2007 International Nuclear Atlantic Conference - INAC 2007
Santos, SP, Brazil, September 30 to October 5, 2007
ASSOCIAÇÃO BRASILEIRA DE ENERGIA NUCLEAR - ABEN
ISBN: 978-85-99141-02-1

A CHALLENGE FOR THE VALIDATION OF THE GEL CLOT TEST IN RADIOPHARMACEUTICALS PRODUCED IN THE RADIOPHARMACY CENTER

Neuza T. O. Fukumori, Adriana V. Fernandes, Domingos G. de Campos, Antonio C. Gomes, Natanael G. da Silva, Nilda P. S. de Pereira, Constancia P. G. da Silva and Margareth M. N. Matsuda

Instituto de Pesquisas Energéticas e Nucleares (IPEN / CNEN - SP)
Av. Professor Lineu Prestes 2242
05508-000 São Paulo, SP
ntfukumo@ipen.br

ABSTRACT

Before the *Limulus* amebocyte lysate (LAL) test, the only available means of pirogenicity testing for parenteral drug products and medical devices was the United States Pharmacopoeia (USP) rabbit pyrogen test. Especially for radiopharmaceuticals, the LAL test is the elective way to determine bacterial endotoxin. The aim of this work is to define and validate the best concentration for some radiopharmaceuticals without measurable interference. The FDA's LAL-Test guideline defines interference as a test condition that causes a significant difference between the endpoints of a positive water control and positive product control series using a standard endotoxin. Experiments were performed in accordance to the USP bacterial endotoxins test in the labeled molecule Iodine-131 m-iodobenzylguanidine and the radioisotopes Gallium-67 and Thallium-201. All the tested products were found compatible with the LAL test at a 1:100 dilution factor. A standard dilution series was performed with a control standard endotoxin (CSE) from 0.5 to 0.03 EU/mL, to confirm the labeled sensitivity of the LAL reagent (0.125 EU/mL) and also with the product in the 1:100 dilution factor, in three consecutive batches of each radiopharmaceutical. As the results of the experiments accomplished the requirements, the gel clot method was validated for the proposed products.

1. INTRODUCTION

Endotoxins, released by gram-negative bacteria, can cause a response in humans ranging from mild fever to shock and even death. A reliable method of detecting bacterial endotoxins in radiopharmaceuticals is, therefore, of vital importance to patients for *in vivo* use in humans [1,2].

In the rabbit pyrogen test approved by the United States Pharmacopeia (USP) in 1942, the temperature rise (a pyrogenic response) in rabbits is monitored after injection of a substance. An alternative method was introduced by Cooper *et al*. (1971) and Yin *et al*. (1972) thirty years later. The *Limulus* amebocyte lysate (LAL) test utilizes lysate of blood cells of the horseshoe crab, *Limulus polyphemus*, which enzimatically interacts with endotoxins, causing the formation of a solid gel. The rate of gelation is proportional to the endotoxin concentration [3,4]. The LAL test is the most sensitive and specific means to detect and measure bacterial endotoxin. The LAL reaction is an enzyme-mediated process which requires a neutral pH environment and a proper balance of monovalent and divalent cations [1].

The first step in creating a LAL test method for a pharmaceutical entity is the identification of a compatible sample concentration for routine testing. LAL compatibility is a test condition where an endotoxin standard is detected with the same efficiency in a test sample as in sterile water for injection [5].

It is necessary to proceed the validation of the Gel Clot Test in radiopharmaceuticals for parenteral administration. Compendial methods of various nations and the FDA's LAL-test Guideline require a minimum of 3 lots of each product for validation with a vendor-specific LAL reagent [6,7].

A validation must demonstrate that samples do not interfere with the LAL assay considering two important factors: (1) determination of LAL sensitivity and (2) determination of LAL test enhancement or inhibition by the test substance [1].

The validity of test results for bacterial endotoxins requires an adequate demonstration that specimens to which the test is to be applied do not by themselves inhibit or enhance the reaction, or otherwise interfere with the test [3].

Other techniques to determine endotoxins are known, such as the chromogenic (color development) and the turbidimetric test, both of which can provide valuable quantitative and qualitative information about the endotoxin concentration in samples [2].

The aim of this work is to define and validate the best concentration for some radiopharmaceuticals without measurable interference in the Gel Clot Test.

2. MATERIALS AND METHODS

Only licensed LAL and matched Control Standard Endotoxin (CSE) with a manufacturer's Certificate of Analysis was used (Limulus Amebocyte Lysate (LAL), Reagent, and CSE, Endosafe, Inc.™, Charleston, SC). The LAL sensitivity (λ) used was 0.125 Endotoxin Unit per milliliter (EU/mL). Depyrogenated plastic tubes with lids (Endosafe, Inc.™, Charleston, SC) were used for all procedures. All dilutions were made in depyrogenated glass vials (heat treated for 30 min at 250°C) using pyrogen-free sterile pipette tips (MLA) and pyrogen-free water (Sterile Water for Injection - Endosafe, Inc.™, Charleston, SC). The validation protocols for Iodine-131 m-iodobenzylguanidine (^{131}I-MIBG), Gallium-67 citrate (^{67}Ga) and Thallium-201 chloride (^{201}Tl) radiopharmaceuticals were established. The samples were prepared from a single vial as described in the USP Bacterial Endotoxins Test (BET). Assays were performed using 0.1 mL of sample and 0.1 mL of LAL per tube, incubating in an Eletrolab™ water bath at 37 ±1°C, for 60±2 minutes [3,8]. The gel formation was observed by carefully withdrawing each tube from the water bath and inverting it 180°. The formation of a solid clot is positive for endotoxin.

2.1. BET Inhibition or Enhancement

^{131}I-MIBG and the radioisotopes ^{67}Ga and ^{201}Tl were tested in serial dilutions of 10, 50 and 100 times. Negative product control (diluted product in water), positive product control (0.25 EU/mL CSE in the product prepared by adding equal parts of 0.50 EU/mL CSE in water and the drug product), positive control (0.25 EU/mL CSE in water) and negative water control were performed in duplicate. The pH of the samples was measured.

2.2. Preparation of the Standard Dilution Series

0.5 EU/mL dilutions of CSE in water were prepared by diluting 100 µL of a 5 EU/mL CSE with 900 µL of water. Then, 500 µL of this was further diluted with 500 µL of water to make the 0.25 EU/mL dilution. Further two-fold dilutions (0.125, 0.06 and 0.03 EU/mL CSE in water) were made. Similarly, dilutions of the product with water were prepared at the same concentrations of CSE. Each endotoxin concentration was incubated in quadruplicate.

3. RESULTS AND DISCUSSION

To avoid interferent test conditions and reduce the probability of detecting clinically insignificant endotoxin levels, the 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 [6].

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 [6]

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

^{67}Ga and ^{201}Tl, which have the endotoxin limit concentration (E.L.) specified in the individual monograph in terms of volume (EU per mL), 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 to obtain the MVD factor [6]. The MDV factor so obtained is the limit dilution factor for the preparation for the test to be valid.

^{131}I-MIBG, which does not have an official USP endotoxin limit concentration, the MVD Equation considers the Minimum Valid Concentration (MVC). The MVC is the lowest concentration of sample that can be tested and still achieve required sensitivity.

$$\mathrm{MVC} = \frac{\lambda \text{ X } \mathrm{M}}{\mathrm{K}} \quad (2)$$

$$\mathrm{MVD} = \frac{\text{Potency of the product}}{\mathrm{MVC}} \quad (3)$$

where M is the maximum human dose for the product (mL per kg) and K is 5.0 EU per kg for parenteral drugs [6].

Table 1. Maximum Valid Dilution (MVD) for ^{131}I-MIBG, ^{67}Ga and ^{201}Tl

Product	E.L. (EU/mL)	Potency (mL/mL)	λ (EU/mL)	M (mL/kg)	K (EU/kg)	MVD
^{131}I-MIBG	25.00	1.00	0.125	0.1	5.0	400
^{67}Ga	25.00	1.00	0.125	---	5.0	200
^{201}Tl	25.00	1.00	0.125	---	5.0	200

3.1. BET Inhibition or Enhancement

Prior to use for release of drug products, a drug must be shown not to inhibit or enhance the ability of LAL to detect endotoxins. Three consecutive batches of ^{131}I-MIBG and the radioisotopes ^{67}Ga and ^{201}Tl were tested using the USP Inhibition or Enhancement Test. Table 2 presents the serial dilution results.

Table 2. ^{131}I-MIBG, ^{67}Ga and ^{201}Tl serial dilution results for BET Inhibition or Enhancement

Product/Lot	Neg.Contr.		Pos.Contr.		pH	Product Dilution
^{131}I-MIBG Lot 1	_	_	+	+	7.0	1:10
^{131}I-MIBG Lot 2	_	_	+	+	7.0	1:50
^{131}I-MIBG Lot 3	_	_	+	+	7.0	1:100
^{67}Ga Lot 1	+	_	+	+	7.0	1:10
^{67}Ga Lot 2	+	_	+	+	7.0	1:50
^{67}Ga Lot 3	_	_	+	+	7.0	1:100
^{201}Tl Lot 1	_	_	+	+	7.0	1:10
^{201}Tl Lot 2	_	_	+	+	7.0	1:50
^{201}Tl Lot 3	_	_	+	+	7.0	1:100

^{131}I-MIBG, ^{67}Ga and ^{201}Tl individually met the requirements of the test without pH adjustment at 1:100 dilution factor.

3.2. Standard Dilution Series

The CSE/Product Standard Series were performed for all in the 1:100 dilution factor in parallel with the CSE/Sterile Water Standard Dilution Series. The results of the 60-min tests of 1:100 diluted samples are shown in Table 3.

Table 3. Results of 60-Minute BET Tests for ^{131}I-MIBG, ^{67}Ga and ^{201}Tl in the 1:100 dilution factor

Product	EU/mL of added CSE in drug product					EU/mL of added CSE in water					Neg. Water Control
	0,5	0.25	0.125	0.06	0.03	0,5	0.25	0.125	0.06	0.03	
^{131}I-MIBG	+	+	+	+	–	+	+	+	+	–	–
^{67}Ga	+	+	+	+	–	+	+	+	+	–	–
^{201}Tl	+	+	+	+	–	+	+	+	+	–	–

Comparing the CSE dilutions in water to CSE dilutions in the product, it is acceptable if the gel endpoint values for each series are within both a two-fold dilution of each other and a two-fold dilution of the LAL claimed sensitivity and the pH of the drug-lysate mixture is within the acceptable range of 6.0-8.0 [8].

The validation test results showed in Table 3 were considered valid because the negative controls did not gel, the positive controls formed a firm gel and pH measurements were 7.0.

Neutral pH, availability of divalent cations and a dispersed endotoxin are also important for the validity of the test [1]. Sample volume dilution, micropipette calibration, sample and endotoxin homogenization, residual endotoxin concentration in vials and pipette tips were critical to obtain reproducible results. It was observed that the presence of calcium ions over a 20 ppm concentration is a potential source of interference in the reaction between the endotoxin and the LAL reagent in neutral pH.

The Limulus Gel Clot Test was shown to be sensitive and accurate, simple to perform and required no specialized equipment. The presented data were validated for ^{131}I-MIBG, ^{67}Ga and ^{201}Tl in the 1:100 product dilution. In the LAL gel clot test, the products showed to contain less than the LAL claimed sensitivity (0.125 EU/mL).

The use of both positive and negative controls serves as daily confirmation for the validation of a particular batch of lysate and CSE. Any change in formulation, reagent or method of production needs to be performed to assess the levels of endotoxin [8]. Qualitative and quantitative information about the endotoxin concentration can be provided by kinetic methods as colorimetric and turbidimetric tests, therefore experiments are going to be performed in the same products.

REFERENCES

1. T. J. A. Pinto, T. M. Kaneko, M. T. Ohara, *Controle biológico de qualidade de produtos farmacêuticos*, *correlatos e cosméticos,* 2 ed., Atheneu Ed., São Paulo, pp. 179-215 (2003).
2. S. Zijlstra, P. Gerhen, C. Rechin, R. Wortmann, G. Nolotramiprodjo, "Validation of the *Limulus* Amebocyte Lysate (LAL) Test for Routine PET Radiopharmaceuticals", *Appl. Radiat. Isot.*, **48**, pp. 51-54 (1997).
3. *United States Pharmacopoeia,* 27 ed., United States Pharmacopeial Convention, Rockville, pp. 2169-2172, 2194 (2004).
4. J. F. Cooper, J. Levin, H. N. Wagner Jr, "New, Rapid, In Vitro Test for Pyrogen in Short-Lived Radiopharmaceuticals", *J. Nucl. Med*., **11**, p. 310 (1970).
5. J. F. Cooper, "LAL Interference Screening of In-process Materials and Finished Products", *LAL Times*, **5**(1), (1998).
6. Guideline on Validation of the Limulus Amebocyte Lysate Test as an End-Product Endotoxin Test for Human and Animal Parenteral Drugs, Biological Products and Medical Devices. U.S. Department of Health and Human Services, Public Health Service, Food and Drug Administration, pp. 22-24 (1987).
7. J. F. Cooper, "Documenting Validation of a BET Application", *LAL Times*, **6**(3), (1999).
8. C. C. Williams, R. D. Borchert, J. A. Clanton, "The Bacterial Endotoxin Test in the PET Facility", *J. Nucl. Med*., **34**, pp. 469-473 (1993).