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RESEARCH REPORT

APPLICATION OF LIMULUS AMEBOCYTE LYSATE (LAL) TEST FOR DETECTING ENDOTOXIN (PYROGEN) IN LARGE VOLUME PARENTERALS

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ABSTRACT

Screening of twenty five large volume parenterals including dextrose, electrolytes, mannitol, metronidazole infusions, haemodialysis solution, water for injections and distilled water for the determination of pyrogen using Limulus Amebocyte Lysate (LAL) test has been carried out. Out of different preparations only one metronidazole injection exhibited positive LAL test, which was found pyrogen free with USP rabbit pyrogen test.

INTRODUCTION

For 37 years, the rabbit pyrogen test was the only practical pyrogen procedure described in regulatory compendia throughout the world (Addendum, 1989; Anonymous, 1980; 1983; Burdon, 1976; Dubczanski, 1873; Hort & Penfold, 1912; Kristansen, 1984; Levin & Bang, 1964a; 1964b and Panum, 1874). Although, it has met its purposes relatively well over the years, it remains an elaborate procedure subject to the variability inherent in all biological assays. The need for a simple, specific and accurate pyrogen test has long been recognized. Although a number of test systems have been devised for endotoxin detection, only the "Limulus Amebocyte Lysate (LAL) Test" is potentially a satisfactory alternative to the USP rabbit pyrogen test.

With the advent of a sensitive, specific, inexpensive and rapid assay for endotoxin, a number of workers investigated its potential use in the pharmaceutical industry as an alternate to the rabbit pyrogen test.

The development of a viable alternative to the rabbit pyrogen test, began with the innovative work of John Hopkins University investigators Levin and Bang (Prior & Spagna, 1979 and Anonymous, 1996) observed that bacterial infection causes intravascular coagulation in the American Horseshoe crab.

Levin and Bang further reported that the agent responsible for clotting phenomenon resided inside the amebocytes (blood cells) for Horseshoe crab. Bacterial endotoxin was found to be responsible for initiating this gelation, characterized by an enzymatic gelation reaction. An enzyme that produces coagulation, exists in the lysate as an inactive proenzyme, becomes activated in the presence of endotoxin. It then reacts with another protein called Coagulogen, that initiate an enzymatic coagulation cascade, ending in a solid clot (Figs. 1 and 2).

The application of Limulus Amebocyte Lysate (LAL) test to parenterals arose because a suitable means of detecting pyrogens in short-lived radioactive drugs was needed. Following a decade of use as a pyrogen screen, the LAL test is now established as a quality assurance measure for parenterals because of its simplicity, sensitivity, specificity and reproducibility. To date, pharmaceutical industry is utilizing the new approaches in different fields primarily for improving drug quality through in-process testing and for trouble shooting specific problems (Roussy, 1889).

The present work will provide a firm bais in Pakistan by introducing and establishing LAL test to national and multinational pharmaceutical industries for the detection of pyrogenic substances.

EXPERIMENTAL

Twenty-five samples of commonly available, locally manufactured and imported LVPs of various manufacturers were procured from different pharmacies of Karachi city. The date and place of samples purchased and batch numbers of each were kept (Table-1). The lyophilized kit of LAL reagent, "PYROGEL^{R}" was procured from M/s. CONCEPT GmBH, Germany.

Preparation of Samples:

All operations in collection and preparation of samples were performed aseptically using depyrogenated apparatus and non-pyrogenic reagents as recommended in British Pharmacopoeia 1999 (Seibert, 1923). Samples were diluted according to U.S. Food and Drug Administration (FDA) screening procedures to make it compatible with the test (Seibert, 1925). The maximum valid dilution (MVC) calculated has been reported in Table-2.

Determination of the Labelled Sensitivity of the Lysate:

The sensitivity of the lysate as calculated by standardized method was equal to the labeled sensitivity. It was expressed in EU/ml (Table-2).

Table-3 represent results of positive and negative controls.

Methodology:

Among various reported methods of endotoxin (pyrogen) detection with LAL assay, following was used during the present study:

Microtube Method:

The method was first reported by Ole Kristensen in 1964 (Seibert and Mendel, 1923). In this method the tubes were replaced by microtubes (capillaries). The details of the procedure are same (Prior & Spagna, 1979; Anonymous, 1996; Roussy, 1889; Seibert, 1923, 1925 and Seibert & Mendel, 1923) except that instead of 0.1 ml, 10 ml of the reagents were used in the test.

RESULTS AND DISCUSSION

The screening of twenty five (Table-1) infusion samples (including dextrose 5%, sodium chloride 0.9%, mannitol 20%, Hartmann (compound sodium lactate) solutions, dextrose saline solutions, metronidazole injections, amino acids solutions, haemodialysis solution, sterile waters for injection manufactured by different companies and distilled water from the storage tank of laboratory of Department of Pharmaceutics has been carried out for the detection of pyrogen using Limulus Amebocyte Lysate test.

Table 1
Results of Microtube Method

S. No.	Drug Product/ Labeled Strength	Manufacturer Name	Batch No.	pH Adjustment	MVD*	Results I	Results II
1.	Normal Saline 0.9%	Otsuka	214 J91	Not Required	4	-	-
2.	Normal Saline 0.9%	B. Braun	049242	Not Required	4	-	-
3.	Normal Saline 0.9%	Medipak	109205	Not Required	4	-	-
4.	Normal Saline 0.9%	Swepak	10526A	Not Required	4	-	-
5.	Dextrose 5%	Otsuka	111 E91	Required	2	-	-
6.	Dextrose 5%	B. Braun	M108442	Required	2	-	-
7.	Dextrose 5%	Medipak	110264	Required	2	-	-
8.	Dextrose 5%	Swepak	10489A	Required	2	-	-
9.	Mannitol 20%	Otsuka	20L 86	Required	4	-	-
10.	Osmofundin 20%	B. Braun	M030443	Required	4	-	-
11.	Aminovel L600 5%	Otsuka	21H 94	Required	68	-	-
12.	Aminoplasmal L5	B. Braun	013262B	Required	68	-	-
13.	Nutrisol 5%	Green Cross	176HS	Required	68	-	-
14.	Perisolution	Otsuka	256 K91	Required	4	-	-
15.	Peritofundin	B. Braun	M908144	Required	4	-	-
16.	Ringolact Solution	Otsuka	234 K90	Not Required	4	-	-
17.	Compound Sodium Lactate Solution	B. Braun	M033141	Not Required	4	-	-
18.	Diasol 34 Standard	Baxter	GR131268	Not Required	34	-	-
19.	HD Concentrate	B. Braun	0361057/8	Not Required	34	-	-
20.	0.9% NaCl 5% Dextrose	B. Braun	M945142	Not Required	4	-	-
21.	Water for Injection	B. Braun	M920111	Not Required	-	-	-
22.	Water for Injection	Squibb	1228	Not Required	-	-	-
23.	Distilled Water	Pharmaceutics Lab	311291	Not Required	-	+	+
24.	Metronidazole Inj.	B. Braun	M935161	Required	13	-	-
25.	Abozole Injection	Abbott	53-801-XV	Required	13	+	+
26.	Flagyl Injection	M & B	DE1857	Required	13	-	-

*Maximum valid dilution.

Table-1 exhibited the results obtained by microtube or microgel methods. Ole Kristensen (Seibert & Mendel, 1923) described a microtechnique using capillaries, but in the present study this method has been modified by using microtubes. Samples were incubated for 55 minutes, after which gel formation was observed in metronidazole injection (sample No. 25) and water sample from laboratory (Sample No. 23).

Table 2
Determination of Labelled LAL Sensitivity
Microtube Method

Replicate No.	Endotoxin Concentration EU/ml				End Point
	0.5	**0.25**	**0.125**	**0.0625**	
1	+	+	-	-	0.25
2	+	+	+	-	0.125
3	+	+	-	-	0.25
4	+	+	+	-	0.125

Geometric Mean: 0.25
Lysate Sensitivity: 0.25 EU/ml

Table 3
Results of Controls - Microtube Method

Positive Control	**I**	**II**
0.25 EU/ml	+	+
0.25 EU/ml	+	+
0.125 EU/ml	+	-
0.0625 EU/ml	-	-
Negative Control	-	-

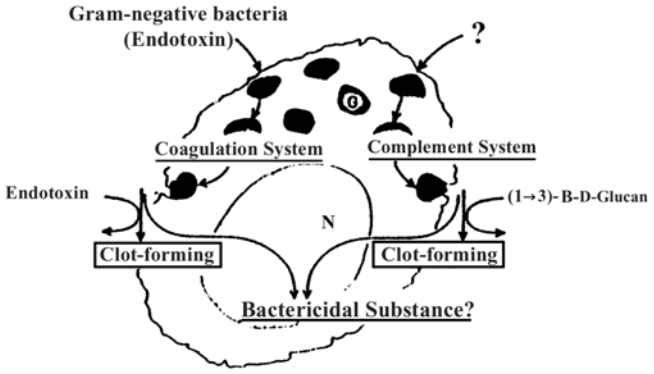

Fig. 1: Schematic visualization of two independent coagulation pathways, endotoxin-mediated and β-D-glucan-mediated pathways, found in Limulus amebocytes (adopted from Iwanaga, 1984).

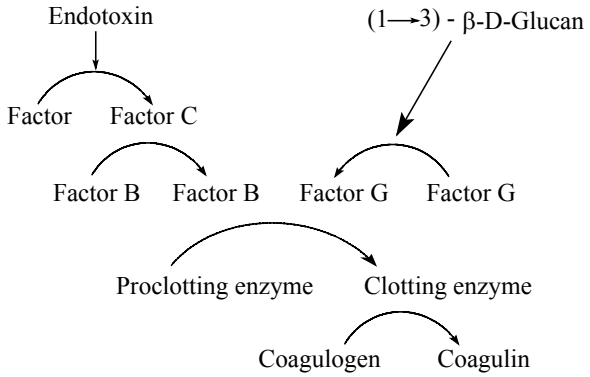

Fig. 2: Tentative mchanism of the *Limulus* coagulation cascade (adopted from Iwanaga, 1984).

The modification infact is a combination of Ole Kristensen method using 10 µl of both lysate and sample and Prior and Spagna method (Seibert and Mendel, 1923), who used microtube U-bottom plates for mixing 50 µl of lysate 50 µl of sample. Glass microtubes available commonly in microbiological laboratories were used in this study. Results indicated no significant difference in sensitivity between parallel clot end point and microtube end point assays, hence the results of present study are in confirmation with the work of Ole Kristensen and Prior and Spagna (Seibert and Mendel, 1923ab).

The results also confirmed the simplicity and rapidity of LAL test as it allows more frequent testing, thus providing an added measure of assurance in pyrogen testing programme. Due to these extra advantages the test has been officially accepted in many countries of the world (Addendum, 1989; Anonymous, 1980; 1983; Burdon, 1976; Dubczanski, 1873; Hort & Penfold, 1912; Kristansen, 1984; Levin & Bang, 1964a; 1964b; Panum, 1874; Prior & Spagna, 1979; Anonymous, 1996; Roussy, 1889; Seibert, 1923, 1925 and Seibert & Mendel, 1923).

It is expected that the present study will act as a milestone in implementing and establishing the Limulus Arnebocyte Lysate (LAL) test, in research institutions and especially in pharmaceutical industries of Pakistan as a latest technique for pyrogen testing.

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