INTRODUCTION

Much of the current success in clinical positron emission tomography (PET) can be attributed to the development of 2-(18F) fluoro-2-deoxy-d-glucose (F18-FDG). It is the most commonly used radiopharmaceutical in the PET, with a half-life of 110 min. Before the release of F18-FDG for human use, the quality control tests need to be performed to check the safety of the product. The bacterial endotoxin test (BET) is one such quality control test. The BET using gel clot method is a 60-min test and typically performed after the decay of the F18-FDG sample to determine the endotoxin content. The gel clot is technically the most simple and was the first BET approved by the Food and Drug Administration (FDA). Endotoxin is a subset of pyrogens that are strictly of gram-negative origin, a natural complex of lipopolysaccharides occurring in the outer layer of the bilayered gram-negative bacterial cell. From the circulating blood cells of Limulus polyphemus, called amebocytes, a clear lysate is obtained that forms an opaque gel in the presence of extremely low concentrations of bacterial endotoxins. The BET is fully described by most pharmacopeial monographs. According to the United states pharmacopeia (USP), pharmacopeia, F18-FDG should not contain more than 175/V USP endotoxin units (EU) per milliliter of F18-FDG injection, in which V is the maximum administered total dose in milliliters, at the expiration time. However, no such standards have been recommended by Indian Pharmacopoeia Commission (IPC) till date. The IPC is taking measures to include radiopharmaceuticals in the Indian Pharmacopoeia.

The objective of this study protocol was to set up a standard pyrogen testing facility and to establish documented evidence if the process employed for BET testing of F18-FDG by gel clot method produces the desired results consistently, when performed as per the standard operating procedures.

MATERIALS AND METHODS

F18-FDG sample preparation

Random samples of the F18-FDG from September 2010 to March 2011 were subjected to the gel clot BET. F18-FDG was synthesized in the in-house medical cyclotron and radiochemistry
Preparation of endotoxin stock solution and endotoxin dilutions

The standard gel clot test for assessing the bacterial endotoxin content consisted of four controls: negative water control (NWC), positive water control (PWC), negative product control (NPC) and positive product control (PPC). The NPC is actually the diluted F18-FDG sample solution. The PWC and PPC contain the standard endotoxin preparation. The NWC is the endotoxin-free Limulus amaebocyte lysate (LAL) reagent water.\(^{[6]}\)

The matched control standard endotoxin (CSE), LAL reagent and LAL reagent water obtained from Charles River Laboratories India Private Limited, Bangalore, India, were used in this study. The sensitivity of the LAL reagent contained in each vial was 0.125 EU/ml. The test for confirmation of lysate sensitivity was carried out with every new batch of the lysate as per the USP<85> recommendations.\(^{[3,4]}\)

The CSE having predetermined amount of endotoxin was prepared according to the directions for use given in the specific certificate of analysis (COA). The CSE stock solution of 20 EU/ml was prepared with endotoxin-free water and vortexed for 5 min. The stock solution was stored at 2°C–8°C for 4 weeks. Before each use, the CSE stock solution was mixed vigorously for 5 min. Endotoxin dilutions were freshly prepared from CSE stock solution for each F18-FDG batch testing.

Glassware for BET

All the dilutions were made in the depyrogenated 16 × 125 mm glass tubes using endotoxin-free water and pyrogen-free sterile pipette tips. Depyrogenations were performed in the dry heat oven at 250°C for 1 hour, when the endotoxin-free glass tubes were not obtained commercially. All assays were performed in the 10 × 75 mm depyrogenated borosilicate glass tubes.

Routine test procedure

The BET was performed using 0.1 ml of F18-FDG sample and 0.1 ml of reconstituted LAL reagent per tube. The LAL reagent was constituted after the addition of LAL reagent water (endotoxin free) in the freeze-dried powder of lysate. The tests were done on all the 10 undiluted F18-FDG batches in duplicates. The PWC and PPC were used for these tests at the endotoxin concentration of 0.5 EU/ml. The four dilutions of the F18-FDG, i.e. 1:10, 1:100, 1:350 and 1:700, were freshly prepared from the batches and subjected to the gel clot assay. The NWC and PWC were common for all the dilutions. The PPC was separately prepared for all the dilutions at the endotoxin concentration of 0.25 EU/ml. The reaction solution was mixed thoroughly and placed immediately in the incubator at 37°C±1°C for 60±2 min. At the end of the incubation period, the tubes were removed from the incubator and inverted.

If the gel had formed and remained intact in the bottom of the reaction tube after an inversion of 180°, the test was considered positive. A positive test indicated that the concentration of endotoxin in the tube is greater than or equal to the sensitivity of the LAL reagent. Any other state of the reaction mixture constituted a negative test, which indicated an endotoxin concentration less than the LAL reagent sensitivity. The test was considered negative when the tube is inverted and the gel breaks or collapses.

RESULTS

A total of 10 different batches of decayed F18-FDG were used in this study. The mean pH was 7.05 (6.5–7.5). The total numbers of tests performed on the F18-FDG were 100 excluding NWC, PWC and PPC.

Table 1 summarizes the results of the undiluted F18-FDG on the positive control solutions at an endotoxin concentration of 0.5 EU/ml (4b). Of the 10 undiluted F18-FDG batches, none gelled after 60-min incubation period at 37°C. This indicated that all the batches did not contain endotoxin greater than 0.5 EU/ml. None of the negative control NWC vials gelled. All the 20 positive endotoxin control PWC vials gelled. Only 18 out of 20 PPC vials gelled after incubation at 37°C for 60 min. This indicated that the undiluted F18-FDG inhibited gel formation.

Table 2 summarizes the results for the 60-min gel clot assay on the four different dilutions of the F18-FDG samples. A total of 80 tests were performed excluding NWC, PWC and PPC vials. None of the negative control NWC vials gelled. All the 20 positive endotoxin control PWC vials gelled. None of the 20 1:10 F18-FDG sample vials gelled after 60-min incubation at 37°C. However, 1 out of 20 PPC vials for the 1:10 dilution did not gel probably due to some interference with the gel formation at the lysate sensitivity of 0.125 EU/ml. These results are similar

| Table 1: Results for undiluted F18-FDG sample |
|---------------------------------------------|
| FDG Lot no. | pH | NWC | FDG undiluted | PWC* | PPC* |
|-------------|----|-----|--------------|------|------|
| 150292010   | 7  | -   | -            | +    | +    |
| 180292010   | 7.5| -   | -            | +    | +    |
| 23092010    | 7  | -   | -            | +    | +    |
| 10012011    | 7  | -   | -            | +    | +    |
| 11012011    | 7  | -   | -            | +    | +    |
| 15022011    | 7  | -   | -            | +    | +    |
| 9032011     | 6.5| -   | -            | +    | +    |
| 10032011    | 6.5| -   | -            | +    | +    |
| 11032011    | 7  | -   | -            | +    | +    |
| 25032011    | 7  | -   | -            | +    | +    |
| 29032011    | 6.5| -   | -            | +    | +    |

EU: endotoxin unit; NWC: negative water control; PWC: positive water control; PPC: positive product control; *Both positive controls contained the standard endotoxin preparation at a concentration of 0.5 EU/ml; +: Firm gel formed; –: No gel formed.
to those obtained from the undiluted FDG samples. All the remaining vials for the 1:100, 1:350 and 1:700 dilutions did not gel. This indicated that all the vials did not contain endotoxin greater than 0.125 EU/ml.

**DISCUSSION**

The gel clot method is often considered as the most accurate and sensitive procedure for testing endotoxin content in injectable radiopharmaceutical products because fewer false-positive and false-negative results are observed when this method is used. The total time taken to complete the gel clot assay was approximately 2 hours.

The LAL reaction requires optimal pH range. In the undiluted samples, the measured pH (7.05) was well within the acceptable range (i.e. 6.0–8.0) for the gel clot assay.

The F18-FDG produced in the Tracerlab MX synthesis module is neutralized in the citrate buffer which contains multivalent ions. Nakao *et al.* reported the effect of the citrate salt concentration on the endotoxin test for the F18-FDG preparations. These authors reported that the undiluted sample showed the recovery less than 20%, beyond the acceptance of the USP. After twofold dilution with sterile water, no interference was observed. Similar results were found in the present study with the undiluted samples as two vials of the PPC (endotoxin concentration equal to 0.5 EU/ml) did not gel, indicating the interference with the gel formation. The interference may be due to the presence of the citrate ions.

At the expiration time, the maximum administered total dose in milliliters of FDG should contain less than 175 EU. The total batch volume of FDG produced is 16 ml in the synthesis module. Therefore, the total F18-FDG preparation at any time did not contain more than 8 EU (0.5 EU/ml × 16 ml). Thus, the product is safe for human administration.

However, the undiluted sample did show the inhibition of the gel formation as shown in Table 1. Therefore, the sample was tested for the various dilutions 1:10, 1:100, 1:350 and 1:700. The 350 and 700 dilution factors were considered according to the limit of 175 EU/ml (single patient dose supplied to the other institute or when the final elution is in 2–3 ml due to some technical errors). The rate of inhibition improved with the dilutions in Table 2.

The gel clot assay has a major drawback which limits its use for the short-lived radiopharmaceuticals. This test is a time consuming test (60 min incubation time).

In addition to this drawback, errors made by technical personnel and the misinterpretation of results are also common problems. The 60-min BET is described in USP<85>, “Bacterial Endotoxins Test”, and is also recommended for pyrogenicity testing in the draft guidance of the FDA on current good manufacturing practice (CGMP) for PET drug products. Because the remainder of the required quality assurance (QA) testing for F18-FDG injection, with the exception of the sterility test, can be completed in approximately 20–30 min, delaying the release of the short-lived F18-FDG injection for an additional 30–40 min is not practical and is, in fact, wasteful. An in-process 20-min BET must be performed before release of the drug product, and a standard 60-min BET must also be completed.

Some other techniques to determine endotoxins are also known, such as the chromogenic (color development) and the turbidimetric (turbidity development) tests, both of which can provide valuable quantitative and qualitative information about the endotoxin concentration in samples. Regardless of which method is used, PET laboratories and pharmacies should ensure that their technicians are well trained and educated in the endotoxin testing method of choice.

**CONCLUSION**

The pyrogen testing facility was successfully established using gel clot procedure. This study suggests that the BET needs to be performed, standardized and documented in each cyclotron.
and PET facility. The undiluted FDG preparations occasionally interfere with the gel formation. The dilution of the test sample is the easiest means to resolve the potential product inhibition/enhancement problem during the gel clot testing procedure. The final F18-FDG preparation at any time point did not contain more than 8 EU, thus making it safe for human administration.

REFERENCES

1. Fludeoxyglucose F 18 injection. In: The United States Pharmacopeia/National formulary. 34 rev/29 ed. Rockville (MD): The United States Pharmacopeial Convention; 2011. p. 2868-9.
2. Hung JC. Comparison of various requirements of the quality assurance procedures for (18)F-FDG injection. J Nucl Med 2002;43:1495-506.
3. Endosafe (package insert): Charles river product and services catalog: 2010.
4. Bacterial endotoxins test USP<85>: In: The United States Pharmacopeia/National formulary. 34 rev/29 ed. Rockville (MD): The United States Pharmacopeial Convention; 2011.
5. Index, Indian Pharmacopoeia Commission. 6th ed. The Indian Pharmacopoeia Commission. 2010(6.0); Ghaziabad. ISBN 81-903436-4-5.
6. Williams CC, Borecht RD, Clanton JA. The bacterial endotoxin test in the PET facility. J Nucl Med 1993;34:469-73.
7. Joiner TJ, Kraus PF, Kupiec TC. Comparison of Endotoxin Testing Methods for Pharmaceutical Products. Int J Pharm Compound 2002;6:408-9.
8. Nakao R, Kida T, Suzuki K. Factors affecting quality control of [18F]FDG injection: bacterial endotoxins test, aluminum ions test and HPLC analysis for FDG and GIDG. Appl Radiat Isot 2005;62:889-95.
9. Hung JC, Iverson BC, Jacobson MS, Mahoney DW. Inhibition evaluation for a 20-min. endotoxin limit test on FDG. Nucl Med Commun 2005;26:869-74.
10. Mitra A, Kulkarni S, Rajan MG. Rapid test for bacterial endotoxin quantification in 18F-FDG by the kinetic chromogenic method. Indian J Nucl Med 2010;25:87.

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