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A Review and Comparative Study of Preservative Efficacy Test in the United States, European, Indian, and Japanese Pharmacopeias and Implications for Asian Pharmacopeias

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ABSTRACT

Introduction: The preservative efficacy test (PET) is a critical tool for assessing the ability of antimicrobial preservatives to prevent microbial contamination in pharmaceutical products, ensuring their safety and stability during production and storage. This study reviews and compares PET standards across four major pharmacopeias—the United States Pharmacopeia (USP), European Pharmacopoeia (EP), Indian Pharmacopoeia (IP), and Japanese Pharmacopoeia (JP)—to identify disparities and propose harmonization strategies, with a focus on implications for Asian pharmacopeias. **Methods:** We systematically compared the PET protocols of the USP, EP, IP, and JP, focusing on product classification, challenge microorganisms, culture media, sample contamination methods, incubation conditions, and acceptance criteria. Data were extracted from the latest editions of each pharmacopeia and analyzed for differences in stringency and methodology. **Results:** Significant variations were identified across the pharmacopeias. For example, the USP requires an inoculum of 10^5 colony-forming units per milliliter (CFU/mL) for certain organisms, while the EP specifies 10^4 CFU/mL. Challenge organisms also differ, with the USP mandating *Pseudomonas aeruginosa* and *Staphylococcus aureus*, and the EP including a broader range, such as *Aspergillus brasiliensis*. Acceptance criteria vary, with the USP requiring a 3-log reduction in *Escherichia coli* within 14 days, compared to the EP's 2-log reduction over the same period. Incubation periods range from 14 days (USP and IP) to 28 days (JP), potentially affecting drug quality assessments. **Conclusions:** These disparities in PET standards may lead to inconsistencies in drug quality and safety across regions, particularly in Asia, where harmonization with global standards is limited. We recommend the development of a unified international framework for PET, incorporating the USP's stringent log-reduction criteria and the EP's comprehensive microbial selection, to enhance global drug safety. For Asian pharmacopeias, adopting such harmonized standards could facilitate regulatory alignment, improve product quality, and support international trade.

INTRODUCTION

Pharmaceutical and cosmetic products require protection against microbial contamination to ensure safety, quality, and suitability for use. These products are susceptible to microbial growth during manufacturing, storage, or consumer use, which can compromise stability and pose health risks [1]. Preservatives, as antimicrobial agents, are incorporated into formulations to inhibit microbial proliferation, ensuring product integrity [2]. Single-dose pharmaceuticals typically do not require preservatives, except in cases like injectable formulations requiring multi-step preparation or extended stability [3]. Some formulations rely on inherent antimicrobial properties of active ingredients or excipients, such as high sugar or alcohol concentrations, which act as osmotic preservatives, or extreme pH levels that inhibit growth [4, 5]. However, most multi-dose products depend on chemical preservatives to prevent microbial spoilage [6].

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At low concentrations, preservatives typically exert bacteriostatic effects, inhibiting growth without eliminating existing microbes [7]. Their use in pharmaceuticals is governed by strict requirements for non-toxicity, chemical stability, and compatibility with formulation components [8]. Preservative selection varies by dosage form. Injectable formulations commonly use benzyl alcohol, methylparaben, propylparaben, phenol, chlorobutanol, or sodium metabisulfite [3]. Ophthalmic products favor benzalkonium chloride, thimerosal, or combinations of methylparaben, propylparaben, and ethylenediaminetetraacetic acid (EDTA) for ocular compatibility [9]. Topical products, such as creams and ointments, employ benzyl alcohol, methylparaben, propylparaben, benzoic acid, sorbic acid, or chlorocresol [10], while oral medications often use sodium benzoate or parabens [11].

To enhance efficacy, preservatives are frequently combined to target diverse microorganisms, including Gram-positive and Gram-negative bacteria, fungi, and yeasts. Non-antimicrobial agents like EDTA may be added to destabilize microbial cell membranes, forming integrated preservative systems [12].

Evaluating preservative efficacy is critical to ensure product safety throughout its shelf life. While chemical methods like high-performance liquid chromatography can quantify preservative concentrations [13], factors such as pH, interactions with formulation components, and adsorption by packaging materials can reduce antimicrobial activity [14, 15].

The preservative efficacy test (PET) evaluates a preservative system's ability to reduce microbial contamination by inoculating products with standardized suspensions of bacteria, yeasts, and molds, followed by periodic microbial counts over a period, typically up to 28 days [16]. A product meets efficacy standards if microbial counts satisfy pharmacopeial criteria, such as log reduction thresholds [17-20].

The PET was formalized in the United States Pharmacopeia (USP) in the late 1960s and became fully integrated in official monographs by USP 18 (1970), later adopted by European pharmacopeias in the 1980s and fully incorporated into the European Pharmacopoeia (EP) by 1992 [21]. Harmonization efforts, such as those aligned with ICH Q4B guidelines, have refined methodologies, but differences persist among the USP 46, EP 10, Indian Pharmacopoeia (IP) 9, and Japanese Pharmacopoeia (JP) 16 in protocols and acceptance criteria [6, 21].

For instance, weak organic acids like benzoic acid and sorbic acid are most effective at low pH, where they remain non-ionized, but lose activity in alkaline conditions [22]. In two-phase products, preservatives partition into the non-aqueous phase, reducing their concentration in the aqueous phase where microbial growth occurs [15]. Similarly, adsorption by suspended solids in suspensions or plastic packaging can diminish preservative efficacy [23, 24]. Thus, biological testing, known as the preservative efficacy test, is essential to assess antimicrobial performance [25].

These discrepancies pose challenges for global pharmaceutical and cosmetic manufacturers. The USP, influential for FDA regulatory processes, contrasts with the EP, overseen by the European Medicines Agency, and the JP, which emphasizes traditional Japanese medicines [21]. Variations in testing standards complicate international trade, increase compliance costs, and risk inconsistent safety levels across markets. For Asian pharmacopeias, alignment with global standards remains limited, hindering regulatory harmonization and product quality assurance [26, 27].

This review compares PET protocols across the USP, EP, IP, and JP to elucidate differences and their implications for manufacturers, regulators, and consumers. By highlighting these variations, it aims to support harmonization efforts, enhance product safety, and strengthen confidence in global pharmaceutical and cosmetic markets [6, 26].

METHODS

Relevant chapters on preservative efficacy testing were sourced from the United States Pharmacopeia (USP 46, Chapter <51> Antimicrobial Effectiveness Testing) [17], European Pharmacopoeia (EP 10, Section 5.1.3 Efficacy of Antimicrobial Preservation) [18], Japanese Pharmacopoeia (JP 16, Section 4.05.1 Preservative Effectiveness Tests) [19], and Indian Pharmacopoeia (IP 9, Section 2.2.2 Effectiveness of Antimicrobial Preservatives under Biological Methods) [20].

This study involves a document analysis of current USP, EP, JP, and IP texts, focusing exclusively on official pharmacopeial protocols for PET without supplementary experimental data. The search was executed across multiple databases, including PubMed, Scopus, and Google Scholar, to ensure a thorough collection of relevant literature. To contextualize the analysis, a literature review was conducted to identify studies on PET methodologies published between 2013 and 2023. Earlier studies were included for historical context via reference and citation tracking of key articles [16, 21, 25].

Data were extracted on test organisms (bacteria and fungi), test procedures (inoculation and incubation conditions), evaluation metrics (log reduction and microbial recovery rates), trial duration, sampling intervals, and acceptance criteria. Articles unrelated to PET or lacking robust methodology (*e.g.*, non-peer-reviewed sources) were excluded. The screening process involved two steps: initial review of titles and abstracts for relevance, followed by full-text evaluation of selected articles. Two independent reviewers screened 200 titles/abstracts using Rayyan and assessed 40 full-texts, resolving discrepancies (<5% of entries) through discussion with a senior researcher.

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Pharmacopeial texts were verified against original sources during final validation [17-20].

A standardized template was used to compare PET protocols across the four pharmacopeias, focusing on product classification, test microorganisms, culture media, incubation conditions, microbial suspension preparation, sample contamination methods, sampling intervals, preservative neutralization, microbial counting, and acceptance criteria. This framework highlights similarities and differences to inform harmonization strategies [6, 26].

Classification of pharmaceutical products

The United States Pharmacopeia (USP), European Pharmacopoeia (EP), Japanese Pharmacopoeia (JP), and Indian Pharmacopoeia (IP) tailor preservative efficacy testing (PET) to pharmaceutical product dosage forms, applying distinct acceptance criteria based on product characteristics. USP, EP, and JP explicitly classify products to guide PET protocols, while IP relies on acceptance criteria that suggest an implicit framework. These classifications are summarized in Table 1.

Table 1. Classification of pharmaceutical products to perform the preservative efficacy test in different pharmacopeias

Pharmacopeia	Classification of Pharmaceutical Products	Reference
USP 46	1. Injections, other parenterals (including emulsions), over-the-counter (OTC) aqueous products, sterile nasal products, and ophthalmic products with aqueous bases or vehicles 2. Topical aqueous products, nonsterile nasal products, and emulsions applied to mucous membranes 3. Oral aqueous products (excluding antacids) 4. Aqueous antacids	[17]
EP 10	1. Oral, rectal, and mucosal products 2. Otic, nasal, topical, and inhalation products 3. Injectable, ophthalmic, intrauterine, and intramammary products	[18]
JP 16	1. Aqueous products: a) Injectable, ophthalmic, and otic products b) Topical, mucosal, inhalation liquids (sterile aqueous solutions for nebulization, excluding pressurized metered-dose inhalers), and nasal drops c) Oral products (excluding antacids) d) Antacids 2. Non-aqueous products	[19]
IP 9	No explicit classification; acceptance criteria imply dosage-form-based categories	[20]

In USP 46, over-the-counter (OTC) products encompass aqueous formulations (*e.g*., oral liquids, nasal sprays) and non-aqueous preparations (*e.g*., antiseptic gels, ointments) [17]. USP applies PET to preserved products but requires sterility testing only for sterile dosage forms, unlike the original text's implication of broader sterility mandates [17]. EP 10 integrates preservative efficacy testing with quality controls, such as low pre-test bioburden and container-closure integrity, without mandating sterility testing for non-sterile products [18]. JP 16 classifies emulsions based on the external phase, categorizing oil-in-water emulsions as aqueous (Category 1) and water-in-oil emulsions as non-aqueous (Category 2), reflecting microbial growth risks associated with the water phase [19]. IP 9 lacks a formal classification but sets acceptance criteria varying by dosage form (*e.g*., stricter for parenterals), suggesting an informal category-based approach [20]. These differences influence PET stringency and highlight harmonization challenges [6, 26].

Microorganisms suggested by pharmacopeias

The United States Pharmacopeia (USP), European Pharmacopoeia (EP), Japanese Pharmacopoeia (JP), and Indian Pharmacopoeia (IP) recommend specific microorganisms for preservative efficacy testing (PET) to assess antimicrobial activity across product categories. These include *Candida albicans*, *Aspergillus niger*, *Aspergillus brasiliensis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, and *Zygosaccharomyces rouxii*, with varying requirements and log reduction criteria summarized in Table 2.

Table 2. Microorganisms recommended for PET by pharmacopeias

Pharmacopeia	Microorganisms							Reference
	S. aureus	*P. aeruginosa*	*E. coli*	*C. albicans*	*A. niger*	*A. brasiliensis*	*Z. rouxii*	
USP 46	Yes	Yes	Yes	Yes	No	Yes	No	[17]
EP 10	Yes	Yes	Oral aqueous products only	Yes	No	Yes	High sugar oral products only	[18]
JP 16	Yes	Yes	Yes	Yes	Yes	No	High sugar oral products only	[19]
IP 9	Yes	Yes	Yes	Yes	Yes	No	High sugar oral products only	[20]

The USP mandates testing against five core microorganisms: *C. albicans*, *A. brasiliensis*, *P. aeruginosa*, *S. aureus*, and *E. coli* for all PET protocols, with flexibility to include product-specific organisms if contamination risks are identified [17]. EP 10 requires *Pseudomonas aeruginosa*, *Candida albicans*,

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Staphylococcus aureus, and *Aspergillus brasiliensis* for all products, adding *Escherichia coli* for non-sterile aqueous oral preparations and *Zygosaccharomyces rouxii* for oral products with high sugar content, such as syrups [18]. JP 16 and IP 9 include *Aspergillus niger* alongside *Candida albicans*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Escherichia coli*, with *Zygosaccharomyces rouxii* for high-sugar oral products. They also recommend testing additional process-related contaminants, such as environmental bacteria, based on manufacturing risks [19, 20]. These variations reflect differing priorities in microbial risk assessment, complicating global harmonization [25, 28].

Preparation of inoculum

Culture media

The United States Pharmacopeia (USP), European Pharmacopoeia (EP), Japanese Pharmacopoeia (JP), and Indian Pharmacopoeia (IP) specify culture media for preservative efficacy testing (PET) to support microbial growth during challenge tests with organisms such as *Candida albicans*, *Aspergillus brasiliensis*, and *Pseudomonas aeruginosa*. Variations in media reflect regional validation practices but ensure comparable microbial recovery, as shown in Table 3.

Table 3. Recommended culture media for preservative efficacy testing across pharmacopeias

Pharmacopeia	Suggested culture media							Reference
	Soybean-Casein Digest Agar	Soybean-Casein Digest Broth	Sabouraud Dextrose Agar	Sabouraud Dextrose Broth	Sabouraud Agar	Glucose Peptone Agar	Potato Dextrose Agar	
USP 46	*E. coli*, *P. aeruginosa*, *S. aureus*	*E. coli*, *P. aeruginosa*, *S. aureus*	*C. albicans*, *A. brasiliensis*	*C. albicans*, *A. brasiliensis*	-	-	-	[17]
EP 10	*E. coli*, *P. aeruginosa*, *S. aureus*	-	*E. coli*, *P. aeruginosa*, *S. aureus*	-	-	-	-	[18]
JP 16	*E. coli*, *P. aeruginosa*, *S. aureus*	*E. coli*, *P. aeruginosa*, *S. aureus*	-	-	*C. albicans*, *Z. rouxii*, *A. niger*	*C. albicans*, *Z. rouxii*, *A. niger*	*C. albicans*, *Z. rouxii*, *A. niger*	[19]
IP 9	*E. coli*, *P. aeruginosa*, *S. aureus*	-	*C. albicans*, *A. niger*	-	-	-	-	[20]

USP 46 recommends Soybean-Casein Digest Agar and Broth for bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*) and Sabouraud Dextrose Agar and Broth for fungi (*Candida albicans*, *Aspergillus brasiliensis*), with Potato Dextrose Agar as an optional alternative for molds [17, 29]. EP 10 specifies Soybean-Casein Digest Agar for bacteria and Sabouraud Dextrose Agar for fungi (*Aspergillus brasiliensis*, *Zygosaccharomyces rouxii*), tailored to product types [18]. JP 16 uses Soybean-Casein Digest media for bacteria and Sabouraud Agar, Glucose Peptone Agar, or Potato Dextrose Agar for fungi (*Candida albicans*, *Zygosaccharomyces rouxii*, *Aspergillus niger*), reflecting broader fungal coverage [19, 29]. IP 9 employs Soybean-Casein Digest Agar for bacteria and Sabouraud Dextrose Agar for fungi (*Candida albicans*, *Aspergillus niger*) [20]. These differences, while practical, underscore challenges in standardizing PET protocols globally [16, 25].

Incubation conditions

The United States Pharmacopeia (USP), European Pharmacopoeia (EP), Japanese Pharmacopoeia (JP), and Indian Pharmacopoeia (IP) specify incubation conditions for preservative efficacy testing (PET) to assess microbial survival in challenged products. Inoculum preparation for bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*) requires incubation at 30–35°C for 18–24 hours to ensure optimal growth. During PET, products are incubated at 20–25°C for 7–28 days to simulate storage conditions, with specific times for *Candida albicans* and *Aspergillus* species (*A. brasiliensis* for USP/EP/JP, *A. niger* for IP) detailed in Table 4 [17-20, 30].

The incubation temperature for *Candida albicans* and *Aspergillus* species is consistently 20–25°C across all four pharmacopeias. For *C. albicans*, EP 10, JP 16, and IP 9 require 48 hours, whereas USP 46 specifies a range of 44–52 hours, allowing slight flexibility in protocol execution [17-20]. For *Aspergillus*, all pharmacopeias standardize the incubation time at 7 days, ensuring comparable assessment of mold survival [17-20, 30]. These minor variations, particularly USP's range for *C. albicans*, may influence test sensitivity but are unlikely to affect overall PET outcomes significantly. Harmonizing incubation times, such as adopting a fixed 48-hour period for *C. albicans*, could enhance global consistency in PET protocols [25, 26].

Preparation of primary microbial suspension

Inoculum preparation for preservative efficacy testing follows pharmacopeia-specific protocols, involving two concentration stages: (1) a stock suspension (10^7–10^8 CFU/mL) and (2) a final test inoculum (10^5–10^6 CFU/mL) after dilution. For stock preparation, liquid cultures are centrifuged and cells washed, while solid cultures are harvested using sterile loops or pipettes. The United States

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Pharmacopeia (USP 46), European Pharmacopoeia (EP 10), and Indian Pharmacopoeia (IP 9) specify sterile normal saline as the diluent for *Candida albicans* and bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*), whereas the Japanese Pharmacopoeia (JP 16) allows either sterile normal saline or 0.1% peptone water [17-20]. For *Aspergillus* strains (*Aspergillus brasiliensis* for USP, EP, JP; *Aspergillus niger* for IP), all pharmacopeias incorporate 0.05% w/v polysorbate 80 in the diluent to enhance spore dispersion, with USP, EP, and IP using sterile normal saline as the base and JP 16 permitting peptone water as an alternative [17-20]. Washing steps are applied across all protocols to standardize microbial concentrations. Prepared suspensions must be used within strict time limits: within 1 hour for JP 16, within 4 hours for IP 9, within 2 hours for USP 46, and within a short period for EP 10 to ensure viability [17-20].

Table 4. Incubation conditions for preservative efficacy testing across pharmacopeias

Pharmacopeia *	Microorganisms						Reference
	Bacteria (*E. coli*, *P. aeruginosa*, *S. aureus*)		*Candida albicans*		*Aspergillus* (*A. brasiliensis* or *A. niger*)		
	Temperature (°C)	Time (hours)	Temperature (°C)	Time (hours)	Temperature (°C)	Time (hours)	
USP 46	30-35	18–24	20-25	44-52	20-25	6-10	[17]
EP 10	30-35	18–24	20-25	48	20-25	7	[18]
JP 16	30-35	18–24	20-25	48	20-25	7	[19]
IP 9	30-35	18–24	20-25	48	20-25	7	[20]

* USP and EP/JP use taxonomically identical strains (ATCC 16404) under different nomenclature. IP may specify *A. niger*.
The incubation time for *Aspergillus* is given as a range in the US and Indian pharmacopoeias, and for *Candida albicans* in the US pharmacopoeia, while other pharmacopoeias suggest a specific time.

Methods for determining the concentration of microbial suspensions

To achieve target microbial concentrations for preservative efficacy testing, pharmacopeias specify distinct quantification methods. The United States Pharmacopeia (USP 46) allows turbidimetry or plate counts, typically calibrated against a standard curve for turbidimetry [17]. The European Pharmacopoeia (EP 10) employs plate counts as the primary method, with membrane filtration used for products containing particulates [18]. The Indian Pharmacopoeia (IP 9) requires pour plate or membrane filtration methods [20]. The Japanese Pharmacopoeia (JP 16) uses plate counts for viable enumeration and turbidimetry for initial suspension adjustments [19].

For *Aspergillus* suspensions (*Aspergillus brasiliensis* for USP, EP, JP; *Aspergillus niger* for IP), diluent compositions vary. USP 46, EP 10, IP 9, and JP 16 specify 0.05% w/v polysorbate 80 in sterile normal saline to enhance spore dispersion [17-20]. JP 16 also permits 0.1% peptone water as an alternative diluent medium [19]. These differences in quantification methods and diluents may affect suspension consistency, highlighting the need for harmonized protocols to ensure reproducible PET outcomes across global pharmacopeias [16, 25].

Inoculation protocol

The inoculation protocols for preservative efficacy testing vary across pharmacopeias, specifying microbial concentrations, container use, and storage conditions. The United States Pharmacopeia (USP 46) requires an inoculum of 10^5–10^6 CFU/mL for products in categories 1–3 (*e.g.*, injections, topical aqueous products, oral products) and 10^3–10^4 CFU/mL for category 4 (antacids with aqueous bases), mixed thoroughly in a single container per microorganism, with plate counts used for post-inoculation enumeration [17]. Samples are stored at 20–25°C and tested at specified intervals. The inoculum volume is 0.5–1% of the product's total volume [17].

The European Pharmacopoeia (EP 10) specifies 10^5–10^6 CFU/mL (or CFU/g for solids) for all products, using multiple containers per microorganism to ensure reproducibility [18]. The inoculum volume is ≤1% of the product's total volume, and samples are stored at 20–25°C, protected from light [18].

The Japanese Pharmacopoeia (JP 16) uses 10^5–10^6 CFU/mL for most products and 10^3–10^4 CFU/mL for antacids, testing five replicates in original containers (or sterile equivalents) plus two uninoculated controls for non-sterile products [19]. Semi-solid products are heated to 45–50°C before inoculation to ensure homogeneity. The inoculum volume is ≤1% of the product's total volume, and changes in color or odor during storage are recorded [19].

The Indian Pharmacopoeia (IP 9) requires 10^5–10^6 CFU/mL for most products and 10^3–10^4 CFU/mL for antacids, using five primary containers per microorganism (or 20 mL aliquots if container volume is <20 mL) [20]. The inoculum volume is ≤1% of the product's total volume, and product changes (*e.g.*, color, odor) during the test period are noted [20].

Across all pharmacopeias, the inoculum volume does not exceed 1% of the product's volume to minimize dilution effects. Variations in replicate requirements and product handling (*e.g.*, heating semi-solids) may influence test consistency, underscoring the need for harmonized protocols to support global PET standardization [6, 16].

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Suggested intervals for sampling

Sampling intervals in preservative efficacy testing reflect pharmacopeial approaches to balancing microbial growth kinetics with product stability. All pharmacopeias require viable microbial counts on Day 0, followed by product-specific intervals, as shown in Table 5. These intervals vary based on product type and risk category, influencing the assessment of preservative efficacy.

Table 5. Comparison of suggested sampling intervals in different pharmacopeias

Pharmacopeia	Sampling intervals	Product category	Reference
USP 46	Days 7, 14, 28	Injections, ophthalmic, sterile nasal	[17]
	Days 14, 28	Oral, topical (non-sterile), rectal	
EP 10	6h, 24h, Days 7, 14, 28	Injectables, ophthalmic, intrauterine	[18]
	Days 2, 7, 14, 28	Otic, nasal, inhalation	
	Days 14, 28	Oral, rectal, mucosal	
JP 16	Days 0, 7, 14, 21, 28	All non-antacid products	[19]
IP 9	Days 0, 7, 14, 28	Standard products	[20]
	Days 14, 28	Antacids	

Day 0 sampling establishes baseline microbial levels, with pharmacopeias allowing a short period post-inoculation to ensure homogeneous mixing: within 1 hour for USP 46, within 2 hours for EP 10 and IP 9, and promptly for JP 16 [17-20]. Subsequent intervals reflect product risk profiles. USP 46 specifies days 7, 14, and 28 for high-risk products (*e.g.*, injections, ophthalmic) and days 14 and 28 for lower-risk products (*e.g.*, oral, topical) [17]. EP 10 requires early sampling (6 h, 24 h) for injectables and intrauterine products, days 2, 7, 14, and 28 for otic, nasal, inhalation, and topical products, and days 14 and 28 for oral and mucosal products [18]. JP 16 applies a uniform schedule of days 7, 14, 21, and 28 for non-antacid products, ensuring conservative monitoring [19]. IP 9 uses days 7, 14, and 28 for most products and days 14 and 28 for antacids [20]. Variations, such as EP's early intervals for rapid kill assessment and JP's unique day 21 sampling, may affect test sensitivity, highlighting the need for harmonized intervals to ensure consistent PET outcomes globally [16, 25, 26].

Eliminating antimicrobial effects and neutralizing preservatives before counting viable microorganisms

To enumerate viable microorganisms in preservative efficacy testing, preservatives must be neutralized to allow microbial growth in appropriate media. Pharmacopeias specify methods such as membrane filtration, dilution, or chemical neutralization to eliminate antimicrobial effects, as outlined in Table 6. These methods ensure accurate colony counts for assessing preservative performance.

Neutralization methods vary by product type and preservative. For example, filtration is preferred for products with particulates, while chemical neutralization suits soluble preservatives. Validation ensures neutralization efficacy without compromising microbial recovery [16-20].

Counting viable microorganisms in samples at suggested intervals

The final step in preservative efficacy testing is enumerating viable microorganisms at specified intervals (see Table 5 for intervals, Table 7 for methods, Tables 8–12 for acceptance criteria). These counts reveal the preservative's ability to inhibit microbial growth over time. Pharmacopeias recommend methods for accurate enumeration, as shown in Table 7.

Table 6. Comparison of preservative neutralization methods across pharmacopeias

Pharmacopeia	Neutralization method	Specified neutralizing agents	Application conditions	Reference
USP 46	Dilution in neutralizing media, chemical neutralization	Polysorbate 80, lecithin, histidine	Quaternary ammonium compounds, parabens, phenols	[17]
EP 10	Filtration, dilution, chemical neutralization	Lecithin, polysorbate 80, Dey-Engley broth	Validated per product-preservative combination	[18]
JP 16	Chemical neutralization, dilution	Sorbitan monooleate, polysorbate 80, lecithin	Emulsification for ointments; dilution for liquids	[19]
IP 9	Filtration, dilution, chemical neutralization	None specified	Validated per product characteristics	[20]

Methods are validated to ensure preservative neutralization and accurate microbial recovery. For instance, membrane filtration suits products with residual preservatives, while plate counts are standard for clear solutions [16-20]. Moreover, Table 13 shows summary of acceptance criteria for preservative efficacy test.

Interpretation of test results

Preservative efficacy is assessed by comparing microbial counts at specified intervals against pharmacopeia-specific acceptance criteria, summarized in Tables 8–12. These criteria, expressed as log reductions or percentages of initial inoculum, vary by product category and pharmacopeia.

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Table 7. Comparison of methods for counting viable microorganisms in preservative efficacy testing

Pharmacopeia	Primary counting method(s)	Conditional alternatives	Reference
USP 46	Plate count	Membrane filtration for turbid or opaque products	[17]
EP 10	Plate count, membrane filtration	Method validated per product characteristics	[18]
JP 16	Pour plate with neutralizer	Membrane filtration	[19]
IP 9	Pour plate, membrane filtration	Method selected based on preservative interference	[20]

Table 8. Acceptance criteria for preservative efficacy testing according to USP 46 [17]

Product category	Microorganism	7 Days	14 Days	28 Days
Injections, parenterals, OTC, sterile nasal, ophthalmic (aqueous)	Bacteria	1 log	3 log	NI
	Fungi	NI	NI	NI
Topical (aqueous), nonsterile nasal, mucosal emulsions	Bacteria	-	2 log	NI
	Fungi	-	NI	NI
Oral (aqueous, non-antacid)	Bacteria	-	1 log	NI
	Fungi	-	NI	NI
Antacids (aqueous)	Bacteria	-	NI	NI
	Fungi	-	NI	NI

Note: NI = no increase (≤ 0.5 log from previous or initial count); - = not applicable.

Table 9. Acceptance criteria for injectable, ophthalmic, intrauterine, and intramammary products according to EP 10 [18]

Microorganism	Logarithmic reduction number (CFU/mL)					
	Criterion	6 h	24 h	7 Days	14 Days	28 Days
Bacteria	A	2 log	3 log	-	-	NR
	B	-	1 log	3 log	-	NI
Fungi	A	-	-	2 log	-	NI
	B	-	-	-	1 log	NI

Note: NR = no recovery; NI = no increase (≤ 0.5 log); - = not applicable; A = recommended; B = justified alternative. B: In justified cases such as where there may be a risk of adverse reactions and an increased risk of side effects

Table 10. Acceptance criteria for otic, nasal, topical, and inhalation products according to EP 10 [18]

Microorganism	Logarithmic reduction number (CFU/mL)				
	Criterion	2 Days	7 Days	14 Days	28 Days
Bacteria	A	2	3	-	NI
	B	-	-	3	NI
Fungi	A	-	-	2	NI
	B	-	-	1	NI

Note: NI = no increase (≤ 0.5 log); - = not applicable; A = recommended; B = justified alternative.

Table 11. Acceptance criteria for oral, rectal, and mucosal products according to EP 10 [18]

Microorganism	Logarithmic reduction number (CFU/mL)	
	14 Days	28 Days
Bacteria	3 log	NI
Fungi	1 log	NI

Note: NI = no increase (≤ 0.5 log).

IP 9 specifies:

1. **Injectable, ophthalmic, nasal, otic (sterile)**: Bacteria ≤10% initial count (7 days), ≤ 0.1% (14 days), further decrease (28 days); yeast/mold ≤initial count (7, 14, 28 days).
2. **Topical (aqueous), nonsterile nasal, mucosal emulsions**: Bacteria ≤1% initial count (14 days), further decrease (28 days); yeast/mold ≤ initial count (14, 28 days).
3. **Oral products**: Bacteria ≤10% initial count (14 days), further decrease (28 days); yeast/mold ≤initial count (14, 28 days) [20].

Preservative efficacy depends on factors like formulation, pH, and storage conditions, which can alter preservative activity [12, 15]. Microbial count variability (typically 10–20%) arises from method limitations and environmental factors, requiring validated protocols and replicate testing to ensure reliable results [31]. Effective preservatives ensure product safety and extend shelf life, but varying criteria across pharmacopeias necessitate tailored formulations. Harmonizing these standards could enhance global consistency and regulatory compliance [6, 17-20, 26, 27].

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Table 12. Acceptance criteria according to JP 16 [19]

Product category	Microorganism	Interpretation criteria	
		14 Days	28 Days
IA (*e.g*., injections)	Bacteria	≤ 0.1%	≤ 14-day level
	Yeast/Mold	≤ Initial count	≤ Initial count
IB (*e.g*., topical)	Bacteria	≤ 1%	≤ 14-day level
	Yeast/Mold	≤ Initial count	≤ Initial count
IC (*e.g*., oral)	Bacteria	≤ 10%	≤ 14-day level
	Yeast/Mold	≤ Initial count	≤ Initial count
ID/II (*e.g*., low-risk)	Bacteria	≤ Initial count	≤ Initial count
	Yeast/Mold	≤ Initial count	≤ Initial count

Table 13. Summary of acceptance criteria for preservative efficacy test

Pharmacopeia	Log reduction	Percentage of initial count	Flexibility	Specificity for product type
USP 46	Yes	No	Limited	High
EP 10	Yes	No	High (A/B criteria)	High
JP 16	No	Yes	Moderate	High
IP 9	No	Yes	Limited	Moderate

Factors affecting repeatability

The PET, while standardized across pharmacopeias, is subject to variability that affects result consistency. Key factors influencing repeatability include microbial enumeration, neutralization efficacy, and incubation conditions.

Microbial enumeration variability arises from counting methods. Manual plate counts introduce 15–20% inter-operator variability due to colony discrimination errors, while automated systems (*e.g*., spiral platers) reduce this to <5% but require calibration to ensure accuracy [32]. Agar depth impacts growth: layers >5 mm limit oxygen for aerobes like *Pseudomonas aeruginosa*, while <3 mm risk desiccation [32]. Neutralization efficacy varies with incomplete neutralization, neutralizer toxicity (*e.g*., lecithin affecting *Staphylococcus aureus*), or inconsistent neutralizer concentrations, potentially skewing counts [16-20]. Incubation conditions, such as temperature fluctuations (±1°C from 20–25°C for fungi or 30–35°C for bacteria), alter growth kinetics of organisms like *Escherichia coli* [33]. Humidity and light exposure also influence fungal recovery, particularly for *Candida albicans* [17-20, 30].

To enhance repeatability, microbiologists should use standardized protocols, implement quality controls (*e.g*., positive/negative controls), train personnel, calibrate equipment, and conduct replicate testing. While pharmacopeias like USP 46, EP 10, JP 16, and IP 9 provide robust frameworks, they differ slightly in guidance (*e.g*., EP's emphasis on validation, JP's focus on environmental controls) [17-20]. Addressing these factors ensures reliable PET outcomes and regulatory compliance [16, 25].

Limitations of preservative efficacy testing

Despite its utility, PET has limitations in simulating real-world product contamination. The test uses standardized monocultures (*e.g., Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* ATCC 6538) rather than mixed cultures, limiting relevance to multi-species contamination during use [17, 34]. Detection challenges arise when preservative-damaged cells form microcolonies below detection limits or product opacity obscures colonies, underestimating survivors in injectables or ophthalmic products [34].

Preservative kill kinetics often deviate from first-order models. Biphasic curves occur with resistant subpopulations (*e.g., P. aeruginosa* in USP 46), while shoulder phases reflect preservative-excipient binding in viscous formulations (EP 10) or cationic preservative interactions (JP 16) [17-19]. These non-linear patterns, influenced by microbial resistance or biofilm formation, complicate interpretation, especially with sparse sampling [17-19, 28]. Survivors after 28 days may require enriched media or extended incubation to recover, as damaged cells (*e.g*., *Candida albicans*) differ from initial inocula, risking underestimation if conditions are suboptimal [35].

Cell aggregation, such as biofilms in eye drop nozzles, shields microbes, producing falsely low counts via plate methods [12]. Studies report microbial contamination in ~10% of preservative-free eye drops due to nozzle biofilms, highlighting PET's inability to mimic such conditions [36]. Inocula of robust, fast-growing microbes contrast with slow-growing contaminants encountered during use, where preservatives are less effective [37]. Nutrient-limited cultures (*e.g.,* low carbon) alter sensitivity, as shown with *S. aureus*, suggesting PET inocula may not reflect real-world challenges [37, 38].

PET cannot fully replicate in-use conditions, prioritizing simplicity and reliability to ensure products resist microbial spoilage [16-20].

CONCLUSIONS

This review compares preservative efficacy testing across USP 46, EP 10, JP 16, and IP 9, revealing shared principles and distinct approaches. All pharmacopeias require at least four indicator microorganisms: *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Candida albicans*, with EP

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10, JP 16, and IP 9 adding *Zygosaccharomyces rouxii* for high-sugar oral products [17-20]. Inoculum volumes are limited to ≤1% of product volume, with USP 46 and EP 10 specifying 0.5–1% and JP 16 and IP 9 adhering strictly to ≤1% [17-20]. Incubation temperatures align at 20–25°C for fungi and yeast and 30–35°C for bacteria, though USP 46 extends inoculum preparation for *C. albicans* to 44–52 hours [17-20]. Differences span product classification, microbial cultivation, suspension preparation, sampling intervals, neutralization, counting methods, and result interpretation [6, 16].

Such variations pose challenges for global regulatory alignment. For instance, the USP employs log-reduction models, while JP applies percentage-based acceptance criteria, potentially leading to inconsistent evaluations [17, 19]. Harmonization could align protocols, creating uniform guidelines for product categories, microorganisms, and acceptance criteria. Benefits include improved result comparability, streamlined approvals, and enhanced trade. Challenges involve reconciling regulatory priorities and scientific methods [6, 26, 27]. Collaboration among pharmacopeias, regulators, and researchers is essential to standardize PET, ensuring reliable, safe pharmaceuticals worldwide.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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DATA AVAILABILITY

This study is a comparative analysis of publicly available pharmacopeial standards (USP 46, EP 10, JP 16, IP 9). No new data were generated. All protocols and criteria are accessible in the cited pharmacopeias.

AUTHORS' CONTRIBUTIONS

SS: Writing, review, and editing; FSh: Writing and review; SH: Supervision. All authors reviewed and approved the final manuscript.

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