COMPARATIVE EVALUATION OF PYROGENS TESTS IN PHARMACEUTICAL PRODUCTS

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ABSTRACT

A comparison of methodologies for detection of pyrogens in pharmaceutical products was performed. The rabbit pyrogen test was optimized and the dose-response curve was obtained for the 2nd International Standard for bacterial endotoxins, establishing 13.81 EU/mL/kg as the concentration of endotoxin necessary to induce a temperature rise of 0.5°C. The 0.5°C cut-off was shown to give results that were more compatible with the pyrogenic doses for humans. The Limulus amoebocyte lysate test (LAL) was standardized with gel-clot and chromogenic endpoints, and used for the comparative evaluation of pharmaceutical products showing good agreement. The use of β-glucan-reactive and non-reactive LAL reagents identified some products with false-positive results. The interference test was carried out and the specifications validated for some new products as the maximum valid dilution. The results emphasized the importance and limitations of the assays recommended for the evaluation of purity and quality control of parenteral medicinal products, improving the existing methodologies in the context of reduction and replacement in the use of animal models.

Key words: bacterial endotoxin, Limulus amoebocyte lysate, pyrogens, rabbit pyrogen test

INTRODUCTION

Pharmaceutical products intended for parenteral use must be free of pyrogens, which can originate from Gram-negative or Gram-positive bacteria, viruses and fungi. Endotoxins (Lipopolysaccharides, LPS) from Gram-negative bacteria are commonly found in parenteral pharmaceuticals and medical devices and are of particular concern to the pharmaceutical industry. Endotoxins are large molecular weight complexes (~10^6 Da) associated with, and shed from, the outer membranes of Gram-negative bacteria (8,14). Endotoxins consist of three distinct chemical regions: a lipid moiety (lipid A) which is linked to a polysaccharide core that is, in turn, linked to O-antigenic side-chains (8,18). Each endotoxin presents a composition and a variable structure that affects its function and biological activity; endotoxin functions include the induction of fever and acute phase proteins, headache and severe hypotensive shock.

There is good evidence that the fever response to various exogenous pyrogens (e.g. endotoxin) is mediated by endogenous pyrogens, i.e. pyrogens generated by the host. Endogenous pyrogens have potent pyrogenic and inflammatory activities and include interleukin-1α (IL-1α), interleukin-1β (IL-1β), tumor necrosis factor α (TNF-α) and interleukin-6 (IL-6) (10,21).

The rabbit pyrogen test based on the intravenous injection of a sterile solution was adopted for many years for the quality control of parenteral preparations (14). Alternatives for the refinement of the test, including the comparison of rabbit responses to two E. coli endotoxin preparations, suggested that the temperature cut-off of 0.6°C should be decreased to 0.5°C, as the criterion for a positive result (9,20). However, the rabbit pyrogen test has several drawbacks including low sensitivity, absence of quantitation, unsuitability for some product categories and the involvement of animals (1,6).

The observation that the blood (haemolymph) of the horseshoe crab became clotted in the presence of the bacterial endotoxins gave rise to the Limulus amoebocyte lysate test (LAL), which is specific and sensitive for endotoxins from Gram-
negative bacteria (11,20). However, the test can give false-negative and false-positive results, and being a test for Gram-negative LPS, it does not detect Gram-positive exotoxins, viruses and fungi (7,13,16). In the LAL test, the LPS-induced reaction can be measured using various approaches, which were adopted together with the rabbit pyrogen test in the collaborative studies that established the 1st and 2nd International Standard for bacterial endotoxins (15,17). However, despite the specification of the LAL test for pharmaceutical products, there remain a number of complex preparations, such as biologicals, without specifications or that cannot be tested by the LAL test (6).

In the context of the development of alternatives for the refinement, replacement and reduction of biological tests, the LAL test and the rabbit pyrogen test are also used for the validation of novel in vitro assays under investigation, based on the use of cell lines, peripheral blood monocytes and whole blood (5,7,13,16).

The aims of the present study were to validate the specifications for the bacterial endotoxins test in parenteral pharmaceutical products, to refine the rabbit pyrogen test, to evaluate the responses of different LAL reagents, and to correlate the results of the two methodologies, thus contributing to the quality control of medicines.

**MATERIALS AND METHODS**

**Reference standard and reagents**

The 2nd International Standard for bacterial endotoxins, 10,000 EU/vial (WHO 94/580), was kindly donated by the National Institute for Biological Standards and Control (NIBSC), Herts, UK. *Limulus* amoebocyte lysate, 0.06 EU/mL was obtained from Endosafe (Charleston; SC, USA), Biowhittaker (Walkersville; MD, USA) and Cape Code (Cape Code; MA, USA). New methylene blue was purchased from Sigma (St. Louis; MO, USA). A number of parenteral pharmaceutical products were used, in some cases different batches of the same product, all within their period of validity. Other reagents and plasticware were purchased as sterile and pyrogen-free and glassware was baked at 250°C for 1 h prior to use.

**Rabbit pyrogen test**

This test was carried out according to the literature (3,20), using the New Zealand white rabbit strain.

**Limulus amoebocyte lysate test (LAL)**

The bacterial endotoxin limits for the parenteral pharmaceutical products without specifications were calculated as the maximum valid dilution (MVD) (2,20).

**Interfering factors test**

The inhibition/enhancement test was performed (2,20) on the sample solutions at a dilution less than the MVD, not containing any detectable endotoxins. The geometric mean endpoint concentration of the solutions was determined using the equation \( M = \exp \left( \frac{\sum \log e}{f} \right) \), where \( \sum \log e \) is the sum of the log endpoint concentrations of the dilution series used, and \( f \) is the number of replicate test tubes. If the sensitivity of the lysate determined in the presence of the sample solution under test is not less than 0.5 \( \lambda \) and not greater than 2 \( \lambda \), the sample solution does not contain factors which interfere under the experimental conditions.

**Gel-clot assay**

The assays were performed in Petri dishes (2,3,20), adjusting the volumes and maintaining constant the relationship between them. Equal volumes of lysate and test solution or standard (usually 10 \( \mu \)L) were added to Petri dishes. The reaction mixture was incubated at 37°C for 1 h. A number of two-fold serial dilutions were tested and the gel-clot endpoint was determined by adding 1 \( \mu \)L of a 0.2% new methylene blue solution, observing the mixing (negative reaction) or distribution on the surface of the gel (positive reaction). The endotoxin concentration was calculated by multiplying the reciprocal of the greatest dilution of the test solution that gave a positive endpoint by the sensitivity (to endotoxin) of the lysate preparation; the results were expressed in EU/mL.

**Chromogenic assay**

The assay was performed in a microplate at 37 ±1°C (2,20). Fifty \( \mu \)L of the standard or samples were dispensed into the appropriate microplate wells. Then, 50 \( \mu \)L of the *Limulus* amoebocyte lysate solution were added and the microplate was incubated for 10 min at 37 ±1°C. One hundred \( \mu \)L of the substrate solution were pipetted and the reaction was stopped after 6 minutes by adding 100 \( \mu \)L of 25% acetic acid. The absorbance was read at 405 nm in a microplate reader and a standard curve plotted. The results were expressed in EU/mL.

**RESULTS**

**Rabbit pyrogen test**

The dose-response curve of the 2nd International Standard for bacterial endotoxins was obtained by recording the rise in temperature at 15 minutes intervals for three hours. The regression line was calculated and the concentrations that produced a temperature rise of 0.5°C and 0.6°C were calculated as 13.81 EU/mL/kg and 18.57 EU/mL/kg, respectively (Fig. 1).

**Limulus amoebocyte lysate test (LAL), gel-clot**

The labeled reagent LAL sensitivity of 0.06 EU/mL was confirmed before carrying out the assays. The interfering factors test was performed for the products without specifications (Table 1), at dilutions not exceeding the MVD spiked with the 2nd International Standard, and the samples returned positive.
results, thereby enabling the determination of the minimum valid dilution of the sample free of interference and the endotoxin content in EU/mL.

The comparative results of LAL reagents using the gel-clot endpoint revealed interference with the β-glucan-reactive LAL reagent for ampicillin samples and erythropoietin C, which gave false-positive results (Table 2).

The LAL gel-clot test, more widely used as a qualitative or semi-quantitative test, was compared to the chromogenic, quantitative assay (Table 3) and produced comparable results for all samples with the exception of methylprednisolone, which gave a lower value for the chromogenic method.

Table 1. Inhibition/enhancement test of pharmaceutical products, by the Limulus amoebocyte lysate (LAL) assay, with a sensitivity of 0.06 EU/mL.

<table>
<thead>
<tr>
<th>Products</th>
<th>Endotoxins limit calculated</th>
<th>Maximum valid dilution</th>
<th>Sample test</th>
<th>Minimum valid dilution</th>
<th>Geometric mean EU/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ciprofloxacin 2 mg/mL</td>
<td>0.87 EU/mg</td>
<td>1:29</td>
<td>-</td>
<td>1:16</td>
<td>0.06</td>
</tr>
<tr>
<td>Ketoprofen 100 mg/2 mL</td>
<td>3.50 EU/mg</td>
<td>1:2917</td>
<td>-</td>
<td>1:32</td>
<td>0.03</td>
</tr>
<tr>
<td>Diclofenac 75 mg/3 mL</td>
<td>4.70 EU/mg</td>
<td>1:1958</td>
<td>-</td>
<td>1:64</td>
<td>0.03</td>
</tr>
<tr>
<td>Dipyrone 500 mg/mL</td>
<td>0.70 EU/mg</td>
<td>1:5833</td>
<td>-</td>
<td>1:10</td>
<td>0.03</td>
</tr>
<tr>
<td>Erythropoietin 2000 IU/vial</td>
<td>2.50 EU/2000 IU</td>
<td>1:42</td>
<td>-</td>
<td>1:2</td>
<td>0.03</td>
</tr>
<tr>
<td>recG-CSF 300 mcg/vial</td>
<td>2.00 EU/mL</td>
<td>1:33</td>
<td>-</td>
<td>1:8</td>
<td>0.06</td>
</tr>
<tr>
<td>Calcium folinate 50 mg/5 mL</td>
<td>0.60 EU/mg</td>
<td>1:100</td>
<td>-</td>
<td>1:1</td>
<td>0.04</td>
</tr>
<tr>
<td>Fluconazol 2 mg/mL</td>
<td>2.33 EU/mg</td>
<td>1:78</td>
<td>-</td>
<td>1:2</td>
<td>0.06</td>
</tr>
<tr>
<td>Mesna</td>
<td>0.87 EU/mg</td>
<td>1:1458</td>
<td>-</td>
<td>1:32</td>
<td>0.06</td>
</tr>
<tr>
<td>Midazolam 50 mg/10 mL</td>
<td>3.50 EU/mg</td>
<td>1:2917</td>
<td>-</td>
<td>1:64</td>
<td>0.06</td>
</tr>
<tr>
<td>Pantoprazol 40 mg/10 mL</td>
<td>8.75 EU/mg</td>
<td>1:5833</td>
<td>-</td>
<td>1:256</td>
<td>0.03</td>
</tr>
<tr>
<td>Tenoxicam 40 mg/mL</td>
<td>8.75 EU/mg</td>
<td>1:2917</td>
<td>-</td>
<td>1:640</td>
<td>0.03</td>
</tr>
</tbody>
</table>

(-) Negative response.

Table 2. Results of pharmaceutical products by the LAL gel-clot test with different reagents.

<table>
<thead>
<tr>
<th>Products</th>
<th>Endotoxin limit EU/mL</th>
<th>Reagent Limulus amoebocyte lysate (LAL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>I^p EU/mL</td>
</tr>
<tr>
<td>Ampicillin 1000 mg/5 mL</td>
<td>30</td>
<td>153.60 – 307.20</td>
</tr>
<tr>
<td>Ampicillin 1000 mg/5 mL</td>
<td>30</td>
<td>30.72 – 61.44</td>
</tr>
<tr>
<td>Insulin 100 U/mL</td>
<td>80</td>
<td>&lt;0.06</td>
</tr>
<tr>
<td>Erythropoietin 2000 IU/vial</td>
<td>2.50</td>
<td>491.50 – 983</td>
</tr>
<tr>
<td>Erythropoietin 4000 IU/vial</td>
<td>5</td>
<td>3.84 – 7.68</td>
</tr>
<tr>
<td>Gentamicin 80 mg/2 mL</td>
<td>68</td>
<td>&lt;0.06</td>
</tr>
<tr>
<td>Oxacillin 500 mg/5 mL</td>
<td>20</td>
<td>&lt;0.06</td>
</tr>
<tr>
<td>Heparin 5000 IU/mL</td>
<td>150</td>
<td>&lt;0.06</td>
</tr>
</tbody>
</table>

^p β-glucans-reactive; ^p β-glucans non-reactive.

The comparative evaluation of pyrogens by the qualitative rabbit pyrogen test and the semi-quantitative LAL test showed a contamination level that, in most cases, was lower than the sensitivity of the lysate used (Table 4); furthermore, there was good correlation within the two assays.

DISCUSSION

The rabbit pyrogen test was studied in the context of the importance of alternatives that could contribute towards its refinement. A dose of 13.81 EU/mL/kg of the 2nd International Standard for bacterial endotoxins was identified as that which
produced a 0.5°C rise in temperature (Fig. 1); this value, although variable according to the strain of the animals used, is recommended as a criterion for positive responses (9,17). Despite its shortcomings, the test is recommended by the Pharmacopoeias (2,3,20), and it is important for the validation of new in vitro assays under development (4,7,16).

The Limulus amoebocyte lysate test (LAL) is recommended (2,3,20) for the quality control of medicines, but the specifications need to be validated for new biological medicines, which are produced mainly through recombinant DNA technology (Table 1). The validation studies indicated geometric means between $\geq 0.5 \lambda$ and $\leq 2 \lambda$, with recovery between 50 and 200% (2,20). This test is important in order to eliminate false-negative and false-positive results (13,14,16) caused by the interference of the active substance or components of the final product formulation. Thus, the establishment and validation of the specifications for the quality control of the medicinal products tested is recommended.

The evaluation of pharmaceutical products using different LAL reagents (Table 2) showed that some batches of ampicillin and human recombinant erythropoietin, which gave positive results with the $\beta$-glucan-reactive reagents, gave negative results when analyzed by the $\beta$-glucan non-reactive reagent, thus demonstrating interference. These observations are important for quality control, assisting in the selection of the LAL reagent and the evaluation of the results obtained for the samples (12,19). It should be recalled that this difference between the reagents could result in the incorrect rejection of batches, mainly when the response levels are near to the maximum valid dilution specified for test compliance.

The comparative tests of pharmaceutical products using the LAL test with chromogenic and gel-clot endpoints gave similar results (Table 3). These experiments are important considering that the gel-clot method, being less expensive and easier to perform, has been used routinely for the quality control of medicines with limits declared as endotoxin units (2,3,20). In the present study, it was also used for comparison with the rabbit pyrogen test, showing good agreement (Table 4) and demonstrating the importance of both methodologies for the development and validation of new pyrogens tests.

Our results show that the rabbit pyrogen test, which is being gradually replaced by the LAL test,
Figure 1. Linear regression line calculated from three independent assays of the 2nd International Standard for Bacterial endotoxins. Concentrations necessary to produce a temperature rise of 0.5°C (...) and 0.6°C (—). \( Y = 0.21 + 0.021 \times \) (R\(^2\) = 0.92; \( p > 0.05 \) for n = 9).

continues to be valid and necessary. However, the LAL test validation and the specifications established for the new recombinant biologicals will contribute towards assuring the quality and safety of parenteral pharmaceutical products.

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RESUMO

Avaliação comparativa de testes de pirogênicos em produtos farmacêuticos

Realizou-se a comparação de metodologia para avaliação de pirogênicos em produtos farmacêuticos. Otimizou-se o teste da hipertermia em coelhos elaborando a curva dose-resposta com o 2º Padrão Internacional de endotoxinas bacterianas, com base na qual determinou-se a concentração de 13,81 UE/mL por kg de peso corporal, necessária para produzir aumento de temperatura de 0,5°C. Observou-se que o limite de 0,5°C forneceu resultados comparáveis com as doses pirogênicas para o homem. Padronizou-se o teste do lisado de amebócitos do Limulus (LAL) com determinação do ponto final cromogênico e por geleificação, que foram utilizados para a avaliação de produtos farmacêuticos obtendo-se resultados concordantes. Avaliaram-se as respostas de reagentes LAL reativos e não-reativos a \( \beta \)-glicanos, observando diferenças que poderiam reprová-los amostras com base em resultados falso-positivos. Executou-se o teste de interferências, validou-se o procedimento e estabeleceu-se a máxima diluição válida para produtos farmacêuticos sem especificações farmacopéicas. Os resultados enfatizam a importância e as limitações dos ensaios preconizados para avaliação da pureza e controle da qualidade de produtos farmacêuticos parenterais, contribuindo para aprimorar as metodologias existentes no contexto da redução e substituição dos modelos animais.

Palavras-chave: endotoxinas bacterianas, lisado do amebócito do Limulus, pirogênicos, hipertermia em coelhos

REFERENCES