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「かさ密度及びタップ密度測定法」及び「エンドトキシン試験法」に係る
第十六改正日本薬局方英文版について

第十六改正日本薬局方は、平成23年3月24日厚生労働省告示第65号により告示されたところですが、当該薬局方中、三薬局方（日本薬局方、米国薬局方及び欧州薬局方）の薬局方検討会議（PDG）での調和合意に基づき規定した「かさ密度及びタップ密度測定法」及び「エンドトキシン試験法」について、英文版を作成しましたので御連絡いたします。

なお、標記試験法の相互利用については、今後、日米EU医薬品規制調和国際会議（ICH）における評価と勧告を得た後、改めてその運用方法を通知する予定です。



3.01 Determination of Bulk and Tapped Densities

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

♦Determination of Bulk and Tapped Densities is a method to determine the bulk densities of powdered drugs under loose and tapped packing conditions respectively. Loose packing is defined as the state obtained by pouring a powder sample into a vessel without any consolidation, and tapped packing is defined as the state obtained when the vessel containing the powder sample is to be repeatedly dropped a specified distance at a constant drop rate until the apparent volume of sample in the vessel becomes almost constant. ♦

1. Bulk density

The bulk density of a powder is the ratio of the mass of an untapped powder sample and its volume including the contribution of the interparticulate void volume. Hence, the bulk density depends on both the density of powder particles and the spatial arrangement of particles in the powder bed. The bulk density is expressed in grams per milliliter (g/mL) although the international unit is kilogram per cubic meter (1 g/mL = 1000 kg/m³) because the measurements are made using cylinders. It may also be expressed in grams per cubic centimeter (g/cm³).

The bulking properties of a powder are dependent upon the preparation, treatment and storage of the sample, i.e. how it was handled. The particles can be packed to have a range of bulk densities and, moreover, the slightest disturbance of the powder bed may result in a changed bulk density. Thus, the bulk density of a powder is often very difficult to measure with good reproducibility and, in reporting the results, it is essential to specify how the determination was made.

The bulk density of a powder is determined by measuring the volume of a known mass of powder sample, that may have been passed through a sieve into a graduated cylinder (Method 1), or by measuring the mass of a known volume of powder that has been passed through a volumeter into a cup (Method 2) or a measuring vessel (Method 3). Method 1 and Method 3 are favoured.

1.1. Method 1: Measurement in a Graduated Cylinder

1.1.1. Procedure

Pass a quantity of powder sufficient to complete the test through a sieve with apertures greater than or equal to 1.0 mm, if necessary, to break up agglomerates that may have formed during storage; this must be done gently to avoid changing the nature of the material. Into a dry graduated cylinder of 250 mL (readable to 2 mL), gently introduce, without compacting, approximately 100 g of the test sample (*m*) weighed with 0.1 per cent accuracy. Carefully level the powder without compacting, if necessary, and read the unsettled apparent volume (V_0) to the

nearest graduated unit. Calculate the bulk density in g per mL by the formula m/V_0 . Generally, replicate determinations are desirable for the determination of this property.

If the powder density is too low or too high, such that the test sample has an untapped apparent volume of either more than 250 mL or less than 150 mL, it is not possible to use 100 g of powder sample. Therefore, a different amount of powder has to be selected as test sample, such that its untapped apparent volume is 150 mL to 250 mL (apparent volume greater than or equal to 60 per cent of the total volume of the cylinder); the mass of the test sample is specified in the expression of results.

For test samples having an apparent volume between 50 mL and 100 mL a 100 mL cylinder readable to 1 mL can be used; the volume of the cylinder is specified in the expression of results.

1.2. Method 2: Measurement in a Volumeter

1.2.1. Apparatus

The apparatus⁽¹⁾ (Fig. 3.01-1) consists of a top funnel fitted with a 1.0 mm sieve. The funnel is mounted over a baffle box containing four glass baffle plates over which the powder slides and bounces as it passes. At the bottom of the baffle box is a funnel that collects the powder and allows it to pour into a cup mounted directly below it. The cup may be cylindrical (25.00 ± 0.05 mL volume with an inside diameter of 30.00 ± 2.00 mm) or a cubical (16.39 ± 0.20 mL volume with inside dimensions of 25.4 ± 0.076 mm).

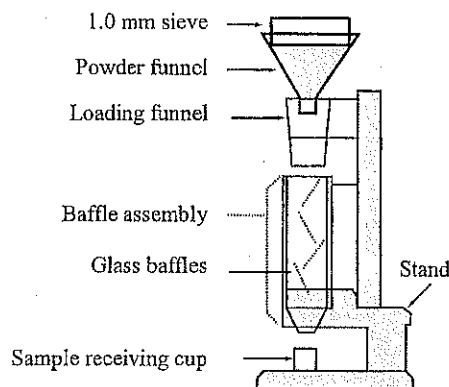


Fig. 3.01-1 Volumeter

1.2.2. Procedure

Allow an excess of powder to flow through the apparatus into the sample receiving cup until it overflows, using a minimum of 25 cm³ of powder with the cubical cup and 35 cm³ of powder with the cylindrical cup. Carefully, scrape excess powder from the top of the cup by smoothly moving the edge of the blade of spatula perpendicular to and in contact with the top surface of the cup, taking care to keep the spatula perpendicular to prevent packing or removal of powder from the cup. Remove any material from the side of the cup and determine the mass (*m*) of the powder to the nearest 0.1 per cent. Calculate the bulk density in g per mL by the formula m/V_0 in which V_0 is the

volume of the cup and record the average of 3 determinations using 3 different powder samples.

1.3. Method 3: Measurement in a Vessel

1.3.1. Apparatus

The apparatus consists of a 100 mL cylindrical vessel of stainless steel with dimensions as specified in Fig. 3.01-2.

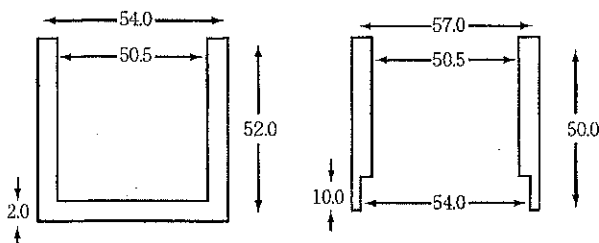


Fig. 3.01-2. Measuring vessel (left) and cap (right)
Dimensions in mm

1.3.2. Procedure

Pass a quantity of powder sufficient to complete the test through a 1.0 mm sieve, if necessary, to break up agglomerates that may have formed during storage and allow the obtained sample to flow freely into the measuring vessel until it overflows. Carefully scrape the excess powder from the top of the vessel as described for Method 2. Determine the mass (m_0) of the powder to the nearest 0.1 per cent by subtraction of the previously determined mass of the empty measuring vessel. Calculate the bulk density (g/mL) by the formula $m_0/100$ and record the average of 3 determinations using 3 different powder samples.

2. Tapped density

The tapped density is an increased bulk density attained after mechanically tapping a container containing the powder sample.

The tapped density is obtained by mechanically tapping a graduated measuring cylinder or vessel containing the powder sample. After observing the initial powder volume or mass, the measuring cylinder or vessel is mechanically tapped, and volume or mass readings are taken until little further volume or mass change is observed. The mechanical tapping is achieved by raising the cylinder or vessel and allowing it to drop, under its own mass, a specified distance by either of 3 methods as described below. Devices that rotate the cylinder or vessel during tapping may be preferred to minimize any possible separation of the mass during tapping down.

2.1. Method 1

2.1.1. Apparatus

The apparatus (Fig. 3.01-3) consists of the following:

- a 250 mL graduated cylinder (readable to 2 mL) with a mass of 220 ± 44 g.

- a settling apparatus capable of producing, in 1 min, either nominally 250 ± 15 taps from a height of 3 ± 0.2 mm, or nominally 300 ± 15 taps from a height of 14 ± 2 mm. The support for the graduated cylinder, with its holder, has a mass of

450 ± 10 g.

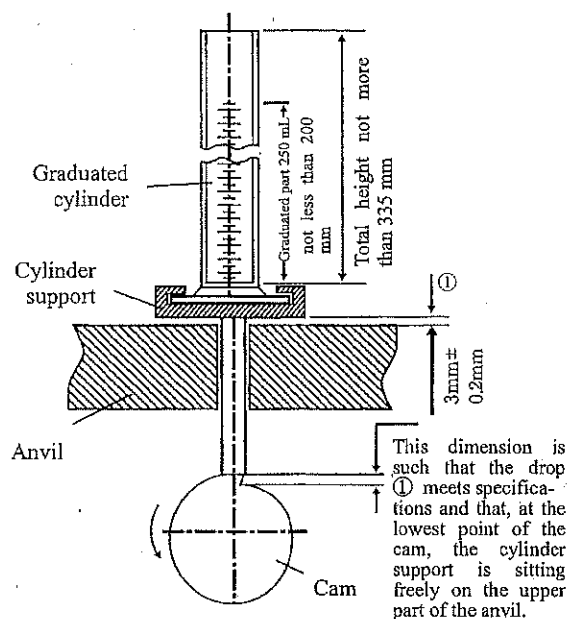


Fig. 3.01-3.

2.1.2. Procedure

Proceed as described above for the determination of the bulk volume (V_0).

Secure the cylinder in the holder. Carry out 10, 500 and 1250 taps on the same powder sample and read the corresponding volumes V_{10} , V_{500} and V_{1250} to the nearest graduated unit. If the difference between V_{500} and V_{1250} is less than or equal to 2 mL, V_{1250} is the tapped volume. If the difference between V_{500} and V_{1250} exceeds 2 mL, repeat in increments such as 1250 taps, until the difference between succeeding measurements is less than or equal to 2 mL. Fewer taps may be appropriate for some powders, when validated. Calculate the tapped density (g/mL) using the formula m/V_f in which V_f is the final tapped volume. Generally, replicate determinations are desirable for the determination of this property. Specify the drop height with the results.

If it is not possible to use a 100 g test sample, use a reduced amount and a suitable 100 mL graduated cylinder (readable to 1 mL) weighing 130 ± 16 g and mounted on a holder weighing 240 ± 12 g. The modified test conditions are specified in the expression of the results.

2.2. Method 2

2.2.1. Procedure

Proceed as directed under Method 1 except that the mechanical tester provides a fixed drop of 3 ± 0.2 mm at a nominal rate of 250 taps per minute.

2.3. Method 3

2.3.1. Procedure

Proceed as described in the method for measuring the bulk density using the measuring vessel equipped with the cap shown in Fig. 3.01-2. The measuring vessel with the cap is lifted 50-60

times per minute by the use of a suitable tapped density tester. Carry out 200 taps, remove the cap and carefully scrape excess powder from the top of the measuring vessel as described in Method 3 for measuring the bulk density. Repeat the procedure using 400 taps. If the difference between the 2 masses obtained after 200 and 400 taps exceeds 2 per cent, carry out a test using 200 additional taps until the difference between succeeding measurements is less than 2 per cent. Calculate the tapped density (g/mL) using the formula $m_t/100$ where m_t is the mass of powder in the measuring vessel. Record the average of 3 determinations using 3 different powder samples. The test conditions including tapping height are specified in the expression of the results.

3. Measures of Powder Compressibility

Because the interparticulate interactions influencing the bulking properties of a powder are also the interactions that interfere with powder flow, a comparison of the bulk and tapped densities can give a measure of the relative importance of these interactions in a given powder. Such a comparison is often used as an index of the ability of the powder to flow, for example the Compressibility Index or the Hausner Ratio.

The Compressibility Index and Hausner Ratio are measures of the propensity of a powder to be compressed as described above. As such, they are measures of the powder ability to settle and they permit an assessment of the relative importance of interparticulate interactions. In a free-flowing powder, such interactions are less significant, and the bulk and tapped densities will be closer in value. For poorer flowing materials, there are frequently greater interparticulate interactions, and a greater difference between the bulk and tapped densities will be observed. These differences are reflected in the Compressibility Index and the Hausner Ratio.

Compressibility Index:

$$100 (V_0 - V_f)/V_0$$

V_0 : unsettled apparent volume

V_f : final tapped volume

Hausner Ratio:

$$V_0/V_f$$

Depending on the material, the compressibility index can be determined using V_{10} instead of V_0 . If V_{10} is used, it is clearly stated in the results.

⁽¹⁾ The apparatus (the Scott Volumeter) conforms to the dimensions in ASTM 329 90.

4.01 Bacterial Endotoxins Test

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

Bacterial Endotoxins Test is a test to detect or quantify bacterial endotoxins of gram-negative bacterial origin using an amoebocyte lysate prepared from blood corpuscle extracts of horseshoe crab (*Limulus polyphemus* or *Tachypleus tridentatus*). There are two types of techniques for this test: the gel-clot techniques, which are based on gel formation by the reaction of the lysate TS with endotoxins, and the photometric techniques, which are based on endotoxin-induced optical changes of the lysate TS. The latter include turbidimetric techniques, which are based on the change in lysate TS turbidity during gel formation, and chromogenic techniques, which are based on the development of color after cleavage of a synthetic peptide-chromogen complex.

Proceed by any one of these techniques for the test. In the event of doubt or dispute, the final decision is made based on the gel-clot techniques, unless otherwise indicated.

The test is carried out in a manner that avoids endotoxin contamination.

1. Apparatus

Depyrogenate all glassware and other heat-stable materials in a hot-air oven using a validated process. Commonly used minimum time and temperature settings are 30 minutes at 250°C. If employing plastic apparatus, such as multi-well plates and tips for micropipettes, use only that which has been shown to be free of detectable endotoxin and which does not interfere with the test.

2. Preparation of Solutions

2.1. Standard Endotoxin Stock Solution

Prepare Standard Endotoxin Stock Solution by dissolving Japanese Pharmacopoeia Reference Standard Endotoxin that has been calibrated to the current WHO International Standard for Endotoxin, using water for bacterial endotoxins test (BET). Endotoxin is expressed in Endotoxin Units (EU). One EU is equal to one International Unit (IU) of endotoxin.

2.2. Standard Endotoxin Solution

After mixing Standard Endotoxin Stock Solution thoroughly, prepare appropriate serial dilutions of Standard Endotoxin Solution, using water for BET. Use dilutions as soon as possible to avoid loss of activity by adsorption.

2.3. Sample Solutions

Unless otherwise specified, prepare sample solutions by dissolving or diluting drugs, using water for BET. If necessary, adjust the pH of the solution to be examined so that the pH of the mixture of the lysate TS and sample solution falls within the specified pH range for the lysate to be used. This usually applies to a sample solution with a pH in the range of 6.0 to 8.0. TSs or solutions used for adjustment of pH may be prepared using water for BET, and then stored in containers free of detectable endotoxin. The TSs or solutions must be validated to be free of detectable endotoxin and interfering factors.

3. Determination of Maximum Valid Dilution

The Maximum Valid Dilution (MVD) is the maximum allowable dilution of a sample solution at which the endotoxin limit can be determined.

Determine the MVD from the following equation:

$$\text{MVD} = \text{Endotoxin limit} \times \text{Concentration of sample solution} / \lambda$$

Endotoxin limit:

The endotoxin limit for injections, defined on the basis of dose, equals K/M , where K is a threshold pyrogenic dose of endotoxin per kg body mass (EU/kg), and M is equal to the maximum bolus dose of product per kg body mass. When the product is to be injected at frequent intervals or infused continuously, M is the maximum total dose administered in a single hour period.

Concentration of sample solution:

mg/mL in the case of endotoxin limit specified by mass (EU/mg)

mEq/mL in the case of endotoxin limit specified by equivalent (EU/mEq)

Units/mL in the case of endotoxin limit specified by biological unit (EU/Unit)

mL/mL in the case of endotoxin limit specified by volume (EU/mL)

λ : the labeled lysate sensitivity in the gel-clot techniques (EU/mL) or the lowest point used (EU/mL) in the standard regression curve of the turbidimetric or chromogenic techniques

4. Gel-clot techniques

The gel-clot techniques detect or quantify endotoxins based on clotting of the lysate TS in the presence of endotoxin. To ensure both the precision and validity of the test, perform the tests for confirming the labeled lysate sensitivity (4.1.1) and for interfering factors (4.1.2) as described under Preparatory testing (4.1).

4.1. Preparatory testing

4.1.1. Test for confirmation of labeled lysate sensitivity

The labeled sensitivity of lysate is defined as the lowest concentration of endotoxin that is needed to cause the lysate TS

to clot under the conditions specified for the lysate to be used.

The test for confirmation of the labeled lysate sensitivity is to be carried out when each new lot of lysate is used or when there is any change in the experimental conditions which may affect the outcome of the test.

Prepare standard solutions having four concentrations equivalent to 2 λ, λ, 0.5 λ and 0.25 λ by diluting the Standard Endotoxin Stock Solution with water for BET. Mix a volume of the lysate TS with an equal volume of one of the standard solutions (usually, 0.1 mL aliquots) in each test tube. When single test vials or ampoules containing lyophilized lysate are used, add solutions directly to the vial or ampoule.

Keep the tubes (or containers such as vials or ampoules) containing the reaction mixture usually at 37 ± 1°C for 60 ± 2 minutes, avoiding vibration. To test the integrity of the gel after incubation, invert each tube or container through approximately 180° in one smooth motion. If a firm gel has formed that remains in place upon inversion, record the result as positive. A result is negative if either a firm gel is not formed, or if a fragile gel has formed but flows out upon inversion.

Making the standard solutions of four concentrations one set, test four replicates of the set.

The test is valid when 0.25 λ of the standard solution shows a negative result in each set of tests. If the test is not valid, repeat the test after verifying the test conditions.

The endpoint is the last positive test in the series of decreasing concentrations of endotoxin. Calculate the geometric mean endpoint concentration of the four replicate series using the following formula:

$$\text{Geometric Mean Endpoint Concentration} = \text{antilog}(\sum e/f)$$

$\sum e$ = the sum of the log endpoint concentrations of the dilution series used

f = the number of replicates

If the geometric mean endpoint concentration is not less than 0.5 λ and not more than 2 λ, the labeled sensitivity is confirmed, and is used in tests performed with this lysate.

4.1.2. Test for interfering factors

This test is performed to check for the presence of enhancing or inhibiting factors for the reaction in sample solutions.

Prepare the solutions A, B, C and D according to Table 4.01-1, and test solutions A and B and solutions C and D in quadruplicate and in duplicate, respectively. Concerning the incubation temperature, incubation time, and procedure for the confirmation of gel formation, follow the procedure described in 4.1.1.

The geometric mean endpoint concentrations of B and C solutions are determined by using the formula described in 4.1.1.

This test must be repeated when there is any change in the experimental conditions which may affect the outcome of the test.

Table 4.01-1

Solution	Endotoxin concentration /Solution to which endotoxin is added	Diluent	Dilution factor	Endotoxin concentration	Number of replicates
A* ¹	0/Sample solution	—	—	—	4
B* ²	2λ/Sample solution	Sample solution	1	2λ	4
			2	1λ	
			4	0.5λ	
			8	0.25λ	
C* ³	2λ/Water for BET	Water for BET	1	2λ	2
			2	1λ	
			4	0.5λ	
			8	0.25λ	
D* ⁴	0/Water for BET	—	—	—	2

*¹ Negative control. Sample solution only.

*² Sample solutions added with standard endotoxin (for testing interfering factors).

*³ Standard endotoxin solutions for confirmation of the labeled lysate sensitivity.

*⁴ Negative control. Water for BET only.

The test is valid if solutions A and D show no reaction and the result for solution C confirms the labeled lysate sensitivity.

If the geometric mean endpoint concentration of solution B is not less than 0.5 λ and not greater than 2 λ, the sample solution being examined does not contain interfering factors and complies with the test for interfering factors. Otherwise the sample solution interferes with the test.

If the sample under test does not comply with the test at a dilution less than the MVD, repeat the test using a greater dilution, not exceeding the MVD. The use of a more sensitive lysate permits a greater dilution of the sample to be examined. Furthermore, interference of the sample solution or diluted sample solution may be eliminated by suitable treatment, such as filtration, neutralization, dialysis or heat treatment. To establish that the treatment chosen effectively eliminates interference without loss of endotoxins, perform the assay described above using the preparation to be examined to which Standard Endotoxin has been added and which has then been submitted to the chosen treatment.

4.2. Limit test

This method tests whether or not a sample contains endotoxins greater than the endotoxin limit specified in the individual monograph based on the gel formation in the presence of endotoxins at a concentration of more than the labeled lysate sensitivity.

4.2.1. Procedure

Prepare solutions A, B, C and D according to Table 4.01-2. Making these four solutions one set, test two replicates of the set.

In preparing solutions A and B, use the sample solutions complying with 4.1.2. Concerning the test conditions including the incubation temperature, incubation time, and

procedure for the confirmation of gel formation, follow the procedure described in 4.1.1.

Table 4.01-2

Solution	Endotoxin concentration /Solution to which endotoxin is added	Number of replicates
A* ¹	0/Sample solution	2
B* ²	2λ/Sample solution	2
C* ³	2λ/Water for BET	2
D* ⁴	0/Water for BET	2

*¹ Sample solution for the limit test. The solution may be diluted not to exceed the MVD.

*² Positive control. Sample solution at the same dilution as solution A, containing standard endotoxin at a concentration of 2λ.

*³ Positive control. Standard endotoxin solution containing standard endotoxin at a concentration of 2λ.

*⁴ Negative control. Water for BET only.

4.2.2. Interpretation

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

When a negative result is found for both replicates of solution A, the sample complies with the Bacterial Endotoxins Test.

When a positive result is found for both replicates of solution A, the sample does not comply with the test.

When a positive result is found for one replicate of solution A and a negative result is found for the other, repeat the test. In the repeat test, the sample complies with the test if a negative result is found for both replicates of solution A. The sample does not comply with the test if a positive result is found for one or both replicates of solution A.

However, if the sample does not comply with the test at a dilution less than the MVD, the test may be repeated using a greater dilution, not exceeding the MVD.

4.3. Quantitative Test

This method measures endotoxin concentrations of samples by determining an endpoint of gel formation.

4.3.1. Procedure

Prepare solutions A, B, C and D according to Table 4.01-3. Making these four solutions one set, test two replicates of the set. When preparing solutions A and B, use sample solutions complying with 4.1.2. Concerning the test conditions, follow the procedure described in 4.1.1.

Table 4.01-3

Solution	Endotoxin concentration /Solution to which endotoxin is added	Diluent	Dilution Factor	Endotoxin concentration	Number of replicates
A* ¹	0/Sample solution	Water for BET	1	—	2
			2	—	
			4	—	
			8	—	
B* ²	2λ/Sample solution	—	1	2λ	2
C* ³	2λ/Water for BET	Water for BET	1	2λ	2
			2	1λ	
			4	0.5λ	
			8	0.25λ	
D* ⁴	0/Water for BET	—	—	—	2

*¹ Sample solutions for the Quantitative test. The dilution range of the dilution series may be changed as appropriate, but not exceeding the MVD.

*² Positive control. Sample solution at the same dilution as the solution A diluted at the lowest dilution factor, containing standard endotoxin at a concentration of 2λ.

*³ Standard endotoxin solutions for confirmation of the labeled lysate sensitivity.

*⁴ Negative control. Water for BET only.

4.3.2. Calculation and interpretation

The test is valid when the following three conditions are met: (a) both replicates of the negative control solution D are negative, (b) both replicates of the positive product control solution B are positive and (c) the geometric mean endpoint concentration of solution C is in the range of 0.5 λ to 2 λ.

The endpoint is defined as the maximum dilution showing the last positive test in the dilution series of solution A, and the endotoxin concentration of the sample solution is calculated by multiplying the endpoint dilution factor by λ.

If none of the dilutions of solution A is positive, report the endotoxin concentration of the sample solution as less than λ × the lowest dilution factor of the sample solution.

If all dilutions are positive, the endotoxin concentration of the sample solution is reported as equal to or greater than the greatest dilution factor of solution A multiplied by λ.

Calculate the endotoxin concentration (in EU/mL, EU/mg, EU/mEq or EU/Unit) of the sample, based on the endotoxin concentration of the sample solution. The sample complies with the Bacterial Endotoxins Test if the endotoxin concentration of the sample in both replicates meets the requirement for the endotoxin limit (in EU/mL, EU/mg, EU/mEq or EU/Unit) specified in the individual monograph.

5. Photometric quantitative techniques

5.1. Turbidimetric technique

This technique measures the endotoxin concentrations of samples based on the measurement of turbidity change accompanying gel formation of the lysate TS. This technique is classified as either endpoint-turbidimetric or

kinetic-turbidimetric.

The endpoint-turbidimetric technique is based on the quantitative relationship between the concentration of endotoxins and the turbidity of the reaction mixture at a specified reaction time.

The kinetic-turbidimetric technique is based on the quantitative relationship between the concentration of endotoxins and either the time needed to reach a predetermined turbidity of the reaction mixture or the rate of turbidity development.

The test is usually carried out at $37 \pm 1^\circ\text{C}$, and turbidity is expressed in terms of either absorbance or transmission.

5.2. Chromogenic technique

This technique measures the endotoxin concentrations of sample solutions based on the measurement of chromophore released from a synthetic chromogenic substrate by the reaction of endotoxins with the lysate TS. This technique is classified as either endpoint-chromogenic or kinetic-chromogenic.

The endpoint-chromogenic technique is based on the quantitative relationship between the concentration of endotoxins and the release of chromophore at the end of an incubation period.

The kinetic-chromogenic technique is based on the quantitative relationship between the concentration of endotoxins and either the time needed to reach a predetermined absorbance (or transmittance) of the reaction mixture or the rate of color development.

The test is usually carried out at $37 \pm 1^\circ\text{C}$.

5.3. Preparatory testing

To assure the precision and validity of the turbidimetric or chromogenic techniques, perform both Test for assurance of criteria for the standard curve (5.3.1) and Test for interfering factors (5.3.2), as indicated below.

5.3.1. Test for assurance of criteria for the standard curve

The test is to be carried out when each new lot of lysate is used or when there is any change in the experimental conditions which may affect the outcome of the test.

Using the Standard Endotoxin Solution, prepare at least three endotoxin concentrations to generate the standard curve within the range of endotoxin concentrations indicated by the instructions for the lysate used. Perform the test using at least three replicates of each standard endotoxin concentration according to the optimal conditions for the lysate used (with regard to volume ratios, incubation time, temperature, pH, etc.). If the desired range is greater than two logs, additional standards should be included to bracket each log increase in the range of the standard curve.

If the absolute value of the correlation coefficient, $|r|$, is greater than or equal to 0.980 for the range of endotoxin concentrations set up, the criteria for the standard curve are valid and the curve complies with the test.

If the standard curve does not comply with the test, repeat the

test after verifying the test conditions.

5.3.2. Test for interfering factors

Prepare solutions A, B, C and D according to Table 4.01-4. Perform the test on these solutions following the optimal conditions for the lysate used (with regard to volume of sample solution and lysate TS, volume ratio of sample solution to lysate TS, incubation time, etc.).

The test for interfering factors must be repeated when any condition changes, which is likely to influence the result of the test.

Table 4.01-4

Solution	Endotoxin concentration	Solution to which endotoxin is added	Number of test tubes or wells
A* ¹	0	Sample solution	Not less than 2
B* ²	Middle concentration of the standard curve	Sample solution	Not less than 2
C* ³	At least 3 concentrations	Water for BET	Each not less than 2
D* ⁴	0	Water for BET	Not less than 2

*¹ Sample solution only (for assaying endotoxin concentration in the sample solution). The sample solution may be diluted not to exceed the MVD.

*² Sample solution at the same dilution as solution A, containing added standard endotoxin at a concentration equal to or near the middle of the standard curve.

*³ Standard endotoxin solutions at the concentrations used in 5.3.1 (for the standard curve).

*⁴ Negative control. Water for BET only.

The test is valid when the following conditions are met.

- 1: The absolute value of the correlation coefficient of the standard curve generated using solution C is greater than or equal to 0.980.
- 2: The result with solution D does not exceed the limit of the blank value required in the description of the lysate employed, or it is less than the endotoxin detection limit of the lysate employed.

Calculate the recovery of the endotoxin added to solution B from the concentration found in solution B after subtracting the endotoxin concentration found in solution A.

When the recovery of the endotoxin added to solution B is within 50% to 200%, the sample solution under test is considered to be free of interfering factors and the solution complies with the test.

When the endotoxin recovery is out of the specified range, the sample solution under test is considered to contain interfering factors. If the sample under test does not comply with the test, repeat the test using a greater dilution, not exceeding the MVD. Furthermore, interference of the sample solution or diluted sample solution not to exceed the MVD may be eliminated by suitable treatment, such as filtration, neutralization, dialysis or heat treatment. To establish that the

treatment chosen effectively eliminates interference without loss of endotoxins, perform the assay described above using the preparation to be examined to which Standard Endotoxin has been added and which has then been submitted to the chosen treatment.

5.4. Quantitative test

5.4.1. Procedure

Prepare solutions A, B, C and D according to Table 4.01-4, and follow the procedure described in 5.3.2.

5.4.2. Calculation of endotoxin concentration

Calculate the mean endotoxin concentration of solution A using the standard curve generated with solution C. The test is valid when all the following requirements are met.

- 1: The absolute value of the correlation coefficient of the standard curve generated using solution C is greater than or equal to 0.980.
- 2: The endotoxin recovery, calculated from the concentration found in solution B after subtracting the concentration of endotoxin found in solution A, is within the range of 50% to 200%.
- 3: The result with solution D does not exceed the limit of the blank value required in the description of the lysate employed, or it is less than the endotoxin detection limit of the lysate employed.

5.4.3. Interpretation

The sample complies with the Bacterial Endotoxins Test if the endotoxin concentration of the sample calculated from the mean endotoxin concentration of solution A meets the requirement of the endotoxin limit (in EU/mL, EU/mg, EU/mEq or EU/Unit) specified in the individual monograph.

General Tests / Reagents, Test Solutions, etc.

9.41 Reagents, Test Solutions

Lysate reagent

A lyophilized product obtained from amebocyte lysate of horseshoe crab (*Limulus polyphemus* or *Tachypleus tridentatus*). Amebocyte lysate preparations which do not react to β -glucans are available: they are prepared by removing the G factor reacting to β -glucans from amebocyte lysate or by inhibiting the G factor reacting system of amebocyte lysate.

Lysate TS

Dissolve a lysate reagent in water for bacterial endotoxins test, or in a suitable buffer, by gentle stirring.

Water for bacterial endotoxins test

Same as the monograph Water for Injection, Sterile Water for Injection in Containers or other waters that do not contain endotoxins at a concentration higher than the detection limit of the lysate employed and that are suitable for the test performed.