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Annex 14 to Note for Evaluation and Recommendation of Pharmacopoeial Texts for Use in the ICH Regions on Bacterial Endotoxins Tests – General Chapter

Step 3

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Annex 14 to Note for Evaluation and Recommendation of Pharmacopoeial Texts for Use in the ICH Regions on Bacterial Endotoxins Tests – General Chapter

Table of contents

1. INTRODUCTION

This annex is the result of the Q4B process for the Bacterial Endotoxins Test General Chapter.

The proposed texts were submitted by the Pharmacopoeial Discussion Group (PDG).

2. Q4B OUTCOME

2.1. Analytical Procedures

The ICH Steering Committee, based on the evaluation by the Q4B Expert Working Group (EWG), recommends that the analytical procedures described in the official pharmacopoeial texts, Ph.Eur. 2.6.14. Bacterial Endotoxins, JP 4.01 Bacterial Endotoxins Test, and USP General Chapter <85> Bacterial Endotoxins Test, can be used as interchangeable in the ICH regions subject to the following conditions:

2.1.1 Any of the three techniques can be used for the test. In the event of doubt or dispute, the gel-clot limit test should be used to make the final decision on compliance for the product being tested.

2.1.2 The Endotoxin Reference Standard should be calibrated to the current WHO (World Health Organization) International Standard for Endotoxin.

2.2. Acceptance Criteria

The evaluated texts did not contain acceptance criteria. Endotoxin limits should be specified in the application dossier unless otherwise specified in an individual monograph.

3. TIMING OF ANNEX IMPLEMENTATION

When this annex is implemented (incorporated into the regulatory process at ICH Step 5) in a region, it can be used in that region. Timing might differ for each region.

4. CONSIDERATIONS FOR IMPLEMENTATION

4.1. General Consideration

When sponsors or manufacturers change their existing methods to the implemented Q4B-evaluated pharmacopoeial texts that are referenced in Section 2.1 of this annex, any change notification, variation, and/or prior approval procedures should be handled in accordance with established regional regulatory mechanisms pertaining to compendial changes.

4.2. FDA Consideration

Based on the recommendation above, and with reference to the conditions set forth in this annex, the pharmacopoeial texts referenced in Section 2.1 of this annex can be considered interchangeable. However, FDA might request that a company demonstrate that the chosen method is acceptable and suitable for a specific material or product, irrespective of the origin of the method.

4.3. EU Consideration

For the European Union, regulatory authorities can accept the reference in a marketing authorisation application, renewal or variation application citing the use of the corresponding text from another pharmacopoeia as referenced in Section 2.1, in accordance with the conditions set out in this annex, as fulfilling the requirements for compliance with the Ph. Eur. Chapter 2.6.14. on the basis of the declaration of interchangeability made above.

4.4. MHLW Consideration

The pharmacopoeial texts referenced in Section 2.1 of this annex can be used as interchangeable in accordance with the conditions set out in this annex. Details of implementation requirements will be provided in the notification by MHLW when this annex is implemented.

4.5. Health Canada Consideration

In Canada any of the pharmacopoeial texts cited in Section 2.1 of this annex and used in accordance with the conditions set out in this annex can be considered interchangeable.

5. REFERENCES USED FOR THE Q4B EVALUATION

5.1 The PDG Stage 5B sign-off document (Rev. 1 – Correction 1): *Japanese Pharmacopoeial Forum*, Volume 18, number 4 (December 2009).

5.2 The pharmacopoeial references for the Bacterial Endotoxins Test General Chapter for this annex are:

5.2.1 *European Pharmacopoeia* (Ph. Eur.):
Supplement 6.6 (official January 1, 2010), Bacterial Endotoxins (reference 01/2010:20614).

5.2.2 *Japanese Pharmacopoeia* (JP):
General Test 4.01 Bacterial Endotoxins Test as it will appear in the JP Sixteenth Edition (March 31, 2011). The draft English version of the JP text provided by MHLW is appended (see Appendix A).

5.2.3 *United States Pharmacopeia* (USP):
Text for <85> Bacterial Endotoxins Test, USP 33 Reissue (published April 2010 and official October 1, 2010).

APPENDIX A

Draft JP XVI English Text Provided by MHLW

4. Biological Tests/Biochemical Tests/Microbial Tests
4.01 Bacterial Endotoxins Test
This test is harmonized with the European Pharmacopoeia and the U. S. Pharmacopeia.

Bacterial Endotoxins Test is a test to detect or quantify bacterial endotoxins of gram-negative bacterial origin using an amoebocyte lysate prepared from blood corpuscle extracts of horseshoe crab (*Limulus polyphemus* or *Tachypleus tridentatus*). There are two types of techniques for this test: the gel-clot techniques, which are based on gel formation by the reaction of the lysate TS with endotoxins, and the photometric techniques, which are based on endotoxin-induced optical changes of the lysate TS. The latter include turbidimetric techniques, which are based on the change in lysate TS turbidity during gel formation, and chromogenic techniques, which are based on the development of color after cleavage of a synthetic peptide-chromogen complex.

Proceed by any one of these techniques for the test. In the event of doubt or dispute, the final decision is made based on the gel-clot techniques, unless otherwise indicated.

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. Commonly used minimum time and temperature settings are 30 minutes at 250°C. If employing plastic apparatus, such as multi-well plates and tips for micropipettes, use only that which has been shown to be free of detectable endotoxin and which does not interfere with the test.

2. Preparation of Solutions
2.1 Standard Endotoxin Stock Solution
Prepare Standard Endotoxin Stock Solution by dissolving Japanese Pharmacopoeia Reference Standard Endotoxin in water for bacterial endotoxins test (BET). Endotoxin is expressed in Endotoxin Units (EU). One EU is equal to one International Unit (IU) of endotoxin.

2.2 Standard Endotoxin Solution
After mixing Standard Endotoxin Stock Solution thoroughly, 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.

2.3 Sample Solutions
Unless otherwise specified, prepare sample solutions by dissolving or diluting drugs, using water for BET. If necessary, adjust the pH of the solution to be examined so that the pH of the mixture of the lysate TS and sample solution falls within the specified pH range for the lysate to be used. This usually applies to a sample solution with a pH in the range of 6.0 to 8.0. TSs or solutions used for adjustment of pH may be prepared using water for BET, and then stored in containers free of detectable endotoxin. The TSs or solutions must be validated to be free of detectable endotoxin and interfering factors.

3. Determination of Maximum Valid Dilution
The Maximum Valid Dilution (MVD) is the maximum allowable dilution of a sample solution at which the endotoxin limit can be determined.
Determine the MVD from the following equation:

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

Endotoxin limit:
The endotoxin limit for injections, defined on the basis of dose, equals K/M, where K is a minimum pyrogenic dose of endotoxin per kg body mass (EU/kg), and M is equal to the maximum bolus dose of product per kg 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.

Concentration of sample solution:

mg/mL in the case of endotoxin limit specified by mass (EU/mg)
mEq/mL in the case of endotoxin limit specified by equivalent (EU/mEq)
Units/mL in the case of endotoxin limit specified by biological unit (EU/Unit)
mL/mL in the case of endotoxin limit specified by volume (EU/mL)
λ: the labeled lysate sensitivity in the gel-clot techniques (EU/mL) or the lowest point used (EU/mL) in the standard regression curve of the turbidimetric or chromogenic techniques

4. Gel-clot techniques
The gel-clot techniques detect or quantify endotoxins based on clotting of the lysate TS in the presence of endotoxin. To ensure both the precision and validity of the test, perform the tests for confirming the labeled lysate sensitivity (4.1.1) and for interfering factors (4.1.2) as described under Preparatory testing (4.1).

4.1 Preparatory testing
4.1.1 Test for confirmation of labeled lysate sensitivity
The labeled sensitivity of lysate is defined as the lowest concentration of endotoxin that is needed to cause the lysate TS to clot under the conditions specified for the lysate to be used.

The test for confirmation of the labeled lysate sensitivity is to be carried out when each new lot 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 having 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 (usually, 0.1 mL aliquots) in each test tube. When single test vials or ampoules containing lyophilized lysate are used, add solutions directly to the vial or ampoule.

Keep the tubes (or containers such as vials or ampoules) containing the reaction mixture usually at 37 ± 1°C for 60 ± 2 minutes, avoiding vibration. To test the integrity of the gel after incubation, invert each tube or container 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 either a firm gel is not formed, or if a fragile gel has formed but flows out upon inversion.

Making the standard solutions of four concentrations one set, test four replicates of the set.

The test is valid when 0.25 λ of the standard solution shows a negative result in each set of tests. If the test is not valid, repeat the test after verifying the test conditions.

The endpoint is the last positive test in the series of decreasing concentrations of endotoxin. Calculate the geometric mean endpoint concentration of the four replicate series using the following formula:

Geometric Mean Endpoint Concentration = antilog ($\sum e/f$)

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

f = the number of replicates

If the geometric mean endpoint concentration 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.

4.1.2 Test for interfering factors
This test is performed to check for the presence of enhancing or inhibiting factors for the reaction in sample solutions.

Prepare the solutions A, B, C and D according to Table 4.01-1, and test solutions A and B and solutions C and D in quadruplicate and in duplicate, respectively. Concerning the incubation temperature, incubation time and procedure for the confirmation of gel formation, follow the procedure described in Table 4.1.1.

The geometric mean endpoint concentrations of B and C solutions are determined by using the formula described in 4.1.1.

This test must be repeated when there is any change in the experimental conditions which may affect the outcome of the test.
Table 4.01-1

Solution	Endotoxin concentration /Solution to which endotoxin is added	Diluent	Dilution factor	Endotoxin concentration	Number of replicates
A*[1]	0/Sample solution	—	—	—	4
B*[2]	2λ/Sample solution	Sample solution	1 2 4 8	2λ 1λ 0.5λ 0.25λ	4
C*[3]	2λ/Water for BET	Water for BET	1 2 4 8	2λ 1λ 0.5λ 0.25λ	2
D*[4]	0/Water for BET	—	—	—	2

*[1] Negative control. Sample solution only.
*[2] Sample solutions added with standard endotoxin (for testing interfering factors).
*[3] Standard endotoxin solutions for confirmation of the labeled lysate sensitivity.
*[4] Negative control. Water for BET only.

The test is valid if solutions A and D show no reaction and the result for solution C confirms the labeled lysate sensitivity.

If the geometric mean endpoint concentration of solution B is not less than 0.5 λ and not greater than 2 λ, the sample solution being examined does not contain interfering factors and complies with the test for interfering factors. Otherwise the sample solution interferes with the test.

If the sample 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 sample to be examined. Furthermore, interference of the sample solution or diluted sample solution 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.

4.2 Limit test
This method tests whether or not a sample contains endotoxins greater than the endotoxin limit specified in the individual monograph based on the gel formation in the presence of endotoxins at a concentration of more than the labeled lysate sensitivity.

4.2.1 Procedure
Prepare solutions A, B, C and D according to Table 4.01-2. Making these four solutions one set, test two replicates of the set.

In preparing solutions A and B, use the sample solutions complying with 4.1.2. Concerning the test conditions including the incubation temperature, incubation time and procedure for the confirmation of gel formation, follow the procedure described in 4.1.1.

Table 4.01-2

Solution	Endotoxin concentration /Solution to which endotoxin is added	Number of replicates
A*[1]	0/Sample solution	2
B*[2]	2λ/Sample solution	2
C*[3]	2λ/Water for BET	2
D*[4]	0/Water for BET	2

*[1] Sample solution for the limit test. The solution may be diluted not to exceed the MVD.
*[2] Positive control. Sample solution at the same dilution as solution A, containing standard endotoxin at a concentration of 2λ.
*[3] Positive control. Standard endotoxin solution containing standard endotoxin at a concentration of 2λ.
*[4] Negative control. Water for BET only.

4.2.2 Interpretation
The test is valid when both replicates of solutions 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 sample complies with the Bacterial Endotoxins Test.

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

However, if the sample 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.

4.3 Quantitative Test
This method measures endotoxin concentrations of samples by determining an endpoint of gel formation.

4.3.1 Procedure
Prepare solutions A, B, C and D according to Table 4.01-3. Making these four solutions one set, test two replicates of the set. When preparing solutions A and B, use sample solutions complying with 4.1.2. Concerning the test conditions, follow the procedure described in 4.1.1.

Table 4.01-3

Solution	Endotoxin concentration /Solution to which endotoxin is added	Diluent	Dilution Factor	Endotoxin concentration	Number of replicates
A*[1]	0/Sample solution	Water for BET	1 2 4 8	— — — —	2
B*[2]	2λ/Sample solution	—	1	2λ	2
C*[3]	2λ/Water for BET	Water for BET	1 2 4 8	2λ 1λ 0.5λ 0.25λ	2
D*[4]	0/Water for BET	—	—	—	2

*[1] Sample solutions for the Quantitative test. The dilution range of the dilution series may be changed as appropriate, but not exceeding the MVD.
*[2] Positive control. Sample solution at the same dilution as the solution A diluted at the lowest dilution factor, containing standard endotoxin at a concentration of 2λ.
*[3] Standard endotoxin solutions for confirmation of the labeled lysate sensitivity.
*[4] Negative control. Water for BET only.

4.3.2 Calculation and interpretation
The test is valid when the following three conditions are met: (a) both replicates of the negative control solution D are negative, (b) both replicates of the positive product control solution B are positive and (c) the geometric mean endpoint concentration of solution C is in the range of 0.5 λ to 2 λ.

The endpoint is defined as the maximum dilution showing the last positive test in the dilution series of solution A, and the endotoxin concentration of the sample solution is calculated by multiplying the endpoint dilution factor by λ.

If none of the dilutions of solution A is positive, report the endotoxin concentration of the sample solution as less than λ × the lowest dilution factor of the sample solution.

If all dilutions are positive, the endotoxin concentration of the sample solution is reported as equal to or greater than the greatest dilution factor of solution A multiplied by λ.

Calculate the endotoxin concentration (in EU per mL, in EU per mg or mEq or in EU per Unit) of the sample, based on the endotoxin concentration of the sample solution. The sample complies with the Bacterial Endotoxins Test if the endotoxin concentration of the sample in both replicates meets the requirement for the endotoxin limit (in EU per mL, in EU per mg or mEq or in EU per Unit) specified in the individual monograph.

5. Photometric quantitative techniques

5.1 Turbidimetric technique

This technique measures the endotoxin concentrations of samples based on the measurement of turbidity change accompanying gel formation of the lysate TS. This technique is classified as either endpoint-turbidimetric or kinetic-turbidimetric.

The endpoint-turbidimetric technique is based on the quantitative relationship between the concentration of endotoxins and the turbidity of the reaction mixture at a specified reaction time.

The kinetic-turbidimetric technique is based on the quantitative relationship between the concentration of endotoxins and either the time needed to reach a predetermined turbidity of the reaction mixture or the rate of turbidity development.

The test is usually carried out at 37 ± 1°C, and turbidity is expressed in terms of either absorbance or transmission.

5.2 Chromogenic technique

This technique measures the endotoxin concentrations of sample solutions based on the measurement of chromophore released from a synthetic chromogenic substrate by the reaction of endotoxins with the lysate TS. This technique is classified as either endpoint-chromogenic or kinetic-chromogenic.

The endpoint-chromogenic technique 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 technique is based on the quantitative relationship between the concentration of endotoxins and either the time needed to reach a predetermined absorbance (or transmittance) of the reaction mixture or the rate of color development.

The test is usually carried out at 37 ± 1°C.

5.3 Preparatory testing

To assure the precision and validity of the turbidimetric or chromogenic techniques, perform both Test for assurance of criteria for the standard curve (5.3.1**)** and Test for interfering factors (5.3.2**)**, as indicated below.

5.3.1 Test for assurance of criteria for the standard curve

The test is to be carried out when each new lot of lysate is used or when there is any change in the experimental conditions which may affect the outcome of the test.

Using the Standard Endotoxin Solution, prepare at least three endotoxin concentrations to generate the standard curve within the range of endotoxin concentrations indicated by the instructions for the lysate used. Perform the test using at least three replicates of each standard endotoxin concentration according to the optimal conditions for the lysate used (with regard to volume ratios, incubation time, temperature, pH, etc.). If the desired range is greater than two logs, additional standards should be included to bracket each log increase in the range of the standard curve.

If the absolute value of the correlation coefficient, $|r|$, is greater than or equal to 0.980 for the range of endotoxin concentrations set up, the criteria for the standard curve are valid and the curve complies with the test.

If the standard curve does not comply with the test, repeat the test after verifying the test conditions.

5.3.2 Test for interfering factors

Prepare solutions A, B, C and D according to Table 4.01-4. Perform the test on these solutions following the optimal conditions for the lysate used (with regard to volume of sample solution and lysate TS, volume ratio of sample solution to lysate TS, incubation time, etc.).

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

Table 4.01-4

Solution	Endotoxin concentration	Solution to which endotoxin is added	Number of test tubes or wells
A*[1]	0	Sample solution	Not less than 2
B*[2]	Middle concentration of the standard curve	Sample solution	Not less than 2
C*[3]	At least 3 concentrations	Water for BET	Each not less than 2
D*[4]	0	Water for BET	Not less than 2

*[1] Sample solution only (for assaying endotoxin concentration in the sample solution). The sample solution may be diluted not to exceed the MVD.
*[2] Sample solution at the same dilution as solution A, containing added standard endotoxin at a concentration equal to or near the middle of the standard curve.
*[3] Standard endotoxin solutions at the concentrations used in 5.3.1 (for the standard curve).
*[4] Negative control. Water for BET only.

The test is 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 recovery of the endotoxin added to solution B from the concentration found in solution B after subtracting the endotoxin concentration found in solution A.

When the recovery of the endotoxin added to solution B is within 50% to 200%, the sample solution under test is considered to be free of interfering factors and the solution complies with the test.

When the endotoxin recovery is out of the specified range, the sample solution under test is considered to contain interfering factors. If the sample under test does not comply with the test, 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.

5.4 Quantitative test
5.4.1 Procedure

Prepare solutions A, B, C and D according to Table 4.01-4, and follow the procedure described in 5.3.2.

5.4.2 Calculation of endotoxin concentration
Calculate the mean endotoxin concentration of solution A using the standard curve generated with solution C. The test is valid when all the following requirements 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 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% to 200%.
3: 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.

5.4.3 Interpretation
The sample complies with the Bacterial Endotoxins Test if the endotoxin concentration of the sample calculated from the mean endotoxin concentration of solution A meets the requirement of the endotoxin limit (in EU per mL, in EU per mg or mEq or in EU per Unit) specified in the individual monograph.