Pyrogen Testing of Parenteral Products—Status Report

Marlys Weary

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ABSTRACT: The current status of the Limulus Amebocyte Lysate (LAL) test for final release of drug products and devices in the United States and abroad is discussed. The principal problem dealt with is determining an endotoxin limit for the LAL assay. This is significant because the LAL test is more sensitive than the USP rabbit pyrogen test, and because, as comparative LAL and rabbit assays indicate, purified endotoxin standard is more pyrogenic than environmental endotoxin.

The pyrogens most commonly found in parenteral products are bacterial endotoxins from the exterior cell membranes of gram-negative bacteria. These lipopolysaccharides are responsible for a wide range of biological activities, the best known of which is initiation of fever if they are injected parenterally in man or animals. In the early 1940s, when the United States Pharmacopeial Convention began to design an official compendial test for pyrogens, this fever-producing property was selected as the indicator of the presence or absence of pyrogen in parenteral solutions. The parameters of the first official compendial

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assay, the USP Rabbit Pyrogen Test (151), were estab-
lished in a large-scale collaborative study jointly designed
and executed by the Food and Drug Administration (FDA),
the National Institutes of Health (NIH), and 14 Pharma-
caceutical manufacturers (1). This rabbit pyrogen test, which
was incorporated into USP XII in 1942, consists of intrave-
nously injecting, under specified conditions, test solutions
into rabbits, and subsequently monitoring and recording
the animals' body temperatures. For over 40 years, the
rabbit inoculation test remained the only official pyrogen
assay. The LAL assay is based on the fact that
bacterial endotoxin initiates a gelation reaction if incubated
with a reagent prepared from lysed blood cells (amebocytes)
from the American horseshoe crab, Limulus polyphemus.
The test is rapid, sensitive, relatively simple to perform, and
is highly cost effective when compared to the official rabbit
pyrogen test. For these reasons, there has been much in-
terest in obtaining USP and regulatory acceptance of the
LAL test as a replacement for the USP pyrogen test.

Six years ago, the FDA published a notice in the Federal
Register describing conditions for the use of LAL as an
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tions, test solutions into rabbits, and subsequently monitor-
ing and recording the animals' body temperatures. For over
40 years, the rabbit inoculation test remained the only official
pyrogen test described in international formularies. In
general, it has served well. However, the test is elaborate,
resulting in high cost and execution problems, and it also
suffers from the variability characteristic of all biological
assays.

During the past decade, the Limulus Amebocyte Lysate
(LAL) test has been widely utilized in the pharmaceutical
industry as an alternative to the rabbit test for end-toxin
pyrogen testing of ingredient waters, raw materials, and
drug products. The LAL assay is based on the fact that
bacterial endotoxin initiates a gelation reaction if incubated
with a reagent prepared from lysed blood cells (amebocytes)
from the American horseshoe crab, Limulus polyphemus.
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Six years ago, the FDA published a notice in the Federal
Register describing conditions for the use of LAL as an
end-product test for endotoxins in biological products and
drug products (2). The notice stated that LAL testing of
drug products for human use would be the subject of a fu-
ture Federal Register publication.

Following this, on March 26, 1979, the Bureau of Med-
ical Devices (BMD) issued a draft guideline setting forth
procedures for LAL as an end-product test of medical de-
vices. Although the guideline was not published in the
Federal Register, it was made available to interested par-
ties, including manufacturers of medical devices, and has been
revised and updated since its original release. As of May
1981, the bureau had approved 168 products from 30 firms
for LAL end-product testing.

Even before the Federal Register announcement, the
Bureau of Biologics (BOB) required the LAL assay as a
release test for a drug product. In the fall of 1974, when the
swine influenza inoculation program became a national
health priority, the Code of Federal Regulations mandated
an LAL general safety release test for embryonated egg-
grown influenza vaccines (3). This requirement remains in
force today. However, even though clearance require-
ments for the use of LAL as an end-product pyrogenicity
test of human biological products subsequently became
available, very few licensed manufacturers of such products
have petitioned for license amendment. To date, two
manufacturers of blood products have received permission
to use the LAL test for end-product release, and the peti-
tions of several manufacturers are currently under active
review.

On January 18, 1980, the FDA issued a notice in the
Federal Register announcing availability of a draft guide-
line for utilizing the LAL test as an end-product pyrogen
test of human and animal injectables (4). In response to
comments received from users of the LAL test, several
major changes were incorporated into the draft guideline.
These changes were discussed by the FDA's Terry Munson
at the International Conference on Endotoxin Standards
and Limulus Amebocyte Lysate Use with Parenteral Drugs
held at the Oceano-graphic Institution, Woods Hole, Mas-
achusetts, in September 1981 (5). At the time, it was an-
ticipated that the draft guideline would be released in a
matter of weeks. However, for a variety of reasons, it was
not. On March 21, 1983, the draft guideline was approved
by the Deputy Commissioner of Food and Drugs and copies
are now available for review and comment. Although the
draft guideline has not been finalized, the FDA office of
New Drug Evaluation is actively reviewing NDA applica-
tions that ask permission to use the LAL test as an alter-
native to the rabbit pyrogen test. Validation requirements
from the guideline are being used as acceptance/rejection
criteria in evaluating these applications. In addition, the
Bureau of Drugs has proposed endotoxin limits for a wide
variety of drug products.

The LAL test has been gaining wide acceptance outside
of the United States for pyrogenicity testing of pharma-
caceutical products and medical devices. Table I indicates
the status of the test in countries where Travenol either con-
ducts pyrogen testing or markets products release-tested
using LAL.

During the 1975–1980 U.S. Pharmacopeia revision, the
Committee of Revision was made aware of the considerable
interest in establishing the LAL test as a replacement for
the USP Pyrogen Test (151). The committee expended
much time and effort in reviewing available data and pre-

<table>
<thead>
<tr>
<th>Country</th>
<th>Medical Devices</th>
<th>Drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Australia</td>
<td>Final Release</td>
<td>In-process</td>
</tr>
<tr>
<td>Belgium</td>
<td>In-process</td>
<td>In-process</td>
</tr>
<tr>
<td>Brazil</td>
<td>Final Release</td>
<td>Final Release</td>
</tr>
<tr>
<td>Canada</td>
<td>Final Release</td>
<td>Final Release</td>
</tr>
<tr>
<td>Columbia</td>
<td>Final Release</td>
<td>Final Release</td>
</tr>
<tr>
<td>Denmark</td>
<td>Final Release</td>
<td>In-process</td>
</tr>
<tr>
<td>France</td>
<td>Final Release</td>
<td>In-process</td>
</tr>
<tr>
<td>Finland</td>
<td>Final Release</td>
<td>Final Release</td>
</tr>
<tr>
<td>Germany</td>
<td>Final Release</td>
<td>In-process</td>
</tr>
<tr>
<td>Ireland</td>
<td>Final Release</td>
<td>In-process</td>
</tr>
<tr>
<td>Israel</td>
<td>Final Release</td>
<td>Final Release</td>
</tr>
<tr>
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<td>Sweden</td>
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<td>In-process</td>
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<tr>
<td>United Kingdom</td>
<td>Final Release</td>
<td>In-process</td>
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<tr>
<td>Yugoslavia</td>
<td>Final Release</td>
<td>NA</td>
</tr>
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paring for the introduction of the test, culminating in the Bacterial Endotoxins Test (BET) (85), first presented in Pharmacopeial Forum and subsequently published in USP XX, which became official on July 1, 1980 (6). The chapter includes preparatory procedures to confirm LAL labeled potency and lack of testing variability, and to prove that the test article neither inhibits nor enhances LAL gelation. It specifies the need for positive and negative controls, and it describes testing procedures. Most important, it establishes a control standard endotoxin (CSE) calibrated to the RSE. However, the BET chapter does not specify endotoxin limits to establish the test as an alternative to the rabbit pyrogen test. The USP subcommittee responsible for the development and revision of the general tests chapter determined that endotoxin limits should be set on an article-by-article basis, recognizing that a specific general limit applied indiscriminately to all relevant compendial articles could not be justified. The first proposals for endotoxin limits appeared in Pharmacopeial Forum (Sept.–Oct. 1982) (7). These proposals established the BET as a replacement for the USP Pyrogen Tests for Water for Injection, Sterile Water for Injection, Bacteriostatic Water for Injection, and Water for Irrigation. The BET was also added as a new requirement for Water for Inhalation, USP. Endotoxin limits for these five compendial articles are shown in Table II.

Despite the unanimous disapproval of these proposals by all groups participating in the USP Open Conference on Parenteral Products, March 28–29, 1983 (8), the unchanged proposals for BET endotoxin limits for pharmaceutical waters were published in Addendum a (9) to Supplement 4 of USP XX (effective November 1, 1983).

The USP endotoxin limits for pharmaceutical waters are highly controversial and raise several important issues of interest to pharmaceutical manufacturers. Of primary concern is the decision to replace the pyrogen test with the BET in these monographs. It is the policy of USP to require that a replacement test for a particular article provide some extra safety or benefit relative to the existing test requirement for that article. Otherwise there is no need to replace one effective test with another, because alternate test methods, appropriately validated, are already permitted by USP. Economic savings or convenience are by themselves insufficient grounds for official replacement. Clearly, a good case can be made for requiring the BET in monographs of products that are not amenable to the use of the rabbit pyrogen test, or for products for which the USP pyrogen test might be replaced with advantage by the BET.

However, no convincing scientific or technical reasons have been given for replacing the USP pyrogen test in the five water monographs cited. It has been the official compendial pyrogen release test for water for over 40 years, and during this period the adequacy of this test for water has remained unchallenged.

On the other hand, there are several excellent reasons that the choice of the BET test for waters should be challenged. It is generally accepted that the BET is much more sensitive to endotoxin than is the rabbit test. The Pharmacopeial Convention is aware of this, and two recent articles appearing in Pharmacopeial Forum state that it would not be desirable to specify excessively low endotoxin limits solely because the lysate test is more sensitive than the rabbit test (10, 11). However, in specifying endotoxin limits of 0.25 EU/ml for Water for Injection, Sterile Water for Injection, and Sterile Water for Irrigation, the USP has done just that. Results of two large-scale rabbit dose-ranging studies (12), using the USP RSE to establish proposed limits, are shown in Table III. As indicated, 0.25 EU/ml is actually less than the calculated ED50 for both rabbit studies. Even the FDA has proposed more acceptable limits of 0.5 EU/ml for Water for Injection and Sterile Water for Injection and 1.0 EU/ml for Bacteriostatic Water for Injection.

The policy of setting endotoxin limits for USP monographed articles based on rabbit test results, using highly purified reference endotoxins, is also open to challenge. Studies have shown that highly purified endotoxins used as LAL test standards are far more pyrogenic than are environmental endotoxins encountered in waters. Recently, Travenol authors published the results of rabbit pyrogen tests conducted on 22 samples of water from common city supplies—drinking fountains, laboratory water outlets, washroom faucets, etc. (13). After the samples were filter sterilized, bacterial endotoxin levels were quantitated using LAL, and dose-ranging experiments were run with rabbits. Results are shown in Table IV. Water samples containing 1.0–4.0 EU/ml consistently passed the USP rabbit test. However, the rabbit pyrogen colony used for these studies consistently demonstrated an ED50 sensitivity of approximately 0.5 EU/ml to the current USP RSE.

### Table II. USP Endotoxin Limits for Compendial Articles

<table>
<thead>
<tr>
<th>Article</th>
<th>Endotoxin Limit (EU/ml)</th>
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<tbody>
<tr>
<td>Water for Injection, USP</td>
<td>0.25</td>
</tr>
<tr>
<td>Bacteriostatic Water for Injection, USP</td>
<td>0.5</td>
</tr>
<tr>
<td>Sterile Water for Inhalation, USP</td>
<td>0.5</td>
</tr>
<tr>
<td>Sterile Water for Injection, USP</td>
<td>0.25</td>
</tr>
<tr>
<td>Sterile Water for Irrigation, USP</td>
<td>0.25</td>
</tr>
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### Table III. Dose-Ranging Studies in Rabbits Using the USP Reference Standard Endotoxin

<table>
<thead>
<tr>
<th>Study</th>
<th>ED50 EU/kg</th>
<th>ED50 EU/ml</th>
<th>ED50 EU/kg</th>
<th>ED50 EU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10.2</td>
<td>1.03</td>
<td>2.6</td>
<td>0.26</td>
</tr>
<tr>
<td>2</td>
<td>15.0</td>
<td>1.50</td>
<td>3.6</td>
<td>0.36</td>
</tr>
</tbody>
</table>


* Study 1 involved 102 rabbits from 4 laboratories. Endotoxin doses ranged from 2.5 to 100.0 EU/kg. ED50 = dose pyrogenic to 50% of the rabbits (0.6 °C rise).

* 95% Confidence Level = 7.5–14.2.

* 95% Confidence Level = 1.0–4.1.

* Study 2 involved 40 rabbits from 1 laboratory. Endotoxin doses ranged from 5.0 to 800 EU/kg. ED50 = dose pyrogenic to 50% of the rabbits (0.55 °C rise).

* 95% Confidence Level = 7.4–25.9.

* 95% Confidence Level = 0.4–7.3.
shown in Table V. Eight hundred sixty solution samples
a manner not possible with the rabbit test and its potential
manufacturer. Its ability to quantitate endotoxin levels in
extremely useful quality-control tool for the pharmaceutical
industry. When properly validated, a negative LAL test will
ensure the absence of a given level of bacterial endotoxin
from test articles. However, the presence of a positive test
does not necessarily indicate the presence of pyrogenic levels
of endotoxin in a test article. It is important that industry
and regulatory agencies understood more clearly the rela­
tionship of “environmental endotoxin” and “purified en­
dotoxin” in rabbit and LAL pyrogen tests. Retaining the
USP rabbit test as an official reference test will allow us to
acquire adequate data with which to establish this rela­
tionship.

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3550 and 3551.
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potency of “environmental” endotoxins as measured by the Limulus
amebocyte lysate test and the USP Rabbit Pyrogen Test.” Endotoxins
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RESUMEN: Se discute el estado actual del ensayo LAL (Limulus Amebocyte Lysate) para la autorización
final de productos medicinales y dispositivos, tanto para los Estados Unidos como para los demás países.
El principal problema que se trata aquí es la determinación del límite de la endotoxina para el ensayo LAL.
Esto es muy significativo puesto que el ensayo LAL es más sensible que el ensayo de la farmacopea ameri­
cana, que usa el conejo, y porque como lo indican ensayos comparativos entre el LAL y el ensayo con el cone­
jo, el patrón de endotoxina purificado es más pirogénico que la endotoxina ambiental.

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