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Comparative Evaluation of Chromogenic Limulus Assay and Rabbit Pyrogen Test for Water For Injection

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A comparative study of application of CLAL (Chromogenic Limulus Amebocyte Lysate) and rabbit pyrogen test on 35 samples of Water for Injection was performed. With the pyrogen test, the accumulated temperature rise ranged from + 0.2° to + 1.3° passing all the samples by the rabbit pyrogen test. The endotoxin content varied from as low as 0 EU/ml (Endotoxin Unit per milliliter) to 0.15 EU/ml as tested by CLAL test suggesting that all samples had endotoxin levels below the prescribed levels of 0.25 EU/ml. No correlation could be established between the endotoxin content and temperature rise.

The preparations for which the LAL (Limulus Amebocyte Lysate) test has proven itself as a final product release test for pyrogen include large volume parenteral¹, radiopharmaceuticals², intravenous fat emulsions³, and iron dextran⁴. However there are no published studies/reports available on application of LAL to parenterals from India. Thus in the present study, comparison of the application of CLAL test and rabbit pyrogen test with respect to a simple parenteral preparation like Water for Injection was performed.

All glassware used in this study was rendered free of endotoxin by heating in an oven at 250° for 2 h. An ELISA reader (FLOW Laboratories, USA) was used for measuring the absorbance at wavelength 405 nm and temperature sensing probe (M/S Electrolab) was used.

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As many as 35 samples of Water for Injection were randomly collected from different commercial sources all over Mumbai. Rabbits having detailed specifications as described later were obtained from M.K. Rangnekar Laboratories, Bombay College of Pharmacy, Kalina, Mumbai. The CLAL assay kit used for this study, was obtained from M/S Bio Whittaker, Inc. 8830, Brggs Ford Road, Walkesville, MD 21793, U.S.A.

All reagents in the kit were reconstituted as per the instructions supplied along with the kit. In each series of the determinations, four standard endotoxin (0111B4; 19 EU/ml) solutions viz., 0.1, 0.25, 0.5, 1.0 EU/ml were prepared as per manufacturer's instructions. Each dilution was vigorously vortexed for at least 1 min before using it in the experiment. Since the chromogenic assay is time-dependent, the reagents were pipetted in the same order

TABLE 1: PROTOCOL FOR CLAL TEST

Reagents	Sample/Standard wells	Blank well
Test sample or endotoxin standard at room temp.(28±2°)	50 µl	—
LAL reagent water	—	50 µl
Limulus Amebocyte Lysate(LAL)	50 µl	50 µl
Mix & incubate at 37±1°	10 min	10 min
Prewarmed reconstituted Chromogenic Substrate at 37±1°	100 µl	100 µl
Mix & incubate at 37±1°	6 min	6 min
Stop reagent (25% Acetic Acid)	100 µl	100 µl
	Mix immediately	
Determine absorbance at wavelength	405 nm in ELISA reader	

— indicates no addition

and at the same rate from well to well as per the procedure described in Table 1. First two wells were used for blank or negative control. Next 8 wells were used for four endotoxin standards in duplicate. The remaining were used for samples in duplicate.

To calculate the endotoxin concentration of unknown sample a graph was plotted taking endotoxin concentration (EU/ml) on the X-axis against absorbance at 405 nm in the range of 0.1 to 1.0 EU.ml on the Y-axis . The absorbance for each endotoxin standard was detected by subtracting the absorbance of blank from the actual value of absorbance of the standards.

Since the endotoxin levels in some of the samples of Water for Injection would be expected to be below 0.1 EU/ml; the CLAL assay was performed by increasing the sensitivity as recommended in the kit and also by the United States Food and Drug Administration⁵ in the Guidelines for LAL test. Following modifications were thus made. Endotoxin standards were prepared between 0.01 to 0.1 EU/ml. The first incubation period was extended to 30 min to enable measuring the concentration of endotoxin in the range of 0.01 to 0.1 EU/ml. The second incubation was same for 6 min. To test for inhibition and enhancement of the assay system, CLAL assay was performed on the samples to be tested for endotoxin levels after spiking them with 0.4 EU/ml of the endotoxin

standard in duplicate. If the detected amount of endotoxin in spiked sample was within ±25% of the spiked value, it was taken to indicate no enhancement or inhibition of the assay system.

For the pyrogen test^{6,7,8}, healthy, adult albino Belgium rabbits, weighing not less than 1.5 kg were selected. They were fed on a complete and balanced diet as per Indian Standard (IS:5654, part 1, 1970) and care was taken to see that the rabbits did not loose any body weight during the week preceeding test. The animals were kept individually in an area of uniform temperature (±2°), with uniform humidity, and free from disturbances that are likely to excite them. The test rabbits, were kept in retaining boxes in such a way that the animals were retained only by loosely fitting neck-stokes; the rest of the body remained relatively free so that the rabbits could sit in a normal position. The animals were introduced into the retaining boxes 1h before the start of the test. When the animals were used for the first time, they were conditioned, 1-3 d before the final test, by injecting them intravenously, in to the marginal ear vein, 10 ml/kg of a pyrogen-free saline (0.9% NaCl) solution. Food was withheld from animals overnight and until the test was completed. Water was withheld during the test. Using the Sham test, preliminary testing of the animals was done when they were used for first time⁷. The main test was

carried out using a group of three rabbits per test. Using accurate temperature-sensing probe for each rabbit, temperature responses were noted. If the sum of the increase in temperature response of the group of three rabbits does not exceed 1.4° and if the response of any individual rabbit is less than 0.6°, the preparation being examined passes the test. If the response of any rabbit is 0.6° or more or if the sum of the responses of the three rabbits exceed 1.4°, the test was repeated using five additional rabbits. If, not more than three of the eight rabbits showed individual responses of 0.6° or more, and if the sum of the responses of the group of eight rabbits did not exceed 3.7°, the preparation being examined passes the test.

With a view to establishing a correlation, if any, between the temperature responses of pyrogens in Water for Injection by rabbit pyrogen test and endotoxin concentration by CLAL assay, in the present work, as many as 35 samples of Water for Injection were selected randomly from all over Mumbai were tested. Before testing for endotoxin by CLAL test as per legal regulation, it is a common practice to carry out an initial quality control test to test for any enhancement or inhibition, likely to be contributed by the test sample in the LAL test and the recovery of spike should be $\pm 25\%$. Accordingly in the present work, four different samples of Water for Injection were checked with and without spike (0.4 EU/ml) for the spike recovery. The amount of spike recovered was found to range from 0.39 EU/ml to 0.4 EU/ml. Thus the percentage of spike recovery varied from 97.5% to 100% indicating no inhibition or enhancement in the system. Thus Water for Injection samples can be used directly for CLAL assay without any pretreatment as was expected due to the nature of the sample. Similar observations are reported earlier⁹⁻¹⁰.

The samples of Water for Injection were tested by CLAL test for their endotoxin content. The samples collected from different sources showed EU/ml levels as low as 0.00 EU/ml as high as 0.15 EU/ml. As many as 6 samples showed complete absence of endotoxin while 14 samples showed 0.099 EU/ml levels or below. Eleven samples showed 0.1 EU/ml and only 2 samples showed 0.2 EU/ml. As per the Phamacopoeia⁶⁻⁷ a level of 0.25 EU/ml is permissible indicating that the locally available

samples were found to pass by CLAL test.

The individual temperature rise of each of the three rabbits used for one sample and also the accumulated temperature rise for these 35 samples subjected to Rabbit pyrogen test were recorded. It was observed that for as many as 35 samples of Water for Injection the accumulated temperature rise ranged from +0.2° to as high as +1.3°. For most of the samples, the response of three rabbits used for testing was found to be substantially variable. Only in two cases no variation was observed in the reading from three rabbits. The variation in individual rabbit response can be attributed to the variable endotoxin susceptibility of individual rabbits on pyrogen test⁸. The accumulated temperature response of + 0.2° to + 0.5° was shown by 17 samples while for 15 samples it ranged between + 0.6° to + 1.0°. For 3 samples it ranged between + 1.0° to + 1.3°. The limit of accumulated response is less than + 1.4° as prescribed by Pharmacopoeia. Therefore all samples studied passed the rabbit pyrogen test⁷. It was observed that for the same accumulated temperature rise, content of endotoxin unit per ml was variable. This suggested no possibility of correlating the temperature rise to the endotoxin levels.

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