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A Highly Sensitive Pyrogen Test for Antibiotics I: Detection of Trace Amounts of Endotoxin in Injectable Sodium Ampicillin Preparations

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Abstract □ The rabbit pyrogen test (specified in the pharmacopeia) and the Limulus amebocyte lysate (LAL) test are influenced by high concentrations of certain antibiotics. Therefore, it has not been possible to detect trace amounts of endotoxin which may contaminate these antibiotics. To detect trace amounts of endotoxin in injectable sodium ampicillin, an ultrafiltration technique was utilized which removed the antibiotic and left a solution which contained predominantly the endotoxin. After ultrafiltration, a trace amount of pyrogen (which otherwise could not be detected) was found using both the rabbit pyrogen and the LAL tests. The endotoxin was also determined quantitatively using a chromogenic endotoxin reagent which is made by combining the Limulus amebocyte lysate and a synthetic substrate with a suitable chromophore.

Keyphrases □ Ampicillin—detection of trace amounts of endotoxin using ultrafiltration with pyrogen tests, chromogenic assay □ Ultrafiltration—of ampicillin solutions, use with pyrogen tests to detect trace amounts of endotoxin in antibiotics □ Chromogenic assay—of ampicillin solution, detection of trace amounts of endotoxin in antibiotics

It is important to develop highly sensitive methods for detecting trace amounts of pyrogen to ensure that pharmaceutical preparations are completely pyrogen free. Several cases of fever have been reported after the injection of β-lactam antibiotics such as methicillin and cloxacillin (1–6). However, in only one case (methicillin) was the presence of a pyrogen detected (6). Fever is caused in most cases by lipopolysaccharide from the outer layer of the cell walls of Gram-negative bacteria. Pyrogens, a type of endotoxin, are often complex, high molecular weight substances containing lipid A (7).

Endotoxins are usually detected by the pyrogen test using rabbits, or by the Limulus amebocyte lysate (LAL) test. The former method is specified in the pharmaceutical compendia of both the U.S. (8) and Japan (9). The latter method is specified in the United States Pharmacopeia XX (10). In the pyrogen test, the rise in the body temperature of rabbits caused by the endotoxin is sometimes inhibited by the pharmacological activity of the coexistent drugs. The sensitivity of the LAL test also is affected by the presence of certain drugs (11). In such cases, it would be desirable to separate the endotoxin from the drug and concentrate the endotoxin.

Minami *et al.* used an ultrafiltration method to separate endotoxins from antipyretics (12). Sullivan *et al.* showed that β-lactam antibiotics such as sodium penicillin G do not combine with endotoxin using ultrafiltration (13).

It was reported that gel formation in the LAL test was induced by the amidase activity of a clotting enzyme in the lysate, which is activated by a bacterial endotoxin (14). Harada *et al.* applied this principle to the colorimetric determination of endotoxins using a synthetic substrate which activates the amidase activity of the enzyme and releases a chromophore (15).

This study reports the detection of trace amounts of

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Table I—Temperature Increase in Rabbits for the Pyrogen Test [a]

Sample	Dose	Rise in Temperature °			Mean ± SE[b]
Test sample	25 mg(potency)/kg	0	0.2	0.3	0.25 ± 0.06
		0.3	0.3	0.4	
	20 mg(potency)/kg	0	0.1	0.2	0.22 ± 0.07
		0.2	0.4	0.4	
Control	25 mg(potency)/kg	0	0.2	0.3	0.25 ± 0.06
		0.3	0.3	0.4	

[a] According to the method specified in the Compendia. [b] $n = 6$.

pyrogen contaminants in injectable sodium ampicillin preparations using ultrafiltration followed by rabbit pyrogen and LAL tests. The quantitative determination of endotoxin by a chromogenic assay method using a reagent made from the Limulus amebocyte lysate and a synthetic substrate is also reported in this paper.

EXPERIMENTAL

Materials—*Samples and Reagents*—An injectable sodium ampicillin preparation in vials of 1 g(potency), suspected to be contaminated with pyrogen, was used as the test sample. A sodium ampicillin preparation for injection manufactured prior to the test sample was used as the control. Commercially available endotoxin from *Escherichia coli* (0111-B4)[1] was dissolved in physiological saline solution immediately before use and diluted to the required concentration, to provide a standard endotoxin. The Limulus amebocyte lysate[2] and chromogenic endotoxin assay[3] reagents were obtained commercially. The chromogenic endotoxin assay reagent is made from Limulus amebocyte lysate and the *p*-nitroaniline derivative of a synthetic oligopeptide as the substrate. Tromethamine hydrochloride[1] (0.1 *M*, pH 8.0) was used as a buffer. The blue dextran solution (2 μg/ml of dye-combined dextran, average molecular weight 2,000,000)[4] was used after it was confirmed to be pyrogen free.

Ultrafiltration Apparatus—The ultrafiltration apparatus[5] (stirring type) was used after washing with pyrogen-free distilled water and drying at 60° for 1 hr. The ultrafiltration membrane[6] had a fraction molecular weight of 10,000 and a diameter of 76 mm. It was used after soaking overnight in 0.1 *N* NaOH solution; the surface of the membrane was washed with distilled water for injection prior to use.

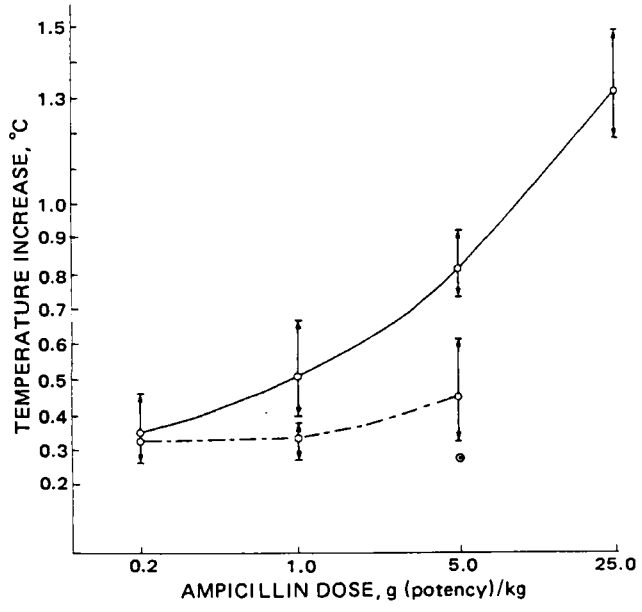

Figure 1—*Dose-response curve of sodium ampicillin in the rabbit pyrogen test. Key: (—) ultrafiltrated sample, n = 10–26; (- · -) without ultrafiltration, n = 9–10; (⊙) control sodium ampicillin, n = 9.*

[1] Seikagaku Kogyo Co. Ltd., Tokyo.
[2] Pregel, Seikagaku Kogyo Co. Ltd., Tokyo.
[3] ETC, Seikagaku Kogyo Co. Ltd., Tokyo.
[4] Blue Dextran 2000, Pharmacia Fine Chemical Co. Ltd., Uppsala, Sweden.
[5] Diaflowcell Model 402, Amicon Corp., Lexington, Mass.
[6] Diaflowmembrane PM10, Amicon Corp., Lexington, Mass.

Table II—Recovery of *E. Coli* 0111-B4 Endotoxin after Ultrafiltration (LAL Test) [a]

Sample	Dilution of Endotoxin Stock Solution[b]				
	× 1	× 2	× 4	× 8	× 16
Untreated solution (I)	++	+	+	±	−
Ultrafiltrated solution (II)	++	++	++ ~ +	±	−

[a] After the mixture was incubated at 37° for 1 hr and then allowed to stand for 5 min. [b] The original concentration of the solution was 5 ng/ml. The key is as listed in the text.

Rabbits—Japanese white, male rabbits weighing 2.0–2.7 kg were bred and maintained in a room kept at 25 ± 2° with a relative humidity of 50 ± 10%. They were fed daily (~100 g of solid food[7]) with an automatic rabbit feeding apparatus. Water was supplied *ad libitum* using an automatic water-supplying system. Only those rabbits that maintained their weight for at least 1 week were used in the experiments. They were conditioned for 1–3 days prior to the pyrogen test by conducting sham tests (*i.e.*, without injection).

Methods—*Ultrafiltration*—All glass apparatus were depyrogenized by heating at 250° for 2 hr. After the ultrafiltration membrane was attached to the apparatus, the apparatus was washed three times with pyrogen-free distilled water and once with 0.01 *N* NaOH solution. The surface of the membrane was coated with 100 ml of dye-combined dextran (average molecular weight 2,000,000) solution to prevent adsorption of the pyrogen. The inside of the apparatus was rinsed with 30 ml of physiological saline solution using manual stirring; the washings were confirmed to be pyrogen free by the LAL and rabbit pyrogen tests (dose: 10 ml/kg).

The samples of sodium ampicillin were dissolved in physiological saline, placed in the ultrafiltration apparatus, and filtered under pressure using nitrogen gas (3.5 kg/cm^2) until the volume of the residual solution was reduced to one-tenth the original volume. If the concentration of sodium ampicillin in the residual solution exceeded 10 mg (potency)/ml, the dilution and filtration were repeated until the concentration of sodium ampicillin became ≤10 mg(potency)/ml. The membrane filter was changed between treatments.

Determination of Endotoxin—For the rabbit pyrogen test, rabbit rectal temperatures were measured using a thermoelectric couple-type thermometer[8]. Temperatures were measured three times at 45-min intervals prior to administration; the third measurement was regarded as the control. Immediately thereafter, the sample solutions (10 ml/kg) were

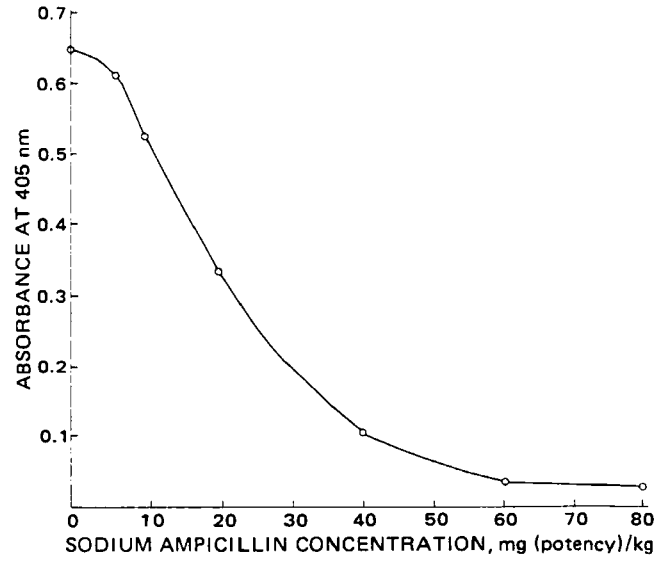

Figure 2—*Influence of the concentration of sodium ampicillin on the determination of endotoxin by the chromogenic assay method. The sample solution was* E. coli *endotoxin at 0.4 ng/ml; the incubation time was 50 min.*

[7] Oriental Solid Feed (ORC-5), Oriental Kobo, Co. Ltd., Tokyo.
[8] Ellab TE3 Type, Ellab Instruments, Copenhagen, Denmark.

Table III—Influence of the Ampicillin Concentration on the LAL Test

Concentration of Endotoxin[a], ng/ml	Concentration of Ampicillin[b], mg(potency)/ml							
	100	60	40	20	10	5	1	0
10	−	−	+	++	++	++	++	++
1	−	−	±	+~±	+	+	+	+

[a] *E. coli* 0111-B4. [b] Key is as given in the text.

administered intravenously through the auricular vein. Rectal temperatures were taken at 1, 1.5, 2, and 3 hr postadministration. The difference between the highest temperature recorded after injection and the control temperature was regarded as the rise in temperature.

For the LAL test, the limulus amebocyte lysate reagent was dissolved in 0.1 ml of pyrogen-free distilled water in an ampule. The sample solutions (0.1 ml) were added to the reagent and the ampules were allowed to stand in an incubator at 37° for 1 hr. After incubation, the ampules were allowed to stand an additional 5 min at room temperature, then were slanted at 45°. Each ampule was judged using the following four grades: (++) a solid gel was formed and it did not move when the ampule was slanted; (+) although a gel was formed, it moved when the ampule was slanted; (±) a coarse granular gel was formed and the viscosity was increased; and (−) the media remained in the liquid state without any change.

For the chromogenic assay method, the contents of each vial of the chromogenic assay reagent were dissolved in 0.1 ml of 0.1 *M* tromethamine buffer (pH 8.0) while being cooled in an ice-water bath. Aliquots (0.1 ml) of the test solution were added to the vials, and the mixtures were incubated for 40 or 50 min in a water bath at 37°. After incubation, the reaction mixture was cooled rapidly in an ice-water bath and then was quenched by adding 1.0 ml of a 12.5% acetic acid solution. The absorbance was measured at 405 nm using a spectrophotometer.

RESULTS

Rabbit Pyrogen Test—Temperature rises in rabbits for samples of sodium ampicillin were measured according to the method in the Japanese Minimum Requirements for Antibiotics [25 mg(potency)/kg] and Code of Federal Regulation (20 mg/kg). No differences in the temperature rise were noted between the test and control samples in the dose range of 20–25 mg(potency)/kg.

After being concentrated by ultrafiltration, the ampicillin solution test samples were administered to rabbits, and the temperature rise was monitored. When the test samples of 0.2–5 g(potency)/kg were administered without ultrafiltration, no significant temperature differences were observed between the test and control samples (Fig. 1). However, when the test samples corresponding to 0.2–25 g(potency)/kg of the original samples were administered after ultrafiltration, temperature rises were clearly observed and were found to be dose dependent. The difference in the temperature rise was highly significant between the test and control samples at 5 g(potency)/kg.

Recovery of Endotoxin After Ultrafiltration—An endotoxin solution was prepared by diluting 200 ng of standard endotoxin (*E. coli* 0111-B4) with 40 ml of physiological saline solution (solution I). This solution was diluted by 400 ml and concentrated again to 40 ml by ultrafiltration (solution II). Solutions I and II were diluted stepwise and tested by the LAL test. As shown in Table II, almost complete recovery of endotoxin was obtained. These results were consistent with the results of Minami *et al.* (12) and Sullivan (13).

Effect of Sodium Ampicillin on the LAL Test—The effect of various amounts of the control sample of sodium ampicillin on the LAL test was studied. As shown in Table III, the gel reaction by 10 ng/ml of endotoxin was inhibited by >40 mg(potency)/ml of sodium ampicillin, and 1 ng/ml of endotoxin was inhibited by >20 mg(potency)/ml of sodium ampicillin. These results indicate that the gel reaction by the LAL test is inhibited by certain concentrations of sodium ampicillin, in agreement with Newsome's data (11).

LAL Test of Samples After Ultrafiltration—Four groups of 60 vials each of injectable sodium ampicillin preparations (1 g of test sample/vial) were treated by ultrafiltration to separate and concentrate the suspected pyrogen contaminant, and then were tested by the LAL test. The results are shown in Table IV. Although the data for the test samples showed some variation, they clearly exhibited a stronger gel reaction than that shown by the control sample. The control sample also showed a slightly positive reaction, possibly due to the sensitivity of this method. Trace amounts of pyrogen were detected in the distilled water used as the blank.

Table IV—LAL Test of Ampicillin After Ultrafiltration

Sample	Test Run	Equivalent[a]/Residual[b] Amount of Ampicillin, mg(potency)/ml: 250[a]/5[b] (×2)	125[a]/2.5[b] (×4)	62.5[a]/1.25[b] (×8)	Endotoxin Reference[c]
Test sample	1	++	++	+	
	2	++	+	±	±
	3	++	+	±	±
	4	++	++	+	+
Blank	1	+	±	−	+
	2	±	−	−	±
	3	+	−	−	±
	4	−	−	−	+
Control		+	±	−	+
Blank		±	−	−	+

[a] The equivalent amount of ampicillin is the initial concentration before ultrafiltration. [b] The residual amount of ampicillin is determined after ultrafiltration. The number in parentheses is the dilution. The key is as in the text. [c] *E. coli* 0111-B4, 1 ng/ml.

Chromogenic Assay Method—This assay is possibly more sensitive and leads to better quantitation than the LAL test. It was used to detect endotoxin in the injectable sodium ampicillin preparations with and without prior ultrafiltration. The effect of sodium ampicillin on this assay method was examined. To 0.4 ng/ml of the standard endotoxin was added 6, 10, 20, 40, 60, and 80 mg(potency)/ml of control sodium ampicillin, and the mixtures were incubated at 37° for 50 min. As shown in Fig. 2, when the concentration of sodium ampicillin was increased, the absorbance decreased depending on the drug dose. It has been reported that the minimal detectable amount of endotoxin varies and depends on the incubation time (15). Therefore, calibration curves were obtained for 0.1–0.6 ng of endotoxin in the presence of the control sodium ampicillin [20 mg(potency)/ml] for incubation periods of 40 and 50 min (Fig. 3). The curves indicated that the absorbance at 405 nm varied depending on the incubation time. The absorbance observed was ~0.3 for 0.6 ng/ml of en-

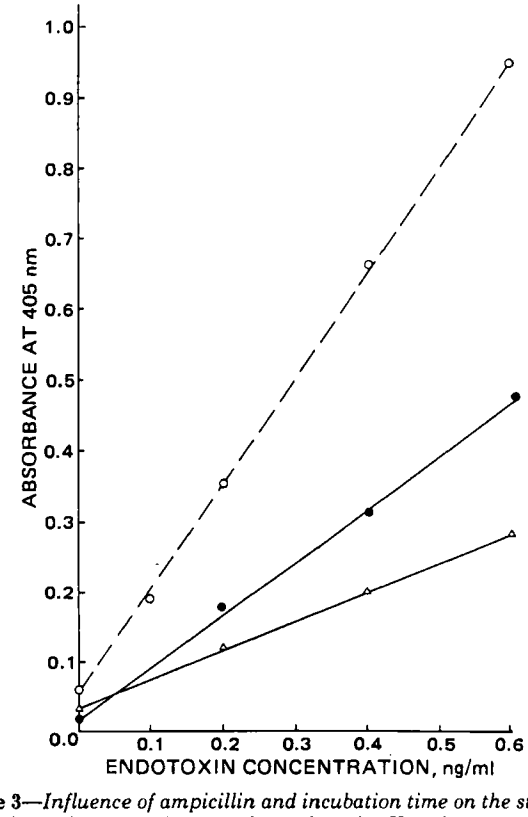

Figure 3—*Influence of ampicillin and incubation time on the standard curve of the chromogenic assay for endotoxin. Key: (○) 50-min incubation without ampicillin; (●) 50-min incubation with 20 mg of ampicillin/ml; (△) 40-min incubation with 20 mg of ampicillin/ml.*

dotoxin after a 40-min incubation and ~0.5 after 50 min. However, for the reaction mixture without ampicillin, the observed absorbance was ~0.95 for 0.6 ng/ml of endotoxin after 50 min.

From the calibration curve obtained in the presence of sodium ampicillin, the amount of endotoxin in the concentrated sample which was used in the rabbit pyrogen LAL tests was determined. It was revealed that 1 g (potency) of sodium ampicillin was contaminated with 6.4 ng of endotoxin (*E. coli* 0111-B4 endotoxin). Furthermore, the endotoxin in each vial was determined quantitatively without ultrafiltration by this chromogenic assay method using 40 vials (1 g/vial) of test samples. The results showed that the sodium ampicillin test samples were contaminated with an average of 4.73 ng of endotoxin (expressed as *E. coli* 0111-B4 endotoxin activity) per vial (gram) with a standard deviation (*SD*) of 1.75 ng/vial.

DISCUSSION

In the injectable sodium ampicillin test sample suspected of contamination, a trace amount of pyrogen (endotoxin) was detected by three methods. For each method, experiments were performed using a pyrogen-free sodium ampicillin preparation and as a control endotoxin derived from *E. coli*. The data indicate the following:

1. The presence of sodium ampicillin interferes with the detection of endotoxin, but sodium ampicillin could be separated from the endotoxin by ultrafiltration. Using ultrafiltration. it is possible not only to eliminate the coexistent drugs but also to concentrate the endotoxin. This makes it possible to detect the endotoxin in minute amounts.
2. The results obtained by the rabbit pyrogen test, LAL test, and chromogenic assay method indicated that the test samples of sodium ampicillin were contaminated with small amounts of endotoxin.
3. The endotoxin could be determined quantitatively in the presence of <20 mg/ml of sodium ampicillin. This study showed that the LAL test and the rabbit pyrogen test gave consistent results, suggesting that the *in vitro* method may become a useful method for the detection of pyrogens in drug preparations.

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Evaluation of the Teratogenicity of Morphine Sulfate Administered *Via* a Miniature Implantable Pump

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Abstract □ A technique has been developed for the implantation of miniature infusion pumps in pregnant mice with minimal teratogenic and toxic side effects. In 7- to 10-day pregnant CF-1 mice receiving constant low doses of morphine sulfate *via* the infusion pump, the results, including fetal weight reduction and various skeletal and soft tissue abnormalities, were similar to those reported in previous investigations using single injections.

Keyphrases □ Delivery systems—implantable infusion pump, teratology studies □ Morphine sulfate—use of implantable infusion pumps for teratology studies, comparison with conventional techniques □ Infusion pump—delivery of test drug in teratology studies, comparison to conventional techniques

Teratology studies have often shown that drugs can affect the developing fetus, thereby dispelling the myth of a fully protective placental barrier. Many of these drugs have been administered to the pregnant animal in a single dose on a specific day of gestation allowing investigators to observe their teratogenic effects at different times of fetal development (1–3). However, with the introduction of a new type of drug dosing, the miniature infusion pump[1], a distinctly different approach can be used to study the possible teratogenic influence of drugs. This instrument is a constant-flow delivery system which allows the long-term steady-state effects of a drug on the developing fetus to be ascertained. Because there has been no reported use of this constant-flow delivery system for teratology studies, the feasibility of using the miniature infusion pump in such a study was investigated.

To determine if the use of the miniature infusion pump was a reasonable alternative to other types of drug dosing, a pilot study was undertaken comparing the fetal effects of saline administered *via* the pump and by hypodermic injection. The pump implantation procedure and the presence of the pump during pregnancy was observed to have a minimal effect on the fetus, thereby providing the

[1] Alzet mini-osmotic pump, Model 2001, Lot No. 07885, Alza Corp., Palo Alto, CA 94304.

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