TECHNICAL COMMUNICATION

Pyrogens in Small-Volume Parenterals Prepared in Hospital Pharmacy

C. LACASA*△ and F. A. VEGA†

** Servicio de Farmacia, Hospital de Basurto, Bilbao, Spain, and † Universidad de Navarra, Pamplona, Spain*

ABSTRACT: Three pyrogen assay methods [the rabbit method, Limulus *Amebocyte Lysate (LAL) gelation and chromogenic substrate, and bioburden prior to sterilization] were evaluated in three standard batches of small-volume parenteral preparations; i.e., water for injection, sodium chloride 0.9% injection, and sodium bicarbonate 8.4% injection. The same preparations were also assayed after contamination with* Escherichia coli *followed by sterilization. All methods gave the same results with the water for injection and the sodium chloride 0.9%. With sodium bicarbonate 8.4%, only the rabbit method was valid. For sodium chloride 0.9%, the chromogenic substrate method was valid; the gelation method was not valid. Endotoxins from the manufacturing plant were less pyrogenic in rabbits than were standard endotoxins or those from hospital* E. coli.

Introduction

Hospital pharmacies require a simple and rapid method for pyrogen control for the parenteral preparations they make and for those supplied by the pharmaceutical industry that have caused pyrogenic reactions in patients. The classic rabbit pyrogen test requires conditions that are very difficult to maintain in a hospital pharmacy.

A review of the general aspects of lipopolysaccharides (LPS) was given in an earlier paper (1). In 1986 the FDA (2) supplied guidelines for validating the *Limulus* Amebocyte Lysate (LAL) test as an endotoxin assay for parenteral drugs and biological products and for medical devices; the *USP XXI/NF XVI* (3) includes the "Bacterial Endotoxin Test" (Supplement No. 1). The European Pharmacopoeia Commission (4) has also adopted the LAL assay for bacterial endotoxin using the gelation method.

The purpose of this study was to test the possibility of substituting the LAL and bioburden pyrogen assay methods for the rabbit test, in hospital pharmacies for use in the following parenteral preparations: water for injection, sodium chloride 0.9%, and sodium bicarbonate 8.4%.

In this paper a comparison is made between the LAL, rabbit, and bioburden methods on 10-mL ampuls prepared by the standard process in the hospital pharmacy and on similar ampuls previously contaminated with *Escherichia coli* followed by sterilization.

Materials and Methods

Instrumentation

Equipment includes Autofarma 2500 Macorra ampul filling and sealing machine; 3M Indos 202B ethylene oxide sterilization apparatus; Atom 334 spectrophotometer; Labora-Adsuar autoclave; Selecta 140-D dry heat stove with forced air circulation; Millipore No. YY3009000 filtration set; and Ellab thermoelectric temperature-recording apparatus. All materials used were disposable, sterile, and pyrogen free.

Reagents

Culture Media: Mueller-Hinton Difco 075701 broth and blood agar with tryptone and soya Oxoid CM 131, for aerobic bacteria; MacConkey Agar Oxoid CM 115, for enteric bacteria; Thioglycolate Difco 025601 and Brucella agar Oxoid CM 691, for anaerobic bacteria; and Mueller-Hinton broth, mentioned above, and modified Sabouraud agar medium, for fungi and yeast.

LAL: "Pyrogent" reagent (Lot No. 4AF, Millinckrodt Inc., St. Louis, MO) and "Coatest Endotoxin" reagent (Lot No. 80907-51, Kabi Vitrum, Mölndal, Sweden).

Endotoxins: *E. coli* 055:B5 endotoxin, 10 EU/ng (Lot No. 5244, Mallinckrodt Inc., St. Louis, MO); *E. coli* 011:B4 endotoxin 12.5 EU/mL (Lot No. 39513-51, Kabi Vitrum, Mölndal, Sweden); and hospital *E. coli* strains of the Microbiology Laboratory, Basurto Hospital, Bilbao, Spain.

Animals

3-4 kg New Zealand albino rabbits.

Sample Preparation

Uncontaminated Parenteral Preparations: Three standard batches of water for injection, sodium chloride 0.9%, and sodium bicarbonate 8.4% injections were prepared. All glass and metallic material in contact with the solutions was rendered sterile and pyrogen-free by treatment at 250°C for 30 minutes (*USP XXI/NF XVI*, p. 1181); rubber and plastic material were sterilized by 1.24 g/L concentration ethylene oxide at 63°C, for 170 minutes with 40–50% relative humidity. Negative controls were performed by LAL assay in all cases. The solutions were

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△ Author to whom correspondence should be addressed: [Present address: Dra. Carmen Lacasa, c/o Hurtado 4, 2º, 1ª, 08023 Barcelona, Spain.]

filtered through a 0.45-μm filter, and the ampuls were filled and sealed. Eighteen ampuls from the beginning and 18 from the end of each batch were separated for bioburden assay. The rest were autoclaved at 121°C for 20 minutes.

Contaminated preparations: *E. coli* dilutions 10,000, 1,000, 100, and 10 colonies/mL were prepared in the same solutions according to Marcus's technique (5). Samples of the dilutions were taken and filtered through a 0.45-μm filter. The remainder was distı ıted into ampuls, sterilized, and assayed by either the LAL or the rabbit method.

Bioburden

European Pharmacopoeia Method (6): The above mentioned specific culture media were used to detect the presence of viable bacteria, fungi, and yeast forms.

Uncontaminated Parenterals: Of the 18 ampuls separated from the beginning and the end of each batch, six were used for the detection of aerobic bacteria, six for anaerobic bacteria, and six for fungi and yeast. Five milliliters of culture broth and 10 mL of each sample were passed through a 0.45-μm filter. Filter readings for aerobic and anaerobic bacteria were taken after incubation of the samples for 14 days at 37°C; filter readings for fungi and yeast were taken after 30 days, with half the samples incubated at room temperature and half at 37°C.

Solutions Previously Contaminated with *E. coli*: As explained above, samples were taken from each of the dilutions prepared in the solutions previously shown to be pyrogen-free: six samples of 0.1 mL for each dilution of 10,000 bacteria/mL and six samples of 1 mL for each one of the 1000-, 100-, and 10-bacteria/mL dilutions. The filters were separated and placed on agar MacConkey plates at 37°C for 24 hours.

LAL Method

The material and instructions provided by the reagent manufacturer were used.

LAL "Pyrogent": Uses the clot-no clot readings. The gel's appearance is uncertain. Positive, negative, and inhibition controls were performed. Sample pH was maintained between 6 and 7.5. The standard endotoxin used was *E. coli* 055:B5.

LAL "Coatest Endotoxin": The standard endotoxin used was *E. coli* 0111:B4. Results were read at 405 nm. Calibration was performed with endotoxin concentrations of 0.15, 0.3, 0.6, 0.9, and 1.2 EU/mL.

Rabbit Assay

***USP XXI/NF XVI* Method (3):** Rabbits were injected with 10 mL/kg water for injection, 10 mL/kg sodium chloride 0.9%, and 5 mL/kg sodium bicarbonate 8.4%.

Results

Tables I and II show the results obtained by the three methods on uncontaminated parenteral preparations and on previously contaminated solutions. Table III shows the gelation study in LAL "Pyrogent" by previously contaminated sodium chloride 0.9%, and the effect that dilution and pH have on the gelation of LAL "Pyrogent" in sodium bicarbonate 8.4% with previously added *E. coli* 055:B5 endotoxin. The calibration curve equations for LAL "Coatest endotoxin" method were (7): Water for injection: $y = 2.80 + 7.18x$; sodium chloride 0.9%: $y = -0.44 + 4.25x$; sodium bicarbonate 3.4%: $y = 0.36 + 2.65x$; ($r = 0.99$ for the three equations).

Discussion

There are three possible means of pyrogenic contamination to be considered: *1.* Bacterial contamination prior to sterilization. This can be avoided by means of a correct in-process control (8). *2.* Endotoxin contamination in the finished parenteral preparation. This is improbable if the above has been avoided. *3.* Other pyrogenic products. Many years of experience with the in-process control of parenteral products and the authorities' supervision have

TABLE I. Results on Uncontaminated Injections

	Bacterial/mL ×		LAL		Rabbits
	B	*E*	"Pyrogent"	"Coatest" (EU/mL)	*USP XXI*
Water for injection	6.3	1.4	–	>0.15 (11) 0.21 (1)	–
Sodium chloride, 0.9%	43.2	3.2	–	>0.15 (8) 0.18 (1) 0.20 (1) 0.23 (1) 0.31 (1)	–
Sodium bicarbonate, 8.4%	0	0	–	>0.15 (12)	–
N	6	6	4	12	3

B = Samples belonging to the beginning of the batch.
E = Samples belonging to the end of the batch.
– = Negative.
( ) = Number of ampuls with that result.
N = Number of ampuls tested for each batch.

TABLE II. Results Obtained with Solutions Previously Contaminated with *E. coli*

	Bacteria/mL		LAL		
	Theoretical	Viable (mean 6 filters)	"Pyrogent" (mean 4 ampuls)	"Coatest" EU/mL	Rabbits
Water for injection	10,000	15	++	0.33	+
	1,000	8	+	0.31	−
	100	1	−	0.24	−
	10	0	−	0.17	−
Sodium chloride, 0.9%	10,000	5187	+	540*	+
	1,000	484	+	31.62*	+
	100	50	−	5.22*	+
	10	4	−	0.31*	+
Sodium bicarbonate, 8.4%	1,000	6	−	>0.15	+
	1,000	2	−	>0.15	+
	100	0	−	>0.15	+
	10	0	−	>0.15	+

1 = Before sterilization.
2 = After sterilization.
* = Estimated by extrapolation.
− = Absence of gel or rabbit negative.
+ = Slight viscosity increase or rabbit positive.
++ = Notable viscosity increase.
+++ = Firm gel.
Correlation between count viable bacteria and endotoxin concentrations in sodium chloride 0.9%: $y = -2.15 + 0.10x$, $r = 0.99$.

proved that possible pyrogenic contamination by agents other than endotoxin is insignificant (9).

USP XXI/NF XVI, p. 1353, states that in water for injection, aerobic bacteria microbial counts should be less than 50 bacteria/mL and that if they approach or exceed this value, it becomes necessary to take corrective actions such as chlorination, filtration, radiation, etc. (3).

Uncontaminated Preparations

The results obtained with the three methods on uncontaminated solutions (Table I) coincide. Low microbial counts were obtained (the bacteria identified were *Pseudomonas putida fluorescens* and negative coagulase *Staphylococcus*). The highest counts were obtained in sodium chloride 0.9%. The counts decrease in water for injection and none were found in sodium bicarbonate 8.4%. After sterilization, endotoxin concentrations were below 0.3 EU/mL, and negative results in the rabbit test were obtained.

TABLE III. LAL "Pyrogent" in Preparations Contaminated with *E. coli* 0.55:B5 Endotoxin (see Table II)

Preparation	Endotoxin (EU/mL)	Results at pH 5.5	6.65	7	7.80	8.3
Sodium bicarbonate (%)						
8.4	1.0		++		+	
0.84	1.0				++	
0.84	0.1		++			++
Sodium chloride (%)	1	+++		+++		
0.9	0.5	++		+++		

Some authors (5) have observed that for bacterial counts below 1000 viable bacteria per milliliter, the pyrogen assay was negative on rabbits. After the solution's sterilization, with a safety factor of 100, quality can be assured with counts below 10 viable bacteria per milliter. Endotoxins of gram-negative bacteria and certain genera and species are more pyrogenic than others (10). Endotoxins of the bacteria commonly isolated at manufacturing plants produce less hyperthermia in rabbits than the control-standard endotoxin (11). This agrees with the results we have observed. Thus, uncontaminated sodium chloride 0.9%, which had the highest microbial counts, gave negative results in LAL and rabbit assays. Concentrations less than 0.3 EU/mL in uncontaminated sodium chloride give negative results in rabbits. Similar concentrations in contaminated solutions give positive results in rabbits (see Tables I and II).

Contaminated Preparations

Similar results were obtained in water for injection by the three methods. Linear correlation ($p = 0.001$) was observed in sodium chloride 0.9% between the number of viable bacteria and the endotoxin concentration by the LAL "Coatest endotoxin" assay (Table II). The results in sodium chloride 0.9% (Table III) at pH 7, were similar to those of water for injection. Viscosity only increased slightly at pH 5.5 with 10 000 bacteria/mL dilution.

This does not agree with the microbiological counts, with the rabbit assay, nor with the high concentrations obtained with LAL "Coatest endotoxin" (Table II). In our tests the LAL "Pyrogent" method was not valid for this preparation. It has already been mentioned that the

gelation reaction was inhibited, in some LAL methods, by sodium chloride 0.9% (12).

The method based on the presence or absence of firm gel is interpreted more subjectively that the one with chromogenic substrate; Levin (1985) (13) stated that the results are not of firm gel but of flocculation and an increased viscosity that can be considered positive. This increases the sensitivity of the analysis if the negative controls are correct. In our experience, however, we consider that the said interpretation would be less definitive and has made it necessary for us to define three positivity degrees.

Inhibition in sodium bicarbonate 8.4%, with standard endotoxin, can be partially avoided by adjusting the pH and diluting the samples 1/10 (Table III). In sodium bicarbonate 8.4% contaminated with hospital *E. coli* (Table II), the results obtained were negative by LAL and positive on rabbits; this means that dilution did not prevent inhibition. This does not agree with Fussellier et al. (14) who considered this LAL method valid for this solution.

We conclude that the LAL assay and bioburden can replace the rabbit pyrogen test in water for injection. In sodium chloride 0.9%, the bioburden and LAL "Coatest endotoxin" methods, but not the LAL "Pyrogent" assay, can replace the rabbit method. However, the LAL method used and the bioburden were not valid for sodium bicarbonate 8.4%; and thus it is recommended that the rabbit assay continue to be used for this solution. A detailed study comparing the three methods, rabbit, LAL and bioburden, should be performed for each parenteral preparation to determine the possibility of substituting the rabbit pyrogen test by LAL and bioburden. The LAL assay can be useful in hospital pharmacies because of its sensitivity and simplicity (if no interference arises). In preparations for spinal administration, its use becomes essential due to its greater sensitivity compared to the rabbit assay. A commercial batch of a product that had produced several cases of aseptic meningitis after spinal anesthesia were also tested. The LAL "Pyrogent" test gave high endotoxin concentrations; the rabbit pyrogen test was negative (15, 16).

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/Editor
Joseph B. Schwartz, Ph.D.
Philadelphia College of Pharmacy and Science
43rd and Woodland Avenue
Philadelphia, PA 19104
(215) 596-8590

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by C. Lacasa and F. Vega

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Dr. Joseph R. Robinson
School of Pharmacy
University of Wisconsin
Madison, Wisconsin 53706

March 30, 1988

Dr. Carmen Lacasa Diaz
Servicio de Farmacia
Hospital Civil De Bilbao
Santo Hospital Civil De Bilbao
Avda, Montevideo 18
48013 - Bilbao
Spain

Dear Dr. Diaz:

This will acknowledge receipt of your manuscript "Pyrogens in Small Volume Parenterals Manufactured in Hospital Pharmacy" by C. Lacasa and F. A. Vega. The manuscript is being sent out for review, and I will be in touch with you upon receipt of the reviewers' comments and recommendation.

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Joseph B. Schwartz, Ph.D.
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43rd and Woodland Avenue
Philadelphia, PA 19104
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Dra. Carmen LaCasa
c/ Hurtado 4,2°1a
08023 Barcelona
Spain

RE: "Pyrogens in Small-Volume Parenterals Prepared in Hospital Pharmacy"
by C. Lacasa and F. Vega

Dear Dra. LaCasa:

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