The Bacterial Endotoxin Test in the PET Facility

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A method by which the gel-clot Limulus amebocyte lysate test may be performed in 20 rather than 60 min with sufficient sensitivity to satisfy the needs of the nuclear medicine or positron emission tomography laboratories has been developed and validated for use as a substitute for the Bacterial Endotoxin Test described in the United States Pharmacopeia, 22nd revision. Using this method, results may be obtained from the test prior to the human administration of radiopharmaceuticals without extensive loss of activity and with increased safety when compared to tests performed after administration. Additionally, studies on the shelf-lives of the reagents used in the test were conducted. When refrigerated between use, control standard endotoxin dilutions of 5 EU/ml or greater may be used for at least 1 mo after preparation and reconstituted lysate retains its labeled sensitivity for at least 10 days, considerably longer than the manufacturer's stated shelf-lives.

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The Bacterial Endotoxin Test (BET) (*1*) is routinely used to assure that unacceptable amounts of endotoxins are not present in radiopharmaceuticals. The United States Pharmacopeia, 22nd revision (USP) typically requires intravenous radiopharmaceuticals to contain endotoxin levels below those which would result in a patient receiving 175 endotoxin units (EU) at all times up to the expiration date and time of the drug product. The USP has recognized the superiority of the BET over the Pyrogen Test by recently substituting the BET for the Pyrogen Test as a monograph requirement for 185 drugs (*2*).

The BET is an in vitro test utilizing lysate of blood cells of the horseshoe crab, which enzymatically interacts with endotoxins and can be analyzed by checking for formation of a solid gel (i.e., gel-clot method) or by incorporation of a turbidometric or colorometric assay. First described in 1970 (*3*), the Limulus amebocyte lysate (LAL) test is a sensitive, relatively easy-to-use test and has been used extensively by nuclear medicine personnel because of the small sample volumes needed for analysis, the speed and the convenience with which it can be performed when compared to the USP Pyrogen Test.

Although the turbidometric or colorometric tests using LAL are acceptable means of performing the BET if properly validated, the majority of nuclear medicine and PET laboratories prefer the USP's gel-clot method. This preference is due to its simplicity and minimal need for additional pieces of expensive laboratory equipment and because it is the method described in the USP.

The gel-clot method requires a 60-min incubation of samples before analysis which presents a problem when evaluating products with limited stability and short-lived radiopharmaceuticals. At the present time, Flurodeoxyglucose ^{18}F, USP (FDG) and Ammonia ^{13}N, USP (ammonia) are radiopharmaceuticals routinely used in positron emission tomography (PET) centers with half-lives of 110 and 9.96 min, respectively. A 60-min incubation period would result in an unacceptable loss of product while waiting for results of the BET to be obtained. The US Food and Drug Administration realizes that this is a problem. The FDA finds it acceptable to release the product for human use prior to completion of the BET, provided that preliminary validation batches of the drug product have been shown to be of acceptable quality and that the BET test is performed on each batch of the product, with the results of the analysis obtained after patients have received the drug. Many PET centers find this less acceptable than a quality control test whose results can be known prior to release of the product for patient use. A method which requires less than a 60-min incubation has been developed (JC) that will produce reliable and reproducible results within 20 min. A 20-min period was chosen since many of the other radionuclidic, radiochemical and chemical quality control procedures performed on each radiopharmaceutical batch require approximately 20 min for completion prior to release of product. Thus, no additional time delay is introduced by waiting for completion of a 20-min BET. It was hoped that this 20-min test could be validated to confirm that

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each patient received less than 175 EU of endotoxin when injected with FDG, ammonia and additional drugs as they become clinically available.

This report presents a description and validation of a modified 20-min BET. Data are presented (CW) from one of the author's laboratories for its use in testing daily batches of FDG and ammonia.

MATERIALS AND METHODS

Only licensed LAL and matched Control Standard Endotoxin (CSE) with a manufacturer's Certificate of Analysis is used (Pyrotell®, Associates of Cape Cod, Inc., Woods Hole, MA and Limulus Amebocyte Lysate (LAL), Reagent, Endosafe, Inc.™, Charleston, SC). Depyrogenated 10 × 75 mm flint glass tubes are used for all assay procedures. Depyrogenations are performed in an oven whose dry heat depyrogenation cycle has been validated. All assays are performed using 0.1 ml of sample and 0.1 ml of LAL per tube. Dilutions are made in the depyrogenated assay tubes using pyrogen-free sterile pipette tips (Medical Laboratory Automation, Inc., Pleasantville, NY). Prior to validation of a 20-min protocol, a test for confirmation of labeled LAL reagent sensitivity (*1*) is performed on each new lot of LAL-CSE received. The laboratory, each analyst and each lot of LAL-CSE must be shown to confirm a lysate's label claim; the acceptable limits being within a two-fold dilution of the manufacturer's stated label claim. Rather than performing this test on four replicate vials of LAL, quadruplicate samples are prepared from a single vial as described in proposed changes to the BET (*4,5*). For a successful 20-min test, lysate of the highest sensitivity should be obtained. The authors have validated this test using LAL with sensitivities of 0.03 and 0.06 EU/ml. The results presented here are based entirely upon work with 0.03 EU/ml LAL using Associates of Cape Cod, Inc. LAL and CSE. Therefore, whenever a CSE dilution of twice the lysate's sensitivity is required, a CSE of 0.06 EU/ml is used.

Twenty-Minute Test and Shelf-life

After confirming label claim, a 20-min test must be developed that demonstrates the ability to confirm that no patient will receive more than 175 EU of endotoxin in a worst-case scenario; i.e., where the patient receives the entire product batch. If no batch exceeds 20 ml, this requirement can be met by demonstrating the ability to detect the presence of 5 EU/ml of CSE at 20 min in both water and drug product, i.e., no more than 100 EU will be present in the entire 20 ml sample. The confirmation that a 20-min test can confirm the presence of 5 EU/ml in water was tested repeatedly in conjunction with the stability testing of the refrigerated lysate and diluted CSE reagents which were used to determine these reagents' shelf-lives. Duplicate LAL tubes were prepared as follows:

1. sterile water (20-min incubation)
2. 5 EU/ml CSE in sterile water (20 min)
3. sterile water (60 min)
4. 0.06 EU/ml CSE in sterile water, prepared from the 5 EU/ml monthly CSE vial (60 min).

For a test to be acceptable, each of the sterile water tubes must not form a gel and each of the CSE dilutions must form a gel when removed from the 37° ± 1°C dry-block incubator at the appropriate time interval.

These reagents can be used for periods of time longer than the shelf-lives stated by the respective manufacturers so long as the reagents can be shown to retain their potency over an extended time period. A 5 EU/ml standard of CSE in sterile water was prepared in a volume sufficient (10 ml) for a month-long test using a depyrogenated 16 × 125 mm flint glass tube. Dilutions to twice the LAL's claimed sensitivity were prepared daily from this 5 EU/ml standard and the excess discarded after use. LAL was similarly reconstituted using the 5 ml (50 test) size vial. When not in use, both were covered with Parafilm® "M" (American National Can™, Greenwich, CT) and stored at 2°–8° C in a refrigerator.

An end-point determination was also performed for the CSE and lysate using the 20-min incubation with new and aged reagents. This was performed by preparing serial two-fold dilutions of the 5 EU/ml CSE in sterile water to determine the lowest concentration of CSE that could confidently be shown to form a gel with lysate.

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. For each new drug product or change in method of preparation of an existing product, three batches of product were tested using the USP Inhibition or Enhancement Test. The USP procedure was used with four replicates at each of the dilutions tested. CSE dilutions in sterile water were compared to CSE dilutions in the product and were determined to be acceptable if the sterile water and product controls did not form a firm gel, the gel endpoint values for each series were 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 was within the acceptable range of 6.0–7.5.

For this test, 0.5 EU/ml dilutions of CSE in the product were prepared by diluting 100 μl of a 5 EU/ml CSE in sterile water with 900 μl of the product. Then, 200 μl of this was further diluted with 1400 μl of the product to make the 0.06 EU/ml dilution in the drug product. Further two-fold dilutions (0.03, 0.016 and 0.008 EU/ml) were made with the product. Similarly, dilutions with sterile water were prepared at the same concentrations of CSE and a BET was performed on both sets of endotoxin dilutions.

Routine Daily QC Tests

Daily testing for endotoxins in FDG and ammonia requires 0.4 ml of the product and 0.8 ml of LAL. The pH of FDG, ammonia and ammonia-LAL samples are tested. For each product batch, duplicate LAL tubes are prepared for the four samples as follows:

1. Sample (undiluted drug product).
2. Negative control (Sterile Water for Injection, USP).
3. Positive control (5 EU/ml CSE in sterile water—prepared monthly).
4. Positive product control (5 EU/ml CSE in the product—prepared by adding 20 μl of a 50 EU/ml CSE in sterile water which is prepared monthly to 180 μl drug product, then divided between the two duplicate tubes).

The tubes are read at 20 min. The test results are valid if the negative controls do not gel and the positive controls form a firm gel. The drug product passes the daily test if the results meet the requirements for validity, the sample tubes do not gel and pH

TABLE 1
LAL and Diluted CSE Shelf-Life Determination*

20-min data		60-min data			
5 EU/ml CSE	Negative control	0.06 EU/ml CSE	Negative control	No. days after preparation of LAL	No. days after preparation of 5 EU/ml CSE in sterile water
++	--	++	--	0†	0
++	--	++	--	1	1
++	--	++	--	2	2
++	--	++	--	3	3
++	--	++	--	4	4
++	--	++	--	0†	7
++	--	++	--	2	9
++	--	++	--	4	11
++	--	++	--	7	14
++	--	++	--	9	16
++	--	++	--	0‡	18
++	--	++	--	3	21
++	--	++	--	5	23
++	--	++	--	7	25
++	--	++	--	12	30
++	--	++	--	14	32
++	--	++	--	17	35

*LAL-0.03 EU/ml sensitivity. CSE prepared in sterile water at 5 EU/ml. Dilutions made to 0.06 EU/ml on the day of use.
†Began using a new vial of LAL (5 ml—50 test size).
‡Two vials of LAL reconstituted for remaining tests.
+Firm gel found.
−No gel.

measurements are acceptable. As an added check, data from the USP 60-min BET test on three samples of each new product or change in method of production of an existing drug and quarterly tests thereafter on products in clinical use are performed to assess the levels of endotoxin with greater sensitivity than is possible with a 20-min test. Dilutions of CSE in product are made to twice the sensitivity of the LAL by adding 10 μl of a 5 EU/ml CSE in Sterile Water to 790 μl of undiluted drug product.

RESULTS

Twenty-Minute Test and Shelf-Life

Table 1 demonstrates that the shelf-lives of CSE dilutions and LAL are considerably longer than that suggested by the manufacturer and that a 20-min test can yield valid results. The 5 EU/ml CSE in sterile water standard will continue to form a firm gel at 20 min with LAL and the 0.06 EU/ml daily dilutions from it form a firm gel at 60 min longer than 1 mo after preparation. Similarly, the reconstituted LAL maintains its ability to form a firm gel with CSE standards for greater than 1 wk after reconstitution.

With freshly prepared CSE and lysate, the geometric mean of the 20-min end-point sensitivity was 1.25 EU/ml (Table 2). This test was repeated using refrigerated lysate over several days with CSE dilutions made from an aged 5 EU/ml CSE dilution. The endpoint was 4.2 EU/ml using 10-day-old lysate and 35-day-old CSE, confirming the ability to detect at least 5 EU/ml in a sample with the aged refrigerated lysate and moderately diluted CSE over this time period.

BET Inhibition or Enhancement

When tested, three batches of both FDG and ammonia met the requirements of this test without pH adjustment or need for dilution of drug as shown in Table 3. FDG is of acceptable pH for the BET (6.0–7.5) itself and continues to be within that range when mixed with lysate. The ammonia is outside of this pH range when tested by itself, but the lysate-ammonia mixture is within the acceptable range. Therefore, no pH adjustment is needed to obtain a valid BET result with either drug.

Routine Daily QC Tests

Results of the 20-min test over the 8-mo time period the PET center has been operational, which include data from 98 batches of FDG and 37 batches of ammonia, appear in Table 4, showing that the 20-min test produces consistently valid results and that, without exception, no batch of FDG or ammonia tested had measurable levels of endotoxins present.

Results of the 60-min tests (Table 5) on undiluted samples show that over the same period of time the batches of FDG and ammonia contain less than 0.03 EU/ml for the samples tested.

TABLE 2
Twenty-Minute Endpoint Sensitivity Test of LAL Reagents*

No. days post-reconstitution of:		EU/ml						
LAL	5 EU/ml CSE dilution in sterile water	5	2.5	1.25	0.625	0.313	0.156	Geometric mean end-point sensitivity (EU/ml)
0	0		++++	++++	----	----	----	1.25
4	24	++++	++++	++++	----			1.25
7	24	++++	+++-	----	----			3.0
10	35	++++	+---	----	----			4.2

*LAL-0.03 EU/ml sensitivity. CSE prepared in sterile water at 5 EU/ml. Further dilutions made on the day of use.
+Firm gel found.
−No gel.

TABLE 3
BET Inhibition and Enhancement Results (60 Minutes) for FDG and Ammonia*

	EU/ml of added CSE in drug product†				EU/ml of added CSE in sterile water			
Lot no.	0.06	0.03	0.016	0.008	0.06	0.03	0.016	0.008
FDG								
F-12-91-10-10	++++	++++	----	----	++++	----	----	----
F-12-91-10-14	++++	----	----	----	++++	++++	----	----
F-12-91-10-15	++++	----	----	----	++++	++++	----	----
Ammonia								
N-01-91-11-08	++++	----	----	----	++++	----	----	----
N-01-91-11-11	++++	++++	----	----	++++	----	----	----
N-01-91-11-12	++++	----	----	----	++++	++++	----	----

*Duplicate negative controls of undiluted drug product alone and sterile water tested for each lot of product and did not gel. LAL sensitivity-0.03 EU/ml.
†pH of all lysate-drug samples was 6.5 for FDG and 6.8–7.0 for ammonia.
+Firm gel found.
−No gel.

TABLE 4
Results of 20-Minute BETs Performed on Daily Batches of Undiluted FDG and Ammonia*

		Results†		
			Valid	
Drug product	No. of batches tested	Invalid	Positive	Negative
FDG	98	0	0	98
Ammonia	37	0	0	37

*BETs were performed between October 1991 and June 1992.
†Results are valid when positive controls form a firm gel and negative controls do not gel. Results are positive if the test is valid and at least one sample tube forms a firm gel, causing rejection of the batch of drug for clinical use. Results are negative if the test is valid and the sample tubes do not form a gel.

TABLE 5
Results of 20- and 60-Minute BET Tests for FDG and Ammonia*

Lot no.	pH		20-min results				60-min results			
	Drug product alone	Lysate-drug sample	Drug sample	Sterile water	5 EU/ml CSE in water	5 EU/ml CSE in drug	Drug sample	Sterile water	0.06 EU/ml CSE in water	0.06 EU/ml CSE in drug
FDG										
F-12-91-10-10	6.0	6.5	--	--	++	++	--	--	++	++
F-12-91-10-14	6.5	6.5	--	--	++	++	--	--	++	++
F-12-91-10-14b	6.5	6.5	--	--	++	++	--	--	++	++
F-12-91-10-15	6.5	6.5	--	--	++	++	--	--	++	++
F-12-91-10-23	7.0	6.8	--	--	++	++	--	--	++	++
F-12-91-10-25	6.0	6.8	--	--	++	++	--	--	++	++
F-12-91-11-28	7.0	†	--	--	++	++	--	--	++	++
F-12-92-01-14	6.5	†	--	--	++	++	--	--	++	++
F-12-92-04-08	6.5	†	--	--	++	++	--	--	++	++
F-12-92-05-26	7.2	†	--	--	++	++	--	--	++	++
F-12-92-05-29	6.8	†	--	--	++	++	--	--	++	++
F-12-92-06-04	6.5	†	--	--	++	++	--	--	++	++
Ammonia										
N-01-91-11-08	5.5	6.8	--	--	++	++	--	--	++	++
N-01-91-11-08	5.7	6.8	--	--	++	++	--	--	++	++
N-01-91-11-08	5.5	6.8	--	--	++	++	--	--	++	++
N-01-91-11-11	5.5	6.5	--	--	++	++	--	--	++	++
N-01-91-11-11	5.7	6.8	--	--	++	++	--	--	++	++
N-01-91-11-11	5.7	6.8	--	--	++	++	--	--	++	++
N-01-91-11-11	5.7	6.8	--	--	++	++	--	--	++	++
N-01-91-11-12	5.5	7.0	--	--	++	++	--	--	++	++
N-01-91-11-12	5.5	7.0	--	--	++	++	--	--	++	++
N-01-92-01-23	5.5	6.8	--	--	++	++	--	--	++	++
N-01-92-05-19	5.8	6.5	--	--	++	++	--	--	++	++

*LAL sensitivity = 0.03 EU/ml and 5 and 50 EU/ml CSE in sterile water prepared monthly. Dilutions from these prepared daily as described in text.

†Data demonstrate that since the product alone is in the acceptable pH range for BET and previous lysate-drug mixtures remain in the appropriate pH range and that further pH tests of the drug lysate mixture are not needed so long as the drug alone is within the acceptable range of 6.0–7.5.

+Firm gel found.

−No gel.

DISCUSSION

A method for performing endotoxin testing on short-lived radiopharmaceuticals has been shown which is convenient, inexpensive and obtains results faster than the test described in the USP. The data presented validate this method as an acceptable alternative to the BET.

Shelf-lives of both reconstituted lysate and CSE dilutions were documented to extend well beyond the manufacturer's claims when refrigerated. The use of both positive and negative controls serves as daily confirmation that a particular batch of lysate or CSE used beyond the manufacturer's recommended shelf-life will yield valid results.

Preliminary and quarterly tests using the 60-min procedure further continue to cross-check validations. For FDG and ammonia, endotoxin levels in these drugs, although only being shown to contain <5 EU/ml on a daily basis, actually contain <0.03 EU/ml (the limit of detectability for this system) when tested at the more sensitive limit. PET centers can therefore confidently release these drug products with prior knowledge that patients will not receive 175 EU or more of endotoxin, rather than testing for endotoxins after administration of the drug.

As in the case of all validation methods, the tests described in this work need to be performed, verified and documented in the records of each nuclear medicine or PET laboratory that intends to use this method.

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