The Essentials of United States Pharmacopeia Chapter <51> Antimicrobial Effectiveness Testing

AND ITS APPLICATION IN PHARMACEUTICAL COMPOUNDING

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INTRODUCTION

Antimicrobial preservatives are excipients added to multi-dose formulas of both sterile and nonsterile drug products for inhibition of microbial growth. Microbial contamination may occur during nonsterile processing or during the period of use due to the repeated withdrawal of individual doses from multi-dose containers.[1] Multi-dose pharmaceutical products containing preservatives offer several advantages over single-dose packages. Multi-dose drugs minimize product wastage and allow flexibility for dosage adjustments; repeated doses may be obtained from the same container without concerns for microbial growth during use; and their packaging is reduced because multiple doses are supplied in a single container.[2] It is general knowledge that unit-dose packaging is the most optimal with respect to the maintenance of sterility, but it is not efficient and cost effective as preserved multi-dose preparations.

Antimicrobial preservatives can be microcidal, microstatic, and sporicidal.

ABSTRACT **Antimicrobial preservatives are excipients added to multi-dose containers of both sterile and nonsterile drug products. Antimicrobial preservatives are used primarily to inhibit growth of microbial contamination occurring during the period of use. Demonstration of antimicrobial preservative effectiveness is required for these functional excipients. This article reviews key factors for consideration in the selection of preservatives, principles of the preservative-effectiveness test, and the significance of requirements for preservative-effectiveness testing in the compounding practice.**

They interfere with various mechanisms in microbial cells causing cellular damage or cell lysis. The mechanisms for antimicrobial effects are not always specific and can be difficult to elucidate. Some preservatives may act at the cell wall, others may target the cytoplasmic membrane or cytosolic components. Their activities may lead to irreversible cell membrane damage, precipitation of cellular proteins, or inhibition of critical pathways for signal induction and cellular transport. Preservatives may also act synergistically with other preservatives or with other components of the formula to enhance the total effects for microbial control. Due to the cytotoxic effects they exert against microbial cells, these preservatives can not be regarded simply as inactive ingredients. Their inclusion in pharmaceutical preparations should be at a concentration that is effective but nontoxic to humans.[3] An ideal preservative should be active against a broad spectrum of microorganisms but nontoxic to human cells and should be tolerable by the intended patient groups; it must also be stable and compatible with the other components of the drug product to be effective. Activities of commonly used antimicrobial agents, which are relatively safe for use in pharmaceutical compounding, will be discussed in the following sections. The principle of antimicrobial effectiveness testing and its requirements in the compounding practice will also be discussed.

GENERAL CONSIDERATIONS IN THE SELECTION OF ANTIMICROBIAL PRESERVATIVES

Most viable cells function optimally within a narrow pH range around neutrality, and growth is slow at pH beyond 6 or 8.[4] This pH range may not always be optimal due to solubility and stability of formulation ingredients. Hence, the pH of a formula is often adjusted to enhance product quality. In terms of solubility, the optimum pH for formulation ingredients can be deduced from their dissociation constants (pK_a) and their oil-water partition coefficients ($LogP_{o/w}$). Both parameters are related to their aqueous solubility, where the antimicrobial effect is required, and their concentration in different phases of a multiphasic system. However, the relationship between pH and antimicrobial activities is more complex. For example, the antifungal activity of benzoic acid is less susceptible to pH than its antibacterial activity. Similarly, sorbic acid has significant antifungal but little antibacterial activity at pH 6.0.[4]

Antacid formulations and multiphase systems are more difficult to preserve than simple aqueous formulas. Such products require additional ingredients that have a high potential for interactions. Interactions of preservatives with formulation ingredients and containers may compromise product stability and preservative efficacy. Interactions do not always lead to structural modification of the preservatives but may occur in the form of complex formation, precipitation, or adsorption to surfaces. Incompatibility among components occurs in the presence of strong oxidizing agents, or between a strong base and acidic preservatives. Cationic preservatives are incompatible with anionic surfactants, and non-ionic surfactants (e.g., polysorbate 80) are incompatible with some alcohol phenolic preservatives. The parabens, benzoic acid, chlorobutanol, m-cresol, etc. are relatively volatile and can be lost during processing and storage. Preservative precipitation in the presence of polyvalent cations was observed with sorbic acid, butylated hydroxyanisole, chlorhexidine, etc. Additionally, reconstitution of Activase, Proleukin, and Leukine with diluents containing preservatives may denature protein and peptide molecules.[4,5] Lab techniques such as size exclusion chromatography (SEC), dynamic light scattering (DLS), fourier transform infrared (FTIR), electron microscopy, histologic analysis, and immunological assay have been used to characterize interactions in small-molecule drug products and in biopharmaceuticals.[2]

In addition to *in vitro* formulation issues, *in vivo* adverse effects may further limit the availability of suitable agents for preserved products. As previously discussed, most preservatives are cytoxic to microbial cells, and their use may impart unintended side effects in patients. Notably, benzyl alcohol is not recommended in neonatal parenteral products, as it has been linked to fatal toxic syndrome in premature neonates. Irritants, such as parabens, were determined unsuitable for ophthalmic preparations, and benzalkonium chloride may not be appropriate for soft contact lenses solutions. Concerns over neurotoxicity have lead to the declined usage of organomecuric compounds in parenteral products, and hexachlorophene in topical products.[5,6]

The above discussion highlights formulation and external factors that must be considered in the preparation of preserved products. Optimization of the preservative system is often conducted during pre-formulation studies, which are not usually performed for compounded preparations, thus emphasizing the requirements for the demonstration of antimicrobial efficacy in dispensing and beyond-use dating (BUD).

COMMONLY UTILIZED ANTIMICROBIAL PRESERVATIVES

In a review conducted by Meyer, et al,[2] the authors observed that macromolecular biotech products such as peptides and proteins usually contain phenol and benzyl alcohol as preservatives. Whereas a combination of parabens are found in small molecule parenterals, and phenoxyethanol is often found in vaccines. M-cresol and chlorobutanol are present in fewer products, and older products may also contain thimerosal or phenylmercurric salts although they are no longer preferred agents in new formulas. Most intraocular and intrathecal products are preservative-free because of safety considerations.[7]

A list of common antimicrobial preservatives with proven performance characteristics in various dosage forms is provided in Table 1. Although limited in content, the table contains historical data that may be useful as a quick reference in a busy pharmacy environ-

ment. *The Handbook of Pharmaceutical Excipients*[3] is a comprehensive source of data describing physicochemical properties and safety profiles of available excipients, including antimicrobial preservatives. Interested readers are directed to this reference for additional information.

ANTIMICROBIAL EFFECTIVENESS TESTING (*UNITED STATES PHARMACOPEIA* CHAPTER <51>)

SUMMARY OF TEST

The *USP* Chapter <51> Antimicrobial Effectiveness Test[1] is conducted by adding specified microorganisms individually to the test product at relatively high concentrations to simulate contamination. The product is held for 28 days, during which time the added microorganisms are enumerated at defined intervals to determine any change in microbial content. Inoculated microorganisms include *Candida albicans, Aspergillus niger, Escherichia coli, Pseudomonas aeruginosa, and Staphylococcus aureus*. The acceptance criteria are specified for each drug product categories. In general, a 1 to 3 log reduction in bacteria from the initial level should occur in one to two weeks, with no further increase in bacteria thereafter at 28 days. For yeast and mold, no increase from the initial inoculum level is permitted at all sampling intervals.

PRODUCT CATEGORIES AND SPECIFICATIONS

Pharmaceutical products are divided into four categories based on product risk.[8] As shown in Table 2,[1] shorter sampling intervals during a 28-day period, and more stringent criteria are associated with Category 1 products, which includes sterile parenteral in aqueous base or emulsions (e.g., injections, otic products, ophthalmic products, nasal products). Adequate preservation is indicated by not less than 1 and 3 log reduction in bacterial count from the initial value at day 7 and day 14, respectively. Subsequently, bacterial counts at day 28 should not increase from counts at day 14. Less stringent criteria are applied to topical and oral products in categories 2 and 3. For oral and topical products, at least 1 log (oral products) and 2 log (topical products) reduction from initial bacterial count at day 14 should be observed, and no increase relative to day-14 counts at day-28 testing. Antacid products are qualified by

TABLE 1. Common Pharmaceutical Preservatives.

PRESERVATIVE	FORMULATION	CONCENTRATION (PERCENTAGE)	OPTIMAL pH	SPECTRUM
4-Chlorocresol	Oral, Topical	Up to 0.2	<9.0	• Bacteria, spores, molds, and yeasts • Active in acidic media
4-Chloroxylenol	Topical	0.1 to 0.8	-----	• Gram (+) bacteria • Less active vs Gram (-) bacteria • Synergistic with EDTA
Benzalkonium	Oral, Ophthalmic, Topical	0.01 to 0.02	4 to 10	• Gram (+) > Gram (-) bacteria • Ineffective vs resistant P. aeruginosa strains • Minimal activity vs bacterial endospores, acid-fast bacteria
Benzethonium chloride	Topical, Ophthalmic	Up to 0.5	4 to 10	• Bacteria, fungi, and molds • Synergistic with ethanol • Reduced efficacy by soaps and other anionic surfactants
Benzoic acid	Oral, Parenteral, Topical	0.1 to 0.2	2.5 to 4.5	• Moderate activity vs Gram (+) < Gram (-) • Moderate activity vs fungal • Moderate activity vs mold
Benzyl alcohol	Oral, Parenteral	Up to 2.0	<5.0	• Moderate activity vs Gram (+) < Gram (-) • Effective vs molds and yeasts
Boric acid	Ophthalmic, Topical	-----	3.5 to 4.1	• Weak bacteriostatic • Weak fungistatic
Cetrimide	Ophthalmic, Topical	• Ophthalmic: 0.005 • Topical: 0.1 to 1.0	Neutral or slightly alkaline	• Gram (+) > Gram (-) bacteria • Synergistic with alcohols • Variable activity vs fungi • Synergistic with EDTA vs resistant strains of P. aeruginosa, A. niger, C. albicans
Chlorhexidine	Ophthalmic	0.01	5 to 7	• Gram (+) > Gram (-) • Weak activity vs Proteus and Pseudomonas • Inactive vs acid-fast bacilli • Weak activity vs molds, yeasts
Chlorobutanol	Parenteral	Up to 0.5	<5.5	• Activity Gram (+), Gram (-), and some fungi
Imidurea	Topical, Ophthalmic	0.03 to 0.5	3 to 9	• Broad-spectrum antibacteria • Some antifungal properties • Synergistic with parabens vs fungi
m-Cresol	Parenteral	0.15 to 0.3	<9.0	• Moderately Gram (+) > Gram (-) • Weak activity vs yeasts and molds
Methylparaben	Oral, Parenteral	0.0018	4 to 8	• Broad spectrum antimicrobial activity • Most effective vs yeasts and molds
Phenols 0.5%	Parenteral	0.01	<9	• Moderate activity vs Gram (+) < Gram (-) • Weak activity vs yeasts and molds
Phenoxyethanol	Parenteral, Topical	0.5 to 2.2	<7	• Antibacterial vs P. aeruginosa < Proteus vulgaris • Weak activity vs Gram (-) • Frequently used in combination with other preservatives
Potassium sorbate	Oral, Topical	0.1 to 0.2	<6	• Predominantly antifungal • Moderate antibacterial
Propionic acid	Oral, Topical	-----	3.9	• Bacteria, fungi, and molds
Propylparaben	Oral, Parenteral	0.0002	4 to 8	• Activity vs yeasts and molds > bacteria • Gram (+) > Gram (-) bacteria
Sodium benzoate	Oral, Parenteral	• Oral: 0.02 to 0.5 • Parenteral: 0.5	2 to 5	• Bacteriostatic • Antifungal
Sorbic acid	Oral, Topical	0.05 to 0.2	4.5	• Primarily antifungal • Weak antimicrobial • Synergy with glycol
Thimerosal	Ophthalmic, Parenteral	0.001 to 0.01	7 to 8	• Bactericidal at acidic pH • Bacteriostatic and fungistatic at alkaline or neutral pH • Ineffective vs spore-forming organisms

separate criteria in Category 4 due to the inherent issues with this product (e.g., high pH, interactions of preservatives with formulation ingredients). Effective antimicrobial activities in antacid products are indicated by no increase in bacterial, yeast, and mold from initial counts when tested at day 14 and day 28. No increase or no change is equivalent to not more than a 0.5 log change from the initial inoculum level to account for variability of the test.

TEST ORGANISMS AND PREPARATION OF STANDARDIZED CELL SUSPENSIONS

A panel of five challenge organisms are used in *USP* <51>, including *Candida albicans* (yeast), *Aspergillus niger* (mold), *Escherichia coli* (Gram-negative enterobacillus), *Pseudomonas aeruginosa* (Gram-negative bacillus), and *Staphylococcus aureus* (Gram-positive coccus). Fresh cultures of each organism are harvested in sterile saline and standardized to about 10^8 colony forming units per mL (cfu/mL). Extensive propagating of microbial cells is discouraged because it could lead to changes in phenotypic expression and antimicrobial susceptibility. Therefore, seed-stock techniques are recommended for long-term storage, and stock cultures of each organism are limited to no more than five passages removed from the original seed stock.[1,9]

The microbial enumeration test is performed to determine the number of viable cells in each cell suspension. Bacteria are grown at 30°C to 35°C on Soybean-Casein Digest Agar, while yeast and mold are grown at 20°C to 25°C on Sabouraud Dextrose Agar. Table 3 describes the culture conditions for the preparation of standardized cell suspensions and microbial recovery study.

CHALLENGE TEST

The standardized cell suspensions are added to the test product in five separate containers, one container for each challenge organism. The concentration of challenge organisms in product Categories 1 through 3 is between 10^5 and 10^6 cfu/mL. The products in Category 4 (antacids) contain between 10^3 to 10^4 cfu/mL of each challenge organism. The inoculum volume should not exceed 1% of the total volume of the product to be tested. Inoculated samples are incubated at 20°C to 25°C for 28 days. The microbial enumeration test is performed at days 7, 14, and 28 by the validated method.

METHOD SUITABILITY TEST

The antimicrobial preservatives in the drug product must be neutralized to recover viable cells in the microbial enumeration test. This neutralization can be accomplished by neutralizing agents, membrane filtration, dilution, or any combination of these methods. Neutralization conditions must be validated for efficiency and suitability by the counting method. All organisms used in the challenge test must be included in the validation of methods. The validation protocol should follow guidelines elaborated in *USP* General Chapters <61> and <1227>. Briefly, the validation study must show that recovery of an inoculum containing ≤100 cfu of the challenge organism is not inhibited by the test sample and by the neutralization method. This is accomplished by comparing recovery results for three treatment groups:

1. The test group: Neutralized product inoculated with 100 cfu of the challenge organism
2. The peptone control group: The same treatment as in the test group but peptone is used instead of the test product
3. Inoculum control containing 100 cfu of the challenge organism, but no neutralization and no product present

The validation study is conducted in three independent experiments. In each experiment, average recovery of viable cells in the test group should be at least 70% relative to the inoculum control.

TABLE 2. Four Categories of Drug Products and Specifications for Antimicrobial Efficacy.[1]

CATEGORY	PRODUCT DESCRIPTION	CRITERIA FOR BACTERIA	CRITERIA FOR YEAST AND MOLD
1	• Parenterals (injections, emulsions) • Otic, ophthalmic, and sterile nasal products in aqueous base	• ≥1.0 log reduction at day 7 relative to initial count • ≥3.0 log reduction at day 14 relative to initial count • No increase at day 28 relative to day-14 count	No increase at days 7, 14, and 28 relative to initial count
2	• Topical products in aqueous base • Nonsterile nasal products • Nonsterile emulsions • Products for mucosal application	• ≥2.0 log reduction at day 14 relative to initial count • No increase at day 28 relative to day-14 count	No increase at days 7, 14, and 28 relative to initial count
3	Oral products in aqueous base (excluding antacids)	• ≥1.0 log reduction at day 14 relative to initial count • No increase at day 28 relative to day-14 count	No increase at days 7, 14, and 28 relative to initial count
4	Antacids in aqueous base	No increase at days 14 and 28 relative to initial count	

TABLE 3. Incubation Temperature and Incubation Time for Preparation of Standardized Cell Suspensions and Microbial Recovery Study.[1]

ORGANISM	CULTURE MEDIUM	TEMPERATURE (°C)	TIME (CELL SUSPENSION)	TIME (RECOVERY)
E. coli	Soybean-Casein Digest (broth, agar)	30 to 35	18 to 24 hours	3 to 5 days
P. aeruginosa	Soybean-Casein Digest (broth, agar)	30 to 35	18 to 24 hours	3 to 5 days
S. aureus	Soybean-Casein Digest (broth, agar)	30 to 35	18 to 24 hours	3 to 5 days
C. albicans	Sabouraud Dextrose (broth, agar)	20 to 25	44 to 52 hours	3 to 5 days
A. niger	Sabouraud Dextrose (broth, agar)	20 to 25	6 to 10 days	3 to 7 days

COMPARISON AMONG COMPENDIA MICROBIAL-EFFICACY TESTS

Procedures for antimicrobial efficacy determination are described in three major compendia: the *USP* (Chapter <51> Antimicrobial Effectiveness Testing), the *European Pharmacopoeia (EP)* (Chapter <5.1.3> Efficacy of Antimicrobial Preservation), and the *Japanese Pharmacopoeia (JP)* (Chapter <19> Preservative Effectiveness Test). These chapters are essentially harmonized in principles, but minor differences exist with respect to the challenge organisms, test intervals, and acceptance criteria.[9] In situations where compliance to three compendia are required, these differences should be incorporated into the test protocol.

PREPARATION OF CHALLENGE MICROORGANISMS

The *EP* does not include *E. coli* in the panel of challenge microorganisms, but does allow supplementing the panel with additional species "that may represent likely contaminants," and recommends the addition of *E. coli* for all oral preparations.[10] *USP* <51> listed only the strains of challenge organisms sourced from American Type Culture Collection (ATCC), while both the *EP* and *JP* recognize additional source strains besides those listed in *USP* <51>. The incubation temperatures of subcultures are harmonized, but the incubation durations are slightly varied for yeast and mold. To comply with three compendia, *C.*

albicans should be harvested at about 48 hours, and *A. niger* should be harvested after 6 to 7 days "when good sporulation is obtained." Standardized cell suspensions should be used within 8 hours, and stored at 2°C to 8°C when not in use.[9]

TEST INTERVALS AND ACCEPTANCE CRITERIA

The acceptance criteria expressed in the *EP* are the most stringent compared to the *USP* and *JP*. The *EP* has two criteria (A and B) for products in Categories 1 (parenteral intrauterine, intramammary preparations) and 2 (ear, nasal, inhalation, cutaneous preparations). The A criteria are "the recommended efficacy to be achieved," and "In justified cases where the A criteria cannot be attained..., the B criteria must be satisfied." The *EP* Category 1-A has approval criteria at 6 hours and 24 hours in addition to days 7, 14, and 28. The *JP* has acceptance criteria expressed as a percentage recovery for days 14 and 28.[9] To comply with three compendia requirements, sampling intervals should start at 6 hours for products in Category 1, and day 2 for products in Category 2. Table 4 shows sampling frequencies and acceptance criteria expressed by the *EP*, *JP*, and *USP* for sterile parenteral products.[9,10]

TABLE 4. Comparison of Acceptance Criteria for Parenteral Products by *JP*, *USP*, and *EP*.

TIME	*JP* (% REDUCTION)		*USP* (LOG REDUCTION)		*EP* (LOG REDUCTION)			
	BACTERIA	FUNGAL AND MOLD	BACTERIA	FUNGAL AND MOLD	BACTERIA		FUNGAL AND MOLD	
					A	B	A	B
6 Hours	NT	NT	NT	NT	2	NT	NT	NT
24 Hours	NT	NT	NT	NT	3	1	NT	NT
7 Days	NT	NT	1	NI	NT	3	2	NT
14 Days	0.1% or less	≤ at day 14	3	NI	NT	NT	NT	1
28 Days	reduction ≤ at day 14	≤ at day 14	NI	NI	NR	NI	NI	NI

NI = no increase; NR = no recovery; NT = not tested
EP = European Pharmacopoeia; JP = Japanese Pharmacopoeia; USP = United States Pharmacopeia

SIGNIFICANCE OF ANTIMICROBIAL-EFFECTIVENESS TEST

The purpose of *USP* <51> is to provide a guide to antimicrobial-effective testing. Preservatives are not meant to replace but to compliment current good manufacturing processes. *USP* <51> testing ensures the efficacy of pharmaceutical products containing preservatives in original, unopened containers made and distributed by the manufacturer. Measurement of preservation during in-use is outside the scope of the current protocol and requires different experimental designs (e.g., broaching study designs). In addition, the panel of five organisms employed in the challenge study does not represent resistant phenotypes that have acquired the ability to withstand the activity of the preservative. The standard preservative test may then be insufficient to demonstrate the survival capacity in pharmaceuticals of strains adapted to low-nutrient environment and low storage temperatures.[11]

WHEN ANTIMICROBIAL-EFFECTIVENESS TEST IS PERFORMED

The antimicrobial-effectiveness test is often performed during drug development for optimization of formulation ingredients. The International Conference on Harmonization (ICH) requires[12]:

> Antimicrobial preservative effectiveness should be demonstrated during development, during scale-up, and throughout the shelf-life..., although chemical testing for preservative content is the attribute normally included in the specification.

The ICH Q6A further specifies[12]:

> The testing for antimicrobial preservative content should normally be performed at release. Acceptance criteria for preservative content should be based upon the levels of antimicrobial preservative necessary to maintain microbiological quality of the product at all stages throughout its proposed usage and shelf life. The lowest specified concentration of antimicrobial preservative should be demonstrated to be effective in controlling microorganisms by using a pharmacopeial antimicrobial preservative effectiveness test.

The ICH further states[13]:

> A single primary stability batch of drug product should be tested for antimicrobial preservative effectiveness (in addition to preservative content) at the proposed shelf life for verification purpose.

In pharmaceutical compounding, *USP* Chapter <51> forms a part of the product quality test for preserved preparations due to limited pre-formulation data. Preservative content and effectiveness testing should be a part of a stability program for BUD of all preserved preparations, "when such a test is performed, the results shall support the BUD assigned to the compounded preparations."[1] One strategy is to prepare formulas with 100% and 70% of the label concentration for the preservative (limit for assay ± 20% of preservative label content). Preservative effectiveness and content are established for these samples at the initial time point, and then content testing will be conducted for the remainder of the stability time intervals. It is also prudent to confirm the preservative effectiveness at the BUD according to *USP* Chapter <51>. Based on the stability results, only the content test will need to be conducted for future batches unless fundamental changes occur in the formulation or compounding procedure. The above discussion pertains to antimicrobial-preservative testing only and does not address other testing requirements for compounded preparations.

CONCLUSION

The *USP* Chapter <51> Antimicrobial Effectiveness Testing is a culture-based method and accuracy of results is dependent upon adequate neutralization of antimicrobial activities in test samples for enumeration testing. The efficiency of the neutralization method employed must be validated for all five challenge organisms. Alternative methods can be substituted if proven to be equivalent to compendia testing.

USP <51> does not address contamination by end users. Evaluating the efficacy of the preservative system for these in-use conditions requires different experimental designs that simulate in-use. The three major compendia (*EP, JP, USP*) are harmonized in principles but different in some aspects, which must be incorporated into the test protocol for compliance.

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