Research Article

DETERMINATION OF ENDOTOXIN IN SMALL-VOLUME PARENTERALS (SVPs): A COMPARISON OF LAL GEL-CLOT METHOD WITH U.S.P. RABBIT PYROGEN TEST

Morteza Rafiee-Tehrani and M.H. Djamshidi

Industrial Pharmacy Research Laboratory, School of Pharmacy, Tehran Medical Sciences University, Tehran, Iran.

Abstract

The simplicity and sensitivity of the Limulus Amebocyte Lysate (LAL) gel-clot method in detection of endotoxin in Small Volume Parenterals (SVPs) were studied and compared with rabbit pyrogen test.

All formulations were artificially spiked with 0.25, 0.5 and 5EU/ml of Escherichia coli 0111:B4 endotoxin (lambda=0.5EU/ml). The Threshold Pyrogenic Dose (TPD) for E.coli 0111:B4 endotoxin was found to be more than 1ng per kg of body weight (0.5EU/ml). However, the sensitivity of LAL test was found to be 0.25 Eu/ml for water for injection and 0.5 EU/ml for other formulations. The observed inhibitory effect of electrolyte containing solutions was deleted by some degree of dilution prior to test. However, none of the samples showed inhibitory at the Maximum Valid Dilution (MVD). The U.S.P. rabbit pyrogen test was insignificant for all the solutions tested. These results suggest, LAL gel-clot test to be useful for determination of bacterial endotoxin as final release test in SVPs. A comparison of the pyrogenicity assays also indicates, that LAL is more sensitive and simple and less time-consuming than the conventional rabbit test.

Introduction

Endotoxins are the lipopolysaccharide constituents of the outer cell wall of gram negative bacteria. These compounds are thought to be responsible for the pathophysiological events associated with gram negative infection, that may ultimately lead to sepsis, septic shock and death (1,2). Both improper production procedures and raw materials might be responsible for pyrogenic substances (3). The gel-clot LAL test to detect these substances was introduced by Levin and Bang in 1968 (4). Since then, numerous studies have shown that the in-vitro LAL test is a valuable alternative to the in-vivo pyrogen test using rabbits in that the former is more expeditious, specific, and conveninent. In 1986 the FDA supplied guidelines for validating the Limulus Amebocyte Lysate (LAL) test as an endotoxins assay for parenteral drugs and biological products and for medical devices (6); the USPXXII / NFXVII included the "Bacterial Endotoxin Test" (7). Numerous parenteral preparations are tested according to these requirements (Supplements No. 5-7) (7). The European Pharmacopoeia Commission (8) has also adopted the LAL assay for bacterial endotoxin using the gelation method. The inhibitory effects caused by the presence of electrolytes are being reported by other investigators (5).

The purpose of this investigation was to assess the possibility of replacing the rabbit pyrogen test with the LAL assay, as a final release test for Small Volume Parenterals (SVPs).

MATERIALS AND METHODS

Materials: All materials were tested for endotoxin before use in the analysis. All glasswares were washed, rinsed and depyrogenated by dry heat sterilizations at 250℃ for over 8 hours.

Reagents: The following commercially available test solutions were used: sodium chloride 0.9%, sodium lactate 2%, sodium phosphate, sodium sulfate 3.89%, sodium bicarbonate 7.5%, sodium citrate 4% calcium chloride 10%, calcium gluconate 10%, magnesium sulfate 10%, aluminum chloride 1%, thorium nitrate 1%, vitamin C injection, vitamin B 12 injection, vitamin B complex injection, betamethasone injectable solution, dexamethasone injectable solution, and gentamycine sulfate injection.

Commercially available USP sterile water for injection was used as the diluent for the LAL test. Various concentrations of HC1 (0.1-6N), and NaOH (0.1-10N) 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 1ng 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 final endotoxin concentrations were 0.5 and 5 EU/ml (0.1 and 1ng per ml).

Rabbit Pyrogen Test: The pyrogenicity test was performed in accordance with USP procedure (1): 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. Calcium gluconate, betamethasone, dexamethasone and gentamycine sulfate were appropriately diluted with pyrogen-free normal saline before injection into the rabbit. The rabbit assay was interpreted in accordance with USP (7) 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 rise does not exceed 1.4° C. In accordance with USP procedure (7), rabbit pyrogen test is not required for detection of pyrogenicity in magnesium sulfate solution (10%) and sodium phosphate.

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 was added to the reconstituted 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. 1ml of lysate and 0.1 ml of sterile non-pyrogenic distilled water, and a positive control consisting of 0.1 ml of sterile pyrogen-free

normal saline containing 0.1ng/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 0.1ng and 1ng for each test solution. Also, a diluent containing 0.1ng endotoxin concentration was used to dilute the spiked test solutions. All LAL tests for different concentrations of endotoxin in each individual test solution were established in duplicate.

RESULTS AND DISCUSSION

Threshold Pyrogenic Dose (TPD): Both the USP rabbit test and the LAL assay can be used to detect endotoxins; however, only the LAL test can rapidly and precisely quantify endotoxin levels. The minimum quantity of endotoxin that is pyrogenic in rabbits, the TPD, must be determined so that the LAL assay can be correlated with the rabbit data.

Humans and rabbits reported to respond equally on a per-kilogram of body weight basis to threshold pyrogenic quantities of endotoxin (9). Therefore, the minimum quantity of endotoxin that is pyrogenic to humans, the TPD, can be determined by the USP rabbit test (7). Table 1 summarizes the results of various parenterals that passed USP three-rabbit test at a dose of 1.0 ml/kg of 01ng/ml endotoxin. These results are consistent with other findings that the TPD for both human and rabbit is equal and approximately 1.0ng/kg of body wight (10).

Hornick and Greisman reported that TPD for both humans and rabbits is 1.0ng/kg of body weight for an E.coli endotoxin (9). Endotoxin preparations from E.coli 055:B5 and Salmonella abortus equi exhibited TPD's of slightly more than 1.0ng per kg of body weight (11). Furthermore, the endotoxin preparations from Klebsiella pneumoniae and Shigella flexneri were less potent, demonstrating TPD's of above 2.0 ng per kg of body weight (11). However, Bleeker et al. found a threshold pyrogenic dose of 2ng/kg when E.coli 055:B5 was used (12). The sensitivity of USP rabbit test using different test solutions is exhibited in Table 2. This table demonstrates that all endotoxin preparatoins tested had TPD's of 2ng/kg of body weight. However, the vitamin B_{12} ampoules containing endotoxin exhibited TPD's of 1ng/kg of body weight. Furthermore, the test solutions containing corticosteroids betamethasone and dexamethasone) showed TPD's of slightly more than 2ng/kg due to the decrease ormation of prostaglandins (13). However, test solutions containing calcium ion (calcium chloride and

calcium gluconate) exhibited TPD's of more than 2ng/kg of body weight, as well, which might be due to the inhibiting effect of calcium ion on endogeneous pyrogen release from leukocytes (14).

LAL: The sensitivity of LAL gel-clot test in detection of endotoxin E.coli 0111:B4 in different test solutions is exhibited in Table 3. The sensitivity of sterile water for injection was found to be 0.25 EU/ml which was consistent with endotoxin limits for this article. However, NaCl showed identical sensitivity when was diluted. The sensitivity of other test solutions was 0.5 EU/ml (0.1 ng/ml). The pH of most of the test solutions was in the range of 5.5-8.0. However, the pH of others was adjusted by NaOH or HCl before test.

Tables 4 and 5 demonstrate the inhibitory effect of electrolytes on endotoxin detection of LAL, eventhough the LAL reagent was capable of detecting less than 0.1ng (0.5 EU) of endotoxin per ml. The inhibiting effect of electrolytes first observed in Rafiee et al. (5) study is of limited importance for low concentrations, i.e. NaCl 0.9% and sodium lactate 2% since this inhibition can be eliminated to an acceptable degree by four folds dilution. However, when higher concentrations are involved i.e. KCl 10% (Rafiee and Khoshbooii unpublished results) a stronger inhibiton occurs and interferes with LAL gelation reaction. This interferance can be deleted by higher dilution. Nevertheless, none of the samples showed inhibitory at the Maximum Valid Dilution (MVD). Furthermore, divalent, trivalent and tetravalent cation (Ca^{2+}, Mg^{2+}, Al^{3+}, Th^{4+}) demonstrated a much stronger inhibition than monovalents cations (Table 5 and Figure 1). These results were consistent with previous report of Rafiee et al. (5). Beggerman et al. stated that stronger inhibitory effects were occured when divalent cations (Ca^{2+}) were involved (16). These investigators stated that electrolytes are responsible for changes of physico-chemical properties of endotoxin resulting in a decrease of activity. Salt effect on endotoxin adsorption by charged depth filter was reported by Hou et al. (17). However the change in the aggregation state as a possible cause for physico-chemical properties of endotoxin was suggested by some investigators (3, 16.18).

The present investigation showed that the inhibiting effect of cations on endotoxin detection by LAL gelation test increase with valency enhancement. Tetravalent cations (Th^{4+}) produced a much stronger inhibitory effect than the monovalent cations. Table 5 also exhibits that a similar inhibition occurring when polyvalent anions (sulfate, citrate and phosphate) were present. Based on this data as well as those cited in other literatures (5, 16, 19, 20, 21) the electric double layer surrounding

endotoxin and lysate is affected by counterions persent in the test solution. As the concentration and valency of cations present (i.e. Na^+, K^+, Ca^{2+}, Mg^{2+}, Al^{3+}, Th^{4+}) in the system are increased the zeta potential of negatively charged endotoxin is also increased. As a consequence these electrokinetic potential shifts may potentially inhibit the LAL reaction. Moreover, weaker inhibition occured when polyvalent anions (sulfate, citrate and phosphate) were present, decreasing the positive zeta potential of lysate.

Lacasa et al. (21) reported that the detection of standard endotoxin in sodium bicarbonate 8.4% solution after dilution were negative by LAL and positive on rabbits, this means that dilution did not prevent inhibition. This does not agree with our observations as well as Fussellier et al. (21). We considered that LAL gel-clot test is valid for detection of endotoxin in sodium bicarbonate 8.4% solution.

Table 1. Three-rabbit test results using different test solutions with an endotoxin concentration of 0.1ng/ml (0.5 EU/ml) at a dose of 10ml/kg

Test Solution	USP Total Temperature increase (C)	Mean Temperature Increase	Standard Deviation (C)	Coefficient of variation (%)
Sterile water for injection	1.2	0.4	0.173	43.25
Sodium chloride 0.9%	1.0	0.33	0.351	106.36
Calcium gluconate 10%	0.6	0.2	0.2	100
Calcium chloride 10%	0.9	0.3	0.0	0.0
Sodium lactate 2%	0.9	0.3	0.1	33.33
Vitamin B-complex	1.5	0.5	0.1	20.0
Vitamin B_{12}	1.4	0.467	0.058	13.39
Vitamin C	1.4	0.467	0.058	12.24
Betamethasone	0.8	0.267	0.153	57.3
Dexamethasone	0.4	0.134	0.05	43.28

Table 2. USP-rabbit test sensitivity using different parenteral solutions

Test Solution	+TPD (EU/kg)	Dilution Factor (with Nacl. 0.9%)
Sterile water for injection	10.0	UND*
Vitamin C	10.0	UND
Vitamin B-complex	10.0	UND
Vitamin B_{12}	5.0	UND
Betamethasone	11.0	1:40
Dexamethasone	11.0	1:40
Gentamycin sulfate	10.0	1:40
Sodium chloride 0.9%	10.0	UND
Sodium lactate 2%	10.0	UND
Calcium chloride 10%	11.5	1:2
Calcium gluconate 10%	11.5	1:2

* Undiluted

+ Threshold Pyrogenic Dose

Table 3. LAL gel-clot sensitiviry using various test solutions

Test Solution	Potency	Positive End Point (EU/ml)	Diluted Before Test	pH Ajustment
Vitamin C		0.5	No	No
Vitamin B-complex		0.5	No	Yes
Vitamin B_{12}		0.5	No	Yes
Betamethasone		0.5	No	Yes
Dexamethasone		0.5	No	No
Sodium chloride	0.9%	0.25	Yes	No
Sodium lacate	2%	0.5	Yes	No
Sodium lacate	7.5%	0.5	Yes	No
Sodium phosphate		0.5	Yes	No
Sodium sulfate	3.89%	0.5	Yes	No
Sodium citrate	4%	0.5	Yes	No
Calcium chloride	10%	0.5	Yes	No
Calcium gluconate	10%	0.5	Yes	No
Magnesium sulfate	10%	0.5	Yes	Yes
Aluminum chloride	1%	0.5	Yes	No
Thorium nitrate	1%	0.5	Yes	No

Table 4. LAL test results different test solutions containing electrolytes with an endotoxin concentration of 0.10g/ml (0.5 EU/ml)

Test Solution	Potency	Ion Consentration (m Eq/L)	Inhibition	pH Adjustment
Sterile water for injection			No	No
Sodium Chloride	0.9%	154	Yes	No
Sodium lacate	2.0%	167	Yes	No
Sodium phosphate			Yes	No
Sodium sulfate	3.89%	242	Yes	No
Calcium chloride	4.0%	408	Yes	No
Calcium gluconate	10.0%	1803	Yes	No
Magnesium sulfate	10.0%	813	Yes	No
Aluminum chloride	1.0%	225	Yes	Yes
Thorium nitrate	1.0%	70	Yes	Yes
Sodium bicabonate	7.5%	85	Yes	No

Table 5. Inhibitory effect of various electrolytes on detection of endotoxin E.coli 0111:B4 by LAL gel-clot method

Test Solution	Potency	Cation	Anion	Ion Concentration Before Dilution (mEq/L)	*MVD	+ DF	Ion Concentration Dilution Without Inhibition Effect (mEq/L)
Sodium bicarbonate	7.5%	1	1	85	1:22	1:20	4.25
Sodium chloride	0.9%	1	1	154	1:4	1:4	38.5
Sodium lactate	2%	1	1	167	1:5	1:4	41.75
Sodium sulfate	3.89%	1	2	242	1:11	1:5	48.4
Sodium citrate	4%	1	3	408		1:10	40.8
Sodium phophate	2/3	1	3			1:80	50.0
	1/3	2		4000			
Calcium chloride	10%	2	2	1360	1:70	1:60	22.7
Calcium gluconate	10%	2	1	465	1:48	1:20	23.25
Magnesium sulfate	10%	2	2	813	1:70	1:40	20.3
Aluminum chloride	1%	3	1	225		1:19	11.8
Thorium nitrate	1%	4	1	80		1:60	5.0

* Maximum Valid Dilution

+ Dilution Factor

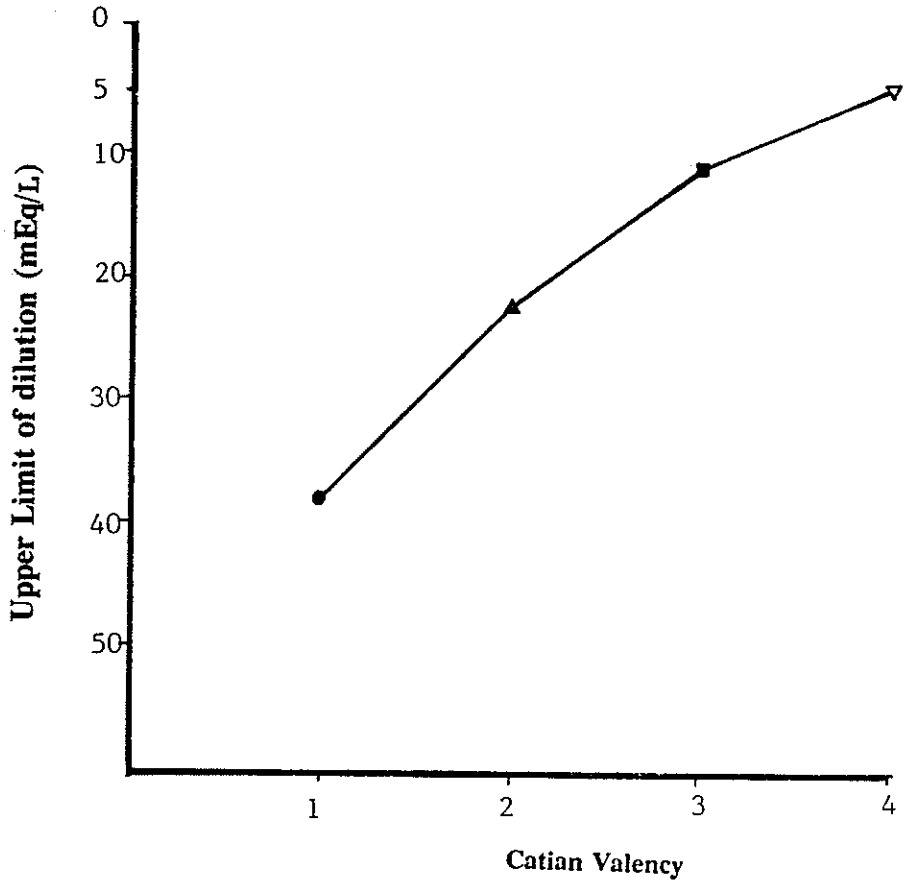

Figure 1. Inhibiting effect of Cation Valency on LAL gel. Clot method, symbols: (●) NaCl; (▲) $CaCl_2$; (■) $AlCl_3$; (Δ) Th $(No3)_4$

Conclusion

The results of this study show that the LAL gel-clot method is useful method for the detection of bacterial endotoxin in small-volume parenterals (SVPs). This system is more sensitive than the conventional rabbit pyrogen test. The simplicity and preciseness of the LAL gel-clot assay enhances the utilization of this method in pharmaceutical analysis of endotoxin as a final release test.

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