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Effect of some penicillins on the sensitivity of limulus amoebocyte lysate test

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Abstract—A group of penicillins have been tested for their effect on the sensitivity of limulus amebocyte lysate test (LAL). Cloxacillin, cephalothin and cefuroxime inhibited the gel formation at concentrations above 2 mg mL^{-1}, while ampicillin, methicillin and mecillinam showed no inhibitory effects upto concentrations of 10 mg mL^{-1}. For benzylepenicillin the maximum non-inhibitory concentration was 10,000 units mL^{-1}. However, the dilutions required to overcome inhibition were within the limits of maximum valid dilutions computed for each product showing that LAL test is valid for these products at their non-inhibitory concentrations.

The limulus amebocyte lysate test (LAL) is under investigation as an alternative to conventional rabbit fever test for the detection of pyrogens in pharmaceuticals (Eibert 1972; Cooper 1975; Hochstein 1981; Yano et al 1986). The test was included as The Bacterial Endotoxin Test (BET) in the USP XX and revised in the USP XXI and its first supplement. At present the LAL test has its widest application for the detection of endotoxins in water for injection, large volume parenterals, radiopharmaceuticals and biologicals. However a variety of solid formulations and small volume parenterals have been found to inhibit the LAL-endotoxin reaction (Twohy et al 1984). Antibiotics, in particular, are known to inhibit the clot formation at concentrations used for parenteral administration (McCullough & Scolnick 1976; Newsome 1977; Harrison et al 1979; Case et al 1983). We have screened some penicillin preparations, as proprietary formulations in their final form, for their inhibitory effects on the LAL test and report the highest concentrations that cause no inhibition under routine conditions.

Materials and methods

The antibiotic formulations tested were those manufactured by Hoechst Co. (benzylpenicillin), Bristol laboratories (ampicillin sodium), Beecham laboratories (methicillin sodium and cloxacillin sodium), Glaxo laboratories (cephalothin sodium and cefuroxime sodium) and Leo Pharmaceuticals (mecillinam). Control Standard Endotoxin, Lot No. 84 BE_1 obtained from Haemachem Inc., strain *Escherichia coli* 055:B_5 with a defined activity of 10 EU ng^{-1} in terms of RSE, EC−5 was used for spiking the antibiotic solutions. Lysate preparations were obtained from Whittaker M. A. Bio-products or Haemachem Inc. Details of the Lysate Lot No. and their sensitivities are given as subscripts to Table 1.

Serial dilutions of endotoxins were prepared in endotoxin-free water for injection containing specified concentrations of antibiotics and then 0·1 mL of each diluted solution was distributed into 10 × 75 mm test tubes. To these 0·1 mL of reconstituted lysate was added, mixed gently and the tubes incubated at 37° C for 60 ± 2 min. The tubes were examinined for the formation of a gel at the end of the incubation period. Those with a firm gel that adhered to the test tube in inverted position were scored as positive (+) and those with no gel or slightly opaque solution flowing freely from the tube on inversion were scored as negative (−).

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Results and discussion

The results on the effects of various concentrations of tested products on the sensitivity of lysate are presented in Table 1 and the data on their maximum non-inhibitory concentrations, dilutions required to overcome inhibition (DROI), maximum valid dilution (MVD) factors and the DROI:MVD ratios are delineated in Table 2. The data on non-inhibitory concentrations was based on direct dilutions of the antibiotics solutions without recourse to pH adjustments. At least two replicate endpoint assays were performed for each compound.

The results show that penicillins as a group vary greatly in their capacity to inhibit the LAL test. Cloxacillin, cephalothin and cefuroxime show greater inhibitory activity than benzylepenicillin, ampicillin, methicillin and mecillinam. Cloxacillin, cephalothin and cefuroxime inhibited the gel formation at

Table 1. Effect of some penicillins on the sensitivity of limulus amebocyte lysate test. Values for endotoxin concentrations below 0·032 EU mL^{-1} and water for injections were all negative.

Penicillins (mg or units* mL^{-1})		Endotoxin concn (EU mL^{-1}) 0·500	0·250	0·125	0·062
Benzylpenicillin[1]*	50000	−	−	−	−
	20000	+	−	−	−
	10000	+	+	−	−
	5000	+	+	−	−
	0	+	+	−	−
Ampicillin[2]	25	+	−	−	−
	10	+	+	+	+
	5	+	+	+	+
	2	+	+	+	+
	0	+	+	+	+
Methicillin[3]	50	−	−	−	−
	20	+	−	−	−
	10	+	+	+	+
	1	+	+	+	+
	0	+	+	+	+
Cloxacillin[3]	25	−	−	−	−
	10	−	−	−	−
	5	−	−	−	−
	2	+	+	+	+
	1	+	+	+	+
	0	+	+	+	+
Cephalothin[4]	5	+	−	−	−
	3	+	+	−	−
	2	+	+	+	−
	0	+	+	+	−
Cefuroxime[4]	50	−	−	−	−
	25	−	−	−	−
	10	−	−	−	−
	5	+	−	−	−
	2	+	+	+	−
	0	+	+	+	−
Mecillinam[4]	40	−	−	−	−
	20	+	+	−	−
	10	+	+	+	−
	0	+	+	+	−

[1] Lysate Lot No. 3KR (Pyrogent) with labelled sensitivity of 0·125 EU mL^{-1} was used for benzylpenicillin.
[2] Lysate Lot No. 74B1 (Limusate) with labelled sensitivity of 0·06 EU mL^{-1} was used for ampicillin.
[3] Lysate Lot No. 101B3 (Limusate) with labelled sensitivity of 0·057 EU mL^{-1} was used for methicillin and cloxacillin.
[4] Lysate Lot No. 14B1 (limusate) with labelled sensitivity of 0.125 EU mL^{-1} was used for cephalothim, cefuroxime and mecillinam.

Table 2. Maximum non-inhibitory concentrations in relation to maximum valid dilutions computed for some penicillins for use with limulus amebocyte lysate test.

Penicillins	Potency of the product (mg or units*mL^{-1})	Human or rabbit dose (M) (mg or units* Kg^{-1})	Endotoxin limit (K/M) (EU mg mL^{-1} or units^{-1}*)	Sensitivity of LAL used (EU mL^{-1})	Max non-inhibitory Concn (mg or units*mL^{-1})	DROI	MVD	DROI / MVD
Benzylpenicillin*	50,000	50,000	0·0001	0·250	10,000	5	20	0·250
Ampicillin	50	20	0·25	0·062	10	5	200	0·025
Methicillin	50	60	0·08	0·062	10	5	65	0·075
Cloxacillin	50	20	0·25	0·062	2	25	202	0·123
Cephalothin	100	50	0·10	0·125	2	50	80	0·625
Cefuroxime	125	21·4	0·23	0·125	2	62·5	230	0·272
Mecillinam	100	40	0·125	0·125	10	10	100	0·100

DROI = Dilution Required to Overcome Inhibition = Potency of the product/maximum non-inhibitory concentration.
Potency of the product = package size (mg/vial)/volume of water for injection for dilution.
MVD = Maximum valid dilution = Endotoxin limit (FDA list) X potency of product/sensitivity of lysate used.
K = 5 EU for any parenteral drug except those administered intrathecally.
M = Maximum human or rabbit dose kg^{-1} that would be administered in a 1h period, whichever is larger (source: Appendix D to FDA guidelines, December 1987).

concentrations above 2mg mL^{-1} while ampicillin, methicillin and mecillinam produced inhibition only above 10 mg mL^{-1}. Benzylpenicillin above 10000 units mL^{-1} interfered with the test. The results are in agreement with those reported by others for methicillin (Newsome 1977; Case et al 1983) and for cloxacillin and ampicillin (Newsome 1977). Case et al (1983) however, reported higher non-inhibitory concentrations for cephalothin (6 mg mL^{-1}) and ampicillin (25 mg mL^{-1}) and lower ones for benzylpenicillin (2000 units mL^{-1}). In the present experiments concentrations of 3 and 5 mg mL^{-1} of cephalothin and 25 mg mL^{-1} of ampicillin inhibited the test while benzylpenicillin showed no inhibition up to 10000 units mL^{-1}. The discrepancies with cephalothin and ampicillin may be due to incompatibility of pH, which was not adjusted in this study, or to formulation effects. For benzylpenicillin, the only apparent difference is the use of potassium salt by other workers (Case et al 1983) while we used the sodium salt.

The results of the present study also show that the inhibitory effects of these selected penicillins can be overcome by dilution (Table 1). To show that such dilutions did not affect the validity of the LAL test for these products, the values for dilutions required to overcome inhibition (DROI) were compared with the values for maximum valid dilution (MVD) for each product. For all the products tested the DROI values were lower than the MVD values (Table 2) showing that the products can be tested with LAL at the maximum non-inhibitory concentrations with lysate sensitivities around 0·06 EU mL.$^{-1}$ The dilution ratios between DROI and MVD ranged from 1:1·6 for cephalothin to 1:40 for ampicillin. With cephalothin and cefuroxime where the DROI/MVD index was lower, a more sensitive lysate would be useful. The results are presented for the purpose of providing information on the applicability of LAL test to some important penicillins. The dilution ratios found to overcome inhibition under specified condition serve only as guidelines and each manufacturer and testing laboratories have to confirm the levels of NIC's on their own before validating the LAL test for each product.

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