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Short communication

Detection of pyrogenicity on medical grade polymer materials using rabbit pyrogen, LAL and ELISA method

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ABSTRACT

The objective of the study is to detect the pyrogenicity of five medical grade gelatinous polymer materials, intended for the manufacturing of capsule for pharmaceutical applications, by an indigenously developed ELISA, LAL and rabbit pyrogen assays. The ELISA methodology includes the incubation of the sample extract with blood from a healthy donor at 37 °C. Any pyrogen present in the extract induces the IL-1β which can be determined by ELISA. The rabbit pyrogen and LAL assays were performed as per standards. The result of the ELISA method indicated that all the materials extract induced high level of IL-1β as a marker for pyrogenicity. The rise in temperature of rabbit pyrogen was above 0.5 °C in all materials extract. LAL assay induced an endotoxin level above 0.5 EU. All the five polymer materials were found pyrogenic in all the assays. The ELISA method is very sensitive because the lowest limit of detection was 10 pg/ml endotoxin. Hence it can be concluded that the ELISA method will be an added advantage for the quality control release of a batch of medical products and improving the existing methodologies in the context of reduction and replacement in the use of animal models.

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1. Introduction

Pyrogens have been measured with the *in vivo* rabbit pyrogen test, where the drugs are injected into at least three rabbits and the fever reaction measured by a rectal probe. The rabbit pyrogen test based on the intravenous injection of a sterile solution was adopted for many years for the quality control of parenteral preparations. In new therapies such as recombinant human proteins or cellular therapeutics, the rabbit pyrogen assay is unviable due to false positives resulting from species specificity in the immunological recognition of these agents [1,2]. The *in vitro* Limulus Amoebocyte Lysate (LAL) test, detects only one class of pyrogen, endotoxin (Lipopolysaccharides: LPS) from gram negative bacteria, leaving the patients at risk from undetected non-endotoxin pyrogens such as gram positive bacterial toxins, viruses and fungi [1]. Endotoxin induces multiple biological effects *in vivo*, for example, fever, leukocytosis, hypoferremia, platelet aggregation, thrombocytopenia and coagulopathies. These effects can be attributed to activation of various endogenous pathways or cascade mechanisms. Toxicity of LPS (endotoxin) is, to a large extent, mediated by the activation of monocytes/macrophages and subsequent release of cytokines, such as IL-1β and tumor necrosis factor alpha (TNF-α). It is known that LPS binds readily to serum lipoproteins and those LPS–lipoprotein complexes are less toxic than unbound LPS [3,4]. Macrophages and mononuclear phagocytes are known to produce and release a variety of cytokines, including Interleukins (IL) and tumor necrosis factor (TNF), in response to endotoxin stimulation both *in vivo* and *in vitro*. These cytokines mediate the harmful effects of the endotoxins *in vivo* leading to endotoxemia which can result in septic shock and multiple system organ failure [5,6].

An ELISA method developed by our group using human whole blood was used for the detection of cytokines, specifically IL-1β. In this assay, the release of IL-1β, on direct contact of the immunocompetent cells (monocytes/macrophage) with the surface of any material of interest can be measured by ELISA. In other words, a heterogeneous phase reaction is the basic physicochemical principle of the assay. This ELISA method is more versatile than the *in vivo* rabbit test with respect to applications to medical devices, pharmaceuticals and blood derived products (unpublished data). Hence an effort was made to detect the pyrogenicity of five medical grade polymer (gelatin) materials, intended for the manufacturing of capsule for pharmaceutical applications, using an indigenously developed ELISA method, LAL and rabbit pyrogen assay.

2. Materials and methods

2.1. Reagents and materials

Physiological saline (Bendtt, India), medical grade polymer (India) intended for the manufacturing of capsule for pharmaceutical applications, ELISA plates (Nunc Maxisorb, USA), Autoclave

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(Relience, India), Glass wares (Borosil, India), LAL reagents and kits (Charle's River, USA), Indigenous ELISA kit (SCTIMST, India). All the reagents and materials employed in the LAL and rabbit pyrogen tests were depyrogenated by washing in detergent solution and by heating at 250 °C for 30 min as per International standards.

2.2. Equipments

ELISA reader (ASYS, Germany), Ultra high speed centrifuge (Eppendoff, USA), High Accuracy Thermometer (Palmer, Germany), Deep freezer (Sanyo, Japan), Incubator (Sanyo, Japan), Hot air oven (Sanyo, Japan), Laminar bench (Elite, India), Endosafe PTS instrument (Charle's River, USA) were used in the study.

2.3. Rabbit pyrogen test

2.3.1. Preparations of materials extract

Four grams each of the five medical grade polymer gelatin materials (GEL1, GEL2, GEL3, GEL4 and GEL5) were taken into separate 250 ml pyrogen free conical flask containing 100 ml of physiological saline each and allowed to soak for 2 h and then kept in a water bath at 60 °C until a clear solution was obtained. The flasks were then covered with clean aluminum foil and autoclaved at 121 °C for 5 h, followed by cooling the solutions to 37 °C and the volume was made up to 100 ml to replace the saline loss during autoclaving. These solutions were used for intravenous injection.

2.3.2. Rabbit pyrogen assay

Rabbit pyrogen assay carried out as per ISO 10993-11 and the evaluation criteria is. If no rabbit shows an individual rise of 0.5 °C or more above its respective control temperature, the material meets the requirement for absence of pyrogen, i.e. non-pyrogenic [7,8]. All the animal experimentations were carried out in accordance with approved institutional protocols, CPSEA Guidelines for the Care and Use of Laboratory Animals and Institutional Animal Ethics Committee.

2.4. Limulus Amoebocyte Lysate (LAL) test

The extract prepared as per Section 2.3.1 was used for the LAL test and ELISA method. LAL test was carried out with the PTS instrument (Endosafe PTS instrument). 25 μl of the 1:4 diluted material extract was pipetted into the four sample reservoirs of the cartridge of the PTS instrument. The instrument automatically draws and mixes the sample with the LAL reagent in two of the channels (sample channels) and with the LAL reagent and positive product control in the other two channels (spike channels). Optical density of the wells was measured and analyzed against an internally archived standard curve in the instrument. The endotoxin level, spike recovery and CV percentage was obtained as a printout [9].

2.5. ELISA method for pyrogen test

The development and assays related with the ELISA method (developed by our group) was briefly detailed here, the aqueous solution of medical grade polymer gelatin prepared as mentioned earlier was incubated with 150 μl of blood to a final concentration of 400 μg/ml and 4000 μg/ml to final volume of 1.0 ml (15%, v/v) in a pyrogen-free microfuge tubes and incubated at 37 °C in an incubator for definite time-period. Reaction standards containing endotoxin at concentrations of 0.1, 0.5, 1, 2 and 5 EU/ml were also performed parallel. The reaction mixture was centrifuged and serum used for the ELISA estimation. This procedure was performed for all the five polymer gelatin materials: GEL1, GEL2, GEL3, GEL4 and GEL5. Anti-human IL-1β in 50 mM carbonate–bicarbonate buffer coated plates was blocked with blocking solution (1% BSA) for 1 h at room temperature. After washing with washing buffer, 50 μl of the diluted serum samples (1:20 in blocking solution) as well as cytokine standards were incubated for 2 h at room temperature. Internal cytokine standards were included in every test. The wells were washed again and diluted HRP-conjugated anti-IL-1β and incubated at room temperature for 2 h. After washing, the wells were incubated with TMB substrate for 30 min and the optical density was measured at 450 nm filter with 620 nm corrective filters using an ELISA reader.

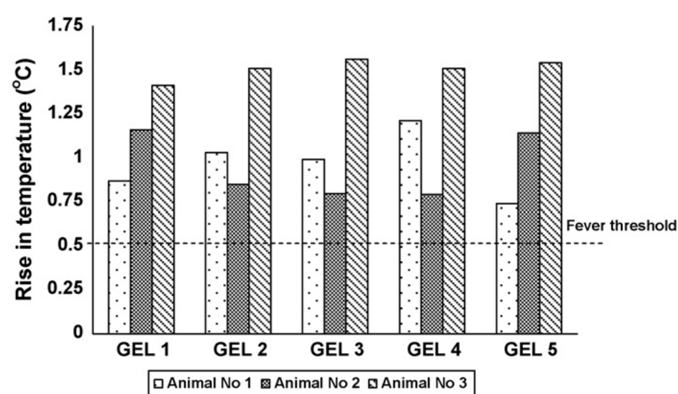

Fig. 1. Rise in temperature following the intravenous injection of five polymer gelatin materials extract.

3. Results and discussion

3.1. Rabbit pyrogen test

The general physical conditions of all the 15 experimental rabbits were normal through out the conditioning period. The body weight and feed intake was also normal during the experimental period. None of the animals showed any abnormality or behavioral changes during this period. The results of the experiments are shown in Table 1. The observed temperatures indicated that a rise in temperature following the intravenous injection of an aqueous solution of polymer gelatin GEL1 administered animals were 0.87 °C, 1.16 °C and 1.41 °C in animal numbers 322♀, 330♂ and 346♀ respectively. The rise in temperature of animal numbers 324♀, 321♂ and 339♀ treated with GEL 2 polymer gelatin material was 1.03 °C, 0.85 °C and 1.51 °C respectively. It was found that the rise in temperature was above 0.5 °C (0.99 °C, 0.80 °C and 1.56 °C) in all the animal numbers, i.e. 340♀, 345♀ and 335♀ treated with polymer gelatin GEL 3 material extract. In animals treated with polymer gelatin GEL 4, the rise in temperature noted was 1.21 °C, 0.79 °C and 1.51 °C in animal numbers 380♀, 378♀ and 357♂ respectively. It was also found that, there was a rise in temperature of 0.74 °C in animal number 333♂, 1.14 °C in animal number 334♀ and 1.54 °C in animal number 326♀ administered with polymer gelatin GEL 5 extract (Table 1 and Fig. 1).

3.2. Limulus Amoebocyte Lysate (LAL) assay

The LAL assay using PTS method is an advanced method and widely used as a quantitative method. The aqueous solutions of all the five polymer gelatin material (GEL 1, GEL 2, GEL 3, GEL 4, and GEL 5) were prepared as mentioned above. All the extracts prepared as described earlier were diluted further (1:4 dilution, polymer extract:pyrogen free physiological saline) and used for the LAL assay as per the Endosafe PTS method. The results of the present study are summarized in Table 2 showing the relationship between polymer samples. The results clearly indicated that an endotoxin

Table 1
Temperature variation during rabbit pyrogen test after intravenous injection of polymer gelatin material.

Material	Rabbit No and body weight	Volume of extract. injected (ml)	Temperature before 30 min (°C)	Temperatures (°C) after minutes of injection						
				60 min	90 min	120 min	150 min	180 min	Rise in temperature (°C)	Result
GEL 1	322♀ 2414 g	24	38.99	39.04	39.86	39.84	38.70	39.65	0.87	Pyrogenic
	330♂ 2093 g	21	39.29	40.02	39.93	40.45	40.36	40.28	1.16	
	346♀ 2790 g	28	38.66	39.50	39.92	39.82	40.07	39.63	1.41	
GEL 2	324♀ 2380 g	24	39.23	39.47	40.00	40.26	40.19	40.02	1.03	Pyrogenic
	321♂ 2000 g	20	39.04	39.10	39.70	39.89	39.77	39.59	0.85	
	339♀ 2126 g	21	39.15	39.63	39.99	40.66	40.28	40.32	1.51	
GEL 3	340♀ 2619 g	26	39.05	39.22	40.04	39.92	39.77	39.82	0.99	Pyrogenic
	345♀ 2531 g	25	39.47	39.53	40.27	40.03	40.02	39.87	0.80	
	335♀ 2377	24	38.30	39.43	39.83	39.86	39.79	39.80	1.56	
GEL 4	380♀ 2462 g	25	39.09	39.44	40.30	40.09	39.85	39.95	1.21	Pyrogenic
	378♀ 2149 g	21	39.19	39.04	39.82	39.98	39.98	39.67	0.79	
	357♂ 2000 g	20	39.01	39.30	40.35	40.41	40.52	40.42	1.51	
GEL 5	333♂ 2042 g	20	39.13	39.29	39.48	39.87	39.69	39.55	0.74	Pyrogenic
	334♀ 2240 g	22	39.52	39.74	40.39	40.66	40.27	40.17	1.14	
	326♀ 2527 g	25	38.34	39.54	39.88	39.84	39.80	39.82	1.54	

GEL: polymer gelatin material; ♂: male; ♀: female.

Table 2
Limulus Amoebocyte Lysate assay using Endosafe-PTS system.

Material	Volume of extract	Dilution (with LAL water)	Endotoxin level (EU/ml)	Spike recovery (%)	CV%
GEL 1	25 μl	1:4	2.91	121	6.7
GEL 2	25 μl	1:4	4.22	66	10.7
GEL 3	25 μl	1:4	2.01	111	1.2
GEL 4	25 μl	1:4	1.87	87	8.1
GEL 5	25 μl	1:4	2.31	66	8.4

GEL: polymer gelatin materials.

level of 2.91, 4.22, 2.01, 1.87 and 2.31 EU/ml were obtained from the aqueous solutions of polymer GEL 1, GEL 2, GEL 3, GEL 4, GEL 5 respectively. The sensitivity of the assay was 0.5–5 EU. The positive and negative control was run simultaneously to evaluate the functioning of the system. It was documented that 0.5 EU/ml is widely accepted as the fever threshold [10–12], and all the polymer extracts subjected were deemed as pyrogenic (Fig. 2). These observations are in par with the *in vivo* pyrogen test mentioned above (Fig. 1).

3.3. Indigenously developed ELISA method for pyrogen assay

The aqueous solutions of medical grade polymer gelatin materials were incubated with small volume of human whole blood for 3 h at 37 °C. After incubation the aliquots were centrifuged and serum were used to evaluate pyrogens by ELISA method ($n = 4$, Fig. 3). The endotoxin standard curve plotted from OD of endotoxin standard reactions (Fig. 4) was used to quantitate the amount of pyrogen present in polymer aqueous solution. The results indicate that all the polymer material extracts induced significant level of IL-1β and were above the fever threshold limit of 0.5 EU/ml [10–12]. Hence, all the five polymer extracts were deemed as pyrogenic at a concentration of 4000 μg/ml. These observations were confirmed by *in vivo* pyrogen assay and LAL test (Figs. 1 and 2).

The pyrogen assay was carried out in the context of the importance of a comparative assessment of pyrogenicity and an

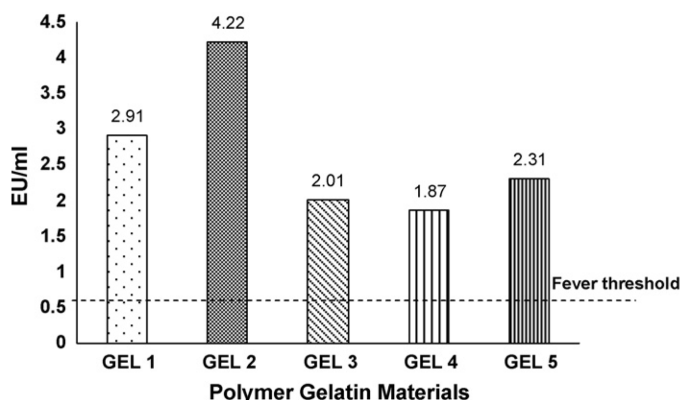

Fig. 2. Endotoxin release level from five polymer gelatin materials extract using LAL test.

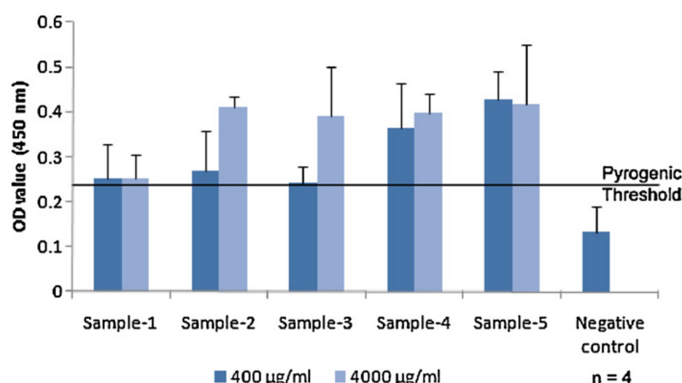

Fig. 3. Interleukin 1β release level from five polymer gelatin materials extract using ELISA.

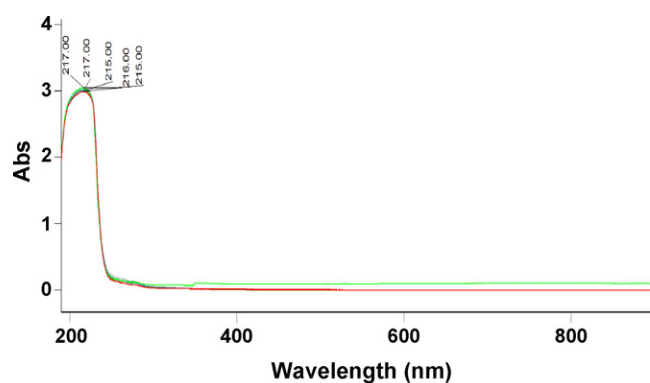

Fig. 5. Absorption level of five polymer gelatin material using a UV spectrophotometer.

alternative, which could contribute towards its refinement. The rabbit pyrogen test has served for drug safety control for more than 50 years and is laborious and expensive. Most biologicals, especially blood derived drugs; the rabbit experiment still represents the only choice, at an annual expense of hundreds of thousands of animals in the European Union [1]. For new therapies such as recombinant human proteins or cellular therapeutics, the rabbit pyrogen assay is not applicable, because in many cases, false positive results were obtained due to the species specificity of the immunological recognition of these agents [1,13].

In the present study an attempt was made to detect the pyrogenicity of commercially available polymer gelatin materials intended for the manufacturing of capsules for pharmaceutical applications and to comparatively assess the traditional *in vivo* rabbit pyrogen test, the USP accepted LAL test and an indigenously developed ELISA method. The polymer gelatin materials (GEL 1, GEL 2, GEL 3, GEL 4 and GEL 5) were soaked for 2 h in physiological saline at 60 °C until a clear solution obtained, autoclaved and used for pyrogen test. The pH of the aqueous solution was 6.7 (GEL 1), 6.7 (GEL 2), 6.5 (GEL 3), 7.6 (GLE 4) and 6.5 in GEL 5 material respectively. The aqueous extract was scanned under a spectrophotometer at UV range indicated that all the extracts were in the same absorbance level (Fig. 5).

The results of the rabbit pyrogen assay are summarized in Table 1 and Fig. 1 showing that the maximum rise in temperatures were 1.41 °C, 1.51 °C, 1.56 °C, 1.51 °C and 1.54 °C, when the animals were administered aqueous solutions of polymer gelatin GEL 1, GEL 2, GEL 3, GEL 4 and GEL 5. The rise in temperature of all the 15 animals treated with the aqueous solutions of polymer gelatin samples (GEL 1, 2, 3, 4 and 5) were above the acceptable level, i.e. 0.5 °C as recommended by USP and ISO 10993-11. The test was not repeated for any of the materials since the average total rise in temperature of all the three animals in a group was above 3.3 °C according to the criteria mentioned by USP and ISO [7,8].

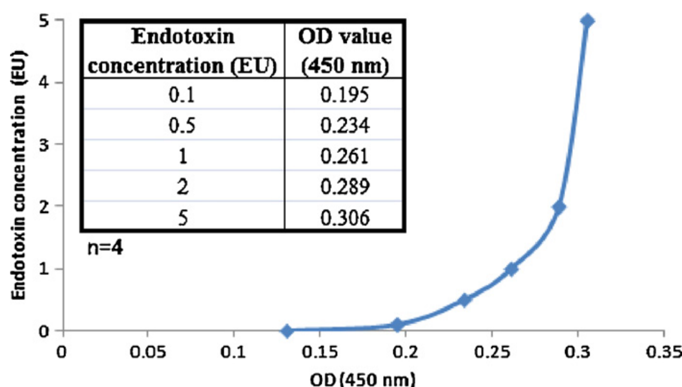

Fig. 4. Endotoxin standard reactions.

The results of the LAL test using PTS method are summarized in Table 2. It was found that the aqueous solution of all the five gelatin materials induced an endotoxin level of above 0.5 EU/ml, which is above acceptable level, as per USP and declared as pyrogenic (Fig. 2). Hochstein et al. reported that a dose of 13.81 EU/ml/kg of the 2nd International Standard for bacterial endotoxins was identified as that which produced a 0.5 °C rise in temperature [10,13]. This value, although variable according to the strain of the animals used, is recommended as a criterion for positive responses. Despite its shortcomings, the test is recommended by the Pharmacopoeias and other standards [7,8], and is important for the validation of new *in vitro* assays under development [1,14–17]. The *in vitro* LAL test, which measures the coagulation in a lysate prepared from the blood of the horseshoe crab, specifically initiated by endotoxins, can only be performed with solutes and detects only one class of pyrogen, i.e. endotoxin from gram negative bacteria, leaving the patients at risk from undetected pyrogens of non-endotoxin origin such as gram positive bacterial toxins, viruses and fungi [1].

The result of the ELISA method (Figs. 3 and 4) indicates that the aqueous extract of all the five polymer gelatin materials induced significant level of IL-1β. It was well documented that 0.5 EU/ml is widely accepted as the fever threshold [10,12] and hence it is suggested that at a higher concentration of 4000 μg/ml, all the five polymer extracts were deemed to be pyrogenic and these results were confirmed by *in vivo* pyrogen test (Fig. 1).

All the five aqueous extracts of polymer gelatin material (GEL 1, 2, 3, 4 and 5) induced a fever threshold above 0.5 °C, which is considered to be pyrogenic according to the International Standards ISO and USP. The requirement of LAL test was also not met by the materials since all showed a value above 0.5 EU/ml, confirming their pyrogenicity. The pyrogenicity of the five gelatin materials was further confirmed by the over threshold limit release of IL-1β by the indigenously developed ELISA method. It was also found that the lowest limit of detection of IL-1β is 10 pg/ml level. The sensitivity and specificity of the indigenously developed ELISA method, Limulus Amebocyte Lysate assay and rabbit pyrogen assay were thus assessed with five medical grade polymer gelatin materials. The result of the study indicates that the aqueous solution of all the five gelatin materials was found pyrogenic in LAL assay and indigenously developed ELISA method and these observations were confirmed by *in vivo* pyrogen test. Recently, an *in vitro* monocyte activation assay using human whole blood, was included in European Pharmacopoeia [18], hence an ELISA based technique will be an alternative to animal experimentation.

4. Conclusion

The observations of the study also demonstrate that, the indigenously developed ELISA method provides more sensitive and accurate results with a lower detection limit of 10 pg/ml (0.1 EU/ml) level. Hence it is emphasized, the importance of the indigenously developed ELISA method for the evaluation of pyrogenicity as a quality control of parenteral medicinal products or medical devices. This ELISA method will provide an added advantage for the quality control release of a batch of medical products, pharmaceuticals, etc., and improving the existing methodologies in the context of reduction and replacement in the use of animal models.

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