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Endotoxin Contamination of Large Volume Parenterals as Detected by Limulus Amebocyte Lysate as an Alternative to Rabbit Pyrogen Test

Key words: LAL, Endotoxin

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Abstract

The possibility of replacing the rabbit pyrogen test by the *Limulus Amebocyte lysate* (LAL) test, as a final release test for Large Volume Parenterals (LVPs) was investigated. The sensitivity and specificity of the LAL test and rabbit pyrogen assay were studied by means of artificially contaminated parenterals. Various LVPs (mostly containing electrolytes) were spiked with 0.5 and 5 EU/ml of *E.coli 0111:B4* endotoxin (Lambda =0.5 EU/ml). The pH of the formulations were measured, and if necessary, adjusted to 5.5 – 8.0 by NaOH or HCl. Four of the products showed significant responses to the LAL test when the endotoxin concentration was 0.1 ng/ml. However, the remaining formulations required some degree of dilution prior to the test to overcome inhibition. The inhibitory effect caused by cations on the LAL reaction is enhanced by increases in valency. Therefore, the difference between the products in responding to the LAL test have resulted from shifts in the electrokinetic potentials between the LAL and the endotoxin. The rabbit test response was insignificant for all the solutions. The LAL test was found to be useful for the detection of bacterial endotoxin in LVPs. It also has the advantage of being more sensitive, rapid and reproducible than the rabbit pyrogen test.

Introduction

Avoiding pyrogen contamination of parenteral dosage forms, like the Large Volume Parenterals (LVPs), is very important in clinical medicine. By far, the most important pyrogenic contaminants are endotoxins, which are the outer cell wall fragments of gram-negative bacteria, chemically characterized as lipopolysaccharides (1,2). The gel-clot LAL test to detect these substances was introduced by Levin and Bang in 1968 (3).

Since then, numerous studies have shown that the *in vitro* LAL test is a viable alternative to the *in vivo* pyrogen test using rabbits in that the former is more sensitive, rapid and less costly (3 – 5). In 1980, the Food and Drug Administration (FDA) announced the availability of draft guidelines for the use of the LAL test for human and veterinary drugs (3). At the same time, the FDA began a human drug surveillance program in which aqueous and lyophilized parenteral drugs were examined for the presence of endotoxin (3). Recently, the LAL test has been widely used by the pharmaceutical manufacturer to monitor production processes and screen final products (1).

In the United States, the LAL test is adopted in the USP-NFXXI as a Bacterial Endotoxin Test (1). The distilled water for injection and some radioactive reagents for diagnostic use are tested according to this requirement (1).

In Europe, a group of experts of the European Pharmâcopoeia Commission is currently elaborating a monograph for a limit test, which will be based on a gel-clot technique similar to the USP method (6).

The inhibitory effects caused by the presence of electrolytes is being reported by other investigators (7). The purpose of this study was to assess the possibility of replacing the rabbit pyrogen test with the LAL assay, as a final release test for LVPs.

Results and Discussion

Specificity and sensitivity of pyrogen test with lysate and rabbits were tested with endotoxin of *E.coli 0111 : B4.*

Test solution	USP Total Temperature Increase (°C)	Mean Temperature Increase (°C)	Standard Deviation (°C)	Coefficient of Variation (%)
Water for injection	1.05	0.35	0.05	14.3
Ringer's solution[a]	1.25	0.417	0.058	13.9
Lactated ringer's[b]	1.30	0.433	0.104	24.0
Sodium lactate[c]	1.10	0.37	0.076	20.5
Saline-dextrose[d]	0.95	0.317	0.076	23.9
NaCl 0.9%	1.2	0.4	0.132	33.0
NaCl 23. 4%	1.15	0.383	0.035	9.1
KCl[e] 14.9%	0. 85	0.283	0.058	20.4
Dextrose 5%	0.80	0.267	0.076	28.5

[a]147. 5 mEq/L Na^+, 4.0 mEq/L K^+, 4.5 mEq/L Ca^{++}
[b]130 mEq/L Na^+, 4.0 mEq/L K^+, 3.0 mEq/L Ca^{++}.
[c]167 mEq/L Sodium lactate.
[d]0.45% NaCl, 2.5% dextrose.
[e]Diluted 1:50 with sodium chloride injection.

Table I. Three-rabbit test results using different test solution with an endotoxin concentration of 0.1 ng/ml (0.5 EU/ml) at a dose of 10 ml/kg

Both the rabbit test and the LAL assay can be used to detect endotoxin; however, only the LAL test can rapidly and more accurately measure the endotoxin levels. Table I summarizes the results of various parenterals that passed USP three-rabbit test at a dose of 10 ml/kg of 0.1 ng/ml endotoxin. These results are conistent with other findings that the *Threshold Pyrogenic Dose* (TPD) for both human and rabbit is equal and is approximately 1.0 ng/kg of body weight (9).

The Health Industry Manufacturers Association collaboration study showed that TPD for both humans and rabbits is 1.0 ng/kg of body weight (10). This value is in agreement with the Greisman and Hornick report which maintains that the threshold pyrogenic response for both humans and rabbits is 1.0 ng/kg of body weight for an *E. coli* endotoxin (10). Pearson and Weary reported that endotoxin preparations from *E.coli 055:B5* and *S. abortus* exhibited TPD's of slightly more than 1.0 ng/kg of body weight (11).

However, other investigators found a threshold pyrogenic dose of 2 ng/kg when *E.coli 055:B5* was used (12). Table II, and III indicate the LAL test results of different parenterals at endotoxin concentrations of 1 ng/ml and 0.1 ng/ml respectively. Solutions such as NaCl 0.9%, saline-dextrose, dextrose 5% and water for injection did not show any inhibition at these endotoxin levels. However, the remaining formulations required some degree of dilution prior to the test to overcome the inhibition. These results are in agreement with those obtained by other investigators (1,3).

Table III also demonstrates the inhibitory effect of electrolytes on endotoxin detection by LAL, even though the LAL reagent was capable of detecting less than 0.1 ng of endotoxin per ml. The inhibitory effect was not observed with NaCl 0.9% solution. However, when higher electrolyte concentrations are involved i.e., KCl 14.9%, and NaCl 23.4%, a strong inhibition is exhibited and interferes with the gelation reaction, which can be eliminated by dilution. Furthermore, divalent cations (Mg^{++}) demonstrated a much stronger inhibition than monovalent cations.

These results were consistent with findings of other investigators (7,13,14). Stronger inhibitory effects were revealed, when trivalent and tetravalent cations were exmained (Rafiee, Jamshidi, unpublished results). Sullivan and Watson stated that NaCl concentrations greater than 154 mEq/L decrease the sensitivity of the lysate (10). Baggerman and Kannegieter reported stronger inhibitory effects produced when divalent cations (Ca^{++}) were involved (7). These authors stated that electrolytes might change the physicochemical properties of endotoxins resulting in a decrease of activity. They also indicated that these physicochemical properties have been shown to be influenced predominantly by divalent cations, and that changes in aggregation-state are involved.

Baggerman and Junginger observed a strong inhibition effect of divalent cations in endotoxin removal from LVPs by various adsorbents (2). These investigators concluded that the adsorption is governed by electrokinetic forces, and increasing inhibition was observed when using cations with increasing valency. Since the inhibitory effect of cations on endotoxin detection by LAL increases with valency enhancement, these data as well as those cited in literature (1,2,3,7,10,14,15), strongly suggest that the electric double layer surrounding endotoxin is effected by gegenions present in the test solution. As the concentration

Test solution	Potency	Inhibition	pH Adjustment
Water for injection		No	No
Ringer's solution[a]		No	No
Lactated ringer's[b]		No	No
Sodium lactate	167 mEq/L	No	No
Saline-dextrose	0.45%, 2.5%	No	Yes
NaCl[c]	0.9%	No	Yes
NaCl[d]	23.4%	Yes	No
KCl[e]	14.9%	Yes	No
Dextrose	5%	No	Yes
Magnesium sulfate[f]	20%	Yes	Yes

[a]147.5 mEq/L Na^+, 4.0 mEq/L K^+, 4.5 mEq/L Ca^{++}.
[b]130 mEq/L Na^+, 4.0 mEq/L K^+, 3.0 mEq/L Ca^{++}.
[c]154 mEq/L Na^+.
[d]4000 mEq/L Na^+.
[e]2000 mEq/L K^+.
[f]1626 mEq/L Mg^{++}.

Table II. LAL test results using different parenteral solution with an endotoxin concentration of 1 ng/ml (5 EU/ml)

and valence of cations present (i.e., K^+, Na^+, Ca^{++}, Mg^{++}) in the system are increased, the zeta potential of the negatively charged endotoxin is also increased. As a result, these electrokinetic potential shifts may potentially inhibit the LAL reaction. Based on our results, it was concluded that the LAL assay is useful for the detection of bacterial endotoxin in LVPs. A comparison of the pyrogenisity assays also indicates that the LAL is more sensitive, reproducible, and rapid, than the conventional rabbit test.

Experimental

Materials: All materials were tested for endotoxin before use in the analysis. All glassware was depyrogenated by dry-heat sterilization.

Reagents: The following commercially available test solutions were used: sterile water for injection, dextrose injection (5%), sodium chloride injection (0.9%), potassium chloride injection (14.9%), sodium chloride injection (23.4%), ringer's injection, saline-dextrose injection, lactated ringer's injection, sodium lactate injection (0.167 M), and Mg sulfate injection (20%). Commercially available USP sterile water for injection was used as the diluent for the LAL test. Various concentrations of HCl (0.1 – 6 N), and NaOH (0.1 – 10 N) were used to adjust the product pH. The LAL reagent, PREGEL TEST (manufactured by Teikoku Hormone, Japan), and *Escherichia coli 0111 : B4* endotoxin (Teikoku Hormone, Japan) were employed, the sensitivity of endotoxin (Lambda) was 0. 5 EU/ml.

The endotoxin stock solution contained 1ng of endotoxin per ml of sterile water for injection.The stock solution was refrigerated at 0° to 4°C and was used within 2 weeks of reconstitution. From this stock solution, fresh samples containing 0.1, and 1 ng of endotoxin of each individual sterile test solution were prepared for use each day.

Sample Preparation: All sterile test solutions were spiked individually with *E. coli 0111: B4* endotoxin so that the final endotoxin concentrations were 0.5 and 5 EU/ml (0.1 and 1 ng per ml).

Rabbit Pyrogen Test: The pyrogenicity test was performed in accordance with USP procedure (8); three rabbits per sample were innoculated at a dose of 10 ml per kg of body weight. The test consists of measuring the rise in body temperature evoked in the rabbits by intravenous injection of the different sterile test solutions to be examined. All the tests were conducted in duplicate for each individual sample. Sterile water for injection (SWFI) was treated with 33.3 ml pyrogen-free sodium chloride 27% to make 1000 ml of isotonic solution. Sodium chloride injection (23.4%) was appropriately diluted with water for injection to make isotonic solution. Potassium chloride was appropriately diluted 1:50 with pyrogen-free normal saline before injection into the rabbit. The rabbit assay was interpreted in accordance with USP (8) as follows. The material is considered non-pyrogenic if no rabbit shows an individual rise in temperature of 0.6°C or more, and if the sum of the three individual maximum temperature rises does not exceed 1.4°C. To perform the LAL test, the pH of all of the test solutions were measured and adjusted when necessary with pyrogen-free HCl or NaOH to pH 5.5 to 8.0. In accordance with USP procedure (8), rabbit pyrogen test is not required for detection of pyrogenicity in Mg sulfate solution (20%).

LAL Assay: For the LAL assay, the manufacturer's procedure was used. For this method, each LAL ampoule was reconstituted with 0.1 ml of pyrogen free water. An aliquot (0.1 ml) of each test sample containing endotoxin

Test solution	Potency	Inhibition	pH Adjustment	Test Solution Dilution
Water for injection		No	No	UND[g]
Ringer's solution[a]		Yes	No	1:2
Lactated ringer's[b]		Yes	No	1:2
Sodium lactate	167 mEq/L	Yes	No	1:4
Saline-dextrose	0.45%, 2.5%	No	Yes	UND
NaCl[c]	0.9%	No	Yes	UND
NaCl[d]	23.4%	Yes	No	1:90
KCl[e]	14.9%	Yes	No	1:50
Dextrose	5%	No	Yes	UND
Magnesium sulfate[f]	20%	Yes	Yes	1:80

[a]147.5 mEq/L Na^+, 4.0 mEq/L K^-, 4.5 mEq/L Ca^{++}.
[b]130 mEq/L Na^+, 4.0 mEq/L K^+, 3.0 mEq/L Ca^{++}.
[c]154 mEq/L Na^+.
[d]4000 mEq/L Na^+.
[e]2000 mEq/L K^+.
[f]1626 mEq/L Mg^{++}
[g]Undiluted.

Table III. LAL test results using different parenteral solutions with an endotoxin concentration of 0.1 ng/ml (0.5 EU/ml)

was added to the reconstituted LAL ampoule. After gentle mixing, the ampoules were incubated in a water bath at 37°C for 1 hour and then at room temperature (25°C) for 5 minutes. At the end of the incubation period, the gelation was determined by carefully inverting the test tube. A hard gel was defined as a solid clot that maintains its integrity and does not move when inverted 45°. For each set of experiments, a negative control consisting of 0.1 ml of lysate and 0.1 ml of sterile non pyrogenic distilled water, and a positive control consisting of 0.1 ml of lysate and 0.1 ml of sterile pyrogen-free normal saline containing 0.1 ng/ml of endotoxin were included. The negative control served as an indicator that the experimental conditions and the water used for dilution were non-pyrogenic. The undiluted product was spiked so that the final endotoxin concentrations were 0.1 ng and 1 ng for each test solution. Also, a diluent containing 0.1 ng endotoxin concentration was used to dilute the spiked test solutions. All LAL tests for different concentration of endotoxin in each individual test solution were established in duplicate.

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