á85ñ BACTERIAL ENDOTOXINS TEST

Change to read:

F Portions of this general chapter have been harmonized with the corresponding texts of the European Pharmacopoeia and/or the Japanese Pharmacopoeia. Those portions that are not harmonized are marked with symbols (F) to specify this fact. F

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" n 2S (USP35) unless otherwise indicated in the monograph for the product being tested. 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. F1 F A commonly used minimum time and temperature is 30 min at 250°. If employing plastic apparatus, such as microplates and pipet tips for automatic pipetters, use apparatus that is shown to be free of detectable endotoxin and does not interfere in the test. [NOTE—In this chapter, the term "tube" includes any other receptacle such as a microtiter well.]

REAGENTS AND TEST SOLUTIONS

a A lyophilized product obtained

tions that 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 endotoxin testing in the presence of glucans.]

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

a Dissolve Amoebocyte lysate in Water for 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.

F1 For a validity test of the procedure for inactivating endotoxins, see Dry-Heat Sterilization under Sterilization and Sterility Assurance of Compendial Articles á1211ñ. Use Lysate TS having a sensitivity of not less than 0.15 Endotoxin Unit per mL. F

Change to read:

PREPARATION OF SOLUTIONS

a a r A Standard Endotoxin Stock Solution is prepared from a USP 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 USP Endotoxin Unit (EU) is equal to one International Unit (IU) of endotoxin.]

a a r After mixing the 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.

a Prepare the Sample Solutions by dissolving or diluting drugs "n 2S (USP35)" using Water for BET. Some substances or preparations may be more appropriately dissolved, "n or diluted" n 2S (USP35) 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 and Sample Solution falls within the pH range specified by the lysate manufacturer, usually 6.0–8.0. The pH may be adjusted by use of an 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 inter fering factors.

Change to read:

DETERMINATION OF MAXIMUM VALID DILUTION (MVD)

The maximum valid dilution is the maximum allowable dilution of a specimen at which the endotoxin limit can be determined. Determine the MVD from the following equation:

drugs, defined on the basis of dose, equals K/M F2 F, 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 inter vals or infused continuously, M is the maximum total dose administered in a single hour period. The endotoxin limit for parenteral drugs is specified in the individual monograph in units such as EU/mL, EU/mg, EU/Unit of biological activity, etc.

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mg/mL: in the case of endotoxin limit specified by weight (EU/mg);

F2 "K is 5 USP-EU/kg of body weight for any route of administration other than intrathecal (for which K is 0.2 USP-EU/kg of body weight). For radiopharmaceutical products not administered intrathecally, the endotoxin limit is calculated as 175 EU/V, where V is the maximum recommended dose in mL. For intrathecally administered radiopharmaceuticals, the endotoxin limit is obtained by the formula 14 EU/V. For formulations (usually anticancer products) administered on a per square meter of body surface, the formula is K/M, where K = 100 EU/m² and M is the maximum dose/m

Table 3. Preparation of Solutions for the Gel-Clot Assay

Solution	Endotoxin Concentration/ Solution to Which Endotoxin Is Added	Diluent	Dilution Factor	Endotoxin Concentration	Number of Replicates
A[a]	None/Sample Solution	Water for BET	1	—	2
			2	—	2
			4	—	2
			8	—	2
B[b]	2 /Sample Solution	—	1	2	2
C[c]	2 /Water for BET	Water for BET	1	2	2
			2	1	2
			4	0.5	2
			8	0.25	2
D[d]	None/Water for BET	—	—	—	2

[a] 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.
[b] Solution B: Solution A containing standard endotoxin at a concentration of 2 (positive product control).
[c] Solution C: Two replicates of four tubes of Water for BET containing the standard endotoxin at concentrations of 2 , , 0.5 , and 0.25 , respectively.
[d] Solution D: Water for BET (negative control).

that the chosen treatment 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.

r r Prepare Solutions A, B, C, and D as shown in Table 2, and perform the test on these solutions following the procedure above for Preparatory Testing, Test for Confirmation of Labeled Lysate Sensitivity.

Table 2. Preparation of Solutions for the Gel-Clot Limit Test

	Endotoxin Concentration/ Solution to Which	

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.

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r r The test quantifies bacterial endotoxins in Sample Solutions by titration to an endpoint. Prepare Solutions A, B, C, and D as shown in Table 3, and test these solutions by following the procedure in Preparatory Testing, Test for Confirmation of Labeled Lysate Sensitivity.

Table 4. Preparation of Solutions for the Inhibition/Enhancement Test for Photometric Techniques

Solution	Endotoxin Concentration	Solution to Which Endotoxin Is Added	Number of Replicates
A[a]	None	Sample Solution	Not less than 2
B[b]	Middle concentration of the standard cur		

PHOTOMETRIC QUANTITATIVE TECHNIQUES

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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°).

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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°).

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To assure the precision or validity of the turbidimetric and chromogenic techniques, preparatory tests are conducted to verify 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 that are likely to influence the test result change.

ra r ra r a ar r The test must be carried out for each lot of lysate reagent. 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.

r r r a r 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 4. Perform the test on Solutions A, B, C, and D at least in 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.

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 reagent employed, or it is less than the endotoxin detection limit of the lysate reagent 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 chosen treatment 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.

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Follow the procedure described for Test for Interfering Factors under Preparatory Testing, immediately above.

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Calculate the endotoxin concentration of each of the replicates of 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 for 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 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 reagent employed.

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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.