PHARMACOPOEIAL DISCUSSION GROUP

SIGN-OFF COVER SHEET

CODE: Q-06

NAME: Bacterial Endotoxins Test

(Version 2 of the sign-off cover sheet to Revision 2 signed on 16 June 2011)

It is understood that sign-off covers the technical content of the draft and each party will adapt it as necessary to conform to the usual presentation of the pharmacopoeia in question; such adaptation includes stipulation of the particular pharmacopoeia's reference materials and general chapters.

Amended Item:

Addition of non-harmonised part for EP with fluorimetric end-point method using recombinant factor C (rFC) as method G.

Non-harmonised parts:

EP	Method G (fluorimetric end-point method using recombinant factor C [rFC]), related text is included in the introduction and sections 2, 5, 6 and 9.
JP	none
USP	none

European Pharmacopoeia

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BACTERIAL ENDOTOXINS TEST
(Rev. 2)

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 techniques for this test: the gel-clot technique, which is based on gel formation; the turbidimetric technique, based on the development of turbidity after cleavage of an endogenous substrate; and the chromogenic technique, based on the development of color after cleavage of a synthetic peptide-chromogen complex.

Proceed by any of the three techniques for the test. In the event of doubt or dispute, the final decision is made based upon the gel-clot limit test unless otherwise indicated in the monograph for the product being tested.

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. 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.

2. Reagents, Test Solutions

(1) Amoebocyte lysate

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

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

(2) Lysate test solution (TS)

Dissolve amebocyte lysate in water for bacterial endotoxins test, 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.

(3) Water for bacterial endotoxins test (BET)

Water for Injection or water produced by other procedures that shows no reaction with the lysate employed, at the detection limit of the reagent.

3. Preparation of Standard Endotoxin Stock Solution

A Standard Endotoxin Stock Solution is prepared from an Endotoxin Reference Standard that has been calibrated to the current 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 Endotoxin Units (EU).

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

4. Preparation of Standard Endotoxin Solution

After mixing Standard Endotoxin Stock Solution vigorously, 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.

5. Preparation of sample solutions

Prepare sample solutions by dissolving or diluting drugs 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 solution to be examined (or dilution thereof) so that the pH of the mixture of the lysate TS and sample solution falls within the pH range specified by the lysate manufacturer, usually 6.0 to 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 for BET in containers free of detectable endotoxin. Buffers must be validated to be free of detectable endotoxin and interfering factors.

6. Determination of Maximum Valid Dilution

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

Determine the MVD from the following equation:

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

Endotoxin Limit:

The endotoxin limit for parenteral drugs, 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.

The endotoxin limit for parenteral drugs is specified in units such as EU/ml, EU/mg, EU/Unit of biological activity, etc., in the individual monograph.

Concentration of sample solution:

mg/ml in the case of endotoxin limit specified by weight (EU/mg)

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

ml/ml when the endotoxin limit is specified by volume (EU/ml)

λ: the labeled lysate sensitivity in the gel-clot technique (EU/ml) or the lowest concentration used

in the standard curve for the turbidimetric or chromogenic techniques

7. Gel-clot technique

The gel-clot technique is for detecting or quantifying endotoxins based on clotting of the lysate TS in the presence of endotoxin. The minimum concentration of endotoxin required to cause the lysate to clot under standard conditions is the labeled sensitivity of the lysate TS. To ensure both the precision and validity of the test, perform the tests for confirming the labeled lysate sensitivity and for interfering factors as described under (1) Preparatory testing.

(1) Preparatory testing

(i) Test for confirmation of labeled lysate sensitivity

Confirm in four replicates the labeled sensitivity, λ, expressed in EU/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 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 (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 endpoint is the lowest concentration in the series of decreasing concentrations of standard endotoxin that clots the lysate. Determine the geometric mean endpoint concentration by calculating the mean of the logarithms of the endpoint concentrations of the four dilution series, take the antilogarithm of this value, as indicated in the following formula:

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

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

f = the number of replicate test tubes

The geometric mean endpoint concentration is the measured sensitivity of the lysate (EU/ml). If this 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.

(ii) 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 under (i) Test for confirmation of labeled lysate sensitivity of (1) Preparatory testing.

The geometric mean endpoint concentrations of B and C solutions are determined by using the formula described in (i) Test for confirmation of labeled lysate sensitivity of (1) Preparatory testing.

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	2 λ / Sample solution	Sample solution	1 2 4 8	2 λ 1 λ 0.5 λ 0.25 λ	4 4 4 4
C	2 λ / Water for BET	Water for BET	1 2 4 8	2 λ 1 λ 0.5 λ 0.25 λ	2 2 2 2
D	None / Water for 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 labeled lysate sensitivity

Solution D : negative control of water for 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 labeled sensitivity.

If the sensitivity of the lysate determined in the presence of solution B is not less than 0.5 λ and not greater than 2 λ, 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.

(2) Limit test

(i) Procedure

Prepare the solutions A, B, C and D according to Table 2, and perform the test on these solutions following the procedure under (i) Test for confirmation of labeled lysate sensitivity of (1) Preparatory testing.

Table 2

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

Note: Prepare the solution A and the positive product control solution B using a dilution not greater than the MVD and treatments as for the (ii) Test for interfering factors in (1) Preparatory testing. The positive control solutions B and C contain the standard endotoxin preparation at a concentration corresponding to twice the labeled lysate sensitivity. The negative control solution D consists of water for BET.

(ii) 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.

(3) Quantitative Test

(i) Procedure

The test quantifies bacterial endotoxins in sample solutions by titration to an endpoint.

Prepare the solutions A, B, C and D following Table 3, and test these solutions according to the procedure under (i) Test for confirmation of labeled lysate sensitivity of (1) 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 for BET	1 2 4 8	- - - -	2 2 2 2
B	2 λ / Sample solution		1	2 λ	2
C	2 λ / Water for BET	Water for BET	1 2 4 8	2 λ 1 λ 0.5 λ 0.25 λ	2 2 2 2
D	None / Water for 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 for 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 2 λ (positive product control)

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

Solution D: Water for BET (negative control)

(ii) 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 λ to 2 λ.

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

The endotoxin concentration in the sample solution is the endpoint 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 x 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 x 8 x 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.

8. Photometric quantitative techniques

(1) 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 endpoint-turbidimetric assay or a kinetic-turbidimetric assay.

The endpoint-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).

(2) 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 endpoint-chromogenic assay or a kinetic-chromogenic assay.

The endpoint-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 color development.

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

(3) 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.

(i) 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.

(ii) 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 TS, volume ratio of sample solution to lysate TS, 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 for BET	Each not less than 2
D	None	Water for 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 under (i) Assurance of criteria for the standard curve of

(3) Preparatory testing (positive controls)

Solution D : Water for 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 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) Test

(i) Procedure

Follow the procedure described in (ii) Test for interfering factors of (3) Preparatory testing.

(ii) 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 control solution C comply with the requirements for validation defined under (i) Assurance of criteria for the standard curve of (3) 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.

(iii) 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.