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A RAPID KINETIC CHROMOGENIC METHOD FOR QUANTIFICATION OF BACTERIAL ENDOTOXINS IN LYOPHILIZED REAGENTS FOR LABELING WITH ^{99m}Tc RADIOPHARMACEUTICALS

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ABSTRACT

A rapid quantitative kinetic chromogenic test in an automated Portable Test System (PTS) has been developed for determination of bacterial endotoxins in water, in-process and end-products using the Limulus amebocyte lysate (LAL). The aim of this work was to validate the method for lyophilized reagents for labeling with ^{99m}Tc radiopharmaceuticals with no interfering factors. Experiments were performed in three consecutive batches of the lyophilized reagents Methylenediphosphonic Acid (MDP) and Pyrophosphate (PYRO) produced at IPEN-CNEN/SP using the PTS from Endosafe, Inc.^{TM}, Charleston, SC. The Maximum Valid Dilution (MVD) was calculated to establish the extent of dilution to avoid interfering test conditions (MVD=500). Better results were obtained above 1:20 dilution factor for MDP and 1:100 for PYRO. The parameters of coefficient correlation (R) $\leq$ -0.980, RPPC between 50 - 200% and coefficient variation (CV) of the samples less than 25% were satisfied and the endotoxin concentration was lower than the lowest concentration of the standard curve (0.05 EU mL^{-1}), therefore less than the established limit in pharmacopoeias. The PTS is a rapid, simple and accurate technique using the quantitative kinetic chromogenic method for bacterial endotoxin determination. For this reason, it is very practical in the radiopharmaceutical area and it trends to be the method of choice for the pyrogen test. For MDP and PYRO, the validation was successfully performed.

1. INTRODUCTION

Pyrogens include any substance capable of eliciting a febrile response upon injection or infection. Endotoxin is a subset of pyrogens that are strictly of gram-negative origin, a natural complex of lipopolysaccharide ocurring in the outer layer of the bilayered gram-negative bacterial cell. From the circulating blood cells of *Limulus polyphemus* (Fig.1), called amebocytes, a clear lysate is obtained which forms an opaque gel in the presence of extremely small concentrations of bacterial endotoxins [1].

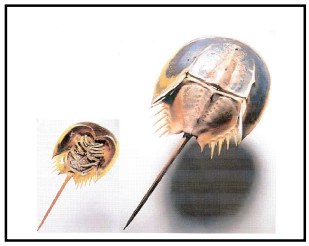

Figure 1. ***Limulus polyphemus.***

The quantitative kinetic chromogenic method measures the chromophore released from a suitable chromogenic peptide by the reaction of endotoxins with the Limulus amebocyte lysate (LAL) Reagent. It is based on the bacterial endotoxin action in activating the coagulating enzyme which reacts with a colorless synthetic substrate. This substrate is constituted of a small peptide linked by the C-terminal arginine to the p-nitroaniline chromophore molecule (pNA). Once the cascading enzymatic reaction is activated, the coagulating enzyme causes the release of the yellow pNA molecule. The color development is proportional to the endotoxin concentration in samples. The chromophore release can be spectrophotometrically monitored at 405 nm OD (Optical Density) and either the onset time needs to reach a predetermined absorbance of the reaction mixture or the rate of color development is measured [1-3].

In order to assure the precision or validity of the chromogenic technique, preparatory tests are conducted to verify that the criteria for the standard curve are valid and that the sample solution does not inhibit or enhance the reaction [2, 4].

A rapid quantitative kinetic chromogenic test in an automated Portable Test System (PTS) has been developed for determination of bacterial endotoxins in water, in-process and end-products using the LAL. The aim of this work was to validate the method for lyophilized reagents for labeling with ^{99m}Tc radiopharmaceuticals with no interfering factors.

2. MATERIALS AND METHODS

Experiments were performed in three consecutive batches of the lyophilized reagents Methylenediphosphonic Acid (MDP) and Pyrophosphate (PYRO) produced at IPEN-CNEN/SP using the PTS (Fig. 2) from Endosafe, Inc.^{TM}, Charleston, SC. Single polystyrene Endosafe cartridges containing dry LAL-reagents, Control Standard Endotoxin (CSE) and synthetic color substrate were used. The LAL sensitivity (λ) was 0.05 EU mL^{-1}. Serial dilutions (1:1; 1:5; 1:10; 1:20; 1:50; 1:100 and 1:200) were carried out by the addition of sterile and pyrogen-free water to the vials. 25 µL samples were pipetted into the cartridge wells and the temperature of the reaction was maintained at 37 ± 1 °C.
Results were obtained for the endotoxin concentration in samples by interpolation of an archived standard curve (5.0; 0.5 and 0.05 EU mL^{-1}) at 405 nm OD (Optical Density), after about 20 minutes.

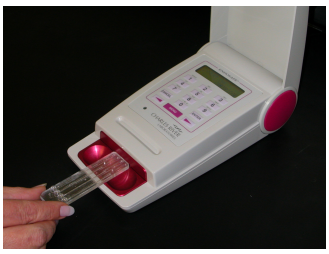

Figure 2. Portable Test System (PTS).

3. RESULTS AND DISCUSSION

To avoid interfering test conditions, the United States Pharmacopeia (USP) allows drug product dilutions based on the established endotoxin limits, such as 175 EU per dose of radiopharmaceuticals. These limits may be used to determine the extent of dilution (Maximum Valid Dilution - MVD) that may be applied to overcome an interference problem without exceeding the limit endotoxin concentration [5-8].

The MVD is the maximum allowable dilution of a drug product at which the endotoxin limit can be determined. The general equation to determine MVD is [5, 6]:

$$MVD = \frac{\text{Endotoxin limit X Potency of Product}}{\text{labelled sensitivity (EU per mL) of the LAL Reagent}} \quad (1)$$

The endotoxin limit is multiplied by the potency (the potency is 1.00 mL per mL for drugs administered in volume per kg) and the product of the multiplication is divided by λ (0.05 EU mL^{-1} or the lowest point of the endotoxin standard curve) to obtain the MVD factor [5]. The calculated MVD was 500, i.e., the limit dilution factor for the preparation for the test to be valid.

In the chromogenic method, product dilution can be greater than in the gel clot method (MVD=200 for radiopharmaceuticals and 0.125 EU mL^{-1} LAL Reagent sensitivity), then it is necessary to perform validation experiments to determine the minimum interfering dilution.

There is a linear quantitative correlation between log of the endotoxin concentration and log of the reaction time, and the product endotoxin concentration (PEC) and the product positive control (PPC) in the analyzed samples were obtained by interpolation on an archived standard curve [9].

The results of the validation experiments for MDP and PYRO are expressed in Tables 1 and 2, and Fig. 3 and 4, respectively.

Table 1. Inhibition or Enhancement Test Results in MDP

Batch	Dilution Factor	PEC (EU mL^{-1})	PPC (EU mL^{-1})	%CV PPC	% RPPC
1	1:1	<0.176	---	---	*Fail*
	1:5	0.583	0.010	18.8	2
	1:10	<0.500	0.266	3.1	66
	1:20	<1.00	0.474	23.4	119
	1:50	<2. 50	0.256	1.0	64
	1:100	<5.00	0.241	13.9	60
	1:200	<10.00	0.370	4.1	92
2	1:1	<0.050	<0.000	0.0	*Fail*
	1:5	0.954	---	---	12
	1:10	<1.30	0.053	13.6	41
	1:20	<1.00	0.178	18.2	74
	1:50	<2.50	0.319	7.1	74
	1:100	<5.00	0.311	4.8	72
	1:200	<10.00	0.774	5.0	180
3	1:1	<0.050	<0.000	0.0	*Fail*
	1:5	<0.250	<0.000	0.0	*Fail*
	1:10	<0.500	0.305	5.4	76
	1:20	<1.00	0.407	3.4	95
	1:50	<2.50	0.435	3.0	101
	1:100	<5.00	0.616	7.4	143
	1:200	<10.00	0.544	2.7	127

PEC – Product Endotoxin Concentration; PPC – Positive Product Control

Table 2. Inhibition or Enhancement Test Results in PYRO

Batch	Dilution Factor	PEC (EU mL^{-1})	PPC (EU mL^{-1})	%CV PPC	% RPPC
1	1:1	<0.050	<0.000	0.0	*Fail*
	1:5	>14.9	1.07	4.8	248
	1:10	30.3	0.557	4.6	130
	1:20	7.97	2.80	4.4	651
	1:50	<2.50	0.740	0.0	172
	1:100	<5.00	0.943	3.4	153
	1:200	<10.00	0.528	4.3	123
2	1:1	<0.050	<0.000	0.0	*Fail*
	1:5	21.9	---	---	*Fail*
	1:10	11.4	1.88	9.6	437
	1:20	3.11	0.935	0.7	217
	1:50	<2.50	0.643	2.9	149
	1:100	<5.00	0.622	1.1	145
	1:200	<10.00	1.250	0.7	139
3	1:1	<0.050	<0.000	0.0	*Fail*
	1:5	>20.7	---	---	*Fail*
	1:10	40.6	---	---	*Fail*
	1:20	<2.21	1.83	0.8	416
	1:50	<2.50	0.903	1.3	208
	1:100	<5.00	0.573	3.7	130
	1:200	<10.00	0.507	1.5	115

Fig. 3 and 4 show %RPPC for serial dilution factors of three batches of MDP and PYRO, respectively.

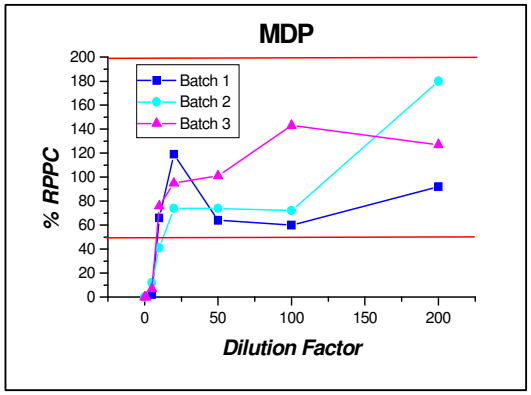

Figure 3. %RPPC versus Dilution Factor for MDP.

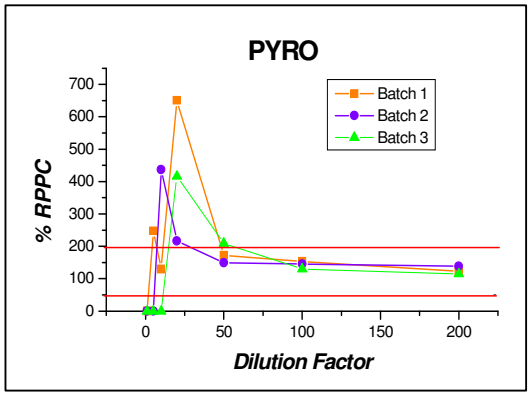

Figure 4. %RPPC versus Dilution Factor for PYRO.

The product sample does not show inhibition or enhancement when it meets the acceptable criteria presented in Table 3.

Table 3. Acceptable Criteria for the Kinetic Chromogenic Method [6]

Parameter	Acceptable Criteria
Coefficient Correlation of the Archived Standard Curve (R)	≤ -0,980
Recovery of the Product Positive Control (RPPC)	50 – 200%
Coefficient Variation (CV)	< 25%

When any criteria, mainly %RPPC, were not in the acceptable range, the test was not valid. PEC values were obtained by multiplying the dilution factor and the lowest concentration of the standard curve (0.05 EU mL^{-1}). An out of specification %RPPC is associated with a calculated PEC that expresses any interference (inhibition or enhancement) [9].

The %CV of the samples demonstrates how trained is the analyst in pippeting into the cartridge wells and small variations show good personal performance.

Experiments showed better results above 1:20 dilution factor for MDP and 1:100 for PYRO. It was observed that there is a specific dilution for each radiopharmaceutical and the profile for the %RPPC versus dilution factor graph was similar in the three analyzed batches of each product. Above those dilution factors, the parameters of $R \leq -0.980$, RPPC between 50 - 200% and CV of the samples less than 25% were satisfied, therefore less than the established limit in pharmacopoeias.

4. CONCLUSIONS

The PTS is a rapid, simple and accurate technique using the quantitative kinetic chromogenic method for bacterial endotoxin determination. For this reason, it is very practical in the radiopharmaceutical area and it trends to be the method of choice for the pyrogen test. For MDP and PYRO, the validation was successfully performed.

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