or, in the case of herbal drugs, of pre-treatment, would not reduce the level of organisms sufficiently to reach the criteria required under B

TAMC (*2.6.12*)	Acceptance criterion: 10^5 CFU/g or CFU/mL Maximum acceptable count: 500 000 CFU/g or CFU/mL
TYMC (*2.6.12*)	Acceptance criterion: 10^4 CFU/g or CFU/mL Maximum acceptable count: 50 000 CFU/g or CFU/mL
Bile-tolerant gram-negative bacteria (*2.6.31*)	Acceptance criterion: 10^4 CFU/g or CFU/mL
Escherichia coli (*2.6.31*)	Absence (1 g or 1 mL)
Salmonella (*2.6.31*)	Absence (25 g or 25 mL)

EXTRACTS

Extracts should fulfill the acceptance criteria for category B herbal medicinal products. However, where it can be demonstrated that the method of processing would not reduce the level of micro-organisms sufficiently to reach the category B criteria, the extracts shall meet the requirements for category C herbal medicinal products.

The recommended acceptance criteria apply to extracts that are to be incorporated into herbal medicinal products for oral use. More-stringent acceptance criteria may be required for extracts that are to be incorporated into pharmaceutical preparations to be administered by other routes in order to satisfy the acceptance criteria for the intended route of administration (*5.1.4*).

It is recognised that for some herbal medicinal products and extracts used in their preparation the criteria given above for TAMC, TYMC and bile-tolerant gram-negative bacteria cannot be met because of the typical level of microbial contamination. Less-stringent acceptance criteria may be applied on the basis of a risk assessment that takes account of qualitative and quantitative characterisation of the microbial contamination and the intended use of the herbal medicinal product or extract.

If it has been shown that none of the prescribed tests for a herbal medicinal product or extract will allow valid enumeration of micro-organisms at the level prescribed, a validated method with a limit of detection as close as possible to the indicated acceptance criterion is used.

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5.1.9. GUIDELINES FOR USING THE TEST FOR STERILITY

The purpose of the test for sterility (*2.6.1*), as that of all pharmacopoeial tests, is to provide an independent control analyst with the means of verifying that a particular material meets the requirements of the European Pharmacopoeia. A manufacturer is neither obliged to carry out such tests nor precluded from using modifications of, or alternatives to, the stated method, provided he is satisfied that, if tested by the official method, the material in question would comply with the requirements of the European Pharmacopoeia.

PRECAUTIONS AGAINST MICROBIAL CONTAMINATION

Aseptic conditions for performance of the test can be achieved using, for example, a class A laminar-air-flow cabinet located within a class B clean room, or an isolator

GUIDANCE TO MANUFACTURERS

The level of assurance provided by a satisfactory result of a test for sterility (the absence of contaminated units in the sample) as applied to the quality of the batch is a function of the homogeneity of the batch, the conditions of manufacture and the efficiency of the adopted sampling plan. Hence for the purpose of this text a batch is defined as a homogeneous collection of sealed containers prepared in such a manner that the risk of contamination is the same for each of the units contained therein.

In the case of terminally sterilised products, physical proofs, biologically based and automatically documented, showing correct treatment throughout the batch during sterilisation are of greater assurance than the sterility test. The circumstances in which parametric release may be considered appropriate are described under *5.1.1. Methods of preparation of sterile products*. The method of media-fill runs may be used to evaluate the process of aseptic production. Apart from that, the sterility test is the only analytical method available for products prepared under aseptic conditions and furthermore it is, in all cases, the only analytical method available to the authorities who have to examine a specimen of a product for sterility.

The probability of detecting micro-organisms by the test for sterility increases with their number present in the sample tested and varies according to the readiness of growth of micro-organism present. The probability of detecting very low levels of contamination even when it is homogenous throughout the batch is very low. The interpretation of the results of the test for sterility rests on the assumption that the contents of every container in the batch, had they been tested, would have given the same result. Since it is manifest that every container cannot be tested, an appropriate sampling plan should be adopted. In the case of aseptic production, it is recommended to include samples filled at the beginning and at the end of the batch and after significant intervention.

OBSERVATION AND INTERPRETATION OF RESULTS

Conventional microbiological/biochemical techniques are generally satisfactory for identification of micro-organisms recovered from a sterility test. However, if a manufacturer wishes to use condition (d) as the sole criterion for invalidating a sterility test, it may be necessary to employ sensitive typing techniques to demonstrate that a micro-organism isolated from the product test is identical to a micro-organism isolated from the test materials and/or the testing environment. While routine microbiological/biochemical identification techniques can demonstrate that 2 isolates are not identical, these methods may not be sufficiently sensitive or reliable enough to provide unequivocal evidence that 2 isolates are from the same source. More sensitive tests, for example molecular typing with RNA/DNA homology, may be necessary to determine that micro-organisms are clonally related and have a common origin.

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5.1.10. GUIDELINES FOR USING THE TEST FOR BACTERIAL ENDOTOXINS

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 (*2.6.14*), information must be available on the 2 following aspects before a test on a sample can be regarded as valid.

- 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.
- 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 text *2.6.14. Bacterial endotoxins* 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 general chapter 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.

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:

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

K = threshold pyrogenic dose of endotoxin per kilogram of body mass;

M = maximum recommended bolus dose of product per kilogram of body mass.

When the product is to be injected at frequent intervals or infused continuously, M is the maximum total dose administered in a single hour period.

The endotoxin limit depends on the product and its route of administration and is stated in the monograph. Values for K are suggested in Table 5.1.10.-1.

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 5.1.10.-1

Route of administration	*K* (IU of endotoxin per kilogram of body mass)
Intravenous	5.0
Intravenous, 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 using the following expression:

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

Concentration of test solution:

- mg/mL if the endotoxin limit is specified by mass (IU/mg);
- Units/mL if the endotoxin limit is specified by unit of biological activity (IU/Unit);
- 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 concentration 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.

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;

c = 50 mg/mL;

K = 5 IU of endotoxin per kilogram of body mass;

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:

- 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;
- the relatively low precision of the rising temperature response in rabbits would call for many replications in rabbits;
- the terms 'pyrogens' and 'endotoxins' denote groups of entities that do not coincide completely.

The text *2.6.14. 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 3 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 6.0-8.0. 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 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 6.0-8.0 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 2 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 determine 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.

Polysulfone 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.

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 administration that may contain toxic amounts of bacterial endotoxins require either a test for bacterial endotoxins or a rabbit pyrogen test. As a general policy:

- in any individual monograph, when a test is required, only one test is included, either that for pyrogens or that for bacterial endotoxins;
- 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;
- 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;
- 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 includes 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 6 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 3 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 administration that have to be tested for the presence of bacterial endotoxins according to the requirements of the Pharmacopoeia.