**MINISTRY OF HEALTH OF THE RUSSIAN FEDERATION**

# GENERAL PHARMACOPOEIA MONOGRAPH

**Bacterial endotoxins** **GPM**.1.2.4.0006.15

Replaces the State Pharmacopoeia of the Russian Federation XII, Part 1 Monograph, GPM 42-0062-07

The present General Pharmacopoeia Monograph describes the methods used to detect bacterial endotoxins in medicinal products designed for parenteral administration and drug substances used to manufacture such products.

The bacterial endotoxins assay is carried out using a reagent, which presents a lysate of blood cells (amoebocytes) from horseshoe crab: *Limulus polyphemus (LAL-reagent)* or *Tachypleus tridentatus (TAL-reagent*). LAL reagents interact specifically with bacterial endotoxins. As a result of an enzymatic reaction, the reactant mixture undergoes a change proportionate to the concentration of the endotoxin.

There are three major methodological approaches to performing this test: the gel clot assay based on gel formation; the turbidimetric principle in which the reactant mixture becomes turbid after degradation of the substrate contained in the LAL reagent; and the chromogenic principle based on the colour induced by degradation of a synthetic peptide – chromogenic complex.

The present Pharmacopoeia Monograph describes the following six tests based on the aforementioned principles:

– Qualitative gel clot assay (Method A);

– Quantitative gel clot assay (Method B);

– Turbidimetric kinetic test (Method C);

– Chromogenic kinetic test (Method D);

– Chromogenic endpoint assay (Method E);

– Turbidimetric endpoint assay (Method F).

The test may be performed using any of the six aforementioned methods. In case of any doubts or discrepancies, the final conclusion should be made on the basis of results obtained using Method A.

***Laboratory utensils and their preparation***

Glass and plastic laboratory ware used in the LAL-test should not contain bacterial endotoxins in quantities detected in this test, and should not affect the course of the reaction.

The recommended depyrogenation regimen is heating at temperature 250 °C over at least 30 minutes in accordance with the validated procedure.

***Endotoxin standards***

Content of bacterial endotoxins is expressed in Endotoxin Units (EU) of the International Endotoxin Standard. One endotoxin International Unit (IU) corresponds to one EU.

Analysis may also be based on the Endotoxin Reference Standard (ERS); its activity is established according to the International Standard for endotoxins. An Endotoxin Reference Standard should be designed for testing a particular LAL-reagent (TAL-reagent) lot[1]. Dissolution and storage of the ERS are carried out according to the manufacturer's Instructions for Use.

***LAL reagent***

The LAL-reagent designed for the selected bacterial endotoxins testing method should be used.

LAL-reagent sensitivity (λ) is expressed in endotoxin units [EU/mL], and corresponds to the minimum International Endotoxin Standard concentration that promotes the formation of a dense gel when reacting with the particular LAL-

1 The Endotoxin Reference Standard (ERS) and LAL-reagent or TAL-reagent should be authorized for use by the Ministry of Health and Social Development of the Russian Federation.

reagent (Methods A and B) or corresponds to the minimum value point on the standard curve (Methods C, D, E, and F).

Dissolution of the lyophilized LAL-reagent and its storage are carried out in accordance with the manufacturer's Instructions for Use.

***Note:*** *Apart from endotoxins, a LAL reagent may also react with some β-glycans, and therefore a specific LAL reagent deprived of the G-factor, which reacts with glycans, may be employed. Use of accessorial solutions that block the G factor reacting system is allowed as well. These reagents may be used for the determination of endotoxins in the presence of glycans.*

***Water for LAL testing***

Water for LAL-testing is used for preparation of all reagents and dilutions of the tested medicinal product. Water for LAL-testing should comply with the requirements established for Water for Injections, and it should not contain bacterial endotoxins in quantities detectable by the test.

***Preparation of the tested sample***

Every selected sample should be tested individually.

Water for LAL-testing is used to dissolve and / or dilute the tested medicinal product, unless otherwise specified in the Pharmacopoeia Monograph. The tested solution should have its pH value within the range specified by the manufacturer of the LAL-reagent, most commonly 6.0 – 8.0. When necessary, the pH value of the tested solution is adjusted using acidic or basic solutions, or a buffer solution. The employed solutions should not contain bacterial endotoxins in quantities detectable by the test, and should not affect the course of the reaction.

***Maximum acceptable dilution of the tested medicinal product***

The maximum acceptable dilution (MAD) is the highest dilution of the tested medicinal product in which the endotoxin concentration corresponding to the maximum content of bacterial endotoxins approved for the particular medicinal product can be detected.

The tested medicinal product can be tested either in one dilution or in a series of dilutions, provided that the final dilution does not exceed the MAD value, which is calculated according to the following equation:

$$MAD = \frac{\text{maximum content of bacterial endotoxins} \cdot \text{concentration of the tested solution}}{\lambda}$$

where: "*maximum content of bacterial endotoxins*" is the admissible content of bacterial endotoxins in the tested medicinal product, as specified in the Pharmacopoeia Monograph;

"*concentration of the tested solution*" is the concentration of the medicinal product or active ingredient for which the maximum content of bacterial endotoxins is established;

λ is the sensitivity of the LAL reagent (EU/mL).

The following equation is used to calculate the maximum content of bacterial endotoxins:

$$\text{maximum content of bacterial endotoxins} = \frac{K}{M},$$

where: K - is the threshold pyrogenic dose equal to 5 EU/kg per hour for the tested medicinal product (if the latter is administered to patients through any parenteral route, except for the intrathecal route). If the drug is administered through the intrathecal route, the K value is 0.2 EU/kg;

M - maximum therapeutic dose of the tested medicinal product administered over a period of one hour (expressed in mg, mL, or units per kg of body weight).

For radiopharmaceutical medicinal products administered intravenously, the maximum content of bacterial endotoxins is calculated as 175 / V, where V is the maximum recommended dose (mL). For radiopharmaceutical medicinal products administered intrathecally, the maximum content of bacterial endotoxins is calculated as 14 / V.

If doses of the medicinal product are expressed per square metre of body surface area (such as antineoplastic drugs), the threshold pyrogenic dose (K) is set at 100 EU/$m^2$.

**Gel clot assay (Methods A and B)**

The gel clot method permits detection or quantification of endotoxins in a sample. The reaction between the LAL reagent and the endotoxin results in an increase in the viscidity of the reaction mixture until a dense gel is formed.

To ensure accuracy and reliability of test results, the claimed sensitivity of the LAL reagent should be verified, and a test for the presence of interfering factors should be performed as described in the "*Preparatory testing*" section.

***Procedure description.*** Transfer equal volumes of the tested solution and the LAL-reagent (0.1 mL of each) into round-bottomed test tubes with a diameter of 10 mm. Mix carefully the reaction mixtures, and incubate at temperature 37 ± 1 °C over 60 ± 2 minutes. During incubation, vibration and mechanical shocks should be avoided. After the specified period of time, results are assessed by visual examination as positive or negative. A positive reaction (+) is characterized by the formation of a dense gel, which is not destroyed by a single careful 180° turn of the test tube. A negative reaction (-) is characterized by the absence of such gel.

## PREPARATORY TESTING

### Confirmation of the claimed LAL-reagent sensitivity

This analysis is carried out for each new batch of the used LAL-reagent, as well as upon any changes in the experimental conditions, used materials and reagents that could have an effect on test results.

***Procedure description.*** For this test, solutions C and D are prepared in accordance with the requirements of Table 1.

Table 1 – **Experiment design, "Confirmation of the claimed LAL-reagent sensitivity"**

| Solution | Original solution | Diluent | Dilution factor | Final ERS concentration in the tested solution | Number of replicates |
|---|---|---|---|---|---|
| *C* | ERS solution in Water for LAL-testing with the 2λ ERS concentration | Water for LAL-testing | 1<br>2<br>4<br>8 | 2λ<br>1λ<br>0,5 λ<br>0,25 λ | 4<br>4<br>4<br>4 |
| *D* | Water for LAL-testing | – | – | – | 2 |

The *Solutions C* series consists of ERS dilutions in Water for LAL-testing (LAL-reagent sensitivity verification);

*Solution D* – Water for LAL-testing (negative control).

The test is carried out as described in the "Procedure description" section.

***Results and interpretation*** An analysis is considered valid if the following conditions are fulfilled:

– for *Solution D* (negative control) - negative results are obtained in all test replicates;

– for *Solution C* with the 2λ concentration – positive results are obtained;

– for *Solution C* with the 0.25λ concentration – negative results are obtained.

The reaction end-point for each replicate of the *Solutions C* series is a positive result obtained for the solution with the lowest ERS concentration. These results are used to calculate the geometric mean value of LAL-reagent sensitivity; the calculation is performed according to the following equation:

$$\text{Geometric mean of ERS concentrations at reaction end-point} = \textit{antilog}\left(\frac{\sum e}{f}\right),$$

where: $\sum e$ is the sum of logarithms of the ERS concentrations at reaction end-points in each of the replicates;

$f$ is the number of replicates.

The claimed LAL-reagent sensitivity is considered validated and can be used in subsequent calculation, provided that the LAL-reagent sensitivity value obtained in the test is not below 0.5λ and does not exceed 2λ.

***Interfering factors***

The tested medicinal product may contain interfering factors intensifying and / or inhibiting the reaction of the LAL-reagent with the bacterial endotoxins. These events may be recognized through comparison of the ability of the used LAL-reagent to react with the ERS solution in Water for LAL-testing and in the

solution of the tested medicinal product under the standard experimental conditions.

A medicinal product may be tested in any dilution not exceeding the MAD value. The samples of the tested medicinal product (or its dilution) used in this analysis should not contain bacterial endotoxins in quantities detectable by the test.

***Procedure description***. For this analysis, Solutions A – D are prepared according to the requirements included in Table 2.

*Solution A* – the tested medicinal product in the selected dilution (control for the absence of bacterial endotoxins).

*Solutions B* – the series of ERS dilutions in the solution of the tested medicinal product (test detecting the possibility of reaction inhibition or intensification).

*Solutions C* – the series of ERS dilutions in Water for LAL-testing (positive control).

*Solution D* – Water for LAL-testing (negative control).

Table 2 – **Experiment design, "Interfering factors"**

| Solution | Original solution | Diluent | Dilution factor | Final endotoxin concentration in the tested solution | Number of replicates |
|---|---|---|---|---|---|
| A | Tested medicinal product | - | – | – | 4 |
| B | Tested medicinal product containing ERS at the 2λ concentration | Tested medicinal product | 1<br>2<br>4<br>8 | 2 λ<br>1 λ<br>0.5 λ<br>025 λ | 4<br>4<br>4<br>4 |
| C | ERS solution in Water for LAL-testing with the 2λ ERS concentration | Water for LAL-testing | 1<br>2<br>4<br>8 | 2 λ<br>1 λ<br>0.5 λ<br>025 λ | 2<br>2<br>2<br>2 |
| D | Water for LAL-testing | - | – | – | 2 |

This test should be carried out as described in the "*Procedure description*" section.

***Results and interpretation.*** A test may be considered reliable if the following conditions are fulfilled:

- for *Solutions A and D* - negative results are obtained in all replicates;
- for *Solutions C* (positive control) – the geometric mean value of the bacterial endotoxins concentration should be not less than 0.5λ and not more than 2λ.

Results obtained in each replicate of the *Solutions B* series are employed to calculate the geometric mean value of LAL-reagent sensitivity. The calculation is carried out as described in the "*Confirmation of the claimed LAL-reagent sensitivity*" section. If the obtained mean sensitivity value is not less than 0.5λ and not more than 2λ, this means that the tested medicinal product in the particular dilution does not contain any interfering factors capable of inhibition and / or intensification of the reaction of the LAL-reagent with bacterial endotoxins, and it therefore may be analyzed for bacterial endotoxins content.

If a presence of interfering factors has been demonstrated for the tested medicinal product analyzed in a dilution lower than the MAD, the test should be repeated for a higher dilution, up to the dilution equal to the MAD. In most cases, additional dilution of the tested medicinal product is capable of eliminating the effects of interfering factors. The use of a LAL-reagent with a higher sensitivity will allow to increase the degree of dilution.

The effects of interfering factors can be overcome with appropriate sample preparation, such as filtration, neutralization, dialysis, or temperature processing. The method selected to remove interfering factors should not change the concentration of bacterial endotoxins in the tested medicinal product, and therefore the ERS with the known concentration is added to the solution of the tested medicinal product before such processing, while the Interfering Factors analysis is carried out afterwards. If the selected method of processing is associated with satisfactory results of the Interfering Factors test, the tested medicinal product may be analyzed for bacterial endotoxins content.

If the interfering factors cannot be removed from the tested medicinal product, the latter cannot be tested for bacterial endotoxins content using the LAL-test.

QUALITATIVE ANALYSIS (Method A)

The objective of this analysis is to demonstrate that the content of bacterial endotoxins in the tested medicinal product sample does not exceed the Maximum Content of Bacterial Endotoxins specified in the Pharmacopoeia Monograph.

***Procedure description.*** For this analysis, *Solutions A – D* should be prepared according to the requirements presented in Table 3.

*Solution A* – the tested medicinal product in the dilution containing no interfering factors, or in a higher dilution, provided that it does not exceed the MAD value.

*Solution B* – the tested medicinal product in the selected dilution, with the Endotoxin Reference Standard added. The final endotoxin concentration in the analyzed solution should be 2λ (positive control of the tested medicinal product).

*Solution C* – the ERS solution in Water for LAL-testing with final concentration 2λ (positive control).

*Solution D* – Water for LAL-testing (negative control).

This analysis is carried out as described in the "Procedure description" section.

Table 3 - **Experiment design, "Qualitative analysis"**

| **Solution** | **Baseline solution** | **Final endotoxin concentration (ERS) in the tested solution** | **Number of replicates** |
|---|---|---|---|
| A | Tested medicinal product | – | 2 |
| B | Tested medicinal product containing ERS at the 2λ concentration | 2 λ | 2 |
| C | ERS solution in Water for LAL-testing with the 2λ ERS concentration | 2 λ | 2 |
| D | Water for LAL-testing | – | 2 |

***Results and interpretation***. A test should be considered reliable if the following conditions are fulfilled:

- for *Solution D* (negative control) – negative results are obtained in both replicates;
- for *Solution C* (positive control) – positive results are obtained in all replicates;
- for *Solution B* (positive control of the tested sample) - positive results are obtained in both replicates.

If negative results are obtained for *Solution A* in both replicates, the medicinal product complies with the requirements of the test.

If a positive result is obtained for both replicates for the tested medicinal product's dilution less than the MAD, the test should be repeated for a higher dilution or the dilution equal to the MAD.

If a positive result is obtained for both replicates for the tested medicinal product's dilution equal to the MAD, such medicinal product does not comply with the requirements of the Bacterial Endotoxins Section of the Pharmacopoeia Monograph.

If a positive result is obtained for one of the replicates for *Solution A*, a repeat test should be carried out. If negative results are obtained for both replicates in the second test, such medicinal product has passed the test.

QUANTITATIVE ANALYSIS (Method B)

This method serves to determine the content of bacterial endotoxins using a series of successive dilutions of the tested medicinal product.

***Procedure description.*** For this analysis, *Solutions A – D* should be prepared according to the requirements presented in Table 4.

*Solutions A* – the dilutions of the tested medicinal product, beginning from the dilution containing no interfering factors to the highest dilution not exceeding the MAD value.

*Solution B* – the lowest dilution of the Solution A serial dilutions to which the ERS solution was added. The final endotoxin concentration in the analyzed solution should be 2λ (positive control of the tested sample).

*Solutions C* – the series of ERS dilutions in Water for LAL-testing (positive control).

*Solution D* – Water for LAL-testing (negative control).

This analysis is carried out as described in the "Procedure description" section.

Table 4 **- Experiment design, "Quantitative analysis"**

| **Solution** | **Original solution** | **Diluent** | **Dilution factor** | **Final ERS concentration in the tested solution** | **Number of replicates** |
|---|---|---|---|---|---|
| A | Tested medicinal product | Water for LAL-testing | 1<br>2<br>4<br>8<br>and so forth to MAD | —<br>—<br>—<br>— | 2<br>2<br>2<br>2 |
| B | Tested medicinal product containing ERS at the 2λ concentration | Tested medicinal product | 1 | 2 λ | 2 |
| C | ERS solution in Water for LAL-testing with the 2λ ERS concentration | Water for LAL-testing | 1<br>2<br>4<br>8 | 2 λ<br>1 λ<br>0.5 λ<br>0.25 λ | 2<br>2<br>2<br>2 |
| D | Water for LAL-testing | - | - | — | 2 |

***Results and interpretation.*** A test should be considered reliable if the following conditions are fulfilled:

- for *Solution D* (negative control) – negative results are obtained in both replicates;

- for the *Solutions C* series (positive control) - the geometric mean value of the bacterial endotoxins concentration should be not less than 0.5λ and not more than 2λ;
- for *Solution B* (positive control of the tested sample) – positive results are obtained in two replicates;
- for the *Solutions A* series – the reaction end-point is a positive result obtained for the highest dilution of the tested medicinal product.

The respective dilution factor multiplied by the LAL-reagent sensitivity value (λ) is equal to the endotoxin concentration in *Solution A* obtained for this particular replicate.

The geometric mean value of the endotoxin concentration is calculated as described in the "*Confirmation of the claimed LAL-reagent sensitivity*" section.

If negative results are obtained for all replicates of the *Solutions A* series, the bacterial endotoxins concentration in the tested medicinal product is below the LAL-reagent sensitivity value multiplied by the lowest dilution factor. If positive results are obtained for all replicates of the *Solutions A* series, the bacterial endotoxins concentration in the tested medicinal product is above the LAL-reagent sensitivity value multiplied by the highest dilution factor.

A medicinal product has passed the test if the mean bacterial endotoxins content value produced by the test is below the Maximum Content of Bacterial Endotoxins value specified in the Pharmacopoeia Monograph.

## PHOTOMETRIC METHODS (Methods C, D, E, and F)

### TURBIDIMETRIC METHODS (C and F)

Turbidimetric methods are a variant of photometric methods based on measurement of the turbidity of the reaction mixture. Depending on the principle underlying the test, this method may be conducted either as an endpoint turbidimetric test or a kinetic turbidimetric assay.

The endpoint turbidimetric test (Method F) is based on measurement of the turbidity of the reaction mixture at the end of the incubation period, which depends on the endotoxin concentration.

The kinetic turbidimetric assay (Method C) is based on determination of the turbidity development rate of the reaction mixture evaluated by the time required to achieve a target optical density value.

This test is conducted at the incubation temperature recommended by the manufacturer of the LAL reagent (usually 37 ± 1 °C).

## CHROMOGENIC METHODS (D and E)

Chromogenic methods are used to determine the amount of the chromophore released from a chromogenic substrate as a result of the reaction between endotoxins and the LAL reagent. Depending on the principle underlying the test, this method may be conducted either as an endpoint chromogenic test or a kinetic chromogenic assay.

The endpoint chromogenic test (Method E) is based on measurement of the colour intensity of the reaction mixture, which depends on the amount of the chromophore released at the end of the incubation period. The amount of the released chromophore depends on the endotoxin concentration.

During the kinetic chromogenic assay (Method D), the rate at which the colour of the reaction mixture develops is determined; it is evaluated by the time required to achieve a target reaction mixture optical density value.

This test is conducted at the incubation temperature recommended by the manufacturer of the LAL reagent (usually 37 ± 1 °C).

### PREPARATORY TESTING

To demonstrate the accuracy and reliability of test results obtained with the turbidimetric or chromogenic method, preliminary tests should be carried out to make sure that the standard curve criteria are reliable and that the tested solution contains no factors interfering with the course of the reaction.

Any changes that can have an effect on results of this experiment require additional confirmation of the reliability and accuracy of this test.

***Standard curve criteria reliability check***

This analysis should be carried out for each new LAL reagent batch.

To obtain a standard curve, not less than three different concentrations of the endotoxin should be prepared from the stock ERS solution in accordance with the recommendations of the LAL reagent's manufacturer. The test should be performed with at least replicates, under the conditions recommended by the manufacturer of the LAL reagent (volume ratios, incubation time, temperature, pH value, etc.).

If the procedure of a kinetic method necessitates a standard curve with an ERS range exceeding 2 lg of the endotoxin concentration value for each measurement range change of an endotoxin concentration value lg, an ERS solution of the appropriate concentration should be included in the design of this experiment.

The absolute correlation coefficient |r| value for the examined endotoxin concentration range should be equal to or more than 0.980.

***Interfering factors***

The test may be performed on any medicinal product in any dilution not exceeding the MAD value.

***Procedure description.*** Solutions A – D should be prepared as specified in Table 5. Solutions A, B, C, and D should be tested on at least two replicates, in accordance with the recommendations of the LAL reagent's manufacturer (volumes and volume ratios of the tested medicinal product and the LAL reagent, incubation time, temperature, pH value, etc.).

Table 5 - **Experiment design, "Interfering factors"**

| Solution | Endotoxin concentration | Solution mixed with endotoxin | Number of replicates |
|---|---|---|---|
| A | – | Tested solution | Not less than 2 |
| B | Mean standard curve concentration | Tested solution | Not less than 2 |
| C | Not less than 3 concentrations (the lowest concentration is denominated λ) | Water for LAL testing | Not less than 2 for each of the concentrations |

| | | | |
|---|---|---|---|
| D | – | Water for LAL testing | Not less than 2 |

*Solution A* — the solution of the tested medicinal product in a dilution not exceeding the MAD value;

*Solution B* — the tested medicinal product in the selected dilution upon addition of the ERS. The final endotoxin concentration of the analyzed solution should be equal or close to the mean value for the ERS concentrations used to plot the standard curve (positive control of the tested sample);

*Solutions C* — the ERS solutions used to plot the standard curve, at the same concentrations that were used during the «*Standard curve criteria reliability check*» (positive control);

Solution D — Water for LAL testing (negative control).

Test results are considered reliable if the following conditions are fulfilled:

– results obtained for the standard curve (Solution C) meet the reliability criteria established for the «Standard curve criteria reliability check» section;

– the result obtained for Solution D (negative control) does not exceed the value specified in the Instructions for Use of the LAL reagent used or is lower than the endotoxin concentration detected by the method used.

The experimental mean concentration of the added endotoxin is calculated by subtracting the mean endotoxin concentration of *Solution A* (if available) from the mean endotoxin concentration of *Solution B* (containing the added endotoxin).

The tested solution is considered to be demonstrated to contain no interfering factors if the measured concentration of the endotoxin added to the tested solution is 50 % to 200 % of the known concentration of the added endotoxin under the test conditions.

If the endotoxin concentration determined in the experiment lies outside of the established limits, a conclusion is made that the tested medicinal product contains factors interfering with the reaction. In this case, the test may be repeated at a higher dilution, up to the dilution equal to the MAD. Apart from higher dilutions of the tested medicinal product, the effects of interfering factors may be

overcome by appropriate processing, such as filtration, neutralization, dialysis, or temperature processing. The method chosen to eliminate interfering factors should not decrease the concentration of bacterial endotoxins in the tested medicinal product, therefore the ERS solution of the known concentration should first be added to the tested solution before such processing, and afterwards the «Interfering factors» analysis should be repeated. If test results obtained after the selected type of processing are deemed satisfactory, the tested medicinal product may be analyzed for the content of bacterial endotoxins.

**Test procedure**

***Procedure description.*** The test should be carried out in accordance with the procedure description included in the «Interfering factors» section.

***Results.*** The endotoxin concentration should be determined for each replicate of *Solution A* using the standard curve obtained using the ERS serial dilutions (*Solution C*).

Test results are considered reliable if the following conditions are fulfilled:

1. results obtained for the standard curve (*Solutions C*) meet the reliability criteria established for the «Standard curve criteria reliability check» section;
2. the experimental concentration of the endotoxin added to *Solution B* after subtracting the endotoxin concentration value found for *Solution A* lies in the range of 50 % to 200 % of the known value;
3. the result obtained for *Solution D* (negative control) does not exceed the value specified in the Instructions for Use of the LAL reagent used or is lower than the endotoxin concentration detected by the method used.

***Interpretation of results.*** A medicinal product passes the test if the experimental mean content of bacterial endotoxins found for the *Solution A* replicates (adjusted for the dilution and concentration of the tested medicinal product) is lower than the bacterial endotoxins content upper limit specified in the Pharmacopoeia Monograph.