BACTERIAL ENDOTOXIN DETECTION IN PHARMACEUTICAL PREPARATIONS: COMPARATIVE PRESENTATION OF PYROGEN TEST ON RABBITS AND LAL-TEST

M.B. Arambašić

"ICN-GALENIKA" - Institute, Quality Control Center, Biological Laboratory, 29. Novembra 111, YU-11.000 Beograd, Yugoslavia.
Tel. ++ 381 11 751 726. Fax. ++ 381 11 752 463.

Key words: bacterial endotoxins, pharmaceutical preparations, pyrogen test on rabbits, LAL-test

Bacterial endotoxins are, by chemical nature, lipopolysaccharides which are present in Gram-negative bacteria. During lifetime, humans are exposed to minimal amounts of endotoxins which are released in the gastro-intestinal tract after bacteria are killed (mostly Escherichia coli Bergey). This small amount of endotoxins induces in the body the formation of moderate amount of antibodies to endotoxins. However, in case of gastro-intestinal crises (i.e. the obstruction of intestines with the occurrence of gangrene and rapid increase of the number of bacteria), a large amount of endotoxins can penetrate into blood inducing anaphylactic reaction resulting in severe shock (endotoxin shock) [1].

Due to the effect that bacterial endotoxins may have on human health, according to pharmacopoeic requirements, one of the conditions for release of drugs for parenteral use is their testing for bacterial endotoxins. This paper presents two methods for bacterial endotoxin detection in pharmaceutical preparations (raw materials, final product and contact package):

1) The in vivo test for pyrogens on rabbits (pyrogen test on rabbit)
   and
2) The in vitro detection of pyrogens using Limulus amoebocyte lysate test (LAL-test).

1) PYROGEN TEST ON RABBITS. This is a classical test for determination of bacterial endotoxin presence based on the fact that bacterial endotoxins cause an increase in body temperature of rabbits above the range of physiological body temperature variance. The rabbit (Oryctolagus cuniculus L.) is selected for the test organism for determining the presence of pyrogenic substances because of its thermo-regulation centre sensitivity which responds to minimal presence of bacterial endotoxin. The presence of bacterial endotoxins in rabbits, similarly to humans, induces fever and high body temperature within the first hours after their introduction into the organism. The reaction is qualitative, "all or nothing" type. The requirements and the general procedure for pyrogen test are prescribed by national pharmacopoeias, i.e. American [2,3], British [4], European [5] etc. World accepted pharmacopoeias are only mentioned. Other countries apply the criteria of some of these pharmacopoeias, or of their modifications (Czech Pharmacopoeia [6], Yugoslav Pharmacopoeia [7]).

Which pharmacopoeias will be used for pyrogen test on rabbits is a major concern, since there are differences and similarities for py-
rogen test procedure among pharmacopoeias.

The common issue for all pharmacopoeias is that the test is performed on a group of 3 Chinchilla rabbits. The tested preparation (in the form of: solution, or autoclaved material, or supernatant, or eluent in redistilled water, or isotonic physiological saline - 0.9% w/v sodium chloride in redistilled water) is injected intravenously in a specified dose (mg/ml/kg bodyweight, or IU/ml/kg bodyweight, or surface/ml/kg bodyweight). The dose is specified by a pharmacopoeial requirement (pharmacopoeic monograph for given preparations), or, if there is no pharmacopoeic requirement, so that the pyrogen test is performed according to internal specification, it is determined based on therapeutic doses and represents the dose (maximal therapeutic dose/kg bodyweight of man or animal (if veterinary preparations are concerned) which does not induce pyrogen response in normal conditions.

Differences among pharmacopoeias exist in criteria and interpretation of results and are reflected in the following: if, after the pyrogen test is performed on a group of 3 rabbits, the response is positive (tested preparation contains pyrogenic substances), the test should be repeated for the safety of results.

A) On groups of 3 rabbits to the total of 12 rabbits. Body temperature should be taken at intervals not longer than 30 minutes during 3 hours after injection and the reported temperature values added [4, 5] (Table 1).

<table>
<thead>
<tr>
<th>Number of rabbits</th>
<th>The preparation passes the test if the sum of responses does not exceed</th>
<th>The preparation does not pass the test if the sum of responses exceeds</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>1.15°C</td>
<td>2.65°C</td>
</tr>
<tr>
<td>6</td>
<td>2.80°C</td>
<td>4.30°C</td>
</tr>
<tr>
<td>9</td>
<td>4.45°C</td>
<td>5.95°C</td>
</tr>
<tr>
<td>12</td>
<td>6.60°C</td>
<td>6.60°C</td>
</tr>
</tbody>
</table>

B) On a group of 5 rabbits. Body temperature is taken at 60-minute intervals, at 1st, 2nd and 3rd hour after injection [2], i.e. body temperature of an animal is taken at 30-minute intervals during 3 hours after injection starting from the 1st hour [3], i.e. body temperature is taken at 45-minute intervals during 3 hours after injection starting from 45th minute [6,7]. The recorded temperature values are added (total 8 rabbits) (Table 2, N = number of rabbits).

<table>
<thead>
<tr>
<th>Pharmacopoeia</th>
<th>The preparation passes the test if individual response does not exceed</th>
<th>The preparation passes the test if the sum of responses does not exceed</th>
<th>The preparation passes the test if the sum of responses does not exceed</th>
</tr>
</thead>
<tbody>
<tr>
<td>USP XXII [2]</td>
<td>0.6°C (N=3)</td>
<td>1.4°C (N=3)</td>
<td>3.7°C (N=8)</td>
</tr>
<tr>
<td>USP XXIII [3]</td>
<td>0.5°C (N=3)</td>
<td>1.4°C (N=3)</td>
<td>3.3°C (N=8)</td>
</tr>
<tr>
<td>Ph.Bs. IV [6]</td>
<td>0.6°C (N=3)</td>
<td>1.4°C (N=3)</td>
<td>3.7°C (N=8)</td>
</tr>
<tr>
<td>Ph.Jug. IV [7]</td>
<td>0.6°C (N=3)</td>
<td>1.4°C (N=3)</td>
<td>3.7°C (N=8)</td>
</tr>
</tbody>
</table>

The result of the pyrogen test is a qualitative data on
whether the tested preparation contains pyrogenic substances ("pyrogenic" or "contains pyrogenic substances") or does not contain pyrogenic substances ("nonpyrogenic" or "does not contain pyrogenic substances" or "meets pharmacopoeic requirements").

2) LAL-TEST. This was once an alternative test for detection of bacterial endotoxins, however, recently, it replaces more and more the pyrogen test on rabbits, since it is at least 5 to 10 times more sensitive than pyrogen test on rabbits. LAL-test is based on the fact that the presence of bacterial endotoxins causes the coagulation of Limulus amebocyte lysate (LAL). The test is quantitative in character, because it can determine the amount of endotoxin in the tested sample. The substrate for LAL test is lysate obtained by processing of horse-shoe crab ameobocyte (Limulus polyphemus L.). Hemolymph of this animal species is characterized by that it has only one type of cells - amebocytes. Those cells are very sensitive and contain procoagulant protein. In case of Gram-negative bacteria penetration into the circulation of the horse-shoe crab, intravascular coagulation occurs and the animal dies. This fact was used to develop in vitro methods for detecting bacterial endotoxins [8]:

A) Method of solid coagulum formation (gel-clot method). In the presence of bacterial endotoxins in the tested preparation, depending on the sensitivity of applied lysate, coagulum is formed.

B) Method of substrate colour change (hromogenous method). In the presence of bacterial endotoxin in the tested preparation, depending on the sensitivity of applied lysate, substrate changes its colour. The intensity of the colour change can be quantified spectrophotometrically at 405 nm.

C) Method of substrate optical density change (kinetical-turbidimetric method). In the presence of bacterial endotoxins in the tested preparation, depending on the sensitivity of applied lysate, substrate changes its optical density. The intensity of optical density change can be quantified spectrophotometrically.

The requirement and procedure for bacterial endotoxin detection test are prescribed by national pharmacopoeia, i.e. American [2, 3], British [4], European [9]. American pharmacopoeia proposed gel-clot method for the official method of bacterial endotoxin detection, suggesting hromogenous and kinetal-turbidimetric methods as alternatives. On the contrary, British pharmacopoeia and European pharmacopoeia propose all three mentioned methods as the methods for bacterial endotoxin detection.

Pharmacopoeic requirement (monography for individual preparations) gives maximum amount of bacterial endotoxin (in EU/mg or EU/ml) allowed to be contained in the tested preparation. The maximum allowed amount of bacterial endotoxin in the tested preparation is the amount of bacterial endotoxins which will not cause pyrogenic response. This amount is determined by a formula K/M where K is upper limit of bacterial endotoxin content for parenteral preparations (5 EU/kg/dose). The upper limit for intratecally applied preparations is 0.2 EU/kg/dose. M in the formula K/M means maximum therapeutic dose/kg body-weight of a man or animal (if veterinary preparations are concerned).

The amount of 5 EU/kg/dose (parenteral), i.e. 0.2 EU/kg/dose (intratecal) represent the least amount of RSE (reference standard endotoxin - purified bacterial endotoxin) which induce pyrogenic response in man and rabbit. The difference between the upper limits of endo-
toxin, which induces pyrogenic response in man and animal after parenteral and intrathecal use, is conditioned by that 25 times lower amount of RSE, given intrathecally, causes pyrogenic response in relation to RSE amount which should be given intravenously to induce pyrogenic response [10].

As the result of bacterial endotoxin test, a quantitative information is obtained in terms of how much bacterial endotoxin the tested preparation contains (in EU/mg or EU/ml).

Bacterial endotoxin test can be used for the control of depirogenization process (sterilization), since the upper limit of bacterial endotoxin content in the eluent from surgical instruments is 0.5 EU/ml. For instrument in contact with cerebrospinal liquid (liquor), the upper limit of bacterial endotoxin content is 0.06 EU/ml [11]. Bacterial endotoxin test can be used for the control (validation) of dry sterilizer operation.

ACKNOWLEDGEMENT:
The author thank Mrs. Zlatica Milutinović for translating the paper into English.

LITERATURE: