Maize starch	1.0 g
Sodium chloride	5.0 g
Agar, according to gelling power	10.0 g to 15.0 g
Purified water	1000 ml

Hydrate the agar, dissolve by heating to boiling with continuous stirring. If necessary, adjust the pH so that after sterilisation it is 7.3 ± 0.2. Sterilise by heating in an autoclave at 121 °C for 15 min. Allow to cool to 45-50 °C; add, where necessary, gentamicin sulphate corresponding to 20 mg of gentamicin base and pour into Petri dishes.

Medium R (Lactose monohydrate sulphite medium)

Pancreatic digest of casein	5.0 g
Yeast extract	2.5 g
Sodium chloride	2.5 g
Lactose monohydrate	10.0 g
Cysteine hydrochloride	0.3 g
Purified water	1000 ml

Dissolve, adjust to pH 7.1 ± 0.1 and fill to 8 ml in 16 mm × 160 mm tubes containing a small Durham tube. Sterilise by heating in an autoclave at 121 °C for 15 min and store at 4 °C.

Before use, heat the medium for 5 min in a water-bath and cool. Add to each tube 0.5 ml of a 12 g/l solution of *sodium metabisulphite R* and 0.5 ml of a 10 g/l solution of ferric ammonium citrate, both solutions being freshly prepared and filtered through membranes (pore size: 0.45 µm).

Agar medium S (R2A)

Yeast extract	0.5 g
Proteose peptone	0.5 g
Casein hydrolysate	0.5 g
Glucose	0.5 g
Starch	0.5 g
Dipotassium hydrogen phosphate	0.3 g
Magnesium sulphate, anhydrous	0.024 g
Sodium pyruvate	0.3 g
Agar	15.0 g
Purified water	1000 ml

Adjust the pH so that after sterilisation it is 7.2 ± 0.2. Sterilise by heating in an autoclave at 121 °C for 15 min.

NEUTRALISING AGENTS

Neutralising agents may be used to neutralise the activity of antimicrobial agents. They may be added to buffered sodium chloride-peptone solution pH 7.0, preferably before sterilisation. If utilised their efficacy and non-toxicity towards micro-organisms are demonstrated.

A typical neutralising fluid has the following composition:

Polysorbate 80	30 g
Lecithin (egg)	3 g
Histidine hydrochloride	1 g
Peptone (meat or casein)	1 g
Sodium chloride	4.3 g
Potassium dihydrogen phosphate	3.6 g
Disodium hydrogen phosphate dihydrate	7.2 g
Purified water	1000 ml

Sterilise by heating in an autoclave at 121 °C for 15 min.

If the solution has insufficient neutralising capacity the concentration of polysorbate 80 or lecithin may be increased. Alternatively, the neutralisers mentioned in Table 2.6.13.-3 may be added.

Table 2.6.13.-3. – *Inactivators for antimicrobial agents to be added to buffered sodium chloride-peptone solution pH 7.0*

Type of antimicrobial agent	Inactivator	Concentration	Comment
Phenolics	Sodium laurilsulfate	4 g/l	Add after sterilisation of buffered sodium chloride-peptone solution pH 7.0
	Polysorbate 80 and lecithin	30 g/l and 3 g/l	
	Egg yolk	5 ml/l - 50 ml/l	
Organo-mercurals	Sodium thioglycolate	0.5 g/l - 5 g/l	
Halogens	Sodium thiosulphate	5 g/l	
Quaternary ammonium compounds	Egg yolk	5 ml/l - 50 ml/l	Add after sterilisation of buffered sodium chloride-peptone solution pH 7.0

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2.6.14. BACTERIAL ENDOTOXINS

The test for bacterial endotoxins is used to detect or quantify endotoxins of gram-negative bacterial origin using amoebocyte lysate from horseshoe crab (*Limulus polyphemus* or *Tachypleus tridentatus*). There are 3 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 colour after cleavage of a synthetic peptide-chromogen complex.

The following 6 methods are described in the present chapter:

Method A. Gel-clot method: limit test

Method B. Gel-clot method: semi-quantitative test

Method C. Turbidimetric kinetic method

Method D. Chromogenic kinetic method

Method E. Chromogenic end-point method

Method F. Turbidimetric end-point method

Proceed by any of the 6 methods for the test. In the event of doubt or dispute, the final decision is made based upon method A unless otherwise indicated in the monograph.

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

Apparatus

Depyrogenate all glassware and other heat-stable apparatus 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 microtitre plates and pipette tips for automatic pipetters, use apparatus shown to be free of detectable endotoxin and of interfering effects for the test.

NOTE: In this chapter, the term 'tube' includes all types of receptacles, for example microtitre plate wells.

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Preparation of the standard endotoxin stock solution

The standard endotoxin stock solution is prepared from an endotoxin reference standard that has been calibrated against the International Standard, for example *endotoxin standard BRP*.

Endotoxin is expressed in International Units (IU). The equivalence in IU of the International Standard is stated by the World Health Organisation.

NOTE: One International Unit (IU) of endotoxin is equal to one Endotoxin Unit (E.U.).

Follow the specifications in the package leaflet and on the label for preparation and storage of the standard endotoxin stock solution.

Preparation of the standard endotoxin solutions

After vigorously mixing the standard endotoxin stock solution, prepare appropriate serial dilutions of this solution using water for bacterial endotoxins test (water for BET).

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

Preparation of the test solutions

Prepare the test solutions by dissolving or diluting active substances or medicinal products using water for BET. Some substances or preparations may be more appropriately dissolved or diluted in other aqueous solutions. If necessary, adjust the pH of the test solution (or dilution thereof) so that the pH of the mixture of the lysate and test solution falls within the pH range specified by the lysate manufacturer. This usually applies to a product with a pH in the range of 6.0 to 8.0. The pH may be adjusted by the use of acid, base or a 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.

Determination of the Maximum Valid Dilution

The Maximum Valid Dilution (MVD) is the maximum allowable dilution of a sample at which the endotoxin limit can be determined. Determine the MVD using the following formulae:

$$\mathrm{MVD} = \frac{\text{endotoxin limit} \times \text{concentration of test solution}}{\lambda}$$

Endotoxin limit: the endotoxin limit for active substances administered parenterally, defined on the basis of dose, is equal to:

$$\frac{K}{M}$$

K = threshold pyrogenic dose of endotoxin per kilogram of body mass in a single hour period,

M = maximum recommended dose of product per kilogram of body mass in a single hour period.

The endotoxin limit for active substances administered parenterally is specified in units such as IU/ml, IU/mg, IU/Unit of biological activity, etc., in monographs.

Concentration of test solution:

- in mg/ml if the endotoxin limit is specified by mass (IU/mg),
- in Units/ml if the endotoxin limit is specified by unit of biological activity (IU/Unit),
- in ml/ml if the endotoxin limit is specified by volume (IU/ml).

λ = the labelled lysate sensitivity in the gel-clot technique (IU/ml) or the lowest point used in the standard curve of the turbidimetric or chromogenic techniques.

GEL-CLOT TECHNIQUE (METHODS A AND B)

The gel-clot technique allows detection or quantification of endotoxins and is based on clotting of the lysate in the presence of endotoxins. The concentration of endotoxins required to cause the lysate to clot under standard conditions is the labelled lysate sensitivity. To ensure both the precision and validity of the test, confirm the labelled lysate sensitivity and perform the test for interfering factors as described under *1. Preparatory testing*.

1. PREPARATORY TESTING

(i) Confirmation of the labelled lysate sensitivity

Confirm in 4 replicates the labelled sensitivity λ, expressed in IU/ml, of the lysate solution prior to use in the test. Confirmation of the lysate sensitivity is carried out when a new batch 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 of at least 4 concentrations equivalent to 2λ, λ, 0.5λ and 0.25λ by diluting the standard endotoxin stock solution with water for BET.

Mix a volume of the lysate solution with an equal volume of 1 of the standard solutions (such as 0.1 ml aliquots) in each tube. When single test vials or ampoules containing lyophilised lysate are employed, add solutions directly to the vial or ampoule. Incubate the reaction mixture for a constant period according to the recommendations of the lysate manufacturer (usually at 37 ± 1 °C for 60 ± 2 min), avoiding vibration. Test the integrity of the gel: for tubes, take each tube in turn directly from the incubator and invert it 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 an intact gel is not formed.

The test is not valid unless the lowest concentration of the standard solutions shows a negative result in all replicate tests.

The end-point is the last positive result in the series of decreasing concentrations of endotoxin. Calculate the mean value of the logarithms of the end-point concentrations and then the antilogarithm of the mean value using the following expression:

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

$\sum e$ = sum of the log end-point concentrations of the dilution series used,

f = number of replicates.

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

(ii) Test for interfering factors

Prepare solutions A, B, C and D as shown in Table 2.6.14.-1, and use the test solutions at a dilution less than the MVD, not containing any detectable endotoxins, operating as described under 1. Preparatory testing, (i) Confirmation of the labelled lysate sensitivity.

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Table 2.6.14.-1

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

Solution A = solution of the preparation being examined that is free of detectable endotoxins.
Solution B = test for interference.
Solution C = control of the labelled lysate sensitivity.
Solution D = negative control (water for BET).

The geometric mean end-point concentrations of solutions B and C are determined using the expression described in 1. Preparatory testing, (i) Confirmation of the labelled lysate sensitivity.

The test for interfering factors is repeated when any changes are made to the experimental conditions that are likely to influence the result of the test.

The test is not valid unless all replicates of solutions A and D show no reaction and the result of solution C confirms the labelled lysate sensitivity.

If the sensitivity of the lysate determined with solution B is not less than 0.5λ and not greater than 2λ, the test solution does not contain interfering factors under the experimental conditions used. Otherwise, the solution interferes with the test.

If the preparation being examined interferes with the test at a dilution less than the MVD, repeat the test for interfering factors using a greater dilution, not exceeding the MVD. The use of a more sensitive lysate permits a greater dilution of the preparation being examined and this may contribute to the elimination of interference.

Interference may be overcome by suitable treatment, such as filtration, neutralisation, dialysis or heat treatment. To establish that the treatment chosen effectively eliminates interference without loss of endotoxins, repeat the test for interfering factors using the preparation being examined to which the standard endotoxin has been added and which has then been submitted to the chosen treatment.

2. LIMIT TEST (METHOD A)

(i) Procedure

Prepare solutions A, B, C and D as shown in Table 2.6.14.-2, and perform the test on these solutions following the procedure described under 1. Preparatory testing, (i) Confirmation of the labelled lysate sensitivity.

Table 2.6.14.-2

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

Prepare solution A and solution B (positive product control) using a dilution not greater than the MVD and treatments as described in 1. Preparatory testing, (ii) Test for interfering factors. Solutions B and C (positive controls) contain the standard endotoxin at a concentration corresponding to twice the labelled lysate sensitivity. Solution D (negative control) consists of water for BET.

(ii) Interpretation

The test is not valid unless both replicates of the 2 positive control solutions B and C are positive and those of the negative control solution D are negative.

The preparation being examined complies with the test when a negative result is found for both replicates of solution A.

When a positive result is found for both replicates of solution A:

- if the preparation being examined is diluted to the MVD, it does not comply with the test,
- if the preparation being examined is diluted to a dilution less than the MVD, the test is repeated at a dilution not greater than the MVD.

Repeat the test if a positive result is found for one replicate of solution A and a negative result is found for the other. The preparation being examined complies with the test if a negative result is found for both replicates of solution A in the repeat test.

3. SEMI-QUANTITATIVE TEST (METHOD B)

(i) Procedure

The test quantifies bacterial endotoxins in the test solution by titration to an end-point. Prepare solutions A, B, C and D as shown in Table 2.6.14.-3, and test these solutions according to the procedure described under 1. Preparatory testing, (i) Confirmation of the labelled lysate sensitivity.

(ii) Calculation and interpretation

The test is not valid unless the following 3 conditions are met:

(a) both replicates of solution D (negative control) are negative,

(b) both replicates of solution B (positive product control) are positive,

(c) the geometric mean end-point concentration of solution C is in the range of 0.5λ to 2λ.

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

The endotoxin concentration in the test solution is the geometric mean end-point concentration of the replicates (see the expression given under 1. Preparatory testing, (i) Confirmation of the labelled lysate sensitivity). If the

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test is conducted with a diluted test solution, calculate the concentration of endotoxin in the original solution by multiplying the result by the dilution factor.

If none of the dilutions of the test solution is positive in a valid test, record the endotoxin concentration as less than λ (or, if a diluted sample was tested, as less than λ × the lowest dilution factor of the sample). If all dilutions are positive, the endotoxin concentration is recorded as equal to or greater than the greatest dilution factor multiplied by λ (e.g. in Table 2.6.14.-3, the initial dilution factor × 8 × λ).

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

PHOTOMETRIC TECHNIQUES (METHODS C, D, E AND F)

1. TURBIDIMETRIC TECHNIQUE (METHODS C AND F)

This technique is a photometric test to measure the increase in turbidity. Based on the test principle employed, this technique is classified as being the end-point-turbidimetric test or the kinetic-turbidimetric test.

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

The kinetic-turbidimetric test (Method C) is a method to measure either the time (onset time) needed for the reaction mixture to reach a predetermined absorbance, or the rate of turbidity development.

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

2. CHROMOGENIC TECHNIQUE (METHODS D AND E)

This technique is used to measure the chromophore released from a suitable chromogenic peptide by the reaction of endotoxins with the lysate. Depending on the test principle employed, this technique is classified as being the end-point-chromogenic test or the kinetic-chromogenic test.

The end-point-chromogenic test (Method E) is based on the quantitative relationship between the endotoxin concentration and the quantity of chromophore released at the end of an incubation period.

The kinetic-chromogenic test (Method D) measures either the time (onset time) needed for the reaction mixture to reach a predetermined absorbance, or the rate of colour development.

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

3. PREPARATORY TESTING

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

Validation of the test method is required when any changes are made to the experimental conditions that are likely to influence the result of the test.

(i) Assurance of criteria for the standard curve

Using the standard endotoxin solution, prepare at least 3 endotoxin concentrations to generate the standard curve. Perform the test using at least 3 replicates of each standard endotoxin solution as recommended by the lysate manufacturer (volume ratios, incubation time, temperature, pH, etc.).

If the desired range is greater than 2 log in the kinetic methods, additional standards must 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 indicated by the lysate manufacturer.

(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 as shown in Table 2.6.14.-4. Perform the test on at least 2 replicates of these solutions as recommended by the lysate manufacturer (volume of test solution and lysate solution, volume ratio of test solution to lysate solution, incubation time, etc.).

Table 2.6.14.-3

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

Solution A = test solution at the dilution, not exceeding the MVD, with which the test for interfering factors was carried out. Subsequent dilution of the test solution must not exceed the MVD. Use water for BET to make two dilution series of 1, 1/2, 1/4 and 1/8, relative to the dilution with which the test for interfering factors was carried out. Other dilutions may be used as appropriate.

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

Solution C = 2 series of water for BET containing the standard endotoxin at concentrations of 2λ, λ, 0.5λ and 0.25λ.

Solution D = water for BET (negative control).

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Table 2.6.14.-4.

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

Solution A = test solution, that may be diluted not to exceed the MVD.

Solution B = preparation to be examined at the same dilution as solution A, containing added endotoxin at a concentration equal to or near the middle of the standard curve.

Solution C = standard endotoxin solution at the concentrations used in the validation of the method as described under 3. Preparatory testing, (i) Assurance of criteria for the standard curve (positive controls.

Solution D = water for BET (negative control).

Calculate the mean recovery of the added endotoxin by subtracting the mean endotoxin concentration in the solution (if any) from that in the solution containing the added endotoxin.

The test solution is considered free of interfering factors if under the conditions of the test, the measured concentration of the endotoxin added to the test solution is within 50-200 per cent 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 ranges, the interfering factors must be removed as described in the section Gel-clot technique, under 1. Preparatory testing, (ii) Test for interfering factors. The efficiency of the treatment is verified by repeating the test for interfering factors.

4. TEST

(i) Procedure

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

(ii) Calculation

Calculate the endotoxin concentration of each replicate of solution A using the standard curve generated by the series of positive controls, solution C.

The test is not valid unless the following 3 requirements are met:

(a) the result obtained with solution D (negative control) does not exceed the limit of the blank value required in the description of the lysate employed,

(b) the results obtained with the series of positive controls, solution C, comply with the requirements for validation defined under 3. Preparatory testing, (i) Assurance of criteria for the standard curve,

(c) the endotoxin recovery, calculated from the endotoxin concentration found in solution B after subtracting the endotoxin concentration found in solution A, is within the range of 50-200 per cent.

(iii) Interpretation

The preparation being examined complies with the test if the mean endotoxin concentration of the replicates of solution A, after correction for dilution and concentration, is less than the endotoxin limit for the product.

5. REAGENTS

(i) Lysate solution

Dissolve amoebocyte lysate in water for BET or in a buffer, as recommended by the lysate manufacturer, by gentle stirring. Store the reconstituted lysate, refrigerated or frozen, as indicated by the manufacturer.

(ii) Amoebocyte lysate

Amoebocyte lysate is a lyophilised product obtained from amoebocyte lysate from Horseshoe Crab (*Limulus polyphemus* or *Tachypleus tridentatus*). This reagent refers only to a product manufactured in accordance with the regulations of the competent authority.

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

(iii) Water for BET (water for bacterial endotoxins test)

Water for BET is *water for injections R* or water produced by other procedures that shows no reaction with the lysate employed at the detection limit of the reagent.

The following section is published for information.

Test for bacterial endotoxins: guidelines

1. INTRODUCTION

Endotoxins from gram-negative bacteria are the most common cause of toxic reactions resulting from contamination of pharmaceutical products with pyrogens; their pyrogenic activity is much higher than that of most other pyrogenic substances. These endotoxins are lipo-polysaccharides. Although there are a small number of pyrogens which possess a different structure, the conclusion is generally justified that the absence of bacterial endotoxins in a product implies the absence of pyrogenic components, provided the presence of non-endotoxin pyrogenic substances can be ruled out.

The presence of endotoxins in a product may be masked by factors interfering with the reaction between the endotoxins and the amoebocyte lysate. Hence, the analyst who wishes to replace the rabbit pyrogen test required in a pharmacopoeial monograph by a test for bacterial endotoxins has to demonstrate that a valid test can be carried out on the product concerned; this may entail a procedure for removing interfering factors.

As indicated in the test for bacterial endotoxins, information must be available on the 2 following aspects before a test on a sample can be regarded as valid.

1.1. The suitability of the material to be used for the test has to be established. The absence of endotoxins in the water for BET and in the other reagents must be assured and the sensitivity of the amoebocyte lysate must be checked to confirm the sensitivity declared by the manufacturer.

1.2. As the product to be examined may interfere with the test, the sensitivity of the amoebocyte lysate is determined in the presence and in the absence of the product under examination. There must be no significant difference between the 2 sensitivity values.

The test for bacterial endotoxins (*2.6.14*) indicates methods for removing interfering factors; in the case of interference, another test must be carried out after such a method has been applied to check whether the interference has indeed been neutralised or removed.

This annex explains the reasons for the requirements in the test for bacterial endotoxins, then deals with the reading and interpretation of the results.

Substitution of the rabbit pyrogen test required in a pharmacopoeial monograph by an amoebocyte lysate test constitutes the use of an alternative method of analysis and hence requires validation; some guidance on how to proceed is given in section 11.

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The reference method for bacterial endotoxins is stated in the monograph on a given product; where no method is stated, method A is the reference method. If a method other than the reference method is to be used, the analyst must demonstrate that the method is appropriate for this product and gives a result consistent with that obtained with the reference method (see also Section 13).

2. METHOD

The addition of endotoxins to amoebocyte lysate may result in turbidity, precipitation or gelation (gel-clot); only the gel-clot method was used in the Pharmacopoeia as an evaluation criterion in the first type of test for bacterial endotoxins. The advantage was the simplicity of basing the decision to pass or fail the product under examination on the absence or presence of a gel-clot, visible with the naked eye. The quantitative methods described as methods C, D, E and F were developed later: they require more instrumentation, but they are easier to automate for the regular testing of large numbers of samples of the same product.

Endotoxins may be adsorbed onto the surface of tubes or pipettes made from certain plastics or types of glass. Interference may appear due to the release of substances from plastic materials. Hence, the materials used should be checked; subsequent batches of tubes or pipettes may have a slightly different composition, and therefore the analyst is advised to repeat such tests on starting with new batches of materials.

The decision to use the test for bacterial endotoxins as a limit test implies first, that a threshold endotoxin concentration must be defined for the product to be tested and second, that the objective of the test is to know whether the endotoxin concentration in the product under examination is below or above this threshold. The quantitative methods C, D, E and F make it possible to determine the endotoxin concentration in the sample under examination, but for compliance with the Pharmacopoeia and in routine quality control the final question is whether or not this concentration exceeds a defined limit.

In setting a threshold concentration of endotoxin for the product to be tested, due attention should be paid to the dose of the product: the threshold should be set so as to ensure that as long as the endotoxin concentration in the product remains below this threshold even the maximal dose administered by the intended route per hour does not contain sufficient endotoxin to cause a toxic reaction.

When the endotoxin concentration in the product exactly equals the threshold value, gelation will occur, as is the case when the endotoxin concentration is much higher, and the product will fail the test, because the all-or-none character of the test makes it impossible to differentiate between a concentration exactly equal to the threshold concentration and one that is higher. It is only when no gelation occurs that the analyst may conclude that the endotoxin concentration is below the threshold concentration.

For products in the solid state, this threshold concentration of endotoxin per mass unit or per International Unit (IU) of product has to be translated into a concentration of endotoxin per millilitre of solution to be tested, as the test can only be carried out on a solution. The case of products that already exist in the liquid state (such as infusion fluids) is discussed below.

Endotoxin limit: the endotoxin limit for active substances administered parenterally, defined on the basis of dose, is equal to, where:

$$\frac{K}{M}$$

K = threshold pyrogenic dose of endotoxin per kilogram of body mass in a single hour period,

M = maximum recommended dose of product per kilogram of body mass in a single hour period.

The endotoxin limit depends on the product and its route of administration and is stated in monographs. Values for K are suggested in Table 2.6.14.-5.

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

Table 2.6.14.-5

Route of administration	*K* (IU of endotoxin per kilogram of body mass per hour)
Intravenous	5.0
Intravanous, for radiopharmaceuticals	2.5
Intrathecal	0.2

Which dilution of the product is to be used in the test to obtain maximal assurance that a negative result means that the endotoxin concentration of the product is less than the endotoxin limit and that a positive result means that the lysate detected an endotoxin concentration equal to or greater than the endotoxin limit? This dilution depends on the endotoxin limit and on the sensitivity of the lysate: it is called the Maximum Valid Dilution (MVD) and its value may be calculated as follows:

$$\mathrm{MVD} = \frac{\text{endotoxin limit} \times \text{concentration of test solution}}{\lambda}$$

Concentration of test solution:

- in mg/ml if the endotoxin limit is specified by mass (IU/mg),
- in Units/ml if the endotoxin limit is specified by unit of biological activity (IU/Unit),
- in ml/ml if the endotoxin limit is specified by volume (IU/ml).

λ = the labelled lysate sensitivity in the gel-clot technique (IU/ml) or the lowest point used in the standard curve of the turbidimetric or chromogenic techniques.

When the value of the maximum valid dilution is not a whole number, a convenient whole number smaller than the MVD may be used for routine purposes (which means preparing a solution of the product which is less diluted than the MVD indicates). In this case, a negative result indicates that the endotoxin concentration of the product lies below the limit value. However, when the endotoxin concentration of the product in such a test is less than the endotoxin limit but high enough to make the reaction with the lysate result in a clot, the test may be positive under these conditions. Hence, when a test with this 'convenient' dilution factor is positive, the product should be diluted to the MVD and the test should be repeated. In any case of doubt or dispute the MVD must be used.

This stresses the importance of the confirmation of the sensitivity of the lysate.

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Example

A 50 mg/ml solution of phenytoin sodium (intended for intravenous injection) has to be tested. Determine the MVD, given the following variables:

M = maximum human dose = 15 mg per kilogram of body mass per hour,

c = 50 mg/ml,

K = 5 IU of endotoxin per kilogram of body mass per hour,

λ = 0.4 IU of endotoxin per millilitre.

$$\mathrm{MVD} = \frac{5 \times 50}{15} \times \frac{1}{0.4} = 41.67$$

For routine tests on this product, it may be expedient to dilute 1 ml of the solution to be tested to 20 ml (MVD/2 rounded to the next lower whole number). However, if this test result is positive the analyst will have to dilute 1 ml to 41.67 ml and repeat the test. A dilution to 41.67 ml is also necessary when the test is performed to settle a dispute.

3. REFERENCE MATERIAL

Endotoxin standard BRP is intended for use as the reference preparation. It has been assayed against the WHO International Standard for Endotoxin and its potency is expressed in International Units of endotoxin per ampoule. The International Unit of endotoxin is defined as the specific activity of a defined mass of the International Standard.

For routine purposes, another preparation of endotoxin may be used, provided it has been assayed against the International Standard for Endotoxin or the BRP and its potency is expressed in International Units of endotoxin.

NOTE: 1 International Unit (IU) of endotoxin is equal to 1 Endotoxin Unit (E.U.).

4. WATER FOR BET

Testing the absence of endotoxin in this reagent by a technique derived from the rabbit pyrogen test was rejected for practical and theoretical reasons:

4.1. The rabbit test is not sensitive enough to detect endotoxin in water for BET intended for tests on products with a very low endotoxin limit.

4.2. The relatively low precision of the rising temperature response in rabbits would call for many replications in rabbits.

4.3. The terms ‘pyrogens’ and ‘endotoxins’ denote groups of entities that do not coincide completely.

The text of the test for bacterial endotoxins indicates that methods other than triple distillation may be used to prepare water for BET. Reverse osmosis has been used with good results; some analysts may prefer to distil the water more than three times. Whatever method is used, the resultant product must be free of detectable endotoxins.

5. pH OF THE MIXTURE

In the test for bacterial endotoxins, optimum gel-clot occurs for a mixture at pH 6.0 to 8.0. However, the addition of the lysate to the sample may result in a lowering of the pH.

6. VALIDATION OF THE LYSATE

It is important to follow the manufacturer’s instructions for the preparation of the solutions of the lysate.

The positive end-point dilution factors in the gel-clot methods A and B are converted to logarithms. The reason is that if the frequency distribution of these logarithmic values is plotted, it usually approaches a normal distribution curve much more closely than the frequency distribution of the dilution factors themselves; in fact it is so similar that it is acceptable to use the normal frequency distribution as a mathematical model and to calculate confidence limits with Student’s *t*-test.

7. PRELIMINARY TEST FOR INTERFERING FACTORS

Some products cannot be tested directly for the presence of endotoxins because they are not miscible with the reagents, they cannot be adjusted to pH 6.0 to 8.0 or they inhibit or activate gel formation. Therefore a preliminary test is required to check for the presence of interfering factors; when these are found the analyst must demonstrate that the procedure to remove them has been effective.

The object of the preliminary test is to test the null hypothesis that the sensitivity of the lysate in the presence of the product under examination does not differ significantly from the sensitivity of the lysate in the absence of the product. A simple criterion is used in methods A and B: the null hypothesis is accepted when the sensitivity of the lysate in the presence of the product is at least 0.5 times and not more than twice the sensitivity of the lysate by itself.

A classical approach would have been to calculate the means of the log dilution factor for the lysate sensitivity with and without the product and to test the difference between the two means with Student’s *t*-test.

The test for interfering factors in gel-clot methods A and B requires the use of a sample of the product in which no endotoxins are detectable. This presents a theoretical problem when an entirely new product has to be tested. Hence, a different approach was designed for quantitative methods C, D, E and F.

8. REMOVAL OF INTERFERING FACTORS

The procedures to remove interfering factors must not increase or decrease (for example, by adsorption) the amount of endotoxin in the product under examination. The correct way of checking this is to apply the procedures to a spiked sample of the product, that is, a sample to which a known amount of endotoxin has been added, and then to measure the recovery of the endotoxin.

Methods C and D. If the nature of the product to be analysed shows interference which cannot be removed by classical methods, it may be possible to carry out the standard curve in the same type of product freed from endotoxins by appropriate treatment or by dilution of the product. The endotoxins test is then carried out by comparison with this standard curve.

Ultrafiltration with cellulose triacetate asymmetric membrane filters has been found to be suitable in most cases. The filters should be properly validated, because under some circumstances cellulose derivatives (β-D-glucans) can cause false positive results.

Polysulphone filters have been found to be unsuitable because false positive results had been obtained by some users.

9. THE PURPOSE OF THE CONTROLS

The purpose of the control made up with water for BET and the reference preparation of endotoxin at twice the concentration of the labelled lysate sensitivity is to verify the activity of the lysate at the time and under the conditions of the test. The purpose of the negative control is to verify the absence of a detectable concentration of endotoxin in water for BET.

The positive control, which contains the product to be examined at the concentration used in the test, is intended to show the absence of inhibiting factors at the time and under the conditions of the test.

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10. READING AND INTERPRETATION OF THE RESULTS

Minute amounts of endotoxin in the water for BET, or in any other reagent or material to which the lysate is exposed during the test, may escape detection as long as they do not reach the sensitivity limit of the lysate. However, they may raise the amount of endotoxin in the solution containing the product under examination to just above the sensitivity limit and cause a positive reaction.

The risk of this happening may be reduced by testing the water for BET and the other reagents and materials with the most sensitive lysate available, or at least one that is more sensitive than the one used in the test on the product. Even then, the risk of such a 'false positive result' cannot be ruled out completely. It should be realised, however, that in this respect the test design is 'fail-safe' in contrast to a test design permitting a false negative result, which could lead to the release of an unsatisfactory product, thus endangering the patient's health.

11. REPLACEMENT OF THE RABBIT PYROGEN TEST BY A TEST FOR BACTERIAL ENDOTOXINS

Monographs on pharmaceutical products intended for parenteral use that may contain toxic amounts of bacterial endotoxins require either a test for bacterial endotoxins or a rabbit pyrogen test. As a general policy:

11.1. In any individual monograph, when a test is required, only one test is included, either that for pyrogens or that for bacterial endotoxins.

11.2. In the absence of evidence to the contrary, the test for bacterial endotoxins is preferred over the test for pyrogens, since it is usually considered to provide equal or better protection to the patient.

11.3. Before including a test for bacterial endotoxins in a monograph, evidence is required that one of the tests described in chapter *2.6.14* can be applied satisfactorily to the product in question.

11.4. The necessary information is sought from manufacturers. Companies are invited to provide any validation data that they have concerning the applicability of the test for bacterial endotoxins to the substances and formulations of interest. Such data include details of sample preparation and of any procedures necessary to eliminate interfering factors. In addition, any available parallel data for rabbit pyrogen testing that would contribute to an assurance that the replacement of a rabbit pyrogen test by the test for bacterial endotoxin is appropriate, must be provided.

Additional requirements are defined in the following sections.

12. USE OF A DIFFERENT BACTERIAL ENDOTOXIN TEST FROM THAT PRESCRIBED IN THE MONOGRAPH

When a test for bacterial endotoxins is prescribed in a monograph and none of the six methods (A to F) described in chapter *2.6.14* is specified, then method A, the gel-clot method limit test, has been validated for this product. If one of the other methods (B to F) is specified, this is the one which has been validated for this product.

13. VALIDATION OF ALTERNATIVE METHODS

Replacement of a rabbit pyrogen test by a bacterial endotoxin test, or replacement of a stated or implied method for bacterial endotoxins by another method, is to be regarded as the use of an alternative method in the replacement of a pharmacopoeial test, as described in the General Notices:

> "The test and assays described are the official methods upon which the standards of the Pharmacopoeia are based. With the agreement of the competent authority, alternative methods of analysis may be used for control purposes, provided that the methods used enable an unequivocal decision to be made as to whether compliance with the standards of the monographs would be achieved if the official methods were used. In the event of doubt or dispute, the methods of analysis of the Pharmacopoeia are alone authoritative."

The following procedures are suggested for validating a method for bacterial endotoxins other than the one implied or indicated in the monograph.

13.1. The procedure and the materials and reagents used in the method should be validated as described for the test concerned.

13.2. The presence of interfering factors (and, if needed, the procedure for removing them) should be tested on samples of at least three production batches. It should be borne in mind that methods D and E, using a chromogenic peptide, require reagents that are absent in methods A, B, C and F, and hence compliance of methods A, B, C or F with the requirements for interfering factors cannot be extrapolated to method D or method E without further testing.

14. VALIDATION OF THE TEST FOR NEW PRODUCTS

The procedures described under 13.1 and 13.2 should be applied to all new products intended for parenteral use that have to be tested for the presence of bacterial endotoxins according to the requirements of the Pharmacopoeia.

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2.6.15. PREKALLIKREIN ACTIVATOR

Prekallikrein activator (PKA) activates prekallikrein to kallikrein and may be assayed by its ability to cleave a chromophore from a synthetic peptide substrate so that the rate of cleavage can be measured spectrophotometrically and the concentration of PKA calculated by comparison with a reference preparation calibrated in International Units.

The International Unit is the activity of a stated amount of the International Standard which consists of freeze-dried prekallikrein activator. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

Prekallikrein activator in albumin BRP is calibrated in International Units by comparison with the International Standard.

PREPARATION OF PREKALLIKREIN SUBSTRATE

To avoid coagulation activation, blood or plasma used for the preparation of prekallikrein must come into contact only with plastics or silicone-treated glass surfaces.

Draw 9 volumes of human blood into 1 volume of anticoagulant solution (ACD, CPD or 38 g/l *sodium citrate R*) to which 1 mg/ml of *hexadimethrine bromide R* has been added. Centrifuge the mixture at 3600 *g* for 5 min. Separate the plasma and centrifuge again at 6000 *g* for 20 min to sediment platelets. Separate the platelet-poor plasma and dialyse against 10 volumes of buffer A for 20 h. Apply the dialysed plasma to a chromatography column containing *agarose-DEAE for ion exchange chromatography R* which has been equilibrated in buffer A and is equal to twice the volume of the plasma. Elute from the column with buffer A at 20 ml/cm^2/h. Collect the eluate in fractions and record the absorbance at 280 nm (*2.2.25*). Pool the fractions containing the first protein peak so that the volume of the pool is about 1.2 times the volume of the platelet-poor plasma.

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