia-1-azabisirlo[4.2.0]oct-2-ene-3-yl]methyl]pyridinium and (2) 7-(α-sulfophenylacetamido)-3-(4′-carbamoylpyridinium)methyl-3-cephem-4-carboxylic acid.

2) Drugs in this class include cefsulodin, cefsulodin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of cefsulodin ($C_{22}H_{20}N_4O_8S_2$) in mass (potency).

4) 1.041 mg of cefsulodin sodium reference standard ($C_{22}H_{19}N_4NaO_8S_2$) is equivalent to a potency of 1 mg.

31. Cefcapene Pivoxil Antibiotic Drugs

1) Cefcapene pivoxil is a pivaloyloxymethyl derivative of a derivative of an antibiotic substance (cefcapene), which is prepared by culturing *Cephalosporium acremonium* or *Cephalosporium salmosynnematum*. It is also known as (6*R*,7*R*)-3-[[(aminocarbonyl)oxy]methyl]-7-[[(2*Z*)-2-(2-amino-4-thiazolyl)-1-oxo-2-phentenyl]amino]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (2,2-dimethyl-1-oxopropoxy)methyl ester and (2) (6*R*,7*R*)-7-[(*Z*)-2-(2)-aminothiazol-4-yl)-2-pentenoylamino]-3-(carbamoyloxymethyl)-3-cephem-4-carboxylic acid pivaloyloxymethyl ester.

2) Drugs in this class include cefcapene pivoxil, cefcapene pivoxil salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of cefcapene ($C_{17}H_{19}N_5O_6S_2$) in mass (potency).

4) 1.372 mg of cefcapene pivoxil hydrochloride reference standard ($C_{23}H_{29}N_5O_8S_2.HCl.H_2O$) is equivalent to a potency of 1 mg.

32. Ceftazidime Antibiotic Drugs

1) Ceftazidime is a derivative of antibiotics prepared by culturing *Cephalosporium acremonium* or *Cephalosporium salmosynnematum*, or through other methods. It is also known as (1) 1-[[(6R,7R)-7-[[(2*Z*)-(2-amino-4-thiazolyl)[(1-carboxy-1-methylethoxy)imino]acetyl]amino]-2-carboxy-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-3-yl]methyl]pyridinium and (2) 1-[[(6R,7R)-7-[2-(2-amino-4-thiazolyl)glyoxylamido]-2-carboxy-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-3-yl]methyl]pyridinium hydroxide 7^2-(*Z*)-[*O*-(1-carboxy-1-methylethyl)oxime].

2) Drugs in this class include ceftazidime, ceftazidime salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of ceftazidime ($C_{22}H_{22}N_6O_7S_2$) in mass (potency).

4) 1.165 mg of ceftazidime reference standard ($C_{22}H_{22}N_6O_7S_2.5H_2O$) is equivalent to a potency of 1 mg.

33. Cefteram Pivoxil Antibiotic Drugs

1) Cefteram pivoxil is a pivaloyloxymethyl derivative of a derivative of an antibiotic substance (cefteram), which is prepared by culturing *Cephalosporium acremonium* or *Cephalosporium salmosynnematum*, or through other methods. It is also known as (1) (+)-(6R,7R)-7-[(Z)-2-(2-amino-4-thiazolyl)-2-(methoxyimino)acetamido]-3-[(5-methyl-2*H*-tetrazol-2-yl)methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid pivaloyloxymethyl ester and (2) 7-[2-(2-aminothiazole)-4-yl)-2-*syn*-methoxyiminoacetamido]-3-[2-(5-methyl-1,2,3,4-tetrazolyl)methyl]-Δ^3-cephem-4-carboxylic acid pivaloyloxymethyl ester.

2) Drugs in this class include cefteram pivoxil, cefteram pivoxil salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of cefteram ($C_{16}H_{17}N_9O_5S_2$) in mass (potency).

4) 1.656 mg of cefteram pivoxil reference standard ($C_{22}H_{28}N_9O_7S_2.C_9H_{11}O_3S$) is equivalent to a potency of 1 mg.

34. Ceftezole Antibiotic Drugs

1) Ceftezole is a derivative of antibiotics prepared by culturing *Cephalosporium acremonium* or *Cephalosporium salmosynnematum*, or through other methods. It is also known as (1) (6*R*,7*R*)-8-oxo-7-[(1*H*-tetrazole-1-ylacetyl)amino]-3-[(1,3,4-thiadiazol-2-ylthio)methyl]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-carboxylic acid and (2) 7-(1*H*-tetrazol-1-yl)acetamido-3-(1,3,4-thiadiazol-2-ylthio)methylceph-3-ene-4-carboxylic acid.

2) Drugs in this class include ceftezole, ceftezole salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of ceftezole ($C_{13}H_{12}N_8O_4S_3$) in mass (potency).

4) 1 mg of ceftezole reference standard ($C_{13}H_{12}N_8O_4S_3$) is equivalent to a potency of 1 mg.

35. Ceftriaxone Antibiotic Drugs

1) Ceftriaxone is a derivative of antibiotics prepared by culturing *Cephalosporium acremonium* or through other methods. It is also known as (1) (6*R*,7*R*)-7-[[(2*Z*)-(2-amino-4-thiazolyl)(methoxyimino)acetyl]amino]-8-oxo-3-[[(1,2,5,6-tetrahydro-2-methyl-5,6-dioxo-12,2,4-triazine-3-yl)-thio]methyl]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid and (2) (6*R*,7*R*)-7-[2-(2-amino-4-thiazolyl)glyoxylamido]-3-[[(2,5-dihydro-6-hydroxy-2-methyl-5-oxo-*as*-triazin-3-yl)thio]methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid 7^2-(*Z*)-(*O*-methyloxime).

2) Drugs in this class include ceftriaxone, ceftriaxone salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of ceftriaxone ($C_{18}H_{18}N_8O_7S_3$) in mass (potency).

4) 1.193 mg of ceftriaxone sodium reference standard ($C_{18}H_{16}N_8Na_2O_7S_3.7/2H_2O$) is equivalent to a potency of 1 mg.

36. Ceftibuten Antibiotic Drugs

1) Ceftibuten is a derivative of antibiotics prepared by culturing *Cephalosporium acremonium* or *Cephalosporium salmosynnematum*, or through other methods. It is also known as (1) (6*R*,7*R*)-7-[(2*Z*)-2-(2-amino-4-thiazolyl)-4-carboxy-1-oxo-2-butenyl]amino]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid and (2) 7β-[(*Z*)-2-(2-aminothiazol-4-yl)-4-carboxy-2-butenoylamino]-3-cephem-4-carboxylic acid.

2) Drugs in this class include ceftibuten, ceftibuten salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of ceftibuten ($C_{15}H_{14}N_4O_6S_2$) in mass (potency).

4) 1.133 mg of ceftibuten hydrochloride reference standard

($C_{15}H_{14}N_4O_6S_2.HCl.H_2O$) is equivalent to a potency of 1 mg.

37. Ceftizoxime Antibiotic Drugs

1) Ceftizoxime is a derivative of antibiotics prepared by culturing *Cephalosporium acremonium* or *Cephalosporium salmosynnematum*, or through other methods. It is also known as (1) (6*R*,7*R*)-7-[(2)-2-(2-amino-4-Thiazolyl)(methoxyimino)acetyl]amino]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid and (2) (6*R*,7*R*)-7-[2-(2-imino-4-thiazolyl)glyoxylamido]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid 7^2-(*Z*)-(*O*-methyloxime).

2) Drugs in this class include ceftizoxime, ceftizoxime salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of ceftizoxime ($C_{13}H_{13}N_5O_5S_2$) in mass (potency).

4) 1 mg of ceftizoxime reference standard ($C_{13}H_{13}N_5O_5S_2$) is equivalent to a potency of 1 mg.

38. Cefpodoxime Proxetil Antibiotic Drugs

1) Cefpodoxime proxetil is an isopropoxycarbonyloxyethyl derivative of a derivative of an antibiotic substance (cefpodoxime), which is prepared by culturing *Cephalosporium acremonium* or *Cephalosporium salmosynnematum*, or through other methods. It is also known as (1) (6*R*,7*R*)-7-[[(2*Z*)-(2-amino-4-thiazolyl)(methoxyimino)acetyl]amino]-3-(methoxymethyl)-8-oxo-5-thia-1-azabicyclo[4.2.0] oct-2-ene-2-carboxylic acid 1-[[(1-methylethoxy)carbonyl]oxy]ethyl ester and (2) 1-(isopropoxycarbonyloxy)ethyl (6*R*,7*R*)-7-[2-(2-amino-4-thiazolyl)-(*Z*)-2-(methoxyimino)acetamido]-3-methoxymethyl-3-cephem-4-carboxylic acid.

2) Drugs in this class include preparations containing cefpodoxime proxetil and cefpodoxime proxetil salts.

3) The potency of drugs in this class is expressed as the amount of cefpodoxime ($C_{15}H_{17}N_5O_6S_2$) in mass (potency).

4) 1.304 mg of cefpodoxime proxetil reference standard ($C_{15}H_{17}N_5O_6S_2$) is equivalent to a potency of 1 mg.

39. Cefprozil Antibiotic Drugs

1) Cefprozil is an antibiotic substance prepared by synthesis. It is also known as (1) (6*R*,7*R*)-7-[[(2*R*)-amino(4-hydroxyphenyl)-acetyl]amino]-8-oxo-3-(1-propenyl)-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid and (2) (6*R*,7*R*)-7-[(*R*)-2-amino-2-(*p*-hydroxyphenyl)acetamido]-8-oxo-3-propenyl-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid.

2) Drugs in this class include cefprozil and preparations containing it.

3) The potency of drugs in this class is expressed as the amount of cefprozil ($C_{18}H_{19}N_3O_5S$) in mass (potency).

4) 1.046 mg of cefprozil (*Z*) isomer reference standard ($C_{18}H_{19}N_3O_5S.H_2O$) is equivalent to a potency of 1 mg.

40. Cefpiramide Antibiotic Drugs

1) Cefpiramide is a derivative of antibiotics prepared by culturing *Cephalosporium acremoniurn* or *Cephalosporium salmosynnematum*, or through other methods. It is also known as (1) (6*R*,7*R*)-7-[[(2*R*)-(4-hydroxy-6-methyl-3-pyridinyl)carbonyl]amino](4-hydroxyphenyl)acetyl]amino]-3-[[(1-methyl-1*H*-tetrazol-5-yl)thio]methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid and (2) 7-[(*R*)-2-(4-hydroxy-6-methylnicodinamido)-2-(*p*-hydroxyphenyl)acetamido]-3-[[(1-methyl-1*H*-tetra zol-5-yl)thio]methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid.

2) Drugs in this class include cefpiramide, cefpiramide salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of cefpiramide ($C_{25}H_{24}N_8O_7S_2$) in mass (potency).

4) 1 mg of cefpiramide reference standard ($C_{25}H_{24}N_8O_7S_2$) is equivalent to a potency of 1 mg.

41. Cefpirome Antibiotic Drugs

1) Cefpirome is a derivative of an antibiotic substance, which is prepared by culturing *Cephalosporium acremonium* or *Cephalosporium salmosynnematum, or* through other methods. It is also known as (1) (-)-1-[(6*R*, 7*R*)-7-[[(*Z*)-2 (2-amino-4-thiazolyl)-2-methoxyiminoacetamide]-2-carboxy-8-oxo-5-thia-1-azabicyclo [4.2.0] oct-2-ene-3-yl]methyl]-6,7-dihydro-5*H*-1-pyrindinium hydroxide and (2) (-)-6*R*, 7*R*)-7-[(*Z*)-2-(2-amino-4-thiazolyl)-2-methoxyiminoacetamide]-3-(6,7-dihydro 5*H*-1-pyrindinium-1-ylmethyl)-3-cephem-4-carboxylic acid.

2) Drugs in this class include cefpirome, cefpirome salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of cefpirome ($C_{22}H_{22}N_6O_5S_2$) in mass (potency).

4) 1.191 mg of cefpirome sulfate reference standard ($C_{22}H_{22}N_6O_5S_2.H_2SO_4$) is equivalent to a potency of 1 mg

42. Cefixime Antibiotic Drugs

1) Cefixime is a derivative of antibiotics prepared by culturing *Cephalosporium acremonium* or *Cephalosporium salmosynnematum* or through other methods. It is also known as (1) (6*R*,7*R*)-7-[[(2*Z*)-(2-amino-4-thiazolyl)[(carboxymethoxy)imino]acetyl]amino]-3-ethynyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid and (2) 7-[2-(2-amino-4-thiazolyl)-2-(carboxymethoxyimino)acetamido]-3-vinyl-3-cephem-4-carboxylic acid.

2) Drugs in this class include cefixime, cefixime salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of cefixime ($C_{16}H_{15}N_5O_7S_2$) in mass (potency).

4) 1.119 mg of cefixime reference standard ($C_{16}H_{15}N_5O_7S_2$) is equivalent to a potency of 1 mg.

VIII. Aminoglycoside Antibiotics

1. Gentamicin Antibiotic Drugs

1) Gentamicin is a mixture of gentamicin C_1, gentamicin C_2, gentamicin C_1, etc., which are prepared by culturing *Micromonospora purpurea* or *Micromonospora echinospora*, or an equivalent substance prepared through other methods.

2) Drugs in this class include gentamicin, gentamicin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of gentamicin C_1 ($C_{21}H_{43}N_5O_7$) in mass (potency).

4) 1.458 mg of gentamicin sulfate reference standard ($C_{21}H_{43}N_5O_7.2H_2SO_4$) is equivalent to a potency of 1 mg.

2. Neomycin Antibiotic Drugs

1) Neomycin is a mixture of neomycin B and neomycin C, which are prepared by culturing *Streptomyces fradiae*, or an equivalent substance prepared through other methods.

2) Drugs in this class include neomycin, salts or derivatives of neomycin, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of neomycin ($C_{23}H_{46}N_6O_{13}$) in mass (potency).

4) 1.479 mg of neomycin sulfate reference standard ($C_{23}H_{46}N_6O_{13}.3H_2SO_4$) dried at 60°C for 3 hours under a reduced pressure of ≤ 0.7 kPa is equivalent to a potency of 1 mg.

3. Netilmicin Antibiotic Drugs

1) A derivative of sisomicin, netilmicin is 1-*N*-ethylsisomicin.

2) Drugs in this class include netilmicin, netilmicin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of netilmicin ($C_{21}H_{41}N_5O_7$) in mass (potency).

4) 1 mg of netilmicin sulfate reference standard ($C_{21}H_{41}N_5O_7.5/2H_2SO_4$) dried at 60℃ for 3 hours under a reduced pressure of ≤ 0.7 kPa is equivalent to a potency of 1 mg.

4. Dibekacin Antibiotic Drug

1) Dibekacin is a dideoxy derivative of bekanamycin at the 3rd and 4th positions (3', 4'-dideoxybekanamycin).

2) Drugs in this class include dibekacin, dibekacin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of dibekacin ($C_{18}H_{37}N_5O_8$) in mass (potency).

4) 1 mg of dibekacin reference standard ($C_{18}H_{37}N_5O_8$) is equivalent to a potency of 1 mg.

5. Ribostamycin Antibiotic Drugs

1) Ribostamycin is a substance prepared by culturing *Streptomyces ribosidificus* or an equivalent substance prepared through other methods.

2) Drugs in this class include ribostamycin, ribostamycin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of ribostamycin ($C_{17}H_{34}N_4O_{10}$) in mass (potency).

4) 1 mg of ribostamycin reference standard ($C_{17}H_{34}N_4O_{10}$) is equivalent to a potency of 1 mg.

6. Micronomicin Antibiotic Drugs

1) Micronomicin is a substance prepared by culturing *Micromonospora sagamiensis* or its variants, or an equivalent substance prepared through other methods.

2) Drugs in this class include micronomicin, micronomicin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of micronomicin ($C_{20}H_{41}N_5O_7$) in mass (potency).

4) 1.529mg of micronomicin sulfate reference standard ($C_{20}H_{41}N_5O_7.5/2H_2SO_4$) is equivalent to a potency of 1 mg.

7. Streptomycin Antibiotic Drugs

1) Streptomycin is a substance prepared by culturing *Streptomyces griseus* or an equivalent substance prepared through other methods.

2) Drugs in this class include streptomycin, streptomycin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of streptomycin ($C_{21}H_{39}N_7O_{12}$) in mass (potency).

4) 1.253 mg of streptomycin sulfate reference standard in an anhydrous form ($C_{21}H_{39}N_7O_{12}.3/2H_2SO_4$) is equivalent to a potency of 1 mg.

8. Sisomicin Antibiotic Drugs

1) Sisomicin is a substance prepared by culturing *Micromonospora inyoensis* or an equivalent substance prepared through other methods.

2) Drugs in this class include sisomicin, sisomicin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of sisomicin ($C_{19}H_{37}N_5O_7$) in mass (potency).

4) 1.548 mg of sisomicin sulfate reference standard ($C_{19}H_{37}N_5O_7.5/2H_2SO_4$) dried at 110℃ for 3 hours under a reduced pressure of ≤ 0.7 kPa is equivalent to a potency of 1 mg.

9. Arbekacin Antibiotic Drugs

1) Arbekacin is a derivative of dibekasin. It is also known as 1-*N*-[(*S*)-4-amino-2-hydroxybutylyl] dibekasin.

2) Drugs in this class include arbekasin, arbekasin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of arbekasin in mass (potency).

4) 1 mg of arbekasin reference standard ($C_{22}H_{44}N_6O_{10}$) is equivalent to a potency of 1 mg.

10. Amikacin Antibiotic Drugs

1) Amikacin is a chemical derivative of kanamycin, which is prepared by culturing *Streptomyces kanamyceticus*. It is also known as (1) *O*-3-amino-3-deoxy-α-D-glucopyranosyl-(1→6)-*O*-[6-amino-6-deoxy-α-D-glucopyranosyl-(1→4)]-N^1-[(2S)-4-amino-2-hydroxy-1-oxobutyl]-2-deoxy-D-Streptamine and (2) 1-*N*-[L(-)-4-amino-2-hydroxybutyryl] kanamycin A.

2) Drugs in this class include amikacin, amikacin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of amikacin ($C_{22}H_{43}N_5O_{13}$) in mass (potency).

4) 1.335 mg of amikacin reference standard ($C_{22}H_{43}N_5O_{13}.2H_2SO_4$) is equivalent to a potency of 1 mg.

11. Astromicin Antibiotic Drugs

1) Astromicin is a substance prepared by culturing *Micromonospora oliuasferospora* or an equivalent substance prepared through other methods.

2) Drugs in this class include astromicin salts and preparations containing it.

3) The potency of drugs in this class is expressed as the amount of astromicin ($C_{17}H_{35}N_5O_6$) in mass (potency).

4) 1.484 mg of astromicin sulfate reference standard ($C_{17}H_{35}N_3O_6.2H_2SO_4$) is equivalent to a potency of 1 mg.

12. Isepamicin Antibiotic Drugs

1) Isepamicin is a derivative of gentamicin B, which is prepared by culturing *Micromonospora purpurea*. It is also known as1-*N*-[(*S*)-3-amino-2-hydroxypropionyl] gentamicin B.

2) Drugs in this class include isepamicin, isepamicin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of isepamicin ($C_{22}H_{43}N_5O_{12}$) in mass (potency).

4) 1.344 mg of isepamicin sulfate reference standard ($C_{22}H_{43}N_5O_{12}.2H_2SO_4$) is equivalent to a potency of 1 mg.

13. Kanamycin Antibiotic Drugs

1) Kanamycin is a substance prepared by culturing *Streptomyces Kanamyceticus* or an equivalent substance prepared through other methods.

2) Drugs in this class include kanamycin, kanamycin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of kanamycin ($C_{18}H_{36}N_4O_{11}$) in mass (potency).

4) 1.24 mg of kanamycin sulfate reference standard ($C_{18}H_{36}N_4O_{11}.H_2SO_4.H_2O$) is equivalent to a potency of 1 mg.

14. Tobramycin Antibiotic Drugs

1) Tobramycin is a substance prepared by culturing *Streptomyces tenebrarius* or an equivalent substance prepared through other methods.

2) Drugs in this class include tobramycin, tobramycin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of tobramycin ($C_{18}H_{37}N_5O_9$) in mass (potency).

4) 1 mg of tobramycin reference standard ($C_{18}H_{37}N_5O_9$) in an anhydrous form is equivalent to a potency of 1 mg.

IX. Anthracycline Antibiotics

1. Daunorubicin Antibiotic Drugs

1) Daunorubicin is an antibiotic substance prepared by culturing *Streptomyces peucetius* or through other methods.

2) Drugs in this class include daunorubicin, daunorubicin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of daunorubicin hydrochloride ($C_{27}H_{29}NO_{10}$.HCl) in mass (potency).

4) 1 mg of daunorubicin hydrochloride reference standard ($C_{27}H_{29}NO_{10}$.HCl) is equivalent to a potency of 1 mg.

2. Doxorubicin Antibiotic Drugs

1) Doxorubicin is a substance prepared by culturing *Streptomyces peucetius var. caesius* or an equivalent substance prepared through other methods.

2) Drugs in this class include doxorubicin, doxorubicin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of doxorubicin hydrochloride ($C_{27}H_{29}NO_{11}$.HCl : 579.99) in mass (potency).

4) 1 mg of doxorubicin hydrochloride reference standard ($C_{27}H_{29}NO_{11}$.HCl) is equivalent to a potency of 1 mg.

3. Idarubicin Antibiotic Drugs

1) Idarubicin is a derivative of daunorubicin. It is also known as (1) 9-acetyl-7-[(3-amino-2,3,6-trideoxy-α-*L*-lyxo-hexopyranosyl)oxy]-7,8,9,10-tetrahydro-6,9,11-trihydroxy-5,12-naphthacenedione and (2) (1S, 3S)-3-acetyl-1,2,3,4,6,11-hexahydro-3,5,12-trihydroxy-6,11-dioxo-1-naphthacenyl-3-amino-2,3,6-trideoxy-α-*L*-lyxo-hexopyranoside.

2) Drugs in this class include idarubicin, idarubicin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of idarubicin hydrochloride ($C_{26}H_{27}NO_9$·HCl) in mass (potency).

4) 1 mg of idarubicin hydrochloride reference standard ($C_{26}H_{27}NO_9$·HCl) dried at 60℃ for 3 hours under a reduced pressure of ≤ 0.7 kPa is equivalent to a potency of 1 mg.

4. Aclarubicin Antibiotic Drugs

1) Aclarubicin is a substance prepared by culturing *Streptomyces galilaeus* or an equivalent substance prepared through other methods.

2) Drugs in this class include aclarubicin, aclarubicin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of aclarubicin ($C_{42}H_{53}NO_{15}$) in mass (potency).

4) 1 mg of aclarubicin reference standard ($C_{42}H_{53}NO_{15}$) is equivalent to a potency of 1 mg.

5. Epirubicin Antibiotic Drugs

1) Epirubicin is a derivative of doxorubicin. It is also known as 4-epirubicin.

2) Drugs in this class include epirubicin, epirubicin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of epirubicin hydrochloride ($C_{27}H_{29}NO_{11}$.HCl) in mass (potency).

4) 1 mg of epirubicin hydrochloride reference standard ($C_{27}H_{29}NO_{11}$.HCl) is equivalent to a potency of 1 mg.

6. Pirarubicin Antibiotic Drugs

1) Pirarubicin is a derivative of doxorubicin. It is also known as (2 " *R)*-4′-*O*-tetrahydropyranyldoxorubicin.

2) Drugs in this class include pirarubicin, pirarubicin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of pirarubicin ($C_{32}H_{37}NO_{12}$) in mass (potency).

4) 1 mg of pirarubicin reference standard ($C_{32}H_{37}NO_{12}$) is equivalent to a potency of 1 mg.

X. Oxacephem Antibiotics

1. Latamoxef Antibiotic Drugs

1) Latamoxef is an antibiotic substance prepared through a semi-synthetic process using 6β-aminopenicillic acid as a raw ingredient. It is also known as (1) (6R, 7R)-7-[2-carboxy-2-(P-hydroxyphenyl)acetamido]-7-methoxy-3[[(1-methyl-1*H*-tetrazol-5-yl)thio]-methyl]-8-oxo-5-oxy-1-azabicyclo[4.2.0]oct-2 -ene-2-carboxylic acid and (2) 7β-[2-carboxy-2-*P*-hydroxyphenyl)acetamido]-7α-methoxy-3-[[(1-methyl-1*H*-tetrazole- 5-yl)thio]-methyl]-1-oxy-1-dethia-3-cephem-4-carboxylic acid.

2) Drugs in this class include latamoxef, latamoxef salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of latamoxef ($C_{20}H_{20}N_6O_9S$) in mass (potency).

4) 1.085 mg of latamoxef sodium reference standard ($C_{20}H_{19}N_6NaO_9S$) is equivalent to a potency of 1 mg.

2. Flomoxef Antibiotic Drugs

1) Flomoxef is an antibiotic prepared thorugh a semi-synthetic process using 6β-aminopenicillic acid as a raw ingredient. It is also known as (1) (-)-(6*R*,7*R*)-7-[2-(difluoromethylthio)acetamido] -7-methoxy-3-[[1-(2-hydroxyethyl)-1*H*-tetrazol-5-yl]thiomethyl]-8-oxo-5-oxa-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid and (2) 7β-[2-(difluoromethylthia)acetamido]-7α-methoxy-3-[[1-(2-hydroxyethyl)-1*H*-tetrazol-5-yl]thiomethyl]-1-oxa-1-dithia-3-cephem-4-carboxylic acid.

2) Drugs in this class include flomoxef, flomoxef salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount as flomoxef ($C_{15}H_{18}F_2N_6O_7S_2$) in mass (potency).

4) 1.044 mg of flomoxef sodium reference standard ($C_{15}H_{17}F_2N_6NaO_7S_2$) is equivalent to a potency of 1 mg.

XI. Cabacephem Antibiotics

1. Loracarbef Antibiotic Drugs

1) Loracarbef is a derivative of beta-lactam antibiotic substance. It is also known as (6*R*,7*S*)-7-[(*R*)-2-amino-2-phenylacetamido]-3-chloro-8-oxo-1-azabicyclo[4.2.0]oct-2-ene-carboxylic acid.

2) Drugs in this class include loracarbef, loracarbef salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of loracarbef ($C_{16}H_{16}ClN_3O_4$) in mass (potency).

4) 1.051 mg of loracarbef reference standard ($C_{16}H_{16}ClN_3O_4$) is equivalent to a potency of 1 mg.

XII. Carbapenem Antibiotics

1. Meropenem Antibiotic Drugs

1) Meropenem is a derivative of antibiotics prepared by culturing *Streptomyces cattleya* or an antibiotic substance prepared by synthesis. It is also known as (-)-(4*R*,5*S*,6*S*)-3-

[[(3*S*,5*S*)-5-(dimethylcarbamoyl)-3-pyrrolidinyl]thio]-6-[(1*R*)-1-hydroxyethyl]-4-methyl-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid.

2) Drugs in this class include meropenem, meropenem trihydrate, meropenem salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of meropenem ($C_{17}H_{25}N_3O_5S$) in mass (potency).

4) 1.141 mg of meropenem reference standard ($C_{17}H_{25}N_3O_5S.3H_2O$) is equivalent to a potency of 1 mg.

2. Ertapenem Antibiotic Drugs

1) Ertapenem is a derivative of antibiotics prepared by culturing *Streptomyces cattleya* or an antibiotic substance prepared by synthesis. It is also known as [4R-[3(3S*,5S*),4α,5β,6β(R*)]-3-[[5-[[(3-carboxyphenyl)amino]carbonyl]-3-pyrrolidinyl]thio]-6-(1-hydroxyethyl)-4-methyl-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid.

2) Drugs in this class include ertapenem, ertapenem salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of ertapenem ($C_{22}H_{25}N_3O_7S$) in mass (potency).

4) 1.046 mg of ertapenem sodium reference standard ($C_{22}H_{24}NaN_3O_7S$) is equivalent to a potency of 1 mg.

3. Imipenem Antibiotic Drug

1) Imipenem is a derivative of antibiotics prepared by culturing *Streptomyces cattleya* or through other methods, or a substance prepared by synthesis. It is also known as (+)-(5*R*,6*S*)-3-[[(2-formimidoylamino)ethyl] thio]-6-[(*R*)-1-hydroxyethyl]-7-oxo-1-c[3.2.0]hepta-2-ene-2-carboxylic acid.

2) Drugs in this class include imipenem, imipenem salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of imipenem ($C_{12}H_{17}N_3O_4S$) in mass (potency).

4) 1.066 mg of imipenem reference standard ($C_{12}H_{17}N_3O_4S.H_2O$) is equivalent to a potency of 1 mg.

4. Panipenem Antibiotic Drugs

1) Panipenem is a derivative of antibiotics prepared by culturing *Streptomyces cattleya* or an antibiotic substance prepared by synthesis. It is also known as (+)-(5*R*,6*S*)-3-[(*S*)-1-(acethymidylpyrrolidine-3-yl)thio]-6-[(*R*)-1-hydroxyethyl]-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid.

2) Drugs in this class include panipenem, panipenem salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of panipenem ($C_{15}H_{21}N_3O_4S$) in mass (potency).

4) 1.224 mg of panipenem reference standard ($C_{15}H_{21}N_3O_4S.CH_4N_2S$) is equivalent to a potency of 1 mg.

XIII. Tetracycline Antibiotics

1. Doxycycline Antibiotic Drugs

1) Doxycycline is a deoxy derivative of oxytetracycline at the 6th position (α-6-deoxy-oxytetracycline).

2) Drugs in this class include doxycycline, doxycycline salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of doxycycline ($C_{22}H_{24}N_2O_8$) in mass (potency).

4) 1.155 mg of doxycycline reference standard ($C_{22}H_{24}N_2O_8 \cdot HCl.1/2C_2H_5OH.1/2H_2O$) is equivalent to a potency of 1 mg.

2. Meclocyline Antibiotic Drugs

1) Meclocycline is a 7-chloro-6-methylene-5-hydroxytetracycline.

2) Drugs in this class include meclocycline, meclocycline salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of meclocycline ($C_{22}H_{21}ClN_2O_8$) in mass (potency).

4) 1 mg of meclocycline reference standard ($C_{22}H_{21}ClN_2O_8$) is equivalent to a potency of 1 mg.

3. Methacycline Antibiotic Drugs

1) Methacycline is a methylene derivative of oxytetracycline at the 6th position (6-methyleneoxytetracycline).

2) Drugs in this class include methacycline, methacycline salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of methacycline hydrochloride ($C_{22}H_{22}N_2O_8.HCl$) in mass (potency).

4) 1 mg of metacycline hydrochloride reference standard ($C_{22}H_{22}N_2O_8.HCl$) dried at 60°C for 3 hours under a reduced pressure of ≤ 0.7 kPa is equivalent to a potency of 1 mg.

4. Minocycline Antibiotic Drugs

1) Minocycline is a deoxy- and demethyl- derivative of tetracycline at the 6th position and also a dimethylamino derivative tetracycline at the 7th position (7-dimethylamino-6-deoxy-6-demethyltetracycline).

2) Drugs in this class include minocycline, minocycline salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of minocycline ($C_{23}H_{27}N_3O$) in mass (potency).

4) 1.159 mg of minocycline hydrochloride reference standard ($C_{23}H_{27}N_3O_7.HCl.2H_2O$) is equivalent to a potency of 1 mg.

5. Oxytetracycline Antibiotic Drugs

1) Oxytetracycline is a substance prepared by culturing *Streptomyces rimosus* or an equivalent substance prepared through other methods.

2) Drugs in this class include oxytetracycline, oxytetracycline salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of oxytetracycline ($C_{22}H_{24}N_2O_9$) in mass (potency).

4) 1.082 mg of oxytetracycline reference standard ($C_{22}H_{24}N_2O_9.2H_2O$) dried under a reduced pressure of ≤ 0.7 kPa for 3 hours is equivalent to a potency of 1 mg.

XIV. Penicillin Antibiotics

1. Nafcillin Antibiotic Drugs

1) Nafcillin is an ethoxynaphthyl derivative of 6-aminopenicillanic acid.

2) Drugs in this class include nafcillin, nafcillin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of nafcillin ($C_{21}H_{22}N_2O_5S$) in mass (potency).

4) 1.089 mg of nafcillin sodium reference standard is equivalent to a potency of 1 mg.

2. Dicloxacillin Antibiotic Drugs

1) Dicloxacillin is a methyl-dichlorophenyl-isoxazolyl derivative of 6-aminopenicillanic acid.

2) Drugs in this class include dicloxacillin, dicloxacillin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of dicloxacillin ($C_{19}H_{17}Cl_2N_3O_5S$) in mass (potency).

4) 1.085 mg of dicloxacillin sodium reference standard is equivalent to a potency of 1 mg.

3. Mecillinam Antibiotic Drugs

1) Mecillinam is also known as (1) 6-[(hexahydro-1*H*-azepin-1-yl)-methylene]amino]-3,3-dimethyl-7-oxo-4-thio]-azabicyclo-[3.2.0]heptane-2-carboxylate and (2) 6-[(hexahydro-1*H*-azepin-1-yl)methyleneamino]penicillic acid.

2) Drugs in this class include mecillinam and preparations containing it.

3) The potency of drugs in this class is expressed as the amount as mecillinam ($C_{15}H_{23}N_3O_3S$) in mass (potency).

4) 1 mg of mecillinam reference standard ($C_{15}H_{23}N_3O_3S$) is equivalent to a potency of 1 mg.

4. Mezlocillin Antibiotic Drugs

1) Mezlocillin is a methyl-sulfonyl-oxoimidazolidin-carbonyl-amino-benzyl derivative of 6-aminopenicillanic acid.

2) Drugs in this class include mezlocillin, mezlocillin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of mezlocillin ($C_{21}H_{25}N_5O_8S_2$) in mass (potency).

4) 1.074 mg of mezlocillin sodium reference standard ($C_{21}H_{24}NaN_5O_8S_2.H_2O$) is equivalent to a potency of 1 mg.

5. Methicillin Antibiotic Drugs

1) Methicillin is a dimethoxyphenyl derivative of 6-aminopenicillanic acid.

2) Drugs in this class include methicillin, methicillin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of methicillin ($C_{17}H_{20}N_2O_6S$) in mass (potency).

4) 1.105 mg of methicillin sodium reference standard ($C_{17}H_{19}N_2NaO_6S.H_2O$) is equivalent to a potency of 1 mg.

6. Metampicillin Antibiotic Drugs

1) Metampicillin is also known as (1) 3,3-dimethyl-6-[[(methyleneamino)phenylacetyl]amino]-7-oxo-4-thia-1-azabicyclo[3.2.0]-heptane-2-carboxylic acid and (2) D-6-[α-(methyleneamino)phenylacetmido]penicillanic acid.

2) Drugs in this class include metampicillin, metampicillin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of metampicillin ($C_{17}H_{19}N_3O_4S$) in mass (potency).

4) 1.061 mg of metampicillin sodium reference standard ($C_{17}H_{18}N_7NaO_4S$) is equivalent to a potency of 1 mg.

7. Sulbenicillin Antibiotic Drugs

1) Sulbenicillin is also known as sulfobenzyl derivative of 6-aminopenicillanic acid. It is also known as (1) (2*S*,5*R*,6*R*)-3,3-dimethyl-7-oxo-6-[[(2*R*)-phenylsulfoacetyl]amino]-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid and (2) α-sulfobenzylpenicillin.

2) Drugs in this class include sulbenicillin, sulbenicillin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of sulbenicillin ($C_{16}H_{18}N_2O_7S_2$) in mass (potency).

4) 1.106 mg of sulbenicillin sodium reference standard ($C_{16}H_{16}N_2Na_2O_7S_2$) is equivalent to a potency of 1 mg.

8. Sultamicillin antibiotic Drugs

1) Sultamicillin is a derivative of 6-aminopenicillanic acid. It is also known as (1) hydroxymethyl(+)-(2*S*,5*R*,6*R*)-6-[(*R*)-(2-amino-2-phenylacetamido]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate (ester) and (2) (2*S*,5*R*)-3,3-dimethyl- 7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate (ester) *S*,*S*-dioxide.

2) Drugs in this class include sultamicillin, sultamicillin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of sultamicillin ($C_{25}H_{30}N_4O_9S_2$) in mass (potency).

4) 1 mg of sultamicillin reference standard ($C_{25}H_{30}N_4O_9S_2$) is equivalent to a potency of 1 mg.

9. Ciclacillin Antibiotic Drugs

1) Ciclacillin is an aminocyclohexyl derivative of 6-aminopenicillanic acid. It is also known as (1) (2*S*,5*R*,6*R*)-6-[[(1-aminocyclohexyl)carbonyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid and (2) (1-aminocyclohexyl)penicillin.

2) Drugs in this class include ciclacillin, ciclacillin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of ciclacillin ($C_{15}H_{23}N_3O_4S$) in mass (potency).

4) 1 mg of ciclacillin reference standard ($C_{15}H_{23}N_3O_4S$) is equivalent to a potency of 1 mg.

10. Amoxicillin Antibiotic Drugs

1) Amoxicillin is an aminohydroxybenzyl derivative of 6-aminopenicillanic acid. It is also known as (1) (2*S*,5*R*,6*R*)-6-[[(2*R*)-amino(4-hydroxyphenyl)acetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid and (2) *p*-hydroxyampicillin.

2) Drugs in this class include amoxicillin, amoxicillin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of amoxicillin ($C_{16}H_{19}N_3O_5S$) in mass (potency).

4) 1.148 mg of amoxicillin reference standard ($C_{16}H_{19}N_3O_5S\cdot3H_2O$) is equivalent to a potency of 1 mg.

11. Aspoxicillin Antibiotic Drugs

1) Aspoxycillin is an N^4-methyl-D-asparaginyl-aminohydroxybenzyl derivative of 6-aminopenicillanic acid.

2) Drugs in this class include aspoxycillin, aspoxycillin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of aspoxycillin ($C_{21}H_{27}N_5O_7S$) in mass (potency).

4) 1.110 mg of aspoxycillin reference standard ($C_{21}H_{27}N_5O_7S\cdot3H_2O$) is equivalent to a potency of 1 mg.

12. Azlocillin Antibiotic Drugs

1) Azlocillin is also known as (1) (2*S*,5*R*,6*R*)-3,3-dimethyl-7-oxo-6-[[(2*R*)-[[(2-oxo-1-imidazolidinyl)carbonyl]amino]phenylacetyl]amino]-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid and (2) D-α-[(imidazolidin-2-on-1-yl)carbonylamino] benzylpenicillin.

2) Drugs in this class include azlocillin, azlocillin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of azlocillin ($C_{20}H_{23}N_5O_6S$) in mass (potency).

4) 1.128 mg of azlocillin sodium reference standard ($C_{20}H_{22}N_5NaO_6S$) is equivalent to a potency of 1 mg.

13. Ampicillin Antibiotic Drugs

1) Ampicillin is an aminobenzyl derivative of 6-aminopenicillanic acid.

2) Drugs in this class include ampicillin, ethoxy-carbonyloxyethyl derivatives of ampicillin (bacampicillin), phthalidyl derivatives of ampicillin (talampicillin), methyl-oxo-dioxolenly methyl derivatives of ampicillin (lenampicillin), and preparations containing any of these substances or their salts.

3) The potency of drugs in this class is expressed as the

amount of ampicillin ($C_{16}H_{19}N_3O_4S$) in mass (potency).

4) 1.155 mg of ampicillin reference standard ($C_{16}H_{19}N_3O_4S \cdot 3H_2O$) is equivalent to a potency of 1 mg.
1.482 mg of ampicillin phthalidyl reference standard ($C_{24}H_{23}N_3O_6S \cdot HCl$) is equivalent to a potency of 1 mg.

14. Cloxacillin Antibiotic Drugs

1) Cloxacillin is a methylchlorphenyl-isoxazolyl derivative of 6-aminopenicillanic acid.

2) Drugs in this class include cloxacillin, cloxacillin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount as cloxacillin ($C_{19}H_{18}ClN_3O_5S$) in mass (potency).

4) 1.092 mg of cloxacillin sodium reference standard ($C_{19}H_{17}ClN_3NaO_5S.H_2O$) is equivalent to a potency of 1 mg.

15. Ticarcillin Antibiotic Drugs

1) Ticarcillin is a carboxythienylmethyl derivative of 6-aminopenicillanic acid.

2) Drugs in this class include ticarcillin, ticarcillin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of ticarcillin ($C_{15}H_{16}N_2O_6S_2$) in mass (potency).

4) 1.114 mg of ticarcillin sodium reference standard ($C_{15}H_{14}N_2Na_2O_6S_2$) is equivalent to a potency of 1 mg.

16. Phenethicillin Antibiotic Drugs

1) Phenethicillin is a phenoxyethyl derivative of 6-aminopenicillanic acid.

2) Drugs in this class include phenethicillin, phenethicillin derivatives, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of *L*-phenethicillin potassium ($C_{17}H_{19}KN_2O_5S$) in unit (potency).

4) 0.68 μg of L-phenethicillin potassium reference standard ($C_{17}H_{19}KN_2O_5S$) is equivalent to 1 unit (potency).

17. Penicillin G Antibiotic Drugs

1) Penicillin G (benzylpenicillin) is a substance prepared by culturing genus *Penicillium* or an equivalent substance prepared through other methods.

2) Drugs in this class include penicillin, penicillin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of penicillin G sodium ($C_{16}H_{17}N_2NaO_4S$) in unit (potency).

4) 0.6 μg of penicillin sodium G sodium reference standard ($C_{16}H_{17}N_2NaO_4S$) is equivalent to 1 unit (potency).

18. Phenoxymethylpenicillin Antibiotic Drugs

1) Phenoxymethylpenicillin (penicillin V) is a substance prepared by culturing genus *Penicillium* or an equivalent substance prepared through other methods.

2) Drugs in this class include phenoxymethylpenicillin, phenoxymethylpenicillin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of phenoxymethylpenicillin ($C_{16}H_{18}N_2O_5S$) in unit (potency).

4) 0.59 μg of phenoxymethylpenicillin reference standard ($C_{16}H_{18}N_2O_5S$) is equivalent to 1 unit (potency).

19. Flucloxacillin Antibiotic Drugs

1) Flucloxacillin is a methyl-chlorofluoro-phenylisoxazolyl derivative of 6-aminopenicillanic acid.

2) Drugs in this class include flucloxacillin, flucloxacillin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of flucloxacillin ($C_{19}H_{17}ClFN_3O_5S$) in mass (potency).

4) 1.088 mg of flucloxacillin sodium reference standard ($C_{19}H_{16}ClFN_3NaO_5S.H_2O$) is equivalent to a potency of 1 mg.

20. Pivampicillin Antibiotic Drugs

1) Pivampicillin is an acyloxymethyl ester of ampicillin. It is also known as pivaloyloxymethyl D-α-aminobenzylpenicillinate.

2) Drugs in this class include pivampicillin, pivampicillin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of pivampicillin ($C_{22}H_{29}N_3O_6S$) in mass (potency).

4) 1 mg of pivampicillin reference standard ($C_{22}H_{29}N_3O_6S$) is equivalent to a potency of 1 mg.

21. Fibracillin Antibiotic Drugs

1) Fibracillin is a chlorophenoxy-2-methylpropionamidobenzyl derivative of 6-aminopenicillanic acid.

2) Drugs in this class include fibracillin, fibracillin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of fibracillin ($C_{26}H_{28}ClN_3O_6S$) in mass (potency).

4) 1 mg of fibracillin reference standard ($C_{26}H_{28}ClN_3O_6S$) is equivalent to a potency of 1 mg.

22. Pivmecillinam Antibiotic Drugs

1) Pivmecillinam is a pivaloyloxymethyl derivative of the hexahydroazepinylmethyridine derivative of 6-aminopenicillanic acid (mecillinam).

2) Drugs in this class include pivmecillinam, pivmecillinam salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of mecillinam ($C_{15}H_{23}N_3O_3S$) in mass (potency).

4) 1.463 mg of pivmecillinam hydrochloride reference standard ($C_{21}H_{35}N_3O_5S.HCl$) is equivalent to a potency of 1 mg.

23. Piperacillin Antibiotic Drugs

1) Piperacillin is a 4-ethyl-2,3-dioxopiperadinecarboxamidebenzyl derivative of 6-aminopenicillanic acid.

2) Drugs in this class include piperacillin, piperacillin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of piperacillin ($C_{23}H_{27}N_5O_7S$) in mass (potency).

4) 1.035 mg of piperacillin reference standard ($C_{23}H_{27}N_5O_7S.H_2O$) is equivalent to a potency of 1 mg.

24. Hetacillin Antibiotic Drugs

1) Hetacillin is an *N*,*N*'-isopropylideneaminobenzyl derivative of 6-aminopenicillanic acid.

2) Drugs in this class include hetacillin, hetacillin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of hetacillin ($C_{19}H_{23}N_3O_4S$) in mass (potency).

4) 1 mg of hetacillin reference standard ($C_{19}H_{23}N_3O_4S$) is equivalent to a potency of 1 mg.

XV. Peptide Antibiotics

1. Gramicidin Antibiotic Drugs

1) Gramicidin is a substance prepared by culturing *Bacillus brevis Dubos* or an equivalent substance prepared through other

methods.

2) Drugs in this class include gramicidin, gramicidin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of gramicidin molecular formula unknown) in mass (potency).

4) 1 mg of gramicidin reference standard dried at 60 °C for 3 hours under a reduced pressure of ≤ 0.7 kPa is equivalent to a potency of 1 mg.

2. Dactinomycin Antibiotic Drugs

1) Dactinomycin is a substance prepared by culturing *Streptomyces parvullus* or an equivalent substance prepared through other methods.

2) Drugs in this class include dactinomycin and preparations containing it.

3) The potency of drugs in this class is expressed as the amount of dactinomycin ($C_{63}H_{86}N_{12}O_{16}$) in mass (potency).

4) 1 mg of dactinomycin reference standard dried at 60 °C for 3 hours under a reduced pressure of ≤ 0.67 kPa is equivalent to a potency of 1 mg.

3. Bacitracin Antibiotic Drugs

1) Bacitracin is a substance mainly composed of bacitracin A, which is prepared by culturing *Bacillus subtilis var. Tracy* or an equivalent substance prepared through other methods.

2) Drugs in this class include bacitracin, bacitracin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of bacitracin A ($C_{66}H_{103}N_{17}O_{16}S$) in unit.

4) 23.8 μg of bacitracin reference standard dried at 60 °C for 3 hours under a reduced pressure of ≤ 0.7 kPa is equivalent to 1 unit.

4. Enviomycin Antibiotic Drugs

1) Enviomycin is a mixture of tuberactinomycin N, tuberactinomycin O, etc., which are prepared by culturing from *Streptomyces griseoverticillatus var. tuberacticus* N6-130, or an equivalent substance prepared through other methods.

2) Drugs in this class include enviomycin, enviomycin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of tuberactinomycin N ($C_{25}H_{43}N_{13}O_{10}$) in mass (potency).

4) 1.16 mg of enviomycin hydrochloride reference standard ($C_{25}H_{43}N_{13}O_{10}.3HCl$) dried at 60℃ for 3 hours under a reduced pressure of ≤ 0.7 kPa is equivalent to a potency of 1 mg.

5. Capreomycin Antibiotic Drugs

1) Capreomycin is a mixture of capreomycin 1A, capreomycin 1B, etc., which are prepared by culturing *Streptomyces capreolus*.

2) Drugs in this class include capreomycin, capreomycin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of capreomycin ($C_{25}H_{44}N_{44}O_{7\sim8}$) in mass (potency).

4) 1.087 mg of capreomycin sulfate reference standards ($C_{25}H_{44}N_{14}O_8.2H_2SO_4$ and $C_{25}H_{44}N_{14}O_7.2H_2SO_4$) dried at 100℃ for 4 hours under a reduced pressure of ≤ 0.7 kPa is equivalent to a potency of 1 mg.

6. Colistin Antibiotic Drugs

1) Colistin is a substance prepared by culturing *Bacillus polymyxa var. colistinus*, and its main components are colistin A and colistin B.

2) Drugs in this class include colistin, salts or derivatives of colistin, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of colistin A ($C_{53}H_{100}N_{16}O_{13}$) in mass (potency) or unit.

4) 1.21 mg of colistin reference standard ($C_{53}H_{100}N_{16}O_{13}.5/2H_2SO_4$) dried at 60 ℃ for 3 hours under a reduced pressure of ≤ 0.7 kPa is equivalent to a potency of 1 mg, and 1 mg (potency) is equivalent to 30000 units (potency).

7. Tyrothricin Antibiotic Drugs

1) Tyrothricin is a mixture of polypeptide antibiotics, which are prepared by culturing bacteria of the *Tyrothrix* group.

8. Polymyxin B Antibiotic Drugs

1) Polymyxin B is a substance mainly composed of polymyxin B1 and polymyxin B2, which are prepared by culturing *Bacillus polymyxa*, or through other methods.

2) Drugs in this class include polymyxin B, polymyxin B salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of polymyxin B ($C_{43}H_{82}N_{16}O_{12}$) in unit (potency).

4) 0.129 μg of polymyxin B sulfate reference standard ($C_{43}H_{82}N_{16}O_{12}.xH_2SO_4$) is equivalent to 1 unit (potency).

XVI. Polyene Macrolide Antibiotics

1. Nystatin Antibiotic Drugs

1) Nystatin is a substance prepared by culturing *Streptomyces noursei* or an equivalent substance prepared through other methods.

2) Drugs in this class include nystatin and preparations containing nystatin.

3) The potency of drugs in this class is expressed as the amount of nystatin ($C_{47}H_{75}NO_{17}$) in unit.

4) 0.27 μg of nystatin reference standard dried at 40 °C for 2 hours under a reduced pressure of ≤ 0.7 kPa is equivalent to 1 unit.

2. Amphotericin B Antibiotic Drugs

1) Amphotericin B is a substance prepared by culturing *Streptomyces nodosus* or an equivalent substance prepared through other methods.

2) Drugs in this class are amphotericin B, amphotericin B salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of amphotericin B ($C_{47}H_{73}NO_{17}$) in mass (potency).

4) 1 mg of amphotericin B reference standard ($C_{47}H_{73}NO_{17}$) dried at 60 °C for 3 hours under a reduced pressure of ≤ 0.7 kPa is equivalent to a potency of 1 mg.

3. Pimaricin Antibiotic Drugs

1) Pimaricin is a substance prepared by culturing *Streptomyces natalensis* or an equivalent substance prepared through other methods.

2) Drugs in this class include pimaricin and preparations containing pimaricin.

3) The potency of drugs in this class is expressed as the amount of pimaricin ($C_{33}H_{47}NO_{13}$) in mass (potency).

4) 1mg of pimaricin reference standard ($C_{33}H_{47}NO_{13}$) is equivalent to a potency of 1 mg.

XVII. Other Antibiotic Substances

1. Griseofulvin Antibiotic Drugs

1) Griseofulvin is a substance prepared by culturing *Penicillium griseofulvum or Penicillium janczewskii*, or an equivalent substance prepared through other methods.

2) Drugs in this class include griseofulvin and preparations containing it.

3) The potency of drugs in this class is expressed as the amount of griseofulvin ($C_{17}H_{17}ClO_6$) in mass (potency).

4) 1 mg of griseofulvin reference standard is equivalent to a potency of 1 mg.

2. Mupirocin Antibiotic Drugs

1) Mupirocin is a substance prepared by culturing *Pseudomonas fluorescens* or an equivalent substance prepared through other methods.

2) Drugs in this class include mupirocin, mupirocin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of mupirocin ($C_{26}H_{44}O_9$) in mass (potency).

4) 1.075 mg of mupirocin reference standard ($C_{26}H_{44}O_9$) is equivalent to a potency of 1 mg.

3. Mitomycin C Antibiotic Drugs

1) Mitomycin C is a substance prepared by culturing *Streptomyces caespitosus* or an equivalent substance prepared through other methods.

2) Drugs in this class include mitomycin C, mitomycin C salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of mitomycin C ($C_{15}H_{18}N_4O_5$) in mass (potency).

4) 1 mg of mitomycin C reference standard ($C_{15}H_{18}N_4O_5$) dried at 60 °C for 3 hours under a reduced pressure of ≤ 0.7 kPa is equivalent to a potency of 1 mg.

4. Sulbactam Antibiotic Drugs

1) Sulbactam is a derivative of 6-aminopenicillanic acid. It is also known as (2*S*, 5*R*)-3, 3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]-heptane-2-carboxylic acid-4, 4-dioxide.

2) Drugs in this class include sulbactam, sulbactam salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of sulbactam ($C_8H_{11}NO_5S$) in mass (potency).

4) 1 mg of sulbactam reference standard ($C_8H_{11}NO_5S$) is equivalent 1 mg (potency).

5. Spectinomycin Antibiotic Drugs

1) Spectinomycin is an antibiotic substance prepared by culturing *Streptomyces spectabilis* or through other methods.

2) Drugs in this class include spectinomycin, spectinomycin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of spectinomycin ($C_{14}H_{24}N_2O_7$) in mass (potency).

4) 1.490 mg of spectinomycin hydrochloride reference standard ($C_{14}H_{24}N_2O_7.2HCl.5H_2O$) is equivalent to a potency of 1 mg.

6. Cycloserine Antibiotic Drugs

1) Cycloserine is a substance prepared by culturing *Streptomyces orchidaceus*, *Streptomyces garyphalus*, or *Streptomyces lavendulae*, or an equivalent substance prepared through other methods.

2) Drugs in this class include cycloserine, cycloserine salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of cycloserine ($C_3H_6N_2O_2$) in mass (potency).

4) 1 mg of cycloserine reference standard ($C_3H_6N_2O_2$) is equivalent to a potency of 1 mg.

5) Terizidone is a [1,4-bis-*D*-(3-oxo-4-isoxazolidinyl-iminomethyl)-benzene, which is prepared by condensing two molecules of cycloserine with terephthalaldehyde.

7. Thiamphenicol Antibiotic Drugs

1) Thiamphenicol is a 2,2-dichloro-*N*-[(α*R*,*βR*)-*β*-hydroxy-α-hydroxymethyl-4-methylsulfonyl-phenmethyl]acetamide.

2) Drugs in this class include thiamphenicol, thiamphenicol salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of thiamphenicol ($C_{12}H_{15}Cl_2NO_5S$) in mass (potency).

8. Clavulanic Acid Antibiotic Drugs

1) Clavulanic acid is (*Z*)-(2*R*,5*R*)-3-(2-hydroxyethylidene)-7-oxo-4-oxa-1-azabicyclo[3.2.0]peptane-2-carboxylic acid., which is prepared by culturing *Streptomyces clavuligenus* ATCC 27064 or through other methods.

2) Drugs in this class include clavulanic acid, clavulanic acid salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of clavulanic acid ($C_8H_9NO_5$) in mass (potency).

4) 1.030 mg of clavulanic acid reference standard ($C_8H_8LiNO_5$) is equivalent to a potency of 1 mg.

9. Chloramphenicol Antibiotic Drugs

1) Chloramphenicol is a substance prepared by culturing *Streptomyces venezuelae* or *Streptomyces omiyaensis*, or an equivalent substance prepared through other methods. It is also known as *D* (-)-threo-1-paranitrophenyl-2-dichloroacetamide-1,3-propanediol.

2) Drugs in this class include chloramphenicol, chloramphenicol derivatives, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of chloramphenicol ($C_{11}H_{12}Cl_2N_2O_5$) in mass (potency).

4) 1 mg of chloramphenicol reference standard ($C_{11}H_{12}Cl_2N_2O_5$) is equivalent to a potency of 1 mg.

10. Fosfomycin Antibiotic Drugs

1) Fosfomycin is (-)-(1*R*,2*S*)-1,2-epoxypropylphosphonic acid, which is prepared by culturing *Streptomyces fradiae* or through other methods.

2) Drugs in this class include fosfomycin, fosfomycin salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of fosfomycin ($C_3H_7O_4P$) in mass (potency).

4) 2.008 mg of fosfomycin reference standard ($C_{11}H_{18}NO_4P.H_2O$) is equivalent to a potency of 1 mg.

11. Fusidic Acid Antibiotic Drugs

1) Fusidic acid is a substance prepared by culturing *Fusidium coccineum* or an equivalent substance prepared through other methods.

2) Drugs in this class include fusidic acid, fusidic acid salts, and preparations containing them.

3) The potency of drugs in this class is expressed as the amount of fusidic acid ($C_{31}H_{48}O_6$) in mass (potency).

4) 1.203 mg of fusidic acid reference standard [$C_{31}H_{48}O_6.(HOCH_2CH_2)_2NH$] is equivalent to a potency of 1 mg.

12. Pyrrolnitrin Antibiotic Drugs

1) Pyrrolnitrin is a 3-chloro-4-(3-chloro-2-nitrophenyl)pyrrole, which is prepared by culturing *Pseudomonas pyrrocinia* or *Pseudomonas aeruginosa*, or through other methods.

2) Drugs in this class include pyrrolnitrin and preparations containing it.

3) The potency of drugs in this class is expressed as the

amount of pyrrolnitrin ($C_{10}H_6Cl_2N_2O_2$) in mass (potency).

4) 1 mg of pyrrolnitrin standard ($C_{10}H_6Cl_2N_2O_2$) is equivalent to 1 mg (potency).

Density of Solids
고체의 밀도측정법

The density of solids means their average mass per unit volume and typically is expressed in grams per cubic centimetre (g/cm^3) although the International Unit is the kilogram per cubic metre (1 g/cm^3 = 1000 kg/m^3).

Unlike gases and liquids whose density depends only on temperature and pressure, the density of a solid also depends on its assembly form, and therefore varies with its crystal structure and degree of crystallinity.

When a solid is amorphous or partially amorphous, its density may further depend upon the method of preparation, treatment and storage.

Therefore, unlike fluids, for 2 chemically equivalent solids, their densities may be different, which reflects a difference in solid-state structure. The density of solid or powder particles is an important physical characteristic of pharmaceutical powders and pharmaceutical ingredients. The density of a solid particle can have different values depending on the method used to measure the volume of the particle. It is useful to distinguish 3 levels of expression of density:

–*True density*, which only includes the solid fraction of the material; in case of crystalline material, the true density is also called *crystal density*;

–*Particle density*, which also includes the volume due to intraparticulate pores;

–*Bulk density*, which further includes the interparticulate void volume formed in the powder bed.

True density

The true density of a certain substance is the average mass per unit volume, excluding all voids that are not fundamental parts of the molecular packing arrangement. It is an intrinsic property of the specified crystal structure of substance, and hence should be independent on the measurement method. The true density is determined by calculation.

For example, it is obtained using crystallographic data (volume and composition of the unit cell) from X-ray diffraction data, either on a single crystal or by refinement of the crystalline structure from X-ray powder diffraction data.

Particle density

The particle density takes into account both the true density and the intraparticulate pores (sealed and/or open pores inside the particle). Thus, particle density depends on the determined volume value, which in turn depends on the method of measurement. The particle density can be determined using one of the 2 following methods:

The *gas pycnometric density* is determined by measuring the volume of a powder whose mass is known, which is equivalent to the volume of gas displaced by the powder using a gas displacement pycnometer. In gas pycnometric density measurements, the volume determined excludes the volume occupied by open pores, but includes the one occupied by sealed pores or pores inaccessible to the gas. As the helium has high diffusivity and is accessible to open pores, it is recommended for measuring particle density. Therefore, the gas pycnometric density of a finely milled powder is generally not much different from the true density. Hence, this density is the best estimate of the true density of an amorphous or partially crystalline sample and is therefore widely applicable for processed pharmaceutical powder samples.

The *mercury porosimeter density* is also called granular density. In this method, the volume determined includes the one occupied by sealed pores or pores inaccessible by mercury, but includes the one only from open pores smaller than some size limit. This pore-size limit or minimal access diameter depends on the maximal mercury intrusion pressure applied during the measurement, and the mercury does not penetrate the finest pores accessible to helium under normal operating pressures. Various granular densities can be obtained from one sample since, for each applied mercury intrusion pressure, a density can be determined that corresponds to the pore-size limit at that pressure.

Bulk and tapped density

The bulk density of a powder is calculated by considering the interparticulate void volume as a portion of its volume. Hence, the bulk density depends on both the density of powder particles and the spatial arrangement of particles in the powder bed.

It is so difficult to measure the bulk density of a powder with good reproducibility since the slightest disturbance of the bed may result in a change of spatial arrangement. Thus, in reporting bulk density, it is essential to specify how the determination was made and the measurement conditions.

The Determination of Bulk and Tapped Densities is specified in the general information of the Korean Pharmacopoeia.

Determination of Bulk and Tapped Densities
겉보기밀도 및 탭밀도측정법

Determination of Bulk and Tapped Densities is a method of measuring the apparent densities of each powdered drug product under loose and tapped packing conditions. Loose packing is defined as the state obtained by pouring a powder into a vessel without any compaction, while tapped packing is defined as the state obtained by repeatedly dropping a vessel containing a powder from a specified height at a specified speed until the apparent volume of the powder in the vessel becomes nearly constant so that the powder is densely filled.

1. Bulk density

The bulk density of a powder is the ratio of the mass of a powder to the volume of the powder, which includes factors of the void volume between particles, in a loose, untapped state. Therefore, the bulk density is determined by the particle density of the powder and the spatial arrangement of the particles in the powder layer. Although expressed in kg/m^3 in the International System of Units, the bulk density is usually expressed in g/mL (1 g/mL=1000k g/m^3), as it is measured using a measuring cylinder. It can also be expressed in g/cm^3.

The volume properties of a powder may vary depending on the powder handling method, which encompasses sample preparation, pretreatment, and storage. Particles can be filled to achieve a specific range of bulk densities, and the bulk density can be changed with even a slight disturbance to the powder layer. As a result, measuring the bulk density of a powder in a way that enables high reproducibility is challenging, and therefore it is important to specify the measurement method when recording the results.

Methods for determining the bulk density of powders are as follows: passing a powder sample with a known mass through a sieve, followed by measuring the volume using a graduated measuring cylinder (Method 1), passing a powder sample with a known volume through a volumeter, then filling it into a measuring vessel to determine the mass (Method 2), and measuring the volume of a powder sample in a stainless steel measuring vessel (Method 3). Of these methods, it is preferable to use Method 1 or 3.

A. Method 1: Constant mass method using a graduated cylinder

(1) Procedure

If necessary, pass an amount of the sample sufficient to complete the test through a sieve with an opening of no less than 1.0 mm to break up agglomerates that may have formed during storage. At this time, carry out this operation gently so that the properties of the substance do not change. Weigh about 100 g of the test sample (m) with an accuracy of 0.1% and pour it carefully into a dry 250-mL graduated cylinder (graduation: 2 mL) without applying pressure. If necessary, flatten the powder gently without applying pressure to compact it and read the apparent volume (V0) value to the smallest unit of the graduation mark. Then, calculate the bulk density (g/mL) using the equation m/V0. To measure this characteristic value, it is generally recommended to repeat the measurement.

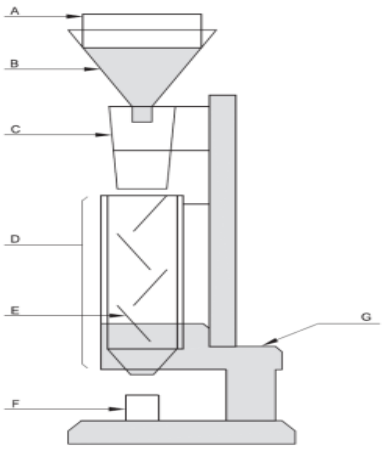
If the powder density is too low or too high, meaning that the apparent volume of the sample is less than 150 mL or more than 250 mL, it is not possible to measure samples with a mass exceeding 100 g. In this case, adjust the amount to achieve an untapped apparent volume of 150 to 250 mL (at least 60% of the graduated cylinder's total volume). When recording measurements, specify the sample mass.

For samples with an apparent volume of 50 to 100 mL, a 100-mL graduated cylinder (graduation: 1 mL) can be used. When recording the measurements, specify the volume of the graduated cylinder.

B. Method 2: Constant volume method using a volumeter

(1) Apparatus

This device (Figure 1) consists of an upper funnel fitted with a sieve with an opening of 1.0 mm, which is fixed to the top of a compartmented box consisting of four glass slats that slide or bounce over the powder as it passes through them. There is another funnel at the bottom of the compartmented box, which collects the powder and allows it to fall into the measuring vessel directly below. The specifications of the measuring vessel are as follows: 25.00 ± 0.05 mL in volume and 30.00 ± 2.00 mm in inner diameter for cylindrical vessels and 16.39 ± 0.20 mL in volume and 25.400 ± 0.076 mm in each side for cubic vessels.



A. Sieve with an opening size of 1.0 mm
B. Funnel for powder
C. Funnel for filling
D Compartmented box
E. Glass slats
F. Measuring vessel
G. Stand

Figure 1. Volumeter

(2) Procedure

Pass excess powder through the measuring vessel until it overflows. Use no less than 25 cm3 of powder for cubic measuring vessels, and no less than 35 cm3 of powder for cylindrical measuring vessels. Place the edge of the flat part of a spatula perpendicular to the upper surface of the vessel and move the edge of the flat part of the spatula smoothly across it while in contact with the upper surface of the measuring vessel to remove excess powder deposited on the upper surface of the measuring vessel. At this time, keep the spatula in a vertical position to prevent the powder in the measuring vessel from being removed or the vessel from being filled with more powder. Remove any powder from the sides of the measuring vessel, and then determine the mass of the sample (M) to a precision of 0.1%. Calculate the bulk density (g/mL) using the equation M/V0 (V0 is the volume of the measuring vessel). Repeat the measurement three times using three different samples and record the average value.

C. Method 3: Applied volume measurement method

(1) Apparatus

This device consists of a 100-mL cylindrical vessel made of stainless steel, with the dimensions shown in Figure 2.

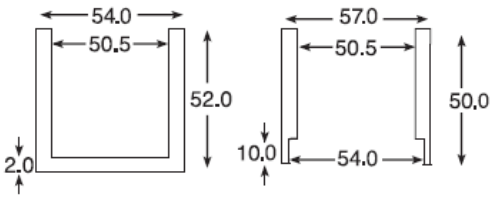


Figure 2. A measuring vessel (left) and an auxiliary cylinder (right) (unit: mm)

(1) Procedure

If necessary, pass an amount of powder sufficient to complete the test through a sieve with an opening of 1.0 mm to break up aggregates formed during storage, and pass the sieved sample through a measuring vessel made of stainless steel until

it overflows. Carefully scrape off the excess powder from the top of the vessel in the same manner described in Method 2. Subtract the mass of the empty measuring vessel, previously weighed, from the total mass to determine the mass of the powder (M0) to a precision of 0.1%. Calculate the bulk density (g/mL) using the equation M0/100. Repeat the measurement three times using three different samples and record the average value.

2. Tapped density

The tapped density is an apparent density obtained by mechanically tapping a measuring vessel containing a powder sample.

The tapped density is determined by mechanically tapping a graduated cylinder or measuring vessel containing a powder sample. After measuring the initial volume or mass of the powder, the graduated cylinder or measuring vessel is mechanically tapped until there is minimum change in volume or mass before the final measurement. To perform mechanical tapping, the graduated cylinder or measuring vessel is lifted, and then dropped from a specific height, using one of the three methods described below, under its own weight. Using a device that rotates the graduated cylinder or measuring vessel during tapping can help minimize the risk of segregation of powder during the tapping process.

A. Method 1

(1) Apparatus

The device (Figure 3) consists of the following parts:

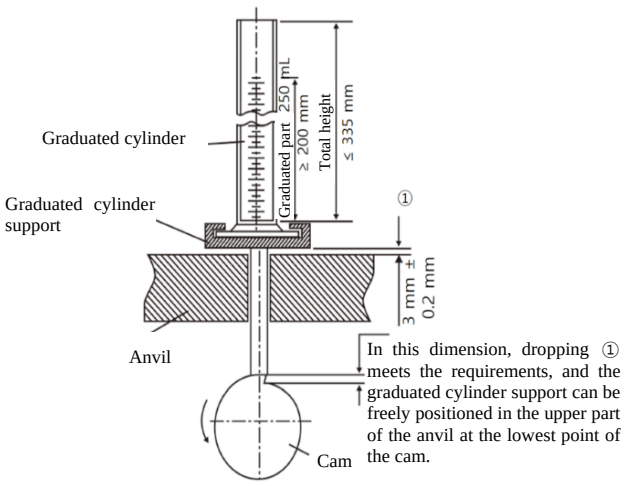


Figure 3. Powder sample dropping device (unit: mm)

- A 250-mL graduated cylinder, 220 ± 44 g in mass (smallest graduation unit: 2 mL)

- A device nominally capable of 250 ± 15 taps per minute at a height of 3 ± 0.2 mm, or of 300 ± 15 taps per minute at a height of 14 ± 2 mm. The mass of the graduated cylinder support equipped with the holder is 450 ± 10 g.

(2) Procedure

Determine the apparent volume (V_0) using the method described above. Mount the graduated cylinder on the support. Tap the same powder sample 10 times, 500 times, and 1250 times, and record the corresponding volumes as V_{10}, V_{500}, and V_{1250}, respectively, to the smallest graduation unit. If the difference between V_{500} and V_{1250} is 2 mL or less, use V_{1250} as the tap volume. If the difference between V_{500} and V_{1250} exceeds 2 mL, repeat the tapping process until the difference in successive measurements becomes no more than 2 mL– for example, by performing sets of 1250 taps. For validated samples, the number of taps require may vary depending on the powder. Calculate the tapped density (g/mL) using the equationm/Vf where Vf corresponds to the final tapped volume. Typically, the tap density is determined through repeated measurements. When recording the measurements, be sure to specify the drop height.

If it is impossible to use a sample of 100 g, reduce the sample amount and use an appropriate 100-mL graduated cylinder weighing 130 ± 16 g, fixed on a support weighing 240 ± 12 g (with the smallest graduation unit at 1 mL). If the difference between V_{500} and V_{1250} is 1 mL or less, use V_{1250} as the tap volume. If the difference between V_{500} and V_{1250} exceeds 1 mL, repeat tapping until the difference in successive measurements becomes no more than 1 mL, such as by performing sets of 1250 taps. When recording the measurements, be sure to specify the modified test conditions as well.

B. Method 2

(1) Procedure

Perform the test in the same manner described in the procedure for Method 1, except using a mechanical measuring device with a fixed drop height of 3 ± 0.2 mm at a nominal speed of 250 taps per minute.

C. Method 3

(1) Procedure

Perform the test in the same manner described in Method 3 for determining bulk density, using a measuring vessel equipped with an auxiliary cylinder as shown in Figure 2. Utilize an appropriate tap density measuring device to tap the measuring vessel equipped with an auxiliary cylinder at a rate of 50 to 60 times per minute. After 200 taps, carefully remove the auxiliary cylinder, and then remove excess powder from the top of the measuring vessel in the manner described in Method 3 for determining bulk density. Repeat the tap operation up to 400 times. If the difference between the two masses obtained after 200 taps and 400 taps exceeds 2%, repeat the test by tapping sets of 200 times until the difference between the two consecutive measurements becomes less than 2%. Calculate the tap density (g/mL) using the equationM_f/100, where M_f corresponds to the mass of the powder in the measuring vessel. Perform the measurement three times using three different samples and record the average value. When recording the measurements, be sure to specify the test conditions, including the tab height, in the results section.

3. Determination of powder compressibility

Because interactions between particles that affect the volume properties of a powder interfere with the fluidity of the powder, comparison of bulk density and tap density is a measure that can be used to evaluate the relative significance of the interactions between particles of the powder. The compressibility index and the Hausner ratio, both of which are indicators of powder fluidity, are often used for such comparison.

The compression index and the Hausner ratio, as explained previously, are indicators of powder compressibility. These indicators reflect the powder's ability to compress (settlement ability index), and as a result, can evaluate the relative significance of interactions between particles. For free-flowing powders, such interactions are not particularly important, and the bulk and tap densities are relatively close in value. Conversely, for powders with poor flowability, greater particle interactions result in a larger difference between the bulk density and the tap

density. These differences are manifested in the compression index and the Hausner ratio.

Compression index: Calculate using the following equation:

$$100\times[(V_o - V_f)/V_o]$$

V_o = Apparent volume without settlement
V_f = Final tapped volume

Hausner ratio: Calculated using the following equation: V_o/V_f

Depending on the sample, the compressibility index can be calculated using V_{10} instead of V_o. If V_{10} is used in place of V_0, specify this with the test results.

Disinfection and Sterilization Methods
소독법 및 멸균법

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 Methods" described in “Terminal Sterilization and Sterilization Indicators”. The expected killing effect on microorganisms or the estimated level of sterility assurance varies significantly depending on the purposes for which this method is applied, making it impractical to specify uniform treatment conditions for each disinfection and sterilization method.

In general, the following methods are typically implemented either singly or in combination after examining suitable selection and operation procedures as well as optimization of conditions according to the nature of the substance to which this method is applied and the contamination state (types of contaminating microorganisms and degree of microbial contamination). The validation of sterilization in accordance with [Terminal Sterilization and Sterilization Indicators] is required when these methods are applied to the manufacturing process of drug products.

1. Disinfection methods

Disinfection methods are used to reduce the number of living microorganisms but do not necessarily remove or kill every microorganism present. Generally, disinfection is classified into chemical disinfection with chemical agents (disinfectants) and physical disinfection with moist heat, ultraviolet rays, or other physical means of disinfection.

A. Chemical disinfection

Chemical disinfection refers to the use of chemical agents to kill microorganisms. The killing effect and mechanisms of the chemical agents differ depending on the type, concentration, operating temperature, operating time, the degree of contamination of the object to be disinfected, and the type and state (e.g., vegetative bacteria or spore bacteria) of microorganisms.

Therefore, when applying this method, care must be taken regarding the sterility and permissible storage period of the prepared chemical agent, the possibility of resistance of microorganisms at the site of application, and the effect of residual chemical agents on the product. When selecting a suitable chemical agent, the following factors should be considered according to the purpose of use:

1) The range of the antimicrobial spectrum
2) Time required to kill 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) Impact on the environment following disposal
12) Frequency of occurrence of resistant bacteria

B. Physical disinfection

Physical disinfection refers to the method of killing microorganisms without chemical agents.

(i) Steam flow method

The steam flow method refers to the method of directly applying heated steam to kill microorganisms. This method is used for products likely to be denatured by the moist heat method. Usually, the product is kept in flowing steam at 100 °C for 30 - 60 minutes.

(ii) Boiling method

The boiling method refers to a method of killing microorganisms by putting the object in boiling water. This method is used for products likely to be denatured by the moist heat method. Usually, the object is placed in boiling water and boiled for at least 15 minutes.

(iii) Intermittent method

The intermittent method refers to a method of killing microorganisms 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 products likely to be denatured by the moist heat method. There is also another method called the low-temperature intermittent method in which heating is repeated in the same way at 60 - 80 °C. During the intermission periods between heating or warming, a suitable temperature must be maintained for the growth of microorganisms of 20 °C or higher.

(iv) Ultraviolet method

The ultraviolet method refers to a method of killing microorganisms by irradiation with ultraviolet rays with a wavelength of around 254 nm. This method is used for materials resistant to UV irradiation, such as smooth surfaces, facilities, equipment, or water and air. This method does not have the risk of the appearance of resistant bacteria observed in chemical disinfection, and it shows a killing effect on bacteria, fungi, and viruses. However, caution is required as direct exposure of the human body to ultraviolet rays can cause harm to the eyes and skin.

2. Sterilization methods

A. Heating method

When applying the heating method, the heating time until the temperature or pressure reaches the specified conditions differs depending on the nature of the subject to which this method is applied, the size of the container, and the packing or storage condition. Furthermore, this method’s duration is counted from when the subject to which this method is applied has reached the specified temperature.

(i) Moist heat method

The moist heat method refers to a method of killing microorganisms by heating in saturated steam at a suitable

temperature and pressure. This method is mainly used for heat-stable materials such as glass, ceramic, metals, rubbers, plastics, papers textiles, and other thermally stable substances, including water, culture media, reagents/test solutions, or liquid samples.

Sterilization is usually carried out under one of the following conditions:

115 - 118 °C for 30 minutes
121 - 124 °C for 15 minutes
126 - 129 °C for 10 minutes

(ii) Dry-heat method

The dry-heat method refers to a method of killing microorganisms by heating in dry-heated air. This method is mainly used for heat-stable materials such as glass, ceramic, metals, or other thermally stable substances, including mineral oils, fat and oils, as well as powder samples. This method is generally conducted through direct heating by gas or electricity or circulating heated air. Sterilization is usually carried out under one of the following conditions:

160 - 170 °C for 120 minutes
170 - 180 °C for 60 minutes
180 - 190 °C for 30 minutes

B. Irradiation methods

(i) Radiation method

The radiation method refers to a method of killing microorganisms by irradiating γ-rays emitted from radioactive isotopes, electron beams generated from an electron accelerator, or controlled radiation (X-rays).

This method is mainly used for radiation-resistant materials such as glass, ceramic, metals, rubbers, plastics, and textiles. The dose is adjusted according to the material properties and the degree of contamination of the product to be sterilized. Special attention is necessary to the possibility of qualitative product change after application.

(Ii) Microwave method

The microwave method refers to a method of killing microorganisms using heat generated by direct irradiation of high-frequency waves. This method is mainly used for microwave-resistant substances such as water, culture media, and reagent solutions capable of withstanding high-frequency irradiation. Usually, a high-frequency radiation of 2450±50 MHz wavelength is used.

C. Gas methods

The gas method refers to a method of killing microorganisms by sterilizing gas. Suitable gases for killing microorganisms include ethylene oxide gas, formaldehyde gas, hydrogen peroxide gas, chlorine dioxide gas, etc. The temperature, humidity, gas concentration, and exposure time vary depending on the types of gas used in sterilization. In addition, utmost caution is required regarding the environment where the gas is used and residual gas concentration, as some gases can harm humans. For some gas methods, it may be difficult to measure or estimate the killing of microorganisms quantitatively.

D. Filtration method

The filtration method refers to a method of removing microorganisms by filtration using a suitable filtering device. This method is mainly used for culture media and test solutions containing gaseous, aqueous, or soluble substances that are unstable at high temperatures. Usually, filters with a pore diameter of 0.22 μm or smaller are used for sterilization. However, this method uses a filter with a pore diameter of 0.45 μm or less.

Endotoxin Test Using Recombinant Factor C
재조합 C 인자를 이용한 엔도톡신 시험법

The test for bacterial endotoxins using recombinant factor C (rFC) is carried out to quantify endotoxins from gram-negative bacteria. It is performed using rFC based on the gene sequence of the horseshoe crab (*Limulus polyphemus, Tachypleus tridentatus, Tachypleus gigas* or *Carcinoscorpius rotundicauda)*, by a fluorimetric method.

The test is carried out in a manner that avoids bacterial endotoxin contamination.

1. Apparatus

All glassware and other heat-stable apparatus are depyrogenated in a dry-heat oven using a validated process. A commonly used minimum time and temperature is 30 min at 250 °C. Where plastic equipment (such as microplates and pipette tips for automatic pipettes) is employed, it must be shown to be free of detectable endotoxin and not to interfere with the test.

2. Reagents

A. Reagents

Recombinant factor C is based on the gene sequence of the horseshoe crab (*Limulus polyphemus, Tachypleus tridentatus, Tachypleus gigas* or *Carcinoscorpius rotundicauda*). All reagents, including the fluorogenic substrate and buffer solution, must be free of detectable endotoxin.

B. Reagent solutions

If necessary, prepare the reagents according to the test kit manufacturer's instructions. Store the reagents, refrigerated or frozen, as indicated by the manufacturer.

C. Water for BET (water for bacterial endotoxins test)

Water for injections R or water produced by other procedures that shows no reaction with the reagent employed at the detection limit of the reagent.

3. Preparation of the Standard Endotoxin Stock Solution

The standard endotoxin stock solution is prepared from an endotoxin reference standard that has been calibrated against the International Standard, for example *endotoxin standard BRP*. Endotoxin is expressed in International Units (IU). The equivalence in IU of the International Standard is stated by the World Health Organization.

Note: 1 *International Unit (IU) of endotoxin is equal to 1 Endotoxin Unit (EU).*

Follow the specifications in the package leaflet and on the label for preparation and storage of the standard endotoxin stock solution.

4. Preparation of the Standard Endotoxin Solutions

After vigorously mixing the standard endotoxin stock solution, prepare appropriate serial dilutions of this solution using water for BET. Use the solutions as soon as possible to avoid loss of activity by adsorption.

5. Preparation of the Test Solutions

After vigorously mixing the standard endotoxin stock solution, prepare appropriate serial dilutions of this solution using water for BET. Use the solutions as soon as possible to avoid loss of activity by adsorption.Prepare the test solutions by dissolving or diluting active substances or medicinal products using water for BET. Some substances or preparations may be more appropriately dissolved or diluted in other aqueous solutions. If necessary, adjust the pH of the test solution (or dilution thereof) so that the pH of the mixture of the reagent(s) and test solution falls within the pH range specified by the test kit manufacturer, usually 6.0 to 8.0. The pH may be adjusted by the use of acid, base or a suitable buffer solution, as recommended by the test kit manufacturer. Acids and bases may be prepared from concentrates or solids with water for BET in containers free of detectable endotoxin. Buffer solutions must be validated to be free of detectable endotoxin and interfering factors.

6. Determination of the Maximum Valid Dilution

The maximum valid dilution (MVD) is the maximum allowable dilution of a sample at which the endotoxin limit can be determined. Determine the MVD using the following equation:

$$\text{MVD} = \frac{\text{endotoxin limit} \times \text{concentration of test solution}}{\lambda}$$

Note: λ = the lowest concentration used in the standard curve.

A. Endotoxin limit

The endotoxin limit for active substances administered parenterally, defined on the basis of dose, is equal to:

$$\frac{K}{M}$$

K = threshold pyrogenic dose of endotoxin per kilogram of body mass;

M = maximum recommended bolus dose of product per kilogram of body mass.

When the product is to be injected at frequent intervals or infused continuously, M is the maximum total dose administered in a single hour period.

The endotoxin limit for active substances administered parenterally is specified in units such as IU/mL, IU/mg, IU/Unit of biological activity, etc., in monographs.

B. Concentration of test solution

The unit is mg/mL if the endotoxin limit is specified by mass (IU/mg); Units/mL if the endotoxin limit is specified by unit of biological activity (IU/Unit); mL/mL if the endotoxin limit is specified by volume (IU/mL).

7. Fluorometric Quantitative Technique

This technique is used to measure the fluorescence (relative fluorescence units; RFU) emitted by a fluorescent substrate (reagent) after cleavage by endotoxin-activated factor C. It is used as an end-point-fluorescent test.

The end-point-fluorescent test is based on the quantitative relationship between the endotoxin concentration and the fluorescence of the reagent mixture at the end of the incubation period, expressed for example as *ΔRFU*:

$$\Delta RFU = RFU_{t_{endpoint}} - RFU_{t_0}$$

$RFU_{t_{endpoint}}$ = fluorescence of the reagent mixture at the end of the incubation period;

RFU_{t_0} = fluorescence of the reagent mixture at the start of the incubation period

The test is carried out at the temperature recommended by the test kit manufacturer (usually 37 ± 1 °C).

8. Preparatory Testing

Preparatory tests are conducted to ensure that the fluorometric technique is valid. These tests demonstrate that the criteria for the standard curve are satisfied and that the test solution does not interfere with the test.

Validation of the test method is required when any changes are made to the experimental conditions that are likely to influence the result of the test.

A. Assurance of Criteria for the Standard Curve

The test must be carried out for each lot of recombinant factor C reagent. Instrument sensitivity must be adjusted in accordance with the recommendations of the test kit manufacturer. Using the standard endotoxin solution, prepare at least 3 endotoxin concentrations within the range indicated by the test kit manufacturer to generate the standard curve. If the desired range exceeds the range indicated by the manufacturer by more than 2 $\log_{10}$, additional standards must be included to bracket each log increase in the range.

Perform the test using at least 3 replicates of each standard endotoxin solution as recommended by the manufacturer (volume ratios, incubation time, temperature, pH, etc.). The absolute value of the correlation coefficient, | r |, must be greater than or equal to 0.980, for the range of endotoxin concentrations prepared.

B. Interfering Factors

As factor G is absent from the test kit, false-positive results due to *β*-glucan activation are not expected to occur. This must be taken into account when the method is compared to other bacterial endotoxin quantification methods. Select an endotoxin concentration at or near the middle of the endotoxin standard curve.

Prepare solutions A, B, C and D as shown in Table 1. Perform the test on at least 2 replicates of these solutions as recommended by the test kit manufacturer (volume of test solution and reagent test kit mixture, volume ratio of test solution to reagent test kit mixture, incubation time, etc.).

Table 1

Solution	Endotoxin concentration	Solution to which endotoxin is added	Number of replicates
A	None	Test solution	Not less than 2
B	Middle concentration of the standard curve	Test solution	Not less than 2
C	At least 3 concentrations (lowest concentration is designated λ)	Water for BET	Each concentration is not less than 2
D	None	Water for BET	Not less than 2

1) Solution A = test solution, which may be diluted but not exceeding the MVD.
2) Solution B (positive product control) = preparation to be examined at the same dilution as solution A, containing added endotoxin at a concentration equal to or near the middle of the standard curve.
3) Solution C = standard endotoxin solution at the concentrations used

in the validation of the method.

4) Solution D (negative control) = water for BET.

This test is considered when the following two conditions are met.

Condition 1

The absolute value of the correlation coefficient of the standard curve generated using solution C is greater than or equal to 0.980.

Condition 2

The result with solution D does not exceed the limit of the blank test required in the description of the reagent mixture employed, or it is less than the endotoxin detection limit of the rFC employed.

Calculate the mean recovery of the added endotoxin by subtracting the mean endotoxin concentration in the solution (if any) (solution A) from that in the solution containing the added endotoxin (solution B). The test solution is considered free of interfering factors if, under the conditions of the test, the measured concentration of the endotoxin added to the test solution is within 50-200 % of the known added endotoxin concentration, after subtraction of any endotoxin detected in the solution without added endotoxin. When the endotoxin recovery is outside the specified range, the test solution is considered to contain interfering factors. Repeat the test using a greater dilution, not exceeding the MVD. Furthermore, interference of the test solution or diluted test solution (not exceeding the MVD) may be eliminated by suitable validated treatment, such as filtration, neutralisation, dialysis, heat treatment or endotoxin-specific binding steps (enrichment of endotoxin from the test solution prior to detection in the absence of the interfering matrix). To establish that the treatment chosen effectively eliminates interference without loss of endotoxins, repeat the test for interfering factors using the preparation being examined to which the standard endotoxin has been added and which has then been submitted to the chosen treatment.

9. Test

A. Procedure

Follow the procedure described in section B) Interfering Factors of 8. Preparatory Testing.

B. Calculation

Calculate the endotoxin concentration of each replicate of solution A using the standard curve generated by the standard endotoxin solution C.

The test is considered valid when the following 3 requirements are met:

(1) the results obtained with solution C comply with the requirements for validation defined in section A) Assurance of Criteria for the Standard Curve of 8. Preparatory Testing;

(2) the endotoxin recovery, calculated from the endotoxin concentration found in solution B after subtracting the endotoxin concentration found in solution A, is within the range of 50-200 %;

(3) the result obtained with solution D (negative control) does not exceed the limit value of the blank test required in the description of the reagent mixture employed, or it is less than the endotoxin detection limit of the rFC employed.

C. Interpretation

The preparation being examined complies with the test if the mean endotoxin concentration of the replicates of solution A, after correction for dilution and concentration, is less than the endotoxin limit for the product.

Enzyme-Linked Immunosorbent Assay (ELISA)
효소 결합 면역흡착 분석법(ELISA)

Enzyme-linked immunosorbent assay (ELISA) is one of the most widely used immunological test methods (ITMs) for characterization, release, and stability testing of biotechnology products to help ensure the quality of biological drug substances and drug products. The term “ELISA” is used here in a broader sense and includes enzyme immunoassays (EIAs), as well as alternative detection methods, e.g., chemiluminescence and fluorescence.

1. Definition

ELISA can be defined as a qualitative or quantitative solid-phase immunological method to measure an analyte following its binding to an immunosorbent surface and its subsequent detection by the use of enzymatic hydrolysis of a reporter substrate, either directly or indirectly. Qualitative results provide a simple positive or negative result for a sample. Converting quantitative to qualitative results based on a cutoff value that separates positive and negative results is common practice. Because the performance properties of the assay depend heavily on the cutoff value, the process used to determine the cutoff should be evidence-based and well documented. Quantitative assays determine the quantity of the analyte based on the interpolation of a standard calibration curve with known analyte concentration, run simultaneously in the same assay. This reference standard should be an appropriate, preferably homologous, reference material or calibration material that is representative of the analyte(s) of interest. ELISA assays are widely used in the biopharmaceutical industry for various applications such as identity, purity, potency, detection or quantitation of antibody or antigen, and other purposes.

2. Basic Principles

The essential steps of an ELISA can be broken down as follows (*Figure 1*):

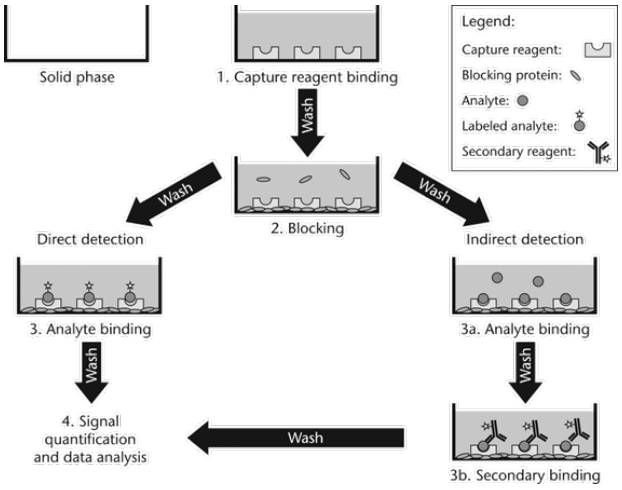


Figure 1. Essential steps for performing an ELISA.

As shown in Figure 1, detector antibody binding and

analysis are the five basic steps of ELISA. The binding of the capture reagent, blocking, and binding of the analyte are each followed by a wash step to remove unbound reagents before the next reagent is added. Prior to analysis, appropriate substrates are added, and measured using suitable equipment for detection. Quantification of unknown substances is achieved by comparison to a standard curve.

1) Binding of the capture reagent (generally an antibody or antigen), which functions as an immunosorbent for capture of the analyte, to a solid surface;

2) Removal of excess, unbound capture reagent followed by blocking of unoccupied binding sites with a blocking protein such as albumin, gelatin, casein, or other suitable material;

3) or 3a. Incubation of the analyte (in the test sample or reference standard) with the capture reagent to bind the analyte onto the solid surface, followed by the washing away of unbound material in the test sample and detection of the analyte.

3) Direct detection occurs when the analyte has enzymatic activity or has been linked to a detector molecule (e.g., enzyme); or

3b. Incubation of the analyte (in the test sample or reference standard) with the capture reagent to bind the analyte onto the solid surface, followed by the washing away of unbound material in the test sample and subsequent detection of the analyte (Figure 1, step 3a). Indirect detection occurs when the analyte is detected by the addition of a secondary enzyme-labeled reagent (*Figure 1*, step 3b); and

4) Quantification of the analyte by addition of a substrate suitable for the detector used [e.g., 3,3′,5,5′-tetramethylbenzidine (TMB)], followed by comparison of the test sample to the reference standard.

3. ASSAY DESIGN

Four general categories of ELISA are described in Table 1 and in the sections that follow. The assay designs are flexible and, depending on specific needs, can be modified from these procedures. The choice of format depends primarily on the amounts and purity of reagents and equipment available. On some occasions the analyte being characterized actually is an antibody, as in the case of a monoclonal antibody that is being developed as a drug. In this case, anti-idiotypic or other antibodies specific for the antibody are used to develop the assays.

4. Direct ELISA

A. Directly Labeled Antibody

In this assay an antigen is coated onto a solid surface and the remaining unbound reactive sites are blocked [*Figure 2* (A)]. Then a solution containing a specific antibody labeled with a detector is added. After incubation, the unbound antibody is washed away, followed by the addition of an appropriate substrate for the detector used.

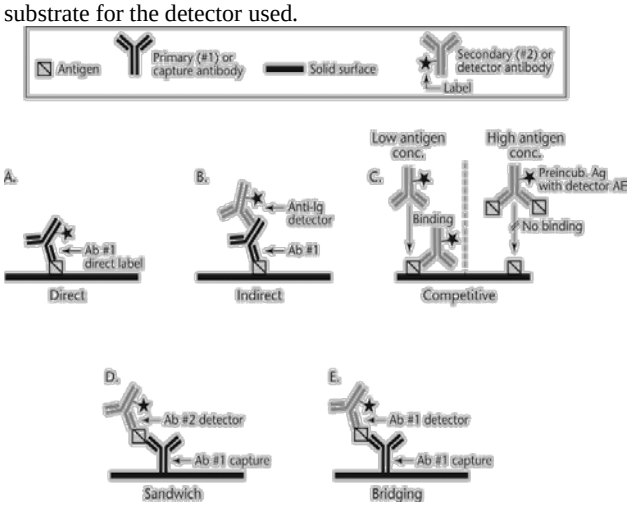


Figure 2. Schematic representations of direct, indirect, competitive, sandwich, and bridging ELISAs.[2] [Ab = antibody; Anti-Ig = anti-immunoglobulin; Ag = antigen (or analyte); Conc = concentration; Preincub = preincubation]

As shown in Figure 2, the type of ELISA method depends on the availability of reagents, the intended use of the analysis, and the physicochemical properties of the target analyte. In bridging ELISA, since the capture and detector antibodies recognize the same epitope, at least two epitopes must be present in the target antibody for binding to occur.

B. Directly Labeled Antigen

This assay is similar to that using a directly labeled antibody, except that the antibody is coated onto the solid surface and a labeled antigen is used as the detector.

5. Indirect ELISA

In this assay an antigen is coated onto a solid surface and then, after blocking, a solution containing a specific antibody is added [*Figure 2* (B)]. After incubation, the unbound antibody is washed away, followed by the addition of an anti-immunoglobulin (anti-Ig) detector antibody. Anti-Ig detectors are available commercially for specific Ig classes and subclasses from a variety of species, which makes this assay format useful for isotyping of antibodies. In addition, the use of a labeled anti-Ig detector amplifies the signal compared to a *Direct ELISA*, thereby increasing assay sensitivity.

Table 1. Representative ELISA Types

ELISA type	Required reagents	Attributes	Disadvantages
Direct detection	- Capture analyte[a] - Labeled primary antibody specific for antigen	- Rapid because only one antibody is used - Uses less reagent - Analyte is immobilized	- May modify the conformation of the analyte - Sensitive to matrix and adjuvant components - Not commonly used - Poor sensitivity
Indirect detection	- Capture analytes[a] - Primary antibody specific for antigen - Labeled secondary detector antibody that binds to the primary antibody	- Versatile because a variety of primary antibodies can be used with the same secondary detector - Improved sensitivity due to signal amplification - Analyte is immobilized	- Longer because of more incubation and washing steps
Competitive	- Analyte can be used as a capture reagent	- Good for assessing antigenic	- Format difficult to troubleshoot

ELISA type	Required reagents	Attributes	Disadvantages
	or can be labeled with a detection label - Antibody specific for analyte can be used for capture or labeled for detection - Labeled secondary antibodies to bind to primary antibody if an indirect format is used	cross- reactivity - Appropriate for smaller proteins with single epitopes - Requires only a single antibody - Analytes in solution competes for binding to primary antibody	- Limited dynamic range
Sandwich	- Primary capture antibody specific for analyte - Sample solution containing analyte - A different primary enzyme-antibody conjugate specific for analyte	- Improved sensitivity - Good for quantitative assays for larger multi-epitope molecules - Analyte measured in solution	- Requires relatively large amounts of pure or semipure specific antibody - Not suitable for smaller proteins that may have only a single epitope or a few closely spaced epitopes

[a] This reagent can be either purified or partially purified. The terms "analyte" and "antigen" are used interchangeably when describing ELISAs.

6. Competitive ELISA

A. Direct Antibody Competitive ELISA

This assay is used to detect or quantitate soluble antigens [*Figure 2* (C)].)]. It requires an antigen-specific antibody that has been conjugated to an appropriate detector, e.g., horseradish peroxidase (HRP), alkaline phosphatase (AP), ruthenium, or fluorescein. It also requires a purified or partially purified antigen for coating. The antigen is coated onto a solid surface, followed by a blocking step. The antibody–conjugate is incubated with the test solution containing soluble antigen. The mixture is then added to the immobilized antigen, incubated, and unbound antigen–antibody complex is washed away. Substrate is added, and the inhibition of the reaction (e.g., chromogenic assay, electrochemiluminescence, fluorescence, or chemiluminescence) is measured relative to the reaction when no competitor antigen is added. The amount of inhibition is inversely proportional to the amount of antigen in the test sample. Competitive assays can also measure small molecules by coating an antibody to the plate that is specific to the small molecule. The small molecule is often biotinylated with a long linker that does not interfere with binding between the capture antibody on the plate and the small molecule. Antigen (the small molecule) in the sample then competes with the labeled small molecule for binding to the capture antibody. After washing, a detection reagent (e.g., streptavidin labeled with HRP) is added to detect the binding complex.

B. Direct Antigen Competitive ELISA

This assay is similar to the *Direct Antibody Competitive ELISA*, except that it is used to detect soluble antibodies. The antigen is conjugated to the detector and the antibody is coated onto the solid surface.

C. Indirect Antibody Competitive ELISA

This assay is similar to the *Direct Antibody Competitive ELISA*, except that instead of directly labeling the antibody, the test uses a labeled anti-Ig reagent for detection.

D. Indirect Antigen Competitive ELISA

This assay is similar to the *Direct Antigen Competitive ELISA*, except that instead of directly labeling the antigen, the test uses a labeled secondary antibody for detection.

7. Sandwich ELISA

A. Direct Sandwich ELISA

In this assay an antibody is immobilized onto a solid surface and blocked, and then a solution containing a specific antigen is added [*Figure 2* (D)]. After an incubation step, the unbound material is washed away, and a labeled detector antibody is added. This assay format requires two antibodies, each of which binds to different epitopes on the surface of the large and complex molecule. The two antibodies are specific for the antigen, and the antigen should be sufficiently large and complex to accommodate the binding of two antibodies.

B. Indirect Sandwich ELISA

Alternatively, instead of directly labeling the detector antibody, an anti-Ig antibody detector can be used. Indirect sandwich immunoassay formats can be considered only if each binding reagent is from a unique species (e.g., a sandwich assay using two mouse monoclonal antibodies for capture and detection could not be detected indirectly because the resulting signal may become independent of the antigen concentration).

C. Bridging ELISA

This subset of *Sandwich ELISA* assays often uses a single antibody for both capture and detection [*Figure 2* (E)]. If a monoclonal antibody is used, it requires that the target antigen have at least two identical epitopes that are adequately spaced to prevent steric hindrance so that one epitope binds to the capture antibody and the other epitope binds to the detector antibody. Alternatively, a polyclonal antibody can be used but still requires that the target antigen be large enough to accommodate the binding of two antibody molecules. With respect to specificity and sensitivity, bridging assays usually are suitable for most large molecules.

8. CHOICE OF ASSAY

Deciding which ELISA procedure or format to use often depends on individual choice and availability of reagents, instruments, and other equipment. For example, sometimes a laboratory repeatedly engineers a particular epitope into multiple fusion proteins. In this case, the laboratory can use certain common qualified reagents (e.g., an antibody to a glutathione S-transferase region in multiple fusion proteins), facilitating rapid sandwich immunoassay development. Small antigens with a limited number of epitopes available for antibody binding restrict ELISA format choices. If there is only one binding epitope, then ELISA methods that use the sandwich/two-site binding or other bridging formats cannot be used because they require at least two available epitopes for antibody binding. In addition, small molecules are not usually used as a capture reagent on a plate because the process may interfere with binding to the detection reagent. Examples of such small molecules are some peptides, oligosaccharides, nucleotides, and antibacterials. Analysts usually adopt a competitive assay format for such small analytes.

Different assays and formats may demonstrate different properties and characteristics, e.g., specificity, precision, accuracy, sensitivity, dynamic range, dose-response ratio, sample throughput, sensitivity to interference, and simplicity or efficiency for automation. Ease of validation also may vary between different assay protocols and formats. Assay designs with replicates in adjacent wells could be biased if there are location effects; hence, in this case, replicates should not be in adjacent wells. Assay designs that are convenient to perform on 96-well plates, using relatively few single-channel pipet actions and more multi-channel pipet actions, are usually easier to adapt to automation. Assays with steep dose-response curves are generally better able to deliver high precision estimates; however, some assays with steep dose-response curves are imprecise in the EC_{50} and require a wider dose range.

9. Procedures

A. Solid phase

Solid phases are available in a variety of forms (e.g., membrane, plate, or bead) and chemistries [e.g., nylon, nitrocellulose, polyvinylidene fluoride (PVDF), polyvinyl, polystyrene, or a chemically derivatized surface]. The selection of the solid phase determines the most likely binding mechanism, i.e., hydrophobic, hydrophilic, or covalent interactions. In general, compared to plates, beads offer higher capacity and are more commonly used in clinical assays whereas plates are more commonly used to test biotechnology products. Additional information on plates is provided below.

(1) coating the solid phase—immobilization of capture reagent

Capture reagents are coated onto a solid phase by adding a solution containing the capture reagent to the surface. The most commonly used solid-phase materials for capture reagent immobilization are plastic 96-well microtiter plates. Those with flat-bottom wells are recommended for spectrophotometric readings, and round-bottom well plates are useful for visual assessment of a dye's color development. The degree of coating is influenced by the concentration of capture reagent, temperature during coating, duration of capture reagent adsorption, the surface properties of the solid-phase material, and the nature of the buffer solution of the capture reagent solution. Although the optimum coating concentration must be determined for each capture reagent, concentrations of 1–10 µg/mL are most commonly used. The volume of capture reagent added to each well usually corresponds to the sample volume that will be analyzed, i.e., 50–100 µL. During the coating procedure analysts should avoid introducing bubbles. Proteins that bind to plastic can be denatured, which alters antigenicity. In such cases, a capture antibody or an intermediary protein such as Protein A or Protein G can be used. In addition, streptavidin can be used if the reagent is biotinylated. The pH of the coating buffer solution should be optimized based on the isoelectric point of the capture reagent and the surface properties of the assay plate chosen.

(2) Microtiter plates

The composition and commercial source of the microtiter plate can influence binding of the capture reagent during coating. Several microtiter plates from different suppliers should be compared using a single coating procedure to select those that provide high specificity for the capture reagent of interest and low nonspecific background. Comparisons of different grades of plates from a single supplier also may be needed. Clear plates typically are used for colorimetric ELISA, and opaque plates often are used for chemiluminescent and fluorometric ELISA. Acidic capture reagents may require a lower pH solution to neutralize repulsive forces between the protein and solid phase. Peptides often require to optimize the pH of buffer solution based on their charge for optimal coating conditions during assay development. Polysaccharides, lipopolysaccharides, or glycoproteins may be difficult to coat directly to the plate and may require a capture antibody or a buffer solution that contains lysine or glutaraldehyde. Coating with an antibody can be enhanced by precoating the microtiter plate with Protein A or Protein G or a combination of the two, which allows binding to the Fc region so that the Fab portion can bind to the analyte of interest. However, care must be taken to ensure that subsequent secondary antibodies do not react with the Protein A- or Protein G-coated wells. In this case, for example, chicken IgY or another appropriate antibody class could be used. Microtiter plate formats other than the 96-well variety, such as half-volume 96-well or 384-well plates, can be used to increase throughput and/or conserve reagents.

(3) Coating time

Coating time depends on binding kinetics, stability, concentration of capture reagent, and incubation temperature. Although different combinations of coating times and temperatures often result in the same coating efficiency, the stability of the capture reagent (which should be determined during method development) influences which conditions to select. Analysts must assess the impact of varying the coating time in order to determine the robustness of the assay procedure.

(4) Coating temperature

Coating temperature and time are closely related assay parameters. The coating temperature depends on the binding kinetics and stability of the antigen. Higher temperatures can increase the rate of adsorption and may shorten the coating time, but they are likely to affect interaction sites and to reduce antigen-antibody affinity. Typical combinations of time and temperature are 1–4 h at ambient temperature, 15 min to 2 h at 37°, or overnight at 4°. Analysts should determine the effects of variations in temperature in order to assess the robustness of the assay procedure.

(5) Buffer solutions

Buffer solutions used for diluents, coating, blocking, and washing plates can affect overall assay performance. Components of buffer solutions can interact with the test sample and inhibit binding. They also can cause low antigen sensitivity or high nonspecific background activity.

(A) Diluent

Buffer solutions [e.g., phosphate-buffered saline (PBS) or imidazole-buffered saline] with polysorbate 20 (0.01%–0.1%) are used commonly for different ELISA steps as a diluent and washing buffer solution.

(B) Coating buffer solutions

Coating buffer solutions should maximize assay consistency and promote binding of the capture reagent to the solid phase. Commonly used coating buffer solutions include 50 mM carbonate, pH 9.6; 20 mM tris-hydrochloride (tris-HCl), pH 8.5; and 10 mM PBS, pH 7.2. The choice of coating buffer solution depends on the nature of the individual antigens and should be determined empirically.

(C) Blocking reagents and buffer solutions

A blocking reagent is a compound (e.g., protein or

detergent) that should saturate the remaining immunosorbent binding sites following capture reagent (antibody or antigen) binding. This reduces nonspecific binding of analyte and nonanalyte components to the immunosorbent matrix and/or the absorbed reagent. Nonspecific binding occurs when protein in the test sample binds to the plastic of the microtiter plate or absorbed reagent instead of specifically binding to the capture reagent of interest. Nonspecific binding can be reduced by adding blocking reagent to the wells and by the addition of another protein such as bovine serum albumin (BSA) to the diluted buffer solution. The choice of blocking reagent should be governed by the nature of the capture reagent, plate, coating buffer solution, test sample diluent, and related factors. If any of these parameters changes, a change in blocking reagent may be needed. Commonly used blocking reagents include BSA, nonfat milk, gelatin, casein, normal horse serum, fetal bovine serum, polysorbate 20, and others. Several grades of BSA are available commercially, and the optimal grade should be empirically determined for each assay. In addition, many commercial blocking and assay diluent reagents are available for ITM.

B. Adding Samples and Reagents

Samples and reagents generally are pipetted into the ELISA plate wells. Care should be taken to avoid cross-contamination, frothing, or bubbles. A sample loading pattern should be included in the test method procedure. For reproducibility and accuracy of results, consistency between the wells of the ELISA plate is very important. This can be achieved by using replicates; however, as mentioned above, care should be taken to avoid replicates in adjacent wells. A common way of avoiding the edge effect is not to use the edge wells at all. Additionally, plate edge effects can also be avoided by reducing assay time, using a low evaporation lid, or sealing the plate with a clear or breathable sterile tape.

Labor-saving equipment such as electronic pipets, automated liquid handlers, plate washers, and robotic pipets also can be used to improve precision, reduce analyst-to-analyst variability, and increase throughput.

C. Washing

Wash steps are included throughout the ELISA procedure to remove the unbound coating antigen, sample, and detection reagents. Washing is critical for assay performance, can be a source of assay failure, and is important to evaluate during method development. Multiple approaches can be used for washing. Manual procedures include using a squeeze bottle, dipping the microtiter plate in washing buffer solution, and adding washing buffer solution with a multi-channel pipet or hand-held multi-channel (8- or 12-pin) manifolds. Analysts should wash carefully to avoid cross-well contamination. Automatic microplate washers generally provide more washing consistency. Strip-well and multi-well washers are available. Most automatic washers can be programmed for different dispensing volumes and speeds, number of washes, speed of buffer aspiration, and amount of residual buffer solution left in the well. Incorrectly programmed or maintained, as well as incompletely cleaned, automatic washers can cause assay variation and elevated assay background.

D. Incubation

ELISAs are incubated following the addition of samples and reagents. The optimal time, conditions, and temperature of each incubation step should be determined during method development. Incubation times vary from minutes to overnight. Commonly used incubation temperatures are ambient temperature, 4°, and 37°. ELISA plates commonly are sealed or placed in a secondary container to avoid evaporation or contamination during incubation. Atmospheric conditions such as dry or humidified incubation should be evaluated during method development. Rocking, shaking, or rotating the microtiter plates may be necessary or desirable depending on the kinetics of binding.

E. Blocking Conditions and Non-specific Reactions

After immobilization and removal of the unbound antigen or antibody, unoccupied binding sites are blocked to ensure that the measured analyte in the test article or subsequent (detection) reagents does not bind nonspecifically to the solid surface or to the coated antigen or antibody. If nonspecific binding occurs, any reported signal could bias the measurement and may reduce the sensitivity and dynamic range of the assay. Blocking is critical to ensure the sensitivity and/or specificity of the assay. Sources of nonspecific binding fall into two general categories:

1) Ionic or hydrophobic interactions occur when binding is mediated by nonspecific ionic or hydrophobic interactions between assay reagents and the solid surface or another assay reagent.

2) Immunological interactions occur when binding is mediated by unintended antigen–antibody interactions. This occurs when antibody preparations used in the assay interact with other assay reagents. For example, if an ELISA was designed to test a serum-derived analyte using murine capture and detection antibodies, antibodies in the test article with reactivity to murine Ig (also known as heterophilic antibodies) could be nonspecifically detected in the assay.

The choice of blocking reagent (examples are found in *Blocking reagents and buffer solutions*) is determined empirically, and the balance between the reduction in nonspecific binding and the impact on assay sensitivity should be assessed during method development. Cross-reactivity with other assay reagents should be considered; for example, endogenous biotin is found in milk and serum, and serum may contain antibody to viral or bacterial proteins. Therefore, screening of serum lots may be necessary. The volume of blocking solution added to the well should be greater than the maximum reaction volume used for later steps so that all of the potential surface area that may interfere with the binding reaction is blocked.

In addition, Ig in the test materials can be removed by using buffer solutions that inhibit antibody conformation or aggregate the heterophilic antibodies, by blocking with nonimmune serum, or by removing Fc regions in critical antibody reagents, thereby reducing or eliminating undesired immunological interactions that cannot be addressed by the blocking reagents described above. Negative control wells can be included to monitor nonspecific reactions. The nature of the negative control wells depends on the assay but can include blocked wells without coating antigen, eliminating the primary or secondary antibody, or using buffer solution in place of sample. Control wells also can be useful as part of system suitability testing.

F. Pretreatment of Samples

Although ELISA methods are designed to measure an analyte in complex mixtures, the presence of other materials can prove problematic if they interfere with analyte detection. In order to ensure assay specificity, the specific procedure to treat samples to remove nonspecific interfering substances (e.g., reducing agents or precipitates) can be determined empirically during method development and then can be incorporated into the validated assay. Any sample-processing step should be evaluated against the potential that the treatment will alter the test article's properties and/or introduce further variability that results

in biased measurements. Samples, reference materials, and controls should be prepared and handled in processes as similar to each other as possible. Analysts should verify that sample pretreatments have not damaged the sample so much that it can no longer be measured (e.g., by spiking experiments).

G. Detector Antibodies

Depending on the ELISA format, detector antibodies labeled with enzyme or other labels can be used as primary or secondary reagents to enable detection of the immobilized analyte. In a direct or competitive ELISA [*Figure 2* (A and C)], after the analyte is bound to the immunosorbent surface, excess analyte is washed away and the immobilized analyte is detected using a detector antibody that is considered to be the primary antibody. In other ELISA formats [*Figure 2* (B, D, and E)], the analyte-specific Ig (nonconjugated primary antibody) is allowed to bind to the immobilized analyte, and any excess antibody is washed away before the addition of a detector antibody, which is termed the secondary antibody.

To facilitate detection, in all ELISA formats that use enzyme-conjugated antibodies, a substrate specific for the conjugated enzyme is introduced into the assay system. An enzymatic reaction ensues, converting a substrate into a soluble product that can be measured using appropriate wavelengths and a suitable reader.

ELISA sensitivity depends on the quality of the reagents and the detection system, including the label and substrate. If multiple differently conjugated antibodies are available, analysts should select one appropriate for the assay. During this evaluation, the dilution of each conjugate that yields desirable sensitivity and specificity should be determined using appropriate control groups.

The most commonly used labeling enzymes for conjugating to antibodies include AP, HRP, and galactosidase. These enzymes are highly specific, sensitive, and stable in catalyzing chromogenic, luminescent, or fluorescent reactions. *para*-Nitrophenyl phosphate (pNPP) is a commonly used substrate for AP. Commonly used substrates for HRP include TMB, *o*-phenylenediamine dihydrochloride (OPD), and [2,2′-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt (ABTS)] (see *Table 2*). The substrates for AP and HRP are chromogenic and result in the formation of a colorimetric product that can be measured using a spectrophotometer. Chemiluminescent and fluorescent substrates for AP and HRP also are available, and in many cases they are available as commercial kits. Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate (CSPD) is a known chemiluminescent substrate for AP (see *Table 2*). Well-known fluorescent substrates for galactosidase include 4-methylumbelliferyl galactoside (MG) and nitrophenyl galactoside (NG). If a chemiluminescent substrate is used, then a luminometer is required to quantitate the formed product. A fluorometer is needed if a fluorescent substrate is used in the ELISA.

Table 2 also provides a summary of the advantages and disadvantages of different types of ELISA substrates. Colorimetric substrates have been prevalent since the origin of ELISAs and may yield robust assays that generally are more cost efficient than assays that use chemiluminescent and fluorescent substrates. Nevertheless, chemiluminescent and fluorescent ELISA methods may yield more rapid and sensitive assays with a wider dynamic range than assays that use a colorimetric readout. The final choice of readout should be governed by the assay's purpose and the requirements of the assay.

ASSAY DEVELOPMENT AND VALIDATION PLAN

1. Critical Reagent Development

Key considerations for critical reagents are source, purity, specificity, and stability. For quality measurements, ITMs use reference standards along with critical reagents for analyte capture and detection. Any changes of critical biological reagents should be evaluated.

A. Source

The availability and quality of the starting material should be controlled so that manufacturing of the (purified) reagent can be reproducibly and consistently performed, potentially over several decades. Because critical reagents are biological molecules, sources can range from chemical synthesis (e.g., peptides) to complex biological matrices (e.g., antibodies prepared from serum, monoclonal antibody from ascites/cell culture, or fermentation/cell culture products). When appropriate for the intended use of the assay, a single lot of a critical reagent can be manufactured to establish a substantial supply and to prevent lot-to-lot variability. In other instances, it may be appropriate to include in the validation multiple lots or multiple suppliers in order to demonstrate that the assay is sufficiently robust for its intended use.

B. Purity

In general, the purity of critical reagents should be assessed to ensure the removal of impurities and manufacturing process residuals that can influence reagent performance and/or stability.

C. Specificity

The specificity of a critical reagent refers to its ability to capture or detect only the analyte of interest. The reagent must be specific to the analyte and should show little nonspecific link or no cross-link to off-target molecules in complex test materials.

Table 2. Enzyme conjugates and substrates

Readout	Principle of the Enzymatic Reaction	Enzyme	Substrate	Reader	Advantages	Disadvantages
Colorimetric	Produces a colored product that yields absorbance values directly proportional to analyte concentration	AP[a], HRP[b]	pNPP[c] TMB[d] OPD[e] ABTS[f]	Spectrophotometer	• Robust • Economical • Reagent availability	• Less sensitive
Chemiluminescent	Produces a light emission that is directly proportional to analyte concentration	AP	CSPD[g]	Luminometer	• Wide assay dynamic range • Lower coating concentrations • More sensitive • Rapid signal generation	• Requires special plates • Costly
Fluorescent	Produces excitation-	Galactosidase	MG[h]	Fluorometer	• Rapid	• Requires

Readout	Principle of the Enzymatic Reaction	Enzyme	Substrate	Reader	Advantages	Disadvantages
	induced light emission that is directly proportional to analyte concentration		NG[i]		• Sensitive	special plates • Costly • Interference by excipients

[a] AP: alkaline phosphatase
[b] HRP: horseradish peroxidase
[c] pNPP: para-nitrophenyl phosphate
[d] TMB: 3,3',5,5'-tetramethylbenzidine
[e] OPD: o-phenylenediamine dihydrochloride
[f] ABTS: 2-2'-azino-bis[3-ethyl-benzothiazoline-6-sulfonic acid]diammonium salt
[g] CSPD: Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate
[h] MG: 4-methylumbelliferyl galactoside
[I] NG: nitrophenyl galactoside

D. Stability

The stability of critical reagents should be empirically determined to ensure assay performance over time (issues include accuracy, precision, reproducibility, and assay drift). Long-term (months to years) stability of critical reagents under required storage conditions (e.g., with defined temperatures and containers) should be determined so that appropriate expiry dating can be assigned. Short-term (minutes to days) stability (and freeze/thaw and room temperature stability for frozen critical reagents) also is required to ensure day-to-day assay accuracy, precision, and reproducibility.

2. Feasibility/Pilot Studies

The steps of the process by which an ELISA method is developed, validated, and used in routine sample analysis are described below:

A. Generate or purchase critical reagents to measure the analyte. Determine storage conditions and stability.
B. Understand the performance goals for the assay system.
C. Develop the assay to the point that there is a detectable concentration response curve.
D. Perform method development/robustness testing.
E. Prepare the reference/calibration standard and control and assess stability.
F. Establish assay procedures, appropriate controls and limits, assay and sample acceptance criteria, and instrumentation.
G. Determine method performance, and qualify method for accuracy, specificity, precision, and robustness, including qualification of all applicable sample types to be analyzed.
H. Validate the assay.
I. Implement the method (technology transfer) in the testing laboratory, including training and qualification of analysts.
J. Monitor assay performance.

During assay development, the critical parameters and reagents that are required for the assay should be assessed and set at levels that yield desired assay performance. In many instances several parameters may be evaluated, and well-designed experiments can accelerate assay development, particularly for assessing the potential interaction of several inputs.

Many ELISA procedures are product specific, and external reference/calibration standards may not be available. The preparation and stability of reference/calibration standards should be considered early in assay development.

3. Assay Validation

Assay validation is executed to demonstrate that the particular test used for an analyte is appropriate for its intended use.

Data Analysis

The analysis of ELISA data can be simple (e.g., a linear calibration with inverse regression) or complex (e.g., a nonlinear calibration curve with inverse regression). The type and rigor of data analysis depend largely on the assay system and the intended uses of the assay. For example, data reduction may estimate a concentration (e.g., ng/mL) of an unknown sample using a calibration curve. Other approaches include estimation of the half-maximal inhibitory concentration (IC_{50}) or effective concentration (EC_{50}), estimation of the amount of a sample that yields the same response as the EC_{50} (or IC_{50}) on a standard curve, and an estimate of the relative activity of a test sample compared to a reference/calibration standard.

In general, ELISA curves are characterized by a nonlinear relationship between the concentration of the analyte of interest and the calculated mean response. Typically, this response curve is defined by a sigmoidal relationship of response to concentration. A wide range of mathematical models can fit standard/calibration curves, and analysts should take care in the selection of an appropriate curve-fitting algorithm. In other cases, ELISA assays are used for qualitative purposes to determine whether a sample is positive or negative based on a sensitivity threshold.

Flow Cytometry
유세포 분석법

Flow cytometry is an analytical method that plays a critical role in the quantitative and qualitative assessment of cell populations in patient and cellular product samples. The power of flow cytometry lies in its ability to rapidly and reliably analyze multiple attributes of individual cells within heterogeneous cell populations.

Principles of Operation, Methods, Quality, and Standardization

The process of flow cytometry requires cells to move past a fixed light source consisting of one or more lasers so that individual cells can be observed or classified by characteristics such as size, particle size, and the presence of antigens or molecules on the cell membrane surface or within the cell.

The cells are suspended in fluid in which movement is controlled by the size and configuration of tubing, chambers, and pumps specific to the flow cytometry instrument. The pattern of light signals produced from the laser light's interaction with the cells is captured by a detection system, also specific to the instrument, and the detected signals are transformed into data elements that can be analyzed and combined with data from other cells in a given sample. Data from a cell suspension can then be expressed and presented in one-, two-, or three-dimensional visual formats, or in numerical formats, to characterize the cellular sample and its subpopulations both qualitatively and quantitatively.

1. Flow Cytometry Instrumentation

A. Fluidics

The fluidics system moves a bulk mixture of cells so that a stream of single cells is formed. Within the flow cytometer, the single-cell suspension passes through a confined region where each cell is sequentially illuminated by a uniform light source at the observation point (interrogation point). Most instruments use a flow chamber (flow cell) that, after the cell sample is drawn into the sample injection tip, combines the cells with isotonic sheath fluid, using a conical nozzle assembly that is geometrically designed to produce a laminar flow of fluid (Figure 1). The fluid outlet nozzle typically has an orifice of 50–250 µm in diameter through which fluid exits at a high flow rate. Differential pressures between the sample stream of cells (lower pressure) and the sheath stream (higher pressure) draw the cells/particles out into a confined stream. The resulting coaxial stream within a stream is highly efficient, and the sample stream at the observation point is typically only slightly larger than the cells or particles contained within.

At least one manufacturer uses an alternative approach in which the coaxial stream strategy is replaced by the use of microcapillaries to focus and direct the cells. The light signal deflected or emitted by the cell is then measured and analyzed.

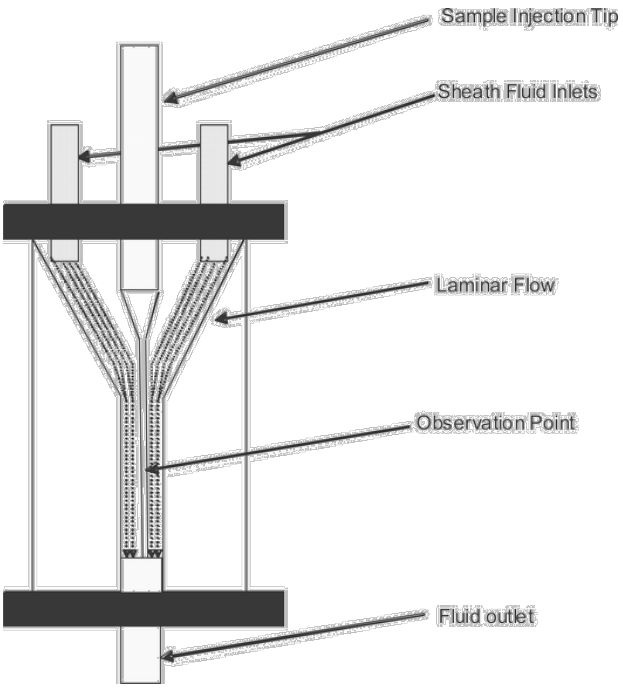


Figure 1. Schematic diagram of a flow cell

B. Optics

When cells are stained with fluorescent dyes or with fluorescent-labeled antibody reagents, light emitted from the laser interacts with the fluorescent dye to generate stimulated emission that has coherent (parallel) light of uniform wavelength, phase, and polarization. The fluorescent light signals generated by the interaction of the laser light with the cells are collected by a series of detectors oriented at 90° in a straight line to the incoming laser beam. The most common commercially available flow cytometer lasers (with the corresponding wavelengths) are the blue argon laser (488 nm), the red diode laser (635 nm), and the violet laser (405 nm).

C. Electronic Signal Processing and Data Output

When a cell passes through the optical system of a flow cytometer, the light-scattering patterns or fluorescence from any fluorochrome on or in the cell are detected by various types of photodetectors or photomultiplier tubes (PMT) that transform the information about the characteristics of the cell into a computerized readout. Each analyzed cell generates an event in each parameter (forward scatter, side scatter, fluorescence) for which it is measured. Figure 2 shows an example of a typical two-color flow cytometer configuration. Different cell types have distinctive sets of signals in the various parameters. For example, when the cell passes through a beam of light, the light deflected in the forward direction (usually about 20° from the forward direction of the laser) is called forward scatter and is collected by a detector known as the forward scatter channel (FSC). The amount of deflection in the FSC is proportional to cell size. Light deflected at a 90° angle is known as side scatter and is collected by the side scatter channel (SSC). This provides a measure of the cell's structural complexity caused by granules, membrane roughness, or nucleus, all of which are associated with higher SSC. The light deflected by other PMTs using a specific band-pass filter is collected by specific fluorescence channels (FL1 and FL2 in Figure 2). The electrical pulses, originating from light detected by the PMTs, are processed by a series of linear and log amplifiers. Logarithmic amplification is often used to measure fluorescence in cells. Figures 3-7 show histograms for cells stained with antibodies conjugated with specific fluorochromes (see Table 1). The antibodies are specific to some of the cluster of differentiation (CD) markers discussed in immunophenotyping (see below).

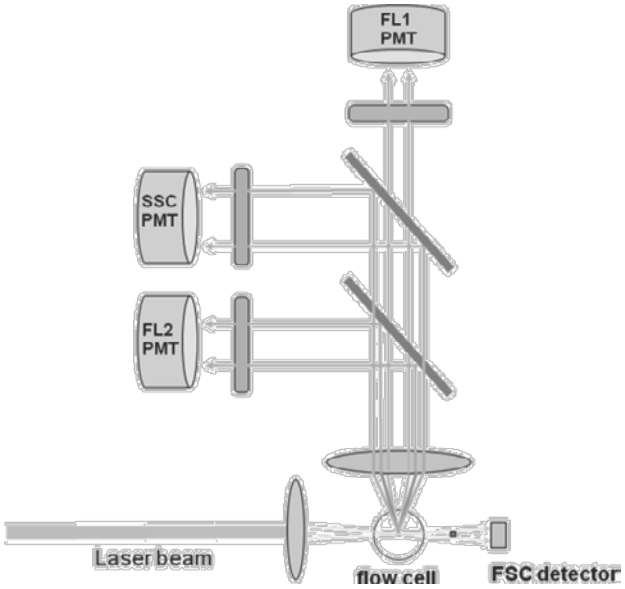


Figure 2. Typical 2-color flow cytometer with detectors for FSC, SSC, and fluorescence
(FL: Fluorescence, PMT: photomultiplier tube)

Table 1. Fluorochromes Commonly Used in Flow Cytometry

Fluorochrome	Typical Excitation Laser (nm)	Emission Peak (nm)
Cascade Blue	375; 401	423
Pacific Blue	403	455
R-Phycoerythrin (R-PE)	480; 565	578
PE-Cy5 conjugates	480; 565; 650	670
PE-Cy7 conjugates	480; 565; 743	767
Red 613 (Texas Red)	480; 565	613
Peridinin Chlorophyll (PerCP)	490	675
Fluorescein (FITC)	495	519
Allophycocyanin (APC)	650	660
APC-Cy7 conjugates	650; 755	767

The versatility of flow cytometry comes from the ability to attach fluorescent tags to the cell's surface, cytoplasm, or nucleus or to products of the cell. Fluorescent markers attached to the cell can be excited by lasers to emit light of specific wavelengths, and this light is then detected and analyzed in the manner described above. The type and amount of fluorescence detected provide both quantitative and qualitative information about the cell.

The photodetectors convert light into an analyzable output by generating a small current of which the voltage has amplitude proportional to the amount of light. The voltage is amplified and converted into electrical signals large enough to be plotted by the

computer in several different ways. Thus, the FSC, SSC, and fluorescent detectors collect the light and convert it into electrical signals that can be analyzed by the computer. In this way, the signals coming from each photodetector can be measured for their intensity (low to high) and sorted into channels. The channels are arranged as a continuum so that a cell population with many large cells will have many events in the higher channels, and one with many small cells will have many events in the lower channels.

D. Data Analysis

Data output from the flow cytometer can be represented in several ways, the most basic of which is the single-parameter histogram (Figure 3), in which events with similar intensity of light (forward scatter, side scatter, or fluorescence) are collected in channels and then plotted. This plot demonstrates the number of cells with similar optical characteristics. Figure 4 is an example of graphs that display two measurement parameters, one on the *x*-axis and one on the *y*-axis, and the cell count as a density (dot) plot or contour map. The parameters could be SSC, FSC, or fluorescence. These parameters can be collected in one channel.

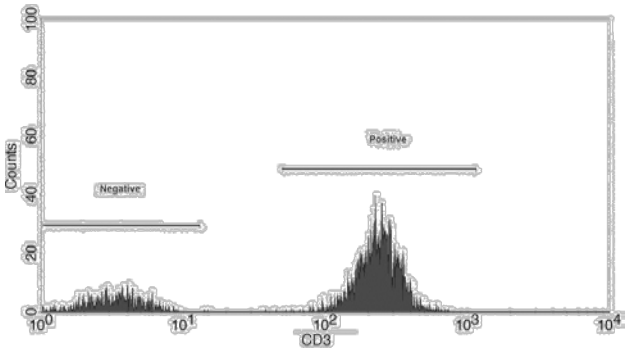


Figure 3. Single-parameter histogram showing expression of the cellular antigen CD3 in a mixture of cells.

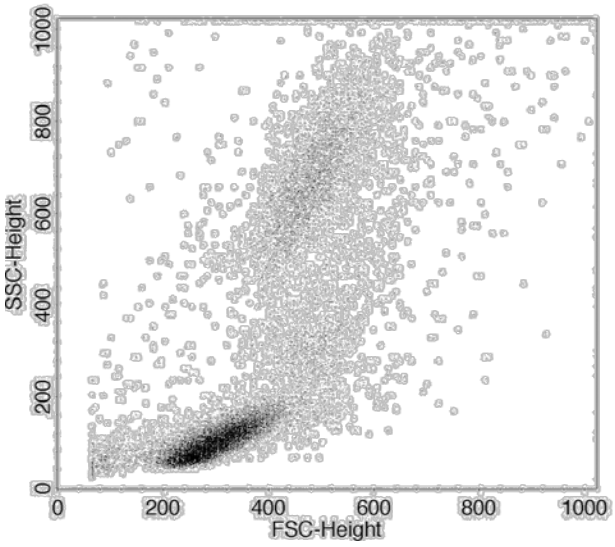


Figure 4. Bivariate dot plot of cells displayed by FSC and SSC.

A dot plot displays a dot for each cell, while density plots and contour plots show a heat map or a topographical linear map, respectively, based on the relative number of cells in each channel. The forward versus side scatter histogram is the most common method of identifying different hematopoietic cell types. Figure 5 shows a contour plot that is a 3-dimensional representation of the relative number of cells in the various channels.

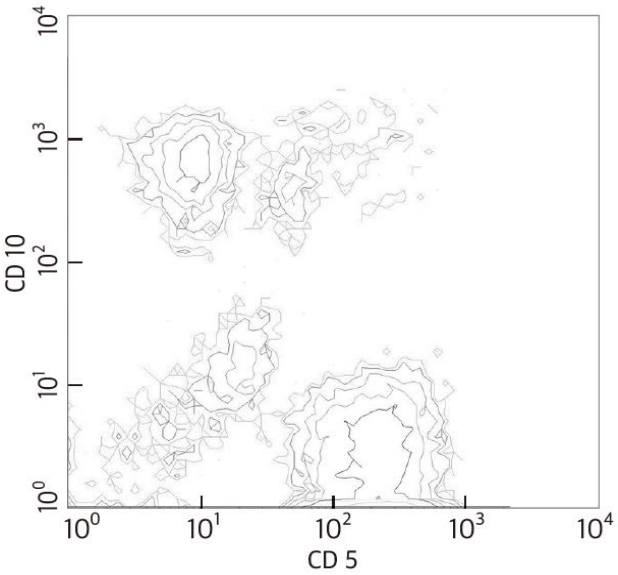


Figure 5. Bivariate contour plot showing relative numbers of cells in each channel that expresses both CD markers (CD5 and CD10)

When cells are stained with antibodies against different epitopes carrying two different fluorochromes, the data are presented as a plot of the two parameters plotted against each other. Cursors can be set on each axis to separate positive populations from negative populations for each of the attributes. This results in a graphical representation of cells that are positive for both markers, negative for both markers and positive for only one of the two markers (Figure 6).

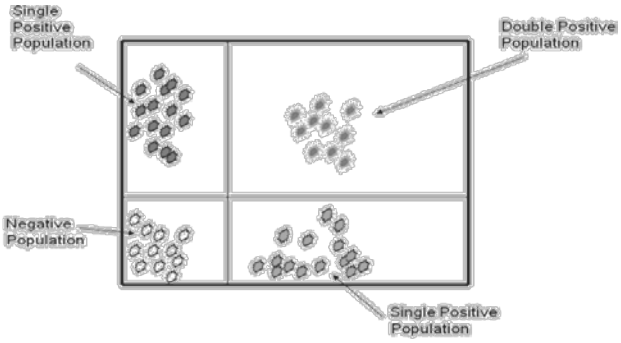


Figure 6. Schematic presentation of a 2-parameter histogram

The flow cytometer allows the user to set positive and negative limits for each marker. Flow cytometric data is collected in list mode, where each electronic signal from individual cells is displayed in the order in which it was collected. List mode files can be edited at a later time to include or exclude any event. A fundamental advantage of flow cytometry is its ability to separate data on cells of interest from both background and dead cells (e.g., non-cellular particles or debris) when dealing with forward and side scatter and cells of other populations. The user must decide which signals are the actual light outputs from the cells and must construct an electronic gate to allow the computer to

count as positive only the events that fall within the gate. Cell populations can vary greatly depending on the tissue or cell source and the characteristics of the flow cytometer used. Gating allows the user to determine which outputs to consider actual events, so this process is of prime importance in standardizing flow cytometry data (Figure 7).

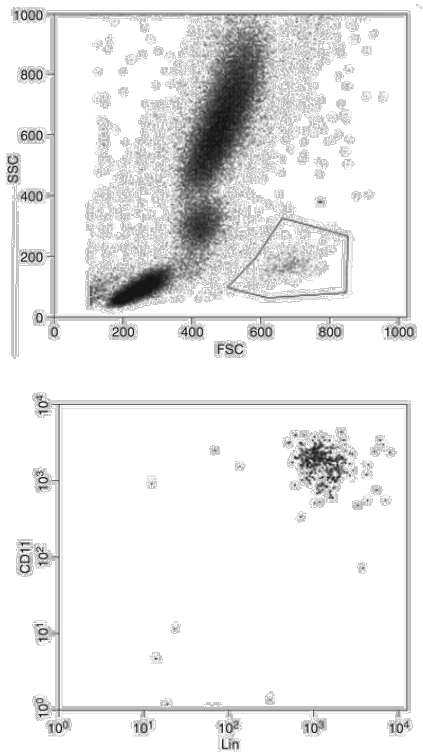


Figure 7. Gating of a cell population with low side scatter and high forward scatter, which is distinct from other cell populations in the sample.

A technique known as compensation can be used to separate spectral overlap of fluorochromes that have similar emission wavelengths. For analog-style flow cytometers manufactured before the late 1990s, compensation must be set before data acquisition. In modern digital instruments, compensation can be set either before or after data acquisition. The adjustment of compensation can be more of an art than a science, and considerable literature has been focused on the relative merits of various methods to determine the correct compensation for cell types or experimental conditions. The analyst should have considerable knowledge of the cell type under analysis in order to prevent errors in phenotyping that can result from improper adjustment of compensation.

The number of events counted should be adequate for statistical confidence in the results. The instrument can be set so that data are collected until a certain number of events in a channel have been measured. This feature allows the operator to vary the length of time or number of events from the sample so that statistically reliable data are generated.

2. Flow Cytometry: Element of Procedure

A. Sample Handling and Staining

(1) Sample collection, handling, and anticoagulation

To perform an accurate analysis of the cell-based drug product, the analyst should ensure that samples extracted from the drug are representative of the entire product as much as possible. Blood, apheresis samples, and cell suspension products should be mixed well before sampling, and care should be taken to obtain an adequate sample volume.

Cell samples containing human blood/plasma must be anticoagulated. Citrate-based anticoagulants (e.g., anticoagulant citrate dextrose solution A) or heparin are recommended rather than ethylenediaminetetraacetic acid (EDTA) because they will optimally preserve samples being held for more than a few hours. For long-term stored samples, special transport/storage media may be required, and validation studies should be performed to ensure that these samples are equivalent to fresh samples when analyzed by flow cytometor.

Samples intended for whole blood lysis and surface antigen staining should be transported and stored, preferably on a slow oscillating mixer at room temperature. Fixed or live cell samples should be stored at 4 °C. If samples are exposed to high temperatures, temperature-regulating materials (room temperature packs, wet ice/cold packs, and insulation) may be required. Validation studies should also be performed to ensure the integrity of the samples. Temperature monitoring equipment may be required during the transport of important or high-value samples.

After acquisition, specimens should be analyzed as soon as possible. Special attention should be paid to situations where cellular proliferation and metabolic depletion of energy sources in the transport/storage media may lead to apoptosis. When accurate counting is not required or if infectious agents are suspected, a commercial lyse/fix solution may extend storage time and reduce the risk of disease transmission. For specimens separated by density-gradient centrifugation, storage in a solution of buffered paraformaldehyde (0.1% - 2.0%) is recommended after cell labeling has occurred.

(2) Sample processing, staining, and fixation

Reagents used in processing, staining, and fixing samples should be qualified for their intended use. Follow the manufacturer's instructions for sample processing when using reagent kits.

Sample processing that involves centrifugation, washing, red blood cell (RBC) removal or lysis, or density gradient segregation is commonly performed in many flow cytometric analyses but can introduce errors and artifacts. Several techniques and reagents are available for red blood cell removal and lysis. Clinical grade [In vitro diagnostics (IVD) or analyte-specific reagents (ASR) reagent] reagents are recommended for optimal quality, but artifacts can still occur. Density-gradient centrifugation may introduce errors related to the loss of viable cells among the subpopulations being measured. These sources of error and artifacts can be avoided by analyzing live whole blood whenever possible.

Most whole blood lysate instructions recommend staining at room temperature and in the dark. Many methods include a dilute fixative to prevent capping and internalization of fluorochrome. In contrast, cell preparations (density-gradient cell preparations, apheresis specimens, tissue culture) should be stained at 4 °C, washed with cold buffer solution, and stored cold until analyzed.

Fixation that also preserves cell surface antigens can be accomplished using commercial leukocyte preservatives or with buffered formaldehyde or paraformaldehyde. Very little validation of storage times, antibody binding, or fluorochrome intensity has been reported. Any laboratory that considers batch analysis of fixed specimens should validate these techniques thoroughly before implementing.

B. Use and choice of Fluorochromes

(1) Fluorochromes

Fluorochromes are used to directly stain cells or as agents bound to antibodies or other reagents to stain cellular antigens or other structures. Table 1 provides examples of common fluorochromes used in flow cytometry and their excitation and emission wavelengths. The wavelengths (nm) may vary slightly depending on the environment. Synthetic probes from certain manufacturers may also be used. Fluorochromes must match the spectral range for the lasers and the filter sets specific to the user's flow cytometer.

When choosing fluorochromes for multicolor phenotyping, the operator should refer to established methods for the particular instrument. In general, the brightest fluorochromes should be matched with the antigens that are expected to have the lowest expression on the cell surface. The brightness of tandem dyes can also be reduced by the use of certain fixatives, some of which are less problematic than others. When designing a multicolor flow and tandem dye procedure that has not been previously validated, the operator should consult the manufacturer's technical service, compare tandem dye/fixative combinations, and validate the final fluorochrome combination to ensure sample-to-sample consistency.

Tandem fluorescent dyes are dual-conjugated fluorescent molecules. When the two labels are in close proximity, energy produced by the laser exciting the donor fluor is transferred to the acceptor fluor, releasing a photon at the emission wavelength of the acceptor fluor (also known as fluorescence resonance energy transfer, or FRET). For example, PECy5 will excite at the excitation wavelength for PE (565 nm), transfer energy to Cy5, and emit at the emission wavelength for Cy5 (670 nm).

(2) Fluorescently labeled antibodies

Most commercially available antibody reagents are monoclonal, but polyclonal reagents may be available, and desirable, for some applications. The quality and specificity of an antibody can vary widely. Antibodies directed at a given antigen may differ in their binding specificity for different antigenic epitopes or in the strength of binding to the same epitope. If possible, use directly conjugated fluorochrome–antibody combinations that are IVD or ASR grade. Optimization of antibody concentration for the desired cell population is protocol specific but is generally accomplished by using increasing concentrations of antibody with a fixed number of cells to bracket the optimal brightness between autofluorescence and quenching. Quenching is caused by the prozone phenomenon, which occurs when excess antibody leads to immunoprecipitation and loss of fluorescence intensity.

(3) Cell Surface Antigen Staining

Techniques for staining surface antigens vary depending on the type of sample. Whole blood lysis techniques typically require surface labeling at room temperature in the dark for 15 - 30 minutes, followed by red blood cell lysis and, if desired, fixation. Generally, lysis of whole blood or bone marrow samples with ammonium chloride (NH_4CI) is used, followed by washing prior to antibody labeling for immunophenotyping of leukemia. Live stained mononuclear cells or cultured cell samples should be stored at 4 °C or in an azide-containing buffer solution to prevent capping and internalization of antibodies.

(4) Intracellular staining

Several standardized procedures are also available for labeling intracellular antigens and cytokines. The operator should consult the manufacturer's protocol and standardized reagents for these procedures. Because permeabilizing reagents vary among procedures and manufacturers, do not mix and match reagents. For cytokine labeling, it is often necessary to use an activation step and a Golgi block to allow sufficient cytokine to accumulate for detection.

(5) Quantitation of antigens

Some applications require quantitation of the average number and density of antigen molecules per cell in order to give a more complete picture of the immunological behavior of cells (e.g., in studies where extracellular antigens are expressed differentially in relation to activating stimuli). The intensity of an antibody/fluorochrome labeled cell preparation is compared to the intensity of a set of microbead fluorescence standards collected at the same PMT voltage settings. The standards are calibrated in molecules of soluble fluorescence (MESF) units, from which one can determine effective fluorescence to antibody (F/P) ratio, the number of antigen molecules per cell, and the density of available sites per cell.

C. Instrument Setup and Operation

(1) Compensation

Most instrument manufacturers provide software and test reagents (usually fluorescent beads) to set PMTs and compensation to determine target values found by the most common clinical tests (e.g., lymphocyte phenotyping, CD34 analysis). The operator should also use a biological control group such as preserved blood or mononuclear cells, which are commercially available. For analog instruments, compensation must be set prior to data collection. For digital instruments, the PMTs must be set correctly as the values for these settings cannot be changed once the list mode file has been created. When examining rare events or using intracellular dyes (e.g., 7-aminoactinomycin D [7-AAD], propidium iodide [PI], Syto-16, etc.) in conjunction with fluorescently labeled antibodies, the balance of PMT voltage and spectral overlap must be closely monitored.

Autofluorescence is fluorescence above baseline in the absence of fluorochrome staining. This occurs in some cells, typically myelocyte (especially alveolar macrophages) and cultured primary cells. If desired or necessary, autofluorescence can be measured directly at a fixed PMT voltage or can be calculated from a reference standard of fluorescent reference bead preparations (see Quantification of Antigens, above). Avoid use of the excitation wavelength of 488 or 532 nm and subsequent spectral compensation of autofluorescence as an additional fluorochrome.

(2) Data acquisition and gating Strategies

When possible, all events should be acquired in list mode, i.e., without selective gating of events. Live gating, defined as selective gating of events during acquisition, should be employed only when the desired subset is sufficiently rare that >2 million total events must be analyzed in order to count a significant (100 or greater) number of events of the desired population. List mode data can be acquired uncompensated when digital instrumentation is used, but most operators find that analysis is much less difficult and time-consuming if the data are in the range of proper compensation before acquisition. In addition, it is often desirable to set thresholds for exclusion of debris. Setting a forward scatter threshold, for instance, excludes events below a predetermined size in order to prevent the large list mode file size that can occur when these events are counted.

(3) Use of control groups

Fluorochrome-conjugated bead samples are used to standardize PMTs and compensation and to quantify the expression of specific markers. The use of biological control groups is also highly recommended. Cell samples are stained with an isotype control group and primary and secondary antibodies to evaluate non-specific binding unless the laboratory has ensured through rigorous validation procedures that non-specific binding does not interfere with test results.

Antigen-positive and -negative cell populations (prepared and stained in the same manner as the test samples) provide an internal system suitability standards. Cell populations of such control group can also be used to evaluate lot-to-lot variations in antibody preparations and staining reagents in the laboratory.

(4) Use of dyes and gating for cell viability

Cell viability dyes such as 7-AAD, PI, and TO-PRO iodide are commonly used to determine the proportion of dead cells in a cell therapy product. These dyes are typically excluded from live cells but pass through the cell membranes of dead cells, staining their DNA. Cell viability staining can be combined with surface membrane or intracellular staining to evaluate subpopulations and the proportion of live and dead cells stained with a given marker. Viability staining can also be used in conjunction with a membrane dye in flow cytometry-based cytotoxicity assays. These viability dyes should not be confused with the many apoptosis-detection reagents now available. Validation techniques for non-IVD viability dyes involve the preparation of a dead cell population that is added in serial dilution to a live cell product, and the cell mixture is then assessed for fidelity to the known proportion by staining with the dye of interest.

(5) Cell enumeration

Absolute cell count, expressed as the number of cells in a given sample volume, can be determined by dual-and single-platform methods. The dual-platform method relies on a separate automated cell counter or a manual counting method to first enumerate the cell population. Then, the percentage of a subset of interest is determined by flow cytometry, that percentage is multiplied by the cell count, and the result is divided by 100. Single-platform methods enumerate the cell population and subsets counts directly by counting the cells in the sample simultaneously while counting reference beads added to the sample volume at a known concentration. The reference beads are often provided as a bead suspension that is added to the sample. Alternatively, a given volume of the sample can be added to a known number of reference beads provided as a solid phase matrix in polystyrene tubes. These approaches are subject to pipetting errors; so extra care must be taken to ensure accuracy and reproducibility.

(6) Instrument setup and quality assurance (QA)

Each laboratory should develop a quality plan that defines the standard operating procedures for instrument setup and calibration, as well as regular monitoring, maintenance, and cleaning of instruments. Instrument logs should document these activities and operators.

Instrument parameters such as laser current, voltage, output, and PMT voltages during calibration should be monitored and recorded whenever the instrument is in use. Careful monitoring of instrument setup parameters can be helpful in detecting trends and predicting laser or PMT failures. Results of biological control group experiments should also be monitored to detect and prevent analytical method drift.

D. Data Management and Statistical Considerations

(1) Data management and storage

Quality control tests and sample analysis in the list mode should be stored in a manner that complies with the regulatory requirements applicable to the laboratory. This can be accomplished by transferring them to fixed drives, removable media, or to a server such as a commercial laboratory information system. Storage of results should always be traceable to the original Flow Cytometry Standard (FCS) list mode file, instrument settings, and quality control parameters for the particular sample. Data should be backed up to prevent data loss. Storage and backup procedures should also be established for manual records that may be used for calculations and summary data.

(2) Data analysis and statistical considerations

For most flow cytometry applications, data analysis involves displaying the data from list mode files or real-time gating on a plot (single-parameter histogram plot, two-parameter dot plot with regions, or three-dimensional plot) and measuring the distribution of events within that plot. Further analysis of data within selected populations can be performed by gating on specific cell populations. The description of the data usually includes the percentage of events within the population with a given characteristic (forward scatter, side scatter, fluorescent marker). The numerator is the number of events with the characteristics, and the denominator is the total number of counted or gated events. For two-dimensional plots, the analysis is usually performed with special software that utilizes quadrant statistics. Since cell population cluster may shift from one data file to the next, cluster analysis software has been developed.

Statistical analysis of quantitative flow cytometry applications differs from qualitative applications where a cell is considered either positive or negative for a given marker. For general quantitative methods where the number of molecules on the cell surface is estimated, the mean or median fluorescence intensity of sample cells labeled with a fluorescent antibody bound to the molecule of interest can be compared to standard curves of cells or an appropriate control groups, containing a known amount of that molecule/antibody.

A common practical consideration for flow cytometric analysis of cell therapy products, especially autologous and related donor allogeneic products, is that sample size for analytical testing is often limited because of the limited cell content of the therapeutic product itself. This creates special challenges if cells containing the flow marker of interest are rare events. In these cases, before making a decision on sample size, the user must consider the expectations for detecting the rare events within a given number of total cells (e.g., prevalence, variability, and sampling error) in relation to the desired precision of the estimate.

3. Quality and Standardization

Standardization of flow cytometry methods and equipment requires validation, quality assurance (QA), and quality control (QC). Although flow cytometry is widely used in both research and clinical laboratories, testing of cells for the development of clinical diagnostic and therapeutic applications is increasing, leading to more comprehensive regulatory requirements and more attention to standardization. For example, flow cytometry operators have traditionally used fluorescent microbeads or cells for instrument setup and QC control, often based on manufacturer's recommendations. However, consistent instructions about how these control group standards should be applied to instrument setup and QC remain unclear.

Properly applied, validation provides documented evidence that the manufacturing or testing process consistently produces product that meets predetermined specifications.

Validation based on a thorough understanding of the critical process parameters helps to define product quality and helps to ensure a consistent and well-controlled manufacturing or test process. Validation of flow cytometric methods should include instrument qualification, validation of analytical methods, and operator qualification.

A. Documentation

Good Laboratory Practice (GLP) and Good Manufacturing Practice (GMP) processes require appropriate documentation, such as standard operating procedures (SOP) for all laboratory processes. SOPs must be regularly updated and approved to reflect current practices. Training and qualifications are required so that operators have an appropriate level of competence for their assigned responsibilities. Operator competencies must be continuously reviewed and assessed in relation to SOPs and policies.

Integrating both internal and external quality processes is an important element of quality assurance. These involve equipment validation, manufacturing controls and limits, and product specifications. Process and equipment validation processes generally require installation qualification (IQ), operational qualification (OQ), and performance qualification (PQ).

B. Equipment and Assay Qualification

IQ ensures that the instrument is properly installed in an appropriate environment. This generally means determining if the flow cytometer can be installed appropriately by checking facility requirements. Qualification factors typically include temperature, humidity, space, and electrical facility capabilities in relationship to the instrument manufacturer's requirements. IQ procedures also ensure that all purchased hardware and software components are properly installed.

OQ demonstrates that an instrument will function according to the manufacturer's specifications. This generally component-level by the instrument manufacturer’s representative or using a manufacturer’s validation package that guides the end user to perform this function. Where possible, these tests should have specifications with corresponding quantitative control limits. This testing ensures that the instrument hardware and software are operational by comparison with the manufacturer’s specifications.

PQ demonstrates that both the instrument and the assay consistently perform according to specifications. For flow cytometry, these specifications are generally determined by the laboratory performing the flow cytometric testing and usually include daily instrument and assay control test specifications. For specific assays, PQ should incorporate standardized methodology, application-specific setup and compensation, and specifications for linearity, precision, and accuracy of reported assay results.

C. Instrument Performance

After identifying the manufacturer's specifications, the laboratory must establish and standardize specifications appropriate for the hardware configuration(s) to be used. For example, the manufacturer might have basic specifications for a four-color dual-laser flow cytometer. If the base specification requires the standard red diode laser of 635 nm, but an air-cooled helium-neon laser of 633 nm is substituted, the base specifications are no longer valid for the system. Similarly, if the specifications are based on the use of a 660 nm bandpass filter, but a 675 nm long pass filter is substituted, the base specifications are no longer valid because of differences in the emission filter characteristics.

D. Performance Monitoring

Instrument performance can be monitored on a daily basis using commercially available fluorescent beads. Light detectors, such as PMTs, photo-diode arrays (PO), and avalanche photo-diode arrays (APD), are used in most systems to detect signals, and their gains can be changed to increase or decrease the sensitivity of the detectors. Therefore, monitoring the settings of these detectors is as important as monitoring the signals, and these settings should be associated with each raw data file. The easiest approach is to maintain the detector settings from day to day and to measure the intensity of the fluorescence signals. This approach should be implemented for all parameters that must be validated with the appropriate beads. Instrument sensitivity is based on ability of the detection system to resolve dim cell or bead population. For this reason, measuring the coefficient of validation (CV) of dim to moderately intense fluorescence bead populations is a means to monitor fluorescence sensitivity on a daily basis. The instrument manufacturer's recommendations should be used to monitor performance.

High ambient temperature can affect laser and PMT performance and should also be monitored on a daily basis.

Incorrect compensation for spectral overlap can significantly affect data during multicolor analysis. Many approaches have been established for this purpose, and recently, mathematical algorithms have been used rather than analog circuitry. Algorithms, such as those that use matrix algebra, enable the operator or investigators to apply objective criteria to compensate for spectral overlap after all data have been collected. On older systems, the standard approach has been to compensate using a subtractive hardware adjustment to the observed preliminary data before all data have been collected. This approach can be subjective and is not as likely to produce accurate results as compensation by newer methods. Antibody-bound capture beads are valuable compensation tools because they can be used with the same antibody and tandem dyes for all fluorophores that will be used on cells. Validate the use of beads in place of cells for compensation purposes.

E. Standards

(1) Type I

Type I standards are alignment standards used to adjust the optical alignment of the instrument. They are usually used to check the optical signal alignment to improve the sensitivity of the instrument. These particles are usually small ($\approx 2\ \mu$m), bright and provide the most uniform illumination.

(2) Type II

Type II standards are reference beads and the most commonly used bead standards. They are generally used on a daily basis, have dim to moderate fluorescence intensity, and can be obtained with various attached fluorophores. They can be used to mimic cells and determine relative instrument sensitivity with dedicated software.

(3) Type III

Type III standards are used for fluorescence calibration. They are used for specialized applications that require calibration of one or more fluorescence detectors for quantification of fluorochrome molecules. Determination of the ratio of

fluorophores to antibody (F/P ratio) allows subsequent calculation of the number of antibodies bound per cell.

F. Instrument Setup and Quality Assurance

Instrument setup and quality assurance should be performed independently of the biological assay. Many variables associated with the instrument can lead to artifacts in the results of the biological assay. Two activities can be used for instrument quality assurance: baseline setup and daily setup.

(1) Baseline setup

On newer digital instruments, it is desirable to establish a baseline setup of instrument settings that provide optimal sensitivity. This setup is not a daily procedure, but should be performed if the instrument configuration is changed or if the instrument is inspected and repaired. Since PMT voltages and instrument configuration can strongly affect the instrument sensitivity, this method should be used to provide objectivity and improved sensitivity. PMT voltages can be increased to a range that provides lower CV (Figure 8). These settings can be used for the biological assay.

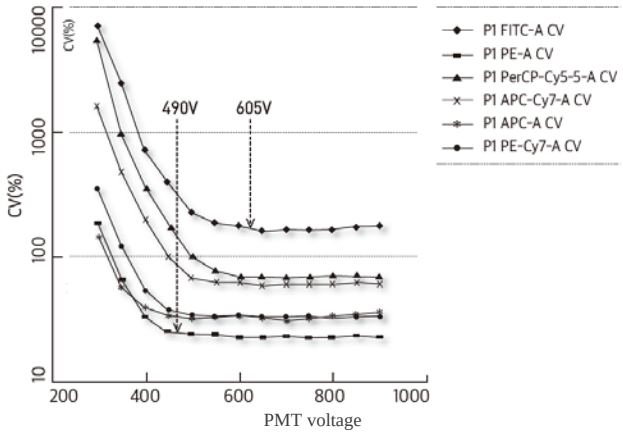


Figure 8. PMT voltages can be increased to a range that provides a lower CV

(2) Daily setup

Type II standards are reference particles and can be used to monitor signal intensity, segregation of moderately bright and dim particles, and signal resolution Fluorophore-matched beads provide a compensation tool as well as a means of knowing that the instrument is able to detect those wavelengths. Fluorophore-matched beads, however, do not have the fluorescence uniformity required for measuring CVs. Based on the optical performance of the instrument, CVs are best measured using dim and moderately bright hard-dyed beads such as coumarin-dyed microparticles that fluoresce in a broad spectral range.

It is easy to confuse assay control groups and instrument control groups. As beads provide a fluorescence uniformity and consistency that cannot be obtained with cells, they are useful for monitoring instrument performance. For this reason, it is better to use a process control cell preparation to verify compensation and fluorophore permeability.

Instrument settings for the daily setup are usually the same as those used for assays. Not all assays can be used with the same instrument settings, and it is not always necessary to perform these activities for every instrument setting unless required by validation.

Daily activities include consistent instrument setting from day to day, use of a broad range of dim to moderate intensity beads, and monitoring key parameters, including bead fluorescence intensity (absolute and % CV), PMT values, temperature, laser power, and laser wavelength.

G. Assay Quality Control and Quality Assurance

Assay-specific instrument settings should be established to demonstrate that all cell populations can be identified in bivariate plots for fluorescence and light scatter. Most importantly, positive populations should be on scale and properly compensated. This is critical when the cells are excited with red lasers, as the red laser do not cause cells to generate significant autofluorescence. For this reason, it is important to verify appropriate PMT settings so that the positive population is in the upper part of its fluorescence scale, because it may be extremely difficult to identify the negative cells.

Fluorescence compensation is a critical adjustment. Digital instruments provide objective offline adjustments during analysis, and detailed instructions for proper compensation settings are available. Using cells or capture beads stained with a single antibody-fluorochrome is generally the best approach. However, specialized fluorochrome-labeled bead mixtures can also be effective to compensate for multicolor acquisition and analysis.

H. Isotype Control Groups

An isotype control group is a negative-control group antibody that should not react with the antigen of interest and is the same isotype as the test antibody. Myeloma protein or immunoglobulin that has no specificity to the species being tested and has the same Ig chain class and subclass as the test antibody is conjugated to a fluorochrome identical to that on the test antibody. Ideally, very little or no binding occurs when the isotype control group is used in parallel with the test. However, idiotypic non-specific binding frequently occurs and is independent of the isotype of the antibody. This is most likely related to other differences in antibody chemistry and can be particularly problematic with rare-events detection assays, such as those for hematopoietic stem cell assays in peripheral blood.

I. Fluorescence Minus One Control Groups

Fluorescence minus one (FMO) control groups are used to control non-specific staining during a multicolor assay. After compensation has been set, a tube containing all of the fluorochrome-labeled antibodies, except one, is run. If the compensation has been properly set, any positive fluorescence in the parameter corresponding to the missing fluorochrome-labeled antibody is caused by non-specific staining and may indicate antibody excess or degradation of the relevant tandem dyes. Although FMO control grups are very useful for estimating the sensitivity of a particular detector in the context of other reagents, the control groups do not consider non-specific binding that may occur with the addition of the test antibody. FMO control group tubes are most appropriately used for troubleshooting or when establishing a new multicolor reagent cocktail.

J. Process Control Groups

Process control groups, also known as system suitability standards, account for sample preparation and data acquisition. They can include commercially available preserved control group cells, cell lines, or primary cells such as normal peripheral blood. Process control groups can also be used to test new lots of antibody reagents against old lots.

K. Biological Control Groups

When treated or stimulated cells are compared to untreated or unstimulated cells, the untreated or unstimulated cells may in some cases be the most useful control group for setting a positive/negative boundary.,

However, use of isotype control groups may also apply to these situations, because stimulation may lead to Fc receptor upregulation, leading in turn to increased background staining, the presence of which can be elucidated by an isotype control group.

Flow Cytometry Applications for Cellular Samples and Cell Therapy Products

A wide variety of flow cytometry applications have been developed for research, clinical diagnosis and monitoring, drug development, and cellular product characterization and quality assessment (i.e., control to allow batch release). Traditional clinical applications include monitoring human immunodeficiency virus (HIV) disease and diagnosis and monitoring leukemia and lymphoma. Both pharmaceutical and academic research laboratories have increasingly broadened the application of flow cytometry from immunophenotyping to functional cellular analysis, as well as microbead-based multiplex assays capable of measuring multiple functional parameters on individual cells. Current functional assays include those that allow direct study of cellular activation status by measuring intracellular cytokine production or secretion of chemokines or cytokines using a ligand-binding sandwich assay on microspheres.

1. Immunophenotyping

Flow cytometry allows the characterization of leukocyte subtypes by labeling cells with fluorochrome-conjugated monoclonal antibodies. The CD system defines monoclonal antibodies that recognize unique cell-surface antigens. Many clinical applications take advantage of the unique capabilities of flow cytometry to measure multiple CD antigens on thousands of individual cells.

A. CD4 Enumeration

HIV infects CD4 T cells and the number of CD4 T cells in a patient's peripheral blood is a useful indicator of immune status. CD4 enumeration has become the most commonly used diagnostic test in HIV-infected patients to determine the need for anti-retroviral therapy and to monitor the effectiveness of anti-retroviral drugs. T-cell subset counts are typically expressed in terms of cells per microliter and as a percentages of lymphocytes using a standardized reagent, software, and instrument system.

B. Leukemia and Lymphoma

Multidimensional flow cytometric analysis enables identification of aberrant cell populations in the bone marrow, lymphatic tissue, and peripheral blood of patients with leukemia or lymphoma. This is accomplished with oncology-relevant and lineage-specific cocktails of monoclonal antibodies. With optimal fluorophores and improved optical/electronic configurations in flow cytometry instruments, additional cell markers can be detected to more accurately identify leukemia or lymphoma cell phenotypes and to improve the physician's assessment of patient status. Rare-event detection methods have improved the ability to detect minimal residual disease.

C. Dendritic Cells

Dendritic cells (DCs) act as antigen-presenting cells that can influence the nature and strength of the immune response to specific antigens. This finding has led to the development of DCs as cell-based therapies for cancer, infectious diseases, and autoimmune disorders. DCs are morphologically and phenotypically diverse and can be derived from several cell types. Two major DC lineages, known as myeloid and plasmacytoid DCs, can be segregated on the basis of their expression of CD11c and CD123, respectively. In addition, the expression of the costimulatory molecules CD80 and CD86 can be monitored to determine DC maturation state.

D. Stem and Progenitor Cells

Expression of CD34 is commonly used to characterize hematopoietic stem cells (HSCs) in peripheral blood, cord blood, bone marrow, and purified HSC preparations from these sources. Flow cytometric Identification and enumeration of HSCs is possible by using monoclonal antibodies specific to the CD34 class III epitope, along with other well-characterized reagents, analysis software, and protocols. The reagent combination of anti-CD45, anti-CD34, and a viability dye such as 7-AAD is widely used for clinical applications. The growing interest in developing cell-based therapies from embryonic, fetal, and adult tissue sources has led to the use of a variety of conventional and novel phenotypic markers for characterization of source cells and their more differentiated progeny. Flow cytometric assays are being developed as part of assay methods to evaluate differentiated cellular products derived from pluripotent stem cell sources. These assays will help define appropriate numbers and types of desired cell populations, as well as the identification of undesired cells such as residual pluripotent cells that could prove tumorigenic in the recipient.

E. Leukocytes

Leukoreduction of blood products is a process used to produce blood products with a residual leukocyte content of less than 5×10^6 per unit. Clinical data suggest that nonhemolytic febrile transfusion reactions can be prevented by leukodepletion. Leukodepletion also prevents alloimmunization to human leukocyte antigens (HLAs) in patients requiring repeated transfusions of blood products. Flow cytometry is routinely used to quantify leukocyte contamination in leukocyte-depleted blood products.

F. Platelets

Flow cytometry is a rapid and useful method for the diagnosis of various primary thrombocytopathies associated with defects in structural or functional glycoproteins (e.g., abnormal expression of gpIIb/IIIa in Glanzmann thrombasthenia or gplb in Bernard-Soulier disease). By using thiazole orange, a fluorescent dye that binds RNA, immature platelets (reticulated platelets) can be quantified. The reticulated platelet count can be used to determine the rate of thrombopoiesis. This measurement can separate unexplained thrombocytopenias into those with increased destruction and those with defects in platelet production.

G. Erythrocytes

Rhesus D-negative women receive prophylactic Rh-immunoglobulin to prevent alloimmunization from Rh(D)+ erythrocytes (RBCs). If fetomaternal hemorrhage is suspected, the mother's blood is examined for the presence and quantity of fetal RBCs using fluorescently labeled antibodies to the Rh(D) antigen or to hemoglobin F.

The reticulocyte count is used to help determine whether the bone marrow is adequately responding to the body's need for RBCs and to help classify the different types of anemia. The reticulocyte count is based on the identification of residual

ribosomes and RNA in immature, non-nucleated RBCs. Fluorescent dyes that bind to residual RNA (e.g., thiazole orange) are used in flow cytometric counting of reticulocytes and their differentiation from mature erythrocytes.

2. Bead-based Immunoassays

Multiplex microsphere-based flow cytometric assays combine a series of particles with discrete sizes and/or fluorescence intensities with matched pairs of antibodies to allow simultaneous detection of multiple soluble analytes on a flow cytometer. The flow cytometer's ability to differentiate particles based on size and color allows the determination of multiple results from a single tube or well. Many researchers use such assays to measure secreted chemokines or cytokines, kinases, and anti-human leukocyte antigen antibodies.

3. Proliferation Assays

A. Dye Incorporation into DNA

Bromodeoxyuridine (BrdU) is a thymidine analog that can be incorporated into the DNA of cells during S phase, and then can be detected using specific labeled monoclonal antibodies. By pulsing a stimulated cell culture with BrdU, the cells that have proliferated (passed through S phase) during the time of pulse can be identified. This assay has become a useful alternative to ^{3}H-thymidine incorporation because it is nonradioactive and can identify phenotypes of proliferating cells by using multiple markers and flow cytometry.

B. Dye Incorporation into Cellular Proteins or Cell Membranes

Cell-tracking dyes such as carboxyfluorescein succinimidyl ester (CFSE) and PKH26 have proven useful in assessing cell proliferation. CFSE binds covalently to cytoplasm and membrane proteins, while PKH26 binds non-covalently to cell membranes. When cells divide, CFSE/PKH26 labeling is evenly distributed among the daughter cells; therefore, they show fluorescence only half as strong as the parent cells. The fluorescence of each cell is further halved with each succeeding generation. This property makes CFSE/PKH26 assays useful not only for determining the fraction of cells that have proliferated in a stimulated culture, but also, under ideal conditions determining the number of generations that have elapsed. In this way, the precursor frequencies of small populations that have proliferated over several days in culture can be calculated.

4. Functional Assays

A. Intracellular Cytokine Expression

Cell surface and intracellular labeling techniques have been applied to the identification of cell subsets with specific functional characteristics. For example, brief stimulation of cells such as peripheral blood mononuclear cells with protein or peptide antigens can result in the expression of activation markers and cytokines that can then be measured along with other phenotypic markers on the surface of responding cells. The use of a secretion inhibitor such as brefeldin A or monensin allows intracellular accumulation of cytokines. Then, the cells are fixed, permeabilized, and detected by a flow cytometric method. Such assays are useful for monitoring T cell subpopulations that respond to vaccines, infectious disease treatments, or cancer. The functional properties of other cell types, including monocytes, dendritic cells, and NK cells, can also be monitored using functional assays with appropriate stimuli.

B. Kinases

Phosphorylation-specific mediators of cell activation can be identified with phosphate-specific antibodies and flow cytometry. These reagents are useful for mapping intracellular signaling mechanisms, often in the context of other cell-surface phenotypic markers. Therefore, multicolor flow cytometry can provide a single-cell assessment of intracellular activation states in complex cell populations. These assays may be useful for detecting altered signaling states in cancer cells or directing appropriate therapies based on the signaling properties of tumor cells in a patient.

C. Apoptosis

Apoptosis, commonly described as programmed cell death, is a process of cell death caused by regulated, physiological processes. The apoptotic process appears as a series of morphological, biochemical, and molecular changes in the cell and can be initiated by external or internal stimuli. A central event during apoptosis is the activation of caspases, a family of proteolytic enzymes. Caspases are synthesized as inactive proenzymes and are activated by other caspases or by similar molecules. They form a cascade that can lead to the cleavage of various cytoplasmic or nuclear proteins. One of the caspases that is reported to be crucial for the apoptotic process is caspase-3, which is activated in the early stages of apoptosis. Flow cytometric methods for detecting apoptotic cells include measuring morphology, changes in cell membrane structure, DNA division by endonucleases, and mitochondrial membrane potential. Natural or artificial caspase substrates or antibodies against the activated form of the enzyme have also been used for this purpose.

D. Cell Viability

Flow cytometry is often used to discriminate live cells from dead cells, and the principle of nucleic acid dye exclusion is the basis of this application. A nucleic acid dye such as propidium iodide (PI) or 7-aminoactinomycin D (7-AAD) is added to cell in suspension. During flow cytometric analysis, cells with fluorescence above background are considered nonviable because they cannot exclude the dye, which fluoresces when it binds to cellular DNA.

5. Flow Cytometry Immunoglobulin Assays

A. Flow Cytometry Cross-matching

Prior to organ transplantation, flow cytometry cross-matching (FCXM) is performed on the recipient to screen for anti-human leukocyte antigen (anti-HLA) antibodies that can cause rejection. Anti-HLA antibodies are detected by incubating HLA-defined leukocytes, B-cell lines, or beads coated with HLA antigens with the serum sample, followed by anti-human immunoglobulin fluorescently labeled antibodies. Leukocytes are immunostained to identify T and B cells in order to distinguish between anti-HLA class I and II activity, respectively. In addition, screening of blood donations for anti-HLA antibodies is also increasingly employed to identify donors whose blood products may have increased risk of causing transfusion-related acute lung injury (TRALI) in recipients.

B. Anti-human Neutrophil Antibodies

Anti-human neutrophil antibodies (anti-HNA) can cause neutropenia and have been implicated in transfusion-related acute lung injury (TRALI). Autoimmune neutropenia may occur in patients who have autoimmune disorders such as Felty syndrome, systemic lupus erythematosus, and Hashimoto thyroiditis. The absence of anti-HNA antibodies narrows the differential diagnosis to non-immune causes such as bone marrow failure, myelodysplasia, or marrow-infiltrative

processes. Flow cytometry can detect anti-neutrophil antibodies and can confirm the origin of neutropenia or TRALI.

C. Anti-human Platelet Antibodies

Anti-human platelet antibodies (anti-HPA) are detected by both indirect and direct flow cytometry-based platelet-associated immunoglobulin assays. In autoimmune thrombocytopenic purpura, free serum antibodies are not found as frequently as platelet-bound antibodies. In case of alloantibody formation, serum antibodies may be detected without evidence of platelet-associated antibodies.

Flow Cytometry Assay Troubleshooting

When developing a flow cytometric method, first determine the ultimate purpose of the assay. For assays intended for research, the cell samples, reagents, and protocols may be difficult to standardize. Assays intended for patient diagnosis or to qualify a cellular product for release prior to administration to a patient require more rigorous assay and sample standardization. Regulatory guidelines, the type and stage of the clinical investigation, and the ultimate purpose of the assay determine the level of assay rigor required.

Flow cytometry assay development should include the establishment and qualification of staining, handling, instrument, and analysis parameters and limits. Assuming that the method has been well developed, operators have been properly trained, the instrument has been properly set up, appropriate test and instrument controls have been performed, and, if necessary, the instrument and method validations have been performed, operators may encounter and address instances when troubleshooting is necessary.

The most common issues in flow cytometry are high fluorescence or side scatter background, abnormal event rates, high fluorescence intensity, and low fluorescence signal. Approaches to address these issues are described below.

1. High Particulate Background

Excessive handling of cells (e.g., vortexing), improper fixation, and bacterial contamination of the cells can all increase the particulate background. In addition, if the forward threshold of the instrument is set too low, cell debris will be detected as events. Gentle handling of cells, fresh reagents, and appropriate instrument settings help ensure consistent side scatter profiles.

2. High Fluorescence Background

High fluorescence intensity can be attributed to excessive antibody concentration, inadequate cell washing, or inadequate blocking of Fc receptors. In addition, improperly high instrument PMT can also result in a high background. Consistent antibody concentration and cell density, adequate washing and blocking, and appropriate instrument settings will help avoid abnormally high fluorescence background.

3. High Event Rate

Abnormally high event rates are often attributed to high cell densities during antibody staining or in the final cell sample. Improper mixing and settling of the cell sample can result in high cell event rate, as can improper or inconsistent gating.

4. Low Event Rate

Cell clumping, low final sample cell densities, blockages in the fluidic system of the instrument, or improper gating can often result in abnormally low event detection. Proper cleaning, maintenance, and setup of the instrument, as well as consistent staining protocols, can help achieve consistent results with sufficient sensitivity.

5. High Fluorescence Intensity

As in the case for high fluorescence background, high mean cell fluorescence can be caused by too much labeled antibody, inadequate or inconsistent cell washing, or inadequate blocking. The addition of detergent to the washing buffer solution, especially during intracellular staining, can help prevent non-specific antibody binding.

6. Weak Fluorescence Intensity

Many factors may result in weak fluorescence intensity. Instrument parameters, such as poor laser alignment, improper compensation, improper setup, inconsistent gain settings, and weak laser output can all negatively affect fluorescence intensity. In addition, cell physiology or reagent preparation issues, such as insufficient antibody concentration, labile or secreted target antigen, poor-quality or improperly stored reagents (resulting in fluorochrome fading), or inaccessible target antigen, can all result in a weak signal. Appropriate assay development, proper instrument maintenance, and adherence to qualified protocols can all improve the intensity of the fluorescence signal.

Glycan Analysis of Glycoproteins
당단백질의 당사슬분석법

1. Introduction

Glycan analysis is a test method for analyzing the glycan moieties of glycoproteins. It may involve intact glycoprotein analysis, segregation and detection of protein glycoforms, analysis of glycopeptides obtained after enzymatic treatment of the glycoprotein, and analysis of released glycans obtained after chemical or enzymatic treatment of the glycoprotein. Monosaccharide analysis may complement information obtained by glycan analysis.

Glycosylation can play a very important role in determining the function, pharmacokinetics, pharmacodynamics, stability, and immunogenicity of biopharmaceuticals. Unlike transcription, glycosylation is a non-template-driven enzymatic modification process that result in glycan heterogeneity. The manufacturing procedure also has an influence on glycan heterogeneity. Therefore, glycan analysis of glycoproteins may be an important test to identify variations in the glycosylation pattern of the glycoprotein and monitor the consistency of glycosylation pattern during production. In addition, glycan analysis can be a comparative procedure because the information obtained compared to a similarly treated reference material confirms product consistency.

This chapter provides approaches used for glycan analysis of glycoproteins and requirements for the application of methods and validation of methods. It should be noted the glycan analysis is not a single general test, but involves the application of specific procedures and the development of specific glycan maps for each unique glycoprotein.

A. Protein Glycosylation

Protein glycosylation is a highly complex biosynthetic process that involves multiple steps. Three primary forms of glycosylation exist in proteins: N-glycosylation (the addition of oligosaccharides to the nitrogen of the terminal amide group of asparagine), O-glycosylation (the addition of oligosaccharides to the hydroxyl groups of serine, threonine, and hydroxyproline), and C-glycosylation (the addition of an α-mannopyranose to the

C2 carbon of the indole ring of tryptophan). Non-enzymatic additions known as glycation can occur when proteins are incubated with reducing sugars. This chapter describes analytical methods used for the N- and O-linked glycosylations, which are the most frequently encountered in glycoprotein drugs.

(1) N-Glycosylation

The biosynthesis of N-glycans in glycoproteins can be described as a four-step process:

1) Lipid-linked glycan chain initiation and elongation;

2) Transfer of oligosaccharide to the protein or polypeptide chain;

3) Processing of the N-glycan chain by removal of specific glucose and mannose residues; and

4) Modification of the N-glycan chain by the addition of residues to the non-reducing ends of the glycan chain.

N-glycans can be categorized as high-mannose, hybrid, and complex (Figure 1). High-mannose structures lack galactose or N-acetylglucosamine (GIcNAc) residues in the antennae, branches at the distal end of the chain. In hybrid structures, both GIcNAc residues and terminal mannose residues are present in the antennae. In complex structures, GIcNAc moieties are linked to mannose residues in the antennae. Hybrid and complex glycans have two or more branches, often referred to as antennae; such glycans are therefore often termed, for example, biantennary, triantennary, or tetraantennary. Both monoantennary and pentaantennary N-glycans are also known to exist. In complex glycans, antennae frequently carry terminal sialic acid (N-acetylneuraminic acid, Neu5Ac) residues. Sialylation has been shown to have a great effect on both the pharmacokinetics and the pharmacodynamics of many therapeutic glycoproteins.

(2) O-Glycosylation

O-glycan chains are built up sequentially via an initial GalNAc residue linked to serine, threonine, and tyrosine, as well as to the less common amino acids, hydroxyproline and hydroxylysine. Multiple glycan core structures are known. The sequence and isomeric linkage of monosaccharides show greater variety than that in N-glycans, and at least eight different types have been identified (Figure 2). Although no consensus amino acid sequence for O-glycosylation has been determined, glycosylation is usually favored when there is a proline one residue before or three residues after the glycosylation site, and in the absence of charged amino acid residues proximal to serine or threonine. The disaccharide unit N-acetyllactosamine (Galβ1,4GlcNAc) is the most common chain extension. Additional modifications, including terminal capping of Gal with sialic acid and fucosylation along the chain, are also frequent.

O-glycosylation can occur in cluster form, the mucin type, which usually forms part of the cell surface extracellular matrix or secreted glycoproteins. Other O-glycosylation, such as O-GlcNAc, is found on many nucloeocytoplasmic proteins; and O-mannose-linked glycosylation is found in some muscular and neural glycoproteins and in yeast. O-Fuc- and O-GIc-linked glycosylation types are found on many epidermal growth factor (EGF)-like proteins that are associated with the Notch signaling pathway.

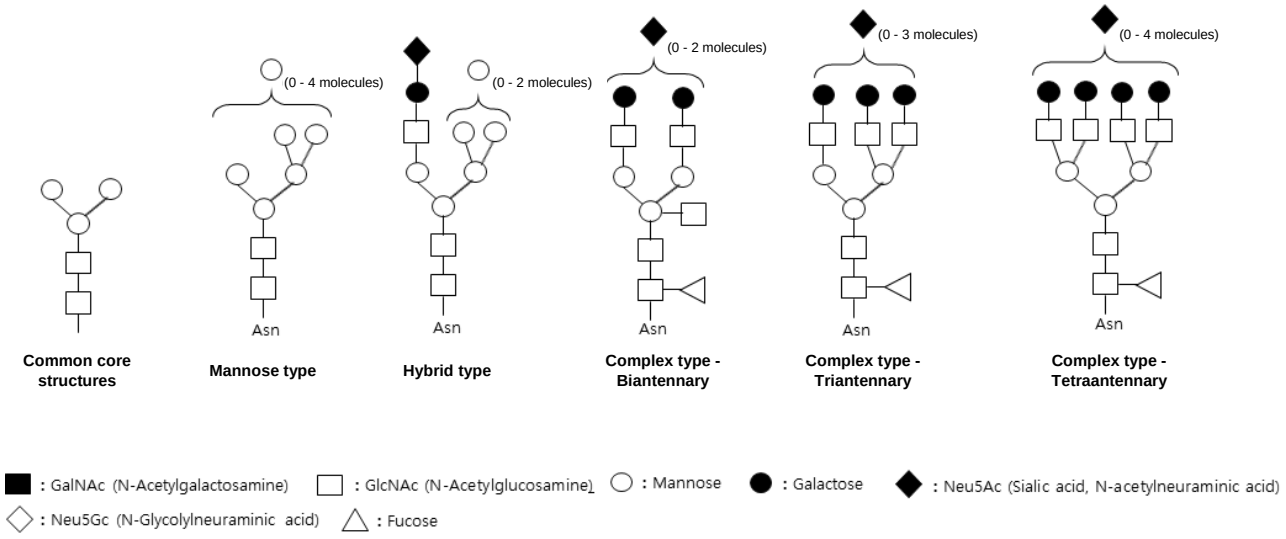


Figure 1. Common types of N-glycans

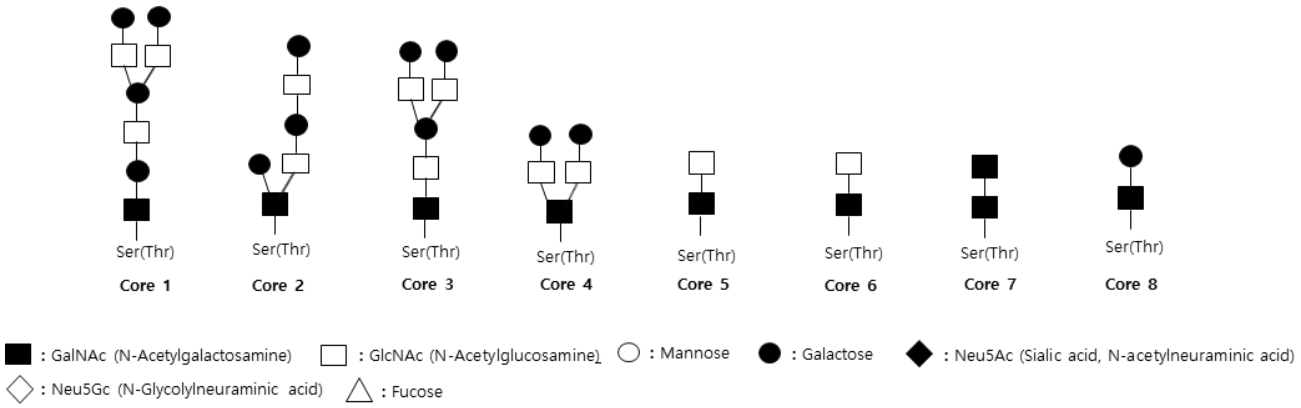


Figure 2. Common core structures of O-glycans

B. Glycan Heterogeneity

Not only the type of glycosylation (N- or O-linked), number of binding site, and site of glycosylation can vary from glycoprotein to glycoprotein, but also the actual oligosaccharide structures (branches and linkages) can differ, even on the same site. This structural variation arises because glycosylation is a process that is not driven by a template. The glycosylation pattern at a given site depends on many factors, including heterogeneity in the Golgi apparatus and glycan chains during biosynthesis. Such heterogeneity results from variability in glycan occupancy (complete, partial, or unoccupied), glycosylation (N- or O-linked), oligosaccharide structure (elongation, branching, and linkage), etc., which leads to different physical and biochemical properties and also to functional diversity. These variations, unlike transcription and translation, occur since glycosylation is a non-template post-translational modification process. The glycosylation pattern at a given site depends on many factors, including the cell-specific and/or growth-dependent availability of glycosyltransferases and exo-glycosidases in the Golgi apparatus and endoplasmic reticulum. Therefore, protein glycosylation is also influenced by protein structure, production process, host-vector expression system, and cell culture conditions.

2. Glycan Analysis for Glycosylated Biological Drugs

Glycosylation of proteins can affect biological activity, either directly or indirectly, and variability in glycosylation arises not only from cellular diversity but also from the manufacturing process. The glycosylation pattern thus may be important as a part of characterization studies in assuring process consistency and may be also important in ensuring the consistent quality of a biological drug product after market access. Appropriately characterized reference materials are needed in order to support biological and physicochemical testing of production batches to ensure batch-to-batch consistency. Glycan analysis may be appropriate for the following: Glycan analysis is suitable for the following application:

1) Characterizing the structure and stability of novel products and their stability to processing steps and storage;

2) Batch release testing and in-process control testing; and

3) Assessing comparability between products (e.g., when one or more process changes have been made).

An understanding of the relationship between glycan structure and biological function is important in determining the information required at each stage of development. For biological products, the characterization criteria and specifications are generally outlined in the guidelines ICH Q6B (Test Procedures and Acceptance Criteria for Biotechnological/Biological Products) and Q5E (Comparability of Biotechnological/Biological Products Subject to Changes in Their Manufacturing Process). Numerous approaches and methods are applied for glycan profiling or mapping. This diversity is a result of the diversity and complexity of glycan structures and the available technologies and detection systems. Meanwhile, N- and O-glycosylation are the most extensively studied types of protein glycosylation that affect biological activity.

Glycan analysis can be used in various fields; and the most important are product characterization, process validation, comparability evaluation, stability testing, monitoring manufacturing process consistency, and release testing. The choice and application of analytical techniques in the development and manufacturing of products depend on many factors, such as the complexity of the glycoprotein, the understanding of the relationships between glycosylation and safety and efficacy, and the overall design the strategy for manufacturing process control. Even when the biological relevance of glycosylation is not certain, control of glycosylation could be considered as a measure of manufacturing consistency.

Figure 3A flow chart assists in the choice of applications for glycan analysis, and Figure 3B provides an overview of the available analytical techniques and the equipment.

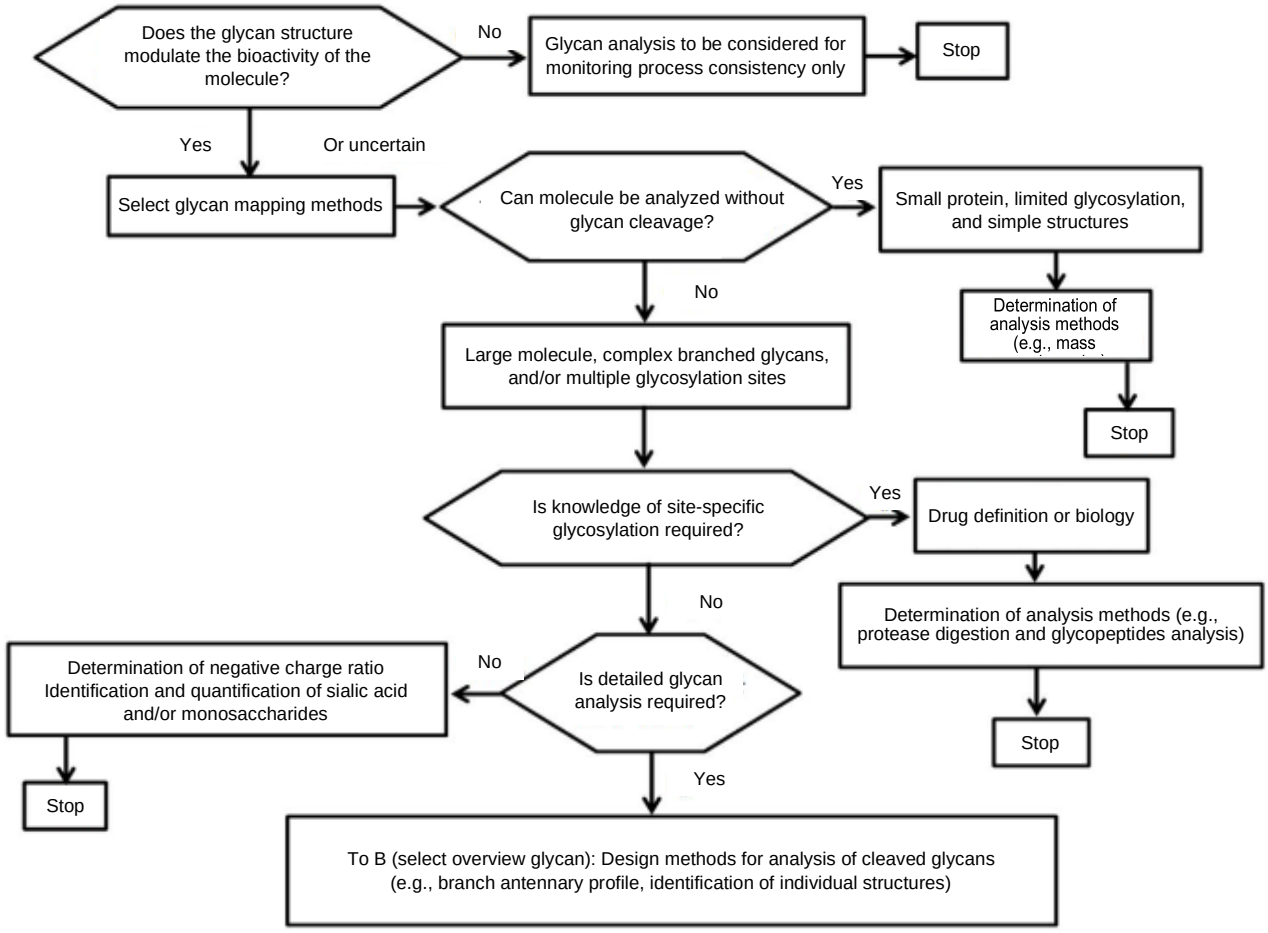


Figure 3A. Flow chart assisting in the choice of options for glycan analysis

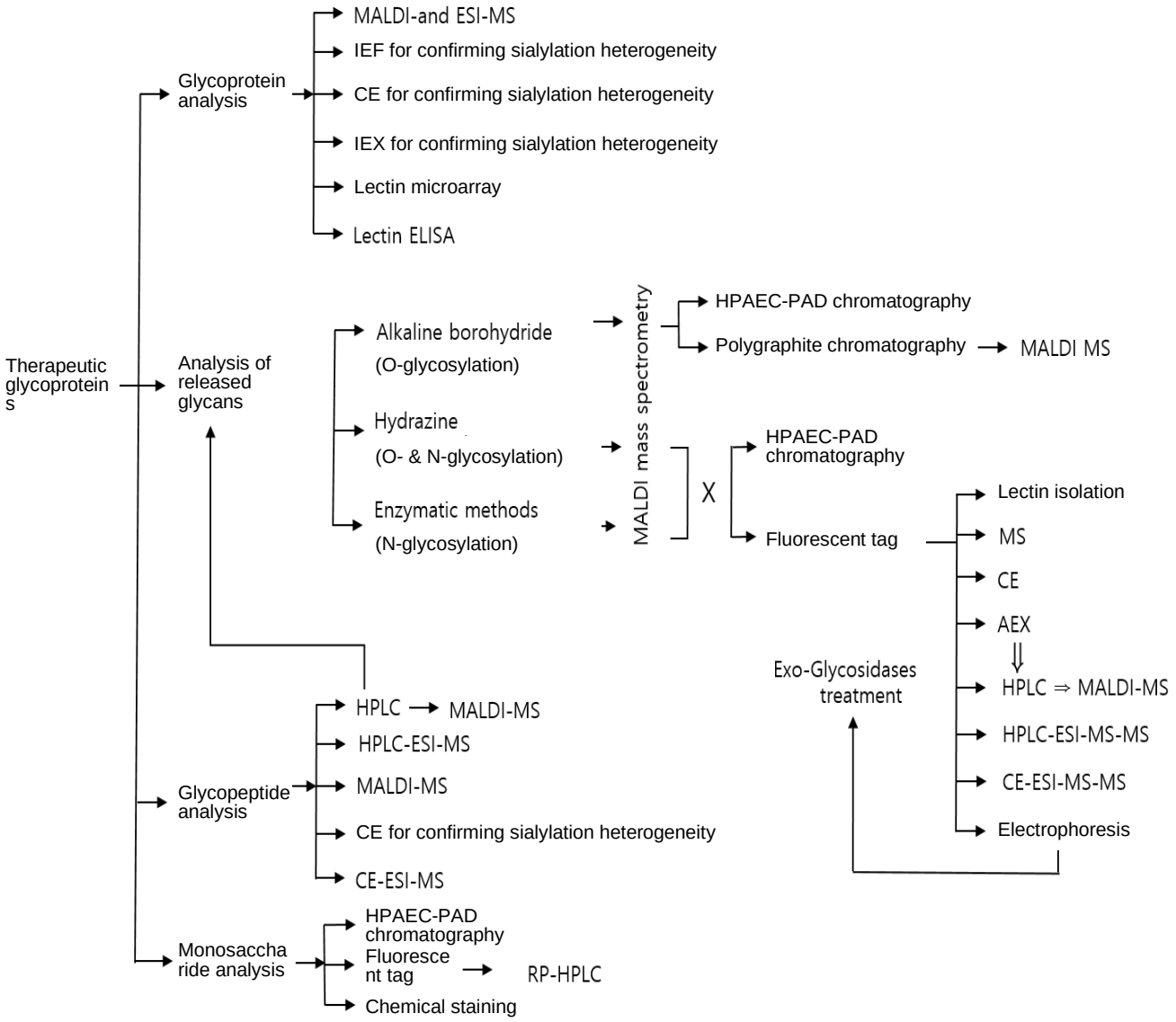
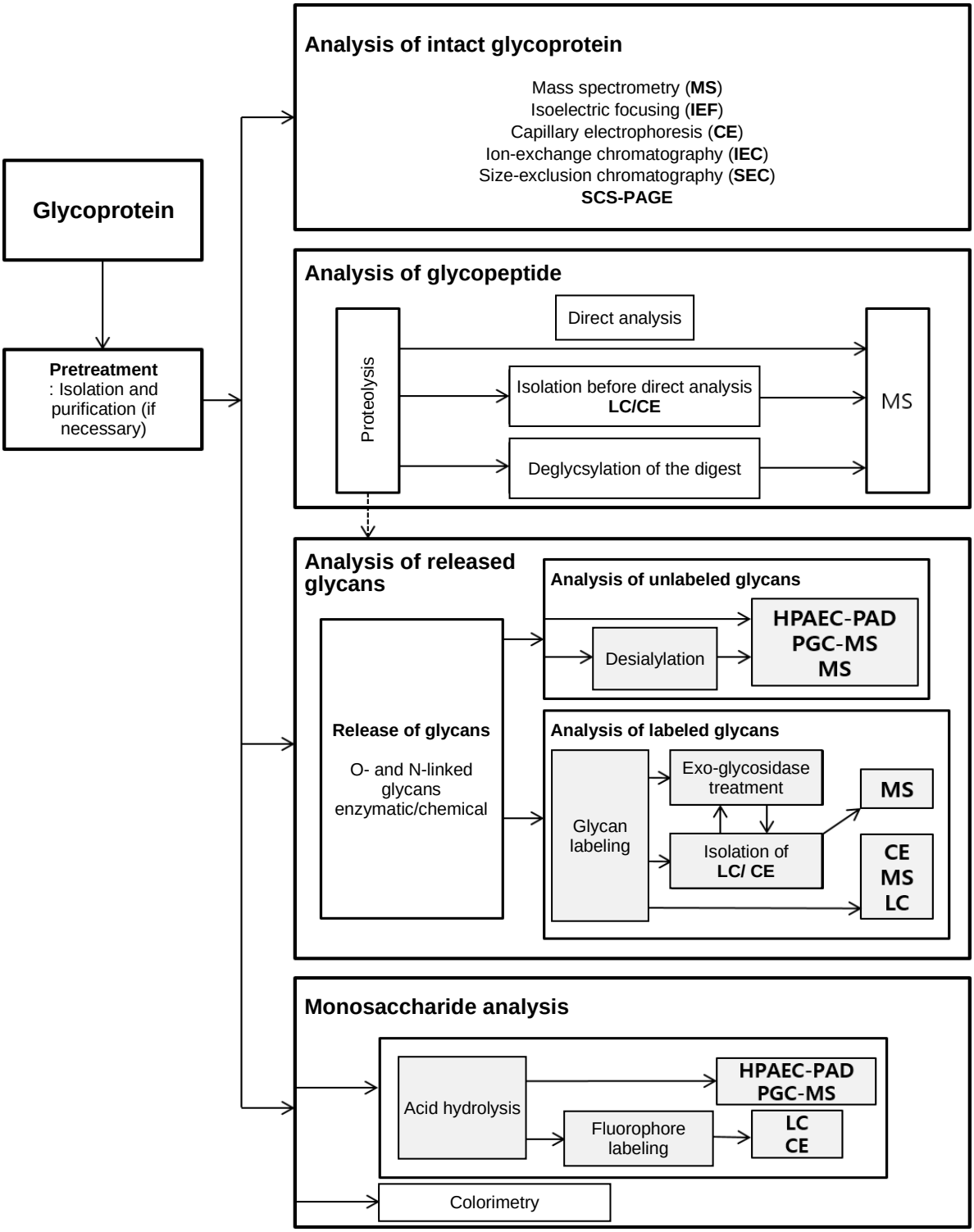


Figure 3B. Overview of glycan analysis methods and equipment

3. Glycan Analysis Procedures

Heterogeneity in glycosylation can be assessed by four distinct and complementary approaches: intact glycoprotein analysis, glycopeptide analysis, released glycan analysis, monosaccharide analysis. This section provides methods and general requirements used for glycan analysis of glycoproteins containing N- and O-linked glycans. Glycan analysis generally involves multi-steps. There are numerous methodologies for glycan analysis. This variety is a consequence of the diversity and complexity of glycan structures, the available analytical techniques, and detection systems, and the wide range of approaches depending on the level of information required.

Figure 4 shows the general glycan analysis procedures and techniques. The analytical techniques and conditions can be modified depending on the structure and origin of the glycan. Isolation and purification of glycoproteins may be necessary for analysis of formulations containing large amount of ingredients or interfering excipients.



CE: Capillary electrophoresis
HPAEC-PAD: High performance anion-exchange chromatography with pulsed amperometric detection
IEF: Isoelectric focusing
LC: Liquid chromatography
MS: Mass spectrometry
PGC: Porous graphite chromatography
SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Figure 4. Glycan analysis procedures

A. Intact Glycoprotein Analysis

The most direct method of analysis is to study the intact molecule directly. This mode provides information about the glycosylation profile of the glycoprotein. However, this approach provides limited information when the molecule is large and contains multiple glycosylation sites. One of the most important glycosylation factors related to biological activity is the degree of sialic acid binding, which commonly determines the half-life of glycoproteins in circulation. Electrophoresis and ion-exchange chromatography are useful for analyzing the degree of sialylation. Almost all types of gel electrophoresis have been used to probe protein glycosylation, including polyacrylamide gel electrophoresis (PAGE), and isoelectric focusing (IEF). Similarly, capillary electrophoresis (CE) has also been found suitable. On the other hand, anion-exchange chromatography has been used for the same purpose, but the resolution is often inferior to that of IEF and CE. Direct mass spectrometry (MS) is another option for the analysis of posttranslational modification. As mass analysis resolution continues to improve, more complex glycoproteins can be characterized directly through this method.

B. Glycopeptide Analysis

Glycopeptide analysis provides information about site-specific glycosylation properties, the degree of occupancy, and oligosaccharide structures. It involves proteolytic digestion of the glycoprotein. After proteolysis of the glycoprotein, the following approaches can be chosen.

(1) Direct analysis by MS

Care should be taken to ensure the glycopeptide signal is not suppressed due to the presence of other peptides, especially where glycopeptides represent a minor portion of the total peptide mixture and where signal intensities are lower than those of non-glycosylated peptides.

(2) Segregation prior to analysis by MS

This additional step overcomes the problems raised above. Enrichment or fractionation techniques can be used either in parallel or sequentially to direct analysis. Segregation techniques such as LC and CE are suitable. These techniques may be interfaced with MS.

(3) Deglycosylation of the glycopeptides

Glycosylation sites on glycoprotein can be identified by comparing the peptide profiles obtained by proteolysis of the intact glycoprotein to those with obtained by deglycosylation.

Calculating the mass difference between the intact glycopeptide and the deglycosylated provides information about the glycosylation sites. Mass values for the linked glycans can provide insights into their composition and heterogeneity. The segregation step can be performed after or before deglycosylation.

C. Released Glycan Analysis

Analysis of released glycans provides a convenient way to obtain information on the various populations of glycans present on the protein (bi-, tri-, and tetra-antennary profile). The degree of sialylation can also be addressed at this stage. Depending on the chosen method, prior derivatization/labeling may be required for the detection of glycans. Released glycan analysis typically involves the purification of glycans from the reaction mixture, followed by labeling/derivatization of the glycans if necessary. The glycans are then profiled.

(1) Release of glycans

The selection of the method used for the release of glycans depends on the specific glycoprotein under test. The selection of the cleaving agent determined by the required cleavage form and t level of information required. Enzymatic or chemical cleavage may be used. Table 1 provides a list of commonly used enzymatic cleavage agents. Digestion efficiency is generally dependent on the accessibility of the glycans on the protein and hence the protein can be denatured to maximize the exposure of glycosylation sites unless it is desirable to distinguish between surface and buried glycans. Alternatively, chemical cleaving agents such as hydrazine or alkaline borohydride may be use.

Table 1. Examples of enzymatic cleavage agents

Agents	Specificity
N-linked glycans release	
Peptide-N4-(N-acetyl-*β*-glucosaminyl)asparagine amidase	Hydrolysis of an N4-(acetyl-*β*-D-glucosaminy l)asparagine residue (Since glucosamine residues usually have additional glycans attached, hydrolysis yields peptides containing glycosylated N-acetyl-*β*-D-glucosaminylamine and aspartate.)
Peptide N-glycosidase F (PNGase F)	Although the N-glycan chain is released, the N-glycan chain containing (α1-3)-linked fucose is not released.
Peptide N-glycosidase A (PNGase A)	The N-glycan chain containing (α1-3)-linked fucose is released.
Mannosyl-glycoprotein endo-*β*-N-acetyl glucosaminidase	Endohydrolysis of the N,N4-diacetylchitobiosyl unit in mannose-type glycopeptides/glycoproteins containing the [Man(GlcNAc)2]Asn structure
Endo-*β*-N-acetylglucosaminidase F (endo F)	Release of mannose, hybrid, and complex oligosaccharides
Endo-*β*-N-acetylglucosaminidase H (endo H)	Release of mannose and hybrid oligosaccharides
O-linked glycans release	
Glycopeptide α-N-acetylgalactosaminidase	Hydrolysis of terminal D-galactosyl-N-acetyl-α-Dgalactosaminidic residues
Due to its high substrate specificity, the use of this enzyme is limited.	

(A) Chemical or enzymatic release of N-glycans

PNGase F (Flavobacterium meningosepticum) is an enzyme of choice for the release of N-glycans for most glycoproteins except for some insect cell and plant glycoproteins that may contain a Fucα,1,3 linked to the chitobiosyl core. N-glycan chains with this structure can only be cleaved by the PNGase A. Chemical release by hydrazine is much less common, primarily due to its limited availability, which is considered a hazardous chemical.

(B) Chemical or enzymatic release of O-glycans

Currently, there is only one enzyme, O-glycanase from Diplococcus pneumoniae, is available for the release of O-glycans. However, its use is limited due to its high substrate specificity; it cleaves only Galβ1,3GaINAcα1-Ser/Thr. In addition, no ideal chemical procedure is available. However, Ser- and Thr-linked O-glycans can usually be released by the reductive alkali-catalyzed β-elimination reaction (alkaline borohydride reaction), in which the released glycans are reduced immediately after they are cleaved in order to prevent degradation. However, this reaction lacks specificity and generally releases approximately 10-20% of N-glycans. The released glycans lack a reducing group used for the attachment of fluorescent labels. Fortunately, with advances in MS techniques, direct identification of reduced glycans is possible. Relatively good quality reducing O-glycans can be obtained by alkali-catalyzed β-elimination using primary amines such as ethylamine and hydrazine. However, both reagents have the potential to produce degradation products. Furthermore, O-

glycan release by ethylamine is not quantitative. Hydrazine, while potentially better for use than ethylamine, requires strict control of reaction conditions and handling.

(2) Analysis of unlabeled glycans

Unlabeled native glycans can be analyzed by high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD), porous graphite chromatography (PGC), and mass spectrometry (MS).

HPAEC-PAD offers high sensitivity and the ability to separate some isomers. However, signal-to-response ratios are not equal for the different oligosaccharide structures. Absolute quantification of the glycan is not possible unless an oligosaccharide reference material library is available. Quantitative analysis can be performed by comparison with a well-characterized reference material library being tested, or by relating the peak area ratio of each glycan to the total peak area of all glycans.

PGC can be used to separate native glycans due to its superior selectivity compared to the conventional non-polar columns. Furthermore, direct glycan analysis of the native glycan can be performed through mass spectrometry coupled with PGC (PGC-MS).

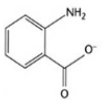
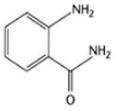
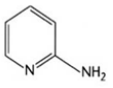
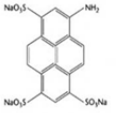
(3) Analysis of labeled glycans

(A) Glycan labeling

Derivatization with fluorescent labels is the most commonly used technique for labeling glycans. One label can be attached to every single mono- and oligo-saccharide, allowing the determination of molar quantities. Table 2 provides a list of commonly used fluorescent labels and suitable analytical techniques.

Permethylation of glycans may also be used when MS is used alone for the detection.

Table 2. Example of fluorescent labels and suitable techniques

Name	Acronym	Structure	Analytical techniques
2-Aminobenzoic acid	2-AA		Liquid chromatography
2-Aminobenzamide	2-AB		Liquid chromatography, mass spectrometry
2-Aminopyridine	2-AP		Liquid chromatography
Trisodium 8-aminopyrene-1,3,6-trisulfonic acid	APTS		Capillary electrophoresis

(B) Glycan analysis

Labeled glycans can be analyzed by analytical techniques such as LC, CE, and MS. According to the segregation properties of the glycans, glycans can be profiled and quantified by various LC systems: reverse-phase (segregation by hydrophobicity), normal-phase (segregation by size) and anion-exchange (segregation by charge) LC.

(C) Use of mass spectrometry (MS)

Profiling of fluorescent-labeled glycans using LC is the most commonly used approach The accuracy of glycan identification can be verified by using glycan reference material or by coupling LC with MS. Particularly, the LC-MS connection allows for both relative quantification and glycan structural analysis in a single analysis. If peak identities have been previously confirmed by alternative methods, and peak homogeneity is ensured, peak naming can be achieved solely based on retention time.

The degree of sialylation of glycan chains can be a crucial factor for clinical efficacy, because sialylation often defines the half-life of the molecules in vivo. Anion-exchange chromatography is the simplest method for its determination, and glycan structures based on charge can then be identified by MS. In some cases, desalting of each fraction is required prior to MS analysis. High-resolution isolation systems such as CE have been used to identify glycan structures without MS when well characterized reference materials are used for comparison. The development of the CE-MS system has further increased the power of glycan analysis using this approach.

(D) Use of micro-enzyme sequencing

When detailed structural information is required, micro-enzyme sequencing is usually used for the analysis. The success of this analysis highly dependent on the specificity and quality of the enzymes used. Recently, tandem MS has become a more prevalent method for confirming glycan structures, particularly when analyzing glycans released from well-known glycoproteins.

D. Monosaccharide analysis

Different quantitative monosaccharide assays are carried out for various purposes. In the glycoprotein analysis, they provide information on the relative amounts of saccharide in a glycoprotein and the degree of sialylation of a glycoprotein. The identification of monosaccharide composition can provide some information on the structure of the glycans. The simplest assays used are chromogenic assays to confirm glycosylation and quantify the total saccharide content in the product. These have poor specificity between different types of sugar residues.

Assays of monosaccharide composition are generally simpler to perform than oligosaccharide profiling, but they provide less information. The most widely used assay is quantification of sialic acid content Loss of sialic acid and exposure of terminal Gal residues can lead to quicker clearance of glycoproteins from circulation.

The assays can be divided into two types: 1) Those that provide information on the composition of the intact sample without prior degradation (e.g., chromogenic assay); and 2) Others that require hydrolysis of the saccharide chains before analysis and provide quantitative information on several different monosaccharide species simultaneously (e.g., chromatography). The hydrolysis step is a significant source of assay variability and may require careful optimization for specific samples.

The presence of certain monosaccharides is a diagnostic of specific glycan structures. For example, observation of GalNAc is usually a marker for the presence of O-linked glycans, and fucose denotes the presence of specific types of chains. As a consequence of the limited diversity of monosaccharide residues present in glycoprotein glycans, accurate quantification of Man, Gal, or GIcNAc residues is required in order to distinguish among a large number of structurally diverse glycans. The monosaccharide N-glycolylneuraminic acid (Neu5Gc) is not produced in humans and is generally regarded as an undesirable and potentially immunogenic component of biopharmaceutical products.

(1) Sample preparation

Glycoprotein samples for monosaccharide analysis should

be free of salts, excipients, and other carrier materials (low molecular weight sugars are commonly used as excipients for biopharmaceuticals). This can be achieved through various methods, including the following:

1) Dialysis against water or a volatile buffer solution, using an appropriate membrane, and lyophilization;

2) HPLC on an appropriate gel-permeation column eluted with water or a volatile buffer solution, monitoring by UV absorbance or refractive index, and followed by lyophilization of the sample; or

3) Sample trapping on a conventional reverse-phase solid phase extraction (SPE) cartridge such as a C18 or C8 SPE system, followed by washing out salts and eluting the required sample.

(2) Quantification

The common method for quantifying of neutral sugars in glycoproteins depends on the color generated by heating glycans or glycoproteins in the presence of aqueous phenol in concentrated sulfuric acid. In many cases, the heat required for this reaction is generated by addition of concentrated sulfuric acid to the glycoprotein-phenol mixture. In this method, rapid and efficient mixing of the solutions is critical for consistent results. Quantitative results are obtained from a calibration curve generated by the simultaneous analysis of reference materials.

(3) Hydrolysis of polysaccharides and glycoprotein glycans

Chromatographic methods for the identification and quantification of monosaccharide components require hydrolysis of the sample prior to the analysis. Appropriate sample preparation is required because excipients or process-related impurities may be saccharides and residual salts may interfere with hydrolysis, chromatographic isolation, or fluorophore labeling. Sialic acid residues can be released through mild acid hydrolysis or enzymatic treatment, leaving other sugar residues attached to the peptide backbone intact, while glycosylating the sialic acid residues. To quantify the sugar, an internal standard is added before or after hydrolysis. The most commonly used standard for sialic acid analysis by HPAEC is 3-deoxy-D-glycero-D-galacto-2-nonulosonic acid (KDN), while 2-deoxyglucose is widely used for neutral sugars. Both of these sugar reference materials are acid labile and should be added after the hydrolysis step. Accurate quantification depends both on stoichiometric hydrolysis and a lack of degradation of monosaccharide products during hydrolysis.

(4) Determination of total sialic acid

Sialic acids are typically found in bacterial polysaccharides and glycoproteins in the form of N-acetyl and N-glycolyl derivatives of neuraminic acid (Neu5Ac and Neu5Gc). The sialic acids can be determined together with other monosaccharides after acid hydrolysis to release the individual monosaccharides, followed by HPLC using an appropriate standard mixture. Alternatively, total sialic acid content can also be determined by chromogenic assay without hydrolysis. A chromogenic assay, commonly referred to as the Warren method, is based on the reaction of thiobarbituric acid (TBA) with the product of periodate oxidation of neuraminic acid released from the glycoprotein. Alternatively, the color can be generated by the reaction of resorcinol with neuraminic acid. To ensure accurate quantification, it is essential to include a reference material in each measurement..

(A) Selective release of sialic acid

Mild acid hydrolysis or enzymatic digestion can be used to selectively release sialic acid from glycoprotein glycans for quantification by chromatographic methods and for quantification of unwelcome forms such as Neu5Gc. More aggressive acid conditions are required to release neutral and amino sugars before chromatographic analysis. The protocol must be optimized for each protein to be analyzed.

(B) Neuraminidase digestion for the release of sialic acid from intact glycoproteins

Several types of neuraminidases have been isolated and studied. The enzyme derived from *Clostridium perfringens* is the one most commonly used for the enzymatic release of sialic acids from glycoproteins. Related recombinant enzymes are also commercially available. Other enzymes with different specificities are available and can be used to distinguish different types of linkages. Hydrolysis conditions should be optimized for each product, because kinetic parameters for different linkages and for Neu5Ac and Neu5Gc may differ. Selective removal of Neu5Acα2→,3-linked and Neu5Acα2→,6- linked from cleaved glycans is a convenient means of defining linkages. For quantitative analyses, a known amount of a suitable internal standard, often 2-deoxyglucose, is added after hydrolysis and removal of the acid.

(5) Segregation and quantification of unlabeled monosaccharides

The HPAEC-PAD analysis method is the only one capable of simultaneously identifying and quantifying unlabeled monosaccharides in hydrolysates. HPAEC-PAD is also applicable to oligosaccharide segregations, and a single instrumental approach can be used for both applications.

HPAEC-PAD enables the analysis of monosaccharides and oligosaccharides without derivatization. Carbohydrates, because they are polyhydric compounds, are weak acids that have pKa values of 12-14. At high pH, even neutral carbohydrates are ionized and can be separated by ion-exchange chromatography. Although segregations can be performed on alkali-stable, porous anionic column (made of polystyrene-divinylbenzene), carbohydrates tend to show broad peaks as a result of mass transfer problems. In microbead pellicular anion-exchange column packings, small porous latex beads (NMT 0.1 μm in diameter) are attached to larger (NMT 10 μm in diameter) nonporous beads. In such packings, the carbohydrate molecules interact with the functional groups on the surface of the latex microbeads, suppressing diffusion into and out of the pores and the associated peak broadening.

PAD is the preferred method for detecting carbohydrates in HPAEC because it relies on the high-pH solutions that HPAEC provides by default. PAD measures the current or charge resulting from the oxidation or reduction of analyte molecules at the surface of a working electrode. In oxidation reactions, electrons are transferred from the analyte to the electrode; in reduction reactions, electrons are transferred in the opposite direction. This process allows sensitive and highly selective detection of analytes that can be oxidized or reduced, whereas molecules that are not electroactive remain undetected. Carbohydrates are easily oxidized at gold and platinum electrodes at high pH, and the current generated is proportional to the carbohydrate concentration.

A typical amperometric detection system contains a working electrode and a reference electrode. Gold electrodes are the most common for carbohydrate analysis, but oxidation products poison the electrode surface and inhibit further oxidation. Maintaining a stable, active electrode surface is accomplished by cyclic pulsing between positive and negative potentials. This timed series of different potentials is referred to as a waveform, and the repeated application of a waveform is the basis of the PAD. Different waveforms are used for different HPAEC-PAD applications and various working electrodes. Disposable gold electrodes require the use of fast quadruple waveforms, while other gold electrodes allow the use of a wider range of waveforms without damaging the electrode surface.

Disposable electrodes and fast waveforms were introduced to minimize the influence of electrode inactivation on the sensitivity and precision of quantitative monosaccharide analysis.

(6) Fluorescent labeling of monosaccharides before segregation and quantification

An alternative approach to the identifying and quantifying monosaccharides present in a hydrolysate is to modify the monosaccharides through reductive amination with an easily detectable fluorophore. This method increases detection sensitivity and improves the chromatographic segregation of monosaccharides. Essentially, standard HPLC equipment can be used, and because the same labeling approaches are applicable to cleaved oligosaccharides, a consistent analytical approach can be applied. However, fluorophore labeling has been much less widely used than HPAEC-PAD for the identification and quantification of monosaccharides. Sialic acid derivatives are typically labeled with 1,2-phenylenediamine or 4,5-methylenedioxy (DMB) derivatives, and the resulting products are separated using a C-18 column and measured with a fluorescence detector.

4. Evaluation and Analysis of Data

Data obtained from analytical methods for glycans can be analyzed and evaluated for three different purposes:

1) Confirmation of identity of individual structures or families of structures;

2) Confirmation of compliance of the substances being tested with qualitative requirements;

3) Confirmation of compliance of the substances being tested with quantitative requirements.

A. Confirmation of identity of individual structures or families of structures

The analytical target for a glycan analysis may be an individual monosaccharide (e.g., sialic acid, fucose), a defined oligosaccharide structure (e.g., tetra-sialylated, tetra-antennary glycan), or a family of structures sharing a common analytical features (e.g., tetra-sialylated isoforms, tri-antennary isoforms). Confirmation of the identity of the analytical target is an essential step in the analysis and evaluation of data. It can be achieved either absolutely, by verification of molecular structure, or comparatively, by comparing with an appropriate reference standard.

(1) Absolute confirmation of identity

Absolute confirmation of the identity of glycan structures is usually achieved during product development, and should not necessarily be the target of routine analysis. It involves assigning the structure name based on a known molecular property of the molecule. Such absolute identification of individual structures can require multi-step approaches using enzymatic and chemical reactions, segregation techniques, and online or offline detection methods, and typically uses charge-to-mass ratios.

(2) Comparative confirmation of identity

The identity of the analytical target may be confirmed by comparison with process or system suitability reference materials. These may be generated from known, well-characterized glycoproteins, which may be the same general class as the test material (e.g., fetuin for complex N-linked glycoproteins) or may be derived from a well-characterized batch of the test material. In comparative identification, you can consider the following:

1) in the case of a validated high reproducibility of the retention time, the absolute retention times can be used for correct assignment;

2) alternatively, a glycan marker can be injected at the beginning and end of the test sequence and checked for any drifts in retention times; based on these reference chromatograms, the glycans of the test samples can be assigned; and

3) in cases where no reference material is available to assign all glycan peaks in the test sample, absolute or normalized retention times can be used to monitor and label unidentified glycan peaks.

B. Confirmation of compliance of the substances being tested with qualitative requirements

To determine if the sample complies with specifications, analytical results are compared with data obtained using a reference material being tested. When evaluating the data, consider the following:

1) to establish that the analytical result obtained using the reference standard is broadly comparable to the expected result, to verify the suitability of the system; For example, in a glycan mapping procedure, this would be achieved by comparison of the map obtained with the reference material with a provided specimen map obtained during establishment of the reference substance, and by ensuring compliance with all stated system suitability criteria;

2) to demonstrate similarity of the map obtained with the reference material and the test substance, using any specific compliance criteria given in the specific monograph.

C. Confirmation of compliance of the substance being tested with quantitative requirements

(1) Quantitative measurement of analyte levels and expression of results

When measuring sialic acid or other monosaccharides, data can be expressed as a molar ratio of sialic acid to glycoprotein. At this time, data is calculated by reference to a reference standard for sialic acid and to a validated method of protein determination.

(2) Quantitative expression of segregation profile

Profiles or distribution patterns may be expressed numerically in various ways, including normalization procedure. The percentage content of each analytical target for example, the glycan entity, can be calculated by determining the response of the glycan entity as a percentage of the total response of all the entities. In addition, numerical values like the Z number, which are method- and product-specific and defined in specific monographs, can be used to quantitatively represent the profiles of the analyzed targets.

5. Reference Standards

Reference standards for glycan analysis serve two functions: the verification of the system suitability and the confirmation that the article under test complies with specified requirements. The reference standards used for system suitability may include:

1) A reference material for the substance being tested;

2) Glycan moieties liberated from a fully characterized reference material of the substance being tested;

3) Well-characterized glycan moieties liberated from glycoproteins (e.g., fetuin, IgG);

4) Glycan markers characterized for identity and purity.

The reference material used for compliance of the glycoprotein under test is a preparation of the substances being tested. It is noted that the glycan analysis procedures described in specific monographs prescribe the use of a reference material for the substance being tested and for which the glycan analysis procedure has been validated.

6. Points to Consider in Method Development

This section covers procedures that are actually carried out during the process of the method development. The extent of method development and analytical validation is selected on the basis of their suitability for a specific product. Depending on the chosen approach, several steps are required. For example, for example:

1) isolation and purification (or desalting) of the glycoprotein;

2) enzymatic (or chemical) treatment of the glycoprotein to selectively release either N- or O-linked glycans from the protein backbone;

3) isolation and purification of the released glycans;

4) verification of released sialic acid and monosaccharide residues;

5) chromophore labeling of the released glycans;

6) segregation of the glycans, native or fluorescence labelled;

7) identification and quantification of glycans

8) determination of site occupancy based on relative quantities of glycosylated and non-glycosylated peptides.

A. Protein isolation and purification

Isolation and purification of the glycoprotein from their matrix may be necessary to remove all interfering substances (e.g., excipients, salts). When required, it will be specified in more detail in the specific monograph. This process must be executed in a reproducible to ensure a quantitative recovery of the protein.

B. Release and isolation of oligosaccharides

The approach chosen for the glycans release will depend on the protein under investigation and will be based on the types of glycosylation (N- or O-linked glycosylation). Non-compendial methods for glycans release must be optimized to ensure a quantitative profiling of all glycan entities. Factors that affect the efficiency of cleavage, such as the ratio of enzyme to protein concentration, temperature, reaction time course, and denaturation of protein prior to digestion, must be optimized.

It should be noted that the enzymatic/chemical reaction must not alter the glycan composition, e.g., not destroy sialic acid residues. In cases where multiple glycosylation sites exist, the enzymatic treatment should proportionally release all oligosaccharide moieties attached to the protein, regardless of their structure and their individual position in the protein. Reproducible recovery of all glycan entities from the reaction mixture must be confirmed.

C. Derivatization of released glycans

Derivatization is usually performed following non-compendial protocols. Therefore, the reproducibility of derivatization methods for all glycans must be verified. This may be achieved by optimizing the reaction conditions, such as the amount of derivatization reagent, reaction temperature and time. The derivatization reaction must not alter the glycan composition, for example, it should not destroy sialic acid residues.

D. Segregation, identification, and system suitability

The methods employed for glycan analysis should be capable of detecting and separating different glycan moieties to ascertain a reliable identification (assignment) and quantification. The acceptance criteria for system suitability depend on the critical test parameters that affect the result. A comparison between the glycan map of the substance under test and that of a reference substance, being treated in the same conditions, is an indicator to evaluate the performance of the analytical procedures. In order to further confirm the obtained results, the analyses may be repeated with an orthogonal method. The use of a reference standard (e.g., reference material of the product being examined, glycan marker for system suitability) is essential in the establishment of the system suitability parameters and validation of the analytical procedure. The reproducibility of the quantitative representation of glycan profiles (e.g., estimation of Z values) must also be verified.

E. Determination of site occupancy based on relative quantities of glycosylated and non-glycosylated peptides

Where site occupancy is estimated by comparing glycosylated and non-glycosylated peptides from an enzymatically digested glycoprotein, reproducibility of enzyme digestion reactions should be verified.

7. Terminology

- CE: Capillary Electrophoresis
- DMB: 4,5-methylenedioxy
- EGF: Epidermal Growth Factor
- GalNAc: N-Acetyl-D-galactosamine
- Galβ1, 4GlcNAc: N-acetyllactosamine
- GlcNAc: N-Acetylglucosamine
- HPAEC-PAD: High Performance Anion-Exchange Chromatograph-PAD
- ICH: International council for Harmonization Technical of Requirement for Pharmaceuticals for Human use
- IEF: IsoElectric Focusing
- KDN: 3-deoxy-D-glycero-D-galacto-2-nonulosonic acid
- LC: Liquid Chromatography
- LC-MS: Liquid Chromatography Mass Spectrometry
- MS: Mass Spectrometry
- Neu5Ac: Sialic acid, N-Acetylneuraminic acid
- Neu5GC: N-glycolylneuraminic acid
- PAD: Pulsed amperometric detection
- PAGE: Polyacrylamide Gel Electrophoresis
- PGC: Porous Graphitic Carbon high-performance liquid chromatography
- PNGase F: Peptide N-glycosidase F
- SDS-PAGE: Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
- SPE: Solid Phase Extraction
- TBA: Thiobarbituric acid

Guideline for Pharmaceutical Quality Control Using Near-Infrared (NIR) Spectroscopy

근적외부스펙트럼(NIR:Near Infrared Spectrosropy)측정법을 이용한 의약품 품질 관리 지침

I. Near-Infrared Spectroscopy

Near-Infrared (NIR) Spectroscopy is a very useful method for identifying organic compounds, which measures the degree to which near-infrared rays are transmitted and reflected when they pass through a sample at a respective wave number or wavelength.

The near-infrared region ranges from 700 to 2500 nm.

Although its intensity is weaker compared to that of the mid-infrared region, it allows identification, qualitative analysis, or quantitative analysis of substances due to the presence of combination bands and overtones of the normal frequency in C-H, N-H, O-H, and S-H bonds.

As with infrared spectroscopy, the spectrum is represented by a graph with wavenumbers (wavelengths) on the x-axis and transmittance or absorbance on the y-axis. When measuring a sample, the spectrum can be affected by factors such as particle size, polymorphism, residual solvents, and humidity. Such effects can be removed through appropriate mathematical pre-processing.

1. Apparatus

It usually consists of a light source unit, a spectrometer unit, a photodetector unit, and a display/recording unit.

- The light source unit uses a light source that provides the whole region or a part of it from 780 nm to 2,500 nm (or 12,821 cm^{-1} to 4,000 cm^{-1}) or a laser that generates monochromatic wavelengths within this region.
- The spectrometer unit uses a monochromator, such as a diffraction grating, filter, or interferometer.
- The photodetector unit consists of a detector and a signal processing system.
- The display/recording unit includes a display and recording device, enabling mathematical pre-processing of the spectrum.

2. Measurement Method

One of the following methods is applied:

1) Transmittance

This method is, in general, applicable to diluted or undiluted liquids or solids in a solution. The sample is measured using a cell that allows near-infrared light to pass through with an appropriate path (generally 0.5 mm to 4 mm) or by immersing a fiber optic probe. When measuring liquid samples, any spectral disturbances, such as temperature, should be considered. Additionally, background interferences should be compensated for in all cases. For example, a reference scan of the air (for liquids) or solvent (for solutions) can be used to compensate for the values in the sample spectrum.

2) Diffuse Reflectance

This method is, in general, useful for solid samples. Samples are analyzed using an appropriate apparatus. When measuring the spectrum by immersing a fiber optic probe in the sample, special care should be taken to ensure that a reproducible spectrum is obtained in each measurement. In all cases, background interferences should be compensated for. For example, a reference scan of internal or external reflection standard materials can be used to compensate for the values in the sample spectrum. Particle size and hydration/solvation state should be considered during measurement.

3) Transflectance

This method is, in general, applicable to diluted or undiluted liquids and solutions, as well as to solids in suspensions. The measurement is conducted by immersing the sample in a cell with a reflector or placing a reflector behind the sample. The reflector should be made of a metal or a chemically inert material (e.g., titanium dioxide). It should not exhibit a spectrum in the near-infrared region and can provide the sample with an absorbance within the quantifiable range.

II. Standards for Validation

These guidelines, based on the content of ICH's General Guidelines for Analytical Methods, are to ensure that comprehensive and highly reliable information on the analytical procedure using NIR spectroscopy is obtained by examining the factors to be considered when applying NIR spectroscopy, focusing on specificity, linearity, range, accuracy, and precision.

1. Specificity

Specificity is the ability to selectively analyze an analyte, even in the presence of potentially interfering substances that are expected to be present. In general, tablets contain many substances in addition to the active ingredients. For example, in an Ambroxol tablets, Ambroxol, the principal component, comprises 30 mg out of the total tablet weight of 210 mg, accounting 12.5%. However, the remaining 87.5% may act as interfering substances in the analysis of Ambroxol. These interfering substances include impurities, degradation products, moisture, residual solvents, and excipients. Their influence can be minimized by conducting chemometric analysis, such as the analysis of the principal component. Specificity can be performed using the following methods:

First, obtain spectra of the active ingredients and all other related substances. When observing the original, untreated spectra, if it is confirmed that the unique wavelengths of the active ingredients do not overlap with the peak wavelengths of other substances, it would be the easiest way to secure specificity in analyzing the active ingredients. However, if such a trend is not observed in the original spectra, specificity can be obtained by performing mathematical pre-processing or chemometric analytical decomposition to remove interfering factors.

Second, check the interference effects during the analysis by adding possible interfering substances present in the mixture before tableting or in the powdered tablets (by increasing the concentration). In other words, it is necessary to check if the wavelengths exhibiting variation depending on the concentration of the additives do not overlap with the unique wavelengths of the active ingredients.

Third, the principal component content in tablets mostly falls within the range of 95 to 105% of the labeled amount. However, if a linear relationship is obtained between the actual value and the predicted value by the NIR spectroscopy by changing the concentration and using the concentration outside the acceptable range of the indicated amount, such as 90%, 100%, and 110%, it would be a good method for identifying specificity.

Fourth, the most reliable approach to specificity is the identification and qualification of the substance. Substance identification is the process of using an appropriate algorithm to compare spectra, measured from test samples with library samples (in powder form) containing variations occurring during the production process. Substance qualitative analysis compares the average spectrum of the samples with the spectra of library samples. Through these processes, a placebo can be identified.

There are several advantages to performing a second derivative spectroscopy on spectral data for analysis. This can maximize the difference between the peaks caused by the active ingredients and interfering substances that may exist, as well as remove baseline variations. The peaks also become sharper and any variation caused by the physical form of the tablets is removed, thereby improving the resolution. In general, since the signal-to-noise ratio is reduced by half when a derivative is performed, the second derivative of the spectrum results in a signal-to-noise ratio that is four times lower than that of the original spectrum. However, with the recent advances in instruments, the effect of such factors is not a significant concern

due to their high stability.

When developing a calibration model, one or more wavelengths can be used for analysis to remove the effects of other components apart from the active ingredient. One example of such chemometric methods is multiple linear regression (MLR). On the other hand, methods that use information about the entire wavelengths or a certain wavelength region include principal components regression (PCR) and partial least squares regression (PLSR).

However, challenges in the analysis are expected when analyzing tablets with a principal component of less than 5% of the total content. Therefore, further study is required to explore methods for improving specificity by minimizing or eliminating 95% of the interfering factors.

2. Linearity

The linearity of an analytical process refers to the ability to obtain results directly proportional to the concentration (amount) of the analyte in the sample. A linear regression analysis is performed within the concentration range that can be predicted from the calibration curve of NIR spectroscopy. In general, liquid chromatography is used to calculate the peak area ratio in the range of 0 to 150% to create a calibration curve. However, the results from NIR spectroscopy express a linear relationship within a narrower range of 90 to 110% between the reference data obtained by conventional methods and the two sets of prediction values acquired by NIR spectroscopy. The linearity relationship is evaluated using appropriate statistical methods, such as calculating the regression line using the least squares. Parameters like the correlation coefficient, y-intercept, slope, and the sum of the squares of the slope and residuals of the regression line are essential information to be documented when analyzing calibration data.

3. Range

The term "range" refers to the area between the maximum and highest values of the concentration, representing the concentration range of the sample used for measuring linearity, precision, and accuracy in the analytical process.

Since it is difficult to collect samples for non-destructive analysis and calibration of tablets, the analysis range is set to twice the allowable range. For example, for the principal components of tablets, a tolerance of ± 5% of the labeled amount is allowed, considering the degradation of the principal components during the effective period of use of tablets and errors in the analysis. In this case, the minimum range of the principal component becomes ± 10% of the labeled amount. A more accurate calibration can be performed if tablets are collected in a wide range of 80 to 120% of the labeled amount. However, it is advisable to set a range suitable for the analysis purpose, considering that the test concentration is 80% to 120% for the assay of drug substances or preparations, 70% to 130% for the content uniformity test, and ±20% for the entire range of the specification for the dissolution test.

4. Accuracy

The accuracy of the analysis process is a scale that expresses the similarity between the values analyzed by the conventional analytical procedures (with proven accuracy) and the values predicted by NIR spectroscopy. Accuracy should be demonstrated throughout the entire range specified by the analytical procedure. Furthermore, accuracy is a parameter that can be predicted only after precision, linearity, and specificity are established.

Accuracy can be evaluated by a minimum of 9 measurements within the specified range, for example, by performing 3 replicates for each of the three different concentrations. The minimum 3 different concentrations are selected by choosing the mean value and the values in both ends, the maximum and minimum values of the range. When quantifying the amount of a sample to which a known amount of an analyte is added, accuracy is expressed as the recovery rate. When compared with true values or values certified or agreed upon as true values, the difference between these values and the average of the measured values is compared in the confidence interval. In the NIR spectroscopy assay, accuracy is expressed as the standard error of prediction in the validation set.

The calibration set should consist of samples with the concentrations of active ingredients produced in batches and should include the entire range of these samples. The selected samples should have a uniform probability distribution rather than a normal distribution, even across the entire calibration range. Additionally, the validation set should consist of samples with the same composition of active ingredients as in the calibration set.

The sample set for parallel tests is used to verify the analysis over time using a new independent production batch. Therefore, the accuracy of the analysis can be confirmed using a set of criteria that is completely independent from the calibration set and the validation set. This test is performed monthly using separate sample sets after developing the calibration model.

5. Precision

Precision of the analysis process refers to the similarity between the results obtained from a series of consecutive analyses of the same sample under specified analysis conditions. The items of precision are repeatability, within-laboratory reproducibility and inter-laboratory reproducibility. Precision is assessed by testing with homogeneous and reliable samples, and if obtaining such samples is difficult, artificially prepared samples or solutions can be used for testing. In addition, precision is often expressed using measures, such as the standard deviation and the coefficient of variation (CV) for a series of measurements.

In the case of repeatability, the entire analysis procedure is repeated at least 6 times over a short time interval at a concentration equivalent to 100% of the test concentration, as long as there is no thermal decomposition of the sample. The relative standard deviation value (CV) should be within 1.0%. The homogeneity of the sample and the surface homogeneity should be considered particularly when the content of the principal component is low. In such cases, the NIR spectroscopy transmittance method is superior to the reflectance method because it covers a larger region that passes through the sample compared to the reflected region. In the transmittance method, on the other hand, if the tablet is thick and the light reaching the detector is weak, more noise may be introduced into the spectrum.

Representative variables that need to be examined in within-laboratory reproducibility include the test date, investigator, and instrument. Inter-laboratory reproducibility is assessed when there is a need to standardize analytical procedure in collaborative experiments between laboratories. If inter-laboratory reproducibility is addressed, it is not necessary to verify within-laboratory reproducibility. However, since inter-laboratory reproducibility is hardly obtained, within-laboratory reproducibility is measured in most cases.

III. Evaluation of an NIR Spectroscopy Spectrophotometer

The purpose of evaluating the qualification of an NIR

spectroscopy spectrophotometer is to ensure that the instrument is suitable for its intended use by comparing it to the specifications. The evaluation procedure includes Design Qualification (DQ), Installation Qualification (IQ), Operational Qualification (OQ), and Performance Qualification (PQ).

Design Qualification (DQ)

DQ provides evidence that the instrument is suitably designed and operates according to its intended use. Various factors influencing the apparatus should be tested to ensure compliance with the device's specifications. At least, it should be verified that the device meets the manufacturer's specifications.

Installation Qualification (IQ)

IQ verifies that the apparatus is installed according to the design and specified requirements. This includes the documentation of hardware serial numbers, and software versions. Additionally, the suitability of the environment and facilities where the apparatus is installed is checked, and the assembly condition and power status are investigated.

Operational Qualification (OQ)

OQ verifies the apparatus once again by checking the methods used in selecting the spectrometer, that is, the accuracy and reproducibility of wavelength, linearity, and noise of the photometer, etc.

Performance Qualification (PQ)

PQ ensures that the instrument continues to operate as intended. It is necessary to inspect the instrument before use or at regular intervals (at least once every 6 months) by applying some of the items from the OQ. The PQ must also be conducted when performing maintenance or replacing lamps.

IV. Quantitative Analysis

In the pharmaceutical industry, drug substances and products are repeatedly tested to control the quality of medicinal products. Nonetheless, since existing analytical procedures for quality control are primarily destructive, it is impossible to control all manufactured products, and only major samples can be covered, which lead to a critical obstacle in ensuring safety. For an optimal quality control process, several principles are required in the analytical process. Accuracy and precision must be achieved, and the sample preprocessing should be non-existent or minimized. Also, it should be capable of analyzing multiple analytes at the same time and allowing for rapid adjustments during the manufacturing process. NIR spectroscopy is one of the analytical methods that satisfies these principles. It enables non-destructive, real-time inspection of all tablets during the production process. The non-destructive assay of tablets will be mainly mentioned among the quantitative analyses of various dosage forms, and the general process includes the following steps:

- Feasibility test
- Reference data
- Sample selection
- Measurement of sample spectra
- Calibration of quantitative models
- Validation of quantitative models
- Validation of model performance

1. Feasibility Study

The feasibility test is a process of identifying whether an assay of drug substances or products can be performed using NIR spectroscopy. The first step involves observing the spectrum's response at the concentration of the analyte used. After measuring the spectra of all drug substances in tablets, the original spectra are observed. If the differences between each drug substance are not distinct, second derivative spectroscopy can be performed. At this time, unique peaks between each drug substance are identified in the differentiated spectra. Additionally, when measuring the spectra of samples with varying concentrations, wavelength at which the spectrum changes according to concentration can be observed in the second derivatives.

The feasibility test is also required when selecting a calibration algorithm. Multiple Linear Regression (MLR) can be used when the major peaks of the active ingredient do not overlap with the peaks of excipients. However, if it is judged that there is a significant interference, other methods, such as Principal Components Regression (PCR) or Partial Least Squares Regression (PLSR), are more effective.

Sample manipulation and measurement methods should also be considered. This includes determining whether transmittance or reflectance mode is used, whether a fiber optic probe is employed, whether the sample measurement exhibits reproducibility when repeated and whether the sample measurement method is robust.

2. Reference Data

The NIR spectroscopy assay is a method that compares measured spectral data to reference data. Therefore, if the reference data is not accurate, the expected values obtained through NIR spectroscopy also cannot be precise. The reference data is acquired through the formulation of actual reference standards or standard analytical procedures, that is, analytical procedures with proven accuracy, such as gravimetry, chromatography, and spectrometry, which are already used as evaluation methods. Linearity, accuracy, and precision of the standard analytical procedures should be verified. NIR spectroscopy and standard analytical procedures should be performed almost simultaneously to minimize environmental errors. The reference values are secured by measuring the standard analytical procedure three times on the same sample.

3. Selection of Samples - Calibration and Validation Test Sets

• Calibration Test Set

This is the criteria used for quantifying the near-infrared spectral responses relative to the reference data. The sample should be carefully selected to ensure that the calibration test set covers the entire possible concentration range of the analytes, that it is robust to changes in the concentration of the excipients, and includes the maximum variation of the analysis. As with other analytical procedures, NIR spectroscopy can be interpolated within the range, but extrapolation is impossible.

• Validation Test Set

This can be considered the first step in validating a calibration model. The validation test set should be used for optimizing the model and should not exceed the entire concentration range of the calibration test set. If variations that are not present in the calibration test set are included, accurate validation values cannot be obtained.

The calibration test set and the validation test set can be partitioned as follows: If tested simultaneously, both sets will have the same instrumental and environmental errors.

• The test set is selected manually or by using a software to ensure a uniform distribution of the targeted drug substance throughout the composition.

• Samples can be selected based on spectral variations using software.

• Regardless of the method chosen, care must be taken to ensure uniform distribution across the concentration range. If the

distribution is not uniform (for example, if samples are concentrated in the central concentration range), appropriate justification must be provided.

The sample size that can be measured by NIR spectroscopy is, in general, significantly smaller than that of the conventional method. This is not due to the measuring apparatus but rather to the region of the sample irradiated by near-infrared rays. Near-infrared rays can detect heterogeneity even at a micro-unit mass, and to accommodate this, an appropriate measurement method is needed. While this characteristic is advantageous in content uniformity tests, most applications require information measured by averaging over a large area. Therefore, measurement methods such as moving or rotating the sample during photo-irradiation are required to obtain the averaged information of the sample.

Among the various dosage forms, tablets can be divided into two categories when selecting samples: using the manufactured product or varying the ratio of the active ingredient to excipient.

A. Using the Manufactured Product

First, this can be applied when transmittance follows Beer-Lambert's law, and can also be employed for tablets have the same composition as the product but have different masse.

Second, it can be applied when samples are collected from products manufactured normally, and each tablet has the required concentration range according to its intended use.

B. Varying the Ratio of the Active Ingredient to Diluent

First, a manufactured product is mixed with a concentration extender to obtain a calibration test set. In general, the concentration range of a drug product lies within 95 to 105% of the labeled amount. Therefore, to expand this range, tablets with extended concentrations are prepared in the laboratory. These concentration extenders have different diluent ratios between the manufactured products and active ingredients, and a range of 90 to 110% or 85 to 115% of the labeled amount is used.

Second, the manufactured product is powdered, and then the active ingredient and diluent are added to adjust the concentration. If the content of the active ingredient is lower than the total dose of the tablet, then adding the active ingredient to the powdered tablet will yield better calibration results.

Third, samples prepared in the laboratory are used to vary the concentration of the active ingredient. This method is similar to using the powdered tablet but allows for a wider concentration range (80-120%) as the amount of the active ingredient can be varied from the time of preparation.

Fourth, the powdered tablet is re-tableted after the active ingredient and diluent are added to adjust the mass. However, this method may fail the specificity test because the total mass of the tablet is different from that of the drug product.

Fifth, the global calibration set, which is a method of creating a calibration model including all existing variable factors, is used to develop a model including all variable factors that occur during the production process. This process largely consists of four steps: laboratory preparation (with the active ingredient within ±15% of the labeled amount), granulation, compression of tablets, and coating of tablets, all of which are included in the calibration test set.

4. Measurement of Samples

Transmittance is applicable to diluted or undiluted liquids or solids in solution. It uses a cell or dip probe with a path length of 0.5 to 4 mm and requires baseline correction. On the other hand, reflectance and diffuse reflectance are primarily applied to solids, and are tested by placing the sample in an appropriate device. When using a fiber-optic tube, it should be properly secured to the sample to obtain a reproducible spectra. As with the transmittance method, baseline correction is required.

For solid particles, physical differences, such as size, shape, and compression level, can affect quantitative analysis. Therefore, it is preferable to prepare the particles to be as small and uniform as possible. Additionally, to ensure uniform effects of moisture and residual solvents remaining in the sample, drying and controlled management over a specific time are implemented to achieve uniform moisture and residual solvent conditions across different samples. When measuring samples using NIR spectroscopy, it is advisable to conduct tests under consistent temperature and humidity conditions by establishing a controlled environment since the spectra is greatly influenced by moisture and temperature. Moreover, if samples have different polymorphic forms or degrees of crystallinity, cautions should be taken since it may also effect quantitative analysis.

Depending on the properties, morphology, and particle size of the sample, an appropriate measurement method is chosen from various instruments to measure the spectra of samples. The precision of the measured continuous spectra is evaluated to prove the validity of the measurement method. The relative standard deviation across the entire wavelength range is calculated from 6 consecutive measurements, and if the maximum value is not more than 1.0%, the measuring method is deemed acceptable.

5. Calibration of Quantitative Models

To develop a quantitative model, appropriate mathematical pre-processing should be performed first. The pre-processing process is an important step in quantitative analysis using NIR spectral data. Preprocessing is defined as the mathematical processing of NIR spectral data to improve the shape of spectra and/or remove unwanted variations before developing the calibration model. Prior to data modeling, spectral data can be tested to select a suitable approach. In other words, various pre-processing methods are performed on the data in parallel to evaluate them, and the optimal pre-processing is selected among them.

There are various pre-processing methods available, which include normalization, smoothing, baseline correction, derivatives, mean centering, variance scaling, and auto-scaling.

After selecting an appropriate pre-processing method, regression analysis of the near-infrared data is performed using various quantitative algorithms. Calibration is the process of constructing a mathematical model that links the response of the analytical instrument and the characteristics (concentration) of the sample. As previously mentioned, major calibration algorithms include Multiple Linear Regression (MLR), Principal Component Analysis (PCA), and Partial Least Squares Regression (PLS). Prediction is the process of estimating the characteristics of an unknown sample from its instrument signal using the developed model. In a broad sense, there are two different approaches to developing calibration models in the field of NIR spectroscopy: univariate analysis and multivariate regression analysis. Univariate analysis, the most general approach in existing analytical methods, links a single signal from the apparatus and the concentration of a single drug substance. This method is not the typical approach used in NIR spectroscopy. In NIR spectroscopy, calibration models are built using multivariate regression analysis, which links multiple signals from the instrument to various sample characteristics.

* Refer to Appendix 1 for algorithm selection when building a quantitative modeling. Appendix 1 focuses on mainly MLR, PCR and PLSR, which are used commonly in NIR

spectroscopy.

6. Validation of Quantitative Models

Quantitative models can be validated internally or externally. An independent validation test set is used to get information on the predictability of the developed model. The accuracy and precision of the NIR spectroscopy are compared with those of the reference analytical procedure. Also, the standard error of calibration (SEC) and standard error of prediction (SEP) are used for evaluating the quality of the quantitative model. While the correlation coefficient, a traditional model evaluation factor, is also used in NIR spectroscopy, it does not have the same significance as in classical univariate analysis.

When analyzing the content of tablets, the percentage (%) of the target substance relative to the mass of the entire tablet is used as the concentration value. This model is recommended when the calculated standard error of prediction, converted to the relative error value based on the amount of the analyte (mg) divided by 100, does not exceed the error range of the existing analytical methods.

7. Validation of Model Performance

To use the model sustainably in industrial settings, it is necessary to periodically validate the performance of the model, rather than simply evaluating it. The validation methods of model performance are largely divided into two: the method of using check samples and that of comparing values obtained from standard analytical procedures. First, to use the check samples, the samples should be stable over time and can be used to evaluate the short-term and long-term accuracy of the model. Second, the method of comparing the values obtained by the standard analytical procedure is to perform paired comparisons within the 95% confidence interval by periodically performing n batches of NIR values and standard values for n months(where n = 1, 2, 3, etc.). If differences are recognized, adjustments or updates to the quantitative model are required.

* Refer to Appendix 2 for Model validation.

* Refer to Appendix 3 for the routine assay and the maintenance of the quantitative model.

V. Qualitative Analysis

Near-Infrared Spectroscopy (NIR spectroscopy) can be used for material identification and qualitative testing.

- It is used when the chemical identification of an identified substance is required.
- After completing the chemical identification of the qualitative substance, the suitability of the sample to the model of substances is measured. This model was developed from samples representing various variations of the substance, including moisture, particle size, solvents, and other chemical and physical information.

  Both identification and qualitative analysis enable the differentiation between substances within the library. Typical qualitative applications of NIR spectroscopy include the following steps:
- Feasibility test
- Selection of samples
- Measurement of samples
- Development of the library
- Validation of the library
- Routine use
- Maintenance and upkeep of the library

1. Feasibility Test

Before model development, a feasibility test is conducted as the initial step. For example, it involves determining the optimal method for sample measurement, the measured amount of sample, and the minimum number of scans required for effective analysis. Prior knowledge of the composition of the library samples and molecular structure is beneficial when analyzing it using NIR spectroscopy. Additionally, when obtaining spectra of representative samples to be analyzed, it should be checked if the second derivative spectra of each substance is different.

2. Selection of Samples

The selection of samples is a crucial step in conducting successful qualitative analysis. It requires both a test set to develop the library and an independent set for validation. All samples used for developing and evaluating the library should be authenticated to a certain level. The level of authentication for each sample may vary depending on its intended use, and the database consists of samples containing various variations of substances.

Samples from different batches are collected over a period of time to reflect changes in the composition, suppliers, processes, and storage conditions. If a sample has been proven for its chemical and physical stability during storage, it can be collected regardless of the storage conditions.

The number of batches to be collected depends on the complexity of the analysis, and the substances to be analyzed should include typical variations of the system. Additionally, the number of batches required for qualitative testing of the substance is larger than the number required for the identification test. There are various types of apparatus for sample measurement using NIR spectroscopy (e.g., cups, vials, fiber optic probes, and customized instruments). The selection of the apparatus is entirely dependent on the user's needs, and the validation of the apparatus is specified during the design qualification (DQ) stage. The apparatus to measure the sample spectra is a potential source of variation during measurement and should be validated as consistently and reproducibly as possible.

3. Sample Measurement

Transmittance is applicable to diluted or undiluted liquids or solids in solution. It uses a cell or dip probe with a path length of 0.5 to 4 mm and requires baseline correction. On the other hand, reflectance and diffuse reflectance are mainly applied to solids, and are tested by placing the sample in an appropriate instrument. When using a fiber-optic probe, it should be properly secured to the sample to obtain reproducible spectra. As in transmittance, baseline correction is required.

For solid particles, physical differences, such as size, shape, and compression degree, can affect the quantitative analysis. Therefore, it is preferable to prepare the particles to be as small and uniform as possible. Additionally, to ensure uniform effects of moisture and residual solvents remaining in the sample, drying and controlled management over a specific period of time are implemented to achieve a uniform moisture and residual solvent conditions across different samples.

When measuring samples using NIR spectroscopy, it is advisable to conduct tests under consistent temperature and humidity conditions by establishing a controlled environment since the spectra is greatly influenced by moisture and temperature. Moreover, if samples have different polymorphic forms or degrees of crystallinity, cautions should be taken since it may also affect quantitative analysis.

Depending on the properties, morphology, and particle size

of the sample, an appropriate measurement method is chosen from various instruments to measure the spectra of samples. The validity of the measuring method is assessed by evaluating the precision of the measured consecutive spectra. The relative standard deviation across the entire wavelength range is calculated from 6 consecutive measurements, and if the maximum value is NMT 1.0%, the measuring method is deemed acceptable.

4. Development of Library

For library development, the following steps are taken:

- Defining the purpose of library development
- Selecting test sets for library development
- Data display
- Selecting test sets for library validation
- Data pre-processing/transformation
- Library construction
- Threshold setting

A. Defining the Purpose of Library Development

Prior to library development, it is important to define the scope and/or effective range of the library based on its intended use. This process applies to substance identification and qualitative analysis, which includes the identification of chemical similarity and the number of groups to be analyzed as well.

B. Selecting Test Sets for Library Development

When developing a library, it is required to consider the variations caused by the following factors. The factors that are particularly important for qualitative analysis in library development are as follows:

- Moisture
- Particle size
- Residual solvents
- Degradation products
- Compositional change of formulated product
- Other chemical/physical properties
- Time
- Alternative sources of material
- Retained samples
- Temperature
- Operator
- Presentation (e.g. probe insertion)
- Between-instrument variation
- Others

These factors and the scope of consideration vary depending on the intended use and the required level of resolution.

C. Data Display

This is an important step as it checks samples with visually abnormal spectra and identifies outliers. Outliers must be investigated whenever possible, and can be removed if there is a valid analytical reasons. Such cases should be properly documented with a clear reason.

D. Selecting Library Validation Set

In some cases, a representative sample should be selected from a large-scale population for analysis. Although the selection can be done with the naked eyes in simple situations, but in more complex situations, the sample is be selected after determining the group of substances using a sample selection tool (e.g., active ingredient analysis or cluster analysis).

The number of samples required for each substance group varies depending on the qualitative algorithm and the system's state (how precisely the group boundaries need to be set).

E. Data Pre-processing/Conversion

To simplify the spectrum, it is necessary to mathematically preprocess the data. For example, differentiation and variance correction algorithms help remove the baseline caused by physical property differences. Spectra are typically used when information derived from the physical form is useful.

It is important to note that mathematical conversions of spectra can result in artificial products and may lead to the loss of important information. It is essential to have a proper understanding of the algorithms for data pre-processing and conversion in order to provide theoretical justifications for each conversion performed.

F. Construction of Library

The structure of the library varies depending on the limitations of the software and the needs of the user. In the simplest case, all substances can be integrated into one library. On the other hand, the library can be divided into sub-libraries to secure the required specificity level.

In the main library, all substance groups undergo the same mathematical conversion process. The conversion may be the same within each sub-library, but may vary across different sub-libraries. For example, when a substance is identified as lactose from an excipient library, the wavelength range can be either the entire range or a reduced range. By using a certain measuring apparatus or removing unnecessary wavelength information (such as a range exceeding the dynamic range or containing excessive noise), the wavelength range can be narrowed. Splitting the wavelength for analysis can be useful to eliminate undesired effects or highlight small but important differences.

Similar to other analytical techniques, NIR spectroscopy cannot distinguish between all substance groups, especially closely related ones. In such cases, it is necessary to either unify the groups or employ alternative methods for substance identification and qualitative processes.

There are various available algorithms such as correlation, Soft Independent Modeling Class Analogy (SIMCA), Mahalanobis distance, and Support Vector Machines (SVM). The user selects the appropriate algorithm considering the effective range of the library. However, when it is easy to differentiate substances, the simplest algorithm is recommended. For example, if the only purpose is substance identification, physical factors may not need to be considered, and after performing the second derivative, wavelength correlation can be chosen as the algorithm.

G. Threshold setting

Internal validation is performed based on values set in the software itself or recommended values by the manufacturer. The threshold values of the library can be adjusted during internal validation of the library or through external evaluation of samples.

5. Validation of Library

The purpose of the validation of an analytical procedure is to ensure that the analysis is suitable for the intended purpose. According to this purpose, the factors affecting the required validation should be determined.

A. Internal Validation

The performance of the library should be evaluated during any spectral database construction process. This is based on the samples constructing the library (ensure that these samples are distinguishable from each other). Internal validation is conducted by software. While the specific procedures may vary depending on the software used, the basic procedure is as follows:

- Validate the spectra used in library development using appropriate methods (correlation or distance).
- Verify if the distribution of substances within the library does not overlap
- Employ cross-validation when building the library.

B. External validation

After successful internal validation, the performance of the database is validated using certified samples that were not used in the database development.

Reproducibility

This is not commonly applied when identifying substances. The reproducibility testing for qualitative analysis should make it possible to reliably distinguish between substances included in the library and those that are not, using defined thresholds.

Robustness

This category relies on the application and sample selection techniques, and tests the effect of subtle variations under normal operating conditions. Using experimental design model can maximize the effectiveness of the information on the analyte. Considerations include:

- The effect of environmental conditions (temperature, humidity) on the analysis
- The effect of the sample temperature on the analysis
- Measurement location within the sample
- Depth of probe insertion and sample compression/loading
- Effects from different sample measuring apparatus
- Effects resulting from instrument component replacements (e.g., lamp)
- Pre-processing effects and algorithm parameters in library development (e.g., differentiation gap/segment, and distance threshold)

6. Routine Use

When evaluating the system, it is adjusted to provide only the necessary features. For example, while system administrators of NIR spectroscopy or library development experts should fully evaluate the software, a routine user would only need to evaluate its performance for routine substance identification.

In terms of developing a spectral identification library, the goal is to include a significant portion of the inherent variations within the substances. However, there are some cases where such variations are not included in the sample sets used for library development. For instance, if the test substance is determined to be outside the boundaries of one model in the library, the NIR external spectrum model is expressed as “inadequate NIR spectrum”. In such cases, either the test substance is confirmed to be within the model or an appropriate alternative testing is conducted to accurately authenticate the substance before including its spectrum in the library.

* Refer to Appendix 4 for the construction of the library for the qualitative analysis.

7. Maintenance of Library

A. Removal of Existing Substances

Removing substances from the library is generally not recommended under normal circumstances. However, if it is proven that there was an error in the sample selection, substances can be removed from the library, and in such cases, the library must be reassessed.

B. Addition of New Substances

To add a new substance to the library, the test set of the substances should be selected in accordance with the details mentioned in the sample selection parameters. The library should be continuously reassessed to validate its specificity.

C. Modification of Groups in the Library

Sometimes, the test set should be modified under the following circumstances:

- Changes in the physical properties of substances
- Changes in the supply source
- Inclusion of a wider range of substances

In each case, authentication should be performed using methods other than NIR spectroscopy before including new samples in the model. Once the samples are included, the library should be modified based on the sample selection criteria and continuously reassessed to validate its specificity.

* Refer to Appendix 5 if there is any problem with the application of the library for the qualitative analysis.

VI. Terms and Definition

Calibration

The process of developing a quantitative model.

Prediction

The process of applying the developed quantitative model to unknown samples to determine the concentrations.

Calibration set

The set of samples used for developing the quantitative model.

Validation test set (validation set)

The set of samples used for validating the quantitative model.

Multiplicative scatter correction

One of the mathematical pre-processing methods that correct variations in the baseline of spectra caused by physical characteristics, such as particle size of the sample.

Multiple linear regression (MLR)

A method of developing a quantitative model using the absorbance of two or more wavelengths.

Principal components regression (PCR)

A type of multivariate analysis that uses factor analysis to analyze patterns and relationships in data.

Partial least squares regression (PLSR)

A type of multivariate analysis that incorporates the concentration information of samples into principal component analysis.

Soft independent modeling by class analogy (SIMCA)

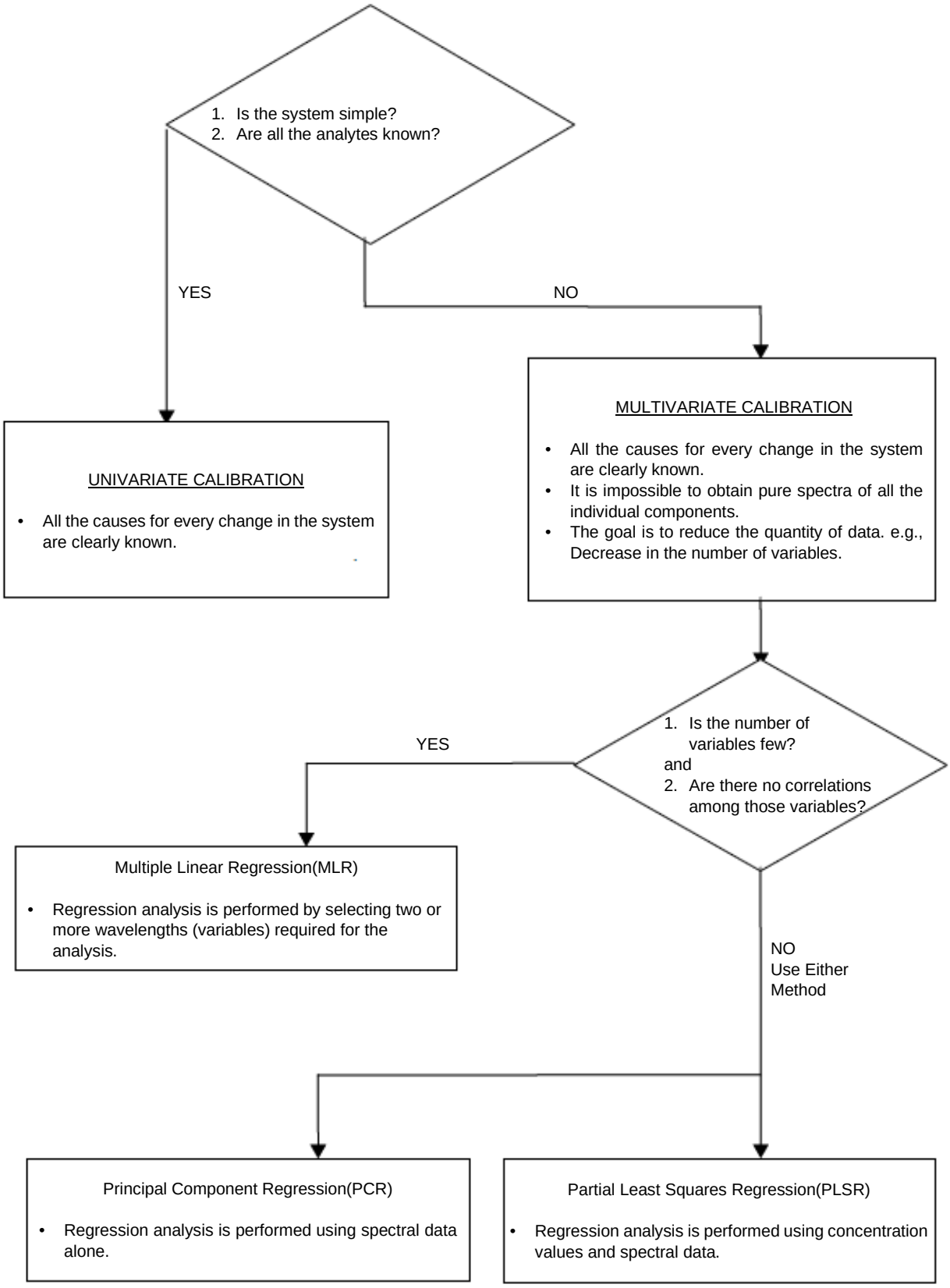
A type of pattern recognition method, an algorithm used for sample identification and qualitative analysis.

NIST SRM

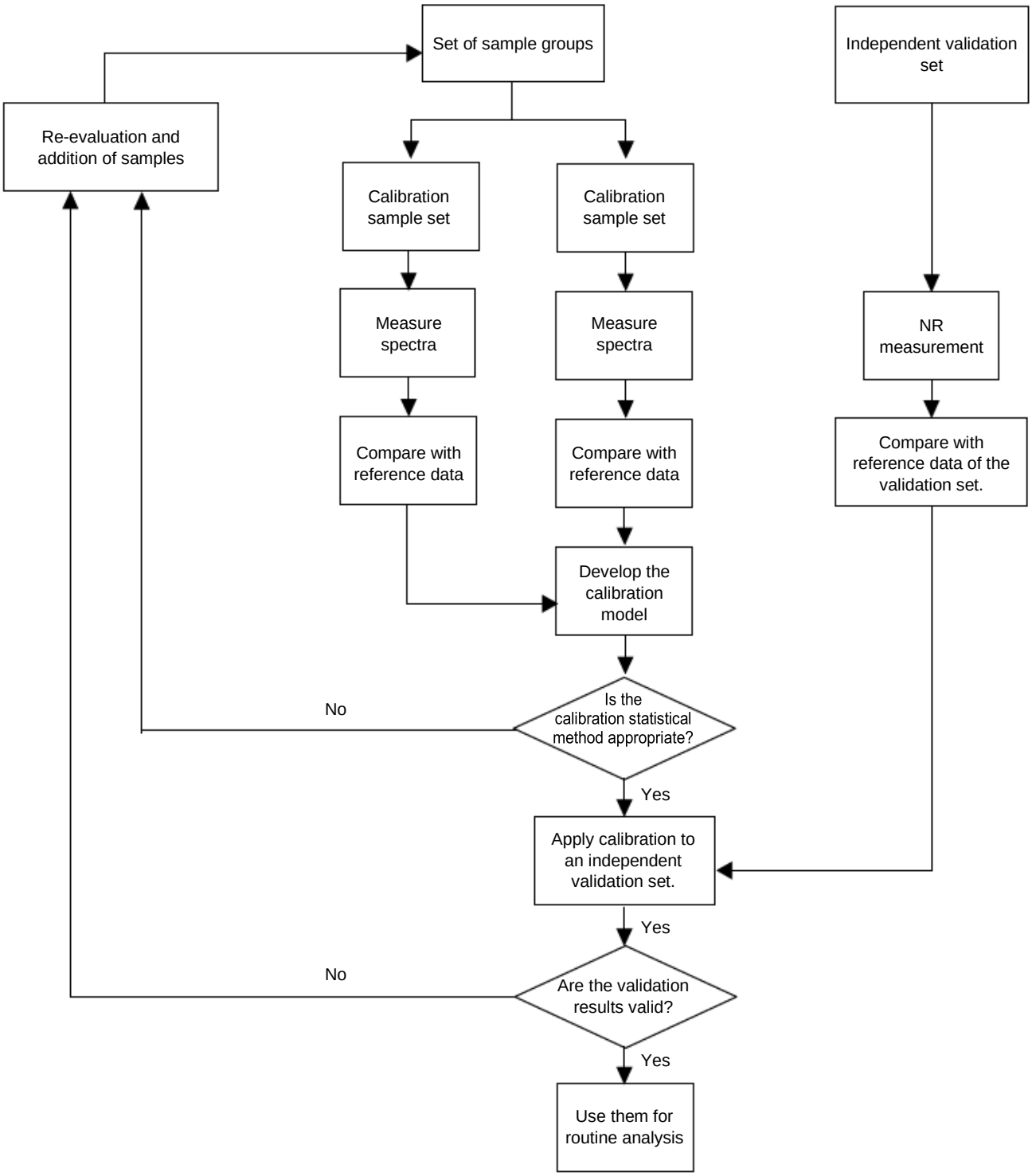
National Institute of Standards and Technology Standard Reference Material

APPENDICES

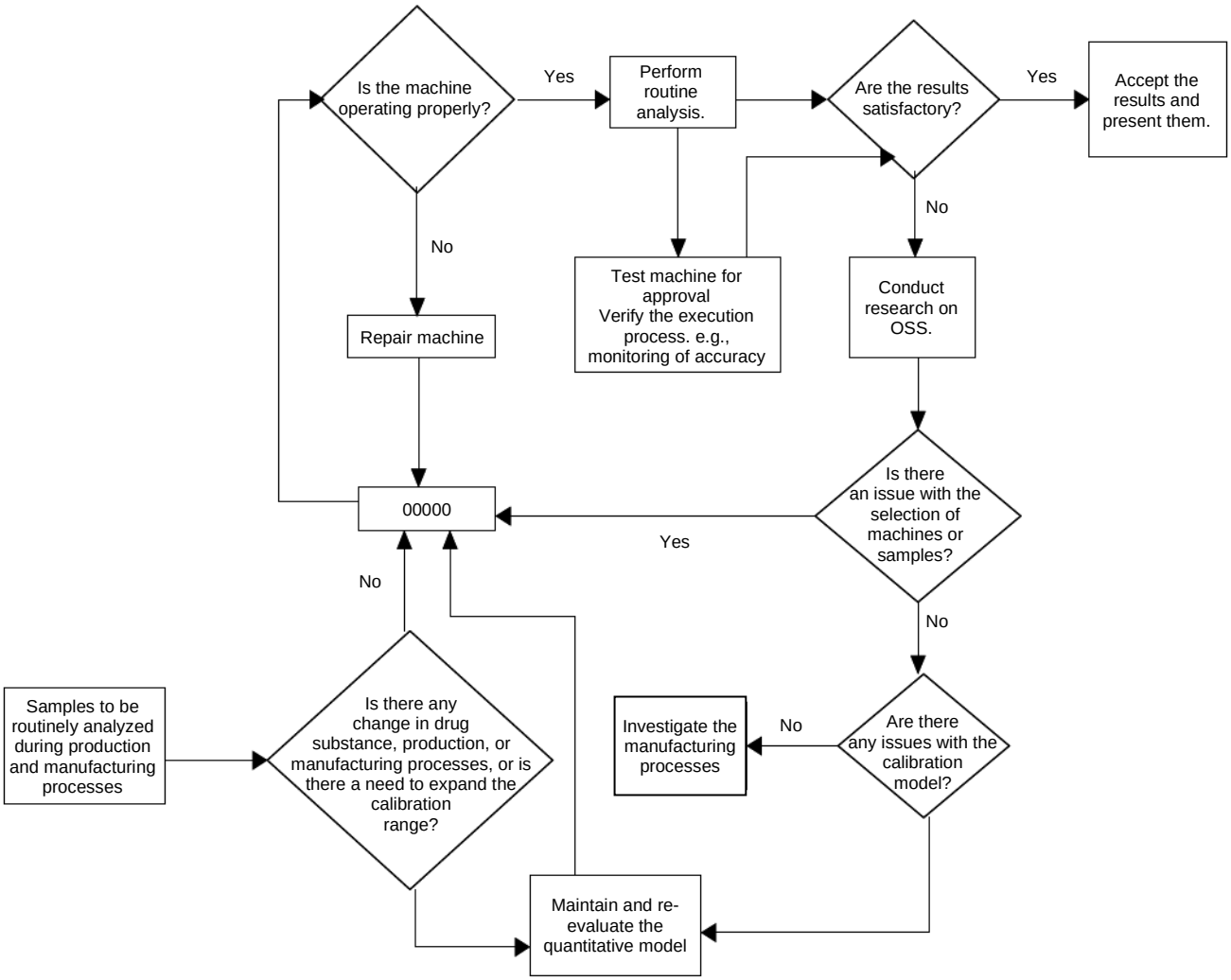
Appendix 1. Selection of Algorithm in Building Quantitative Model



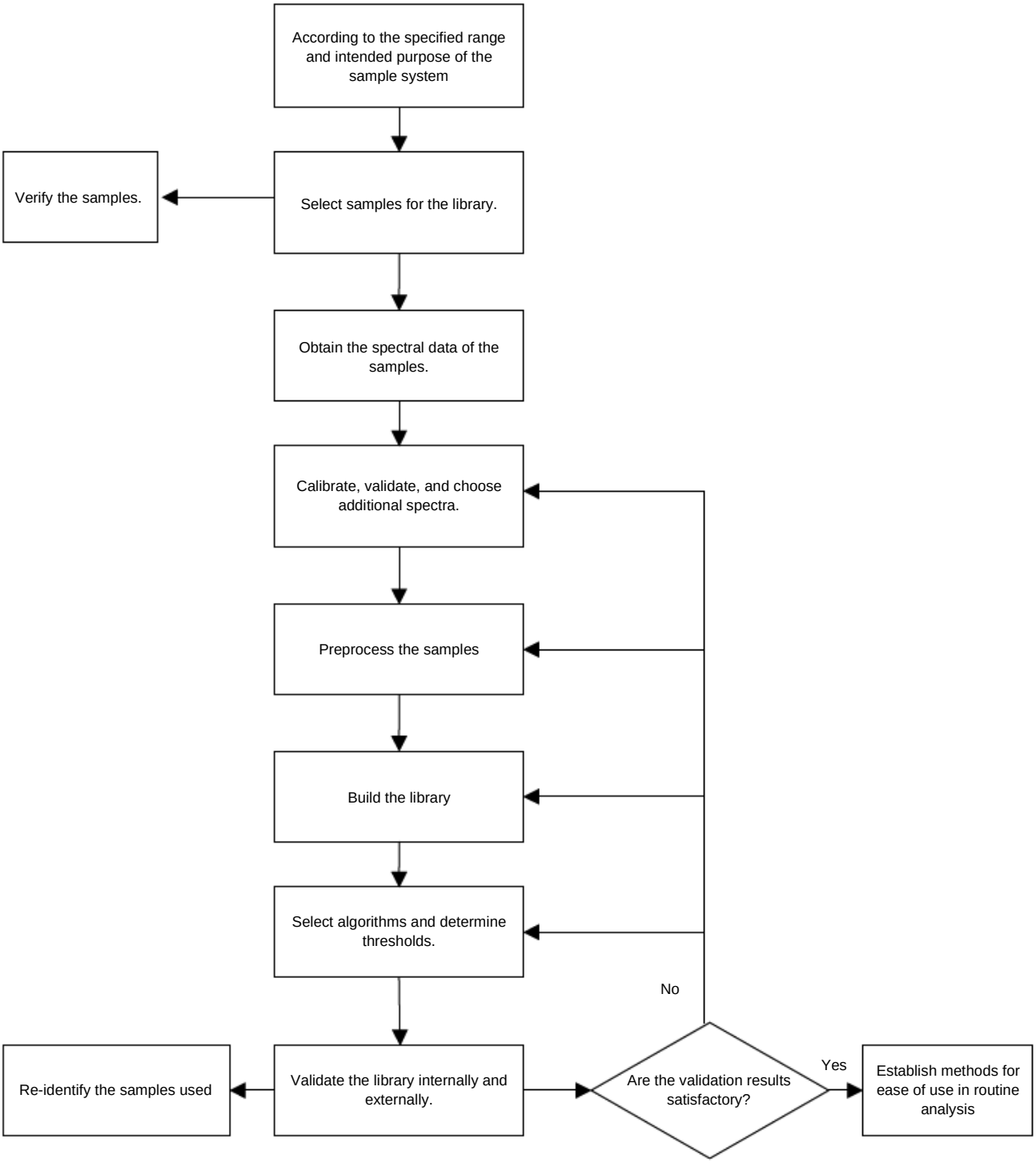
Appendix 2. Validation and Evaluation of a Quantitative Model



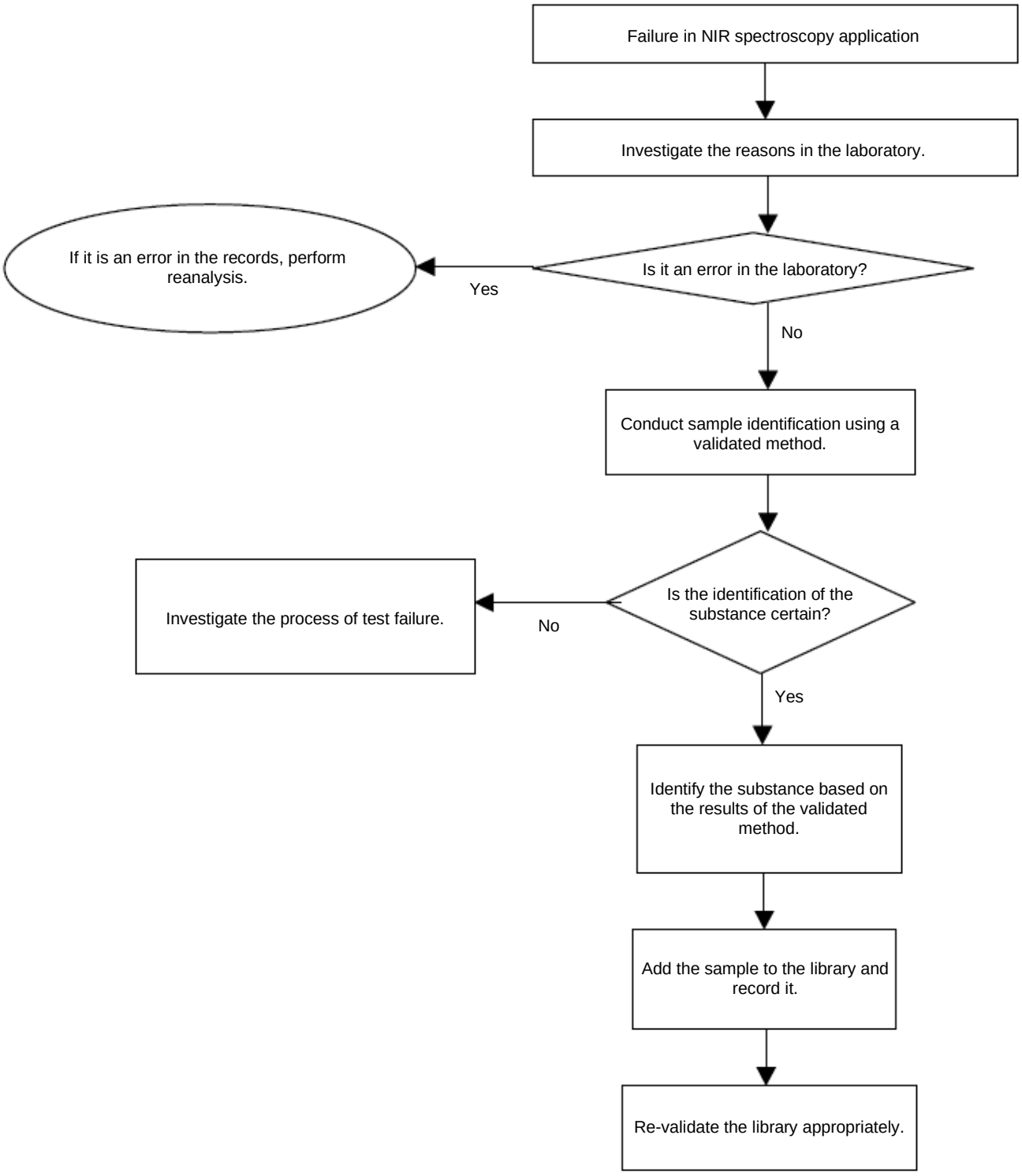
Appendix 3. Routine Quantitative Analysis and Maintenance of Quantitative Model



Appendix 4. Construction of a Library for Qualitative Analysis



Appendix 5. Failure in Applying Library in Qualitative Analysis



Guideline for Setting Dissolution Specification of Oral Dosage Forms
경구용의약품의 용출규격 설정 가이드라인

1. Purpose

The purpose of this guideline is to contribute to the production of good pharmaceuticals by providing specified approaches for setting the dissolution specifications of solid oral dosage forms, such as tablets and capsules, and thereby ensuring quality uniformity of drug products.

2. Significance of Dissolution Test

The absorption of a drug after its oral administration is affected by the release properties of the drug from the dosage form, the degree of dissolution or solubilization of the drug under a given physiological condition, and the intestinal permeability of the drug. The dissolution test is to determine not only the degree of release of a drug, but also the degree of its dissolution or solubilization, which enables the estimation of how an orally-administered drug product works in the biological system. When a dissolution test is performed, it is desirable to consider solubility, dissolution, intestinal permeability, formulation characteristics, pharmacokinetic characteristics, and physiological factors of the drug. In order to establish a correlation with a drug product administered to the human body, similar physiological conditions for the dissolution test are set, such as using several test solutions representing the pH of the gastrointestinal tract, selecting an appropriate rotational speed representing the peristalsis of the gastrointestinal tract, and adding lipids, enzymes, or surfactants to the test solution. Through these approaches, it is sometimes possible to predict the *in vivo* drug behaviors using the results obtained from a dissolution test (*in vitro-in vivo* correlation). However, it is not always necessary to attempt to reflect the physiological conditions in the gastrointestinal tract to the dissolution test. This is because, even under a condition that does not reflect the condition of the human gastrointestinal tract, the following useful information can be obtained to determine whether the quality control and the quality assurance of the preparation are guaranteed, through the dissolution test.

1) Uniformity of quality by lot

2) Composition of a drug product and formation development

3) Information on the drug-release mechanism of a drug product

4) Determine whether the pharmaceutical quality equivalence is maintained during storage until the expiry date.

5) Determine the pharmaceutical equivalence of the preparation before and after the change in the active pharmaceutical ingredients, composition, manufacturing method, manufacturing site, and manufacture scale after the approval of the preparation.

3. Glossary

A. (Conventional) immediate release dosage forms

Conventional immediate release dosage form shows the dissolution pattern of a drug in a drug product determined according to its unique characteristics, and the degree of dissolution does not change significantly depending on the quantity of raw material in the product or the change of manufacturing method.

B. Delayed-release preparations

Delayed-release preparation is configured to delay the dissolution of a drug for a certain period of time after administration, and is generally prepared by a special formulation or manufacturing method to prevent the drug from dissolution in the acidic condition of the stomach. It shows the same dissolution pattern as the conventional release dosage form after a certain period of time while the pharmacokinetic criteria remain unchanged.

C. Extended-release preparations

Extended-release preparation exhibits slower dissolution of the drug than conventional immediate release dosage forms which share the same administration route. It refers to a dosage form typically manufactured by a special formulation or manufacturing method to reduce the dosing frequency compared to conventional release dosage forms.

4. General Considerations in Setting the Dissolution Specifications

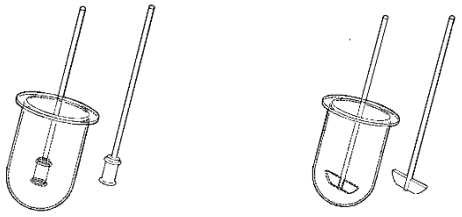
A. Classification of dosage forms

In this guideline, dosage forms are classified into conventional release dosage forms, delayed-release preparations, and extended-release preparations, and their definitions are provided in section 3. Glossary.

B. Conditions for dissolution test

(1) Apparatus for dissolution test

Currently, seven types of dissolution apparatuses are listed in the official compendium, which are illustrated in the following Figure (Figure 1). A suitable apparatus may be selected to perform dissolution test depending on the characteristics of the dosage form. The most commonly used methods for dissolution test are Method 1, the basket method, and Method 2, the paddle method. Both the basket method and the paddle method are simple, robust, standardized well, as well as used widely around the world. Therefore, unless a special dissolution test is required, either the basket method or the paddle method is used in dissolution test. Dissolution apparatuses used for dissolution test either by the basket method or by the paddle method must comply with the dissolution test regulations as specified in the section of General Tests in the Korean Pharmacopoeia. During the development of a drug product, either the rotation basket method or the paddle method should be determined in consideration of *in vitro* dissolution patterns and *in vivo* pharmacokinetic aspects of the drug. In general, the basket method is recommended for capsules, dosage forms that tend to float on the dissolution medium, or dosage forms that tend to disintegrate slowly. Although the paddle method is preferably used for tablets, the basket method is recommended when the disintegrated drug debris is settled down at the bottom of the dissolution vessel, thereby delaying the dissolution process. Also, it is desirable to conduct a suitability test for dissolution apparatuses used for dissolution test (see Appendix I) at regular intervals.



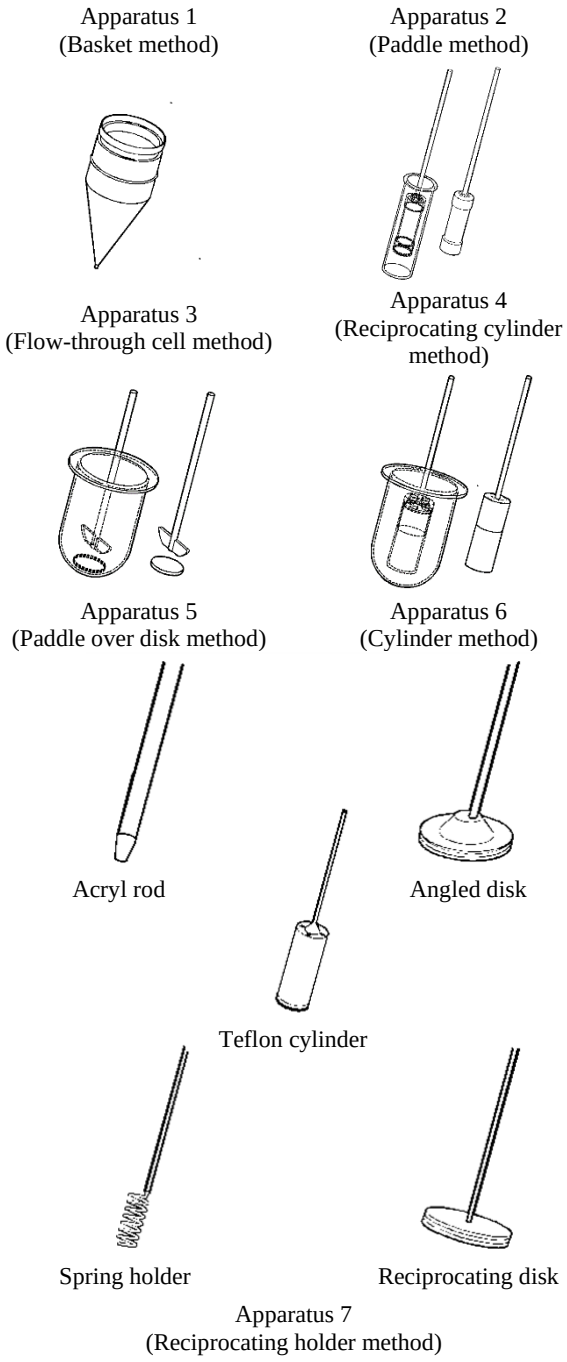


Figure 1. Types of dissolution apparatuses

(2) Rotational speed

In general, the rotational speed is set to 50 rpm in the paddle method, and 100 rpm in the basket method. A faster rotational speed, such as more than 100 rpm, is generally required for the extended-release preparations tested by the paddle method. However, it is not always necessary to set the rotational speed as suggested above. For example, if 100% of the drug is dissolved within 15 minutes when a dissolution test is performed using the basket method at the rotational speed of 100 rpm, it is desirable to reduce the rotation speed and/or to change the composition of the dissolution medium so that the dissolution pattern can be discerned better. In other words, the rotational speed in the dissolution test should be set to the test condition with the greatest discrimination power that can recognize the pharmaceutical non-equivalence of a drug product.

(3) Dissolution medium

(A) General considerations

1) In general, dissolution media with pH 1.2, 4.0, 4.5, 6.8, and 7.4 are used, but the pH and composition of it can be modified according to the characteristics of the drug product (e.g., drug stability or solubility) or for special experimental purposes. At this time, the stability and solubility of the drug should be considered.

2) Select a dissolution medium that is able to properly identify the difference in the dissolution rate between drug products or between lots that may be caused by factors related to the production process, such as the composition of the drug product, manufacturing process, equipment, and raw materials. In general, dissolution media which exhibit too fast dissolution rates are not suitable because of their low discrimination power, so it is recommended to select the one with relatively slower dissolution rates.

3) If a drug adheres to the surface of the dissolution apparatus, thereby interfering with the analysis, use a dissolution medium containing an adsorption inhibitor that can prevent such adhesion.

4) Suitable surfactants may be added to the dissolution medium to enhance the solubility of poorly soluble drugs. However, the use of organic solvents is not recommended for dissolution test.

5) It is not always necessary to deaerate the dissolution medium before dissolution test of all drug products. However, if the same results are not obtained in the tests using deaerated and non-deaerated dissolution media, the deaeration process is necessary (see Appendix II).

(B) Types of dissolution medium

1) Water

Water has the disadvantage that the pH and surface tension is easily changed by drugs or additives during the dissolution test as it has no buffering power, which might affect the test. Nevertheless, it also has an advantage of being the most convenient and environmentally friendly medium. So it is most widely used if it does not affect the dissolution pattern of the drug product.

2) pH 1.2 Dissolution medium

Use the first fluid under the Disintegration described in the Korean Pharmacopoeia. This medium is prepared using hydrochloric acid and sodium chloride, and the concentration of hydrochloric acid in the medium is about 0.1 mol/L. In cases where a particular drug becomes unstable in this medium, it is necessary to set the testing conditions that can help maintain the stability of the drug. In fact, when dissolution test is performed under acidic conditions, the concentration of hydrochloric acid is set differently within the range from 0.001 to 0.1 mol/L in many drug products.

3) pH 4.0/4.5 Dissolution medium

- pH 4.0 Dissolution medium: Use 0.05 mol/L sodium acetate buffer solution [a mixture of 0.05 mol/L acetic acid and 0.05 mol/L sodium acetate (82:18)].
- pH 4.5 Dissolution medium: Use a buffer solution prepared by dissolving 2.99 g of sodium acetate trihydrate and 1.66 g of glacial acetic acid in water to make 1000 mL.
- In cases where undesirable results occur due to interaction with drugs, etc., other buffer solutions such as citrate acid or phosphoric acid may be used alternatively.

4) pH 6.0 Dissolution medium

Use the sodium dihydrogen phosphate and citric acid buffer solution (pH 6.0) described in the Korean Pharmacopoeia.

5) pH 6.8 Dissolution medium

Use the second fluid under the Disintegration described in the Korean Pharmacopoeia.

6) Simulated gastric fluid

Use the simulated gastric fluid TS described in the section of Test Solutions in the US Pharmacopoeia (USP).

7) Simulated intestinal fluid

Use the simulated intestinal fluid TS described in the section of Test Solutions in the USP.

8) Other dissolution medium

In general, dissolution media with pH 1.2, 4.0, 4.5, 6.0, and 6.8 are used, but the pH and composition of it in the dissolution test can be modified if necessary, according to the characteristics of the drug product or for special experimental purposes. For example, it can be modified if a particular drug is most stable or selective analysis of the drug is possible in an alkaline dissolution media, or if sufficient drug solubility is guaranteed in an alkaline dissolution medium. When analyzing a drug according to the liquid chromatography method using a C_{18} column after a dissolution test with an alkaline dissolution medium, it may be necessary to neutralize or sufficiently dilute the column with mobile phase before injecting the test solution, because the packing material of C_{18} columns is unstable at high pH conditions.

(C) Volume of dissolution medium

Although the volume of the dissolution medium is usually 900 mL in the test, it may be adjusted to 500 mL - 1 L depending on the testing condition. Currently, a small dissolution vessel with a capacity of 150 mL or a large one with a capacity of 1 L or more is also used. General considerations for setting the volume of the dissolution medium are as follows:

1) Solubility of drug

The volume of the dissolution medium should satisfy the sink condition. In general, the sink condition refers to use a volume of dissolution medium at least three times (generally 5 to 10 times) greater than the volume required to completely dissolve the drug. In other words, it means that under such conditions, the concentration of the drug when it is completely dissolved in the test solution should always be less than 30% of the intrinsic solubility of the drug. It ensures that the sink condition is maintained during the dissolution test so that the dissolution process of the drug does not affect the rate-limiting step of the dissolution rate.

2) Use of surfactant

When a surfactant is added to the medium to improve the solubility of a poorly soluble drug, it is desirable to use a sufficient volume of the dissolution medium.

3) Ease of assay

In the dissolution test of a drug product with a very small amount of drug, if the concentration of the dissolved drug is lower than the quantification limit, thereby making the quantification of drug difficult, it is recommended to use as little volume of the dissolution medium as possible.

(D) Temperature of dissolution medium

In general, the temperature of the dissolution medium is maintained at 37 ± 5 °C for the dissolution test of oral solid dosage forms.

(4) Dissolution test of a poorly soluble drug product

(A) Impact of pH changes of the dissolution medium to the dissolution rate

The dissolution tests are performed using water, pH 1.2 dissolution medium, pH 4.0/4.5 dissolution medium, and pH 6.8 dissolution medium, etc., at a faster rotational speed (e.g. rotational speed of 120 rpm for the basket method and 75 rpm for the paddle method). After starting the dissolution test, take a portion of the dissolution medium at an appropriate time point to measure the dissolution rate of the drug, and plot a graph with the results of dissolution test. The dissolution medium is selected based on the results of this test. If the dissolution rate of the drug is below the range of 70-85 %, select the dissolution medium that shows the most rapid dissolution rate among the above media and consider adding a surfactant to it.

(B) Selection of suitable surfactant

The drug-surfactant interaction is influenced by the physicochemical properties of the drug and the surfactant. Therefore, for a given drug, conduct the test on various surfactants such as non-ionic, anionic, and cationic as follows. In this case, it is recommended to set the concentration of surfactant to approximately 2% at first; the types of surfactants are as follows:

- Anionic surfactant: Sodium lauryl sulfate (SLS)
- Cationic surfactant: Cetyltrimethyl ammonium bromide (CTAB)
- Non-ionic surfactants: Polysorbates 80, 40, and 20, lauryl dimethylamine N-oxide (LDAO)

(C) Determination of surfactant concentration

When the dissolution test is completed within the specified time (within 2 hours or 6 hours), select the surfactant that shows a sufficient dissolution rate at the lowest concentration. To accomplish this, carry out the dissolution test while gradually decreasing the concentration of surfactant from 2.0% to 1.5, 1.0, 0.75, 0.5, and to 0.25%, etc.

(5) Dissolution test for delayed-release preparations

In order to determine the role of the gastric-resistant coating agent or the effect of diffusion possibility of the drug through coating, carry out the dissolution test under two conditions: an acidic condition (i.e., acid stage) and a buffer condition (i.e., buffer stage) which is similar to the environment of the intestinal tract.

As an example of the dissolution test for delayed-release preparations listed in the official compendium, it is performed with 0.1 mol/L hydrochloric acid under acidic conditions at first, and then another test is performed with the pH 6.8 phosphate buffer solution under buffer conditions. At this time, the following methods can be used.

(A) Conduct a dissolution test at 37 ± 0.5℃ for 2 hours with 750 mL of 0.1 mol/L hydrochloric acid. After 2 hours, take a portion of the dissolution medium to measure the drug concentration. To the above 0.1 mol/L hydrochloric acid, add 250 mL of 0.2 mol/L sodium phosphate solution pre-set to 37 ± 5 °C. If necessary, adjust the pH of the medium to 6.8 using 2 mol/L hydrochloric acid or 1 mol/L sodium hydroxide solution before the dissolution test (Adding 0.2 mol/L sodium phosphate solution and adjusting pH should be completed within 5 minutes).

(B) Conduct a dissolution test at 37 ± 0.5 ℃ for 2 hours with 1 L of 0.1 mol/L hydrochloric acid. After 2 hours, take a portion of the dissolution medium and discard the remaining medium in the vessel. Add 1 L of pH 6.8 phosphate buffer solution pre-set to 37 ± 5 °C in this dissolution vessel, and then continue with the dissolution test. (Prepare the pH 6.8 phosphate buffer solution by adding 250 mL of 0.2 mol/L sodium phosphate to 750 mL of 0.1 mol/L of hydrochloric acid. If necessary, adjust the pH of the medium to 6.8 by using 2 mol/L hydrochloric acid or 1 mol/L sodium hydroxide solution.).

Alternatively, add 1 L of pH 6.8 phosphate buffer solution to a new dissolution vessel, and continue the dissolution test by inserting the paddle or the rotating basket taken out of the dissolution vessel with 0.1 mol/L hydrochloric acid.

(6) Two-stage dissolution test for gelatin capsules

Two-stage dissolution test is recommended for hard- or soft-gelatin capsules. Carry out the test for the first stage using the typical dissolution medium as described above. When the dissolution rate is too slow to be tested properly, add an enzyme before proceeding to the second stage of the dissolution test. The enzyme used is purified pepsin or pancreatin, which is selected according to the pH of the test solution. Add pepsin in case of water or an acidic dissolution medium with a pH of less than 6.8, and the activity of pepsin should be not more than 750,000 units per 1 L of the dissolution medium. In this case, the amount of pepsin to be added should be not more than 3.2 g per 1 L of the medium. Add pancreatin in case of the dissolution medium with a pH of more than 6.8, and the activity of pancreatin should be not more than 1,750 units per 1 L of the dissolution medium. In this case, the amount of pancreatin to be added should be less than 50 mg per 1 L of the medium. If the water is used as the dissolution medium at the first stage, 0.1 mol/L hydrochloric acid solution containing pepsin or pH 6.8 phosphate buffer solution containing pancreatin may be used as the medium for the second stage. However, it should be noted that if the dissolution medium used at the first stage contains a high concentration of a surfactant, such as sodium lauryl sulfate, denaturation of the enzyme may occur due to the surfactant during the second stage test.

5. Dissolution Specifications

A. Conventional release dosage forms

There are two approaches for setting the dissolution specifications for conventional release dosage forms.

(1) Single-point specifications:

It is typically applied to highly soluble drug products (Biopharmaceutics Classification System (BCS) Classes I and III) for the purpose of routine quality control (e.g., “When tested according to the following test method, more than 80% of acetaminophen should be dissolved within 30 minutes.”). The single-point specifications may be set for the dissolution test of a drug product in which more than 80% of the drug is dissolved in 20 to 30 minutes, even when there is a lag time (mainly caused by film-coating or capsules) after the initiation of dissolution. It is not necessary to attempt to construct *in vitro-in vivo* correlation in the development stage of such drug products.

(2) Two-point specifications:

Two-point specifications: It is typically applied to poorly soluble products (BCS Classes II and IV) for the purpose of routine quality control. The two-point specifications are usually required for a drug product with a dissolution time of at least 45 minutes. In general, the two-point specifications are more useful than the single-point specifications.

B. Delayed-release preparations

The dissolution specifications are set to not more than 10% at the acid stage, but to the levels specified for conventional immediate release dosage forms at the buffer stage.

C. Extended-release preparations

The dissolution specifications should be set using at least three time points. In this case, these time points should represent the initial, middle, and end points of the dissolution test. The average dissolution rate at the end point should reach 80%, if possible.

The initial time point (usually 1 to 2 hours after the start of the dissolution test) is selected to investigate whether the drug is released at once; in general, about 20 to 30% of the drug should be dissolved at the initial time point. The time point when 50% of the drug is dissolved is selected as the middle point, while the time point when 80% of the drug is dissolved is selected as the end point, and the majority of the drug should be dissolved by the final point. However, in cases where the average dissolution rate does not reach 80% by the end of the test, the time point when the dissolution rate remains unchanged is set as the end point of dissolution test.

Conventional release dosage forms, delayed-release preparations, and extended-release preparations should always conform to the dissolution specifications within expiry date.

6. Approaches to Setting the Dissolution Specifications

In general, the dissolution specifications are set according to the following procedures. However, in cases where more appropriate method is available or the characteristics of the drug or the dosage form are different, other suitable dissolution test conditions (e.g., dissolution apparatus, rotational speed, dissolution medium, etc.) may be adopted in the test.

A. Selection of test specimen

(1) Test specimens should be selected from a lot that has been used for clinical trials, bioavailability studies, or pharmaceutical equivalence studies (or proven equivalent).

(2) Select a lot within 5% content difference of the indicated amount of the drug or within 5% of the content difference between the reference drug and the test drug.

B. Stage 1 (preliminary test)

(1) Number of test specimens

Perform a dissolution test using six test specimens of lots selected as directed in the previous section.

(2) Interval of taking dissolution solution

Take the dissolution solution at appropriate intervals (e.g., typically at 10, 15, 30, 45, 60, 90, and 120 minutes) up to 2 hours in the test. However, the test may be terminated at a point when the final dissolution rate reaches 85% or more.

(3) Conditions for dissolution test

(A) Dissolution apparatus

1) The paddle method described in the section of Dissolution Test in the Korean Pharmacopoeia is preferentially applied.

2) If the paddle method is hard to apply due to agglomeration of particles caused by disintegration of test specimens at the bottom of vessel, use other methods, such as the basket method.

3) A sinker may be used if good reproducibility results cannot be obtained because of the floating specimens.

(B) Dissolution medium

1) pH 1.2: the first fluid under the disintegration described in the Korean Pharmacopoeia.

2) pH 4.0/4.5: 0.05 mol/L sodium acetate buffer solution* 0.05 mol/L sodium acetate buffer solution*: a mixture of 0.05 mol/L acetic acid solution and 0.05 mol/L sodium acetate solution (82:28). However, the pH and composition of the dissolution medium may be modified depending on the pKa of the drug.

3) pH 6.8: the second fluid under the disintegration described in the Korean Pharmacopoeia.

4) Water

5) Dissolution medium containing a surfactant: A surfactant can be added to formulations with a dissolution rate of less than 85% in all dissolution medium in 1) to 4) above.

(C) Rotational speed

In the paddle method, apply 50 rpm at first, and then increase the speed to 75 rpm if the rate of dissolution is slow. The

rotational speed may be set to100 rpm or higher in some cases, but more than 150 rpm is not recommended.

(4) Selection of dissolution test conditions

(A) Conventional immediate release dosage forms

Select test conditions that show a dissolution rate in the range of 70-85%. In general, test conditions that show too fast a dissolution rate are often poor in discernment, so it is recommended to select a dissolution medium and a rotation speed that show a relatively low dissolution rate (within 1 hour).

(B) Delayed-release preparations

The test conditions are same as described in (A) Conventional release dosage forms above. Refer to the test method in B. Delayed-release preparations in Section 5. Dissolution Specifications. In this case, use the pH 1.2 or pH 6.8 dissolution medium for the test.

(C) Extended-release preparations

At least 3 time points are selected, where 20-30% of the drug is dissolved at the initial time point (1-2 hours), about 50% of the drug is dissolved at the middle time point, and about 80% of the drug at the end point.

C. Stage 2 (main test)

(1) Proceed with the test under the test conditions selected in the Stage 1 (preliminary test).

(2) Number of test specimens

Perform the test with 3 lots, and 12 specimens per lot.

(3) Dissolution specifications

(A) Conventional immediate release dosage forms and delayed-release preparations

1) Time span of test

It should be not more than 1 hour for conventional release dosage forms. For delayed release dosage forms, it should be not more than 2 hours in the acid condition and not more than 1 hour in the buffer condition.

2) Setting the dissolution specifications

- At the time when the dissolution test result graph of the lot showing the intermediate dissolution rate among 3 lots reaches a plateau, a value of about 10% or less than the average dissolution rate is used as the dissolution specification.
- Single-point specifications
  Single-point specifications are applied to drugs which are highly soluble and dissolved quickly (e.g., BCS Classes I or III). The lower limit is set at the time point when the dissolution rate is in the range of 70 to 85%.
- Two-point specifications
  Two-point specifications are applied to drugs which are lowly soluble and dissolved slowly (e.g., BCS Class II). In this case, 15 minutes is generally set as the first time point, and then 30, 45, or 60 minutes as the second time point. In cases where a rapid dissolution may affect the efficacy or adverse effects of the drug, or the drug substance has a narrow therapeutic index (see Appendix 2 Requirements for Bioequivalence Test), two-point specifications are set (if necessary, set both upper and lower limits).

(B) Extended-release preparations

Select at least three time points, where 20-30% of the drug is dissolved at the initial time point (1-2 hours), about 50% of the drug is dissolved at the middle time point, and about 80% of the drug is dissolved or dissolution rate remains unchanged at the end point of dissolution. The dissolution rates at the initial and middle points are set to 'within the average dissolution rate ± 15%' and the dissolution rate at the end point is set to 'within the average dissolution – 10%'.

(C) Perform a dissolution test using the reference drug according to the established specifications (draft) to check the validity of the specifications (draft) including the analysis method.

7. How to Prepare the Validation Data for Dissolution Test

A. Dissolution medium

Briefly summarize the reason for selecting the dissolution medium.

B. Dissolution test procedure

(1) Describe the following items for the dissolution test and the analysis methods.

- Dissolution apparatus
- Preparation of the standard solutions
- Preparation of the test solutions: Collection time of the test solution, dilution, filtration (if there is a filtration procedure involved, check the recovery rate.)
- Analytical procedure for dissolution medium (e.g., HPLC etc.)
- Equation used for the calculation
- Dissolution specifications

(2) Submit chromatograms or spectra of the blank test solution, the standard solution, and the test solution.

C. Validation

Describe the dissolution test process and validation of the analysis method.

(1) Data related to the stability of the test solution during the analysis

(2) Analytical procedure

Perform the analysis according to the 'Guideline of Validation of Analytical Procedures for Pharmaceuticals, etc.'.

- Range
  : It should demonstrate the linearity, accuracy, and precision within the dissolution specifications ± 20%. (For example, if the dissolution specifications for a extended-release preparation are less than 20% after 1 hour and more than 90% after 9 hours, submit the data demonstrating the linearity, accuracy, and precision in the range of 0-110% of the labeled amount).

Appendix I
Suitability test for dissolution apparatus

Dissolution apparatus used on a regular basis should be tested for its suitability to determine whether it can be properly used for dissolution test through an adequate test procedure. Usually, it is recommended to conduct twice a year, but it should be performed even when the dissolution apparatus is moved or a significant change has been made to it. The suitability test is also required when the test method is changed from the basket method to the paddle method or vice versa. As for the dissolution apparatus that relies on the basket method and the paddle method, the suitability test depends on two types of standard calibrators (USP dissolution calibrators: www.usp.org). At this time, one tablet of the non-dissolvable salicylic acid standard tablet and one tablet of the dissolvable prednisone standard tablet are used. For the test of the salicylic acid tablet, 50 mmol/L phosphate buffer solution of pH 7.4 is used as the dissolution medium, while for the test of the prednisone tablet, water is used as the medium.

1. Suitability test for dissolution apparatuses using the 300 mg salicylic acid standard tablet (Lot No. O)

A. Use 900 mL of 50 mmol/L phosphate buffer solution as

the dissolution medium. In this case, the pH of the dissolution medium should be 7.4 ± 0.05 at room temperature.

B. Immediately after heating the dissolution medium to a temperature of 41℃ while stirring to deaerate, filter it using a filter paper with a pore diameter of 0.45 ㎛ under vacuum. At this time, the filtrate should be stirred during the filtration process. After the filtration process is over, block the flask and stir in a vacuum for another 5 minutes.

C. Place the dissolution medium in the vessel of the dissolution apparatus, while caring not to contain air during the process. Allow the dissolution medium to reach an equilibrium temperature at 37 °C (at this time, it is not recommended to rotate the paddle in order to facilitate the temperature equilibrium of medium).

D. Once the temperature of the dissolution medium reaches 37 °C, start the dissolution test at a rotational speed of 100 rpm.

E. At 30 minutes after the dissolution test, take the dissolution medium, filter it, measure the absorbance at 296 nm to calculate the dissolution rate (%) of salicylic acid. For analytical reasons, the filtrate may be diluted with the dissolution medium.

F. If the dissolution rate (%) is within the range specified in the table below, the dissolution apparatus is considered suitable.

Note: After dissolving the reference standard of salicylic acid in 1% or less ethanol solution, dilute it with the dissolution medium to prepare the standard solutions with a series of known concentrations, and then use them for the analysis. When filtering the dissolution medium, the adsorption of salicylic acid to the filter paper should be investigated.

Table 1. Dissolution specifications of the salicylic acid standard tablet (Lot No. O)

Type of dissolution apparatus	Dissolution rate at 30 minutes after the start of dissolution (%)
Apparatus 1 (Basket method)	23 ~ 29
Apparatus 2 (Paddle method)	17 ~ 26

2. Suitability test for dissolution apparatuses using the 10 mg prednisone standard tablet (Lot No. N).

A. Use 500 mL of water as the dissolution medium.

B. Immediately after heating the dissolution medium to a temperature of 41 ℃ while stirring to deaerate, filter it using a filter paper with a pore diameter of 0.45 ㎛ under vacuum. At this time, the filtrate should be stirred during the filtration process. After the filtration process is over, block the flask and stir in a vacuum for another 5 minutes.

C. Place the dissolution medium in the vessel of the dissolution apparatus, while caring not to contain air during the process. Allow the dissolution medium to reach an equilibrium temperature at 37 °C (at this time, it is not recommended to rotate the paddle in order to facilitate the temperature equilibrium of the medium).

D. Once the temperature of the dissolution medium reaches 37 °C, start the dissolution test at a rotational speed of 50 rpm.

E. At 30 minutes after the dissolution test, take the dissolution medium, filter it, measure the absorbance at 242 nm to calculate the dissolution rate (%) of prednisone. For analytical reasons, the filtrate may be diluted with the dissolution medium.
F. If the dissolution rate (%) is within the range specified in the table below, the dissolution apparatus is considered suitable.

Note: After dissolving the reference standard of prednisone in 5 % or less ethanol solution, dilute it with water to prepare the standard solutions with a series of known concentrations, and then use them for the analysis. When filtering the dissolution medium, the adsorption of prednisone to the filter paper should be investigated.

Table 2. Dissolution specifications of the prednisone standard tablet (Lot No. N)

Type of dissolution apparatus	Dissolution rate at 30 minutes after the start of dissolution (%)
Apparatus 1 (Basket method)	54 - 78
Apparatus 2 (Paddle method)	28 - 54

The dissolution rates (%) indicated in Tables 1 and 2 do not represent the average dissolution rate, but the dissolution rates in 6 or 12 dissolution vessels, respectively. Also, the specified dissolution rates differ depending on standard tablet manufacturing lot No., and they are indicated in Table 3. Furthermore, if a dissolution apparatus is used for dissolution test solely by the paddle method, the suitability of the dissolution apparatus should be tested using only the paddle method.

Table 3. Specifications of the suitability test for the prednisone standard tablet (Lot No. J) and the salicylic acid standard tablet (Lot No. K)

Standard tablet	Dissolution rate measured at 30 minutes after the start of dissolution (%)			
	Apparatus 1 (Basket method)		Apparatus 2 (Paddle method)	
	50 rpm	100 rpm	50 rpm	100 rpm
Prednisone	6 - 23	43 - 63	46 - 59	58 - 69
Salicylic acid	14 - 21	23 - 29	13 - 22	16 - 27

Appendix II
Deaeration of dissolution medium

It is not always necessary to deaerate the dissolution medium before dissolution test of all drug products. If the same results are obtained in the tests using deaerated and non-deaerated dissolution media, the deaeration process is not necessary. However, in certain cases, the air dissolved in the dissolution medium may participate in a chemical reaction with the drug or form air bubbles, thereby affecting the results of the dissolution test. For example, in the case of captopril, air dissolved in the dissolution medium promotes the oxidation of captopril, causing a stability problem. Also, in general, the dissolution rate of a drug is often significantly reduced when many bubbles are present in the rotating basket during the dissolution test. Therefore, in these cases, the air dissolved in the dissolution medium must be removed before the dissolution test begins. Typical drug products that require deaeration of the dissolution medium are listed in Table 4.

Table 4. List of drug products that require deaeration of the dissolution medium

Dosage form	Drug substance
Tablet	Captopril
Tablet	Clonazepam
Tablet	Ergotamine tartrate
Capsule	Etoposide

Capsule	Isotretinoin
Tablet	Meprobamate

1. Deaeration method I

(1) Immediately after heating the dissolution medium to a temperature of 41 ℃ while stirring to deaerate, filter it using a filter paper with a pore diameter of 0.45 ㎛ under vacuum. During the filtration process, continue stirring the filtrate with a magnetic stirrer.

(2) After the filtration is completed, continue stirring for 5 additional minutes (the filtrate can be sonicated instead of being stirred).

(3) Transfer the dissolution medium to the dissolution vessel, and allow the temperature to reach 37 °C. As for poorly soluble drugs, the dissolution medium contains a surfactant, such as polysorbate 80 or sodium lauryl sulfate. In this case, foam may be generated during the above deaeration process, especially during the vacuum filtration stage. Therefore, in the case of dissolution medium for insoluble or prolonged release dosage form, it is recommended to deaerate the dissolution medium without surfactants first, and then dissolve the surfactant later.

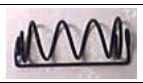
2. Deaeration method II

The other typical method for the deaeration of dissolution medium is helium sparging. The main components of air dissolved in the dissolution medium are oxygen and nitrogen. Helium gas, which is more inert and less water-soluble than these gases, may be injected to purge and remove oxygen and nitrogen from the dissolution medium. In general, a helium sparging rate of 50 mL/min is sufficient for the initial 10-30 minutes; the rate may be reduced to 5-10 mL/min later. The deaeration process by helium sparging generally takes 20-40 minutes. In fact, when helium sparging is used for deaeration of the dissolution medium, it is expected that the relative standard deviation of the dissolution rate is reduced in many cases and that the temperature equilibrium is achieved more rapidly in the dissolution vessel. An alternative way to deaerate the dissolution medium involves sonication of the dissolution medium containing a surfactant under vacuum.

Appendix III
Sinkers used in dissolution test

When performing the dissolution test for capsules using the paddle method, they sometimes do not sink to the bottom of the dissolution vessel while floating on the surface of the dissolution medium. In most cases, capsules may be placed in a sinker made of wire during the dissolution test. Most of the sinkers are made of wire helix products made of wire rope, but various products, including prong sinkers and basket sinkers specified in the Korean Pharmacopoeia, are currently being used for dissolution test.

Table 5. Sinkers used in dissolution test

Types of sinkers	Specifications
	Basket sinker specified in the Korean Pharmacopoeia (2.4 × 1.2 cm)
	Basket sinker (2.6 in radius × 1.7 cm)
	Spiral sinker with 6 coils (typically 2.5 × 1 cm)
	Spiral sinker with 4 coils (typically 2.5 × 1 cm)
	Time-release type sinker (3.8 × 2.7 cm; the size of the clamp is 5 mm × 7 mm.)
	3-prong sinker

Guideline of Limits for Residual Solvents of Pharmaceuticals
의약품 잔류용매 기준 가이드라인

1. Introduction

The objective of this guideline is to recommend acceptable amounts of residual solvents in pharmaceuticals for the safety of patient. The guideline recommends the use of less toxic solvents and describes levels considered to be toxicologically acceptable for some residual solvents.

“Residual solvent" in pharmaceuticals herein refers to volatile organic compounds that are used or produced in the manufacturing process of drug substances [active pharmaceutical ingredients (APIs) of pharmaceuticals], excipients (components other than APIs), or drug products (finished products that are actually administered, such as tablets, capsules, and suppositories containing APIs). These solvents cannot be completely removed by techniques used in practical manufacturing processes. The selection of an appropriate solvent improves the yield in the synthesis of drug substances, determines physical properties such as crystal form, purity and solubility, and sometimes acts as a decisive factor in the synthesis process. This guideline does not address solvents and solvates deliberately used as excipients. However, in this case, the content of solvents in drug products should also be evaluated and justified.

Since all residual solvents have no therapeutic benefits at all, they should be removed to a level that is compliant with product specifications, Good Manufacturing Practices (GMP), or other quality standards requirements. Drug products should not contain residual solvents above the level recognized by safety data. Solvents to be prohibited for use (Class 1, and Table 1) should be avoided in the manufacturing of drug substances, excipients, or drug products, unless they are clearly justified through the risk-benefit assessment. Solvents associated with relatively low toxicity (Class 2, and Table 2) should have their residual amounts regulated to protect patients from potential adverse reactions. Ideally, less toxic solvents (Class 3, and Table 3) should be used. The complete list of solvents included in this guideline is given in APPENDIX 1.

These lists are not exhaustive, so other solvents may also be used, and added to the list. Recommended values for Classes 1 and 2 or the class of solvents may change based on new safety data. When applying for the approval of a pharmaceutical product containing a new solvent, it is necessary to attach supporting data that can assure the safety of that solvent based on this guideline, ICH Q3A (Impurities in New Drug Substances) or Q3B (Impurities in New Drug Products), or all three guidelines.

2. Scope of the guideline on residual solvents

This guideline applies to residual solvents in drug substances, excipients, and drug products. Therefore, if a solvent is present even after the manufacturing or purification process, a residual solvent test should be conducted. It needs to be performed only on solvents that are used or produced in the manufacturing or purification process of drug substances, excipients, or drug products. Pharmaceutical manufacturers may test the drug product or use a cumulative method to calculate the content of residual solvents in the product by summing them in its individual ingredients used in the preparation of the product. If the calculation results are equal to or below the recommended limit values in this guideline, test of residual solvents in drug product is not required. If, however, the calculated value is above the recommended limit value, the drug product should be tested to determine whether the residual solvent content has been reduced below the permitted level during the formulation process. And if a solvent is used during its manufacturing process, the drug product should also be tested.

This guideline does not apply to new drug substances, new excipients, or new drug products used at the clinical trial stages.

This guideline applies to pharmaceutical products of all dosage forms and routes of administration. For the product with a short-term administration (not more than 30 days) or topical application, higher residual solvent levels may be permissible if the justification is recognized in all cases.

Additional information on residual solvents is provided in APPENDIX 2.

3. General principles

A. Classification of residual solvents by risk assessment

The term ‘‘tolerable daily intake’’ (TDI) is used by the International Program on Chemical Safety (IPCS) to define the exposure limits of toxic chemicals. The term ‘‘acceptable daily intake’’ (ADI) is used by the World Health Organization (WHO) and other international health authorities and relevant organizations. In order to avoid confusion arising from different values for ADIs for the same substance as established by organizations such as the WHO, this guideline defines and uses a new term called “permitted daily exposure” (PDE) to refer to the acceptable intake of residual solvents derived from pharmaceutical sources.

In this guideline, residual solvents are classified into three classes as follows by their potential risk to human health, and their common names and structural formulas are presented in APPENDIX 1.

Class 1: Solvents to Be Prohibited for Use
Solvents that are known or strongly suspected to be carcinogenic to humans and pose environmental hazards.

Class 2: Solvents to Be Regulated for their Residual Levels
Solvents that do not show genotoxicity but carcinogenicity in animal testing; that show irreversible toxicity other than neurotoxicity or teratogenicity, etc.; and that are suspected of serious but reversible toxicities.

Class 3: Solvents with Low Toxic Potential
Solvents that are considered to have low toxic potential to humans. No exposure limit is needed for health issues. Class 3 residual solvents have PDEs of more than 50 mg/day.

B. Procedure for setting exposure limits

The procedure used to set daily exposure limits for residual solvents is presented in APPENDIX 3. The toxicological data used in determining exposure limits are published in Pharmeuropa, Vol. 9, No. 1, Supplement, April 1997.

C. Options for setting limits of Class 2 Solvents

Two options are available to determine concentration limits for class 2 residual solvents.

Option 1: Use the concentration limits indicated in ppm in Table 2. These values are calculated through equation (1) below, assuming a daily administered dose of 10 g.

Equation (1):

$$\text{Concentration (ppm)} = \frac{1{,}000 \text{ x PDE}}{\text{Dose}} \qquad (1)$$

Here, PDE is indicated with mg/day, and the dose is g/day.

These concentration limits apply to all drug substances, excipients, and drug products. Therefore, this option is applied if the daily administered dose is unclear or inconsistent. If the concentration of all drug substances and excipients meet the limits given in Option 1, then these components may be used in any proportion. No further calculation is necessary if the daily administered dose does not exceed 10 g. For products with a daily administered dose greater than 10 g per day, Option 2 should be applied.

Option 2: It is not necessary for each component of the drug product to comply with the limits indicated in Option 1. The PDE expressed in mg/day as stated in Table 2 can be used with the known maximum daily administered dose to determine the concentration of residual solvent allowed in a drug product, through equation (1) above.

If it can be demonstrated that the residual solvent content of each component in the drug product has been reduced to the calculated minimum value, such a concentration limit may be acceptable. The concentration limits should be realistic considering the precision of the analysis, manufacturing capability, and deviations that may occur in the manufacturing process, and also reflect the current standards of pharmaceutical manufacturing.

To apply Option 2, the total amount of residual solvents present in each component of the drug product should be such that the sum of the daily exposure to solvents remains below the permitted daily exposure (PDE) limit.

The following is an example of applying Options 1 and 2 to determine the residual acetonitrile amount in the drug product. The permitted daily exposure (PDE) for acetonitrile is 4.1 mg/day (with a concentration limit of 410 ppm in Option 1).

The maximum daily administered dose of this drug product is 5.0 g, and it contains two types of excipients. The estimated value of acetonitrile content, obtained from the composition of this drug product through calculation, is as follows (equivalent to the maximum value among the actually achievable values):

Component	Amount in formulation	Acetonitrile content	Daily exposure
Drug substance	0.3 g	800 ppm	0.24 mg
Excipient 1	0.9 g	400 ppm	0.36 mg
Excipient 2	3.8 g	800 ppm	3.04 mg
Drug product	5.0 g	728 ppm	3.64 mg

Excipient 1 meets the concentration limit in Option 1, but the drug substance, excipient 2, and drug product do not meet it. By the way, this drug product meets 4.1 mg of permitted daily exposure (PDE) in Option 2. Thus, in this case, it conforms to the

recommendations of this guideline.

The following is another example using acetonitrile as a residual solvent. The maximum daily administered dose of this drug product is also 5.0 g, and it contains two types of excipients as well. The estimated value of acetonitrile content, obtained from the composition of this drug product through calculation, is as follows (equivalent to the maximum value among the actually achievable values):

Component	Amount in formulation	Acetonitrile content	Daily exposure
Drug substance	0.3 g	800 ppm	0.24 mg
Excipient 1	0.9 g	2,000 ppm	1.80 mg
Excipient 2	3.8 g	800 ppm	3.04 mg
Drug product	5.0 g	1,016 ppm	5.08 mg

The sum of residual solvent contents for each component in this drug product neither satisfies the concentration limit in Option 1 nor the permitted daily exposure (PDE) in Option 2. The manufacturer should conduct tests to check if the level of acetonitrile can be reduced during formulation process. If the acetonitrile level does not decrease below the recommended limit during the formulation process, the manufacturer of drug product should change the process to reduce the level of acetonitrile in the drug product. If all of these steps fail to meet the recommended value in this guideline, the manufacturer could provide a summary of efforts made to reduce the solvent level to the recommended level in the guideline and provide risk-benefit evaluation data indicating the need to use the solvent even though it has more than the recommended value.

D. Analytical methods

Residual solvents are generally analyzed using chromatography, such as Gas Chromatography. As a standardized method to determine the levels of residual solvents, the method described in compendia, such as pharmacopoeia, should be used if possible. However, in special cases, manufacturers are free to select other validated and most appropriate methods. If only Class 3 solvents are present, non-specific methods such as loss on drying may be used.

Validation of analytical methods for residual solvents should conform to the Guideline of Validation of Analytical Procedures for Drugs.

E. Reporting levels of residual solvents

Manufacturers of drugs require information about the content of residual solvents in excipients or drug substances in order to meet the criteria outlined in this guideline. The following statements are examples of information that a supplier of excipients or drug substances could provide to drug manufacturers. The supplier may choose appropriate statements from the following:

- If it is thought that only Class 3 solvents are present, the loss on drying should be no more than 0.5%.
- If it is thought that only Class 2 solvents (X, Y, etc.) are present, all solvents should be less than the concentration limits specified in Option 1 (in this case, the supplier should provide the Class 2 solvent names, such as X, Y).
- If it is thought that both Class 2 (X, Y, etc.) and Class 3 solvents are present, the residual levels of Class 2 solvents should not exceed the concentration limits specified in Option 1, and the residual levels of Class 3 solvents should be no more than 0.5%

If it is thought that Class 1 solvents are present, they should be identified and quantified.

" It is thought to be present" refers to solvents that have been utilized in the final manufacturing step as well as preceding manufacturing steps but cannot be assured to be completely removed by a validated process.

If the residual solvent levels of Class 2 and Class 3 solvents exceed the concentration limits specified in Option 1 or 0.5%, respectively, they should be identified and quantified.

4. Limits of residual solvents

A. Solvents to be prohibited for use

Class 1 solvents should not be used in the manufacturing process of drug substances, excipients, and drug products due to their severe toxicities and deleterious environmental effects. If the use of these solvents is unavoidable in order to manufacture a drug product with significant therapeutic effects, the level for use should be below the concentration limits specified in Table 1, unless otherwise specified. Trichloroethane (1,1,1-trichloroethane) is included in Table 1 because it is a hazardous substance to the environment. The limit of 1500 ppm specified in Table 1 is based on a review of safety data.

Table 1. Class 1 solvents (Solvents to be prohibited for use)

Solvent	Concentration limit (ppm)	Remarks
Benzene	2	Carcinogen
Carbon tetrachloride	4	Toxic and environmental hazard
1,2-Dichloroethane	5	Toxic
1,1-Dichloroethene	8	Toxic
1,1,1-Trichloroethane	1,500	Environmental hazard

B. Solvents to be regulated for their residual levels

The solvents listed in Table 2 are regulated for their residual levels in pharmaceuticals because of their inherent toxicity. The PDEs are indicated up to a unit of 0.1 mg/day, and the concentration limits to a unit of 10 ppm. The values specified in the table do not reflect the analytical method precision required for measurement. Precision should be determined as part of the analytical method validation.

Table 2. Class 2 Solvents in Pharmaceuticals

Solvent	PDE (mg/day)	Concentration limit (ppm)
Acetonitrile	4.1	410
Chlorobenzene	3.6	360
Chloroform	0.6	60
Cumene	0.7	70
Cyclopentyl methyl ether	15	1,500
Cyclohexane	38.8	3,880
1,2-Dichloroethene	18.7	1,870
Dichloromethane	6.0	600
1,2-Dimethoxyethane	1.0	100
N,N-Dimethylacetamide	10.9	1,090
N,N-Dimethylformamide	8.8	880
1,4-Dioxane	3.8	380
t-Butanol	35	3,500
2-Ethoxyethanol	1.6	160
Ethyleneglycol	6.2	620
Formamide	2.2	220

Solvent	PDE (mg/day)	Concentration limit (ppm)
Hexane	2.9	290
Methanol	30.0	3,000
2-Methoxyethanol	0.5	50
Methylbutyl ketone	0.5	50
Methylcyclohexane	11.8	1,180
Methyl isobutyl ketone	45	4500
N-Methylpyrrolidone	5.3	530
Nitromethane	0.5	50
Pyridine	2.0	200
Sulfolane	1.6	160
Tetrahydrofuran	7.2	720
Tetralin	1.0	100
Toluene	8.9	890
1,1,2- Trichloroethene	0.8	80
Xylene[1)]	21.7	2,170

Note 1) Usually 60% m-xylene, 14% p-xylene, 9% o-xylene, with 17% ethyl benzene.

C. Solvents with low toxic potential

Class 3 solvents (Table 3) are less toxic and have a lower risk to human health. Class 3 does not include solvents known as a human health hazard within the levels normally accepted in pharmaceuticals. However, there are no studies to show long-term toxicity or carcinogenicity in most Class 3 solvents. According to the data obtained so far, Class 3 solvents are less toxic in acute or short-term and negative in genotoxicity. If the amount of residual solvent is no more than 50 mg per day (equivalent to 5,000 ppm or 0.5% in Option 1), it may be considered acceptable even without providing supporting data for its justification. Residues higher than this may be permitted if deemed practical and acceptable in relation to the manufacturer's production capability and good manufacturing practices (GMP) for pharmaceutical products.

Table 3. Class 3 solvents (Solvents to be regulated in compliance with GMP for pharmaceutical products or other quality standards)

Acetic acid	Isobutyl acetate
Acetone	Isopropyl acetate
Anisole	Methyl acetate
1-Butanol	3-Methyl-1-butanol
2-Butanol	Methylethyl ketone
Butyl acetate	2-Methyltetrahydrofuran
t-Butylmethyl ether	2-Methyl-1-propanol
Dimethyl sulfoxide	Pentane
Ethanol	1-Pentanol
Ethyl acetate	1-Propanol
Diethyl ether(ether)	2-Propanol
Ethyl formate	Propyl acetate
Formic acid	Triethylamine
Heptane	

Additionally, in case of Class 2 or Class 3 solvents used prior to the last step of a manufacturing process of drug substances, residual solvent limits for the drug substances may not be set if the supporting data demonstrating the absence of solvent residue in the final drug substances is submitted. (Example of submission data) Analytical data from three actual production batches demonstrating non-detectable levels, along with data on quantification limit.

D. Solvents with insufficient toxicological data

The following solvents (Table 4) might lack of interest to manufacturers of excipients, drug substances, or drug products, or have insufficient toxicological data to calculate a PDE. Therefore, manufacturers must provide valid data on the residual levels of these solvents in pharmaceutical products.

Table 4. Solvents with insufficient toxicological data

1,1-Diethoxypropane	Methylisopropyl ketone
1,1-Dimethoxymethane	Petroleum ether
2,2-Dimethoxypropane	Trichloroacetic acid
Isooctane	Trifluoroacetic acid
Isopropyl ether	

5. Glossary

1) Genotoxic carcinogens: Carcinogens that produce cancer by affecting genes or chromosomes.

2) Lowest-observed effect level (LOEL): The lowest dose at which there is a biologically significant increase in frequency or severity of a particular effect when exposed to humans or animals.

3) Modifying factor: A factor determined by the professional judgment of a toxicologist and used to extrapolate actual data to human safety.

4) Neurotoxicity: The property of a substance that can cause adverse effects on the nervous system.

5) No-observed-effect level (NOEL): The highest dose of a substance at which there are no biologically significant increase in frequency or severity of a particular effect when exposed to humans or animals.

6) Permitted daily exposure (PDE): The maximum amount of residual solvent permitted to take per day in pharmaceutical products.

7) Reversible toxicity: The harmful effects that are caused by a certain substance and that disappear after the end of exposure to that substance.

8) Strongly suspected human carcinogen: A substance with no epidemiological evidence of carcinogenesis in humans but positive genotoxicity data and clear evidence of carcinogenesis in rodents.

9) Teratogenicity: The occurrence of morphological abnormalities in a developing fetus when a substance is administered during pregnancy.

APPENDIX 1. List of solvents included in the guideline

Solvent	Other Names	Classification
Acetic acid	Ethanoic acid	Class 3
Acetone	2-Propanone ; Propan-2-one	Class 3
Acetonitrile		Class 2
Anisole	Methoxybenzene	Class 3
Benzene	Benzol	Class 1
1-Butanol	n-Butyl alcohol ; Butan-1-ol	Class 3
2-Butanol	sec-Butyl alcohol ; Butan-2-ol	Class 3
t-Butanol	Tertiary-butyl alcohol ; t-butyl alcohol ; tert-butanol ; TBA	Class 2
Butyl acetate	Acetic acid butyl ester	Class 3
t-Butylmethyl ether	2-Methoxy-2-methyl-propane	Class 3
Carbon tetrachloride	Tetrachloromethane	Class 1
Chlorobenzene		Class 2
Chloroform	Trichloromethane	Class 2
Cumene	Isopropylbenzene ; (1-Methyl)ethylbenzene	Class 2
Cyclopentyl methyl ether	CPME	Class 2
Cyclohexane	Hexamethylene	Class 2
1,2-Dichloroethane	sym-Dichloroethane ; Ethylene dichloride; Ethylene chloride	Class 1
1,1-Dichloroethene	1, 1-Dichloroethylene ; Vinylidene chloride	Class 1
1,2-Dichloroethene	1, 2-Dichloroethylene ; Acetylenedichloride	Class 2
Dichloromethane	Methylene chloride	Class 2
1,2-Dimethoxyethane	Ethyleneglycol dimethyl ether ; Monoglyme ; Dimethyl Cellosolve	Class 2
N,N- Dimethylacetamide	DMA	Class 2
N,N-Dimethylformamide	DMF	Class 2
Dimethyl sulfoxide	Methylsulfinylmethane ; Methyl sulfoxide ; DMSO	Class 3
1,4-Dioxane	p-Dioxane ; [1.4] Dioxane	Class 2
Ethanol	Ethyl alcohol	Class 3
2-Ethoxyethanol	Cellosolve	Class 2
Ethyl acetate	Acetic acid ethyl ester	Class 3
Ethyleneglycol	1,2-Dihydroxyethane ; 1.2-Ethanediol	Class 2
Ethyl ether	Diethyl ether ; Ethoxyethane; 1.1'-Oxybisethane	Class 3
Ethyl formate	Formic acid ethyl ester	Class 3
Formamide	Methanamide	Class 2
Formic acid		Class 3
Heptane	n-Heptane	Class 3
Hexane	n-Hexane	Class 2
Isobutyl acetate	Acetic acid isobutyl ester	Class 3
Isopropyl acetate	Acetic acid isopropyl ester	Class 3
Methanol	Methyl alcohol	Class 2
2-Methoxyethanol	Methyl Cellosolve	Class 2
Methyl acetate	Acetic acid methyl ester	Class 3
3-Methyl-1-butanol	Isoamyl alcohol ; Isopentyl alcohol ; 3-Methylbutan-1-ol	Class 3
Methylbutyl ketone	2-Hexanone ; Hexan-2-one	Class 2
Methylcyclohexane	Cyclohexylmethane	Class 2
Methylethyl ketone	2-Butanone ; MEK ; Butan-2-one	Class 3
Methyl isobutyl ketone	4-Methylpentan-2-one ; 4-Methyl-2-pentanone ; MIBK	Class 3
2-Methyltetrahydrofuran	2-Methyloxolane ; Tetrahydrosylvan ; Tetrahydro-2-methylfuran ; 2-MTHF	Class 3
2-Methyl-1-propanol	Isobutyl alcohol ; 2-Methylpropan-1-ol	Class 3
N-Methylpyrrolidone	1-Methylpyrrolidin-2-one ; 1-Methyl-2-pyrrolidinone	Class 2

Solvent	Other Names	Classification
Nitromethane		Class 2
Pentane	n-Pentane	Class 3
1-Pentanol	Amyl alcohol ; Pentan-1-ol ; Pentyl alcohol	Class 3
1-Propanol	Propan-1-ol ; Propyl alcohol	Class 3
2-Propanol	Propan-2-ol ; Isopropyl alcohol	Class 3
Propyl acetate	Acetic acid propyl ester	Class 3
Pyridine		Class 2
Sulfolane	Tetrahydrothiophene 1.1-dioxide	Class 2
Tetrahydrofuran	Tetramethylene oxide ; Oxacyclopentane	Class 2
Tetralin	1,2,3,4-Tetrahydro-naphthalene	Class 2
Toluene	Methylbenzene	Class 2
1,1,1-Trichloroethane	Methylchloroform	Class 1
1,1, 2-Trichloroethene	Trichloroethene	Class 2
Triethylamine	N,N-Diethylethanamine	Class 3
Xylene[note)]	Dimethybenzene ; Xylol	Class 2

Note) Usually 60% m-xylene, 14% p-xylene, 9% o-xylene with 17% ethyl benzene.

APPENDIX 2. Additional background

1. Environmental regulation of volatile organic solvents

Some residual solvents used in the production of pharmaceuticals are listed as toxic chemicals in the Environmental Health Criteria (EHC) monograph and in the Integrated Risk Information System (IRIS). The objectives of organizations such as the International Programme on Chemical Safety (IPCS), the United States Environmental Protection Agency (US EPA), and the United States Food and Drug Administration (US FDA) include the setting of permitted exposure levels. The ultimate goal is the protection of human health and the environment from the harmful effects that can be caused when chemicals are exposed to the environment for a long time. The establishment of maximum safe exposure limits is generally based on long-term toxicity test data.

When long-term toxicity test data are unavailable, the approaches, such as the use of larger safety factors in toxicology data from shorter-term studies, can be attempted. The approaches outlined in these environmental regulations primarily consider long-term or lifetime exposure of the general public in everyday environments such as ambient air, food, drinking water, and other media.

2. Residual solvents in pharmaceuticals

The exposure limits in this guideline are established based on the methodologies and toxicity data described in the EHC and IRIS monographs. However, the following specific assumptions about the distinct characteristics of residual solvents used in the synthesis and formulation of pharmaceutical products should be taken into account in establishing exposure limits.

1) The pharmaceuticals are used for patients (not the general population) to treat their diseases or for prophylaxis to prevent infection or disease.

2) Although patients are not exposed to most medicines during their lifetime, the hypothesis of lifetime exposure can be applied to reduce the risk to human health.

3) Residual solvents are unavoidable in pharmaceutical production and can often be a part of drug products.

4) Residual solvents should not exceed recommended levels except in exceptional circumstances.

5) Data from toxicological studies used to determine the permitted levels for residual solvents should be tested based on appropriate protocols such as those described for example by OECD, EPA, and the FDA Red Book.

APPENDIX 3. Methods for establishing exposure limits

The Gaylor-Kodell method of risk assessment (Gaylor, D. W. and Kodell, R. L.: Linear Interpolation Algorithm for Low Dose Assessment of Toxic Substance. Journal of Environmental Pathology and Toxicology, 4: 305, 1980) is appropriate for Class 1 carcinogenic solvents. Only in cases where reliable carcinogenicity data are available, extrapolation using mathematical models should be applied in order to establish exposure limits. Exposure limits for Class 1 solvents can be determined through the use of a large safety factor (i.e., 10,000 - 100,000) in the no-observed-effect level (NOEL). Detection and quantification of these residual solvents should be performed by state-of-the-art analytical techniques.

The permitted exposure levels in this guideline for Class 2 residual solvents were established by calculation of PDE values according to the procedures for setting exposure limits in pharmaceuticals stated in US Pharmacopoeia Forum [Pharmacopeial Forum (PF), Nov.–Dec. 1989], and the method adopted by IPCS for Assessing Human Health Risk of Chemicals (Environmental Health Criteria 170, WHO, 1994). These procedures are similar to those used by the U.S. EPA (IRIS) and the U.S. FDA (Red Book) and others. The calculation method of PDE is outlined in this guideline to give a better understanding of the basic for calculation of PDE values. It is not necessary to perform these calculations when using the PDE values presented in Table 2 (Class 2 Solvents in Pharmaceutical Products) in this guideline as they are.

PDE is derived from the no-observed-effect level (NOEL), or the lowest-observed effect level (LOEL), in the most relevant animal study as follows:

$$PDE = \frac{NOEL \times Weight\ Adjustment}{F1 \times F2 \times F3 \times F4 \times F5} \quad (1)$$

The Permitted Daily Exposure (PDE) is preferably calculated based on the No-Observed-Effect Level (NOEL), and if NOEL is not available, the Lowest-Observed-Effect Level (LOEL) can be used. Modified factors proposed herein is for extrapolating the values in animal tests to humans, which include the "uncertainty factors" used in EHC (Environmental Health Criteria 170, World Health Organization, Geneva, 1994), and "modifying factors" or "safety factors" in PF (Pharmacopeial Forum). The assumption of 100% “systemic exposure” is used in all calculations regardless of the route of administration.

The modified factors are as follows:

F1 is a factor to account for extrapolation between species

F1 = 5, in case of extrapolation from rats to humans

F1 = 12, in case of extrapolation from mice to humans

F1 = 2, in case of extrapolation from dogs to humans

F1 = 2.5, in case of extrapolation from rabbits to humans

F1 = 3, in case of extrapolation from monkeys to humans

F1 = 10, in case of extrapolation from other animals to humans

F1 is determined by considering the specific surface area (the ratio of body surface area to body weight) of animals and humans used in the test. Body surface area (S) is calculated as:

$$S = kM^{0.67} \quad (2)$$

where M = body mass, and the constant k is 10. The body weights used in the equation are those shown below in Table 1.

F2 is a factor considering the variation between individuals, and is generally given as 10 in all organic solvents. In this guideline, 10 is used collectively, too.

F3 is a variable applied when the toxicity test period is short.

F3 = 1, in case of a test with at least one-half lifetime (1 year for rodents or rabbits; 7 years for cats, dogs, and monkeys).

F3 = 1, in case of a reproductive toxicology test in which the whole period of organogenesis is covered.

F3 = 2, in case of a 6-month test in rodents, or a 3.5-year test in non-rodents.

F3 = 5, in case of a 3-month test in rodents, or a 2-year test in non-rodents.

F3 = 10, in case of a test with shorter duration.

In all cases, the higher factor has been used when the test period are between the above reference periods, e.g. a factor of 2 has been used in a 9-month rodent test.

F4 is a factor that may be applied in cases of severe toxicity, e.g. non-genotoxic carcinogenicity, neuro-toxicity, or teratogenicity. In reproductive toxicology test, the following factors are used:

F4 = 1, in case of fetal toxicity associated with maternal toxicity.

F4 = 5, in case of fetal toxicity without maternal toxicity.

F4 = 5, in case of teratogenic effect with maternal toxicity.

F4 = 10, in case of a teratogenic effect without maternal toxicity.

F5 is a variable applied if the no-effect level NOEL is not established. When only the LOEL is available, a factor of up to 10 is used depending on the severity of the toxicity.

The weight adjustment is based on the assumption that an adult human body weight for either sex is 50 kg. The use of this relatively low weight provides an additional safety factor, compared to the standard weights of 60 kg or 70 kg that are often used in this type of calculation. It is considered that the factors for some adult patients who weigh less than 50 kg are all included in safety factors used to determine a PDE. If the residual solvent is present in a drug product specifically intended for pediatric use, an adjustment for a lower body weight would be appropriate.

As an example of application of equation (1), consider a toxicity test of acetonitrile in mice that is summarized in Pharmeuropa, Vol. 9, No. 1, Supplement, April 1997, page S24. The NOEL is calculated to be 50.7 mg/kg/day. The PDE for acetonitrile in this study is calculated as follows:

$$PDE = \frac{50.7mg/kg/day \times 50kg}{12 \times 10 \times 5 \times 1 \times 1}$$

$$= 4.22mg/day$$

In this example,

F1 = 12, in case of the extrapolation from mice to humans.

F2 = 10, in case of the differences between individual humans.

F3 = 5 because the test period is only 13 weeks.

F4 = 1 because no severe toxicity is encountered.

F5 = 1 because the no-effect level is determined.

Table 1. Values used in the calculations in this guideline

Rat body weight	425 g
Pregnant rat body weight	330 g
Mouse body weight	28 g
Pregnant mouse body weight	30 g
Guinea-pig body weight	500 g
Rhesus monkey body weight	2.5 kg
Rabbit body weight (pregnant or not)	4 kg
Beagle dog body weight	11.5 kg
Rat respiratory volume	290 L/day
Mouse respiratory volume	43 L/day
Rabbit respiratory volume	1,440 L/day
Guinea-pig respiratory volume	430 L/day
Human respiratory volume	28,800 L/day
Dog respiratory volume	9,000 L/day
Monkey respiratory volume	1,150 L/day
Mouse water consumption	5 mL/day
Rat water consumption	30 mL/day
Rat food consumption	30 g/day

The equation for an ideal gas, PV = nRT, is used to convert the unit of gas concentration used in inhalation toxicity test from ppm to mg/L or mg/m^3. The example of the rat reproductive toxicity test by inhalation of carbon tetrachloride (molecular weight: 153.84) summarized in Pharmeuropa, Vol. 9, No. 1, Supplement, April 1997, page S9, is as follows:

$$\frac{n}{V} = \frac{P}{RT} = \frac{300 \times 10^{-6}\text{atm} \times 153{,}840\text{mg/mol}}{0.082 \times \text{Latm}/mol \times 298\text{K}}$$

$$= \frac{46.15\text{mg}}{24.45\text{L}} = 1.89\text{mg/L}$$

The relationship 1,000 L = 1 m^3 is used to convert to mg/m^3.

Guideline of Validation of Analytical Procedures for Drugs
의약품등 시험방법 밸리데이션 가이드라인

I. Objective

The purpose of this guideline is to provide detailed guidance on how to conduct a validation of the analytical procedures necessary for the application for approval(report) of manufacturing/import items and quality control of drugs and quasi-drugs according to the Korean Ministry of Food and Drug Safety's regulations on Pharmaceuticals Approval, Notification and Review.

II. Introduction

The objective of validation of an analytical procedure for drugs, etc. is to demonstrate that the procedure, applied in the quality control testing of the drugs, etc. is suitable for the intended purpose.

The purpose of this guideline is to provide guidance on how to establish validation parameters for each analytical procedure. The scope of analytical procedures that are subject to this guideline is as follows:

1) Confirmation test established in the specification for drugs

2) Purity test: Quantitative test and limit test for impurities

3) Quantitative test: Assay for the active ingredient of drug substance or drug product, other specific ingredient(s) in drug products, uniformity of dosage units test or dissolution test

The purpose of the analytical procedure should be clear, since the validation parameters to be evaluated are determined depending on this purpose. Validation parameters include specificity, accuracy, precision, detection limit (DL), quantitation limit (QL), linearity, range, and robustness. Appropriate validation parameters are selected and evaluated for the purpose of each analytical procedure.

Meanwhile, when the manufacturing method of drug substances, the composition of the preparation, and the analytical procedure are changed, revalidation is performed, and the degree of revalidation varies depending on the details and extent of the change.

In addition, validation methods, other than those described in this guideline, may be used, although the appropriateness of the alternative method must be demonstrated.

III. Glossary

1. Analytical Procedure

The analytical procedure refers to the series of detailed test procedures required to perform an analysis. The analytical procedure includes analyte, sample, reference standard reagents

and solutions used in identification tests, the use of analysis equipment, generation of calibration curves, purity test, assay; use of calculation formulas, etc.

- Validation of analytical procedure is the process of confirming the validity of the analytical procedure for quality control of drugs in advance.

- Identification test is the test that identifies analytes in a sample and generally compares the physicochemical characteristics (e.g., spectrum, information obtained from chromatographic methods, and chemical reactivity, etc) of a sample with those of a reference standard.

- Purity test is the test that accurately measures the presence of impurities, such as related substances, heavy metals, and residual solvents content in the sample. There are quantitative test and limit test.

- Assay (Content or potency) test is the test that accurately measures the content or potency of the analyte in the sample. In other words, the assay is used to measure the content of the major ingredient(s) (e.g., active ingredients and bioactive ingredients) or other certain ingredient(s) (e.g., additives such as stabilizers and preservatives) in the drug substance or in the drug product. This also includes the analytical process of dissolution tests.

2. Specificity

Specificity refers to the ability to selectively and accurately assess the analyte substances in a mixed state of impurities, degradation products, matrix, etc. Lack of specificity of an analytical procedure may be compensated by other supporting analytical procedure(s).

3. Accuracy

Accuracy of an analytical procedure refers to the degree to which a measured value is close to a known true value or standard value.

4. Precision

Precision of an analytical procedure refers to the proximity (degree of dispersion) between each measurement values obtained by repeatedly measuring samples taken from a homogeneous sample under specified conditions. Precision may be reviewed at three levels: repeatability, intermediate precision, and reproducibility.

- Repeatability is the precision of measurements obtained by the same analyst under the same operating conditions (e.g., the same apparatus and equipment and the same lot number of reagents, etc.) over a short interval of time within the same laboratory. It is also referred to as intra-assay precision.

- Intermediate Precision refers to the proximity between measurements obtained under the different operating conditions including analysts, experimental dates, apparatus and instruments and lot number of reagents, etc, within the same laboratory.

- Reproducibility refers to the proximity between measurements obtained from the same sample in different laboratories. It is generally applied to collaborative studies using standardized test methods.

5. Detection Limit

Detection limit refers to the minimum detectable amount of the analyte in a sample, and does not necessarily need to be quantifiable.

6. Quantitation Limit

Quantitation limit refers to the minimum amount of the analyte in a sample which can be quantitatively determined with a suitable precision and accuracy. The quantitation limit is a validation parameter a quantitative test used to determine a sample containing a small amount of the analyte, or, in particular, and is used particularly for the determination of impurities and/or degradation products.

7. Linearity

Linearity of an analytical procedure is the ability to obtain linear measurement values within a certain range in proportion to the amount (or concentration) of the analyte in the sample.

8. Range

Range of an analytical procedures is the interval between the lower and upper limit of the amount or concentrations of the analyte in a sample for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy, and linearity.

9. Robustness

Robustness of an analytical procedure is a measure of the capacity to remain unaffected by small, but deliberate variations in method parameters. It provides an indication of its reliability during normal use.

IV. Validation of Analytical Procedures

For the validation of analytical procedures, first, a comprehensive and reliable experimental plan for the appropriateness of the analytical procedure is established, and all data and validation parameters obtained during the validation process are used to evaluate its appropriateness. The calculation formula used to calculate all relevant data and validation parameters obtained from the validation process should be submitted and justified as appropriate. Validation parameters to best set for each analytical procedure such as identification test, purity test, and assay, etc. are shown in Appendix 1.

The physical, chemical, and biological characteristics of the reference standard used during the validation should be clearly described, including purity. The level of purity required for the reference standards depends on the purpose of the analytical procedure.

1. Specificity

The specificity should be assessed in the validation of the identification test, the purity test, and quantitative test. The procedure used to demonstrate specificity depends on the purpose of the analytical procedure.

It is not always possible to demonstrate that an analytical procedure is specific and perfectly distinguishable for a particular analyte.

In this case, a combination of two or more analytical procedures is recommended to sufficiently distinguish the analytes.

A. Identification

The identification test should be able to differentiate between compounds of closely related structures when they coexist. The discrimination ability of an analytical procedure may be confirmed by obtaining positive results (perhaps by comparison with the known reference standard) from samples containing the analyte, and by obtaining negative results from samples which do not contain the analyte. In addition, the identification test may be applied to materials structurally similar to or closely related to the analyte to confirm that a positive response is not obtained. The choice of such potentially interfering materials should be based on sound scientific judgment, with a consideration of the interferences that could

occur.

B. Quantitative tests and Purity tests

For chromatographic procedures, representative chromatograms should be used to demonstrate the specificity, and individual components should be labeled appropriately. Similar considerations should be given to other segregation techniques.

Critical separations in chromatography should be evaluated at an appropriate level to demonstrate that the substances are adequately separated. To demonstrate specificity, the separation limit can be expressed using the resolution of the two components that elute closest to each other.

In cases where a non-specific assay is used, other supporting analytical procedures may be used to demonstrate the overall specificity. For example, in cases where a titration is adopted to assay the drug substance for release, specificity can be proven by conducting and reviewing a purity test appropriate for the assay.

The same approach can also be applied to both quantitative test and purity test.

(1) Reference standards of impurities are available

In cases where, impurities and/or excipients, assay should be specific to the analyte. Practically, specificity can be proven by spiking pure substances (drug substance or drug product) with appropriate levels of impurities and/or excipients and demonstrating that the assay result is unaffected by the presence of these materials (by comparison with the assay result obtained on un-spiked samples).

For the purity test, specificity may be verified by spiking drug substances or drug product with appropriate level of impurities, and demonstrating the separation of these impurities individually and/or from other components in a sample matrix.

(2) Reference standards of impurities are not available

If reference standards of impurities are unavailable, specificity may be proven by comparing the test results of samples containing impurities or degradation with the results measured by other analytical procedures that have already been proven, including the methods specified in *Pharmacopoeia* or other validated analytical procedure (independent procedure). As appropriate, this should include samples exposed to harsh conditions, including light, heat, humidity, acid/base hydrolysis and oxidation.

- For the quantitative test, the two results should be compared.

- For the purity test, the profiles of related substance should be compared.

In order to prove that the peak of the analyte on the chromatogram does not originate from other components, a peak purity test using a diode array or mass spectrometer (MS) as a detector is useful.

2. Linearity

A linear relationship must be confirmed for all ranges specified (refer to Section 3) in the analytical procedure. Linearity can be directly demonstrated in the drug substance by diluting the standard stock solution, and by using a mixture prepared by individually weighing the product components. The latter aspect can be considered when setting the scope.

Linearity should be evaluated visually by graphing it as a function of the concentration or content of the analyte. If the linearity is confirmed, measured results should be evaluated using statistical methods such as calculation of a regression line using the least squares method. In some cases, to obtain linearity between assays and sample concentration, the test data may need to be subjected to a mathematical transformation prior to the regression analysis.

Data which can be obtained from the regression line are helpful when evaluating the level of linearity mathematically.

The correlation coefficient, y-intercept, slope of the regression line and residual sum of squares should also be recorded. Analyzing the difference between the actual values and the predicted values on the regression line is also helpful in evaluating linearity.

Some analytical procedures, such as immunoassays, do not show linearity even after mathematical conversion. In this case, the analysis results are expressed as an appropriate function (theoretical or approximate equation) of the concentration (or amount) of an analyte in a sample.

To demonstrate linearity, a minimum of 5 concentrations is recommended. Other approaches should be justified.

3. Range

The specified range is normally determined from the linearity evaluation and depends on the purpose for which the analytical procedure is applied. The validity of the range is proven by confirming the linearity, accuracy and precision of the analytical procedure using samples containing amounts of the analyte within or at the lower and upper concentrations limits of the specified range.

The following minimum specified ranges should be considered:

A. Assay of drug substances or preparations

- Typically, 80 – 120% of the test concentration.

B. Formulation uniformity test

- At least70 to 130% of the test concentration, unless a wider more appropriate range based on the nature of the dosage form (e.g., metered dose inhalers), is justified.

C. Dissolution Test

- +/- 20 % of the specified range indicated in the dissolution specification of the Specifications and Test Procedures of the drug product.

- For example, if the specifications of a controlled release product is20% after 1 hour, and up to 90% after 24 hours, the validated range would be 0 to 110% of the labeled amount.

D. Quantitative testing of impurities

- From the reporting level of the related substance to 120% of the specification.

E. For impurities known to be unusually potent or produce toxic or unexpected pharmacological effects, the detection/quantitation limit should be set in consideration of the level at which the impurities must be controlled. For validation of impurity test procedures carried out during the development, it is recommended to evaluate the range around the expected limit of the related substances.

F. If assay and purity test are performed simultaneously in one test and only samples containing 100% of the indicated amount of the active ingredient are used, linearity must be evaluated from the reporting level of the impurities to 120% of the assay specification.

4. Accuracy

Accuracy must be proven over the entire range specified by the analytical procedure.

A. Assay

(1) Drug Substance

Several methods can be used to determine accuracy:

(a) When the true value is known

Apply an analytical procedure to the analyte of known purity (e.g., reference standard)

(b) When another analytical procedure with known

accuracy is available

Compare the results measured by the analytical procedure to be validated with results from an existing analytical procedure for which the accuracy is known.

(c) Accuracy may be inferred once precision, linearity and specificity have been proven.

(2) Drug product

Several methods can be used to evaluate accuracy:

(a) A known amount of the drug substance being analyzed is added to the mixture of pharmaceutical ingredients and is used as a sample to apply the analytical procedure to be validated.

(b) In cases where it is impossible to obtain samples of all drug product components,

1) Method of adding a known amount of analyte to the preparation

2) Comparing the results measured using an analytical procedure intended to validate a drug with the results of an existing test method with known accuracy.

(c) Accuracy may be inferred once precision, linearity and specificity have been proven.

B. Impurities (Quantification)

Accuracy should be assessed by quantifying samples such as drug substances/drug product spiked with a known amount of impurities.

If it is impossible to obtain samples of certain impurities and/or degradation products, results can be compared with the results of an existing test method of known accuracy. The response factor of the drug substance can be used.

The method used to determine individual or total impurities such as weight/area percent, must be clearly stated in all cases with respect to the major analytes.

C. Recommended Data

Accuracy should be assessed from the results of repeating all operations of the analytical procedure at least 9 times for at least 3 concentrations, including the specified range (e.g., 3 concentrations/3 replicates each of the total analytical procedure).

Accuracy should be expressed as recovery rate (%) by the assay of a known added amount of the analyte in a sample or as the difference between the mean and the accepted true value together with the confidence intervals.

5. Precision

Precision evaluation is included when validating assay and quantitative determination of impurities.

A. Repeatability

Repeatability should be assessed using:

(1) at least 9 times for concentrations including the specified range for the procedure (e.g., 3 concentrations/3 replicates each).

(2) at least 6 times at 100% of the test concentration.

B. Intermediate Precision

The extent to which intermediate precision should be established depends on the circumstances under which the procedure is intended to be used, and the impact of various factors on the precision. Representative variables that require evaluation include experiment date, analysts, equipment, etc. It is not necessary to test each of these factors separately.

C. Reproducibility (Interlaboratory precision)

Reproducibility is assessed through an inter-laboratory trial. Reproducibility should be considered if it is necessary to standardize analytical procedures; such as listing analytical procedures in pharmacopoeia. Interlaboratory precision data are not included in the data submitted for approval.

D. Recommended Data

Standard deviation, relative standard deviation (coefficient of variation) and confidence interval should be recorded for each precision evaluation data.

6. Detection Limit

Depending on whether the procedure is instrumental or not, several methods are available to determine the detection limit. Approaches other than those listed below may be used.

A. Based on Visual Evaluation

Instrumental analysis methods as well as non-instrumental test methods can be visually evaluated.

The detection limit is determined by the analysis of samples with a known concentration of the analyte and by establishing the minimum level at which the analyte can be reliably detected.

B. Based on Signal-to-Noise

This approach can only be applied to analytical procedures which exhibit baseline noise. The signal-to-noise ratio is determined by comparing measured signals from samples with known low concentrations of the analyte with those of blank samples, and establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio of 3:1 or 2:1 is generally considered acceptable for estimating the detection limit.

C. Based on the Standard Deviation of the Response and the Slope of the calibration curve

The detection limit (DL) may be determined using the following formula:

$$DL = 3.3 * \sigma / S$$

where

σ = the standard deviation of the response
S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte. There are several ways to calculate the standard deviation σ, as follows:

(1) Based on the standard deviation of blank samples

The background response level of the analytical procedure is measured by analyzing an appropriate number of blank samples and calculating the standard deviation of these responses.

(2) Based on the calibration curve

A calibration curve should be created using samples containing an analyte in the range of DL. The residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines may be used as the standard deviation σ.

D. Data to submit

The detection limit and the method used for determining the DL should be described. If DL is determined based on visual evaluation or based on signal-to-noise ratio, a chromatogram is submitted to prove its validity.

When the estimated value for the detection limit is obtained by calculation or extrapolation, this estimate may subsequently be validated by analyzing a suitable number of samples prepared at or near the detection limit concentration.

7. Quantification Limit

Several methods can be used to determine the quantification limit, depending on whether the procedure is non-instrumental or instrumental. Approaches other than those listed

below may be acceptable.

A. Based on Visual Evaluation

Visual evaluation may be used for instrumental methods as well as non-instrumental methods.

The quantitation limit is generally determined by analyzing samples with a known concentration of the analyte and by setting the lowest concentration level at which the analyte can be quantified with acceptable accuracy and precision.

B. Based on Signal-to-Noise Approach

This approach can only be applied to analytical procedures that exhibit baseline noise. The signal-to-noise ratio is determined by comparing measured signals from samples with known low concentrations of the analyte with those of blank samples, and by setting the lowest concentration at which the analyte can be reliably quantified. A typical signal-to-noise ratio is 10:1.

C. Based on the Standard Deviation of the Response and the Slope

The quantification limit (QL) may be determined using the following formula:

$$QL = 10 * \sigma / S$$

where,

σ = the standard deviation of the response
S = the slope of the calibration curve.

The slope S may be estimated from the calibration curve of the analyte. There are several measurement methods that can be used for calculating σ, and examples of these are as follows:

(1) Based on the standard deviation of blank samples

The background response level of the analytical procedure is measured by analyzing an appropriate number of blank samples and calculating the standard deviation of these responses.

(2) Based on the calibration curve

A specific calibration curve should be prepared using samples containing analytes in the range of QL. The residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines may be used as σ.

D. Data to submit

The quantification limit and the method used to obtain the quantification limit should be presented.

The limit should be subsequently validated through the analysis of a suitable number of samples prepared at a concentration at or near the limit of quantification.

8. Robustness

Robustness should be evaluated during the development phase, and the evaluation method varies depending on the type of the procedure. It should demonstrate the reliability of the analysis when the analysis conditions are intentionally changed.

If measurements are susceptible to variations in analytical conditions, the analytical conditions should be suitably controlled, or a precautionary statement should be included in the procedure. By assessing robustness, a set of parameters regarding system suitability (e.g., resolution test) is established. By verifying these parameters, it is possible to ensure the validity of the analytical method in routine analysis.

Representative variable factors are as follows:

A. Variable Factors Common to Different Analytical Procedures

- Stability of analytical procedures
- Extraction time

B. Typical Variable Factors for Liquid Chromatography

- pH in a mobile phase
- Variations in mobile phase composition
- Change of columns (different lots and/or suppliers)
- Temperature
- Flow rate

C. Typical Variables Factors for Gas Chromatography

- Change of columns (different lots and/or suppliers)
- Temperature
- Flow rate

9. System Suitability Testing

System suitability testing is an integral part of many analytical procedures. It is based on the concept that test equipment, electronics, analytical procedures and samples to be analyzed constitute an integral system that can be evaluated as such. Parameters of system suitability test to be established for a particular procedure vary depending on the type of procedure being validated. Refer to the Pharmacopoeias for additional information.

Appendix 1) Validation parameters to be assessed per test procedure

Type of analytical procedure / Validation parameter	Identification	Purity test		Assay - Dissolution test (for assay only) - Content/ Potency test
		Quantitative test	Limit test	
Accuracy	−	+	−	+
Precision				
Repeatability	−	+	−	+
Intermediate precision	−	+(1)	−	+(1)
Specificity(2)	+	+	+	+
Detection limit	−	−(3)	+	−
Quantitation limit	−	+	−	−
Linearity	−	+	−	+
Range	−	+	−	+

- indicates that this characteristic is not normally evaluated
+ indicates that this characteristic is normally evaluated

(1) If the interlaboratory precision (reproducibility) is evaluated, intermediate precision is not required.

(2) If specificity cannot be proven with one analytical procedure, other supporting analytical procedure(s) can be used to prove specificity.

(3) It may be needed in some cases.

Immunoblot Analysis
면역 블롯 분석법

Immunoblot analysis is defined as any method in which an

antibody is used for detection of one or more analytes (e.g., proteins, polysaccharides) that has been transferred to a test membrane surface. Immunoblot methods are typically classified by whether electrophoretic segregation occurs as a part of the immunoblot procedure. Electrophoretic segregation is based on molecular weights and charge differences of a population of molecules. An example of immunoblot analysis involving electrophoretic segregation is the Western blot. Another approach for immunoblot analysis is to perform molecule detection using an antibody without prior electrophoretic segregation. Examples of this nonelectrophoretic type of approach are the slot or dot blot (slot/dot). The scope of this chapter includes only those methods in which an antibody is used for the detection of a molecule bound to a membrane.

ASSAY SELECTION

1. Nonelectrophoretic Assay (Slot/Dot Blot)

The slot/dot blot method is a simplified, nonelectrophoretic method in which a mixture containing the analyte(s) for detection is first applied directly to the membrane using a vacuum manifold machined to contain regularly spaced rectangular slots. The slot/dot blot method is faster and simpler because there is no electrophoretic segregation of the multiple, individual analytes that may be present in the mixture. For these reasons, it can be readily adapted for automated analysis of multiple samples, for which a number of systems are commercially available, but it offers no information about the molecular weight and only limited information regarding the quantity of sample. Although it can be set up in a quantitative format, the method usually is used to produce a qualitative result, e.g., confirming identity by demonstrating the presence or absence of specific antigens by means of an immunocomplex detection system. After the analytes are bound to the membrane and unbound sites are blocked, analysts use a detector antibody to determine the presence or absence of the analyte or analytes of interest. The uniform shape of the slot blot and its greater surface area for analyte binding make it better suited than the dot blot for quantitative applications and analysis by densitometry.

2. Electrophoretic Assay

Electrophoretic blotting methods (commonly called Western blots) are widely used for analyzing mixtures of proteins. The Western blot is a powerful tool to study the identification, relative concentration, relative molecular weight, and posttranslational modifications of specific proteins. In Western blots, the proteins of the sample are separated using gel electrophoresis. Protein segregation may be based on molecular weight alone or on isoelectric point (pI) and molecular weight. Proteins migrate either in one dimension (1D) or in two dimensions (2D) through a gel. When proteins are separated by their molecular weights, the smaller proteins migrate faster and separate according to molecular weight. When analysts use a 2D gel, proteins are separated according to pI in the first dimension, and then according to their molecular weights in the second dimension. After segregation, the proteins are transferred to a membrane, the membrane is blocked to avoid nonspecific binding of subsequent assay reagents, and the protein of interest is detected using specific antibodies.

A bound antibody can be detected by different methods, including chromogenic assay, fluorescence detection, chemiluminescent detection, and radioactive detection. Upon detection of the protein(s) of interest, immunoblot quantitation can be indirectly performed by densitometry.

A. 1D Electrophoresis

In 1D electrophoresis, individual proteins or groups of proteins are separated by molecular weight for further analysis by Western blot. Using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), proteins migrate in response to differences in electrical charge through a 3D network of fibers and pores. The network is formed as the bifunctional bisacrylamide, or other cross-linker, cross-links adjacent polyacrylamide chains to form a gel. The combination of gel pore size and protein characteristics determines the migration of proteins. Separated proteins are detected subsequently by Western blot analysis using antibodies specific to the target proteins. By means of Western blot analysis, a test sample can be compared to a standard, and the appearance of degradants and impurities specifically related to the target proteins can be monitored if the detection antibody can still recognize the altered forms of the protein. Although a high level of sensitivity can be achieved by this approach, segregation of individual proteins at similar molecular weights may not be achieved. If analysts must probe individual proteins at similar molecular weights, 2D segregations may be required.

B. 2D Electrophoresis

In 2D electrophoresis, individual proteins or groups of proteins are separated in the first dimension by isoelectric focusing (IEF; charge) and in the second dimension by electrophoresis in the presence of SDS (molecular weight). Separating proteins this way allows information to be obtained not only about molecular weight, as in 1D gels, but also about the charge of a protein. Two-dimensional gels are a useful choice for resolving complex mixtures and for assessing protein antibody specificity (e.g., evaluation of host cell proteins).

3. Membrane, Reagent, and Detection Options

A. MEMBRANES

Generally, both nitrocellulose and polyvinylidine fluoride (PVDF) membranes are used for immunoblot methods. For cost considerations, nitrocellulose membranes are often preferred over PVDF membranes for slot/dot blots (or vacuum blotting), but due to their greater mechanical strength, PVDF membranes should be considered if stripping and reprobing are required.

B. Blocking Reagents

Following transfer or binding of protein to membranes, the unoccupied binding sites on the membranes must be blocked to prevent nonspecific binding of subsequent reagents. Most detection probes are proteins that also can bind to the membrane. Failure to appropriately block the membrane sites can result in nonspecific binding and high background. A number of blocking reagents are available, including gelatin, nonfat milk, and bovine serum albumin (BSA). Proteins should be unrelated to the antigens used in the study. Because these reagents often have lot-to-lot variability, they may require qualification. They must be evaluated with the detection system selected and optimized using that detection system for minimal background with no loss of signal. If the blocking reagent is derived from a biological source, it must not contain trace levels of the protein under measurement, because the latter can increase the background.

C. Methods of Detection

Immunological detection of analytes in any type of immunoblot can be direct or indirect. The choice of format depends on a combination of the level of sensitivity required and the quality of the antisera available. For identity or product detection, sensitivity usually is not critical, and direct detection via a conjugated antibody is commonly used, which often

simplifies and shortens the time required to execute the method. Alternatively, indirect detection, usually by the use of a conjugated anti-species reagent, can be used to improve sensitivity. On some occasions, the analyte being detected is actually an antibody, as in the case of a monoclonal antibody that is being developed as a drug. In this case, antibodies specific for the antibody (e.g., anti-idiotypic antibodies) can be used.

1) Primary antibody: The primary antibody is selected based on its specificity for the analyte or protein. Although polyclonal anti-sera can offer a broad range of detection against a potentially large set of epitopes, an unwanted cross-reaction resulting in decreased specificity may occur. If this cannot be overcome by assay optimization, a monoclonal antibody or groups of selected monoclonal antibodies can be used. Monoclonal antibodies are often advantageous for long-term studies, because they yield a consistent supply of antibody against a specific epitope. The use of monoclonal antibodies directly limits the number of epitopes involved in the detection of the target. This must be evaluated for each application. The primary antibody may be directly conjugated or used in conjunction with a secondary antibody and an appropriate detection system. The optimal antibody concentration usually is considered to be the greatest dilution of antibody that results in a strong positive signal with minimal background. This must be optimized in conjunction with the block and detection system selected. The primary antibody should be qualified before assay use.

2) Secondary antibody: The secondary antibody typically is directed against the species of the primary antibody immunoglobulin (which is specific for the analyte, e.g., goat anti-mouse IgG). Enzymes such as horseradish peroxidase (HRP) or alkaline phosphatase (AP) typically are linked to the secondary antibody, but other labels such as fluorophores or gold particles can be used for detection. If the secondary antibody is biotinylated, biotin–avidin–HRP or –AP complexes can be used for detection.

3) Detection enzyme and substrate: Once an immunocomplex containing the enzyme–conjugate reagent has formed, analysts add a suitable substrate to the assay. This reaction results in production of a colored precipitate or a fluorescent or chemiluminescent product that can be recorded, measured, and analyzed further. A broad range of detection options is available to best fit individual applications and intended uses.

Test Method Development

The scope of method development, and eventually method validation, are dictated by the purpose of the method. The purpose determines the format for the assay and other requirements for the test, and therefore, the purpose should be determined first. The following sections explore considerations for each method's purpose.

1. Intended Purpose of the Method

A. IDENTITY TESTING

In the case of identity tests, analysts want to detect the presence of a protein; therefore, demonstration of specificity is essential and required. For this purpose, analysts also control the quantity of protein in the sample. Thus, the limits of detection (LOD), limits of quantitation (LOQ), and other measures of quantity are not required attributes of the method. Examples include material identity assays that demonstrate the isotype of an IgG and, in some cases, demonstrate the specificity of an antibody in a method validation. If there is no interference from the matrix or potential cross-reaction with other materials present in the sample, then a simple slot/dot blot may suffice. If multiple proteins in the sample display immunoreactivity and must be distinguished from each other, another segregation procedure must be used before blotting and immunostaining. The complexity of the proteins in the sample and the usefulness of the additional information gained using an electrophoretic segregation help determine if a slot/dot blot can meet the needs of the test.

Table 1. Detection Reagents and Methods

Readout	Principle of the Enzymatic Reaction	Enzyme	Substrate	Detection	Advantages	Disadvantages
Colorimetric	Produces a colored product that yields absorbance values directly proportional to analyte concentration	AP[a] HRP[c]	pNPP[b] TMB[d] OPD[e] ABTS[f]	Spectro photometer	• Robust • Economical • Reagent availability	• Time-consuming • Less sensitive than other methods
Chemiluminescent	Produces a light emission that is directly proportional to analyte concentration	AP, HRP	CSPD[g]	Luminometer, photographic film (CCD[h] camera)	• Wide assay dynamic range • Very sensitive • Rapid signal generation	• Reproducibility can be challenging
Fluorescent	Produces excitation-induced light emission that is directly proportional to analyte concentration	Galactosidase, fluorescently labeled antibody	MG[i] NG[j]	Fluorometer (CCD camera with filters)	• Rapid • Sensitive	• Interference by excipients
Radioactive	Antigen is labeled with a radioactive isotope. Radiation is proportional to analyte concentration.	—	—	Scintillation counter	• Easy to quantitate • Rapid	• Safety risk with exposure • Radioactive waste

[a] Alkaline phosphatase.
[b] *para*-Nitrophenyl phosphate.
[c] Horseradish peroxidase.
[d] 3,3',5,5'-Tetramethylbenzidine.
[e] o-Phenylenediamine dihydrochloride.
[f] 2,2'-Azino-bis[3-ethyl-benzothiazoline-6-sulfonic acid]diammonium salt.

[g] Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.13,7]decan}-4-yl)phenyl phosphate.
[h] Charge-coupled device.
[i] 4-Methylumbelliferyl galactoside.
[j] Nitrophenyl galactoside.

B. LIMIT TESTING

In other applications, analysts may want to show that an impurity has been removed to a level below toxicological concern. In many cases, a limit test is used when it is possible to say yes or no about the presence or absence of a protein below a predetermined level. This simplifies the development and validation of the method. With densitometry (scanning or imaging) equipment, the intensity of spots or bands can be determined relative to a standard curve, resulting in an estimate of concentration. An LOD should be determined to establish the appropriate limit threshold for the method. A dot blot may be suitable for either circumstance if the specificity of the antibody in the sample matrix can be demonstrated.

Another common purpose for an immunoblot is to show the presence or absence of a protein expressed from a culture. In this situation, analysts want to establish the identity of the protein by immunostaining, as well as verify that the protein has the expected molecular weight. This provides further assurance of no nonspecific interactions with other proteins in a complex matrix that generates the signal in the blot.

C. SPECIFICITY TESTING

Characterizing the specificity of the reagents for an ELISA impurity test or an immunoaffinity column also is a common immunoblot purpose. This is another form of an identity test in which the desired endpoint is demonstration of the specificity of binding between the antigen and the antibody. The result of the measurement is a demonstration of binding to a select group of the total protein population in the sample or binding to the whole population of proteins in the sample, as required for host-cell protein assays. In order to demonstrate the specificity of an antibody relative to a population of proteins, analysts typically must carry out electrophoretic or other segregations. Showing, by means of immunostaining, that a protein of the right molecular weight or pI can be recognized by the antibody is a powerful demonstration both of specificity toward a given protein and the absence of binding to other proteins. In addition, having, within the same experiment, the appropriate positive control samples that are known to contain the protein, and the appropriate negative samples that are known not to contain the protein, makes a convincing argument for the specificity and selectivity of the antibody when analysts validate an ELISA method for protein impurities. Electrophoretic segregations can be done in one dimension using either SDS–PAGE (for molecular weight) or isoelectric focusing (IEF; for isoelectric point) for a limited number of proteins with known molecular weights. Electrophoretic segregations also can be done in two dimensions (e.g., IEF followed by SDS–PAGE) to show selectivity and specificity toward a more heterogeneous population of proteins. A 2D Western blot commonly is used to demonstrate the specificity of a polyclonal antibody candidate directed against a host-cell protein (HCP) antigen preparation before development of a quantitative ELISA for that purpose.

2. Assay Mode and Sample Introduction

After considering the critical elements required for each purpose of the method, the analyst can use this information to select the most appropriate assay mode. The main points to consider when developing an immunoblot method are shown in Table 2. If appropriate, spotting samples on a membrane or applying by vacuum is the easiest and most convenient way to introduce a sample to an immunoblot membrane. However, low levels of nonspecific binding from multiple proteins can create additive nonspecific interference in dot blots or slot blots, resulting in background levels that appear to be the desired analyte.

Table 2. Points to consider in the development of immunoblot analysis

Key factors	Points to consider in development of immunoblot assay mode
Decide assay mode/Sample introduction	• Slot/dot blot • 1D segregation • 2D segregation
Assay control groups/ Reference materials	• reference materials • Negative control groups • Sensitivity control groups
Membrane selection	• Nitrocellulose • PVDF • Show binding of the antigen to the membrane with a chemical stain
Optimize blot transfer	• Transfer time (gel electrophoresis) • Vacuum time • Spot volume
Antibody specificity	• Primary antibody • Secondary or detection antibody matched to the primary antibody • Secondary or detection antibody matched to the selected substrate/detection system • Selected matrix spikes and control groups
Select blocking reagent	• Absence of antigen analyte • Blocks nonspecific binding of antibody reagents and secondary detection system
Titrate primary and secondary antibodies	• Primary antibody • Secondary antibody • Simultaneous titration with matrix design
Substrate incubation and data acquisition	• Substrate incubation time • Data acquisition time • Data analysis

Electrophoretic segregations, although time consuming, can be useful for separating and further distinguishing specific and nonspecific binding. Analysts must trade sensitivity for selectivity in going from a single dimension to two dimensions because of the further segregation of immunoreactive species from a single band into multiple spots, as is the case with the heterogeneity seen in sialylated proteins or deamidated species.

3. Assay Control Groups and Reference materials

Control groups and reference materials are selected based on the purpose of the assay and the information needed during development. Protein molecular weight markers can be used to obtain an accurate estimate of the molecular weight of immunoreactive species. The use of positive and negative control groups is helpful for troubleshooting throughout the experimental design process. Reference materials or positive and negative control groups can be used to assess system suitability and to establish method performance. A positive control group can confirm appropriate protein migration and can confirm that membrane transfer has reached completion. A negative control group is useful for assessing nonspecific interactions. A method sensitivity control group near the LOQ can be used to measure the consistency of the method near the LOQ to evaluate changes

in assay performance.

4. Membrane Selection

A membrane is selected based on the application and the protein being measured. Membranes with various pore sizes should be available and should be suitable for the molecular weight of the protein of interest to aid in appropriate transfer of different sizes of proteins. If a chemical staining method is known to work on a specific membrane with a specific protein, then it is advantageous to show that the protein binds to the membrane and is stained before analysts work on the immunostaining steps for the assay. Electrophoretic segregations followed by transfer to a membrane should be optimized with chemical staining, e.g., with sensitive fluorescent stains or silver stains and at potentially higher loads before analysts work on lower load levels required for blot optimization. Stains such as Coomassie or colloidal Coomassie may not have sufficient sensitivity to detect a low level of impurities required for certain applications.

5. Optimize Blot Transfer

Analysts should optimize transfer times from the gel to the membrane. Larger proteins require more time for transfer than smaller proteins. Small proteins may be lost during long transfer times and can transfer from the gel all the way through the membrane and be lost on the other side. The density of the gel and gradient gels can result in nonuniformity of transfer from the top to the bottom of the gel. During transfer optimization, many method developers use multiple membranes in order to capture proteins that transfer through the first membrane. Chemical staining of both the gel and the membranes can provide useful information about the location of the proteins transferred from the gel to support, either extending or reducing the transfer time.

After they select the assay mode, analysts next investigate spotting of the antigen or transfer from a gel to the appropriate membrane at various relevant concentration levels. Levels of analyte above the concentration needed for a Western blot may be required at first to determine if transfer and recognition by the antibodies is possible. If the analyte is present in low concentrations, spiking may be necessary to show its location during transfer optimization. Because of the potential variability of immunostaining and transfer, a sensitivity control or several levels of control groups should be incorporated into the method based on the analyte titration above the background level. This can be adjusted as method development progresses.

6. Antibody Specificity

Analysts should demonstrate antibody specificity early in immunoblot method development. If possible, they should test samples of the matrix without the analyte and should show an absence of response. In contrast, samples that contain analyte spiked into the matrix should show a positive response, demonstrating the specificity of the antibodies.

Analysts also should demonstrate the specificity of the secondary antibody conjugate or label. Control immunoblots with lanes or spots of primary antibody and control matrix samples containing the analyte as a negative control can show that the secondary antibody is binding to the primary antibody and not to proteins found in the matrix. Commercial sources for enzyme conjugates or fluorescent-labeled anti-species antibodies are readily available. The secondary antibody or detection system must be matched with the detection equipment and the desired sensitivity of the assay, e.g., fluorescence, colorimetric precipitating substrates, or chemiluminescence.

7. Select Blocking Reagent

Replicate membranes can be blocked with previously described blocking reagents as analysts select the most appropriate blocking reagent and the amount of time required to minimize background by means of subsequent primary and secondary antibody incubations. Analytes titrated at multiple concentrations on the membrane allow analysts to assess the amount of signal to the amount of noise (background) with various blocking reagents followed by immunostaining with the primary antibody, labeled secondary antibody, and substrate, if needed, for visualization. This titration also serves as the starting point for examining LOD and LOQ for limit tests and quantitative measurements. The LOD for immunoblots is determined by the level of nonspecific background relative to the specific signal from the analyte. As is the case with any other analytical method, if the background and signal are equal, there is no distinction between the signal and the noise.

8. Titrate Primary and Secondary Antibodies

Titrating the level of primary and secondary antibody from low to high dilutions can also, as with a blocking reagent, be used to select an antibody concentration that reduces background binding in the blank regions surrounding protein spots or bands, and can optimize the signal from the analyte. A matrix grid that varies the level of primary signal against secondary signal can be useful for optimizing the background, improving analyte signal, and reducing the consumption requirements for expensive antibody reagents.

Immunoaffinity chromatography against a highly purified antigen can be used to reduce the level of nonspecific interference for all of the immunological reagents used in an immunoblot. The method developer must be cautious that the selectivity, specificity, and affinity of the primary antibody are not lost in affinity purification because of high-affinity antibodies that remain on the antigen column or because of the destruction of antibody binding caused by elution conditions. For the secondary antibody, immunoaffinity-purified anti-species antibodies are available commercially with a variety of possible labels conjugated to the antibody.

9. Substrate Incubation and Data Acquisition

Analysts can optimize substrate development time for enzymes in order to minimize background and improve the LOD and LOQ. Excessive substrate development times for precipitating substrates can result in an intensification of the background level relative to the specific signal from the desired analyte. If the blot is agitated during substrate incubation, undesired swirling patterns of product from precipitating substrates can form. Too short an incubation time results in a less-specific signal, but too long a time can result in high background and poor resolution. Most enzyme conjugates have an optimum development time. Fluorescent labels and chemiluminescent labels have the advantage of acquisition by scanning instrumentation that can store data electronically and perhaps acquire image signals in an additive manner. Fluorescent labels have the added advantages that the signal is stable with time, numerous experiments for development time can be obtained with a single blot, and optimization of signal acquisition can be performed on a single blot.

PROCEDURES

1. Slot/Dot Blots

Using an appropriate slot/dot apparatus, analysts can make antigens of interest adhere to a suitable membrane (e.g., nitrocellulose) by gravity or vacuum filtration, followed by

addition and incubation of antigen-specific antibodies that bind to epitopes on the antigens. Remaining binding sites on the membrane are blocked by the addition of nonspecific antigen (e.g., BSA), followed by probing of the antigen-specific antibodies with a detection system [e.g., protein A/G conjugated to HRP binds to the antibodies that then are visualized using a 4-chloro-naphthol (4-CN) peroxidase substrate]. Positive identification is the development of dots or bands on the membrane. A negative result remains white or exhibits faint bands that are considerably lighter than positive bands.

2. 1D Immunoblotting

A. Preparation of SDS–PAGE Gels

Analysts should choose an SDS–PAGE gel with a content of acrylamide–bisacrylamide suitable for the molecular weight(s) of the protein(s) of interest; i.e., the smaller the molecular weight of the protein, the higher the percentage of mono- or bisacrylamide, and conversely, the larger the molecular weight of the protein, the lower the percentage of mono- or bisacrylamide.

Uniform-concentration gels have segregation ranges as shown in Table 3, and gradient gels have a segregation range as shown in Table 4. Gels can be purchased ready-made or can be produced in the laboratory.

Table 3. Linear range of segregation (kD) for uniform-concentration gels

Acrylamide concentration (%)	Linear range of segregation (kD)
5	57 - 212
7.5	36 - 94
10	20 - 80
12	12-60
15	10 - 43

Table 4. Linear range of segregation (kD) for gradient gels

Acrylamide concentration (%)	Protein range (kD)
5 - 15	20 - 250
5 - 20	10 - 200
10 - 20	10 - 150
8 - 20	8 - 150

B. Samples and Reference Materials

To prepare samples, analysts typically must lyse cells and tissues in order to release the proteins of interest. The main consideration when choosing a lysis buffer solution is whether the antibody chosen for detection of the protein(s) of interest can recognize denatured samples. When this is not the case, analysts use buffer solutions without detergent or with relatively mild, nonionic detergent.

Samples should be treated (e.g., reduced, nonreduced, or denatured), and when a sample of unknown protein content is used, a series of dilutions should be loaded onto the gel.

C. Electrophoresis

Before applying samples to the stacking gel wells according to ⟨1056⟩, analysts denature samples (e.g., heat at 95°–100° for 5 min). An appropriate volume of sample is loaded onto the gel, and a voltage of 8 V/cm applied until the dye has moved into the resolving gel. Afterward, the voltage is increased to 15 V/cm, and the segregation is run until the bromophenol blue reaches the bottom. If a commercially available gel is used, the manufacturer's recommendations are followed. Table 5 shows common sample-loading volumes for particular gels.

Table 5. Common sample-loading volumes

Well	Gel thickness (mm)	Maximum sample load volume (µL)
10	1.0	25
10	1.5	37
12	1.0	20
15	1.0	15
15	1.5	25

D. Transfer

After electrophoresis, the proteins of interest can be blotted to a membrane such as nitrocellulose or PVDF with a pore size that is appropriate for the molecular weight of the proteins of interest. Both nitrocellulose and PVDF have a protein-binding capacity of about 100–200 $\mu g/cm^2$. PVDF is more chemically resistant than nitrocellulose and is easier to handle. Transfer can be done in wet or semi-dry conditions. Semi-dry transfer generally is faster, but wet transfer is especially recommended for large proteins >100 kD. For both kinds of transfer, the membrane is placed next to the gel. The two are sandwiched between absorbent materials, and the sandwich is clamped between solid supports to maintain tight contact between the gel and membrane.

A standard buffer solution for transfer is the same as the buffer solution used for the migration or running buffer solution without SDS, but with the addition of methanol to a final concentration of 20%. For proteins larger than 80 kD, SDS should be included at a final concentration of 0.1%. Lowering methanol in the transfer buffer solution also promotes swelling of the gel, allowing large proteins to transfer more easily. Table 6 contains common buffers used for Western blot methods.

Methanol is necessary only if analysts use nitrocellulose. If they use PVDF, they can remove methanol from the transfer buffer solution and need only to activate the PVDF before they assemble the gel and membrane sandwich.

In semidry transfer, a sandwich of paper/gel/membrane/paper wetted in transfer buffer solution is placed directly between the cathode and anode. During wet transfer the membrane should be closest to the positive electrode, and the gel should be closest to the negative electrode. The composition of the transfer buffer solution is not necessarily the same as the migration or running buffer solution. Analysts should consult the apparatus manufacturer's protocol, and it is common to add both SDS and methanol. The balance of SDS and methanol in the transfer buffer solution, the proteins' molecular weights, and the gel percentage can affect transfer efficiency for both wet and semidry transfers.

Table 6. Common Western Blot Buffer Formulations Buffer Content

Buffer solution	Content
Sample buffer solution 2× (nonreducing) 1D electrophoresis	1.89 g of Tris 5.0 g of SDS 50 mg of bromophenol blue 25.0 mL of glycerol 100 mL of water Adjust with HCl to a pH of 6.8. Add water to 125 mL.
Sample buffer solution 2× (reducing) 1D electrophoresis	To nonreducing sample buffer solution: Add 12.5 mL of 2-mercaptoethanol before adjusting the pH. Alternatively, use 1.93 g of Tris, and add a suitable quantity of DTTa to obtain a final concentration of 100 mM DTT.

Buffer solution	Content
Running buffer solution 10× 1D electrophoresis	151.4 g of Tris 721.0 g of glycine 50.0 g of SDS Add water to 5000 mL. Adjust to a pH of 8.1–8.8.
Transfer buffer solution 10×	151.4 g of Tris 721.0 g of glycine Add water to 5000 mL. Adjust to a pH of 8.1–8.8.
Transfer buffer solution 1×	100 mL of 10× stock 500 mL of water 200 mL of methanol Add water to 1000 mL.
TBS 10×	24.23 g of Tris base 80.06 g of NaCl Mix in 800 mL of ultrapure water. Adjust with pure HCl to a pH of 7.6. Add water to 1000 mL.
TBS-T	100 mL of TBS 10× 900 mL of water 1 mL of polysorbate 20
8.5 M urea stock	510 g of urea Add water to 1000 mL.
Sample buffer solution 2D electrophoresis	47 mL of 8.5 M urea stock 385 mg of tributyl phosphine (TBP) 2 g of CHAPS[a] 25 mg of bromophenol blue 1% carrier ampholytes of choice

[a] 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate.

E. Blocking

Blocking the membrane prevents nonspecific background binding of the primary and secondary antibodies to the membrane. Traditionally, one of two blocking reagents is used: nonfat milk or BSA (Cohn fraction V). Milk is cheaper but is not recommended for studies of phosphoproteins. To prepare a 5% milk or BSA solution, weigh 5 g/100 mL of Tris-buffered saline containing polysorbate 20 buffer solution (TBS-T; see Table 5). Mix well, and filter. Failure to filter can lead to spotting in which tiny dark grains contaminate the blot during development. Incubate at 4° for 1 h with gentle shaking. Rinse in TBS-T after the incubation.

F. Primary Antibody and Incubation Buffer Solution

Dilute the antibody with blocking reagent at a proper dilution (1:100–1:3000, depending on antibody titer), and optimize the dilution according to the results. Too much antibody can result in nonspecific bands.

G. Incubation Time

Incubation time can vary between a few hours and overnight, and depends on the binding affinity of the antibody for the protein and the abundance of protein. A more dilute antibody with a prolonged incubation may improve specific binding.

H. Incubation Temperature

It is best to incubate under cold temperatures. When analysts incubate in blocking reagent overnight, they should incubate at 4° to prevent contamination from bacterial growth, and should gently agitate the antibody solution to enable adequate homogeneous covering of the membrane.

I. Secondary Antibody and Incubation Buffer Solution

Handle the secondary antibody and incubation buffer solution as follows. Wash the membrane several times in TBS-T while agitating to remove residual primary antibody. Dilute the secondary antibody with TBS-T at the suggested dilution. Too much secondary antibody can result in nonspecific bands. Incubate the blot at room temperature for 1–2 h with gentle agitation. Table 1 shows multiple options for secondary detection reagents and methods. More details are available in the Immunoblot Data Analysis section below.

3. Slot/Dot Blot

The procedure is similar to the procedure for 1D immunoblotting, but differs because protein samples are not separated electrophoretically but are spotted directly onto the membrane either manually or by use of a blotting unit (dot or slot blot format).

A. Procedure Using Manual Spotting

Handle manual spotting as follows. Place a dry filter paper on a stack of dry paper towels. Place filter paper that is pre-wet with transfer buffer solution on top of the dry filter paper. Place a pre-wet membrane on top of the pre-wet filter paper. Samples are spotted onto the pre-wet membrane and are allowed to absorb into the membrane. After the sample is absorbed, place the membrane on a clean, dry filter paper to dry.

B. Procedure Using a Vacuum-Blotting Unit

Analysts typically use a vacuum-blotting unit as follows. Prepare a membrane, and place it in the blotting unit according to the manufacturer's instructions. Apply vacuum to the blotting unit to remove excess buffer solution. To improve solubility, dissolve the sample in a buffer solution, and if it is not clear, remove precipitates by centrifugation. If the sample is too viscous to pipet, then dilute it further with buffer solution. With the vacuum off, carefully pipet samples into the wells, and apply vacuum to the blotting unit. After all the samples have filtered through the membrane, turn off the vacuum, add buffer solution to each well to wash down the sides, and apply vacuum again. Remove the membrane, and proceed with immunoblotting.

4. 2D Immunoblotting

A. Sample Preparation

The compounds used to solubilize proteins must not increase the ionic strength of the solution. For example, a common sample solubilization solution is the following: 8 M urea, 50 mM dithiothreitol (DTT) or 2 mM tributyl phosphine (TBP), 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 0.2% carrier ampholytes, and 0.0002% bromophenol blue. The addition of carrier ampholytes enhances the solubility of proteins as they approach their isoelectric points. The use of ampholytes produces an approximately uniform conductivity across the pH gradient without affecting its shape, meaning that the concentration of carrier ampholytes should be optimized.

B. Charge Segregation

Several vendors produce and sell immobilized pH gradient (IPG) strips, or they can be made in-house. The choice of IPG strips depends on the pI of the proteins of interest. The size of the IPG should match the size of the second-dimension gel. The amount of protein in each sample should be determined, and the amounts loaded on the IPG strips should be in the range of 10–300 µg, depending on the size of the IPG.

C. Molecular Weight Segregation

After charge segregation, analysts must equilibrate the strip in SDS-containing buffer solution before segregation in the

second dimension to determine molecular weight. Analysts should position the strip directly on top of the gel, then secure the strip by overlaying it with 0.5%–1.0% agarose prepared in SDS–PAGE running buffer solution. To track the ion front in the second dimension, analysts can add bromophenol blue to the agarose.

Immunoblot Data Analysis

The presence or absence of bands usually is determined by comparison to a control group (highly characterized antigens known or qualified to give a precise or expected response) of a type that is similar to the antigen being processed. Although analysts usually perform a qualitative comparison, bands or dots can be quantitated using a detection system (e.g., after incubating in a solution containing 4-CN peroxidase substrate; also see Table 1), and are compared to the control bands run in parallel (e.g., in the same gel).

1. Detection Options

A. Enhanced Chemiluminescence

Enhanced chemiluminescence is a popular method for detection in immunoblot analysis because it is highly sensitive (detection to pg or lower levels), and can be used to quantitate the relative concentration of the protein of interest. The method depends on incubation of the blot with a substrate that luminesces when exposed to the reporter on the secondary antibody. The light is detected using either photographic film or a charge-coupled device (CCD) camera. The image then is analyzed by densitometry to evaluate the relative amount of protein staining in terms of optical density. By using an appropriate set of molecular weight reference materials as markers, analysts can estimate molecular weight.

B. Fluorescence Detection

Direct fluorescence can be used to detect proteins on blots. Direct fluorescence is simple, rapid, sensitive, and has a greater linear range than enhanced chemiluminescent detection. The advantage of direct fluorescence is the ability to detect many different fluorescent signals. This analysis avoids the need to reprobe the blot. Compared to enhanced chemiluminescence, fluorescence methods are easier to visualize and quantitate on CCD or laser-scanning imaging systems. Some data-acquisition systems permit extending the time of data acquisition to optimize signal-to-noise levels. Fluorescence-labeled blots that can be re-examined are useful for this purpose.

Enhanced chemifluorescence (ECF) is another common fluorescence method. ECF uses secondary antibodies conjugated to either HRP or AP. The enzyme-conjugated antibodies react with specific substrates that produce fluorescence after enzymatic cleavage. Analysts visualize the resulting signals using UV epi-illumination and capture digital images. An ECF signal has a greater linear range than traditional enhanced chemiluminescence. For example, direct fluorescence has a limit of detection in the pg range, and also has about 2 logs of linear dynamic range.

Quantum dots also are an alternative to detect proteins in immunoblot analysis. Quantum dots are a type of probe that can be conjugated to antibodies simultaneously or sequentially to detect multiply labeled antigens, without the need for blot stripping. Similarly, near-infrared (NIR) fluorophore–linked antibody is a method for antibody detection whereby light produced from the excitation of a fluorescent dye is measured in a static state. Light measured in a static state allows more precise and accurate detection than light measured in a dynamic state (e.g., chemiluminescence).

C. Radioactive Detection

Proteins also can be detected by labeling an antigen with a radioactive isotope (e.g., iodine). On the one hand, this method has the advantage that the radioactivity in a band is easy to quantitate by means of time exposure to film and densitometry, or by directly excising the band from the membrane and counting using a scintillation counter. On the other hand, radioactivity also introduces the disadvantage of safety because analysts must manage radioactive material, and analytical laboratories must have a program to control and monitor waste management and individual exposure.

2. Immunoblot Quantitation

A. Nonelectrophoretic Quantitation

The quantitation of a specific protein is achieved when the blot procedure is properly optimized and generates a linear response range over a particular time frame. Immunoblot quantitation includes several elements: adequate antigen and antibody concentrations and purity, antibody specificity, blocking conditions, sufficient washes, and the duration and intensity of the signals. Once the exposures are captured on a film or electronically under optimized conditions, analysts use densitometric methods to quantitate results by comparing a specific protein on the blot and on the standard. Analysts can correct results for background by including a negative control.

The intensity of the bands depends on the amount of protein. Different commercial software packages are available for image analysis of bands on a film. Alternatively, digital imaging systems containing CCD cameras usually include software designed to perform data analysis.

B. Electrophoretic Quantitation

Proteins of various molecular weights are identified by the extrapolation of plots of relative mobilities of prestained proteins of known molecular weight and can be compared to the positive control group. Positive control groups are trended to determine the limit range of the densitometry results compared to the nominal concentration results. Independent of the detection method, the following criteria must be met for a valid Western blot result.

1) Ensure adequate development by minimizing membrane overexposure and visualizing staining controls.

2) The prestained molecular weight markers must be visible and must cover the anticipated range.

3) The band(s) should have the appropriate location and intensity for the standard, the control, and the protein of interest.

4) There should be no blot or staining artifacts that obscure the visualization and interpretation of bands.

Method Validation

A qualitative assay such as the slot/dot blot requires validation of specificity. Specificity is the ability to detect the analyte in the presence of other components. For validation, it should be shown that the particular steps of the slot/dot blot method can detect the antigen when present and do not report false positive results when the antigen is absent. In addition, demonstration of the specificity of the antigen-specific antibodies is part of the specificity evaluation.

All methods require a demonstration of the specificity of the antibody to the antigen and the lack of recognition of other proteins and reagents in the matrix. Identity tests require only specificity. Limit tests require specificity and LOD. A sensitivity control incorporated into each test can show that the LOQ is met on each determination to account for potential changes in the

sensitivity of the method. A quantitative test requires all ICH validation parameters, including robustness testing.

Demonstration of electrophoretic immunoblot specificity should include the following: stained gels to show protein segregation, stained blots to show adequate protein transfer to the membrane, blots with control samples to show the specificity of the conjugate to the primary antibody, and blots that show the binding of the antibody to the appropriate antigen. Method validation also can identify the need for control membranes for each assay, as well as protein sensitivity controls as measures of system suitability.

Instrumental Measurement of Coloration of Liquids

색의 비교시험(기기적 측정법)

PRINCIPLE

The observed color of an object depends primarily on its light-absorbing characteristics. However, a variety of conditions such as light-source differences, spectral energy of the light-source, visual sensitivity of the observer, size differences, background differences and directional differences affect the way of showing color. Hue, lightness (or brightness) and saturation are 3 attributes of the color. Under the certain conditions, instrumental measurement allows numerical expression of a color. Instrumental measurement of color is based on the fact that the human eye can detect color via 3 types of receptors.

Instrumental measurement of color provides more objective data than the subjective measurement of observing the color with naked eye. With adequate maintenance and calibration, instrumental methods can provide accurate, precise and consistent measurements of color that do not drift with time. Through the wide range of color-matching experiments on human subjects having normal color vision, distribution coefficients (weighting factors) are calculated at each wavelength in the visible spectrum, and then the relative amount of stimulation of each receptor type caused by the light of that wavelength is calculated.

The International Commission on Illumination (CIE) has developed models taking into account the light source and the angle at which the observer is looking at the target (field of view). In a visual test for coloration of solutions, the use of a 2° field of view and diffuse daylight (illuminant C) are necessary. The mean sensitivity of the human eye is represented by the distribution coefficients $\bar{x}_\lambda$, $\bar{y}_\lambda$and $\bar{z}_\lambda$ (Figure 1).

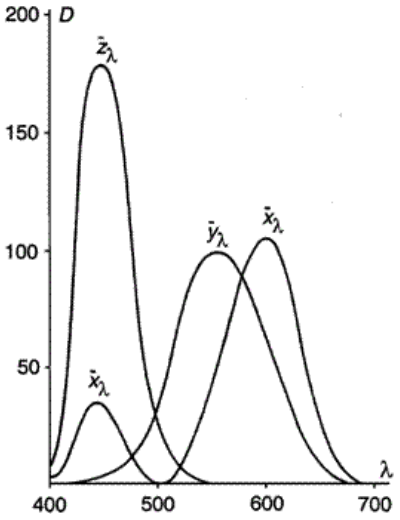


Figure 1. *Mean sensitivity of the human eye represented by distribution coefficients for the Standard Observer with CIE 2° field of view (D = distribution coefficient; λ = wavelength in nanometers)*

For any color, the amount of stimulation of each receptor type is defined by the set of tristimulus values (*XYZ*).

The relationship between the distribution coefficients and the tristimulus values (X, Y and Z) is given by the following integral equations, expressed in terms of integrals. In general, tThe short wavelength limit of the visible light wavelength range can generally be considered to be between 360 – 400 nm and the long wavelength limit is between 760 – 830 nm.

$$X = k\int_0^\infty f_\lambda\,\bar{x}_\lambda S_\lambda d_\lambda$$
$$Y = k\int_0^\infty f_\lambda\,\bar{y}_\lambda S_\lambda d_\lambda$$
$$Z = k\int_0^\infty f_\lambda\,\bar{z}_\lambda S_\lambda d_\lambda$$
$$k = 100/\int_0^\infty \bar{y}_\lambda S_\lambda d_\lambda$$

k: normalizing constant characterizing the stimulation of one receptor type and the light source

S_λ: elative spectral power distribution of the light source

$\bar{x}_\lambda$, $\bar{y}_\lambda$ and $\bar{z}_\lambda$: color matching distribution coefficients for the Standard Observer with CIE 2° field of view

f_λ: spectral transmittance T_λ of the material

λ: wavelength (nm)

In practical calculations of tristimulus values, the integration is approximated by a summation, as follows:

$$X = k\sum_\lambda T_\lambda\bar{x}_\lambda S_\lambda\Delta_\lambda$$
$$Y = k\sum_\lambda T_\lambda\bar{y}_\lambda S_\lambda\Delta_\lambda$$
$$Z = k\sum_\lambda T_\lambda\bar{z}_\lambda S_\lambda\Delta_\lambda$$
$$k = \frac{100}{\sum_\lambda S_\lambda\bar{y}_\lambda\Delta_\lambda}$$

The tristimulus values can be used to calculate the CIE *Lab* color space co-ordinates: *L*(lightness or brightness), a*(red-green)* and *b*(yellow-blue), which are defined by:*

$$L^* = 116f(Y/Y_n) - 16$$
$$a^* = 500[f(X/X_n) - f(Y/Y_n)]$$
$$b^* = 200[f(Y/Y_n) - f(Z/Z_n)]$$

where X_n, Y_n and Z_n are the tristimulus values of water and

$$If\ X/X_n > (6/29)^3,\ f(X/X_n) = (X/X_n)^{1/3}$$
$$\text{otherwise}\quad f(X/X_n) = 841/108(X/X_n) + 4/29$$

$$If\ Y/Y_n > (6/29)^3,\ f(Y/Y_n) = (Y/Y_n)^{1/3}$$
$$\text{otherwise}\quad f(Y/Y_n) = 841/108(Y/Y_n) + 4/29$$

$$If\ Z/Z_n > (6/29)^3,\ f(Z/Z_n) = (Z/Z_n)^{1/3}$$
$$\text{otherwise}\quad f(Z/Z_n) = 841/108(Z/Z_n) + 4/29$$

In the spectrophotometric method, transmittance is obtained at a whole range of wavelengths of the visible spectrum. Then, the transmittance is used to calculate the tristimulus values through the use of weighting factors $\bar{x}_\lambda$, $\bar{y}_\lambda$ and $\bar{z}_\lambda$ for the Standard Observer with a 2° field of view and CIE standard light source C (see the current International Commission on Illumination publication, CIE).

Spectrophotometric method

Using a suitable spectrophotometer according to the manufacturer's instructions attached to it, determine the transmittance (*T*) from at least 400 to 700 nm, at intervals of not greater than 10 nm. The transmittance is expressed as a percentage. Calculate the tristimulus values *X*, *Y*, and *Z* and the color co-ordinates *L**, *a** and *b**.

Determination of Coloration

Calibrate the instrument according to the manufacturer's recommendations attached to it. Carry out system performance tests prior to each measurement or at regular intervals, depending on the use situation of the instrument. For this purpose, use certified reference materials (certified filter or standard solution required by the instrument manufacturer) within the measurement range.

Operate the apparatus according to the manufacturer's instructions and measure the test and standard solutions under the same conditions (e.g. path length of the cuvette, temperature).

For transmittance measurements, use water as the control solution, assigning it a transmittance of 100.0% at all wavelengths in the visible spectrum. Then the weighting factors $\bar{x}_\lambda$, $\bar{y}_\lambda$ and $\bar{z}_\lambda$ for CIE standard light source C are used to calculate the tristimulus values corresponding to color co-ordinates $L^* = 100$, $a^* = 0$ and $b^* = 0$.

Control solution can be measured using the color co-ordinates of water or freshly prepared "matching fluids for color", or using the respective color co-ordinates stored in the instrument manufacturer's database, provided the latter have been obtained under the same testing conditions.

If the test solution is turbid or hazy, it is filtered or centrifuged. If it is not filtered or centrifuged, any haziness or turbidity should be reported with the results together. Air bubbles are to be avoided or, where applicable, removed.

The instrumental method is used to compare 2 solutions in terms of their color or color difference, or a deviation from a defined color. Calculate the color difference (ΔE^*_{tr}) between the test solution (*t*) and matching fluids for color (*r*) using the following equation:

$$\Delta E^*_{tr} = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

where ΔL^*, Δa^* and Δb^* are the differences in color co-ordinates.

The CIE *LCh* color co-ordinates may be used instead of the CIE *Lab* color co-ordinates.

Identification of location within the L*a*b* color space.

Instruments may provide information on the actual location of the test solution within the L*a*b* color space. Using appropriate algorithms, the corresponding matching fluids for color (such as 'test solution equals matching fluids for color XY', 'test solution close to matching fluids for color XY' or 'test solution between matching fluids for color XY and XZ') can be obtained.

Isoelectric Focusing
등전점 전기영동법

General Principles

Isoelectric focusing (IEF) is to separate proteins by using the difference in their isoelectric points (pI, isoelectric pH). Proteins are separated in a plate of polyacrylamide or agarose gel containing 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, immobilized pH gradient-formed gels are used, which are prepared by introducing weak acids and bases into specific regions of the gel network during gel preparation. When the added protein reaches the gel fraction which has the same pH as its isoelectric point, its charge is neutralized and migration is interrupted. Depending on the ampholyte mixture chosen, gradients can be established across different pH ranges.

Theoretical Aspects

When a protein is at the same pH as its isoelectric point in electric field gel, it has no net charge and cannot be moved. In other words, the pH gradient forces a protein to remain in its isoelectric point position, thereby concentrating it. However, some movements by diffusion might be happened. Such concentrating effect is called "focusing." The resolution of the bands can be improved by increasing the applied voltage to the gel or reducing the sample load. However, the applicable voltage range is limited due to the heat generated by electric field. On the other hand, the use of a cooling plate connected to a thermostat or thin gels prevents heating and improves resolution. Resolution (R) is estimated by determining the minimum pI difference (ΔpI), required to separate two adjacent protein bands, as follows:

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

where D is the diffusion coefficient of the protein, dpH/dx is the pH gradient, E is the intensity of the electric field (V/cm), and -dμ/dpH is the change rate of protein mobility according to pH in the region near the pI. As the diffusion coefficient (D) and the change rate of mobility (-dμ/dpH) for a given protein are constant, the resolution can be improved by using a narrower pH

range and increasing the intensity of the electric field. Resolution between protein bands on an IEF gel prepared with ampholytes can be quite good. However, better resolutions can be achieved by using immobilized pH gradients where the buffering species, which are similar to the ampholytes, are combined in the gel matrix. Proteins with pI values that differ by only 0.02 pH units can be resolved with a gel prepared with ampholytes, while immobilized pH gradients can resolve proteins that differ by approximately 0.001 pH units.

Procedures

Special attention must be paid to the characteristics of the sample and its preparation. Samples containing salt can be problematic. Therefore, it is best to prepare the sample by using the deionized water that went through dialysis or gel filtration or 2% ampholytes solution, if necessary.

The time required to complete focusing in thin-layer polyacrylamide gels is determined by placing colored proteins (e.g., hemoglobin) at various positions on the gel surface and applying the voltage. In other words, the completion of focusing is determined when the band positions of chromoproteins which were placed on the other sites become the same. In some protocols, the completion of the focusing is set as the time elapsed after start of electrophoresis. The isoelectric focusing can be used for the identification test of target protein by comparing the electrophoretic pattern with an appropriately prepared standard solution or marker protein for IEF. In addition, the isoelectric focusing can be used for a limit test when the density of a band on the IEF gel is compared to the density of bands appearing in a standard solution. Or, it can also be used as an assay when the density is measured using a densitometer or similar instrument to determine the relative concentration of a target protein in the bands.

Apparatus

The apparatus for isoelectric focusing includes a power supply for controlling voltage, current, and power. A voltage of 2500 V is commonly used, but is considered optimal under certain operating conditions. In general, a constant power of up to 30 W is recommended.

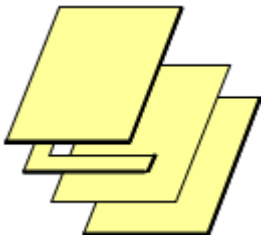
The apparatus also includes a plastic chamber and cover. The chamber contains a cooled plate of suitable material to support the gel, while the cover has platinum electrodes connected to the gel via paper wicks, which are immersed in solutions of anodic and cathodic electrolytes.

Isoelectric Focusing in Polyacrylamide Gels: Detailed Procedure

The following method describes detailed procedures of an IEF using polyacrylamide slab gels, which is used unless otherwise specified in the monograph.

1. Preparation of the Gels

Mold The mold (see Figure) consists of a glass plate (A), polyester film (B) to facilitate handling of the gel, one or more spacers (C), glass plate (D), and clamps to hold these parts together.



7.5% Polyacrylamide gel Dissolve 29.1 g of acrylamide and 0.9 g of N,N'-methylenebisacrylamide in 100 mL of water. To 2.5 volumes of this solution, add the mixture of ampholytes specified in the monograph, and dilute to 10 volumes with water. Mix carefully, and degas the solution.

Assembly of the mold Place the polyester film on the lower glass plate, apply the spacer, place the glass plate, and fix these components with the clamps. Mix the 7.5% polyacrylamide gel prepared above with a magnetic stirrer, and add 0.25 volumes of a 10 % solution of ammonium persulfate and 0.25 volumes of N,N,N',N'-tetramethylenediamine (TEMED). Immediately fill the space between the glass plates of the mold with the solution.

2. Method

Dismantle the mold, and using the polyester film attached to the gel, transfer the gel onto the cooled support wetted with a few mL of a suitable liquid, taking care not to form any air bubbles. Prepare the sample solution and standard solutions. Place paper strips (approx. 10 mm x 5 mm) for dripping on the gel and drip the prescribed amount of the sample and standard solutions. Also, apply the prescribed amount of protein solution with known isoelectric point as pH marker to calibrate the gel. In some procedures, the gel with slot for dripping is used instead of paper strips. Cut two paper strips to the length of the gel and immerse them in the acid anolyte and alkaline catholyte respectively. The compositions of acid anolyte and alkaline catholyte depend on the specific test method. Place a portion of the paper strip over the gel, thereby overlapping the ends of gel in a few millimeters. Attach the cover so that the electrodes are in contact with the paper strips (with respect to the anodic and cathodic poles). Proceed with isoelectric focusing by applying the electrical parameters described in the monograph.

Turn off the current and remove the paper strip for dripping sample solution and electrodes using forceps when the electrophoresis of the protein standard solution has finished. Then, immerse the gel in the fixing solution. After stirring slowly for 30 minutes at room temperature, remove the fixing solution. Add 200 mL of the destaining solution and allow to stir for 1 hour. Remove the destaining solution, add "Coomassie staining solution," and allow the mixture to stand for 30 minutes. Immerse the gel in the "destaining solution" again, and destain it until the bands are clearly visible against a clear background. After destianing, analyze the position and intensity of the stained protein band.

3. Modifications to the detailed procedure (subject to validation)

Modifications to the methodology or procedure must be subject to validation, which includes:

(1) the use of commercially available pre-cast gels and of commercial staining and destaining kits,

(2) the use of immobilized pH gradients gels,

(3) the use of bar-shaped gels,

(4) the use of gel templates with different dimensions (e.g., ultra-thin 0.2 mm gels),

(5) the change of sample dripping procedure (e.g., different

sample volumes, materials other than paper),

(6) the change of electrophoresis conditions (e.g., changes in electric field depending on gel dimensions and equipment, the use of fixed migration times rather than subjective interpretation of band stability),

(7) the inclusion of a prefocusing step,

(8) the use of automated instrumentation,

(9) the use of agarose gels.

4. Validation of the modifications to the detailed procedure

If alternative methods to the detailed procedure are used, they must be validated. The following criteria can be used for validation:

(1) assessment on whether a stable pH gradient is formed by using colored pH markers with known isoelectric points,

(2) comparison of electrophoretic patterns of the reference material and the sample,

(3) any other validation criteria specified in the monograph.

Other specified modifications

Modifications to the test required for the analysis of specific substances may be specified in detail in the monograph., These include:

(1) the addition of urea to the electrophoresis gel (a concentration of 3 mol/L is often used, but up to 8 mol/L may be used): For the proteins that precipitate at their isoelectric point, urea is added to the gel formulation to prevent precipitation. If the addition of urea is required, solutions should be used immediately to prevent carbamylation of the protein;

(2) the use of alternative staining methods;

(3) the use of gel additives such as non-ionic detergents (e.g., octyl glucoside) or zwitterionic detergents (e.g., 3-[(3-cholamidopropyl)di-methylammonio]-1 -propanesulfonate (CHAPS) or 3-[(3-cholamidopropyl)di-methyl ammonio]-2-hydroxy-1-propanesulfate (CHAPSO));

(4) the addition of ampholyte to the sample to prevent proteins from aggregating or precipitating.

Points to Consider

(1) Sample solution can be applied to any area of the gel, but should not be dripped near either electrode to protect proteins from extreme pH environments. During test method development, samples may be dripped to three different positions on the gel (e.g., the middle and both ends), but protein electrophoresis patterns on both ends of the gel may not be identical.

(2) If a gel is focused for too long, pH gradient may be collapsed, leading to a phenomenon known as cathode drift. Although that mechanism has not been fully investigated, it is thought that electroendoosmosis and the absorption of carbon dioxide could be factors to cause cathode drift. Cathode drift is observed as focused protein migrates away from the cathode end of the gel. Immobilized pH gradients can be used to address this issue.

(3) Efficient cooling (approximately 4 °C) of the bed that the gel lies on during focusing is important. High electric field during isoelectric focusing can lead to overheating and affect the quality of the focused gel.

Reagents and Test Solutions

Fixing solution for polyacrylamide gel IFE Dissolve 35 g of 5-sulfosalicylic acid dihydrate and 100 g of trichloroacetic acid with 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).

Particle Size Analysis by Dynamic Light Scattering
동적 광산란에 의한 입자크기 분석법

This method is based on ISO standard 22412:2017 Particle Size Analysis-Dynamic light scattering (DLS).

1. INTRODUCTION

Dynamic light scattering (DLS) can be used to determine the average hydrodynamic particle size and the broadness of the size distribution of submicron particles dispersed in a liquid. Particle size distribution is an important characteristic of dispersed systems such as emulsions, suspensions and liposome formulations.

DLS can be used to determine hydrodynamic size of particles in the submicron range and is therefore particularly suitable for the particle size analysis of dispersed systems that are composed of randomly moving particles measuring up to approximately 1 μm.

2. PRINCIPLE

Submicron particles dispersed in a liquid, which remain free from sedimentation, undergo perpetual random motion known as Brownian motion. When these particles are irradiated with a laser, scattered light intensity from the moving particles fluctuates depending on their diffusion coefficients. The intensity of the scattered light from larger particles fluctuates more slowly, because larger particles move more slowly and conversely the intensity of the scattered light from smaller particles fluctuates more rapidly.

In dynamic light scattering measurements the diffusion dependent fluctuations of the scattered light intensity are measured and analyzed. The translational diffusion coefficient and the particle equivalent spherical diameter are related by the Stokes-Einstein equation.

$$x = \frac{kT}{3\pi\eta D}$$

x: hydrodynamic diameter of an equivalent spherical particle (nm)
k: Boltzmann constant (1.38×10^{-23} J·K^{-1})
T: absolute temperature (K)
η: viscosity of the dispersing medium (mPa·s)
D: translational diffusion coefficient (m^2·s^{-1})

The intensity fluctuations of the scattered light can be evaluated either as a time dependent phase shift or as a spectral frequency shift.

Based on these concepts, the time-dependent intensity of the scattered light is processed either by photon correlation spectroscopy (PCS) or by frequency analysis.

In PCS, the time-dependent intensity of the scattered light is correlated with a time-delayed copy of itself (autocorrelation function) or with the signal from a second detector (cross-correlation function). Both the auto- and cross-correlation function of a disperse particle system decreases with increasing

correlation time. This can be described by an exponential decay. The decay rate depends on the fluctuation of the scattered light as a function of particle size (slower for large particles and faster for small particles).

In frequency analysis, the frequency-based power spectrum of the scattered light is analyzed. For a disperse particle system, the power spectrum can be described by a Lorentzian type function.

These two methods are mathematically equivalent. The time-based autocorrelation function in pcs is equal to the Fourier transform of the frequency-based power spectrum in frequency analysis. Therefore, the average diameter *(X_{DLS})* and the polydispersity index (PI), which indicates the broadness of the particle size distribution, can be evaluated with each method.

Different mathematical approaches are applied for data evaluation, including a Laplace inversion for particle size distribution or the cumulants method to evaluate the time-based autocorrelation function.

Three types of optical detection are used with DLS instruments: homodyne detection, in which only the scattered light is measured and heterodyne detection, in which the scattered light and a portion of the incident light are combined for interference and cross-correlation setup which corresponds to two simultaneous homodyne experiments.

3. INSTRUMENT

The measuring system typically consists of:

(i) A laser: a monochromatic arid coherent laser beam polarized with its electric field component perpendicular to the plane formed by the incident light beam and the light-receiving optical axes (vertical polarization), illuminating the sampler in the measuring cell.

(ii) A sample holder: the sample holder must maintain the temperature of the sample within the appropriate range (for example, ±0.3 ℃).

(iii) Optics and a detector: a beam splitter, used for heterodyne detection or cross-correlation setup, positions a light detector at a fixed angle relative to the incident laser beam to measure the apparent scattered light intensity (i.e., the sum of the scattered light from all the particles in the scattering volume) at appropriate intervals. When a polarization analyzer is included, it is positioned so that the transmittance of the vertically polarized light is maximized.

(iv) A correlator (photon correlation spectroscopy) or spectrum analyzer (frequency analysis).

(v) A computation unit and data processing software (some computation units also function as correlators or spectrum analyzers).

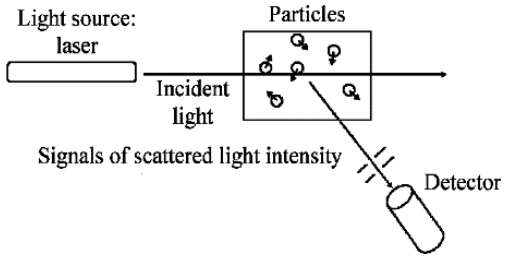


Fig. 1 Schematic illustration of the measurement principle

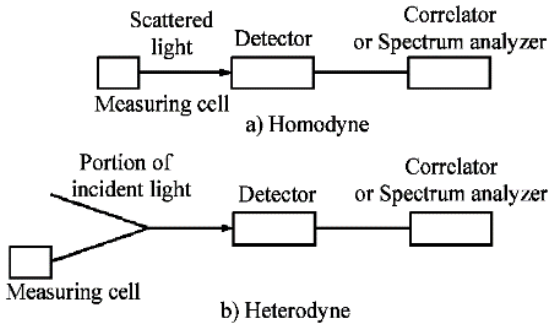


Fig. 2 Different optical arrangements of the apparatus

4. CONTROL OF INSTRUMENT PERFORMANCE/ QUALIFICATION

As the particle sizes obtained by DLS are not relative values calculated using standard particles but absolute values based on the first principle, calibration cannot be performed.

However, the performance of the instrument must be checked after it is first installed or if abnormal performance is suspected using particles with a certified diameter; it is recommended to repeat this check at least once a year thereafter. The use of certified reference materials with appropriate average particle size verified by DLS is recommended or electron microscopy if applicable.

Dispersions of polystyrene latex with narrow size distribution with certified particle diameter of about 100 nm or other suitable size can be used. The measured average particle size must be within the stated range of the certified reference material expanded by 2% on each side. Using cumulant analysis, the polydispersity index must be not more than 0.1 and the relative standard deviation of at least five repeated measurements on a sample must be not more than 2%.

5. PROCEDURE

A. SAMPLE PREPARATION

(i) Test Samples consist of the article well-dispersed in a liquid. The dispersion medium must:

a. be non-absorbing at the wavelength of the laser;

b. be compatible with the materials used in the instrument;

c. not induce particle dissolution, swelling or agglomeration/aggregation;

d. have a known refractive index different from that of the test substance;

e. have known value of viscosity within ±2 per cent at the measuring temperature;

f. be clean and free of particulate contamination e.g., dust, for low background scattering.

(ii) To eliminate the influence of multiple light scattering, their concentration must be within an appropriate range. When applicable, the particle concentration range is determined prior the analysis based on the measurements of systematically diluted samples to ensure that the results of the measurements do not vary significantly. The lower limit of the particle concentration range is determined mainly to prevent scattered light from the dispersion medium and foreign particles from affecting the measurement. Typically, scattered light signals from the dispersion medium used for sample dilution must be undetectable or very weak.

It is also important to remove dust since it may affect the measurement, and to prevent its re-introduction during preparation. If large fluctuations in the scattered light signals accompanied by abnormally strong signals are recorded or if light spots appear in the path of the laser light in the sample, foreign

or other intrinsic large particles are likely to be present in the sample. In such cases, further purification of the dispersion medium is necessary (by filtration, distillation, etc.) before use.

When water is chosen as the dispersion medium, use of fresh distilled water or desalted and filtered (nominal pore size 0.2 μm) water is recommended.

Long-range electrostatic interactions arising between highly charged particles may affect the measurement result. In such cases, a small amount of salt (for example, about 10^{-2} mol/L sodium chloride) may be added to the dispersion medium to reduce the effect. Air bubbles may also appear in the test sample, particularly when measuring an initially refrigerated sample at room temperature, and are to be avoided.

If measured values are dependent on the particle concentration, ensure that the concentration range is appropriate for the sample of interest.

B. TEST PROCEDURE

Switch the instrument on and allow it to warm up.

Clean the measurement cell if necessary. The degree of cell washing required depends on the conditions of the measurement. When an individually packaged clean disposable cell is used, cleaning is not necessary. When a cell is intended to be washed, it is rinsed with water or an organic solvent. If required, a non-abrasive detergent may be used.

Place the measurement cell containing the sample in the sample holder, and wait until temperature equilibrium is reached between the sample and the sample holder. It is recommended to measure and maintain the temperature to within ±0.3 °C.

Perform a preliminary measurement of the sample, and set the particle concentration within the appropriate range (see Sample preparation).

Perform the measurement with the appropriate measuring time and number of acquisitions.

Record the average particle diameter and the PI for each measurement.

Confirm that no significant settling has occurred in the sample at the end of the measurement. The presence of a sediment indicates that the sample may have agglomerated/aggregated or precipitated, or that it may not be a suitable candidate for DLS.

5.3 REPEATABILITY

The repeatability achievable in the testing method mainly depends on the characteristics of the test substance (emulsion/suspension, robustness/fragility, broadness of size distribution, etc.), while the required repeatability depends on the measurement's purpose.

Mandatory limits cannot be specified in this chapter, as repeatability (different sample preparations) may vary appreciably from one substance to another. However, it is good practice to aim for repeatability at a relative standard deviation of not more than 10 per cent [n ≥ 3] for average particle diameter ($\bar{X}_{DLS}$).

6. RESULTS

The test report must include the average particle diameter and PI.

It should specify the dispersion medium used, the refractive index, viscosity of the dispersion medium and temperature of the test sample, and give sufficient information about the measurement system, including the principle of measurement (PCS or frequency analysis), optical configuration (homodyne or heterodyne), laser wavelength and observation angle. The measuring time or number of acquisitions, the sample (nature, concentration and preparation method), the dispersion conditions, the instrument settings, and the measurement cell type must also be described. As the results depend also on the data analysis program, these details must be provided as well.

7. GLOSSARY

(i) Average particle diameter, $\bar{X}_{DLS}$*:* Harmonic mean particle diameter weighted by scattered light intensity, expressed in nanometers. $\bar{X}_{DLS}$ is also commonly known as the z-average diameter or Cumulants diameter.

(ii) Polydispersity index, PI: dimensionless measure of the broadness of the particle size distribution.

(iii) Scattering volume: a section of the incident laser beam viewed by the detector optics. Its order of magnitude is typically 10^{-12} m^3.

(iv) Scattered intensity, a count rate: intensity of the light scattered by the particles in the scattering volume as measured by a detector. In PCS, the number of photon pulses per unit time expressed in counts per second. In frequency analysis, the photodetector current which is proportional to the scattered light intensity.

(v) Viscosity, η: viscosity of the dispersion medium in mPa·s.

(vi) Refractive index, *n:* dimensionless refractive index of the dispersion medium at the wavelength of the laser.

Particle Size Distribution Test
입자크기측정법

Particle size analysis is a method of analyzing that directly or indirectly measures the morphological appearance, shape, size and size distribution of powdered drug substances and excipients to determine their particle size characteristics. Depending on the purpose of measurement and the properties of the sample, optical microscopy or sieve analysis may be employed.

Method 1 Optical Microscopy

Optical microscopy is a method of directly observing the appearance and shape of individual particles with the naked eye or microscopic images to measure their size. This method can also be used to determine the particle size distribution. Using this method, it is possible to measure the size of each solid particle even when several different types of solid particles are mixed, provided they can be optically distinguished. In addition, data processing such as image analysis is useful when determining particle size distribution.

Optical microscopy for particle characterization can generally be applied to particles 1 μm or larger. The lower limit is imposed by the resolving power of the microscope. The upper limit is less definite and is determined by the increased difficulty associated with the characterization of larger particles. Various alternative techniques are available for particle characterization outside the applicable range of optical microscopy. Optical microscopy is particularly useful for characterizing particles that are not spherical. This method may also serve as a foundation for the calibration of faster and more routine methods that may be developed.

Apparatus

Use a microscope that is stable and protected from vibration. The microscope magnification (product of the objective magnification, ocular magnification, and additional magnifying components) must be sufficient to allow adequate

characterization of the smallest particles to be classified in the test specimen. The greatest numerical aperture of the objective should be sought for each magnification range. Polarizing filters may be used in conjunction with suitable analyzers and retardation plates. Color filters of relatively narrow spectral transmission should be used with achromatic objectives, are preferable with apochromats, and are required for appropriate color rendition in photomicrography. Condensers corrected at least for spherical aberration should be used in the microscope substage and with the lamp. The numerical aperture of the substage condenser should match that of the objective under the conditions of use and is affected by the actual aperture of the condenser diaphragm and by the presence of immersion oils.

Adjustment

Both the precise alignment of all elements of the optical system and proper focusing are essential. The focusing of the elements should be done in accordance with the recommendations of the microscope manufacturer. Critical axial alignment is recommended.

Illumination

A requirement for good illumination is a uniform and adjustable intensity of light over the entire field of view; Köhler illumination is preferred. When dealing with colored particles, choose the color of the filters used to control the contrast and detail of the image.

Visual Characterization

The magnification and numerical aperture should be sufficiently high to allow adequate resolution of the images of the particles to be characterized. Determine the actual magnification using a calibrated stage micrometer in order to calibrate an ocular micrometer. Errors can be minimized if the magnification is sufficient that the image of the particle is at least 10 ocular divisions. Each objective must be calibrated separately. To calibrate the ocular scale, the stage micrometer scale and the ocular scale should be aligned. In this way, a precise determination of the distance between ocular stage divisions can be made.

When measuring the particle size, the ocular micrometer is placed at aperture position of the ocular lens, and then the objective micrometer is placed at the center of the stage and fixed in place. The ocular lens is placed on the microscope body tube and focused on the scale of the objective micrometer. Then, by comparing the distance between these two micrometer scales, the size of the sample corresponding to one ocular division in this superimposition is calculated using the following formula:

Size of the sample corresponding to one ocular division (μm) = Length of the objective micrometer (μm) / Number of divisions of the ocular micrometer

After removing the objective micrometer and placing the sample, the focus is adjusted, and the particle size is determined based on the measured number of ocular divisions. Several different magnifications may be necessary to characterize materials with a wide particle size distribution.

Photographic Characterization

If particle size is to be determined by photographic methods, take care to ensure that the object is sharply focused at the plane of the photographic emulsion. Determine the actual magnification by photographing a calibrated stage micrometer, using photographic film of sufficient speed, resolving power, and contrast. Exposure and processing should be identical for photographs of both the test specimen and the determination of magnification. The apparent size of a photographic image is influenced by the exposure, development, and printing processes as well as by the resolving power of the microscope.

Preparation of the Mount

The mounting medium will vary according to the physical properties of the test specimen. Sufficient but not excessive contrast between the specimen and the mounting medium is required to ensure adequate detail of the specimen's edge. The particles should rest in one plane and be adequately dispersed to distinguish individual particles of interest. Furthermore, the particles must be representative of the distribution of sizes in the material and must not be altered during preparation of the mount. Selection of the mounting medium must include consideration of the analyte's solubility.

Crystallinity Characterization

The crystallinity of a material may be characterized to determine compliance with the crystallinity requirement where one is stated in the individual monograph of a drug substance. Unless otherwise specified in the individual monograph, mount a few particles of the specimen in mineral oil on a clean glass slide. Examine the mixture using a polarizing microscope: the particles show birefringence (interference colors) and extinction positions when the microscope stage is revolved.

Limit Test of Particle Size by Microscopy

Weigh a suitable quantity of the powder to be examined (for example, 10 to 100 mg), and suspend it in 10 mL of a suitable medium in which the powder does not dissolve, adding a wetting agent if necessary. A homogeneous suspension of particles can be maintained by suspending the particles in a medium of similar or matching density and by providing adequate agitation. Introduce a portion of the homogeneous suspension into a suitable counting cell, and scan under a microscope an area corresponding to not less than 10 µg of the powder to be examined. Count all the particles having a maximum dimension greater than the prescribed size limit. The size limit and the permitted number of particles exceeding the limit are defined for each substance.

Particle Size Characterization

Measurement of particle size varies in complexity depending on the shape of the particle, and the number of particles characterized must be sufficient to ensure an acceptable level of uncertainty in the measured parameters.[1)] For irregular particles, a variety of definitions of particle size exist. In general, when dealing with irregularly shaped particles, characterization of particle size must also include information on the type of diameter measured as well as information on particle shape. Several commonly used measurements of particle size are defined below (see Figure 1):

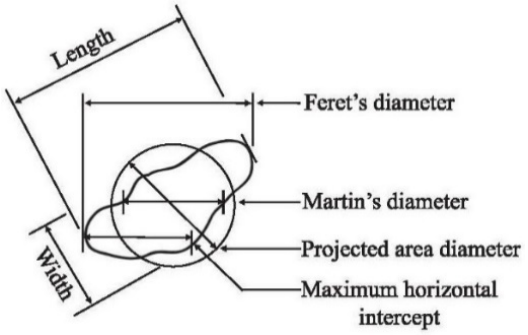


Figure 1. Commonly used measurements of particle size

Feret's diameter (forward tangential diameter): The distance between imaginary parallel lines tangent to a randomly oriented particle and perpendicular to the ocular scale.

Martin's diameter (forward area bisector): The diameter of the particle at the point that divides a randomly oriented particle into two equal projected areas.

Heywood diameter (projected area diameter): The diameter of a circle that has the same projected area as the particle.

Length: The longest dimension from edge to edge of a particle oriented parallel to the ocular scale.

Width: The longest dimension of the particle measured at right angles to the length.

Particle Shape Characterization

When dealing with irregularly shaped particles, characterization of particle size must also encompass information on particle shape. The homogeneity of the powder should be checked using appropriate magnification. The following are some commonly used descriptions of particle shape (see Figure 2):

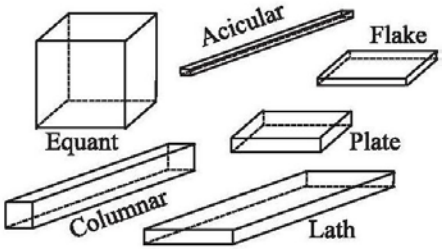


Figure 2. Commonly used descriptions of particle shape

Acicular: Slender, needle-like particle of similar width and thickness.

Columnar: Long, thin particle with a width and thickness greater than those of an acicular particle.

Flake: Thin, flat particle of similar length and width

Plate: Flat particles of similar length and width but with greater thickness than flakes.

Lath: Long, thin, and blade-like particle

Equant: Particles of similar length, width, and thickness; includes both cubical and spherical particles.

General Observations

A particle is generally considered the smallest discrete unit. A particle can take the form of a liquid or semisolid droplet; a single crystal or polycrystalline; amorphous or an agglomerate. Particles may be associated.

This degree of association may be described using the following terms:

Lamellar: Stacked plates.

Aggregate: Mass of adhered particles.

Agglomerate: Fused or cemented particles.

Conglomerate: Mixture of two or more types of particles.

Spherulite: Radial cluster.

Drusy: Particle covered with tiny particles.

Particle condition may be described using the following terms:

Edges: Angular, rounded, smooth, sharp, fractured.

Optical: Color (using proper color-balancing filters), transparent, translucent, opaque.

Defects: Occlusions (in which impurities are trapped in a specific area within the particle), inclusions (in which impurities are randomly distributed within the particle)

Surface characteristics may be described as:

Cracked: Partial split, break, or fissure.

Smooth: Free of irregularities, roughness, or projections.

Porous: Having openings or passageways.

Rough: Bumpy, uneven, not smooth.

Pitted: Small indentations.

Method 2 Sieve Analysis

Sieve analysis is a method of measuring the particle size distribution of powdered drugs using a sieve, and is essentially a two-dimensional measurement method for characterizing size. The particle size measured using the sieve analysis method is expressed as the smallest sieve opening through which particles can pass. Sieving is one of the oldest methods of classifying powders and granules by particle size distribution. When using a woven sieve cloth, sieving will essentially sort the particles by their intermediate size dimension (i.e., breadth or width). Mechanical sieving is most suitable where the majority of the particles are larger than about 75 µm. For smaller particles, the light weight provides insufficient force during sieving to overcome the surface forces of cohesion and adhesion that cause the particles to stick to each other and to the sieve, thus causing particles that would be expected to pass through the sieve to be retained. For such materials, other means of agitation such as air-jet sieving or sonic sifting may be more appropriate. Nevertheless, sieving can sometimes be used for some powders or granules having median particle sizes smaller than 75 µm where the method can be validated. In pharmaceutical terms, sieving is usually the method of choice for classification of the coarser grades of single powders or granules. It is a particularly attractive method, in that powders and granules are classified only on the basis of particle size, and in most cases the analysis can be carried out in the dry state.

Among the limitations of the sieving method are the need for an appreciable amount of sample (normally at least 25 g, depending on the density of the powder or granule, and the diameter of test sieves) and the difficulty in sieving oily or other cohesive powders or granules that tend to clog the sieve openings. The method is essentially a two-dimensional estimate of size because passage through the sieve aperture is frequently more dependent on maximum width and thickness than on length.

This method is intended for estimating the total particle size distribution of a single material. It is not intended for determining the proportion of particles passing or retained in one or two sieves.

Estimate the particle size distribution as described under Dry Sieving Method, unless otherwise specified in the individual monograph. When difficulty is encountered in reaching the endpoint (i.e., material does not readily pass through the sieves) or when it is necessary to use the finer end of the sieving range (below 75 µm), serious consideration should be given to the use

of an alternative particle-sizing method.

Sieving should be performed under conditions that do not cause the test sample to gain or lose moisture. The relative humidity of the environment in which the sieving is carried out should be controlled to prevent moisture uptake or loss by the sample. Unless there is evidence to the contrary, analytical test sieving is normally carried out at ambient humidity. Any special conditions that apply to a particular material should be detailed in the individual monograph.

Principles of Analytical Sieving

Analytical test sieves are constructed from a woven-wire mesh, which is of simple weave that is assumed to give nearly square apertures and is sealed into the base of an open cylindrical container. The basic analytical method involves stacking the sieves on top of one another in ascending degrees of coarseness, and then placing the test powder on the top sieve.

The nest of sieves is subjected to a standardized period of agitation, and then the weight of material retained on each sieve is accurately determined. The test provides the weight percentage of powder in each sieve size range. This sieving process for estimating the particle size distribution of a single pharmaceutical powder is generally intended for use where at least 80 % of the particles are larger than 75 μm. The size parameter involved in determining particle size distribution by analytical sieving is the length of the side of the minimum square aperture through which the particle will pass.

Test Sieves

Test sieves suitable for pharmacopeial tests conform to the current edition of International Organization for Standardization (ISO 3310-1[2)]). Unless otherwise specified in the monograph, use those ISO sieves listed in Table 1 as recommended in the particular region.

Sieves are selected to cover the entire range of particle sizes present in the test specimen. A nest of sieves having a progression of the area of the sieve openings is recommended. The nest of sieves is assembled with the coarsest screen at the top and the finest at the bottom. Use micrometers or millimeters when denoting test sieve openings. (Note: sieve numbers are provided in the table for conversion purposes only.) Test sieves are made from stainless steel, or less preferably, from brass or other suitable nonreactive wire.

Calibration and recalibration of test sieves is in accordance with the most current edition of ISO 3310-1. Sieves should be carefully examined for gross distortions and fractures, especially at their screen frame joints, before use. Sieves may be calibrated optically to estimate the average opening size, and opening variability, of the sieve mesh. Alternatively, for the evaluation of the effective opening of test sieves in the size range of 212 to 850 μm, Standard Glass Spheres are available. Unless otherwise specified in the individual monograph, perform the sieve analysis at controlled room temperature and at ambient relative humidity.

Table 1. Sizes of standard sieve series in range of interest

ISO Nominal Aperture			USP	Recommended USP	European Sieve	Korean Sieve No.
Principal sizes	Supplementary sizes		Sieve No.	Sieve No. (microns)	No.	
R 20/3	R 20	R 40/3				
11.20 mm	11.20 mm	11.20 mm			11200	
	10.00 mm					
8.00 mm		9.50 mm				
	9.00 mm					
	8.00 mm	8.00 mm				
	7.10 mm					
5.60 mm		6.70 mm				
	6.30 mm					
	5.60 mm	5.60 mm			5600	3.5
	5.00 mm					
4.00 mm		4.75 mm				4
	4.50 mm					
	4.00 mm	4.00 mm	5	4000	4000	4.7
	3.55 mm					
2.80 mm		3.35 mm	6			5.5
	3.15 mm					
	2.80 mm	2.80 mm	7	2800	2800	6.5
		2.50 mm				
2.00 mm		2.36 mm	8			7.5
		2.24 mm				
	2.00 mm	2.00 mm	10	2000	2000	8.6
		1.80 mm				
1.40 mm		1.70 mm	12			10
	1.60 mm					
	1.40 mm	1.40 mm	14	1400	1400	12
	1.25 mm					
1.00 mm		1.18 mm	16			14
	1.12 mm					
	1.00 mm	1.00 mm	18	1000	1000	16
	900 μm					
		850 μm	20			18
710 μm	800 μm					
	710 μm	710 μm	25	710	710	22
	630 μm					
		600 μm	30			26
500 μm	560 μm					
	500 μm	500 μm	35	500	500	30

ISO Nominal Aperture			USP	Recommended USP	European Sieve	Korean Sieve No.
Principal sizes	Supplementary sizes		Sieve No.	Sieve No. (microns)	No.	
355 μm	450 μm					
		425 μm	40			36
	400 μm					
	355 μm	355 μm	45	355	355	42
250 μm	315 μm					
		300 μm	50			50
	280 μm					
	250 μm	250 μm	60	250	250	60
180 μm	224 μm					
		212 μm	70			70
	200 μm					
	180 μm	180 μm	80	180	180	83
125 μm	160 μm					
		150 μm	100			100
	140 μm					
	125 μm	125 μm	120	125	125	119
90 μm	112 μm					
		106 μm	140			140
	100 μm					
63 μm	90 μm	90 μm	170	90	90	166
	80 μm					
		75 μm	200			200
	71 μm					
45 μm	63 μm	63 μm	230	63	63	235
	56 μm					
		53 μm	270			282
	50 μm					
	45 μm	45 μm	325	45	45	330
	40 μm					
		38 μm			38	391

Cleaning Test Sieves: Ideally, test sieves should be cleaned using only an air jet or a liquid stream. If some apertures remain blocked by test particles, gentle brushing may be used as a last resort.

Test Specimen

If the test specimen's weight is not specified in the monograph for a particular material, use a test specimen weighing between 25 and 100 g, depending on the bulk density of the material, and test sieves having a diameter of 200mm or 203mm. For sieves of 75-mm or 76-mm diameter, the amount of material that can be accommodated is approximately 1/7 what can be accommodated on a 200-mm or 203-mm sieve. Determine the most appropriate weight for a given material by test sieving accurately weighed specimens of different weights, such as 25, 50, and 100 g, for the same time period on a mechanical shaker. (Note: If the test results are similar for the 25-g and 50-g specimens, but the 100-g specimen shows a lower percentage through the finest sieve, the 100-g specimen size is too large.) Where only a specimen of 10 to 25 g is available, smaller diameter test sieves conforming to the same mesh specifications (Table 1) may be substituted, but the endpoint must be redetermined. The use of test samples having a smaller mass (e.g., down to 5 g) may be needed. For materials with low apparent particle density, or for materials mainly comprising particles with a highly isodiametrical shape, specimen weights below 5 g for a 200-mm or 203-mm sieve may be necessary to avoid excessive blocking of the sieve. During validation of a particular sieve analysis method, it is expected that the problem of sieve blocking will have been addressed.

If the test material is prone to picking up or losing significant amounts of water with varying humidity, the test must be carried out in an appropriately controlled environment. Similarly, if the test material is known to develop an electrostatic charge, careful observation must be made to ensure that such charging is not influencing the analysis. An antistatic agent, such as colloidal silicon dioxide and/or aluminum oxide, may be added at a 0.5 percent (m/m) level to minimize this effect. If both of the above effects cannot be eliminated, an alternative particle-sizing technique must be selected.

Agitation Method

Several different sieve and powder agitation devices are commercially available, all of which may be used to perform sieve analyses. However, the different methods of agitation may give different results for sieve analyses and endpoint determinations because of the different types and magnitude of the forces acting on the individual particles under test. Methods using mechanical agitation or electromagnetic agitation, and which can induce either a vertical oscillation or a horizontal circular motion, or tapping or a combination of both tapping and horizontal circular motion are available. Entrainment of the particles in an air stream may also be used. The results must indicate which agitation method was used and the agitation parameters used (if they can be varied), because changes in the agitation conditions will give different results for the sieve analysis and endpoint determinations, and may be sufficiently different to give a failing result under some circumstances.

Endpoint Determination

The test sieving analysis is complete when the weight on any of the test sieves does not change by more than 5 % or 0.1 g (10 % in the case of 75-mm or 76-mm sieves) of the previous weight on that sieve. If less than 5 % of the total specimen weight is present on a given sieve, the endpoint for that sieve is increased to a weight change of not more than 20 % of the previous weight on that sieve. If more than 50 % of the total specimen weight is found on any one sieve, unless this is indicated in the monograph, the test should be repeated, but with the addition to the sieve nest of a more coarse sieve, intermediate between that carrying the

excessive weight and the next coarsest sieve in the original nest, i.e., addition of the ISO series sieve omitted from the nest of sieves.

Sieving Methods

1) Mechanical Agitation

Dry Sieving Method

Determine tare weight of each test sieve to the nearest 0.1 g. Place an accurately weighed quantity of test specimen on the top (coarsest) sieve, and replace the lid. Agitate the nest of sieves for 5 minutes. Then, carefully remove each from the nest without loss of material. If there is some fine powder on the lower surface of each sieve, carefully remove it with the brush and combine it with the sieve fraction on the next lower sieve. Reweigh each sieve, and determine the weight of material on each sieve. Determine the weight of material in the collecting pan in a similar manner. Reassemble the nest of sieves, and agitate for 5 minutes. Remove and weigh each sieve as previously described. Repeat these steps until the endpoint criteria are met (see Endpoint Determination under Test Sieves). Upon completion of the analysis, reconcile the weights of material. Total losses must not exceed 5% of the weight of the original test specimen.

Repeat the analysis with a fresh specimen, but using a single sieving time equal to that of the combined times used above. Confirm that this sieving time conforms to the requirements for endpoint determination. When this endpoint has been validated for a specific material, then a single fixed time of sieving may be used for future analyses, providing the particle size distribution falls within normal variation.

If there is evidence that the particles retained on any sieve are aggregates rather than single particles, the use of mechanical dry sieving is unlikely to give results with good reproducibility, and a different particle size analysis method should be used.

2) Air Entrainment Methods

Air Jet and Sonic Sifter Sieving

Different types of commercial equipment that use a moving air current are available for sieving. A system that uses a single sieve at a time is referred to as air jet sieving. It uses the same general sieving methodology as that described under the Dry Sieving Method, but with a standardized air jet replacing the normal agitation mechanism. It requires sequential analyses on individual sieves starting with the finest sieve to obtain a particle size distribution. Air jet sieving often includes the use of finer test sieves than those used in ordinary dry sieving. This technique is more suitable where only oversize or undersize fractions are needed. In the sonic sifting method, a nest of sieves is used, and the test specimen is carried in a vertically oscillating column of air that lifts the specimen and then carries it back against the mesh openings at a given number of pulses per minute. It may be necessary to lower the sample amount to 5 g, when sonic sifting is employed.

The air jet sieving and sonic sieving methods may be useful for powders or granules when mechanical sieving techniques are incapable of giving a meaningful analysis. These methods are highly dependent upon proper dispersion of the powder in the air current. This requirement may be hard to achieve if the method is used at the lower end of the sieving range (i.e., below 75 μm), when the particles tend to be more cohesive, and especially if there is any tendency for the material to develop an electrostatic charge. For the above reasons endpoint determination is particularly critical, and it is very important to confirm that the oversize material comprises single particles and is not composed of aggregates.

Interpretation

The raw data must include the weight of the test specimen, the total sieving time, and the precise sieving methodology and the set values for any variable parameters, in addition to the weights retained on the individual sieves and in the pan. It may be convenient to convert the raw data into a cumulative weight distribution, and if it is desired to express the distribution in terms of a cumulative weight undersize, the range of sieves used should include a sieve through which all the material passes. If there is evidence on any of the test sieves that the material remaining on it is composed of aggregates formed during the sieving process, the analysis is invalid.

Note 1) For additional information on particle size measurement, sample size, and data analysis, see ISO 9276.

Note 2): International Organization for Standardization (ISO) Specification ISO 3310-1; Test sieves - Technical requirements and testing

Polymorphism
결정다형

Polymorphism (or crystal polymorphism) is a phenomenon related to the solid state; it is the ability of a compound in the solid state to exist in different crystalline forms having the same chemical composition. Substances that exist in a non-crystalline solid state are said to be amorphous.

When this phenomenon is observed for a chemical element (for example, sulfur), the term allotropy is used instead of polymorphism.

The term pseudopolymorphism is used to describe solvates (including hydrates), where a solvent is present in the crystal matrix in stoichiometric proportions; the term may also be extended to include compounds where the solvent is trapped in the matrix in variable proportions. However the term pseudopolymorphism is ambiguous because of its use in different circumstances. It is therefore preferable to use only the terms “solvates” and “hydrates”.

Where a monograph indicates that a substance shows polymorphism, this may be true crystal polymorphism, occurrence of solvates, allotropy or occurrence of the amorphous form.

The identity of chemical composition implies that all crystalline and amorphous forms of a given species have the same chemical behaviour in solution or as a melt; in contrast, their physico-chemical and physical characteristics (solubility, hardness, compressibility, density, melting point, etc.), and therefore their reactivity and bioavailability may be different at the solid state.

When a compound shows polymorphism, the form for which the free enthalpy is lowest at a given temperature and pressure is the most thermodynamically stable. The other forms are said to be in a metastable state. At ordinary temperature and pressure, a metastable form may remain unchanged or may change to a thermodynamically more stable form.

If there are several crystalline forms, one form is thermodynamically more stable at a given temperature and pressure. A given crystalline form may constitute a phase that can reach equilibrium with other solid phases and with the liquid and gas phases.

If each crystalline form is the more stable within a given temperature range, the change from one form to another is reversible and is said to be enantiotropic. The change from one

phase to another is a univariate equilibrium, so that at a given pressure this state is characterized by a transition temperature. However, if only one of the forms is stable over the entire temperature range, the change is irreversible or monotropic.

Different crystalline forms or solvates may be produced by varying the crystallization conditions (temperature, pressure, solvent, concentration, rate of crystallization, seeding of the crystallization medium, presence and concentration of impurities, etc.).

The following techniques may be used to study polymorphism.

- Powder X-ray diffraction
- X-ray diffraction of single crystals
- Thermal analysis (differential scanning calorimetry, thermogravimetry analysis, thermomicroscopy)
- Microcalorimetry
- Moisture absorption analysis
- Optical and electronic microscopy,
- Solid-state nuclear magnetic resonance
- Infrared absorption spectrophotometry
- Raman spectroscopy
- Measurement of solubility and intrinsic dissolution rate
- Density determination

These techniques are often complementary and it is indispensable to use several of them.

Pressure/temperature and energy/temperature diagrams based on analytical data are valuable tools for fully understanding the energetic relationship (enantiotropism, monotropism) and the thermodynamic stability of the individual modifications of a polymorphic compound.

For solvates, differential scanning calorimetry and thermogravimetry are preferable, combined with measurements of solubility, intrinsic dissolution rate and X-ray diffraction.

For hydrates, water sorption/desorption isotherms are determined to demonstrate the zones of relative stability.

In general, hydrates are less soluble in water than anhydrous forms, and likewise solvates are less soluble in their solvent than unsolvated forms.

Powder Fineness
분체미세도표시법

The particle size distribution is measured by Method 2 Analytical Sieving under Particle Size Distribution Test or by other appropriate methods. For practical reasons, sieves are commonly used to measure powder fineness. Sieving is most suitable where a majority of the particles are larger than about 75 µm, although it can be used for some powders having smaller particle sizes, if it has been validated. Light diffraction is also a widely used technique for measuring the size of a wide range of particles.

1. CLASSIFICATION OF POWDER FINENESS

Where the cumulative distribution has been determined by analytical sieving or other methods, powder fineness is classified in the following manner:

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

x_{50} = median particle size (i.e., 50% of the particles are smaller and 50% of the particles are larger)

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

It is recognized that the symbol d is also widely used to indicate particle size. Therefore, the symbols d_{90}, d_{50}, and d_{10} may be used.

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

$Q_R(x)$ = cumulative distribution ratio of particles with size less than or equal to x where the subscript R reflects the distribution type.

R	Distribution Type
0	Number
1	Length
2	Area
3	Volume

Therefore, defined as follows:
$Q_R(x) = 0.90$ when $x = x_{90}$
$Q_R(x) = 0.50$ when $x = x_{50}$
$Q_R(x) = 0.10$ when $x = x_{10}$

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

1.1 Classification of Powders by Fineness

Descriptive Term	X_{50} (µm)	Cumulative Distribution Ratio by Volume Basis, $Q_3(x)$
Coarse	>355	$Q_3(355) < 0.50$
Moderately Fine	180–355	$Q_3(180) < 0.50$ and $Q_3(355) \geq 0.50$
Fine	125–180	$Q_3(125) < 0.50$ and $Q_3(180) \geq 0.50$
Very Fine	≤125	$Q_3(125) \geq 0.50$

Powder Flow
분체유동성 측정법

The widespread use of powders in the pharmaceutical industry has generated a variety of methods for characterizing powder flow. Not surprisingly, scores of references appear in the pharmaceutical literature, attempting to correlate the various measures of powder flow to manufacturing properties. The development of such a variety of test methods was inevitable; powder behavior is multifaceted and thus complicates the effort to characterize powder flow.

The purpose of this chapter is to review the methods for characterizing powder flow that have appeared most frequently in the pharmaceutical literature. In addition, while it is clear that no single and simple test method can adequately characterize the flow properties of pharmaceutical powders, this chapter proposes the standardizing test methods that may be valuable during pharmaceutical development.

4 commonly reported methods for testing powder flow are:

- Angle of Repose,
- compressibility index or Hausner ratio,
- flow rate through an orifice,
- shear cell.

In addition, numerous variations of each of these basic methods are available. Given the number of test methods and variations, standardizing the test methodology, where possible,

would be advantageous.

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

Angle of Repose

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

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

Basic methods for Angle of Repose

A variety of Angle of Repose test methods are described in the literature. The most common methods for determining the static Angle of Repose can be classified based on 2 important experimental variables:

– the height of the 'funnel' through which the powder passes may be fixed relative to the base, or the height may vary as the pile forms;

– the base upon which the pile forms may be of fixed diameter, or the diameter of the powder cone may be allowed to vary as the pile forms.

Variations in Angle of Repose methods

Variations of the above methods have also been used to some extent in the pharmaceutical literature:

– *drained Angle of Repose*: this is determined by allowing an excess quantity of material positioned above a fixed diameter base to 'drain' from the container. Formation of a cone of powder on the fixed diameter base allows determination of the drained Angle of Repose.

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

General scale of flowability for Angle of Repose

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

Table 1. Flow properties and corresponding angles of repose[2]

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

Experimental considerations for Angle of Repose

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

– the peak of the cone of powder can be distorted by the impact of falling powder from above Minimizing distortion due to impact can be achieved by carefully shaping the cone.

– the nature of the base upon which the powder cone is formed influences the Angle of Repose. It is recommended that the powder cone be formed on a 'common base', which can be achieved by forming the cone of powder on a layer of powder. This can be done by using a base of fixed diameter with a protruding outer edge to retain a layer of powder upon which the cone is formed.

Recommended procedure for Angle of Repose

Form the Angle of Repose on a fixed base with a retaining lip to retain a layer of powder on the base. The base must be free of vibration. Vary the height of the funnel to carefully build up a symmetrical cone of powder. Care must be taken to prevent vibration as the funnel is moved. The funnel height is maintained at approximately 2-4 cm from the top of the powder pile as it is being formed in order to 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, (α) using the following equation:

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.

Compressibility index and Hausner ratio

While there are some variations in the method of determining the compressibility index and Hausner ratio, the basic procedure is to measure the unsettled apparent volume, (V_0), and the final tapped volume, (V_f), of the powder after tapping the material until no further volume changes occur.

The compressibility index and the Hausner ratio are calculated as follows:

$$Compressibility\ Index = 100 \times \frac{V_0 - V_f}{V_0}$$

$$Hausner\ Ratio = \frac{V_0}{V_f}$$

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

$$Compressibility\ Index = 100 \times \frac{\rho_{tapped} - \rho_{bulk}}{\rho_{tapped}}$$

$$Hausner\ Ratio = \frac{\rho_{tapped}}{\rho_{bulk}}$$

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

Table 2. Scale of flowability[2]

Compressibility index (per cent)	Flow character	Hausner ratio
1-10	Excellent	1.00-1.11
11-15	Good	1.12-1.18
16-20	Fair	1.19-1.25
21-25	Passable	1.26-1.34
26-31	Poor	1.35-1.45
32-37	Very poor	1.46-1.59
> 38	Very, very poor	> 1.60

Experimental considerations for the compressibility index and Hausner ratio

Compressibility index and Hausner ratio are not intrinsic properties of the powder, that is to say, they are dependent upon the methodology used. The existing literature points out several important considerations affecting the determination of the unsettled apparent volume, V_0, of the final tapped volume, V_f, of the bulk density, ρ_{bulk}, and of the tapped density, ρ_{tapped}:

– the diameter of the cylinder used,

– the number of times the powder is tapped to achieve the tapped density,

– the mass of material used in the test,

– rotation of the sample during tapping.

Recommended procedure for compressibility index and Hausner ratio

Use a 250 mL volumetric cylinder with a test sample mass of 100 g. Smaller amounts and volumes may be used, but variations in the method must be described with the results. An average of 3 determinations is recommended.

Flow through an orifice

The flow rate of a material depends upon many factors, some of which are particle-related, and some are 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 relate flow rate to the diameter of the opening, particle size, and particle density have been determined. However, the method of determining flow rate through an orifice is useful only for free-flowing materials.

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

Basic methods for flow through an orifice

There are a variety of methods described in the literature. The most common for determining the flow rate through an orifice can be classified based on 3 important experimental variables:

– the type of container used to contain the powder. Common containers are cylinders, funnels, and hoppers from production equipment.

– the size and shape of the orifice used. The orifice diameter and shape are critical factors in determining powder flow rate.

– the method of measuring powder flow rate. Flow rate can be measured continuously using an electronic balance with some sort of recording device (strip chart recorder, computer). It can also be measured in discrete samples (for example, the time it takes for 100 g of powder to pass through the orifice to the nearest tenth of a second or the amount of powder passing through the orifice in 10 s to the nearest tenth of a gram).

Variations in methods for flow through an orifice

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

General scale of flowability for flow through an orifice

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

Experimental considerations for flow through an orifice

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

– the diameter and shape of the orifice,

– the type of container material (metal, glass, plastic),

– the diameter and height of the powder bed.

Experimental considerations for flow through an orifice

Flow rate measurement through an orifice is applicable only to materials with some flowability and is not suitable for cohesive materials. If the height of the powder bed (referred to as the "head" of the powder) is significantly greater than the orifice diameter, the flow rate is nearly independent of the powder head. It is advisable to use a cylinder as the container, because the walls of the container must have little effect on flow. This configuration results in flow rate being determined by the

movement of powder over powder, rather than powder along the wall of the container. Powder flow rate often increases when the height of the powder column is less than twice the diameter of the column. The orifice must be circular and the cylinder must be free of vibration. General guidelines for dimensions of the cylinder are as follows:

– diameter of the orifice should be greater than 6 times the diameter of the particles,

– diameter of the cylinder should be greater than twice the diameter of the orifice.

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.

Shear Cell Methods

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

Basic methods for shear cell

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

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

Recommendations for shear cell

The many existing shear cell configurations and test methods provide a wealth of data and can be used very effectively to characterize powder flow. They are also useful in the designing equipment such as hoppers and storage containers. 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 assessment using shear cell methodology include a comprehensive description of the equipment and methodology used.

Powder Particle Density Determination
분체의 입자밀도측정법

The Powder Particle Density Determination is to determine particle density of powdered pharmaceutical drugs or raw ingredients of drugs, which is generally performed using a gas displacement pycnometer.

The gas pycnometric density is calculated based on the assumption that the volume of gas displaced by the powder is equal to the volume occupied by the powder whose mass is known. When using the gas pycnometric density method, open pore in powder through which gas can penetrate are not considered as part of the powder's volume, while closed pores or pores into which gas cannot penetrate are considered as the volume of the powder. Typically, helium has high diffusivity into minute open pores, so it is used as the gas for the measurement. Particle densities obtained using a gas other than helium may differ from those obtained using helium, as the gas's ability to penetrate the powder depends on the open pores and the cross-sectional area of the gas molecule.

The density measured by the pycnometric method is the volume-weighted mean density of the densities of individual powder particles. It is usually referred to as particle density, which is distinguished from the true density of solids or the bulk density of powders.

Although the international unit of particle density of solids is the mass per unit volume (kg/m^3), it is more commonly expressed in g/cm^3 (1 g/cm^3 = 1000 kg/m^3).

1. Apparatus

The composition of the particle density measurement apparatus (Figure 1) using the pycnometric method is as follows:

- A sealed test cell with an empty cell volume of V_c is connected to an expansion cell with a volume of V_r through a valve.

- In this apparatus, the pressure can be applied to the test cell until the pressure gauge displays the specified pressure (P).

- This apparatus is connected to a gas for measurement, usually helium, unless otherwise specified.

Density determination using the pycnometric method is conducted at 15 to 30 ℃, and the temperature should not change by more than 2 ℃ during the measurement.

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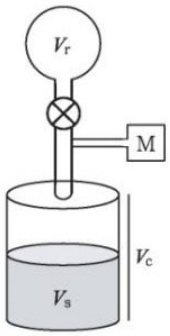
The volumes of the test cell (V_c) and the expansion cell (V_r) must be precisely determined to 0.001 cm^3. To ensure the required accuracy in volume measurement, a calibration tool for measuring particle density with known volume is usually used to calibrate the apparatus as follows:

First, measure the final pressure (P_f) for an empty test cell,

and then for a test cell containing the calibration tool for measuring particle density, according to the procedure below. Then, obtain the volumes of the test cell (V_c) and the expansion cell (V_r) by applying the equation provided in the procedure. Additionally, in the first step of the procedure, the volume of the sample is calculated, assuming $V_s = 0$.



A: Valve
V_r: Volume (cm^3) of an expansion cell
V_c: Volume (cm^3) of a test cell
V_s: Volume (cm^3) of a sample
M: Pressure gauge

Figure. A schematic diagram of a gas displacement pycnometer (apparatus for measuring the particle density)

2. Procedure

Remove any volatile impurities in the powder by injecting the gas for measurement (helium) at a constant rate before measurement. Removal of volatile contaminants is sometimes carried out under reduced pressure; volatile substances may be generated during the measurement, so measure the final mass of the sample after measuring the volume of it.

First, weigh and record the mass of the test cell. Weigh the amount of the sample specified in the Monograph, place it in the test cell, and then seal the cell.

Open the valve (A) connecting the test cell and the expansion cell, check the pressure gauge (M) to see if the pressure within the system is constant, and then read the reference pressure (P_r). Next, close the valve connecting 2 cells, introduce the gas for measurement into the test cell, pressurize it, check whether the pressure gauge indication remains constant, and then read the initial pressure (P_i). Next, open the valve to connect the expansion cell to the test cell, check whether the pressure gauge indication remains constant, then read the final pressure (P_f), and calculate the sample volume (V_s) using the following equation:

Repeat the above procedures for the same sample to check whether the consecutively measured volumes of sample are consistent within a variation of 0.2%, and set the average value as the sample volume (V_S). Finally, separate the test cell, weigh the mass (m) of the final sample, and express it in grams (g). If the operating method or the configuration of the pycnometer is different from that illustrated in Figure 1, follow the manufacturer's instructions.

3. Equations used for the calculation

Calculate the sample volume (V_S) using the following equation:

$$V_s = V_c - V_r\left[\left\{(P_i - P_r)/(P_f - P_r)\right\} - 1\right]$$

V_r: Volume of the expansion cell (cm^3)
V_c: Volume of the test cell (cm^3)
V_s: Volume of the sample (cm^3)
V_s: Initial pressure (kPa)
P_f: Final pressure (kPa)
P_r: Reference pressure (kPa)

Calculate the particle density (ρ) of the powder using the following equation:

$$\rho = m/V_s$$

ρ: Particle density of powder (g/cm^3)
m: Final mass of the sample (g)
V_s: Volume of the sample (cm^3)

Record the pretreatment method for the sample along with the test results. For example, indicate whether the sample was tested as is or dried under a specific condition, such as loss on drying.

Preservatives-Effectiveness Tests
보존력 시험법

The purpose of the Preservatives-Effectiveness Tests is to microbiologically assess the preservative efficacy, either due to the action of product components themselves or any added preservatives for multiple containers. The efficacy of preservatives is assessed by purposely inoculating and mixing the test strains with the product, and tracing the elimination and growth of test strains over time.

Preservatives must not be used solely to comply with GMP for drugs or to reduce viable aerobic counts. In fact, preservatives themselves are toxic substances Therefore, preservatives must not be added to products in amounts that might jeopardize the safety of human beings, and consideration must be given to minimizing the amounts of preservatives used. These tests are commonly used to verify the preservative effectiveness of products at the prescription design phase or in the case of regular monitoring. Although these tests are not performed for lot releasing testing, the efficacy of the preservative present in the product packaged in final containers should be verified throughout the entire effective 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, while Category II products are those made with non-aqueous bases or vehicles. The products made with oil-in-water bases are included in Category I products, while those made with water-in-oil bases are included in Category II products.

Category I is further divided into three sub-types depending on the dosage form.

Category I A: Injections and other sterile parenteral products

Category I B: Non-sterile parenteral products

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

Category II: Products made with non-aqueous bases or vehicles, including all the dosage forms listed under Category I.

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
Asperigillus niger ATCC 16404, NBRC 9455

These test microorganisms are representative of those that might be introduced from humans or the environment during manufacture, use or storage of the product, 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. The test microorganisms can be obtained from microbiological preservation institutions. One passage is defined as the transfer of microorganisms from an established culture to a fresh medium, and microorganisms subjected to not more than 5 passages should be used for the tests. Single- strain challenges rather than mixed cultures should be used. For the cultivation of test strains, either solid agar or broth is selected.

Culture on agar plate media: Inoculate each of the above 5 test strains on the surface of agar plates or agar slants to cultivate them. For growth of bacteria, use Soybean-Casein Digest Agar, and for fungi, use Sabouraud Agar, Glucose-Peptone Agar or Potato Dextrose Agar Medium. Incubate bacterial cultures at 30-35℃ for 18-24 hours, the culture of *C. albicans* at 20-25℃ for 40-48 hours and the culture of *A. niger* at 20-25℃ for one week or until good sporulation is obtained.

Collect these cultured cells aseptically using a platinum loop, etc. Suspend the collected cells in sterile physiological saline or in 0.1% peptone water to prepare suspension with the viable cell count of about 10^8 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 to prepare the suspension with the spore of about 10^8 per mL. Use these suspensions as the inocula.

Liquid cultures: After culturing each of the above 4 strains (except for *A. niger*) in a suitable broth, remove the medium by centrifugation. Wash the cells in sterile physiological saline or 0.1% peptone water to prepare the inoculum with viable cell or spore of about 10^8 per mL in the same solution.

When strains other than the above 5 strains 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 either agar plate media or broth. Store the inoculum suspensions in a refrigerator if it is not possible to inoculate them into the test specimen within 2 hours. Measure the viable cell count of the inocula immediately before use, and then calculate the theoretical viable cell count per mL (g) of the product just after inoculation.

3. Test Procedure

A. Category I products

Inject each of inoculum aseptically into five containers containing the product and mix uniformly. When it is difficult to inject the inoculum into the container aseptically or the amount of product in each container is too small to be tested, aseptically transfer a sufficient amount of the product into each of alternative sterile containers, and mix with the inoculum. When the product is not sterile, incubate additional containers containing the non-inoculated product as controls and calculate their viable cell counts (the viable counts of bacteria and fungi). A sterile syringe, spatula or glass rod may be used to mix the inoculum uniformly in the product. The amount of inoculum mixed in the product must not exceed 1/100 of the amount of product. Generally, the inoculum is inoculated and mixed so that the concentration of viable cells is 10^5-10^6 cells per mL or per gram of the product. Store these containers at 20-25℃ away from light, and calculate the viable cell count of 1 mL or 1 g of the product taken after 0, 14 and 28 days. During this time, record any significant changes (e.g., changes in color or the development of a bad odor) in the mixed samples and review the preservative effectiveness of product. Express sequential changes in the viable counts as percentages, with the count at the start of the test taken as 100. Measurement of the viable cell counts is based, in principle, on the plate-count methods in “Microbiological Examination of Non-sterile Products”. In this case, confirm whether any antimicrobial substance is present in the test specimen. If a confirmed antimicrobial substance needs to be eliminated, effective inactivator of the substance may be added to the buffer solution or broth to be used for dilution of the test specimen, as well as in the agar plate count medium. However, it is necessary to ensure that the inactivator has no effect on the growth of the microorganisms. When 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 “Microbiological Examination of Non-sterile Products”.

B. Category II products

The procedures are the same as those described for Category I products. However, special procedures and considerations should be taken at the uniform dispersion process of the test microorganism in the product and measurement of viable cell counts in the samples.

For semisolid ointment bases, heat the sample to 45-50℃ until it becomes oily, add the suspension and disperse the inoculum uniformly with a sterile glass rod or spatula. Surfactants may also be added to achieve uniform dispersion, but it should ensure that the surfactant added has no effect on survival or growth of the test microorganisms and that it does not potentiate the preservative effectiveness of product. It may be desirable to add a surfactant or an emulsifier when dispersing the product uniformly in the buffer solution or broth for the measurement of viable cell count. Especailly, sorbitan monooleate, polysorbate 80 or lecithin is recommended to disperse uniformly microorganisms which were inoculated in a semi-solid ointment product or an oil-based product in a broth. Sometimes, these agents also serve to inactivate or neutralize many of the most commonly used preservatives.

4. Interpretation

Interpret the preservative effectiveness of the product according to Table 1. When the results described in Table 1 are obtained, the product is considered to be effectively preserved. When microorganisms other than the inoculated ones are found in the sterile product, it is highly likely that the critical microbial contamination has occurred, and caution is required in the test procedures and/or the control of the manufacturing process of the product. Caution is also required in the test procedures and/or the control of the manufacturing process of the product when the contamination level in a non-sterile product to be examined exceeds the microbial enumeration limit specified in “Microbial Quality Characteristics of Non-Sterile Drugs”.

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
	Fungi	Same or less than	Same or less than

Product Category	Microorganisms	Interpretation criteria	
		After 14 days	After 28 days
		inoculum	inoculum count
Category IB	Bacteria	1% of inoculum count or less	Same or less than level after 14 days
	Fungi	Same or less than inoculum	Same or less than inoculum count
Category IC	Bacteria	10% of inoculum count or less	Same or less than level after 14 days
	Fungi	Same or less than inoculum	Same or less than inoculum count
Category II	Bacteria	Same or less than inoculum	Same or less than inoculum count
	Fungi	Same or less than inoculum	Same or less than inoculum count

5. Culture media

Culture media 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

Pancreatic digest of casein	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, sterilize at 121℃ for 15-20 minutes in an autoclave, and adjust the PH to 7.1-7.3.

Sabouraud/Glucose Agar

Peptone (animal tissue an casein)	10.0 g
Glucose	40.0 g
Agar	15.0 g
Water	1000 mL

Mix all of the components, sterilize at 121℃ for 15-20 minutes in an autoclave, and adjust the pH to 5.4 to 5.8.

Glucose/ Peptone (GP) Agar

Glucose	20.0 g
Yeast extract	2.0 g
Magnesium sulfate heptahydrate	0.5 g
Peptone	5.0 g
Potassium dihydrogen phosphate	1.0 g
Agar	15.0 g
Water	1000 mL

Mix all of the components, sterilize at 121℃ for 15-20 minutes in an autoclave, and adjust the pH after to 5.6 to 5.8.

Potato/Dextrose Agar

Potato extract	4.0 g
Glucose	20.0 g
Agar	15.0 g
Water	1000 mL

Mix all of the components, sterilize at 121℃ for 15-20 minutes in an autoclave, and adjust the pH to 5.4 to 5.8.

0.1% Peptone Water

Peptone	1.0 g
Sodium chloride	8.0 g
Water	1000 mL

Mix all of the components, sterilize at 121℃ for 15-20 minutes in an autoclave, and adjust the pH to 7.2 to 7.4.

Specific Surface Area Determination Method
비표면적측정법

Principle

The specific surface area determination is to calculate the specific surface area (the total surface area of a powder per unit mass) of a powdered pharmaceutical product by using the gas adsorption method. The specific surface area of a powdered sample is determined by measuring the physical adsorption of a gas into a solid surface, which is calculated by quantifying the amount of adsorbate gas corresponding to a mono-layer of the molecules on the surface. Physical adsorption is caused by van der Waals forces between the adsorbate gas molecules and the surface of the powder sample. The measurement is typically conducted at the boiling point of liquid nitrogen, and the amount of absorbed gas is determined using either the dynamic flow method or the volumetric method.

The Brunauer, Emmett, Teller (BET) Theory and Specific Surface Area Determination

Multi-point measurement

The result values are calculated according to the following Brunauer, Emmett and Teller (BET) adsorption isotherm equation:

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

P: Partial vapor pressure of adsorbate gas in equilibrium with the surface of the sample at -195.8 ºC (boiling point of liquid nitrogen) (Pa)

P_0: Saturated vapor pressure of adsorbate gas (Pa)

Va: Volume of adsorbate gas under the standard condition (0 ºC, 1.013 × 105Pa) (mL)

V_m: Volume of adsorbate gas forming an apparent mono-layer of molecules on the sample surface under the standard condition (mL)

C: A constant related to the adsorption enthalpy for the adsorbate gas on the surface of the sample

In the multi-point measurement, V_a is measured at more than 3 P/P0s. At this time, the BET value, $1 / [V_a \times \{(P_0/P) - 1\}]$, is plotted against P/P_0 according to Equation (1). This plot usually yields a straight line within the relative pressure range of 0.05 to 0.30. It should meet the requirements that the correlation coefficient, r, for the linear regression is not less than 0.9975, that is, r^2 is not less than 0.995. From the linear plot generated, the values of the slop which is equal to $(C - 1) / (V_m C)$, and the intercept, which is equal to $1 / (V_m C)$, can be obtained through a linear regression analysis. From these values obtained above, V_m = 1 / (slope + intercept) and C = (slope / intercept) + 1 are obtained. From the V_m value obtained above, the specific surface area, S (m^2/g), is calculated using the following equation:

$$S = (V_m N_a) / (m \times 22400) \qquad (2)$$

N: Avogadro constant (6.022×10^{23}/mol)
a: Effective cross-sectional area of one adsorbate gas molecule (m^2)
N_2: 0.162×10^{-18}
Kr: 0.195×10^{-18}
m: Mass of the powder sample (g)
22400: Volume occupied by 1 mol of absorbate gas under the standard condition (0°C, 1.013 × 105 Pa), where a slight deviation from the ideal gas is allowed in mL unit.

At least 3 measurements are necessary. It is important to note that if linearity is not observed around 0.3 of P/P_0 value, additional measurements will be needed. Given that non-linearity often occurs at P/P_0 values below 0.05, measurements in this range are not recommended. Linearity testing, data processing, and calculation of the specific surface area of the sample are performed in the same manner as described above.

Single-point measurement

Normally, it is necessary to measure V_a at more than 3 different P/P_0s for the determination of specific surface area when utilizing either the dynamic flow method (Method I) or by the volumetric method (Method II). Alternatively, it may be acceptable to determine the specific surface area by obtaining V_m from V_a measured around P/P_0 0.300 (equivalent to 0.300 mol of nitrogen and 0.001038 μmol/mol of Krypton) using the following equation:

$$V_m = V_a \{1 - (P/P_0)\} \quad (3)$$

From the V_m obtained above, the specific surface area is calculated using Equation (2).

The single-point method can be employed for a powder sample for which corresponding constant C is much greater than 1. Close similarity between the values obtained by the single-point measurement and the multiple-point measurement suggests that 1 / C is close to zero. For some powder samples for which the constant C is expected to show a large value, C = (1 + [slope / intercept]) is calculated for one of such samples using the multi-point measurement, through which the error regarding V_m can be reduced. Then, V_m is calculated from the V_a measured at P/P_0 using the following equation:

$$V_m = V_a \left(\frac{P_0}{P} - 1\right)\left(\frac{1}{C} + \frac{C-1}{C} \times \frac{P}{P_0}\right) \quad (4)$$

From the V_m obtained above, the specific surface area is calculated using Equation (2).

Experimental Procedure

This chapter describes the preparation of samples and the procedures used in the dynamic flow method (Method 1) and the volumetric method (Method 2).

Sample preparation

Outgassing Before proceeding with the determination of the specific surface, it is necessary to remove gases that have been physically adsorbed onto the surface of the powder sample during its storage and handling. Insufficient outgassing may result in decreasing or fluctuating the specific surface area values due to the effect of gas adsorbed onto part of the surface of sample. Considering the reactive nature of the surface of a substance, establishing a proper outgassing process is critical to achieve the required precision and accuracy of the measurement of specific surface area for the powdered pharmaceutical products.

Condition The outgassing condition should meet the following requirements; to guarantee that it yields reproducible BET plots, that the mass of the sample remains constant, and that there is no detectable physical or chemical changes in the sample. For the outgassing condition which is determined by the temperature, pressure, and time, it should be selected appropriately so that the original surface state of the powder sample is reproduced as similarly as possible. Most commonly used outgassing methods include outgassing under vacuum, putting the sample in a flowing stream of a non-reactive and dry gas, and desorption-adsorption cycling method. Additionally, heat may be applied to increase the rate at which the impurities leave the surface. When heating a powder sample, caution should be exercised so as not to affect the surface property and condition of sample; in order to maintain the reproducibility of the specific surface area measurement, it is desirable to shorten the outgassing time and lower the temperature as much as possible. For heat-sensitive samples, other outgassing methods, such as the desorption-adsorption cycling method, may be used.

Adsorption The standard method for physical adsorption is nitrogen-adsorption at the boiling point of liquid nitrogen. For samples with low specific areas (< 0.2 m^2/g), adsorption of krypton with low vapor pressure is used. In such cases, using krypton at the temperature where nitrogen exists in a liquid state may reduce errors due to the low vapor pressure of the gas. When measuring small surface areas, errors can be corrected by using a large amount of the sample (equivalent to not less than 1 m^2 of the total surface area using nitrogen). All gases used must be free from moisture.

Amount of sample Accurately weigh the mass of powder sample so that the total surface is at least 1 m^2, if the adsorbate gas is nitrogen, and at least 0.5 m^2, if the adsorbate is krypton. Lower quantities of samples may be used after an appropriate validation.

Measurement method Absorption measurement is usually conducted at low temperatures because the amount of the gas adsorbed at a given pressure tends to increase with decreasing temperature. Measurements are performed at –195.8 °C, which is the boiling point of liquid nitrogen.

Method I: Dynamic flow method

Principle In the dynamic flow method (see Figure 1), the recommended adsorbate gas is dry nitrogen or krypton. Helium is used for dilution, as it is not adsorbed. At least three different gas mixtures are prepared by changing the mixing ratios of the adsorbate gas and helium within the P/P_0 range of 0.05 to 0.30. Under the defined temperature and pressure conditions, the gas concentration detector outputs a signal that is near proportional to the volume of gas passing through. As a detector, thermal conductivity detector with a built-in electronic integrator is usually used. No less than 3 data are measured within the P/P_0 range of 0.05 to 0.30.

Procedure The mixture of nitrogen and helium passes through the detector, then is introduced into the test cell, and passes through the detector again. When the test cell is immersed in liquid nitrogen, the sample adsorbs nitrogen from the mobile phase, which is recorded on the recorder as a pulse through the thermal conductivity detector. Next, the test cell is removed from

the coolant. In this way, a desorption peak with the same area is created on the opposite side of the adsorption peak. Because this desorption peak is more clear than the adsorption peak, it is used for the measurement. For calibration, an amount of gas that generates a peak with the same size as the desorption peak is injected to determine the proportional relation between the unit peak area and the gas volume.

In the single-point measurement, a nitrogen/helium mixture is used for measurement, while in the multi-point measurement, two gases are mixed in advance or the mixture of several gases is used for measurement. The calculation method is the same as described in the volumetric method (Method II).

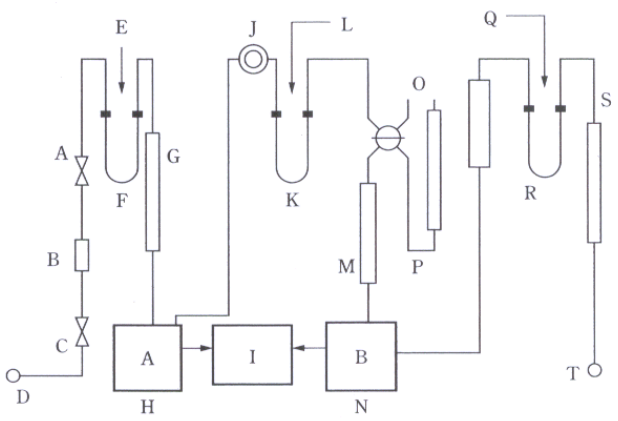
Method II: Volumetric method

Principle In the volumetric method (Figure 2), the most widely used adsorbate gas is nitrogen, which is introduced into the space over the powder sample, previously outgassed, until the specified equilibrium pressure, P, is reached. Helium is used to measure the dead volume. In this method, the interference effect of heat diffusion can be avoided due to the use of pure adsorbate gas alone instead of a mixture of gases.

Procedure To prevent contamination on the surface of the sample, add a small amount of dry nitrogen into the sample tube and separate the tube to insert a stopper. Weigh the stoppered tube to calculate the mass of the sample. Mount the sample tube to the measuring device and carefully reduce the pressure inside the sample tube down to the specified pressure (2 to10 Pa). Alternatively, depressurize some devices at a defined decompression rate (e.g., less than 13 Pa/30s) and maintain it for a certain period of time before proceeding to the next step. For example, if the operating principle of device is to measure the dead volume in the sample tube by injecting a non-adsorbate gas, such as helium, a measurement is taken at this point, and then the sample is discharged. Measurement of the dead volume can be skipped by employing differential measurement, that is, by using the reference and sample tubes connected by a differential transducer. The adsorption of nitrogen gas is measured as follows:

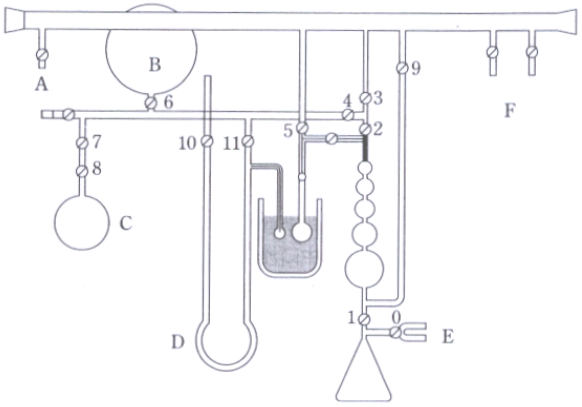
Raise the Dewar vessel containing liquid nitrogen at –195.8 ℃ to the designated position over the sample tube, introduce a sufficient amount of nitrogen to reach the required P/P_0, and measure V_a, the volume of the absorbed gas. In multi-point measurements, repeat the measurement of V_a at successively incrementing P/P_0 conditions. When nitrogen is used as the adsorbate gas, the recommended P/P_0 are 0.10, 0.20, and 0.30.

Reference material Periodically verify the functioning of the apparatus using a reference substance having a specific surface area similar to that of the sample to be examined, such as α-alumina for specific surface area determination.



A: Flow control valve
B: Differential flow controller
C: On-off valve
D: Gas inlet
E: O-ring seals
F: Cold trap
G: Thermal equilibration tube
H: Detector
I: Digital display
J: Calibration septum
K: Test cell
L: Ground-joint tube
M: Short-path ballast
N: Detector
O: Path selection valve
P: Long-path ballast
Q: Flow meter
R: Outgassing port
S: Diffusion baffle
T: Vent

Fig. 1. A schematic diagram of a device used for the dynamic flow method



A: Vacuum gauge
B: Nitrogen reservoir
C: Helium reservoir
D: Vapor pressure manometer
E: Vacuum / atmospheric air
F: Cold trap / vacuum pump

Statistical Analysis Technique of Biological Test Result

생물학적 시험 결과의 통계분석기법

This chapter, “Statistical Analysis of Results of Biological Assays and Tests” provides guidance for appropriate methods of statistical analysis (referred as “statistical analysis,” hereafter) applied to measurement or observation data obtained from experiments (referred as “data,” hereafter) of biological assays and tests (referred as “bioassays,” hereafter), in combination with the chapter, “Design of Biological Experiments and Data Preprocessing Methods.” The principle applied wherever possible throughout the bioassays is that of comparison with a

standard preparation so as to determine how much of the substance to be examined produces the same biological effect as a given quantity, the Potency Unit, of the standard preparation. The statistical tools for obtaining quantitative information with appropriate biological meaning by interpreting results from the bioassays include mainly methods of regression analysis and the related statistics, and any other analytical methods can be used. Statistical analysis given in this chapter is intended for use by those whose primary training and responsibilities are not in statistics, but who have responsibility for analysis or interpretation of the results of bioassays. The methods of calculation described in this chapter are not mandatory for the bioassays which themselves constitute a mandatory part of the Korean Pharmacopoeia, and therefore, application methods of statistical analysis and calculation can be realized in many ways and a wide range of computer software algorithm is available and may be useful depending on the facilities available to, and the expertise of, the analyst.

In the cases of regression analysis as statistical tools for interpreting results of bioassays, two approaches are often utilized. The first one is the utilization of General Linear Model, applicable when the error term of the data constituting results of the bioassay may be normally distributed or may be properly transformed to show a near normal distribution. The second one is the utilization of Generalized Linear Model, which provides standardized methods approaches when the error term shows significantly non-normal distribution or completely different probability distribution by fundamental characteristics of the data, such as a binomial distribution.

For the *General Linear Model*, the *Least Squares Method* is applied, with which a regression model is obtained as it minimizes the sum of squares of errors of dependent variable.

The *Generalized Linear Model* comprises various methods of bioassays. The *Generalized Linear Model* comprises various regression methods applicable by distribution characteristics of data of dependent variable and the Maximum Likelihood Method is applied. The *Maximum Likelihood Method* means a method of estimating the approximation of model equation with the maximum likelihood of the highest probability distribution explaining values of dependent variable with given some observed data of independent variable. The equations for the maximum likelihood can vary by the distribution model of selected error term. The *Maximum Likelihood Method* may give the same results as those of the *Least Squares Method*, when the data are normally distributed.

An essential condition of methods of bioassay is that the tests on the reference material and on the substance to be examined be carried out at the same time and under identical conditions. Bioassay methods are applied for the assay of certain biological substances and preparations whose potency cannot be adequately assured by chemical or physical analysis. The principle applied wherever possible throughout these bioassays is that of comparison with a standard preparation so as to determine how much of the substance to be examined produces the same biological effect as a given quantity, the Unit, of the standard preparation.

Any estimate of potency derived from a bioassay is subject to random error due to the inherent variability of biological responses and calculations of error should be made, if possible, from the results of each assay. Therefore, methods for the design of assays and the calculation of their errors are described below. In every case, before a statistical method is adopted, a preliminary test is to be carried out with an appropriate number of assays, in order to ascertain the applicability of this method.

The confidence interval for the potency gives an indication of the precision with which the potency has been estimated in the assay. It is calculated with due regard to the experimental design and the sample size. The 95 per cent confidence interval (referred as "95% CI," hereafter) is usually chosen in bioassays. Mathematical statistical methods are used to calculate these limits so as to warrant the statement that there is a 95 per cent probability (e.g. 19 out of 20 repeated cases of the procedure) that these limits include the true potency. Whether this precision is acceptable to the Korean Pharmacopoeia depends on the requirements set in the monograph for the preparation concerned.

The terms "mean" and "standard deviation" as estimated statistics of potency are used here as defined in most general textbooks of statistics, but "mean" may indicate "arithmetic mean," "harmonica mean" or "geometric mean" by the used method of statistical analysis and/or the method of data transformation. The terms "stated potency" or "labelled potency," "assigned potency," "assumed potency," "potency ratio" and "estimated potency" are used to indicate the following concepts:

- Labelled potency: in the case of a formulated product, a nominal value assigned from knowledge of the potency of the bulk material; in the case of bulk material, the potency estimated by the manufacturer;
- Assigned potency: the potency of the standard preparation;
- Assumed potency: the provisionally assigned potency of a preparation to be examined which forms the basis of calculating the doses that would be equipotent with the doses to be used of the standard preparation;
- Potency ratio: applied to an unknown preparation; the ratio of equipotent doses of the standard preparation and the unknown preparation under the conditions of the assay;
- Estimated potency: the potency calculated from assay data.

Results from biological tests can be analyzed on the bases of assays depending upon quantitative responses or assays depending upon quantal responses.

1. Assays Depending upon Quantitative Responses

The bioassays have been conceived as "dilution assays," which means that the unknown preparation to be assayed is supposed to contain the same active principle as the standard preparation, but in a different ratio of active and inert components. In such a case the unknown preparation may in theory be derived from the standard preparation by dilution with inert components. To check whether any particular assay may be regarded as a dilution assay, it is necessary to compare the dose-response relationships of the standard and unknown preparations. If these dose-response relationships differ significantly, then the theoretical dilution assay model is not valid. Significant differences in the dose-response relationships for the standard and unknown preparations may suggest that one of the preparations contains, in addition to the active principle, other components which are not inert but which influence the measured responses. To make the effect of dilution in the theoretical model apparent, it is useful to transform the dose-response relationship to a linear function on the widest possible range of doses. Two statistical models are of interest as models for the bioassays prescribed: the parallel-line model and the slope-ratio model. The application of either is dependent on the fulfilment of the following conditions:

- **Condition 1 (Randomization design)**
  The different treatments have been randomly assigned to the experimental units,
- **Condition 2 (Normal distribution of data)**
  The responses to each treatment are normally distributed,
- **Condition 3 (Homogeneity of variance)**
  The standard deviations of the responses within each treatment group of both standard and unknown preparations do not differ significantly from one another.

Condition 1 can be fulfilled by application of any randomization method according to the experimental design as given in the chapter, "Design of Biological Experiments and Data Preprocessing Methods." For Condition 2, minor deviations from this assumption will in general not introduce serious flaws in the analysis as long as several replicates per treatment are included. In case of doubt, a test for deviations from normality such as the Kolmogorov–Smirnov test or the Shapiro-Wilk test may be performed. Condition 3 can be checked with a test for homogeneity of variances such as Bartlett's test or Cochran's test. The F-test also can be performed when two normally distributed data sets are simply test for homogeneity of variances.

Inspection of graphical representations of the data can also be very instructive for the purpose of checking Condition 2 and/or Condition 3. When conditions 2 and/or 3 are not met, a transformation of the responses may bring a better fulfilment of these conditions. According to the data characteristics, a proper one may be selected from the transformation methods given in the chapter, "Design of Biological Experiments and Data Preprocessing Methods."

When an assay is in routine use, it is seldom possible to check systematically for the above conditions, because the limited number of observations per assay is likely to influence the sensitivity of the statistical tests. But in symmetrical balanced assays, small deviations from homogeneity of variance and normality do not seriously affect the assay results.

Additional conditions necessary depend on the statistical model to be used, which are the parallel-line model and the slope-ratio model.

Conditions for the parallel-line model

- **Condition 4A (Logarithmic dose-response relationship)**
  The relationship between the logarithm of the dose and the response can be represented by a straight line over the range of doses used,
- **Condition 5A (Parallelism)**
  For any unknown preparation in the assay the straight line is parallel to that for the standard.

Conditions for the slope-ratio model

- **Condition 4B (Dose-response relationship)**
  The relationship between the dose and the response can be represented by a straight line for each preparation in the assay over the range of doses used,
- **Condition 5B (Same intercept)**
  For any unknown preparation in the assay the straight line intersects the y-axis (at zero dose) at the same point as the straight line of the standard preparation, that is, the response functions of all preparations in the assay must have the same intercept as the response function of the standard.

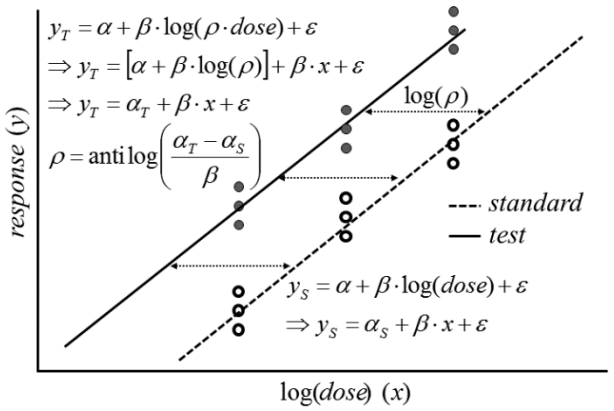
These conditions can be verified only in assays in which at least 3 dilutions of each preparation have been tested. The use of an assay with only 1 or 2 dilutions may be justified when experience has shown that linearity and parallelism or equal intercept are regularly fulfilled. After having collected the results of an assay, and before calculating the relative potency of each test sample, an analysis of variance (ANOVA) is performed, in order to check whether conditions 4A and 5A (or 4B and 5B) are fulfilled. For this, the total sum of squares is subdivided into a certain number of sum of squares corresponding to each condition which has to be fulfilled. The remaining sum of squares represents the residual experimental error to which the absence or existence of the relevant sources of variation can be compared by a series of F-ratios. When validity is established, the potency of each unknown relative to the standard may be calculated and expressed as a potency ratio or converted to some unit relevant to the preparation under test e.g. an International Unit. If any of the above conditions of the selected model are not fulfilled, the methods of calculation described here are invalid and an investigation of the assay technique should be made. The analyst should not adopt another transformation unless it is shown that non-fulfilment of the requirements is not incidental but is due to a systematic change in the experimental conditions. In this case, testing should be repeated before a new transformation is adopted for the routine assays.

It is not always feasible to take account of all possible sources of variation within one single assay. In such a case, the confidence intervals from repeated assays on the same sample may not satisfactorily overlap, and care should be exercised in the interpretation of the individual confidence intervals. In order to obtain a more reliable estimate of the confidence interval it may be necessary to perform several independent assays and to combine these into one single potency estimate and confidence interval.

The following three restrictions are normally imposed on the assay design, for they have advantages both for ease of computation and for precision. First, each preparation in the assay must be tested with the same number of dilutions. Second, in the parallel-line model, the ratio of adjacent doses must be constant for all treatments in the assay. For example, when 5 dose levels are used, they may have the ratios of 1, 2, 4, 8 and 16 in an ascending order of doses. In the slope-ratio model, the interval between adjacent doses must be constant for all treatments in the assay. For example, when 4 dose levels are used, they may have the ratios of 2, 4, 6 and 8 of dose levels. Third, there must be an equal number of experimental units to each treatment. If a design is used which meets these restrictions, the calculation method and the formulae become relatively simple, but assay designs not meeting the above mentioned restrictions may be both possible and correct, using more complicated formulae.

1) The Parallel-line Model

For the parallel-line model illustrated in the following figure, the logarithm of the doses are represented on the horizontal axis with the lowest concentration on the left and the highest concentration on the right. The responses are indicated on the vertical axis. The individual responses to each treatment are indicated with black dots. The 2 lines are the calculated ln(dose)-response relationship for the standard and the unknown. The doses represented on the horizontal axis are usually given in the natural logarithm shown as *In(dose).* However, the "common" logarithm shown as $\log_{10}(dose)$ can equally well be used. Wherever the term "antilogarithm" is used, the quantity e^x is meant for the natural logarithm, while the corresponding antilogarithm is 10^x for the "common" logarithm.



For a satisfactory assay, the assumed potency of the test sample should be close to the true potency. On the basis of this assumed potency and the assigned potency of the standard, equipotent dilutions are prepared, i.e. corresponding doses of standard and unknown are expected to give the same response. If no information on the assumed potency is available, preliminary assays are carried out over a wide range of doses to determine the range where the curve is linear. The more nearly correct the assumed potency of the unknown, the closer the two lines will be together, for they should give equal responses at equal doses. The horizontal distance between the lines represents the true potency of the unknown, relative to its assumed potency. The greater the distance between the two lines, the poorer the assumed potency of the unknown. If the line of the unknown is situated to the right of the standard, the assumed potency was overestimated, and the calculations will indicate an estimated potency lower than the assumed potency. Similarly, if the line of the unknown is situated to the left of the standard, the assumed potency was underestimated, and the calculations will indicate an estimated potency higher than the assumed potency.

(1) Calculation principle of potency ratio

In the parallel-line model, dose–response relationship can be made linear by representing log(dose)-response relationship and two linear regression equations, one for standard sample and the other for test sample. After checking the linearity and the parallelism of both lines, the horizontal distance (shown as double arrow in-between two lines in the figure) is obtained to give the potency ratio and to estimate the potency of the test sample. First of all, the assumption of linear log(dose)-response relationship means that the following equation is assumed. In the equation, α is the y-intercept and β is the slope of the line and ϵ represents error term.

$$y = \alpha + \beta \cdot \log(dose) + \epsilon \;\Rightarrow\; \alpha + \beta \cdot x + \epsilon$$

In the resulting equations, y-intercept and slope will often differ between standard and test samples. But with the parallelism test confirming non-significant difference in two values of slope (common slopes) and then the common slope can be found. The equations for standard and test samples become as following:

Regression equation for standard sample:

$$y_S = \alpha + \beta \cdot \log(dose) + \varepsilon$$
$$\Rightarrow\; y_S = \alpha_S + \beta \cdot x + \varepsilon$$

Regression equation for test sample:

$$y_T = \alpha + \beta \cdot \log(\rho \cdot dose) + \varepsilon$$
$$\Rightarrow\; y_T = [\alpha + \beta \cdot \log(\rho)] + \beta \cdot x + \varepsilon$$
$$\Rightarrow\; y_T = \alpha_T + \beta \cdot x + \varepsilon$$

where ρ denotes the potency ratio between standard and test samples.
As shown in the figure, this horizontal difference is numerically $\log(\rho)$, the logarithm of the potency ratio, and is found as the vertical distance between two lines divided by the common slope. For this, each of the regression equations is found as best-fit by the *Least Squares Method.*

Potency ratio:

$$\rho = \text{antilog}\left(\frac{\alpha_T - \alpha_S}{\beta}\right)$$

For optimizing the precision of the assay, the ratio between the slope and the residual error should be as large as possible, and the range of doses should be as large as possible, and the lines should be as close together as possible, i.e. the assumed potency should be a good estimate of the true potency. And the allocation of experimental units (animals, tubes, etc.) to different treatments may be made in various ways, such as Completely randomised design, Randomised block design, Latin square design and Cross-over design as given in the chapter, "Design of Biological Experiments and Data Preprocessing Methods."

(2) Test of Validity

Assay results are said to be "statistically valid" if the outcome of the analysis of variance for the regression model is as follows.

- The linear regression term is significant, i.e. the calculated probability (*P*) is less than α (commonly 0.05). If this criterion is not met, it is not possible to calculate 95 per cent confidence limits.
- The term for non-linearity is not significant, i.e. the calculated probability (*P*) is not less than α (commonly 0.05). { $P \geqq \alpha(=0.05)$ } This indicates that condition 4A is satisfied.
- The term for non-parallelism is not significant, i.e. the calculated probability (*P*) is not less than α (commonly 0.05). { $P \geqq \alpha(=0.05)$ } This indicates that condition 5A is satisfied.

The elements for the analysis of variance table can be given as the following formulae. In the formulae, *n* is the number of replicates, and for a Latin square design, it is equal to the number of rows or columns. $\overline{y}$ is the mean of all responses, *d* is the number of doses, *h* is the number of sample (standard and test), and $H_P = \frac{n}{d}$, $H_L = \frac{12n}{d^3 - d}$, $K = \frac{n(P_S + P_T + \cdots)^2}{hd}$ are defined as shown.

	Reference standard (*S*)	**1st Test sample (*T*)**	**More Tests (*U*, *V* etc.)**
Mean response lowest dose	S_1	T_1	U_1
Mean response	S_2	T_2	U_2

2nd dose			
...	...	...	...
Mean response highest dose	S_d	T_d	U_d
Total preparation	$P_S = S_1 + S_2 + \cdots + S_d$	$P_T = T_1 + T_2 + \cdots + T_d$	$P_U = \cdots$
Linear contrast	$L_S = 1S_1 + 2S_2 + \cdots + dS_d - \frac{1}{2}(d+1)P_S$	$L_T = 1T_1 + 2T_2 + \cdots + dT_d - \frac{1}{2}(d+1)P_T$	$L_U = \cdots$

Calculation of the sum of squares and degrees of freedom can be done with the formulae given in the following two tables.

Source of variation	Degree of freedom (*f*)	Sum of squares (*SS*)
Preparations	$h-1$	$SS_{\text{prep}} = H_P(P_S^2 + P_T^2 + \cdots) - K$
Linear regression	1	$SS_{\text{reg}} = \frac{1}{h}H_L(L_S + L_T + \cdots)^2$
Non-parallelism	$h-1$	$SS_{\text{par}} = H_L(L_S^2 + {L_T}^2 + \cdots) - SS_{reg}$
Non-linearity*	$h(d-2)$	$SS_{\text{lin}} = SS_{\text{treat}} - SS_{\text{prep}} - SS_{\text{reg}} - SS_{\text{par}}$
Treatment	$hd-1$	$SS_{\text{treat}} = n(S_1^2 + \cdots + S_d^2 + T_1^2 + \cdots + T_d^2 + \cdots) - K$

* Not calculated for 2-dose assays

Source of variation		Degree of freedom (*f*)	Sum of squares (*SS*)
Block (row)*		$n-1$	$SS_{\text{block}} = hd(R_1^2 + \cdots + R_n^2) - K$
Column**		$n-1$	$SS_{\text{col}} = hd(C_1^2 + \cdots + C_n^2) - K$
Residual error***	Completely randomized	$hd(n-1)$	$SS_{\text{res}} = SS_{\text{tot}} - SS_{\text{treat}}$
	Randomized block	$(hd-1)(n-1)$	$SS_{\text{res}} = SS_{\text{tot}} - SS_{\text{treat}} - SS_{\text{block}}$
	Latin square	$(hd-2)(n-1)$	$SS_{\text{res}} = SS_{\text{tot}} - SS_{\text{treat}} - SS_{\text{block}} - SS_{\text{col}}$
Total		$nhd-1$	$SS_{\text{tot}} = \sum(y - \overline{y})^2$

For Latin square designs, these formulae are only applicable if $n = hd$.
* Not calculated for completely randomized designs.
** Only calculated for Latin square designs.
*** Depends on the type of design.

In the above table of analysis of variance, each sum of squares (*SS*) is divided by the corresponding number of degrees of freedom (*f*) to give mean squares (*MS*). The mean square for each variable to be tested is now expressed as a ratio to the residual error (s^2) and the significance of these values, *F*-ratios are assessed for the significance by obtaining corresponding *P* values for *F*-ratios.

A significant deviation from parallelism in a multiple assay may be due to the inclusion in the assay-design of a preparation to be examined that gives a line with a slope different from those for the other preparations. Instead of declaring the whole assay invalid, it may then be decided to eliminate all data relating to that preparation and to restart the analysis from the beginning.

(3) Calculation of Potency Ratio

Estimation of Potency ratio When statistical validity of the regression equation is established in terms of linearity and parallelism of regression lines, potencies and their confidence limits (commonly 95% confidence limits) may be estimated as follows. If *I* is the logarithm of the ratio between adjacent doses of any preparation, the common slope (β) for assays with d doses of each preparation is obtained from:

$$\beta = \frac{H_L(L_S + L_T + \cdots)}{\in h}$$

and the logarithm of the potency ratio of a test preparation, for example *T* is:

$$M_T{}' = \frac{P_T - P_S}{d\beta}$$

The calculated potency is an estimate of the "true potency" of each unknown. Confidence limits may be calculated as the antilogarithms of:

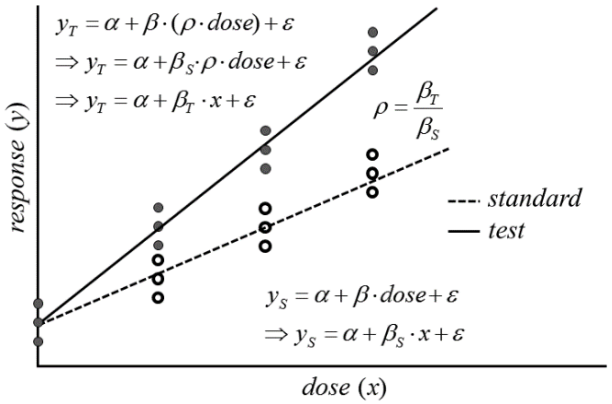
$$CM_T{}' \pm \sqrt{(C-1)(CM_T{}'^2 + 2V)}$$

where $C = \frac{SS_{\text{reg}}}{SS_{\text{reg}} - s^2t^2}$ and $V = SS\frac{\text{reg}}{\beta^2 dn}$ are calculated as shown. The value of *t* may be obtained as a *t*- critical value from the Student's *t*-distribution for α (=0.05, when to obtain 95% confidence limits) and degrees of freedom equal to the number of the degrees of freedom of the residual error.

2) The Slope-ratio Model

For the slope-ratio model, the doses are represented on the horizontal axis with the lowest concentration on the left and the highest concentration on the right. The responses are indicated on the vertical axis. The two lines are the calculated dose-response relationship for the standard and the unknown under the assumption that they intersect each other at zero-dose. Unlike the parallel-line model, the doses are not transformed to logarithms. Just as in the case of an assay based on the parallel-line model, it is important that the assumed potency is close to the true potency, and to prepare equipotent dilutions of the test preparations and

the standard, if feasible. The more nearly correct the assumed potency, the closer the two lines will be together. The ratio of the slopes represents the "true" potency of the unknown, relative to its assumed potency. If the slope of the unknown preparation is steeper than that of the standard, the potency was underestimated and the calculations will indicate an estimated potency higher than the assumed potency. Similarly, if the slope of the unknown is less steep than that of the standard, the potency was overestimated and the calculations will result in an estimated potency lower than the assumed potency. For the slope-ratio model, an extra group of experimental units receiving no treatment may be tested (blank controls), and 3 doses each for the standard and the test samples are used with blank controls in common. This design is called (*hd*+1)-design. However, a linear relationship cannot always be assumed to be valid down to zero-dose. With a slight loss of precision, a design without blank controls may be adopted and the other doses are uniformly spaced with an extra dose group. his design is called (*hd*)-design. The standard is given in a high dose, near to but not exceeding the highest dose giving a mean response on the straight portion of the dose-response line, the other doses are uniformly spaced between the highest dose and zero dose, the test samples are given in corresponding doses based on their assumed potency. And also, the allocation of experimental units (animals, tubes, etc.) to different treatments may be made in various ways, such as Completely randomised design, Randomised block design, Latin square design and Cross-over design as given in the chapter, "Design of Biological Experiments and Data Preprocessing Methods."



The slope-ratio model can be illustrated as shown in the above figure.

(1) Principle for Potency Ratio Calculation

The Slope-ratio Model can be represented as shown in the figure above. This model is a method to estimate the potency of sample by linearizing dose-response relationship between the reference standard and sample to obtain the linear repression equations for reference standard and sample, respectively. Testing the linearity of two straight lines and consistency of intercept between two straight lines, obtaining the slope ratio between two straight lines once the linearity and consistency of intercepts are determined, and then obtaining the potency ratio between the reference standard and sample. Assuming the dose-response relationship as a straight line refers to the assumption of following equation. In the equation, α refers to the y-intercept of straight line; β refers to the slope' and ϵ refers to the error term.

$$y = \alpha + \beta \cdot dose + \epsilon \Rightarrow \alpha + \beta \cdot x + \epsilon$$

The regression equations obtained from both the reference standard and the sample will generally have different y-intercepts and slopes, however, in the Slope-ratio Model, a common intercept can be determined through a test demonstrating no significant difference in intercepts of the two equations. Therefore, the regression equations of the reference standard and sample are represented as follows, respectively. In the equations, ρ refers to the potency ratio between the reference standard and sample.

Reference standard:

$$y_S = \alpha + \beta \cdot dose + \epsilon \Rightarrow y_S = \alpha + \beta_S \cdot x + \epsilon$$

Sample:

$$y_T = \alpha + \beta \cdot (\rho \cdot dose) + \epsilon$$
$$\Rightarrow y_T = \alpha + \beta \cdot \rho \cdot dose + \epsilon$$
$$\Rightarrow y_T = \alpha + \beta_T \cdot x + \epsilon$$

The slope ratio obtained from the reference standard equation and the sample regression equation numerically corresponds to the ρ potency ratio, which is calculated from the slopes when the y-intercepts of two straight lines are matched. In this case, each regression equation is obtained from the optimal straight line by the method of least squares.

$$\text{Potency ratio: } \rho = \frac{\beta_T}{\beta_S}$$

(2) Tests of Validity

The following conditions for ANOVA should be met in the applicable regression model to confirm that the result of biological analysis by the slope-ratio model is statistically valid.

- If the experiment is performed in accordance with the (*hd*+1) design, variation caused by blank test should not be significant. To do so, the probability (*P*) calculated for blank test should not be less than α (0.05 in general). {$P \geqq \alpha$ (=0.05)} It means that the response from blank test is not significantly different from the common intercept and that the linear relationship is valid at up to dose zero (0).
- In analysis where at least 3 doses are included for each treatment, non-linearity tendency should not be significant. To do so, the probability (*P*) should not be less than α (0.05 in general). As such, the Condition 4B of Slope-ratio Model is met.
- To confirm that the variance caused by intercepting section is not significant, the probability α should not be less than (generally 0.05). { $P \geqq \alpha$ (=0.05)} As such, the Condition 5B of Slope-line Model is met.

The components of the ANOVA Table for $(hd + 1)$-design can be expressed in the following equations.

	reference standard (*S*)	First sample (*T*)	Additional samples (*U*, *V* etc.)
Mean response at the lowest dose	S_1	T_1	U_1
Mean response at the second dose	S_2	T_2	U_2
⋮	⋯	⋯	⋯
Mean response at the highest dose	S_d	T_d	U_d
Total preparation	$P_S = S_1 + S_2 + \cdots + S_d$	$P_T = T_1 + T_2 + \cdots + T_d$	$P_U = \cdots$
Linear product	$L_S = 1S_1 + 2S_2 + \cdots + dS_d$	$L_T = 1T_1 + 2T_2 + \cdots + dT_d$	$L_U = \cdots$
Intercept value	$\alpha_S = (4d+2)P_S - 6L_S$	$\alpha_T = (4d+2)P_T - 6L_T$	$\alpha_U = \cdots$
Slope value	$\beta_S = 2L_S - (d+1)P_S$	$\beta_T = 2L_T - (d+1)P_T$	$\beta_U = \cdots$
Treatment value	$G_S = S_1^2 + \cdots + S_d^2$	$G_{T=} T_1^2 + \cdots + T_d^2$	$G_U = \cdots$
Non-linearity*	$J_S = G_S - \frac{P_S^2}{d} - \frac{3b_S^2}{d^3 - d}$	$J_T = G_T - \frac{P_T^2}{d} - \frac{3b_T^2}{d^3 - d}$	$J_U = \cdots$

* Not calculated for 2-dose assays.

Linear combination is calculated by the equations provided in the following table. Note that the following definitions are used for this calculation.

$$H_B = \frac{nhd^2 - nhd}{hd^2 - hd + 4d + 2}, H_I = \frac{n}{4d^3 - 2d^2 - 2d}, \alpha = \frac{\alpha_S + \alpha_T + \cdots}{h(d^2 - d)}, K = \frac{n(B + P_S + P_T + \cdots)^2}{hd + 1}$$

Source of variation	Degree of freedom (*f*)	Sum of squares (*SS*)
Regression	h	$SS_{\text{reg}} = SS_{\text{treat}} - SS_{\text{blank}} - SS_{\text{int}} - SS_{\text{lin}}$
Blank test	1	$SS_{\text{blank}} = H_B(B - \alpha)^2$
Intercepting	$h - 1$	$SS_{\text{int}} = H_I((\alpha_S{}^2 + \alpha_T{}^2 + \cdots) - h(d^2 - d)^2\alpha^2)$
Non-linearity*	$h(d - 2)$	$SS_{\text{lin}} = n(J_S + J_T + \cdots)$
Treatment	hd	$SS_{\text{treat}} = n(B^2 + G_S + G_T + \cdots) - K$

* Not calculated by 2-dose assays.

As a blank test is excluded from the (*hd*)-design, and *B* is excluded from all equations; SS_{blank} is excluded from ANOVA; and the equation of $K = \frac{n(P_S + P_T + \cdots)^2}{hd}$ is used. The degree of freedom for treatments is $hd - 1$. Residual errors, total variation, and their degree of freedom are calculated in the same method as presented in the Parallel-line Model.

Variable		Degree of freedom (*f*)	Sum of squares (*SS*)
Block (row)*		$n - 1$	$SS_{\text{block}} = hd(R_1{}^2 + \cdots + R_n{}^2) - K$
Column**		$n - 1$	$SS_{\text{col}} = hd(C_1{}^2 + \cdots + C_n{}^2) - K$
Residual error***	Completely randomized	$(hd + 1)(n - 1)$	$SS_{\text{res}} = SS_{\text{tot}} - SS_{\text{treat}}$
	Randomized block	$hd(n - 1)$	$SS_{\text{res}} = SS_{\text{tot}} - SS_{\text{treat}} - SS_{\text{block}}$
	Latin square	$(hd - 1)(n - 1)$	$SS_{\text{res}} = SS_{\text{tot}} - SS_{\text{treat}} - SS_{\text{block}} - SS_{\text{col}}$
Total		$nhd + n - 1$	$SS_{tot} = \sum (y - \overline{y})^2$

For Latin square designs, these formulae are only applicable if n = hd
* Not calculated for completely randomized designs
** Only calculated for Latin square designs
*** Depends on the type of design

In the ANOVA Table above, significance is determined by dividing each sum of squares (*SS*) by each degree of freedom (*f*) to obtain the mean square (*MS*); dividing each mean square by the mean square of residual error (s^2) to obtain each *F* ratio; and the *P* value for this *F* ratio is obtained to determine its significance.

(3) Calculation of Potency Ratio

Once the efficacy for the regression equation is confirmed through ANOVA, the potency and its confidence interval (95% CI in general) are calculated as follows. In case of using $(hd + 1)$-design, the common intersection of the preparations α' is calculated by the following equation.

$$\alpha' = \frac{(2d+1)B + (2d-3)h\alpha}{h(2d-3) + 2d + 1}$$

The slope of the reference standard and similarly for each of the other preparation is calculated by the following equation, respectively.

$$\beta_S' = \frac{6L_S - 3d(d+1)\alpha'}{2d^3 + 3d^2 + d} \qquad \beta_T{}' = \frac{6L_T - 3d(d+1)\alpha'}{2d^3 + 3d^2 + d}$$

The potency ratio of each of the test preparations can be calculated by the following equation.

$$\rho_T{}' = \frac{\beta_T{}'}{\beta_S{}'}$$

This value is multiplied by the assumed potency of the test preparation, A_T to obtain the estimated potency, R_T. If the step between adjacent doses was not identical for the reference standard and the test preparation, the potency has to be multiplied by I_S/I_T. The 95% CI for potency ratio of the test preparation, $\rho_T{}'$, is calculated by the following equation.

$$C\rho_T{}' - K' \pm \sqrt{(C-1)(C\rho_T{}'^2 + 1)(K'(K' - 2C\rho_T{}')}$$

where $C = \frac{\beta_S{}'^2}{\beta_S{}'^2 - s^2 t^2 V_1}$ and $K' = (C-1)V_2$, V_1 and V_2 are related to the variance and covariance of the numerator and denominator of R_T, which is calculated by the following equation.

$$V_1 = \frac{6}{n(2d+1)}\left(\frac{1}{d(d+1)} + \frac{3}{2(2d+1) + hd(d-1)}\right),$$
$$V_2 = \frac{3d(d+1)}{(3d+1)(d+2) + hd(d-1)}$$

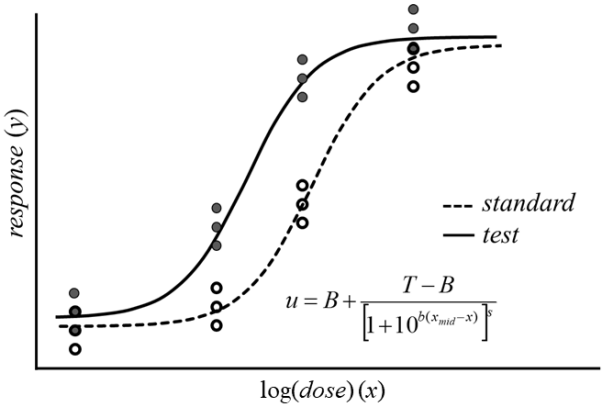
The confidence interval is obtained by multiplying A_T (or multiplying I_S/I_T if needed).

For (hd)-design, the equation is almost the same as in the case of using the $(hd+1)$-design. Use the modified expression as follows:

$$\alpha' = \alpha$$
$$V_1 = \frac{6}{nd(2d+1)}\left(\frac{1}{d+1} + \frac{3}{h(d-1)}\right),$$
$$V_2 = \frac{3(d+1)}{3(d+1) + h(d-1)}$$

3) Expanded Sigmoid Dose-Response Model

The Expanded Sigmoid Dose-Response Model is suitable, for example, for some immunoassays when analysis is required of extended sigmoid dose-response curves.



In this case, the horizontal axis is the logarithms of the dose, with lowest concentration n the left and highest concentration on the right, and the vertical axis represents the responses. The individual responses to each treatment are indicated with black dots. The curves are the calculated log(dose)-response relationship for the standard and the test preparation. The general shape of the curves can usually be described by a logistic function but other shapes are also possible. Each curve represents 5 parameters including the upper asymptote (T), the lower asymptote (B), the slope factor (b), the horizontal location of inflection point (x_{mid}), and asymmetry coefficient (s). These log (dose)-response curves can be represented by the following equation, called 5-parameter logistic Model.

$$u = B + \frac{T-B}{[1 + 10^{b(x_{mid}-x)}]^s}$$

When using the symmetry sigmoid curve model, the asymmetry coefficient, s is 1, resulting in a 4-Parameter Logistic Model. In general, to have valid analysis when using the model with asymmetry coefficient 1, For a valid assay it is necessary that the curves of the standard and the test preparations have the same slope-factor, and the same maximum and minimum response level at the extreme parts. Only the horizontal location (x_{mid}) of the curves may be different. The horizontal distance between two curves is related to the "true" potency of the unknown. If the assay is used routinely, it may be sufficient to test the condition of equal upper and lower response levels when the assay is developed, and then to retest this condition directly only at suitable intervals or when there are changes in materials or assay conditions. The method of the logistic model can vary depending on the applied software and generally can obtain a 5-parameter or 4-parameter logistic model for the given data, using the functions provided. if "reasonable" estimates of the upper limit (T) and lower limit (B) are available, select for all preparations the doses with mean of the responses (u) falling between approximately 20% and 80% of the limits, transform responses of the selected doses to the following equation.

$$y = log_{10}(\frac{u-B}{T-u})$$

And use the parallel line model for the analysis, select an range of doses for which the responses (u) or suitably transformed responses, for example { $\log_{10}(u)$ } are approximately linear when plotted against $\log_{10}(dose)$, the parallel line model may then be used for analysis.

2. Quantal Response-based Analysis

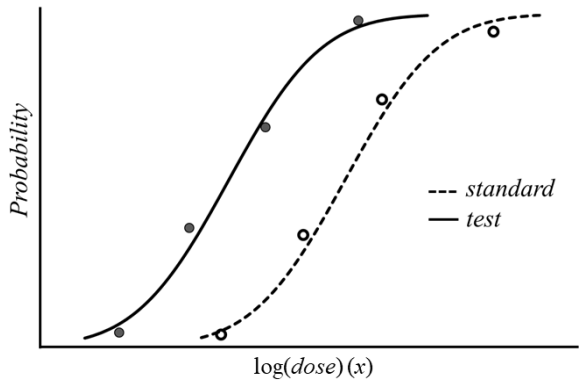
For some analytical methods, it is not feasible to determine the effects on each experimental unit on a quantitative scale. In this case, such effects can be measured by whether a specific symptom or death occurred in each experimental unit. The result depends on the number of experimental units that exhibited the corresponding reaction. These analytical methods are called quantal response-based analysis. It is to record the ratio of units showing responses per specific criteria (e.g., the number of units showing responses among n units, r/n with r ratio), rather than to record each response of individual n experimental units per specific treatment (dose). Schematizing such a ratio with logs (doses) assigned to the horizontal axis, the sigmoid curve can be obtained. The mathematical function showing this sigmoid curve is used to estimate the dose-response curve. In this case, the probability function of the cumulative normal distribution is usually used. The regression equation representing the dose-

response curve can be obtained by using the maximum likelihood method. However, for the convenience of calculation, the alternative method presented below can be used. For the use of this alternative method, the following conditions must be met.

- The relationship between the logarithm of the dose and the response can be represented by a cumulative normal distribution curve.
- The curves for the reference standard and the test preparation are parallel, i.e. they are identically shaped and may only differ in their horizontal location.
- In theory, there is no natural response to extremely low doses and no natural non-response to extremely high doses.

1) Probit Method

The sigmoid curve can be linear by replacing each responses, i.e. the fraction of positive responses per group, by the corresponding value of the cumulative standard normal distribution. Such replacement corresponds to the Probit transformation of "Biological Test Design and Data Preprocessing Method." Once the responses have been linearised, it should be possible to apply the parallel-line analysis. Unfortunately, the validity condition of homogeneity of variance for each dose is not fulfilled. The variance is minimal at normit = 0 and increases for positive and negative values of the normit. It is therefore necessary to give more weight to responses in the middle part of the curve, and less weight to the more extreme parts of the curve.



(1) Presentation of Experiment Results

For the experiment results according to the probit model, enter the data at the location corresponding to the following numbers on the table.

① Doses of reference standard or test preparation, $dose$

② Number of units submitted to that treatment, n

③ Number of units giving positive response to treatment, r

④ Logarithm value of dose, x

⑤ Fraction of positive response per group, $p = r/n$ The first cycle starts here.

⑥ Column Y is filled with zeros at the first iteration.

⑦ The corresponding value $\Phi = \Phi^{-1}(Y)$ of the cumulative standard normal distribution is calculated.

⑧ $Z = \frac{e^{-Y^2/2}}{\sqrt{2\pi}}$

⑨ $y = Y + \frac{p-\Phi}{Z}$

⑩ $w = \frac{nZ^2}{\Phi-\Phi^2}$

⑪ to ⑮ rows are calculated by the values wx, wy, wx^2, and wy^2 from x, y, and w of ④, ⑨, and ⑩ rows; the sum of ⑩ to ⑮ $(\sum)$ is calculated for each treatment.

The sum values are moved to the ① to ⑥ rows of the second table, and then ⑦ to ⑫ rows are calculated as follows.

⑦ $S_{xx} = \sum wx^2 - \frac{(\sum wx)^2}{\sum w}$

⑧ $S_{xy} = \sum wxy - \frac{(\sum wx)(\sum wy)}{\sum w}$

⑨ $S_{yy} = \sum wy^2 - \frac{(\sum wy)^2}{\sum w}$

⑩ $\overline{x} = \frac{\sum wx}{\sum w}$

⑪ $\overline{x} = \frac{\sum wy}{\sum w}$

Calculation Table 1

	①	②	③	④	⑤	⑥	⑦	⑧	⑨	⑩	⑪	⑫	⑬	⑭	⑮
	$dose$	n	r	x	p	Y	Φ	Z	y	w	wx	wy	wx^2	wy^2	wxy
S	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮
										$\sum$	$\sum$	$\sum$	$\sum$	$\sum$	$\sum$
T	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮
etc.										$\sum$	$\sum$	$\sum$	$\sum$	$\sum$	$\sum$

Calculation Table 2

	①	②	③	④	⑤	⑥	⑦	⑧	⑨	⑩	⑪	⑫

	$\sum w$	$\sum wx$	$\sum wy$	$\sum wx^2$	$\sum wy^2$	$\sum wxy$	S_{xx}	S_{xy}	S_{yy}	$\overline{x}$	$\overline{y}$	α
S	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮
T	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮
etc							$\sum$	$\sum$				

The common slope β and intercept values are obtained by the following equation, and the same calculation applies to the sample.

$$\beta = \frac{\sum S_{xy}}{\sum S_{xx}}, ⑫\ \alpha = \overline{y} - \beta \cdot \overline{x}$$

Column ⑥ of the first table can be replaced by $Y = \alpha + \beta \cdot x$, and the cycle is repeated until the difference between two cycles has becomes small (for example, maximum difference of Y between 2 consecutive cycles is smaller than 10^{-8}).

(2) Tests of Validation

Before calculating the potencies and confidence intervals, validity of the assay must be assessed. If at least 3 doses for each preparation have been included, the deviations from linearity can be measured as follows: add a 13th column to above second table and fill it with $S_{yy} - \frac{S_{xy}^2}{S_{xx}}$. The column total is a measure of deviations from linearity and is approximately χ^2 distributed with degrees of freedom equal to $N - 2h$. Significance of this value can be assessed by comparing it to the χ^2- critical value, ${\chi_{critical}}^2(\alpha, f)$ or calculated corresponding probability. If the value is significant at the 0.05 probability level, the assay must probably be rejected. When the above test gives no indication of significant deviations from linear regression, the deviations from parallelism are tested at the 0.05 significance level with following χ^2 value and $h - 1$ degrees of freedom.

$$\chi^2 = \sum \frac{S_{xy}^2}{S_{xx}} - \frac{(\sum S_{xy})^2}{\sum S_{xx}}$$

(3) Calculation of Potency Ratio

When there are no indications for a significant departure from parallelism and linearity, the ln (potency ratio), ${M_T}'$, is calculated as follows.

$${M_T}' = \frac{\alpha_T - \alpha_S}{\beta}$$

The corresponding CI is calculated by the following equation.

$$CM_T' - (C-1)(\overline{x}_S - \overline{x}_T) \pm \sqrt{(C-1)(V\sum S_{xx} + C({M_T}' - \overline{x}_S + \overline{x}_T)^2)}$$

Where, $C = \frac{b^2 \sum S_{xx}}{b^2 \sum S_{xx} - s^2 t^2}$ and $V = \frac{1}{\sum_S w} + \frac{1}{\sum_T w}$.

And the potency ratio and confidence intervals are finally obtained by calculated as the antigogarithm. If the test for deviations from linearity is significant, the assay should normally be rejected. If there are reasons to retain the assay, the formulae are slightly modified. *t* becomes the *t*-value (p = 0.05) with the same number of degrees of freedom as used in the check for linearity and s^2 becomes the χ^2 value divided by the same number of degrees of freedom (and thus typically is greater than 1). In addition, The test for parallelism is also slightly modified. The χ^2 value for non-parallelism is divided by its number of degrees of freedom. The resulting value is divided by s^2 calculated above to obtain an F-ratio with $h - 1$ and $N - 2h$ degrees of freedom, which is evaluated in the usual way at the 0.05 significance level ($\alpha = 0.05$).

2) Logit Motel and Gompit Model

The logit method may sometimes be more appropriate. The name of the method is derived from the logit function which is the inverse of the logistic distribution. The procedure is similar to that described for the probit method with the following modifications in the formulae for Φ and Z.

$$\Phi = \frac{1}{1 + e^{-Y}}, Z = \frac{e^{-Y}}{(1 + e^{-Y})^2}$$

, if it can be shown that the curve is not symmetric, the Gompertz distribution may be appropriate (the gompit method). In this case, the equations for calculating Φ and Z are as follows.

$$\Phi = 1 - e^{-e^Y}, Z = e^{Y - e^Y}$$

3) The Median Effective Dose

In some types of assay, it is desirable to determine a median effective dose which is the dose that produces a response in 50% of the units. The probit method can be used to determine this median effective dose (ED_{50}), but since there is no need to express this dose relative to a standard, the formulae are slightly different. A reference standard can optionally be included in order to validate the assay. Usually the assay is considered valid if the calculated ED_{50} of the reference standard is close enough to the assigned ED_{50}. What "close enough" in this context means depends on the requirements specified in the monograph. The tabulation of the responses to the test samples, and optionally a standard, is as described in the probit method. The test for linearity is as described in the probit method, and a test for parallelism is not necessary for this type of assay. The ED_{50} of test sample T, and similarly for the other samples, is obtained

with the following modifications.

$$M_T' = \frac{-\alpha_T}{\beta}$$

$$CM_T' - (C-1)\overline{x}_T \pm \sqrt{(C-1)(V\sum S_{xx} + C(M_T' - \overline{x}_T)^2)}$$

where $V = \frac{1}{\sum_T w}$, and C is left unchanged.

Tablet Friability Test
정제의 마손도시험법

This chapter provides guidelines for the friability determination of compressed, uncoated tablets. The test procedure presented in this chapter is generally applicable to most compressed tablets. Physical strength, such as hardness of a tablet is measured in this test.

Apparatus

Use a plastic drum with an internal diameter between 283.0 and 291.0 mm and a depth between 36.0 and 40.0 mm, of transparent synthetic polymer with polished internal surfaces, and subject to minimum electrostatic build-up (see figure for a typical apparatus). One side of the drum is removable. The tablets are tumbled with each revolution of the drum by a curved projection with an inside radius between 75.5 and 85.5 mm that extends from the middle of the drum to the outer wall. The outer diameter of the central ring is between 24.5 and 25.5 mm. The drum is attached to the horizontal axis of a device that rotates at 25 ± 1 rpm. Thus, at each turn the tablets roll or slide and fall onto the drum wall or onto each other (See Figure 1. Plastic drum).

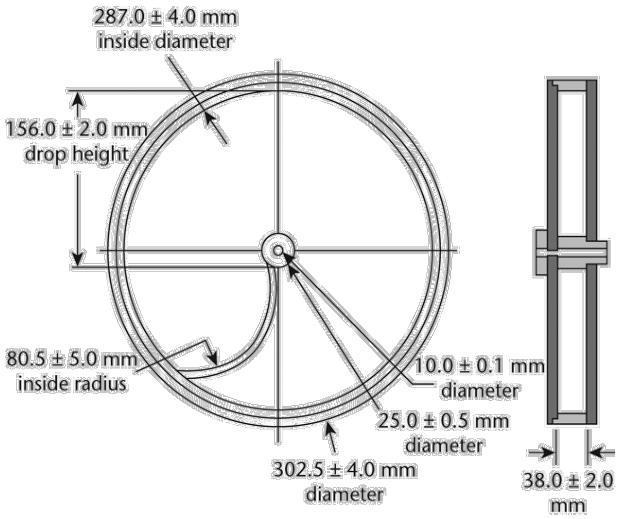


Fig. 1. Tablet Friability Apparatus

Procedure

For tablets with a unit weight equal to or less than 650 mg, take a sample of whole tablets corresponding as near as possible to 6.5 g. For tablets with a unit weight 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 at 25 ± 1 rpm, 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 weight loss from a single test or mean of three tests of not more than 1.0% is considered acceptable for most products. Typically, in the case of effervescent and chewable tablets, the friability specification may be different.

If tablet size or shape causes irregular tumbling, adjust the drum base so that the base forms an angle of about 10° with the horizontal and the tablets no longer bind together when lying next to each other, which prevents them from falling freely.

In the case of hygroscopic tablets, an appropriate humidity-controlled environment is required for testing.

Drums with dual scooping projections or an apparatus with more than one drum, for the running of multiple samples at one time, are also permitted.

Terminal Sterilization and Sterilization Indicators
최종 멸균법 및 멸균 지표체

Sterilization is a process of killing or removing all forms of viable microorganisms from substances. There are two methods of sterilization: terminal sterilization and filtration method. For products to which terminal sterilization is appropriate, a careful selection process is required to determine the most appropriate sterilization method. This selection considers the product's properties and packaging, as well as a thorough evaluation of the advantages and disadvantages of each sterilization method (heating, irradiating, and gas sterilizing methods). After installing the sterilizer (including the design and development of the sterilization process), it is necessary to verify that the process is operating precisely as designed with scientific rationale and validity under both loaded and unloaded conditions of the non-sterile product. After the sterilization process is established, it should be properly managed, and the qualification of the equipment should be validated on a regular basis.

When applying terminal sterilization, it is necessary to measure the bioburden of the non-sterile product regularly or at specified intervals (such as after processing certain batch units) and determine the bioburden for each non-sterile product. Please refer to the ISO standard (ISO 11737-1) for the bioburden measurement method. For products to which terminal sterilization is appropriate, sterilization should be performed under conditions in which a sterility assurance level of NLT 10^{-6} can be achieved. The suitability of sterilization should be determined by conducting appropriate sterilization process control, using appropriate sterilization indicators suitable each sterilization method, and if necessary, based on the results of the sterility test. Filtration method is used for the sterilization of liquid products, to which terminal sterilization cannot be applied. Refer to "Disinfection and Sterilization Methods" for the methods of killing all forms of viable microorganisms, which are required when conducting microbial-related testing, as specified in GMP and monographs.

1. Definitions

The terms used herein are defined as follows:

Terminal sterilization: A sterilization method in which the

non-sterile product, in its final container or packaging, undergoes sterilization that allows quantitative measurement or estimation of the killing (reduction) of the microbial population after the process.

Product: Refers to a non-sterile product that is produced in an intermediate stage of manufacturing and becomes a final product after subsequent manufacturing processes.

Bioburden: Refers to the number and types of viable microorganisms in a substance and/or product subject to sterilization.

Sterility assurance level (SAL): The maximum survival probability of contaminating bacteria presumed to exist among sterilized products processed through an appropriate sterilization process. It is expressed in $10^{-n.}$

Integrity test: A non-destructive method used to predict the filtration sterilization performance of a filter measured by a microorganism challenge test.

D value: A value representing the death rate of microorganisms, which refers to the time (Decimal Reduction Time) or the dose (Decimal Reduction Dose) required to reduce the test microorganism population by 90% or reduce the survival rate to 1/10.

Sterilization indicator: Indicators used to monitor the sterilization process as an index of sterility, such as biological indicators (BI), chemical indicators (CI), and dosimeters.

2. Terminal sterilization

A. Heating method

Heating method is a sterilization method that kills viable microorganisms using heat.

(i) Moist heat method

This refers to a method of killing viable microorganisms in high-pressure saturated steam. Factors that may affect sterilization include temperature, steam pressure and exposure time. Therefore, in the sterilization process control, it is necessary to continuously monitor the temperature, steam pressure and exposure time, and this must be included in the specifications of the sterilizer.

(ii) Dry heat method

Dry heat method is a method of killing viable microorganisms using dry heated air. Usually, a batch-type dry heat sterilizer or a funnel-type dry heat sterilizer is used. In this method, factors that may affect the sterilization include temperature and exposure time. Therefore, in routine sterilization process control, it is required to continuously monitor the temperature and exposure time, and this must be included in the specifications of the sterilizer.

B. Irradiation methods

There are two methods of irradiation: one is the radiation method, which directly kills microorganisms by using ionizing radiation, and the other is the microwave method, which uses high-frequency waves to generate heat and kill microorganisms.

(i) Radiation method

Ionizing radiation includes gamma rays emitted from a radioisotope such as cobalt 60 (^{60}Co), electron rays generated from an electron accelerator, and bremsstrahlung radiation (X rays). Although this procedure can be applied to heat-labile products, the possibility of material degradation should be considered. The sterilization dose of 25 kGy has been widely used so far, but there are several other methods that can be used to calculate the dose, and these are as follows. Method 1 of the ISO standard (ISO 11137) involves measuring the bioburden of the non-sterile product and calculating the sterilization dose based on the mean bioburden and standard resistance distribution. Method 2 in the ISO standard (ISO 11137) involves not measuring the bioburden, but instead calculating the resistance of viable microorganisms from the sterility test results of each cumulative dose exposure time, and then calculating the sterilization dose based on the resistance of viable microorganisms. The Log method (refer to 5-3) involves calculating the sterilization dose based on the bioburden and the D-value of the most resistant microorganism. In this method, factors affecting sterilization include dose (absorbed dose). Therefore, in the gamma-ray sterilization process control, it is required to measure the dose (the absorbed dose) at appropriate intervals, and to continuously monitor the exposure time in terms of the operation parameters (the conveyor speed and the cycle time). The dose control mechanism should be included in the specifications of the sterilizer. When using electron beam or bremsstrahlung irradiation, it is necessary to monitor the acceleration voltage, the beam current and beam scanning width, in addition to the above-mentioned items.

(ii) Microwave method

Microwave method refers to the method of killing microorganisms using heat generated by direct irradiation of microwave radiation. Usually, a high frequency of 2450 ± 50 MHz is used. This method is applied to liquids or water-rich products in hermetic containers. Since a glass or plastic container may be destroyed or deformed as the inner pressure increases, it is necessary to use containers that can withstand the heat and the inner pressure. Radio wave leakage generated from the microwave method must be at a sufficiently low level that does not cause any harm to humans and no interference with radio communications. In this method, factors that may affect sterilization include temperature, processing time and microwave output. Therefore, in the routine sterilization process control, it is required to continuously monitor the temperature, time and microwave output power, and this must be included in the specifications of the sterilizer.

C. Gas methods

Ethylene oxide (EO) gas is widely used as a sterilization gas. Since EO gas is explosive by nature, it is usually diluted with carbon dioxide to 10-30%. Ethylene oxide (EO) gas is a highly reactive alkylating agent, so it cannot be used for sterilizing products that are prone to react with or easily absorb EO gas. In addition, since ethylene oxide (EO) gas has residual toxicity, including mutagenicity, so for products that have undergone sterilization using EO gas, measures such as aeration to reduce the concentration of residual EO gas or other secondary toxic gases to a safe level must be taken before shipment. In this method, factors that may affect the sterilization include temperature, humidity, gas concentration (pressure), and exposure time. Therefore, the routine sterilization process control must include continuous monitoring of the temperature, humidity, gas concentration (pressure), and exposure time, and this must be included in the specifications of the sterilizer.

3. Filtration method

Filtration method refers to the method of killing viable microorganisms using a sterilizing filter made of an appropriate material. In addition, filter sterilization of microorganisms smaller than bacteria is not subject to this method. Generally, sterilizing filters for the purpose of sterilization contain at least 10^7 indicator bacteria *Brevundimonas diminuta* (AATCC 19146, NBRC 14213, and JCM 2428) or smaller bacteria cultured under appropriate conditions per square centimeter (cm^2) of effective filtration area of the membrane. In this method, factors that may affect the sterilization include filtration pressure, flow rate, and

characteristics of the filter. Therefore, in routine filtration process control, it is necessary to perform an integrity test of the sterilizing filter after each filtration process (also before the filtration process, if necessary).

4. Sterilization Indicators

A. Biological Indicator (BI)

BIs are prepared using indicator bacteria that show strong resistance to a specific sterilization method, and are used to determine sterilization conditions and control for the relevant sterilization process. The dry-type BI is classified into two types depending on the carrier. One has its spores applied, dried and packaged using filter paper, glass, or plastic as a carrier, and the other has its spores applied and dried using a product or similar product as a carrier. For packaging, materials with good thermal conductivity should be used in the dry heat method, and materials with excellent gas or saturated water vapor permeability should be used in the gas method and the moist heat method. Regardless of which carrier is used, it must be confirmed that it does not affect the D value of the spores of the biological indicators. If the product is in liquid form, the spores may be suspended in the same solution as the product, or in a solution with an equivalent sterilization effect on biological indicator. However, when the spores of the biological indicators are suspended in a solution, it is necessary to ensure that the resistance characteristics are not affected by germination.

Typical examples of biological indicators are shown in Table 1.

Table 1 Typical Examples of Biological Indicators

Sterilization methods	Biological indicator*	Strain name
Moist heat method	*Geobacillus stearothermophilus*	ATCC 7953, NBRC 13737, JCM 9488, KCTC 2107, ATCC 12980, NBRC 12550, JCM 2501, KCTC 1752
Dry heat method	*Bacillus atrophaeus*	ATCC 9372, NBRC 13721, KCTC 1022
Gas method	*Bacillus atrophaeus*	ATCC 9372, NBRC 13721, KCTC 1022

* In addition to these, other microorganisms with the greatest resistance to the relevant sterilization method in the bioburden can be used as biological indicators.

(1) D value of BI

Methods for measuring the D value include the survival curve method, the fraction negative method, Stumbo Murphy and Cochran Procedure (SMCP), and the Limited Spearman-Karber Procedure (LSKP). When using marketed BIs, it is not usually necessary 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. In most cases, the D value indicated on the label is allowed to vary within ±30 seconds.

(2) Installation method of BI

(i) When sterilizing dry materials

The dry-type BI is placed in the most difficult-to-sterilize area within a predetermined product or an appropriate similar product that demonstrates an equivalent sterilization effect. The BIs are usually packaged in the same manner as the product, including secondary packaging, if applicable.

(ii) When sterilizing 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 in the most difficult-to-sterilize area.

(3) Culture conditions of BI

Generally, soybean-casein digest broth is used. General culture conditions are *G.* at 55 - 60 ℃ for 7 days for *G. stearothermophilus,* and at 30 - 35 ℃ for 7 days for *B. atrophaeus.*

B. Chemical indicator (CI)

Chemical indicator (CI) refers to three types of substances applied or printed on paper or similar materials that show a change in color due to chemical or physical changes when exposed to heat, gas, or radiation, depending on their purposes. The first is used to identify whether sterilization has already been performed, the second is used to control the sterilization process (e.g., its color changes after sterilization for a sufficient time), and the third is the Bowie & Dick type, which is used when testing the vacuum exhaust ability of a vacuum-type sterilization device.

C. Dosimeter

Since the sterilization effect in the radiation (gamma) method depends on the absorbed dose of the product to be sterilized, the sterilization process control is usually performed by measuring the dose. Since the installation location of a dosimeter has a clear quantitative relationship with the minimum radiation region of the radiated container or the minimum radiation region, control and maintenance are easy. Measurement is performed for each radiation lot. If there are many containers in the same lot, NLT one dosimeter is always used in the effective irradiation section of the irradiation chamber. Care should be taken, as dosimeters may be affected by environmental conditions (such as temperature, humidity, ultraviolet light, and time to reading) before, during and after irradiation. Practical dosimeters for measuring the absorbed dose of the gamma ray and bremsstrahlung sterilization include the dyed polymethylmethacrylate dosimeter, clear polymethyl methacrylate dosimeter, ceric-cerous sulfate dosimeter, and alanine-EPR dosimeter. A dosimeter for gamma radiation is generally not suitable for sterilization process control in which electron beam energy of less than 3 MeV is used. Dosimeters for electron beam sterilization include cellulose acetate dosimeter and radiochromic film dosimeter. A practical dosimeter must be calibrated using an appropriate national or international standard dosimetry system.

5. Setting of sterilization conditions using microorganism as an indicator

Considering the characteristics of the sterilization method and the bioburden of the sterilized material, the appropriate method is selected from the following options and the sterilization conditions are established.

A. Half-cycle method

This method employs a sterilization time that is twice as long as the time required to kill all 10^6 counts included in the BI, regardless of the bioburden count present in the material being sterilized or the resistance of the detected bacteria to the corresponding sterilization method..

B. Overkill method

The overkill method assumes that sterilization is performed under conditions that can achieve a sterility assurance level of NMT 10^{-6}, regardless of the bioburden count or the resistance of the detected bacteria to the relevant sterilization method. Generally, the overkill method is a method that applies sterilization conditions equivalent to reducing the bioburden by 10^{-12} (12 D) using a validated BI with a known D value of 1.0 or more.

C. Combination method of BI and bioburden

This method calculates the sterilization time (or sterilization dose) using the BI based on the target sterility assurance level, typically using the average bioburden obtained from an extensive bioburden estimate added to three times the standard deviation as the maximum bioburden count. When using this method, the bioburden of the material to be sterilized must be frequently investigated and the resistance of the detected bacteria to the relevant sterilization method must be measured regularly. If a more resistant strain compared to the BI spore is found in the bioburden estimation, it should be used as the BI.

$$\text{Sterilization time (or radiation dose)} = D \times \log(N_0/N)$$

D: D value of the BI
N: Sterility assurance level
N_0: Maximum bioburden count in the product

D. Absolute bioburden method

In this method, a resistance test is conducted on bacteria detected in the material to be sterilized or the manufacturing environment, based on their resistance to the relevant sterilization method. Among these microorganisms, the most resistant ones are selected, and their D-values are used to establish the sterilization conditions, considering the bioburden of the material to be sterilized. Generally, the bioburden is usually calculated by adding three times the standard deviation to the average bioburden obtained from a wide range of bioburden surveys. If this method is adopted, bacterial count measurement and resistance measurement of the detected bacteria to the relevant sterilization method must be frequently performed in routine bioburden estimation.

Verification of Drug Test Method
의약품 시험방법 베리피케이션

1. Objective

Verification of a test procedure is the process that is performed to obtain suitable results under actual conditions of use, including the personnel, equipment, and reagents when it is newly introduced. Although validation of a compendial test procedure is not required to verify its suitability under actual conditions of use, in general, some of the analytical (procedure) performance characteristics (APPCs) listed in 5. Implementation of Verification of Test Procedures may be used. In addition, whether or not the test procedure is suitable for its intended purpose for a specified drug substance and/or drug product matrix should be assessed and then documented.

2. Scope

The intent of this chapter is to provide general information on the verification of compendial test procedures for drug products that are being performed for the first time to yield acceptable results utilizing the personnel, equipment, and reagents available. However, verification of biological test procedures is not covered in this chapter because their details can be modified unless their nature is interfered. This chapter is also not intended for retroactive application to already successfully established test procedures.

3. Introduction

Since compendial test procedures cannot be considered as validated for all impurities, which can vary depending on the formulation and synthesis methods, problems may arise in the application of test procedures for the following reasons:

- different impurity profiles depending on the manufacturers of raw materials
- the use of various excipients for each drug product
- different recovery of drug substance from drug product matrix due to the influence of excipients, antioxidants, buffer solutions, or container extractives
- variability of the analyzer's experience, analysis equipment, test environment, etc. among laboratories

Therefore, it is necessary to demonstrate the accuracy and reliability with which a compendial test procedure can be performed under actual conditions of use by conducting a verification that reflects potential variations when the procedure is introduced. Verification is not required for basic compendial test procedures that are routinely performed unless there is an indication that the compendial procedure is not appropriate for the article being tested. Examples of basic compendial procedures include, but are not limited to, loss on drying, residue on ignition, various wet chemical procedures such as acid value, and simple instrumental determinations such as pH measurements.

4. Overview of Verification of Test Procedures

A. Selection of Analytical Procedure Performance Characteristics

Verification consists of assessing selected analytical procedure performance characteristics (APPCs) to demonstrate the suitability of test procedures under conditions of actual use. The APPCs can be properly selected by comprehensively considering the test method and sample, and reflecting the following conditions:

- the level of training and experience of the analyzer;
- the type of test procedure;
- the analytical equipment and/or instrumentation being used;
- the specific procedural steps;
- Characteristics of the samples.

Since the evaluation parameters for the verification of the test method are part of validation, it is necessary to prove that some of the parameters included in validation conform to the specifications through verification of test method. As the assessment of APPCs for the verification of test procedures is part of the validation, some of the APPCs included in the validation should be demonstrated as conforming to their specifications through the verification of test procedures. For example, APPCs such as linearity do not need to be included in the verification of test procedures if there is no variation between laboratories, whereas APPCs that vary depending on laboratories, such as reproducibility, should be included in the verification of test procedures.

B. General Requirements

Analyzers are required to complete the training necessary for performing tests and training on the verification of test procedures, and to have the appropriate knowledge and experience on analysis technologies and instrument operations. The entire verification process for test procedures should be documented and approved. The APPCs must be described in detail, and the acceptance criteria used as the basis for determining the suitability of test procedures should also be included.

C. Classification of Test Procedures

Since various test procedures are used depending on their purposes (quantitative test, identification test, etc.), it is reasonable to perform the different verification items for their different purposes. This chapter presents five test purposes which require the verification of test procedures and the APPCs necessary for each purpose.

1) Identification tests
2) Quantitative tests (low concentration)
3) Quantitativetests (high concentration)
4) Limit tests (reference concentration close to the limit of quantitation)
5) Limit tests (reference concentration much higher than the limit of quantitation)

5. Conduct Verification of Test Method

A. Identification

APPCs	Verification	Note
Specificity	required	- Applied except for tests by simple chemical reactions (e.g., precipitation as a result of the reaction of Ag and Cl)

B. Quantitative Tests (low concentration, e.g., quantitative tests in purity tests, etc.)

APPCs	Verification	Note
Specificity	required	-
Accuracy	required	
Precision	required	
Limit of detection (LOD)	required	- Analysis of samples with concentrations close to the LOD
Limit of quantitation (LOQ)	required	- Analysis of samples with concentrations close to the LOQ

C. Quantitative tests (high concentration, e.g. content, dissolution test, etc.)

APPCs	Verification
Specificity	required
Accuracy	required
Precision	required

D. Limit tests (reference concentration close to the LOQ)

APPCs	Verification	Note
Specificity	required	-
Accuracy	required	- Analysis of samples with concentrations close to the LOQ
Precision	required	- Analysis of samples with limit range of concentrations

E. Limit tests (reference concentration much higher than the LOQ)

APPCs	Verification
Specificity	required
Accuracy	required
Precision	required

Viral Safety Evaluation for Biopharmaceutical Products from Cell Lines
세포주로부터 생산된 생물의약품의 바이러스 안전성 평가

1. Introduction

All biological products derived from cell lines have a risk of viral contamination. This contamination originates from contamination of the cell line itself (cell substrate) or occurs due to the introduction of foreign viruses during the production process. The safety of these medicinal products with respect to viral contamination can be reasonably ensured through the application of virus detection tests and evaluation of virus removal and inactivation during the manufacturing process.

Three main, complementary approaches have been developed to control the potential viral contamination of biological products:

(1) Selecting and testing raw materials, including cell lines and other media components, to control viruses that may be infectious and/or pathogenic to humans;

(2) Assessing the capacity of the production processes to clear infectious viruses;

(3) Testing of the product at appropriate stages of production to confirm the absence of contaminating infectious viruses.

Quantitative analysis of viruses has limitations. In other words, for statistical reasons, the detection ability for low-concentration viruses depends on the size of the sample. Therefore, no single approach can guarantee the safety of biological products. In addition to direct testing for the presence of infectious viruses, the absence of infectious viruses in the final product can be assured by demonstrating the ability of the purification process to remove and inactivate viruses. The type and scope of viral tests and clearance methods required for each production step will depend on various factors and should be considered on a case-by-case and step-by-step basis. Factors that should be taken into account include the characterization and scope of suitability of the cell bank, nature of detected viruses, culture medium constituents, culture methods, facility and equipment design, the results of viral tests after cell culture, the ability of the process to clear viruses, and the product type and its intended clinical use. This document applies to interferons produced from cell lines, antibody drugs, and other products made from genetically recombinant DNA, excluding inactivated vaccines and live vaccines. This document describes the general framework for the selection and performance of virus trials and the core concepts for evaluating virus clearance during the process.

2. Potential Factors of Viral Contamination

Viral contamination of biological products may occur from the original source of the cell lines or through introduction of adventitious viruses during production processes.

A. Viruses that Could contaminate the master cell bank (MCB)

Cells may have latent or persistent virus infection (e.g., herpesvirus) or endogenous retrovirus which may be transmitted vertically from one cell generation to the next, since the viral genome persists within the cell. These viruses can become infectious either persistently or unexpectedly. Viruses can be introduced into the MCB through several routes such as: (1) Derivation of cell lines from infected animals; (2) Establishment of cell lines using viruses; (3) the use of contaminated biological reagents such as animal serum components; (4) contamination during cell handling.

B. Adventitious viruses that could be introduced during the production process

Adventitious viruses can be introduced into the final product through several routes including: (1) the use of contaminated biological reagents such as animal serum components; (2) the use of viruses to induce target protein expression; (3) the use of a contaminated reagent, such as monoclonal antibody affinity columns; (4) the use of a contaminated excipients during formulation; and (5) contamination occurring during cell and medium handling. Monitoring of cell culture parameters can be helpful in the early detection of potential adventitious viral contamination.

3. Cell Line Qualification: Testing for Viruses

Appropriate testing for the presence of viruses is important to confirm the suitability of cell lines used in the production of biological products.

A. Suggested Virus Testing Methods for MCB, Working Cell Bank (WCB) and Cells at the Limit of In Vitro Cell Age Used in Production

Table 1 provides examples of virus testing methods to be performed once at each cell stage, including MCB, WCB, and cells at the limit of in vitro cell age used in production.

Table 1. Examples of viral tests to be Performed at each cell stage

	MCB	WCB	EOPC
Tests for retroviruses and other endogenous viruses			
Infectivity	+	-	+
Electron microscope[1]	+[1]	-	+[1]
Reverse transcriptase [2]	+[2]	-	+[2]
Other virus-specific tests [3]	+[3]	-	+[3]
Tests for non-endogenous or adventitious viruses			
In vitro assays	+	-[4]	+
In vivo assays	+	+[4]	+
Antibody production tests[5]	+[5]	-	-
Other virus-specific tests [6]	+[6]	-	-

1. Other contaminants can also be detected.
2. Not necessary if retrovirus infectivity test is positive.
3. Test suitable for cell lines known to be infected with a specific virus.
4. For the first WCB, this test should be performed on cells at the limit of in vitro cell age derived from WCB. For new WCB derived from the same master cell bank, a single in vitro test (e.g., using one of the recommended indicator cells) and in vivo test (e.g., using one of the recommended animal tests) are sufficient.
5. There are MAP, RAP, HAP, etc., which are usually applied to rodent cell lines.
6. Testing of cell lines derived from human, or other primate origin, etc.

(1) Master cell bank (MCB)

In-depth testing for both endogenous and nonendogenous viral contamination should be performed on the MCB. Hetero-hybrid cell lines containing cells of human or other primate origin should be tested in order to detect viruses of human or other primate origin because viral contamination resulting from these cells may pose a particular hazard. With reference to the cell line passage history, non-endogenous virus testing should be able to detect possible contaminating viruses by conducting in vitro and in vivo inoculation tests and any other specific tests, including species-specific tests such as the mouse antibody production (MAP) test.

(2) Working Cell Bank (WCB)

WCB should be tested for adventitious virus either by direct testing or by analyzing cells at the limit of in vitro cell age. If appropriate nonendogenous virus testing have been performed on the MCB, and adventitious virus testing has been performed after cell lines have been cultured to or beyond the limit of shelf life, there is no need to conduct similar testing in the associated initial production cell bank. Antibody production tests are usually not necessary for the WCB. On the other hand, it may also be considered to perform all tests in a WCB rather than a MCB.

(3) Cells at the limit of *in vitro* cell age used in the production

The limit of *in vitro* cell age of cells used in the production should be based on data obtained from production cells expanded under pilot-plant scale or commercial-scale conditions to the proposed in vitro cell age or beyond. Generally, the production cells are prepared from cell proliferation in the WCB, but may also be prepared from a MCB. Cells at the limit of in vitro cell age should be evaluated once in cells at the limit of in vitro cell age because endogenous viruses may not be detected in the MCB and WCB. Additionally, by conducting appropriate tests for adventitious viruses (e.g., in vitro and in vivo) at least once on cells at the limit of in vitro cell age, it is possible to further ensure that the production process is not vulnerable to contamination by adventitious viruses. If any adventitious viruses are detected at this level, the process should be carefully checked in order to determine the cause of the contamination, and should be completely redesigned if necessary.

B. Recommended viral detection and identification tests

There are various test methods that can be used to detect endogenous and adventitious viruses, and Table 2 provides examples of these testing methods. Those methods are currently recommended, and may be replaced by validated new testing methods in line with the advancement of science and technology. Consultation with the regulatory authorities is recommended for such alternative testing methods. If other test methods other than the ones listed in Table 2 are required, an appropriate control group should be included to ensure sufficient sensitivity and specificity. If it is anticipated that certain viruses are more likely to be present based on the origin species of the cell substrate, a specific testing method or approach is necessary. In other words, if the cell line used in production is derived from humans or other primates, additional testing for human viruses known to cause immunodeficiency or hepatitis should be conducted, unless specific exceptions apply. Polymerase chain reaction (PCR) is suitable for the detection of various human viruses or certain viruses.

(1) Tests for retroviruses

Retroviral tests, such as infectivity tests in cell culture susceptible to infection and electron microscopy, are performed on master cell banks and production cells cultured at or beyond the limit of in vitro cell age. If infection is not detected and no retrovirus or retrovirus-like particles have been observed by EM, reverse transcriptase (RT) or other appropriate assays should be performed to detect retroviruses that may be noninfectious. Viral induction studies were not found to be useful.

(2) *In vitro* assay

In vitro tests are performed by inoculating and culturing test samples into various sensitive indicator cells capable of detecting a wide range of human and relevant animal viruses. The choice of cells used in the test is based on the species of origin of the cell bank to be tested, but should include a human and/or a nonhuman primate cell line (The FDA recommends including both types of cells and cell lines used in production as well) susceptible to human viruses and/or other primate cells. Test methods and samples are selected considering the type of virus that may possibly be present depending on the origin or handling of the cells. Both cytopathic effect and hemadsorption must be confirmed(The FDA additionally recommends checking for hemagglutination).

(3) *In vivo* assay

Samples should be inoculated into animals, including suckling and adult mice, and in embryonated eggs to detect viruses that cannot grow in cell cultures. Additional animal species may be used, depending on the nature and origin of the cell lines being tested. The health of the animals should be monitored and any abnormality should be investigated to establish the cause of the illness.

Table 2. Examples of the Use and Limitations of Assays Which May be Used to Test for Virus

Test		Test Article	Detection Capability	Detection Limitation
Antibody production		Cell Lysates cells and culture medium	Specific viral antigens	Antigen that are not infectious for test animals.
In vivo virus screen		Cell lysates and culture medium	Broad range of pathogenic viruses in humans	A virus that does not multiply or cause disease in a test system
In vitro virus screen for:	**Cell bank characterization**	Lysate of cells and their culture medium (for co-cultivation, intact cells should be in the test article)		
	Production screen	Crude recovery or cell lysate and cell culture medium from production reactors		
TEMOn:	**Cell substrate**	Viable cells	Virus and virus-like particles	Qualitative assay with assessment of identity
	Cell culture supernatant	Cell-free culture supernatant		
Reverse transcriptase (RT) assay		Cell-free culture supernatant	Retrovirus and expressed retroviral RT	Measurement is possible only when optimal activity is achieved under selected conditions. Interpretation may be difficult due to presence of cellular enzymes due to concentrated samples
Retrovirus infectivity assay		Cell-free Cell culture supernatant	Infectious retrovirus	RV failing to replicate or form clear focuses or plaques in the chosen test system
Co-culture assay		Viable cells	Infectious retrovirus	RV failing to replicate
1. Infectivity endpoint				1. Refer to the RV infectivity above.
2. TEM endpoint				2. Refer to the TEM above.
3. RT endpoint				3. Refer to the RT above.
Polymerase chain reaction (PCR)		Cells, culture fluid, Other materials	Specific virus sequences	The Primer sequence must be present, and the infectivity of the virus cannot be confirmed.

(4) Antibody production tests

Species-specific viruses present in rodent cell lines may be detected by inoculating samples into virus-free animals and examining the serum antibody concentration or enzyme activity after a specified period. These tests include mouse antibody production (MAP) test, rat antibody production (RAP) test, and hamster antibody production (HAP) test. The viruses currently detectable in the antibody production test are shown in Table 3.

Table 3. Viruses Detected in Antibody Production Test

MAP	HAP	RAP
Ectromelia virus[2,3]	Lymphocytic Choriomeningitis virus(LCM)[1,3]	Hantaan Virus[1,3]
Hantaan Virus [1,3]	Pneumonia virus of Mice (PVM)[2,3]	Kilham rat virus (KRV)[2,3]
K virus[2]	Reovirus Type 3 (Reo3)[1,3]	Mouse encephalomyelitis virus(Theilers, GDVII)[2]
lactate dehydrogenase virus (LDM)[1,3]	Sendai virus[1,3]	Pneumonia Virus of Mice (PVM)[2,3]
Lymphocytic Choriomeningitis Virus (LCM)[1,3]	SV5	Rat coronavirus(RCV)[2]
Minute virus of mice[2,3]		Reovirus type 3(Reo3)[1,3]
Mouse adenovirusvirus[2,3]		Sendai virus[1,3]
Mouse cytomegalovirus(MCMV)[2,3]		Sialadenitis virus(SDAV)[2]
Mouse encephalitis virus(Theilers, GDVII)[2]		Tulane virus(HI)[2,3]
Mouse-infection virus(MHV)[2]		
Mouse rotavirus(EDIM)[2,3]		
Pneumonia Virus of Mice (PVM) [2,3]		
Polyoma virus[2]		
Reovirus type 3(Reo3)[1,3]		
Sendai virus[1,3]		
Thymus virus[2]		

1. It has been reported to be infectious to humans and primates
2. No cases of human infection have been reported
3. The virus can proliferate in cells derived from humans or primates

C. Considerations when using cell lines

It is recognized that some cell lines used for the manufacture of product contain endogenous retroviruses, other viruses, or viral sequences. In such circumstances, virus clearance and virus testing in the purified concentrate are recommended. In order to use cell lines containing viruses other than endogenous retroviruses, the product's benefit and clinical use, type of contaminating virus, potential to cause infection or disease in humans, drug purification process (e.g., virus clearance evaluation data), purified stock solution, and the results of the risk/benefit analysis based on the scope of virus testing performed in the test should be considered.

4. Testing for Viruses in Unprocessed Bulk

The unprocessed bulk constitutes of one or multiple pooled harvests of cells and culture media. Testing of unprocessed bulk is one of the most appropriate steps to determine the possibility of adventitious virus contamination. It is appropriate for virus testing to be performed on at least three lots of the unprocessed bulk. If the unprocessed bulk is not suitable for testing, such as showing toxicity in cell culture for virus testing, the virus test can be performed with the stock solution that has gone through the initial purification process. In certain instances, retrieve and examine a mixture of cells, disrupted cells, and cell culture supernatants from the production incubator.

It is recommended that manufacturers develop a program for the ongoing assessment of adventitious viruses in production batches. The scope, extent, and frequency of virus testing from the unprocessed bulk should be determined by considering several factors, including the characteristics of the cell lines , the results and extent of virus testing on the production cell line, the culture method, raw material sources, and results of viral clearance. Testing of unprocessed bulk typically involves in vitro testing using one or more cell lines (FDA recommends using three cell lines). If appropriate, a PCR test or other suitable methods may be used.

Generally, crude solutions in which adventitious virus has been detected should not be used in product manufacturing. If any adventitious viruses are detected at this level, the process should be carefully checked to determine the cause of the contamination, and appropriate measures must be taken.

5. Evaluation and Characterization of Viral Clearance Procedures

The purification process consists of several purification steps that may have the ability remove/inactivate the virus, and it also may include steps aimed solely at removing/inactivating the virus. For this reason, the purification process plays an important role in ensuring that the drug product is not contaminated with viruses. Therefore, it is crucial to accurately evaluate the virus removal/inactivation ability of each relevant step through research.

The purpose of evaluating viral clearance ability is to investigate and assess the characteristics of process steps that are effective in removing/inactivating viruses, as well as to quantitatively calculate the overall reduction level of viruses resulting from the manufacturing process.

Characterization of the process steps is conducted by investigating the robustness of virus clearance. Robustness is to measure the acceptable worst conditions or scope for several variables such as temperature, protein concentration, pressure, mobile phase flow rate, and pH. In general, it measures consistent removal/inactivation ability against non-specific model viruses with a wide range of physicochemical resistance properties.

Studies assessing virus clearance ability are performed using relevant viruses or specific model viruses and measuring their level of reduction. Whether these evaluations should be conducted depends on findings from the tests on cell lines and the unprocessed bulk. In other words, if no viruses or virus-like particles are detected in the cell line and unprocessed bulk, this evaluation may be omitted. However, if a virus or virus-like particle is detected, this study should be performed.

Infectivity tests using animals or cells or PCR tests are mainly used as virus verification tests required when investigating virus removal characteristics and evaluating removal ability.

6. Virus Tests on Purified Bulk

Virus tests on purified bulk are the final evaluation step to ensure that the final product is free from viral contamination. The absence of viruses should be confirmed in the purified bulk. To this end, the test should be performed using an appropriate testing method with high specificity and sensitivity enough to detect the virus in question. For product approval, test results must be presented from 3 lots of purified bulk manufactured on a pilot or commercial production scale.

The necessity and scope of the test may vary depending on the results of viral tests on cell substrates and unprocessed bulk.

If viruses or virus-like particles are not detected in the cell substrate and unprocessed bulk, viral tests of the purified bulk may not be necessary. However, if a virus or a retrovirus-like particle is detected in the cell substrate and unprocessed bulk, appropriate testing is required for the virus (or virus-like particle) identified in the purified bulk, even if the virus removal/inactivation step has been performed through the purification process.

A production system contaminated with viruses other than rodent retroviruses is usually not used. However, if there is a valid and justifiable reason to use such a system, consultation with regulatory authorities is required. In this case, it is necessary to include proven steps that will certainly remove/inactivate the virus in question in the purification process.

7. Conclusion

This document presents fundamental evaluation concepts to ensure the absence of viral contamination in biological products. It describes the factors assessing potential viral contamination, the importance and methods of virus tests in the quality analysis of the production cell line, as well as the viral tests and viral clearance competency evaluation studies in unprocessed and purified bulk. Additional information can be referenced from the foreign compendium, the International Conference on Harmonization (ICH) guidelines, and the FDA guidelines, etc.

In addition to what is presented in this document, cutting-edge methods that reflect the development of science and accumulation of experiences can be considered to ensure the quality and safety of biological products. In addition, other reasonable alternatives can be applied for each case, considering the origin, derivation, type, manufacturing method, characteristics, and clinical use, etc. of the product.

Terms and Definition

Cell substrate

Cell used in the product manufacture.

Indicator cell

Indicator cell refers to cells of various species, used to detect unintended adventitious contaminants. For example, MRC-5 is commonly used as a human diploid cell and Vero cells are widely used as a simian renal cell.

Master cell bank (MCB)

Cells from animals or other sources for which characterization has been sufficiently analyzed are cultured in the parent cell line to a certain passage level, distributed into several containers and frozen for preservation, and a certain amount of uniform composition is frozen under specified conditions such as the vapor phase or liquid phase of liquid nitrogen. The MCB is used to derive all working cell banks.

Working cell bank (WCB).

Cells from animals or other sources for which characterization has been sufficiently analyzed are cultured in a master cell bank to a certain passage level, dispensed into several containers and frozen for preservation, and a certain amount of a uniform composition is stored under specified conditions such as the vapor phase or liquid phase of liquid nitrogen.

Production cells

Cell substrate used to manufacture product.

Cells at the limit of *in vitro* cell age

Cells at the limit of *in vitro* cell age refer to cells that have reached or surpassed the proliferative stage where they are no longer suitable for further product production, and they are also known as end-of-production cells (EOPC). The limit of *in vitro* cell age is determined based on data obtained from pilot or commercial-scale production.

Unprocessed bulk

Cells and culture medium harvested once or more times. When cells cannot be easily obtained, the unprocessed bulk would consists of liquid harvested from the fermenter.

Adventitious virus

Unintentionally introduced contaminant virus.

Endogenous virus

Viral entity whose genome is part of the germ line of the species of origin of the cell line and is covalently integrated into the genome of animal from which the parental cell line was derived. For the purposes of this document, intentionally introduced, nonintegrated viruses such as EBV used to immortalize cell substrates or Bovine Papilloma Virus fit in this category.

Nonendogenous virus

Virus from external sources present in the MCB.

Virus-like particles

Particles that appears morphologically similar to a known virus when observed under an electron microscope.

Nonspecific model virus

Common types of viruses used in studies evaluating the ability (robustness, etc.) of a process to remove/inactivate viruses.

Relevant virus

Virus used in studies evaluating the ability (robustness, etc.) of the process to remove/inactivate viruses, which is either the identified virus, or of the same species as the known virus, or likely to contaminate the cell substrate or any other reagents or materials used in the production process.

Specific model virus

Virus that is closely related to the known or suspected virus (same genus or family), having similar physical and chemical properties to those of the observed or suspected virus, which is used in studies evaluating the ability (robustness, etc.) of the process to remove/inactivate viruses.

Viral clearance

A process or method to eliminate target virus by removing viral particles or inactivating viral infectivity.

Virus removal

Physical separation of virus particles.

Virus inactivation

Reduction of viral infectivity caused by chemical or physical modification.

Water-Solid Interactions: Determination of Sorption-desorption Isotherms and of Water Activity

수분-고체 상호작용: 수착-탈착 등온곡선 및 수분 활성도 측정법

INTRODUCTION

Pharmaceutical solids as drug substances or as constituents of dosage forms most often come in contact with water during processing and storage. This may occur (a) during crystallization, lyophilization, wet granulation, or spray drying; and (b) because of exposure upon handling and storage to an atmosphere containing water vapor or exposure to other materials in a dosage form that contain water capable of distributing it to other ingredients. Some properties known to be altered by the binding of solids with water include rates of chemical degradation in the "solid-state", crystal growth and dissolution, dispersibility and wetting, powder flow, lubricity, powder compactibility, compact hardness, and microbial contamination.

Although precautions can be taken when water is perceived to be a problem, i.e., eliminating all moisture, reducing contact with the atmosphere, or controlling the relative humidity of the atmosphere, such precautions generally add expense to the process with no guarantee that during the life of the product further problems associated with moisture will be avoided. It is also important to recognize that there are many situations where a certain level of water in a solid is required for proper performance, e.g., powder compaction. It is essential for both reasons, therefore, that as much as possible is known about the effects of moisture on solids before strategies are developed for their handling, storage, and use.

Some of the more critical pieces of required information concerning water–solid interactions are:

- total amount of water present;
- the extent to which adsorption and absorption occur;
- whether or not hydrates form;
- specific surface area of the solid, as well as such properties as degree of crystallinity, degree of porosity, and glass transition and melting temperature;
- site of water interaction, the extent of binding, and the degree of molecular mobility;
- effects of temperature and relative humidity;
- essentially irreversible hydration;
- kinetics of moisture uptake;
- various factors that might influence the rate at which water vapor can be taken up by a solid;
- for water-soluble solids capable of being dissolved by the sorbed water, under which conditions dissolution will take place.

PHYSICAL STATES OF SORBED WATER

Water can physically interact with solids in different ways. It can interact at the surface (adsorption) or it can penetrate the bulk solid structure (absorption). When both adsorption and absorption occur, the term sorption is often used. Adsorption is particularly critical in affecting the properties of solids when the specific surface area is large. Large values of specific surface area are seen with solids having very small particles, as well as with solids having a high degree of intraparticle porosity. Absorption is characterized by an association of water per gram of solid that is much greater than that which can form a monomolecular layer on the available surface, and an amount that is generally independent of the specific surface area.

Most crystalline solids will not absorb water into their bulk structures because of the close packing and high degree of order of the crystal lattice. Indeed, it has been shown that the degree of absorption into solids exhibiting partial crystallinity and partial amorphous structure is often inversely proportional to the degree of crystallinity. With some crystalline solids, however, crystal hydrates may form. These hydrates may exhibit a stoichiometric relationship, in terms of water molecules bound per solid molecule, or they may be non-stoichiometric. Upon dehydration, crystal hydrates may either retain their original crystal structure, lose their crystallinity and become amorphous, or transform into a new anhydrous or less-hydrated crystal form.

Amorphous or partially amorphous solids are capable of taking up significant amounts of water because there is sufficient molecular disorder in the solid to permit penetration, swelling, or dissolution. Such behavior is observed with most amorphous polymers and with small-molecular-mass solids rendered amorphous during preparation, e.g., by lyophilization, or after milling. The introduction of defects into highly crystalline solids will also produce this behavior. The greater the chemical affinity of water for the solid, the greater the total amount that can be absorbed. When water is absorbed by amorphous solids, the bulk properties of the solid can be significantly altered. It is well established, for example, that amorphous solids, depending on the temperature, can exist in at least one of two states: "glassy" or "fluid"; the temperature at which one state transforms into the other is the glass transition temperature, T_g.

Water absorbed into the bulk solid structure, by virtue of its effect on the free volume of the solid, can act as an efficient plasticizer and reduce the value of T_g. Because the rheological properties of "fluid" and "glassy" states are quite different, i.e., the "fluid" state exhibits much less viscosity as the temperature rises above the glass transition point, it is not surprising that a number of important bulk properties dependent on the rheology of the solid are affected by moisture content. Because amorphous solids are metastable relative to the crystalline form of the material, with small-molecular-mass materials, it is possible for absorbed moisture to initiate reversion of the solid to the crystalline form, particularly if the solid is transformed by the sorbed water to a "fluid" state. This is the basis of "cake collapse" often observed during the lyophilization process. An additional phenomenon noted specifically with water-soluble solids is their tendency to deliquesce, i.e., to dissolve in their own sorbed water, at relative humidities, RH_i, in excess of the relative humidity of a saturated solution of the solid, RH0. Deliquescence arises because of the high water solubility of the solid and the significant effect it has on the colligative properties of water. It is a dynamic process that continues to occur as long as RH_i is greater than RH_0.

The key to understanding the effects water can have on the properties of solids, and vice versa, rests with an understanding of the location of the water molecule and its physical state. More specifically, water associated with solids can exist in a state that is directly bound to the solid, as well as in a state of mobility approaching that of bulk water. This difference in mobility has been observed through such measurements as heat of sorption, freezing point, nuclear magnetic resonance, dielectric properties, and diffusion.

Such changes in mobility have been interpreted as arising because of changes in the thermodynamic state of water as more and more water is sorbed. Thus, water bound directly to a solid is often thought as unavailable to affect the properties of the solid, whereas larger amounts of sorbed water may become more

clustered and form water more like that exhibiting solvent properties. In the case of crystal hydrates, the combination of intermolecular forces (hydrogen bonding) and crystal packing can produce very strong water–solid interactions. Recognizing that the presence of water in an amorphous solid can affect the glass transition temperature and hence the physical state of the solid, at low levels of water, most polar amorphous solids are in a highly viscous glassy state because of their high values of T_g. Hence, water is "frozen" into the solid structure and is rendered immobile by the high viscosity, e.g., 1013 Pa·s. As the amount of water sorbed increases and T_g decreases, approaching ambient temperatures, the glassy state approaches that of a "fluid" state and water mobility along with the mobility of the solid itself increases significantly. At high *RH*, the degree of water plasticization of the solid can be sufficiently high so that water and the solid can now achieve significant amounts of mobility. In general, therefore, this picture of the nature of sorbed water helps to explain the rather significant effect moisture can have on a number of bulk properties of solids such as chemical reactivity and mechanical deformation. It suggests strongly that methods of evaluating chemical and physical stability of solids and solid dosage forms take into account the effects water can have on the solid when it is sorbed, particularly when it enters the solid structure and acts as a plasticizer.

Rates of Water Uptake

The rate and extent to which solids exposed to the atmosphere might either sorb or desorb water vapor can be a critical factor in the handling of solids. Even the simple act of weighing out samples of solid on an analytical balance and the exposure, therefore, of a thin layer of powder to the atmosphere for a few minutes can lead to significant error in, for example, the estimation of loss on drying values. It is well established that water-soluble solids exposed to relative humidities above that exhibited by a saturated solution of that solid will spontaneously dissolve via deliquescence and continue to dissolve over a long time period. The rate of water uptake in general depends on a number of parameters not found to be critical in equilibrium measurements because rates of sorption are primarily mass-transfer controlled with some contributions from heat-transfer mechanisms. Thus, factors such as vapor diffusion coefficients in air and in the solid, convective airflow, and the surface area and geometry of the solid bed and surrounding environment, can play an important role. Indeed, the method used to make measurements can often be the rate-determining factor because of these environmental and geometric factors.

DETERMINATION OF SORPTION-DESORPTION ISOTHERMS

Principle

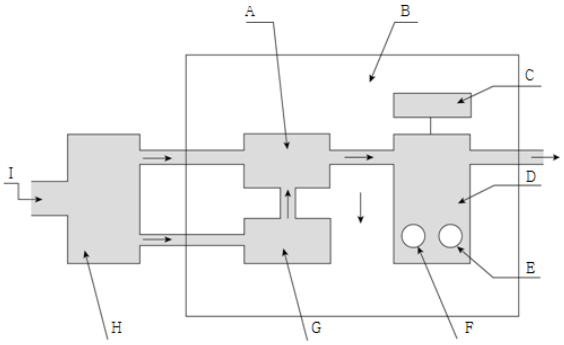
The tendency to take up water vapor is best assessed by measuring sorption or desorption as a function of relative humidity, at constant temperature, and under conditions where sorption or desorption is essentially occurring independently of time, i.e., equilibrium. Relative humidity, RH, is defined by the following equation:

$$P_C / P_0 \times 100$$

P_c = pressure of water vapor in the system

P_0 = saturation pressure of water vapor under the same conditions.

The ratio P_c/P_0 is referred to as the relative pressure. Sorption or water uptake is best assessed starting with dried samples and subjecting them to a known relative humidity. Desorption is studied by beginning with a system already containing sorbed water and reducing the relative humidity. As the name indicates, the sorption–desorption isotherm is valid only for the reference temperature, hence a special isotherm exists for each temperature. Ordinarily, at equilibrium, moisture content at a particular relative humidity must be the same, whether determined from sorption or desorption measurements. However, it is common to see sorption–desorption hysteresis.



A. Humidity controller
B. Constant temperature chamber
C. Balance module
D. Humidity regulated module
E. Reference
F. Sample
G. Vapor humidifier
H. Flow control module
I. Dry gas

Figure 1. Example of an apparatus for the determination of the water sorption (other designs are possible)

Methods

Samples may be stored in chambers at various relative humidities. The mass gained or lost for each sample is then measured. The major advantage of this method is convenience, while the major disadvantages are the slow rate of reaching constant mass, particularly at high relative humidities, and the error introduced in opening and closing the chamber for weighing. Dynamic gravimetric water sorption systems allow the on-line weighing of a sample in a controlled system to assess the interaction of the material with moisture at various programmable levels of relative humidity at a constant temperature. The major benefit of a controlled system is that isothermal conditions can be more reliably established and that the dynamic response of the sample to changing conditions can be monitored (see Figure 1).

Data points for the determination of the sorption isotherm (e.g., from 0% to approximately 95% relative humidity, noncondensing) are only taken after a sufficiently constant signal indicates that the sample has reached equilibrium at a given level of humidity. In some cases (e.g., deliquescence), the maximum time may be restricted although the equilibrium level is not reached. The apparatus must adequately control the temperature to ensure a good baseline stability as well as accurate control of the relative humidity generation. The required relative humidities can be generated, e.g., by accurately mixing dry and saturated vapor gas with flow controllers. The electrostatic behaviour of the powder must also be considered. The verification of the temperature and the relative humidity (controlled with, for example, a certified hygrometer, certified salt solutions, or deliquescence points of certified salts over an adequate range) must be consistent with the instrument specification. The balance must provide a sufficient mass resolution and long-term stability.

It is also possible to measure amounts of water uptake not detectable gravimetrically using volumetric techniques. In some cases, direct analysis of water content by different methods such

as determination of the boiling point, determination of water by distillation, loss on drying or gas chromatography may be advantageous. In the case of adsorption, to improve sensitivity, the specific surface area of the sample can be increased by reducing particle size or by using larger samples to increase the total area. It is important, however, that such comminution of the solid does not alter the surface structure of the solid or render it more amorphous or otherwise less ordered in crystallinity. For absorption, where water uptake is independent of specific surface area, only increasing sample size will help. Increasing sample size, however, will increase the time to establish some type of equilibrium. To establish accurate values, it is important to get desolvation of the sample as thoroughly as possible. Higher temperatures and lower pressures (vacuum) facilitate this process; however, care must be taken to note any adverse effects this might have on the solid such as dehydration, chemical degradation, or sublimation. Using higher temperatures to induce desorption, as in a thermogravimetric apparatus, likewise must be carefully carried out because of these possible pitfalls.

Report and Interpretation of the Data

Sorption data are usually reported as a graph of the apparent mass change in percent of the mass of the dry sample as a function of relative humidity or time. Sorption isotherms are reported both in tabular form and as a graph. The measurement method must be traceable with the data.

Adsorption–desorption hysteresis can be interpreted, for example, in terms of the porosity of the sample, its state of agglomeration (capillary condensation), the formation of hydrates, polymorphic change, or liquefying of the sample. Certain types of systems, particularly those with microporous solids and amorphous solids, are capable of sorbing large amounts of water vapor. Here, the amount of water associated with the solid as relative humidity is decreased, is greater than the amount that originally sorbed as the relative humidity was increased. For microporous solids, vapor adsorption–desorption hysteresis is an equilibrium phenomenon associated with the process of capillary condensation. This takes place because of the high degree of irregular curvature of the micropores and the fact that they “fill” (adsorption) and “empty” (desorption) under different equilibrium conditions. For nonporous solids capable of absorbing water, hysteresis occurs because of a change in the degree of vapor–solid interaction due to a change in the equilibrium state of the solid, e.g., conformation of polymer chains, or because the time scale for structural equilibrium is longer than the time scale for water desorption. In measuring sorption–desorption isotherms, it is therefore important to establish that something close to an equilibrium state has been reached. Particularly with hydrophilic polymers at high relative humidities, the establishment of water sorption or desorption values independent of time is quite difficult, because one is usually dealing with a polymer plasticized into its “fluid” state, where the solid is undergoing significant change.

In the case of crystal hydrate formation, the plot of water uptake versus pressure or relative humidity will in these cases exhibit a sharp increase in uptake at a particular pressure and the amount of water taken up will usually exhibit a stoichiometric mole:mole ratio of water to solid. In some cases, however, crystal hydrates will not appear to undergo a phase change or the anhydrous form will appear amorphous. Consequently, water sorption or desorption may appear more like that seen with adsorption processes. X-ray crystallographic analysis and thermal analysis are particularly useful for the study of such systems.

For situations where water vapor adsorption occurs predominantly, it is very helpful to measure the specific surface area of the solid by an independent method and to express adsorption as mass of water sorbed per unit area of solid surface. This can be very useful in assessing the possible importance of water sorption in affecting solid properties. For example, 0.5% m/m uptake of water could hardly cover the bare surface of 100 m2/g, while for 1.0 m2/g this amounts to 100 times more surface coverage. In the case of pharmaceutical solids that have a specific surface area in the range of 0.01 m2/g to 10 m2/g, what appears to be low water content could represent a significant amount of water for the available surface. Because the “dry surface area” is not a factor in absorption, sorption of water with amorphous or partially amorphous solids can be expressed on the basis of unit mass corrected for crystallinity, when the crystal form does not sorb significant amounts of water relative to the amorphous regions.

DETERMINATION OF THE WATER ACTIVITY

Principle

Water activity, (A_w), is the ratio of vapor pressure of water in the product (P) to saturation pressure of water vapor (P_0) at the same temperature. at the same temperature. It is numerically equal to 1/100 of the relative humidity (*RH*) generated by the product in a closed system. *RH* can be calculated from direct measurements of partial vapor pressure or dew point, or from indirect measurement by sensors whose physical or electric characteristics are altered by the *RH* to which they are exposed. Ignoring activity coefficients, the relationship between Aw and equilibrium relative humidity (*ERH*) are represented by the following equations:

$$A_W = P / P_0$$
$$\mathrm{ERH}(\%) = A_W \times 100$$

Method

The water activity is determined by placing the sample in a small airtight cup inside which the equilibrium between the water in the solid and the headspace can be established. The volume of the headspace must be small in relation to the sample volume in order not to change the sorption state of the sample during the test. The equilibration as a thermodynamic process takes time but may be accelerated by forced circulation within the cell. The acquired water activity value is only valid for the simultaneously determined temperature. This requires a precise temperature-measuring device as part of the equipment. Furthermore, the probe must be thermally insulated to guarantee a constant temperature during the test. The sensor measuring the humidity of the headspace air above the sample is a key component. Theoretically, all types of hygrometers can be used, but for analytical purposes miniaturization and robustness are a precondition. The A_w measurement may be conducted using the dew point/chilled mirror method. A polished, chilled mirror is used as a condensing surface. The cooling system is electronically linked to a photoelectric cell into which light is reflected from the condensing mirror. An air stream, in equilibrium with the test sample, is directed at the mirror which cools until condensation occurs on the mirror. The temperature at which this condensation begins is the dew point from which the ERH is determined. Commercially available instruments using the dew point/chilled mirror method or other technologies need to be evaluated for suitability, validated, and calibrated when used to make water activity determinations.

These instruments are typically calibrated over an adequate range, for example, using some saturated salt solutions at 25 °C such as those listed in Table 1.

Table 1. Standard Saturated Salt Solutions

Saturated Salt Solutions at 25 °C	***ERH* (%)**	**A_w**
Potassium sulfate (K_2SO_4)	97.3	0.973
Barium chloride ($BaCl_2$)	90.2	0.902
Sodium chloride (NaCl)	75.3	0.753
Magnesium nitrate ($Mg(NO_3)_2$)	52.9	0.529
Magnesium chloride ($MgCl_2$)	32.8	0.328
Lithium chloride ($LiCl_2$)	11.2	0.112