3.4 Test for bacterial endotoxins

This text is based on the internationally-harmonized texts developed by the Pharmacopoeial Discussion Group (PDG). Some editorial modifications have been made in order to be in line with the style used in The International Pharmacopoeia*.*

The bacterial endotoxins test (BET) is a test to detect or quantify endotoxins from Gram-negative bacteria using amoebocyte lysate from the horseshoe crab (*Limulus polyphemus* or *Tachypleus tridentatus*). There are three methods for this test:

- Method A. The gel-clot technique, which is based on gel formation;
- Method B. The turbidimetric technique, based on the development of turbidity after cleavage of an endogenous substrate;
- Method C. The chromogenic technique, based on the development of colour after cleavage of a synthetic peptide-chromogen complex.

Unless otherwise indicated in the individual monograph proceed by Method A.

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

APPARATUS

Depyrogenate all glassware and other heat stable materials in a hot air oven using a validated process. A commonly used minimum time and temperature is 30 minutes at 250 °C. If employing plastic apparatus such as microplates and pipet tips for automatic pipetters, use apparatus shown to be free of detectable endotoxin and which does not interfere in the test.

Note: In this chapter the term "tube" includes any other receptacle such as a micro-titre well.

REAGENTS AND TEST SOLUTIONS

- **Amoebocyte lysate**
  A lyophilized product obtained from the lysate of amoebocytes (white blood cells) from the horseshoe crab (*Limulus polyphemus* or *Tachypleus tridentatus*).

  Note: Amoebocyte lysate reacts to some β-glucans in addition to endotoxins. Amoebocyte lysate preparations which do not react to glucans are available: they are prepared by removing the G factor reacting to glucans from amoebocyte lysate or by inhibiting the G factor reacting system of amoebocyte lysate and may be used for the endotoxin testing in the presence of glucans.

- **Lysate solution**
  Dissolve amoebocyte lysate in water BET or in a buffer recommended by the lysate manufacturer, by gentle stirring. Store the reconstituted lysate, refrigerated or frozen, according to the specifications of the manufacturer.

- **Water BET (water for bacterial endotoxins test)**
  Water for injections or water produced by other procedures that shows no reaction with the lysate employed, at the detection limit of the reagent.

Preparation of Standard Endotoxin Stock Solution

A Standard Endotoxin Stock Solution is prepared from the WHO International Standard for endotoxin (available from the National Institute for Biological Standards and Control (NIBSC), United Kingdom of Great Britain) or an endotoxin reference standard that has been calibrated against the WHO International Standard for endotoxin. Follow the specifications in the package leaflet and on the label for preparation and storage of the Standard Endotoxin Stock Solution.

Endotoxin is expressed in International Units (IU) of endotoxin.

Note: One International Unit (IU) of endotoxin is equal to one Endotoxin Unit (EU).

Preparation of Standard Endotoxin Solution

After mixing the Standard Endotoxin Stock Solution vigorously, prepare appropriate serial dilutions of Standard Endotoxin Solution, using water BET.

Use dilutions as soon as possible to avoid loss of activity by adsorption.

Preparation of sample solutions

Prepare sample solutions by dissolving or diluting the pharmaceutical substance or the finished preparation using water BET. Some substances or preparations may be more appropriately dissolved or diluted in other aqueous solutions. If necessary, adjust the pH of the solution to be examined (or dilution thereof) so that the pH of the mixture of the Lysate solution and sample solution falls within the pH range specified by the lysate manufacturer, usually 6.0–8.0. The pH may be adjusted by the use of acid, base or suitable buffer as recommended by the lysate manufacturer. Acids and bases may be prepared from concentrates or solids

with water BET in containers free of detectable endotoxin. Buffers must be validated to be free of detectable endotoxin and interfering factors.

DETERMINATION OF 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 from the following equation:

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

Endotoxin limit

The endotoxin limit for parenteral preparations, defined on the basis of dose, equals K/M, where K is a threshold pyrogenic dose of endotoxin per kg of body weight and M is equal to the maximum recommended bolus dose of product per kg of body weight. 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.

Note: The endotoxin limit depends on the product and its route of administration and is stated in the individual monograph. Suggested values for K are:

- intravenous route: K = 5 IU endotoxin per kg body weight;
- intravenous route for radiopharmaceuticals: K = 2.5 IU endotoxin per kg body weight;
- intrathecal route: K = 0.2 IU endotoxin per kg body weight.

For other routes of administration the acceptance criterion for bacterial endotoxins is generally determined on the basis of results obtained during development of the preparation.

The endotoxin limit for parenteral preparations is specified in units such as IU/mL, IU/mg, IU/Unit of biological activity, etc., in the individual monograph.

Concentration of sample solution:

- mg/mL in the case of endotoxin limit specified by weight (IU/mg);
- units/mL in the case of endotoxin limit specified by unit of biological activity (IU/Unit);
- mL/mL when the endotoxin limit is specified by volume (IU/mL).

λ: the labelled lysate sensitivity in the gel-clot technique (IU/mL) or the lowest concentration used in the standard curve for the turbidimetric or chromogenic techniques.

METHOD A: GEL-CLOT TECHNIQUE

The gel-clot technique is for detecting or quantifying endotoxins based on clotting of the Lysate solution in the presence of endotoxin. The minimum concentration of endotoxin required to cause the lysate to clot under standard conditions is the labelled sensitivity of the Lysate solution. To ensure both the precision and validity of the test, perform the tests for confirming the labelled lysate sensitivity and for interfering factors as described below under Preparatory testing.

Preparatory testing

Test for confirmation of labelled lysate sensitivity

Confirm in four replicates the labelled sensitivity, λ, expressed in IU/mL of the lysate prior to use in the test. The test for confirmation of the lysate sensitivity is to be carried out when a new lot of lysate is used or when there is any change in the test conditions which may affect the outcome of the test.

Prepare standard solutions having at least four concentrations equivalent to 2λ , λ, 0.5λ and 0.25λ by diluting the Standard Endotoxin Stock Solution with water BET.

Mix a volume of the Lysate solution with an equal volume of one of the standard solutions (such as 0.1 mL aliquots) in each tube. When single test vials or ampoules, containing lyophilized lysate are employed, add solutions of standards directly to the vial or ampoule. Incubate the reaction mixture for a constant period according to directions of the lysate manufacturer (usually at 37±1°C for 60 ± 2 minutes), avoiding vibration. Test the integrity of the gel for tests carried out in tubes, take each tube in turn directly from the incubator and invert it through approximately 180 degrees 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 an intact gel is not formed.

The test is considered valid when the lowest concentration of the standard solutions shows a negative result in all replicate tests.

The end-point is the lowest concentration in the series of decreasing concentrations of standard endotoxin that clots the lysate. Determine the geometric mean end-point concentration by calculating the mean of the logarithms of the end-point concentrations of the four dilution series, take the antilogarithm of this value, as indicated in the following formula:

$$\text{Geometric mean end-point concentration} = \text{antilog}\ \frac{\sum e}{f}$$

$\sum e$= the sum of the log end-point concentrations of the dilution series used
f = the number of replicate test tubes

The geometric mean end-point concentration is the measured sensitivity of the lysate (IU/mL). If this is not less than 0.5λ and not more than 2λ, the labelled sensitivity is confirmed and is used in tests performed with this lysate.

Test for interfering factors

Usually prepare the solutions (A-D) in Table 1 and perform the inhibition/enhancement test on the sample solutions at a dilution less than the MVD not containing any detectable endotoxins, operating as described above under Test for confirmation of labelled lysate sensitivity.

The geometric mean end-point concentrations of B and C solutions are determined by using the formula described above under Test for confirmation of labelled lysate sensitivity.

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

Table 1

Solution	Endotoxin concentration/Solution to which endotoxin is added	Diluent	Dilution factor	Endotoxin concentration	Number of replicates
A	None/Sample solution	–	–	–	4
B	2λ/Sample solution	Sample solution	1	2λ	4
			2	1λ	4
			4	0.5λ	4
			8	0.25λ	4
C	2 λ/Water BET	Water BET	1	2λ	2
			2	1λ	2
			4	0.5λ	2
			8	0.25λ	2
D	None/Water BET	–	–	–	2

Note:

– Solution A: a sample solution of the preparation under test that is free of detectable endotoxins

– Solution B: test for interference

– Solution C: control for labelled lysate sensitivity

– Solution D: negative control of water BET.

The test is considered valid when all replicates of solutions A and D show no reaction and the result of solution C confirms the labelled sensitivity.

If the sensitivity of the lysate determined in the presence of solution B is not less than 0.5λ and not greater than 2λ, the sample solution does not contain factors which interfere under the experimental conditions used. Otherwise the sample solution to be examined interferes with the test.

If the preparation 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 preparation to be examined and this may contribute to the elimination of interference.

Interference may be overcome 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.

Limit test

Procedure

Prepare the solutions A, B, C and D according to Table 2 and perform the test on these solutions following the procedure in Test for confirmation of labelled lysate sensitivity under Preparatory testing.

Table 2

Solution	Endotoxin concentration/Solution to which endotoxin is added	Number of replicates
A	None/Diluted sample solution	2
B	2λ/Diluted sample solution	2
C	2λ/Water BET	2
D	None/Water BET	2

Note: Prepare solution A and the positive product control solution B using a dilution not greater than the MVD and treatments as for the Test for interfering factors under Preparatory testing. The positive control solutions B and C contain the standard endotoxin preparation at a concentration corresponding to twice the labelled lysate sensitivity. The negative control solution D consists of water BET.

Interpretation

The test is considered valid when both replicates of solution 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 preparation under test complies with the test.

When a positive result is found for both replicates of solution A the preparation under test 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 preparation under test complies with the test if a negative result is found for both replicates of solution A. The preparation does not comply with the test if a positive result is found for one or both replicates of solution A.

However, if the preparation 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.

Quantitative test

Procedure

The test quantifies bacterial endotoxins in sample solutions by titration to an end-point.

Prepare the solutions A, B, C and D following Table 3 and test these solutions according to the procedure in Test for confirmation of labelled lysate sensitivity under Preparatory testing.

Table 3

Solution	Endotoxin concentration/Solution to which endotoxin is added	Diluent	Dilution factor	Endotoxin concentration	Number of replicates
A	None/Sample solution	Water BET	1	–	2
			2	–	2
			4	–	2
			8		2
B	2λ/Sample solution		1	2λ	2
C	2λ/Water BET	Wate	1	2λ	2

		r BET	2 4 8	1λ 0.5λ 0.25λ	2 2 2
D	None/Water BET	–	–	–	2

Note:

– Solution A: Sample solution under test at the dilution, not to exceed the MVD, with which the test for interfering factors was completed. Subsequent dilution of the sample solution must not exceed the MVD. Use water BET to make a dilution series of four tubes containing the sample solution under test at concentrations of 1, 1/2, 1/4 and 1/8 relative to the concentration used in the test for interfering factors. Other dilutions up to the MVD may be used as appropriate.

– Solution B: Solution A containing standard endotoxin at a concentration of 2λ (positive product control).

– Solution C: A dilution series of four tubes of water BET containing the standard endotoxin at a concentration of 2λ, λ, 0.5λ and 0.25λ, respectively.

– Solution D: Water BET (negative control).

Calculation and interpretation

The test is considered valid when the following three conditions are met.

1. Both replicates of the negative control solution D are negative.
2. Both replicates of the positive product control solution B are positive.
3. The geometric mean endpoint concentration of the solution C is in the range of 0.5–2λ.

To determine the endotoxin concentration of solution A, calculate the end-point concentration for each replicate by multiplying each end-point dilution factor by λ.

The endotoxin concentration in the sample solution is the end-point concentration of the replicates. If the test is conducted with a diluted sample solution, calculate the concentration of endotoxin in the original sample solution by multiplying by the dilution factor.

If none of the dilutions of sample solution is positive in a valid assay, report the endotoxin concentration as less than λ (if diluted sample was tested, report as less than the smallest dilution factor of the sample multiplied by λ). If all dilutions are positive, the endotoxin concentration is reported as equal to or greater than the largest dilution factor multiplied by λ (e.g. initial dilution factor multiplied by 8 and by λ in Table 3).

The preparation under test meets the requirements of the test if the concentration of endotoxin in both replicates is less than that specified in the individual monograph.

PHOTOMETRIC QUANTITATIVE TECHNIQUES

METHOD B. TURBIDIMETRIC TECHNIQUE

This technique is a photometric assay measuring increases in reactant turbidity. On the basis of the particular assay principle employed, this technique may be classified as either an end-point-turbidimetric assay or a kinetic-turbidimetric assay.

The end-point-turbidimetric assay is based on the quantitative relationship between the concentration of endotoxins and the turbidity (absorbance or transmission) of the reaction mixture at the end of an incubation period.

The kinetic-turbidimetric assay is a method to measure either the time (onset time) needed to reach a predetermined absorbance or transmission of the reaction mixture or the rate of turbidity development.

The test is carried out at the incubation temperature recommended by the lysate manufacturer (which is usually 37 ± 1°C).

METHOD C. CHROMOGENIC TECHNIQUE

This technique is an assay to measure the chromophore released from a suitable chromogenic peptide by the reaction of endotoxins with lysate.

On the basis of the particular assay principle employed, this technique may be classified as either an end-point-chromogenic assay or a kinetic-chromogenic assay.

The end-point-chromogenic assay 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 assay is a method to measure either the time (onset time) needed to reach a predetermined absorbance of the reaction mixture, or the rate of colour development.

The test is carried out at the incubation temperature recommended by the lysate manufacturer (which is usually 37 ± 1°C).

Preparatory testing

To assure the precision or validity of the turbidimetric and chromogenic techniques, preparatory tests are conducted to show that the criteria for the standard curve are valid and that the sample solution does not interfere with the test.

Validation for the test method is required when conditions change which are likely to influence the result of the test.

Assurance of criteria for the standard curve

The test must be carried out for each lot of the lysate. Using the Standard Endotoxin Solution, prepare at least three endotoxin concentrations within the range indicated by the lysate manufacturer to generate the standard curve. Perform the assay using at least three replicates of each standard endotoxin concentration according to the manufacturer's instructions for the lysate (volume ratios, incubation time, temperature, pH, etc.).

If the desired range is greater than two logs in the kinetic methods, additional standards should be included to bracket each log increase in the range of the standard curve.

The absolute value of the correlation coefficient, |r|, must be greater than or equal to 0.980, for the range of endotoxin concentrations set up.

Test for interfering factors

Select an endotoxin concentration at or near the middle of the endotoxin standard curve.

Prepare solutions A, B, C and D shown in Table 4. Perform the test on solutions A–D in at least duplicate according to the instructions for the lysate employed, for example, concerning volume of sample solution and Lysate solution, volume ratio of sample solution to Lysate solution, incubation time, etc.

Table 4

Solution	Endotoxin concentration	Solution to which endotoxin is added	Number of replicates
A	None	Sample solution	Not less than 2
B	Middle concentration of the standard curve	Sample solution	Not less than 2
C	At least 3 concentrations (lowest concentration is designated λ)	Water BET	Each not less than 2
D	None	Water BET	Not less than 2

Note:

–Solution A: The sample solution may be diluted not to exceed the MVD.

–Solution B: The preparation under test at the same dilution as solution A, containing added endotoxin at a concentration equal to or near the middle of the standard curve.

–Solution C: The standard endotoxin at the concentrations used in the validation of the method described in Assurance of criteria for the standard curve under Preparatory testing (positive controls).

–Solution D: Water BET (negative control).

The test is considered 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 mean recovery of the added endotoxin by subtracting the mean endotoxin concentration in the solution, if any

(Solution A, Table 4), from that containing the added endotoxin (Solution B, Table 4).

In order to be considered free of factors that interfere with the assay under the conditions of the test, the measured concentration of the endotoxin added to the sample solution must be 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 out of the specified range the sample solution under test is considered to contain interfering factors. Then 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 validated 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.

Test

Procedure

Follow the procedure described in Test for interfering factors under Preparatory testing.

Calculation

Calculate the endotoxin concentration of each of the replicates of test solution A using the standard curve generated by the positive control solution C. The test is considered valid when the following three requirements are met.

1. The results of the positive control solution C comply with the requirements for validation defined in Assurance of criteria for the standard curve under Preparatory testing.
2. The endotoxin recovery, calculated from the concentration found in solution B after subtracting the concentration of endotoxin found in the solution A, is within the range of 50–200%.
3. The result of the negative control 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.

Interpretation

In photometric assays the preparation under test 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.