Review

A comparative analysis of pharmacopeial quality standards for antibiotics with respect to bacterial endotoxin limits

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Abstract

Background: The bacterial endotoxin test (BET) is an important quality control measure for active pharmaceutical ingredients (APIs), injections, and antibiotics used in parenteral preparations. This test confirms that products do not contain hazardous amounts of endotoxins released from the outer membrane of Gram-negative bacteria upon cell lysis. The detection and quantification of endotoxins are crucial for patient safety, as they can cause severe pyrogenic reactions. A pharmacopeia is a book of drug standards that ensures the quality, safety, and efficacy of pharmaceutical products. **Objective:** This study aims to assess the differences and correlations among the Indian Pharmacopeia (IP), British Pharmacopeia (BP), Japanese Pharmacopeia (JP), European Pharmacopeia (Ph. Eur.), United States Pharmacopeia (USP), and International Pharmacopeia (Ph. Int.) regarding injection preparation specifications, antibiotic and API specifications, and acceptance criteria for bacterial endotoxins. BET guidelines are provided by these pharmacopeial organizations, including the BP, JP, IP, Ph. Eur., Ph. Int., and USP. The permissible endotoxin limits vary depending on the route of administration. It was found that bacterial endotoxin limits for antibiotic injections and APIs are specified in the IP, BP, JP, Ph. Eur., USP, and Ph. Int. The findings suggest that the similarities and variations in bacterial endotoxin limits and their acceptance criteria, as outlined in these pharmacopeias, should be harmonized to promote regulatory consistency and patient safety. **Conclusion:** The comparative data obtained from this study will be useful for developing strategies to harmonize pharmacopeial standards concerning antibiotic preparation requirements, including bacterial endotoxin limit specifications.

Keywords: Bacterial endotoxin limits, Antibiotics, Pharmacopeia, Harmonization, Safety

1. Introduction

For thousands of years, people have been increasingly exposed to numerous diseases. Millions of people died from these illnesses, which frequently escalated into epidemics. During this period, people began to think about infectious diseases and their causes. However, efforts to prevent, treat, or control the spread of such diseases were initially unsuccessful.[1] One of the most effective medical breakthroughs against infectious diseases is the discovery of antibiotics. The number of lives they have saved and their profound impact on combating infectious diseases—which throughout most of human history were the main causes of morbidity and mortality—cannot be overstated.[2]

Salvarsan, the first antibiotic, was introduced in 1910. Antibiotics have significantly transformed modern medicine and increased average human life expectancy by approximately 23 years within just over a century.[3] Salvarsan has remained an enigmatic compound due to its extensive use. By 1920, two million doses of Salvarsan were produced annually in the United States (US) alone, and it was also demonstrated to be

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effective against certain parasitic diseases. Salvarsan remained in high demand until 1943, when penicillin, a more tolerable and equally effective drug, became available.[4] Following Fleming's discovery of penicillin in 1928, numerous other antibiotics were subsequently developed and approved for human use. However, both Gram-positive and Gram-negative bacteria have developed resistance to many of these agents.[5]

Given the slow pace in the discovery of novel antibiotic classes over recent decades,[6] there is a growing concern about their effectiveness and the emergence of resistant pathogens. Recent *in vitro* studies have demonstrated that certain antibiotics, particularly those targeting penicillin-binding protein 3, can promote endotoxin release.[7,8] It is well established that cell wall–active antibiotics facilitate the release of endotoxins. The study of endotoxins began in the late 19th century, when Richard Pfeiffer, a student of Robert Koch, discovered that lysates of heat-inactivated *Vibrio cholerae* contained a toxic substance capable of inducing death in experimental animals.[9] In addition, two other scientists, Eugenio Centanni and Hans Buchner, independently isolated the same toxin.[10] Centanni made two important contributions; first, he observed that the toxin could be isolated from lysates of many Gram-negative bacteria,[7] but not from Gram-positive species.[11] Second, he emphasized the remarkable pyrogenic properties of endotoxin.

Since it is well recognized that pyrogenic substances elevate body temperature, bacterial endotoxins exert multiple physiological effects.[12,13] Endotoxins are lipopolysaccharide (LPS) components derived from the outer membrane of Gram-negative bacteria.[14,15] They are released at a low, steady rate from living bacteria; however, substantially larger amounts of endotoxins are released following bacterial cell lysis.[16]

The rabbit pyrogen test (RPT) and bacterial endotoxin test (BET) are compendial methods that share the same origin—"injection fever[17]"—a quality defect that was once prevalent in parenteral therapy. Injection fever results from contamination with Gram-negative bacterial endotoxins, as evidenced by patient febrile reactions caused by contaminated medicinal products, biologicals, and medical devices over the past century.[18] All parenteral drug products and devices administered via intravenous, intraventricular, intra-arterial, intra-articular, subcutaneous, intramuscular, intrathecal, intracisternal, and intraocular routes must comply with an appropriate endotoxin limit specification to obtain marketing authorization from health authorities.[19]

There are variations in the pharmacopeial guidelines and standard limits for bacterial endotoxin testing across different pharmacopeias—including the Indian Pharmacopeia (IP), British Pharmacopeia (BP), Japanese Pharmacopeia (JP), European Pharmacopeia (Ph. Eur.), US Pharmacopeia (USP), and International Pharmacopeia (Ph. Int.)—which directly and indirectly affect the cost of testing, increase the complexity of compliance, and hinder the global transport and trade of pharmaceutical products.

Therefore, the harmonization of pharmacopeial standards is urgently needed, involving the exchange and alignment of methods and requirements to yield comparable results and ensure patient safety by upholding quality standards for pharmaceuticals. Specifically, the harmonization of BET guidelines and limits among different pharmacopeias is crucial to achieve consistency and uniformity in the manufacturing and regulatory processes of pharmaceutical products. Based on the recent updates to the core Q4B guideline and its annexes by the Pharmacopeial Discussion Group, the BET procedures described in the USP, JP, and Ph. Eur. can now be used interchangeably, leading to the recognition of pharmacopeial BET methods by regulatory authorities within the International Council for Harmonization (ICH) regions.[20]

2. Pyrogen and endotoxin

Pyrogens are substances, such as endotoxins (LPS), released from Gram-negative bacteria that can cause fever. They pose a serious risk when present in pharmaceutical products.[21] Pyrogen can be either endotoxins or non-endotoxins, such as non-endotoxin pyrogens (NEPs); however, bacterial endotoxins are the most prevalent and pose the greatest threat to patient safety.[22,23] Endotoxins are intrinsic components of the outer membrane of Gram-negative bacteria.[24,25] They are considered a heterogeneous group of biomolecules that are released upon bacterial cell lysis, resulting in toxic effects such as fever, septic shock, multiple organ failure, and death.[26] Due to the growing number of resistant bacterial strains, endotoxin-induced septic shock syndrome continues to pose a significant global health threat and is associated with an unacceptably high mortality rate.[27]

The primary component of the outer membrane of Gram - negative bacteria, LPS, becomes hazardous when it enters the bloodstream, or when released into the environment, or when used in industrial applications such as pharmaceuticals.[28] Examples of LPS producing bacteria include species of *Escherichia, Salmonella, Yersinia, Shigella* as well as *Enterobacter, Proteus*, and pathogens such as *Vibrio cholerae, Yersinia pestis*, and *Brucella abortus.*[29]

Endotoxin units (EU) are used to measure endotoxin levels. The Second International Endotoxin Standard—which establishes that 1 EU is equivalent to 1 International Unit (IU)—was approved by the World Health Organization's Expert Committee on Biological Standardization. The expression K/M is used to determine the endotoxin limit for a particular test preparation, where M is the maximum dose administered to an adult (estimated to be 70 kg for this purpose) per kilogram of body weight per hour, and K is

the threshold pyrogenic dose of endotoxin per kilogram of body weight. *K* has a value of 5.0 EU/kg for parenteral drugs (except those administered intrathecally) and 0.2 EU/kg for preparations intended for intrathecal administration.[29-31] It has been reported that approximately 2×10^6 LPS molecules, equivalent to approximately 20 femtograms, can be found in a single *E. coli* cell.[29] The physiologically active LPS is associated with or embedded in phospholipids, lipoproteins, and other proteins that constitute the Gram-negative bacterial outer membrane.[32,33] LPS is a structural component of the cell wall of Gram-negative bacteria and plays a major role in the pathogenesis of septic shock in humans. The hydrophobic lipid region of the membrane contains a portion of the LPS molecule embedded within it, while the hydrophilic polysaccharide portion is exposed to the external environment of the cell.[34,35]

The most widely used test for detecting endotoxins is the *Limulus* amebocyte lysate (LAL) assay.[36,37] In this test, horseshoe crab-derived LAL reacts with bacterial endotoxin (LPS) to form a measurable gel clot.[37] The unit of measurement for endotoxins is EU/mL, with 1 EU equivalent to approximately 0.1–0.2 ng of endotoxin/mL of solution.[38,39] The LAL test is currently available in three formats, each with distinct sensitivity levels. While the kinetic turbidimetric and chromogenic LAL assays can detect concentrations as low as 0.01 EU/mL, the gel-clot method detects concentrations as low as 0.03 EU/mL.[24]

3. Overview of endotoxin testing

Endotoxin testing of parenteral medications and implantable devices is essential for detecting contamination and ensuring patient safety. It is also a mandatory release test for the batch production of therapeutic products.[40]

3.1. Rabbit Pyrogen Test (RPT)

The US Food and Drug Administration approved the RPT as the first method for detecting LPS. It was developed in the 1920s, based on the observation that rabbits exhibit an increase in body temperature following the intravenous injection of a test solution.[41] In practice, the RPT has been gradually replaced by the LAL assay due to its greater efficiency in terms of cost and testing time.[26,42] However, the RPT has been phased out in many regions. According to the recent updates to the Ph. Eur., the RPT has been officially removed to promote animal welfare and scientific advancement.[43-45]

3.2. *Limulus* amebocyte lysate assay or BET

In 1964, Levin and Bang made the groundbreaking discovery that the lysate of the Atlantic horseshoe crab (*Limulus polyphemus*) coagulates upon exposure to bacterial endotoxins. Their findings were instrumental in the development of the LAL test.[46] Despite being classified as "vulnerable," Atlantic horseshoe crabs are crucial for maintaining global public health. Currently, humans use these blue-blooded marine invertebrates to ensure that endotoxins do not contaminate vaccines, injectable medications, and medical devices used in both human and veterinary care.[47] Over the past decade, LAL has been used to quantify endotoxin levels in pharmaceutical products and biological fluids.[48] The LAL assay can detect not only Gram-negative bacterial endotoxins but also fungal components containing β-D-1,3-glucan.[49]

All major pharmacopeias worldwide recommend the LAL test for the detection of bacterial endotoxins.[50] Endotoxins are also contaminants found in certain organic dusts and environmental media that support the growth of Gram-negative bacteria.[51] Among the pharmacopeias—IP, BP, JP, Ph. Eur, Ph. Int, and USP—three assay methods are described: (i) the turbidimetric method, which is based on the development of turbidity following the cleavage of an endogenous substrate; (ii) the chromogenic method, which is based on color development that occurs after a synthetic peptide–chromogen complex is cleaved; and (iii) the gel-clot method, which is based on gel formation (Figure 1).[52]

Several ethical concerns are associated with the harvesting of LAL from horseshoe crabs. Overharvesting LAL from crabs may cause injury and mortality, leading to population decline and adverse ecological impacts. Although the crabs are returned to the ocean after blood collection, a significant number of crabs die because of the procedure or suffer from adverse impacts. These ethical issues emphasize the need for alternative methods for the BET.[47]

3.2.1. Legislative classification of Limulus amebocyte lysate assay

The LAL test is categorized at an equivalent level across national pharmacopeias, including the 2024 BP,[53] 2025 USP,[54] JP,[55] 2022 Ph. Eur.,[56] 2022 Ph. Int.,[57] and 2022 IP[58] and its Addendum.

3.3. Qualitative analysis

3.3.1. Gel-clot limit test method

Gel-clot testing is a manual process. The BET performed using the gel-clot method is a 60-min test conducted at an incubation temperature of 37°C.[59] LAL reagent water, LAL, and control standard endotoxin (CSE) are combined to create an endotoxin standard series for the experiment. The sensitivity unit of the LAL employed for the test is lambda (λ), which is printed on the LAL vial and the certificate of analysis. The standard curve dilutions tested are 2λ, 1λ, ½λ, and ¼λ. The standard curve must be positive within a two-fold dilution of λ to be valid.[53-58]

3.3.2. Specific reagents

(a) Endotoxin reference standard (ERS) and control standard endotoxin
The ERS and commercially produced CSE are isolated from Gram-negative bacterial cell membranes using the Westphal hot phenol method and processed to eliminate membrane components.[60] Furthermore, the purified preparations are typically stabilized with suitable agents. A CSE that is appropriately standardized against the ERS may be used for routine bacterial endotoxin testing.[61]

(b) Lysate
Lysate is an aqueous extract of blood cells (amoebocytes) from one of the following horseshoe crab species[62]—*L. polyphemus*, *Tachypleus gigas*, *Tachypleus tridentatus*,[63] or *Carcinoscorpius rotundicauda*—and is reconstituted according to the instructions on the label.[64]

(c) BET-grade water
The BET, which is used to identify and measure endotoxins produced by Gram-negative bacteria, depends heavily on water (comprising 80–95% of the test system).[65] If endotoxins contaminate certain products, particularly those used in medications and medical equipment, they may pose a risk to human health.[66] It is crucial to use water that is free from endotoxins and other impurities that could skew the test results to ensure reliability. If endotoxins are present in this water, they should be below 0.005 EU/mL to prevent false-positive results.[67]

3.3.3. Endotoxin limit calculation for the gel-clot method

(a) Endotoxin limit calculation
The product's dosage and route of administration are considered when calculating the endotoxin limit. The standard equation is given below (Equation [I]):

$$EL = \frac{K}{M} \quad \text{(I)}$$

where:
(i) *EL* is the endotoxin limit (EU/mL or EU/unit).
(ii) *K* is the threshold pyrogenic dose of endotoxin per kilogram of body weight. Standard *K* values are 0.2 EU/kg for intrathecal medications and 5 EU/kg for intravenous injections, depending on the administration route.[68]
(iii) *M* is the maximum dose of the product per kilogram of body weight per hour.

(b) Dilution preparation
A series of dilutions must be prepared to determine whether the product exhibits any interfering effects. The dilution factor should be selected so that the endotoxin concentration falls within the LAL reagent's detection range.

The maximum valid dilution can be calculated using Equation (II):

$$MVD = \frac{EL \times concentration\ of\ the\ test\ solution}{\lambda} \quad \text{(II)}$$

Where:
(i) *MVD* is the maximum dilution that still permits the detection of endotoxins
(ii) *EL* is the endotoxin limit
(iii) λ is the sensitivity of the LAL reagent.

3.4. Photometric quantitative techniques

3.4.1. Kinetic turbidimetric method

The kinetic turbidimetric method—a photometric assay—measures the increase in turbidity resulting from the reaction between endotoxin and lysate. It also determines either the rate of turbidity development or the time required to reach a predefined absorbance or transmission value of the reaction mixture. The test is conducted at the incubation temperature recommended by the lysate manufacture (37 ± 1°C).[53-55,58]

3.4.2. Kinetic chromogenic method

The kinetic chromogenic method is a photometric assay that measures the rate of color development or the time (onset time) required to reach a predetermined absorbance, which results from the reaction between endotoxin and lysate. This reaction releases chromophores from a chromogenic substrate.[53-55,58]

3.4.3. End-point chromogenic assay

The end-point chromogenic assay measures the color intensity at the end of the incubation period, after the reaction has been stopped by the addition of a suitable reagent.[53-55,58]

4. Evidence acquisition

In this study, data were collected from multiple databases. The official websites of national and regional pharmacopeial authorities were examined using the World Health Organization index,[52] and library resources were utilized to obtain additional information. Furthermore, databases such as Google Scholar and PubMed were also assessed to identify relevant materials.

5. Findings and harmonization recommendations

Based on the comparative data obtained from this study, recommendations for harmonizing pharmacopeial updates related to BET are presented in Table 1. In addition, acceptance criteria, specifications, and endotoxin limits for various antibiotics are summarized in Table 2.

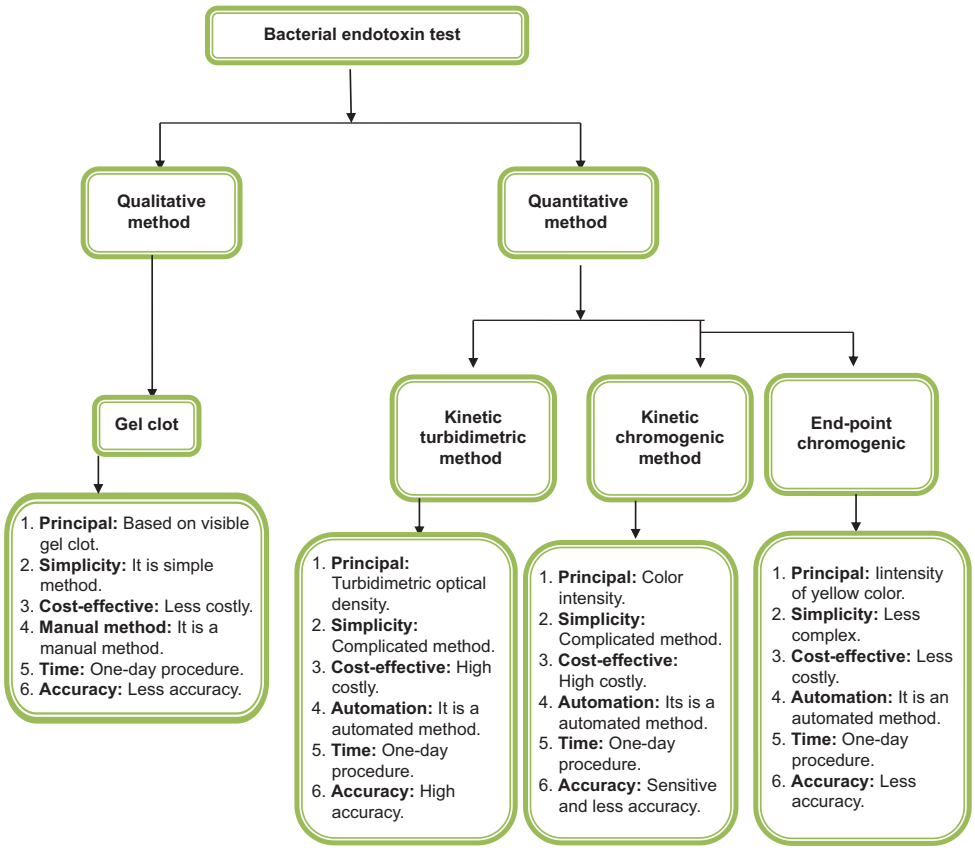

Figure 1. Bacterial endotoxin test methods

Table 1. Comparative status of endotoxin test methods in different pharmacopeias

Test	Pharmacopeia					
	2022 IP	2025 USP	2024 BP	2021 JP	2022 Ph. Eur.	2022 Ph. Int.
Rabbit Pyrogen Test	Chapter 2.2.8	Chapter 151	Appendix: XIV D	General chapter 4.04	Chapter 2.6.8 page no. 215	Chapter 3.5
BET	Chapter 2.2.3	Biological Texts<85>	Appendix: XIV C	General chapter 4.01	Chapter 2.6.14	Chapter 3.4
MAT	Chapter 2.2.25	NA	Appendix: XIV H	NA	Chapter 2.6.30	NA
rFC	NA	Chapter 86	Appendix: XIV C	NA	Chapter 2.6.32	NA
Summary/remarks	IP lacks rFC methods; USP lacks MAT methods; JP lacks MAT and rFC methods; and Ph. Int. lacks MAT and rFC methods.					

Abbreviations: BET: Bacterial endotoxin test; BP: British Pharmacopeia; IP: Indian Pharmacopeia; JP: Japanese Pharmacopeia; MAT: Monocyte activation test; NA: Not available; Ph. Eur.: European Pharmacopeia; Ph. Int.: International Pharmacopeia; rFC: Recombinant factor C; USP: United States Pharmacopeia.

6. Endotoxin test methods in different pharmacopeias

In terms of microbiological requirements for bacterial endotoxin testing, the 2024 BP, 2022 IP, 2021 JP, 2022 EP, 2022 Ph. Int., and 2025 USP were reviewed and compared with respect to bacterial endotoxin limit acceptance criteria and microbial enumeration specifications for antibiotics and antibiotic drug preparations (Table 1).

Since all comparisons in this study were based on data from previously published pharmacopeias, ethical approval was not required.

(i) BP: The BP published similar monographs and contained the same microbiological standards as those described in *General Text 5.8*.[53]

(ii) IP: The *IP Chapter 2.2.3*, "Biological Methods," provides BET values for all types of antibiotics listed in Table 1.[58]

(iii) USP: The *USP Biological Text <85>* and *General Chapter 2025* describe the microbiological characteristics of both nonsterile and sterile products. Each product type should have distinct bacterial endotoxin limits.[54]

(iv) JP: The *JP General Test 4.01* (page V-A764) provides BET methods[55] for all types of antibiotics listed in Table 1.

Table 2. Bacterial endotoxin acceptance criteria for antibiotic preparations in different pharmacopeias

Antibiotics	Pharmacopeia					
	2022 IP	2025 USP	2024 BP	2021 JP	2022 Ph. Eur.	2022 Ph. Int.
Penicillin						
Penicillin G (benzathine) injection	Not more than 0.13 EU/mL	Not more than 0.01 EU/100 penicillin G units	<0.13 IU/mg	NA	NA	NA
Penicillin G (benzathine)	Not more than 0.13 EU/mL	NA	NA	NA	NA	Not more than 0.01 IU of endotoxin RS per mg of benzylpenicillin
Fortified benzathin penicillin injection	Not more than 0.13 EU/mL	NA	NA	NA	NA	NA
Penicillin G (potassium) injection	Not mentioned	Not more than 0.01 EU/100 penicillin G units	Not mentioned	<1.25 EU/mg	NA	NA
Benzyl penicillin potassium	Not more than 0.16 EU/mL	NA	NA	NA	NA	NA
Benzyl penicillin G potassium	Not more than 0.16 EU/mL	NA	NA	NA	NA	NA
Amoxicillin injection	Not more than 0.25 EU/mg of amoxicillin	Not more than 0.25 EU/mg of amoxicillin	<0.25 IU/mg	NA	NA	NA
Amoxicillin sodium	Not more than 0.25 EU/mg of amoxicillin	NA	<0.25 IU/mg	NA	<0.25 IU/mg	NA
Ampicillin injection	Not more than 0.15 EU/mg of ampicillin.	Not more than 0.15 EU/mg of ampicillin	NA	NA	NA	NA
Ampicillin sodium for injection	NA	NA	NA	<0.075 EU/mg of ampicillin	NA	NA
Ampicillin sodium	Not more than 0.15 EU/mg	NA	NA	NA	<0.15 IU/mg	Not more than 0.15 IU of endotoxin RS per mg of ampicillin
Nafcillin injection	NA	Not more than 0.13 EU/mg of Nafcillin	NA	NA	NA	NA
Oxacillin sodium	Not more than 0.20 EU/mg of oxacillin sodium	Not more than 0.2 EU/mg of oxacillin	<0.20 IU/mg	NA	Less than 0.20 IU/mg	NA
Oxacillin sodium for injection	NA	Not more than 0.2 EU/mg of oxacillin	<0.20 IU/mg	NA	-	NA
Oxacillin sodium monohydrate	NA	NA	<0.20 IU/mg	NA	NA	NA
Piperacillin sodium	Not more than 0.07 EU/mg of piperacillin sodium	NA	NA	NA	NA	NA
Piperacillin	Not more than 0.07 EU/mg	NA	NA	NA	NA	NA
Piperacillin hydrate	NA	NA	NA	<0.07 EU/mg	NA	NA
Piperacillin sodium for injection	NA	NA	NA	<0.04 EU/mg	NA	NA
Piperacillin intravenous infusion	Not more than 2.5 EU/mL of a solution	Not more than 0.07 EU/mg of piperacillin	NA	NA	NA	NA
Piperacillin and tazobactam injection	Not more than 0.08 EU/mg	Not more than 0.08 EU/mg	NA	NA	NA	NA
Ticarcillin and clavulanic acid injection	Not more than 0.07 EU/mg of ticarcillin	Not more than 0.07 EU/mg of ticarcillin	NA	NA	NA	NA
Ticarcillin sodium	NA	NA	<0.05 IU/mg	NA	Less than 0.05 IU/mg	NA
Ticarcillin for injection	NA	Not more than 0.05 EU/mg of ticarcillin	NA	NA	NA	NA
Ticarcillin monosodium	Not more than 0.05 EU/mg of ticarcillin	Not more than 0.05 EU/mg of ticarcillin	<0.05 IU/mg	NA	NA	NA
Ticarcillin disodium	NA	Not more than 0.05 EU/mg of ticarcillin	NA	NA	NA	NA

(Cont'd...)

Table 2. ***(Continued)***

Antibiotics	Pharmacopeia					
	2022 IP	**2025 USP**	**2024 BP**	**2021 JP**	**2022 Ph. Eur.**	**2022 Ph. Int.**
Cephalosporins						
Cefazolin sodium	Not more than 0.15 EU/mg cefazolin	Not more than 0.15 EU/mg of cefazolin	<0.15 IU/mg	NA	<0.15 IU/mg	NA
Cefazolin sodium hydrate	NA	NA	NA	<0.10 EU/mg	NA	NA
Cefazolin sodium for injection	Not more than 0.15 EU/mg of cefazolin	NA	NA	<0.05 EU/mg	NA	NA
Cefuroxime injection	Not more than 0.1 EU/mg of cefuroxime	Not more than 0.10 EU/mg of cefuroxime	<0.10 IU/mg	NA	NA	NA
Cefuroxime sodium	Not more than 0.1 EU/mg of cefuroxime	NA	<0.10 IU/mg	NA	<0.10 IU/mg	NA
Ceftriaxone sodium	Not more than 0.20 EU/mg of ceftriaxone sodium	Not more than 0.20 EU/mg of ceftriaxone sodium	<0.08 IU/mg	NA	<0.08 IU/mg	not more than 0.08 IU of EU/mg of ceftriaxone sodium
Ceftriaxone injection	Not more than 0.2 EU/mg of ceftriaxone	Not more than 0.20 EU/mg of ceftriaxone	NA	NA	NA	NA
Cefotaxime sodium injection	Not more than 0.20 EU/mg of cefotaxime	Not more than 0.20 EU/mg of cefotaxime	<0.05 IU/mg (injection not mentioned)	NA	NA	NA
Cefotaxime sodium	Not more than 0.20 EU/mg of cefotaxime	NA	<0.05 IU/mg (injection not mentioned)	NA	<0.05 IU/mg	NA
Ceftazidime injection	Not more than 0.10 EU/mg	Not more than 0.1 EU/mg of Ceftazidime	NA	<0.067 EU/mg	NA	NA
Ceftazidime pentahydrate with sodium carbonate	NA	NA	NA	NA	NA	NA
Ceftazidime pentahydrate	NA	NA	<0.10 IU/mg	NA	<0.10 IU/mg	NA
Ceftazidime pentahydrate with sodium carbonate injection	NA	NA	<0.10 IU/mg	NA	<0.10 IU/mg	NA
Cefepime injection	Not more than 0.06 EU/mg	Not more than 0.06 EU/mg of cefepime	NA	NA	NA	NA
Cefepime dihydrochloride hydrate	NA	NA	NA	<0.04 EU/mg	NA	NA
Cefepime dihydrochloride injection	NA	NA	NA	<0.06 EU/mg	NA	NA
Cefepime hydrochloride/ monohydrate	Not more than 0.04 EU/mg	NA	<0.04 IU/mg	NA	<0.04 IU/mg	NA
Carbapenems						
Imipenem	Not more than 0.17 EU/mg of imipenem	Not more than 0.17 EU/mg of imipenem	<0.17 IU/mg	NA	NA	NA
Imipenem and cilastatin injection	Not more than 0.17 EU/ mg of imipenem and not more than 0.17 EU/mg of cilastatin	Not more than 0.17 EU/mg of imipenem and not more than 0.17 USP EU/mg of cilastatin	NA	NA	NA	NA
Imipenem monohydrate	NA	NA	<0.17 IU/mg	NA	<0.17 IU/mg	NA
Meropenem Injection	Not more than 0.125 EU/ mg of meropenem	Meets the requirements	NA	<0.12 EU/mg	NA	NA
Meropenem trihydrate	NA	NA	<0.125 IU/mg	NA	<0.125 IU/mg	NA

(Cont'd...)

Table 2. ***(Continued)***

Antibiotics	Pharmacopeia					
	2022 IP	**2025 USP**	**2024 BP**	**2021 JP**	**2022 Ph. Eur.**	**2022 Ph. Int.**
Monobactam						
Aztreonam	NA	Not more than 0.17 EU/mg of aztreonam	NA	<0.10 EU/mg	NA	NA
Macrolides						
Azithromycin	NA	Meets the requirements	NA	NA	NA	NA
Tetracyclins						
Tetracycline hydrochloride	Not more than 0.5 EU/mg	Not more than 0.5 EU/mg of tetracycline hydrochloride	<0.5 IU/mg	NA	<0.5 IU/mg	NA
Oxytetracycline	Not more than 0.4 EU/mg of oxytetracycline	-	NA	NA	NA	NA
Oxytetracycline injection	Not more than 0.4 EU/mg of oxytetracycline	-	NA	NA	NA	NA
Oxytetracycline hydrochloride injection	Not more than 0.4 EU/mg of oxytetracycline	-	NA	NA	NA	NA
Doxycycline hydrochloride	Not more than 1.14 EU/mg	NA	<1.14 IU/mg	NA	NA	NA
Aminoglycosides						
Gentamycin sulfate/ injection	Not more than 1.67 EU/mg of gentamycin	Not more than 0.71 EU/mg of gentamycin	<0.71 IU/mg (injection not mentioned)	<0.50 EU/mg	NA	NA
Gentamycin sulfate	NA	NA	<0.71 IU/mg	NA	<0.71 IU/mg	Not more than 1.70 IU of endotoxin RS per mg of gentamicin
Tobramycin injection	Not more than 2.00 EU/mg of tobramycin	Not more than 2.00 EU/mg of tobramycin	NA	<0.50 EU/mg	NA	NA
Tobramycin	Not more than 2.00 EU/mg	NA	NA	NA	<2.00 IU/mg	NA
Amikacin sulfate injection	Not more than 0.33 EU/mg	Not more than 0.33 EU/mg of amikacin	<2.00 IU/mg	<0.50 EU/mg of amikacin	NA	NA
Amikacin sulfate	NA	NA	NA	NA	NA	Not more than 0.33 IU of EU/mg of amikacin
Streptomycin sulfate/ injection	Not more than 0.25 EU/mg	Not more than 0.25 EU/mg	<0.25 IU/mg	<0.10 EU/mg	NA	NA
Streptomycin sulfate	Not more than 0.25 EU/mg	NA	NA	NA	<0.25 IU/mg	Not more than 0.25 IU of endotoxin RS per mg of streptomycin
Fluorquinolones						
Ciprofloxacin injection	Not more than 0.25 EU/mg of ciprofloxacin	Not more than 0.50 EU/mg of Ciprofloxacin	NA	NA	NA	NA
Levofloxacin injection	Not more than 2.0 EU/mg of levofloxacin	NA	NA	<0.60 EU/mg	NA	NA
Ofloxacin infusion	Not more than 0.88 EU/mg of ofloxacin	NA	NA	NA	NA	NA
Glycopeptides						
Vancomycin hydrochloride	Not more than 0.25 EU/mg	Not more than 0.33 EU/mg vancomycin	NA	NA	NA	NA
Vancomycin hydrochloride for injection	NA	NA	NA	<0.25 EU/mg	NA	NA
Vancomycin intravenous infusion	The maximum allowable endotoxin concentration of the solution is 2.5 units of endotoxin per mL	Not more than 0.33 EU/mg of vancomycin	NA	NA	NA	NA
Teicoplanin	Not more than 0.31 EU/mg of teicoplanin	NA	NA	NA	NA	NA
Teicoplanin injection	Not more than 0.30 EU/mg of teicoplanin	NA	NA	NA	NA	NA

(Cont'd...)

Table 2. ***(Continued)***

Antibiotics	Pharmacopeia					
	2022 IP	2025 USP	2024 BP	2021 JP	2022 Ph. Eur.	2022 Ph. Int.
Lincosamides						
Clindamycin phosphate/injection	Not more than 0.6 EU/mg	Not more than 0.58 EU/mg of clindamycin	<0.6 IU/mg	<0.1 EU/mg	<0.61 IU/mg	Not more than 0.6 IU of endotoxin RS per mg of clindamycin
Nitroimidazoles						
Metronidazole injection	Not more than 0.35 EU/mg	Not more than 0.35 EU/mg	NA	NA	NA	NA
Metronidazole	NA	NA	NA	NA	NA	Not more than 0.35 IU of endotoxin RS per mg
Rifamycins						
Rifampin injection	NA	Not more than 0.5 USP EU/mg	NA	NA	NA	NA
Rifampin sodium	NA	NA	NA	NA	<0.50 IU/mg	NA

Abbreviations: EU: Endotoxin unit; IU: International unit; NA: Not available; RS: Reference standard.

(v) Ph. Eur.: The *2022 Ph. Eur. Chapter 2.6.14* (page 226) provides bacterial endotoxin testing guidelines for all types of antibiotics listed in Table 1.[56]

(vi) Ph. Int.: The *2022 Ph. Int. Text 3.4* provides bacterial endotoxin testing procedures for all types of antibiotics listed in Table 1.[57]

7. BET limits for antibiotic preparations in different pharmacopeias

This comparative study focused on pharmacopeial quality standards concerning the BET for antibiotics. It aims to evaluate the differences and similarities among the IP, BP, JP, Ph. Eur., Ph. Int., and USP regarding the endotoxin limit specifications for injectable antibiotics (Table 2).

The methods used for endotoxin detection, such as the RPT and LAL assay, are discussed in detail. These methods ensure patient safety by preventing pyrogenic reactions caused by endotoxins, which are LPS released from Gram-negative bacteria. This study compares the endotoxin limits across different antibiotics and identifies both commonalities and gaps among the pharmacopeial standards.

Comparison of different pharmacopeias revealed significant variations in the guidelines and standard limits for BET methods, indicating regional disparities in the BET specifications across different pharmacopeias (Table 2). Differences in regulatory guidelines, policies, historical developments, and national health priorities have resulted in discrepancies between the pharmacopeias. Additionally, epidemiological factors such as disease outbreaks and pandemics may also drive pharmacopeial content, contributing to these disparities. Therefore, updating and aligning pharmacopeial guidelines to ensure better regulation of bacterial endotoxin limits across various pharmacopeias from different regions is crucial. This harmonization of bacterial endotoxin standards across different pharmacopeias is essential, as it provides consistent and comparable results for the same product at different locations, ensures patient safety, and reduces trade barriers by establishing universally recognized standards for pyrogen detection.

8. Recombinant factor C (rFC) assay

The rFC assay is an animal-free alternative method used for bacterial endotoxin testing. The rFC enzyme, cloned from the horseshoe crab, is used in this assay. The solution emits fluorescence when the activated rFC enzyme cleaves a synthetic fluorogenic substrate in response to endotoxin binding. Without the use of horseshoe crabs, the rFC test provides the same level of reliability as the LAL approach, using a single enzymatic step. Compared with conventional LAL test methods, rFC exhibits higher affinity and sensitivity to LPS, which enhances assay specificity.[69] Compared with the LAL test, the rFC assay provides reliable results and is more readily accepted than other bacterial endotoxin testing methods.[70]

9. Discussion

The methods used for endotoxin detection have evolved over time, with the initial RPT being replaced by more efficient and reliable methods, such as the LAL test and the BET.[71] These methods— including the qualitative gel-clot limit test and photometric quantitative techniques such as kinetic turbidimetric, kinetic chromogenic, and end-point chromogenic methods—have become essential tools for ensuring the safety and effectiveness of medical and pharmaceutical products through the detection of bacterial endotoxins.[72]

The comparative study of BET standards for antibiotics across the 2022 IP, 2025 USP, 2021 JP 2022, Ph. Eur. 2022, Ph. Int., and 2024 BP reveals both similarities and differences in the allowable endotoxin limits for different

antibiotics. Overall, while there are slight differences between the pharmacopeias, the USP generally provides the most detailed and comprehensive specifications for endotoxin limits across a wide range of antibiotics,[53] whereas the IP, JP, and BP occasionally lack specific details for certain drugs. This comparison underscores the importance of harmonizing pharmacopeial standards to ensure consistent quality control and patient safety in antibiotic production worldwide.

10. Harmonization of microbiological bacterial endotoxin acceptance

10.1. Criteria for antibiotics

From the above review, it can be seen that all pharmacopeias demonstrate both similarities and differences in bacterial endotoxin acceptance criteria, including those for antibiotics and their API. The similarities and differences in endotoxin limit and their acceptance criteria, as specified in the various pharmacopeias, need to be harmonized and streamlined to ensure that if a test is within the specified limit according to the harmonized monographs, it satisfies the requirements of all pharmacopeias, as well as the regulatory stipulations of the relevant countries. The ICH has already harmonized pharmacopeial texts for use in the ICH regions through the *BET General Chapter Q4B Annex 14*. These ICH quality guidelines can be used interchangeably within ICH regions.[20]

10.2. Practical barriers to harmonization

Each pharmacopeia has evolved independently, which has led to discrepancies in pharmacopeial standards. In addition, variations in legal frameworks, rigid regulatory requirements, and national priorities act as major hindrances to achieve consistency in drug quality and testing standards. This regulatory inertia serves as a significant practical barrier to pharmacopeial harmonization. Furthermore, harmonization of pharmacopeias also affects manufacturing processes, testing methods, regulatory compliance, and international trade, ultimately influencing production costs.

11. LPS variability complicating standardization

LPSs released by bacteria are complexed with LPS-binding protein, a plasma protein primarily produced by hepatocytes and one of the most extensively studied soluble proteins with LPS-binding capacity.[28] LPS is composed of an O-specific polysaccharide chain, a core oligosaccharide region, and lipid A, which contributes to its toxicity. Lipid A, an amphiphilic glycolipid, exhibits structural variations under different temperature and pH conditions.[28] Thus, LPS variability in chemical structure and molecular interactions leads to complications in the quantification of endotoxin measurements.[73]

12. Clinical impacts of divergent endotoxin limits

When administered to patients, pharmaceutical drugs containing high concentrations of bacterial endotoxins—a type of pyrogen—can cause severe and sometimes fatal immune reactions. Excess endotoxins in injectable drugs, contaminated medical equipment, or implanted materials can result in septic shock and systemic inflammation.[74]

When Gram-negative bacteria die, their cell walls release endotoxins, which are LPS. Lipid A, the toxic component of LPS, binds to host immune cells such as macrophages through Toll-like receptor 4. This interaction triggers a cascade of hyperinflammatory responses that may lead to the following detrimental clinical effects:[74]

(i) Flu-like symptoms and fever (septic fever)
(ii) Shock accompanied by hypotension
(iii) Disseminated intravascular coagulation
(iv) Multiple organ dysfunction syndrome
(v) Inflammatory diseases.

13. Cost impact of harmonization on manufacturers

The main benefits of harmonizing bacterial endotoxin standards for manufacturers include reduced production costs due to simplified procedures, streamlined regulatory compliance, and enhanced market accessibility. However, several drawbacks persist, such as the initial expenses associated with implementing harmonized procedures and the potential challenges in validating complex samples that require specialized testing. The currently employed qualitative BET method (gel-clot limit test) is labor-intensive; however, the universal acceptance of quantitative methods by all pharmacopeias may alleviate costly manufacturing challenges through prior standardization.[40]

14. Recommendations for key stakeholders

While regulatory authorities should implement Good Pharmacopeial Practices and the Common Technical Document format for data submission, pharmacopeial commissions should adopt Good Pharmacopeial Practices and align with ICH guidelines. Collaboration should be fostered through forums such as the Pharmacopeial Discussion Group. In cooperation with their stakeholders, pharmacopeias and regulatory bodies worldwide have been actively and effectively pursuing compendial harmonization for decades. These continuing efforts to harmonize compendial standards provide perspectives that may be useful when considering pharmacopeias' future trajectory.

15. Conclusion

It can be concluded that the test methods and specifications used to ensure the bacterial endotoxin limits of antibiotic

preparations in various pharmacopeias need to be harmonized. A harmonized pharmacopeia would provide a globally standardized procedure for establishing bacterial endotoxin acceptance criteria for antibiotics and antibiotic preparations. This approach would offer a more convenient option for global drug manufacturers involved in the import and export of antibiotic raw materials or injections and would also eliminate the burden of performing multiple analytical methods to comply with different endotoxin acceptance criteria set by various pharmacopeias.

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References

1. Mohr KI. History of antibiotics research. *Curr Top Microbiol Immunol.* 2016;398:237-272. doi: 10.1007/82_2016_499
2. Aminov RI. A brief history of the antibiotic era: Lessons learned and challenges for the future. *Front Microbiol.* 2010;1:134. doi: 10.3389/fmicb.2010.00134
3. Hutchings MI, Truman AW, Wilkinson B. Antibiotics: Past, present and future. *Curr Opin Microbiol.* 2019;51:72-80. doi: 10.1016/j.mib.2019.10.008
4. Lloyd NC, Morgan HW, Nicholson BK, Ronimus RS, Riethmiller S. Salvarsan: The first chemotherapeutic compound. *Chem N Zealand*. 2005;69(1):24-27.
5. Levy SB. *The Antibiotic Paradox: How Miracle Drugs are Destroying the Miracle*. Berlin: Springer; 2013.
6. Sebilleau CO, Sucheck SJ. Lipopeptide adjuvants for antibiotics and vaccines: The future step in the fight against multidrug-resistant and extensively drug-resistant pathogens. *Explor Drug Sci.* 2024;2:203-233. doi: 10.37349/eds.2024.00043
7. Holzheimer RG. The significance of endotoxin release in experimental and clinical sepsis in surgical patients-evidence for antibiotic-induced endotoxin release? *Infection.* 1998;26:77-84. doi: 10.1007/BF02767765
8. Prins JM, Van Deventer SJ, Kuijper EJ, Speelman P. Clinical relevance of antibiotic-induced endotoxin release. *J Antimicrob Chemother*. 1994;38(6):1211-1218. doi: 10.1128/AAC.38.6.1211
9. Morrison DC, Bucklin SE. Evidence for antibiotic-mediated endotoxin release as a contributing factor to lethality in experimental gram-negative sepsis. *Scand J Infect Dis Suppl*. 1996;101:3-8.
10. Bayston KF, Cohen J. Bacterial endotoxin and current concepts in the diagnosis and treatment of endotoxaemia. *Med Microbiol*. 1990;31(2):73-83. doi: 10.1099/00222615-31-2-73
11. Mayer H, Rietschel ET, Westphal O, Lüderitz O, Galanos C. In *Advances in Immunopharmacology: Proceedings of the Third International Conference on Immunopharmacology,* Florence, Italy. Elsevier; 2016. p. 13.
12. Singh N, Mishra T, Singh K, Singh J. Microbial and non-microbial pyrogens in healthcare products: Risks, quality control and regulatory aspects. *Appl Clin Res Clin Trials Regul Affairs*. 2017;4(1):4-15.
13. Oyewo EA. *A Study of Exogenous and Endogenous Pyrogens in Malaria Fever.* England: The University of Manchester (United Kingdom); 1986.
14. Wang X, Quinn PJ. Endotoxins: Lipopolysaccharides of gram-negative bacteria. In: *Endotoxins: Structure, Function and Recognition*. Dordrecht: Springer Netherlands; 2010. p. 3-25.
15. Peterson JW, Baron S. Bacterial pathogenesis. In: *Medical Microbiology.* 4th ed., Ch. 7. Galveston, TX: University of Texas; 1996.
16. Sheehan JR, Sadlier C, O'Brien B. Bacterial endotoxins and exotoxins in intensive care medicine. *BJA Educ*. 2022;22(6):224-230. doi: 10.1016/j.bjae.2022.01.003
17. Associates of Cape Cod, Inc. *Functional Challenges for Alternative Bacterial Endotoxins*; (n.d.). Available from: https://www.acclusa.com/pdfs/supplements/AP.pdf [Last accessed on

12 Aug 2024].
18. Gorbet MB, Sefton MV. Review: Biomaterial-associated thrombosis: Roles of coagulation factors, complement, platelets and leukocytes. *Biomaterials.* 2004;25:219-241. doi: 10.1016/b978-008045154-1.50025-3.
19. Tidswell ECA. A nontrivial analysis of patient safety risk from parenteral drug- and medical device-borne endotoxin. *Drugs.* 2023;23:65-76. doi: 10.1007/s40268-023-00412-y
20. ICH. *Harmonized Tripartite Guideline. Evaluation and Recommendation of Pharmacopoeial Texts for Use in the ICH Regions on Bacterial Endotoxins Test General Chapter-Q4 B Annex 14. Current Step 4 Version*. Geneva: ICH; 2012.
21. Spoladore J, Gimenes I, Bachinski R, *et al*. Standardized pyrogen testing of medical products with the bacterial endotoxin test (BET) as a substitute for rabbit pyrogen testing (RPT): A scoping review. *Toxicol In Vitro.* 2021;74:105160. doi: 10.1016/j.tiv.2021.105160
22. Tindall B, Demircioglu D, Uhlig T. Recombinant bacterial endotoxin testing: A proven solution. *BioTechniques.* 2021;70(5):290-300. doi: 10.2144/btn-2020-0165
23. Cavaillon JM. Exotoxins and endotoxins: inducers of inflammatory cytokines. *Toxicon.* 2017;1888:1-9.
24. Alexander C, Rietschel ET. Invited review: Bacterial lipopolysaccharides and innate immunity. *J Endotoxin Res*. 2001;7(3):167-202.
25. Rita FM, Isabel S, Miguel AV. Lipopolysaccharide modification in Gram-negative bacteria during chronic infection. FEMS Microbiol Rev. 2016;40(4):480-493. doi: 10.1093/femsre/fuw007
26. Su W, Ding X. Methods of endotoxin detection. *J Lab Autom.* 2015;20(4):354-364. doi: 10.1177/2211068215572136
27. Heine H, Rietschel ET, Ulmer AJ. The biology of endotoxin. *Mol Biotechnol*. 2001;19:279-296. doi: 10.1385/MB:19:3:279
28. Basauri A, Gonzalez-Fernandez C, Fallanza M, *et al*. Biochemical interactions between LPS and LPS-Binding molecules. *Crit Rev Biotechnol*. 2020;40(3):292-305. doi: 10.1080/07388551.2019.1709797
29. Caroff M, Novikov A. Lipopolysaccharides: Structure, function and bacterial identification. *OCL*. 2020;27:31. doi: 10.1051/ocl/2020025
30. Indian Pharmacopoeia Commission. *Revised General Chapter 2.2.3: Bacterial Endotoxins*. Indian Pharmacopoeia Commission; n.d. Available from: https://ipc.gov.in [Last accessed on 26 Aug 2024].
31. Munford RS. Sensing gram-negative bacterial lipopolysaccharides: A human disease determinant? *Infect Immunity*. 2008;76(2):454-465. doi: 10.1128/IAI.00939-07
32. Maldonado RF, Sá-Correia I, Valvano MA. Lipopolysaccharide modification in Gram-negative bacteria during chronic infection. *FEMS Microbiol Rev*. 2016;40(4):480-493. doi: 10.1093/femsre/fuw007
33. Farhana A. Biochemistry, lipopolysaccharide. In: *StatPearls*. StatPearls Publishing. Available from: https://www.ncbi.nlm.nih.gov/books/NBK554414 [Last accessed on 2025 Aug 27].
34. Schletter J, Heine H, Ulmer AJ, Rietschel ET. Molecular mechanisms of endotoxin activity. *Arch Microbiol*. 1995;164(6):383-389. doi: 10.1007/BF02529735
35. Muroi M. The polysaccharide portion plays an indispensable role in lipopolysaccharide-induced macrophage activation. *J Immunol.* 2002;168(7):3729-3735.
36. American Pharmaceutical Review. *Limulus Amebocyte Lysate (LAL) Endotoxin Detection*. American Pharmaceutical Review; 2024. Available from: https://www.americanpharmaceuticalreview.com [Last accessed on 26 Aug 2025].
37. Iwanaga S. Biochemical principle of Limulus test for detecting bacterial endotoxins. *Proc Jpn Acad Ser B Phys Biol Sci.* 2007;83(4):110-119. doi: 10.2183/pjab.83.110
38. Hartmann NB, Jensen KA, Baun A, *et al*. Techniques and protocols for dispersing nanoparticle powders in aqueous media-is there a rationale for harmonization? *J Toxicol Environ Health B Crit Rev*. 2015;18(6):299-326. doi: 10.1080/10937404.2015.1074969
39. Baines ALAN. Endotoxin testing. In: Baird R, Hodges N, Denyer S, editors. *Handbook of Microbiological Quality Control*. New York: Taylor & Francis; 2000. p. 144-167.
40. Porzio R, Hosie J. The importance of data integrity in the endotoxin test. In: *Endotoxin Detection and Control in Pharma, Limulus, and Mammalian Systems*. Cham: Springer; 2019. p. 419-466. doi: 10.1007/978-3-030-17148-3_10
41. Sondhi P, Maruf MHU, Stine KJ. Nanomaterials for biosensing lipopolysaccharide. *Biosensors (Basel)*. 2019;10(1):2. doi: 10.3390/bios10010002
42. Indian Pharmacopoeia Commission. *Pyrogens*. Indian Pharmacopoeia. Vol. 1. India: Indian Pharmacopoeia Commission; 2022. p. 38-39.
43. European Pharmacopoeia to Put an End to the Rabbit Pyrogen Test. *European Directorate for the Quality of Medicines and HealthCare*; 2021. Available from: https://www.edqm.eu/en/-/european-pharmacopoeia-to-put-an-end-to-the-rabbit-pyrogen-test [Last accessed on 2025 Aug 26].
44. European Pharmacopoeia Commission. *Supplement 10.3, Chapter 2.6.32, and the Revised General Chapter 5.1.10. Test for Bacterial Endotoxins using Recombinant Factor C Describes a Test for Bacterial Endotoxins (BET) (effective on 1 January 2021)*. European Pharmacopoeia (Ph. Eur.). 10th ed. Council of Europe: European Pharmacopoeia Commission; 2021.
45. Cirefice G, Schütte K, Spreitzer I, *et al*. The future of pyrogenicity testing: Phasing out the rabbit pyrogen test. A meeting report. *Biologicals.* 2023;84:101702. doi: 10.1016/j.biologicals.2023.101702
46. Tamura H, Reich J, Nagaoka I. Outstanding contributions of LAL technology to pharmaceutical and medical science: Review of methods, progress, challenges, and future perspectives in early detection and management of bacterial infections and

invasive fungal diseases. *Biomedicines*. 2021;9(5):536. doi: 10.3390/biomedicines9050536
47. Gorman R. Atlantic horseshoe crabs and endotoxin testing: Perspectives on alternatives, sustainable methods, and the 3Rs (replacement, reduction, and refinement). *Front Mar Sci*. 2020;30(7):1877. doi: 10.3389/fmars.2020.582132
48. Nakamura T, Morita T, Iwanaga S. Lipopolysaccharide-sensitive serine-protease zymogen (factor C) found in Limulus hemocytes-Isolation and characterization. *Eur J Biochem*. 1986;154:511-521. doi: 10.1111/j.1432-1033.1986.tb09427.x
49. Novitsky TJ. Biomedical applications of limulus amebocyte lysate. In: *Biology and Conservation of HorseshoeCrabs*. Berlin: Springer; 2009. p. 315-329. doi: 10.1007/978-0-387-89959-6_20.
50. Mehmood Y. *What is Limulus Amebocyte Lysate (LAL) and its Applicability in Endotoxin Quantification of Pharma Products. Growing and Handling of Bacterial Cultures*. London: Intechopen; 2019. doi: 10.5772/intechopen.81331
51. Lundin JI, Checkoway H. Endotoxin and cancer. *Environ Health Perspect*. 2009;117(9):1344-1350. doi: 10.1289/ehp.0800439
52. Franco E, Garcia-Recio V, Jimenez P, *et al*. Endotoxins from a pharmacopoeial point of view. *Toxins (Basel)*. 2018;10(8):331. doi: 10.3390/toxins10080331
53. British Pharmacopeia Commission. *Bacterial Endotoxin*. United Kingdom: British Pharmacopeia. British Pharmacopeia Commission; 2024. p. V-A473-A478.
54. United States Pharmacopoeial Convention. *Bacterial Endotoxin*. United States: United States Pharmacopeia (online). United States Pharmacopoeial Convention; 2025.
55. Japanese Pharmacopoeia Commission. *Bacterial Endotoxin Japan. Japanese Pharmacopoeia*. 18th ed. Japanese: Japanese Pharmacopoeia Commission; 2021. p.124-127.
56. European Pharmacopoeia (Ph. Eur.). *European Directorate for the Quality of Medicines and HealthCare (EDQM). European Pharmacopoeia*. 11th ed. Strasbourg: Council of Europe; 2023.
57. World Health Organization. *International Pharmacopoeia*. 10th ed. Geneva: World Health Organization; 2022.
58. Indian Pharmacopoeia Commission. *Bacterial Endotoxins*. Vol. 1. India: Indian Pharmacopoeia. Indian Pharmacopoeia Commission; 2022. p. 30-35.
59. Sharma S, Mittal BR, Vatsa R, Singh B. Gel clot bacterial endotoxin test of FDG: Indian scenario. *Indian J Nucl Med.* 2011;26(3):149-152. doi: 10.4103/0972-3919.103998
60. McCullough KZ, Tirumalai R, Hussong D, *et al*. Endotoxin standards and their role in recovery studies: The path forward. *Bio Pharma Asia*. 2016;5:14-23.
61. Dawson ME. Endotoxin standards and CSE potency. *LAL Update*. 1993;11(4):2-5.
62. Nakamura S, Iwanaga S, Harada T, Niwa M. A clottable protein (coagulogen) from amoebocyte lysate of Japanese horseshoe crab (*Tachypleus tridentatus*). Its isolation and biochemical properties. *J Biochem*. 1976;80(5):1011-1021.
63. Liu C, Liu X, Wang Y, *et al*. Research progress and prospect of tachypleus amebocyte lysate in China. *International Horseshoe Crab Conservation and Research Efforts: 2007-2020: Conservation of Horseshoe Crabs Species Globally*. Berlin: Springer; 2022. p. 661-668.
64. McClymont K. *Structural Studies of the Endotoxin Sensing Protein Factor C* (Doctoral Dissertation, University of Glasgow); 2017.
65. Sattar AA, Good CR, Saletes M, Brandão J, Jackson SK. Endotoxin as a marker for water quality. *Int J Environ Res Public Health*. 2022;19(24):16528. doi: 10.3390/ijerph192416528
66. Li Y, Boraschi D. Endotoxin contamination: A key element in the interpretation of nanosafety studies. *Nanomedicine (Lond)*. 2016;11(3):269-287. doi: 10.2217/nnm.15.196
67. Mangini M, Verde A, Boraschi D, Puntes VF, Italiani P, De Luca AC. Interaction of nanoparticles with endotoxin Importance in nanosafety testing and exploitation for endotoxin binding. *Nanotoxicology*. 2021;15(4):558-576. doi: 10.1080/17435390.2021.1898690
68. Williams KL. Endotoxin: Relevance and control in parenteral manufacturing. In: *Microbial Contamination Control in the Pharmaceutical Industry*. United States: CRC Press; 2004. p. 201-268.
69. Di Paolo A, Liberti R, Anzalone L, *et al*. Recombinant factor C as an *in vitro* assay for the residual pathogenicity evaluation of veterinary autogenous vaccines. *Vet Sci.* 2024;11:673. doi: 10.3390/vetsci11120673
70. Kang DH, Yun SY, Eum S, *et al*. A study on the application of recombinant factor C (rFC) assay using biopharmaceuticals. *Microorganisms.* 2024;12:516. doi: 10.3390/microorganisms12030516
71. Park CY, Jung SH, Bak JP, Lee SS, Rhee DK. Comparison of the rabbit pyrogen test and Limulus amoebocyte lysate (LAL) assay for endotoxin in hepatitis B vaccines and the effect of aluminum hydroxide. *Biologicals.* 2005;33(3):145-151. doi: 10.1016/j.biologicals.2005.04.002
72. *Pros and Cons of Different Endotoxin Detection Readouts*; 2024. Available from: https://www.wakopyrostar.com/blog/kit-lal/post/pros-and-cons-of-different-endotoxin-detection-readouts [Last accessed on 03 Sep 2025].
73. Fux AC, Casonato Melo C, Michelini S, *et al.* Heterogeneity of lipopolysaccharide as source of variability in bioassays and LPS-binding proteins as remedy. *Int J Mol Sci.* 2023;24(9):8395. doi: 10.3390/ijms24098395
74. John AK, Claudio R. The role of endotoxin in septic shock. *Crit Care*. 2023;27(1):400. doi: 10.1186/s13054-023-04690-5