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⟨51⟩ ANTIMICROBIAL EFFECTIVENESS TESTING

INTRODUCTION

Antimicrobial preservatives are substances added to aqueous pharmaceutical products. Nonsterile dosage forms may have preservatives added to protect them from growth of microorganisms inadvertently introduced during or subsequent to the manufacturing process. In the case of sterile articles packaged in multiple-dose containers, antimicrobial preservatives are added to inhibit the growth of microorganisms that may be introduced from repeatedly withdrawing individual doses. One or more antimicrobial preservative(s) are expected in all sterile multidose units.

Antimicrobial preservatives should not be used as a substitute for good manufacturing practices, solely to reduce the viable microbial population of a nonsterile product, or control the presterilization bioburden of a multidose formulation during manufacturing. Antimicrobial preservatives in compendial dosage forms meet the requirements for *General Notices, 5.20 Added Substances*.

All useful antimicrobial agents are toxic substances. For maximum protection of patients, the concentration of the preservative shown to be effective in the final packaged product should be below a level that may be toxic to human beings based on the recommended dosage of the medicinal product.

The concentration of an added antimicrobial preservative can be kept to a minimum if the active ingredients of the formulation possess an intrinsic antimicrobial activity. Antimicrobial effectiveness, whether inherent in the product or produced because of the addition of an antimicrobial preservative, must be demonstrated for all injections packaged in multiple-dose containers or for other products containing antimicrobial preservatives. Antimicrobial effectiveness must be demonstrated for aqueous-based, multiple-dose topical and oral dosage forms and for other dosage forms such as ophthalmic, otic, nasal, irrigation, and dialysis fluids (see *Pharmaceutical Dosage Forms* ⟨1151⟩). For the purpose of the test, aqueous is defined as a water activity of more than 0.6 (see *Application of Water Activity Determination to Nonsterile Pharmaceutical Products* ⟨1112⟩).

Challenge organisms are generally based on likely contaminants to a drug product while considering its physical attributes, formulation, and intended use. The standard battery of challenge organisms described in this test need not prevent the inclusion of other species of microorganisms if deemed useful to measure the biological activity of the preservative system for a specific product. These supplemental challenge organisms are not within the scope of this chapter, but may be added in addition to the described test organisms.

GENERAL PROCEDURES

This chapter provides procedures to demonstrate the effectiveness of added antimicrobial preservatives. Such antimicrobial preservatives must be declared on the label. The procedures and acceptance criteria for effectiveness apply to a product in the original, sealed container in which it was distributed by the manufacturer (see *Table 1* for categories of products). The test need not be conducted in these containers, but care should be taken to avoid using materials that can interact with the preservative in the containers that are used for antimicrobial effectiveness testing.

Growth Promotion Procedure and Suitability of the Recovery Method

GENERAL CONSIDERATIONS

The ability of the procedure to detect challenge microorganisms in the presence of a suitably neutralized product to be tested must be established. The suitability of the procedure must be reconfirmed if a change is made in materials or methods or if a change is made in the product or direct product contact materials that may affect the outcome of the test.

The growth-promoting capabilities of media used in this procedure must be established.

PREPARATION OF TEST STRAINS

Use standardized suspensions of test strains or prepare as stated below. Seed-lot culture maintenance techniques (seed-lot systems) are used so that the viable microorganisms used for inoculation are NMT five passages removed from the original master seed lot. Grow each of the bacterial and fungal test strains separately (see *Table 2*).

Use cultures of the following microorganisms:[1] *Candida albicans* (ATCC No. 10231), *Aspergillus brasiliensis* (ATCC No. 16404), *Escherichia coli* (ATCC No. 8739), *Pseudomonas aeruginosa* (ATCC No. 9027), and *Staphylococcus aureus* (ATCC No. 6538). The viable microorganisms used in the procedure should be part of a freshly growing culture (e.g., in logarithmic growth phase) with the exception of *A. brasiliensis* spores. The culture conditions for the inoculum culture are described (see *Table 2*) in which the suitable media are Soybean–Casein Digest or Sabouraud Dextrose Agar Medium.

To harvest the bacterial and *C. albicans* cultures, use sterile saline TS to wash the surface growth, and collect it in a suitable vessel. To harvest the spores of *A. brasiliensis,* use sterile saline TS containing 0.05% of polysorbate 80. The spore suspension should be aseptically

treated (e.g., filtration through sterile glass wool) to remove hyphae. All microbial suspensions should be prepared to ensure that there is no carry over of residual growth medium from the inoculum (e.g., centrifugation followed by resuspension in appropriate sterile suspending fluid.)

Alternatively, the stock culture organisms may be grown in a suitable liquid medium (i.e., Soybean–Casein Digest Broth or Sabouraud Dextrose Broth) and the cells harvested by centrifugation, then washed and resuspended in appropriate sterile suspending fluid. The microbial suspensions used for inoculations should be adjusted to obtain a microbial count of about 1×10^8 cfu/mL. Use the bacterial and yeast suspensions within 2 h, or within 24 h if stored between 2° and 8°. A stable spore suspension can be prepared and then may be maintained at 2°–8° for up to 7 days. [Note—The estimate of inoculum concentration may be obtained by turbidimetric procedures for the challenge microorganisms and later confirmed by plate count.]

GROWTH PROMOTION OF THE MEDIA

Media used in this procedure must be capable of supporting microbial growth. Test each batch of ready-prepared medium and each batch of medium prepared either from dehydrated medium or from the ingredients described.

For solid media, counts obtained must be at least 50% of the calculated value for a standardized inoculum. For a freshly prepared inoculum, growth of the microorganisms occurs comparable to that previously obtained with a previously tested and approved batch of medium.

Suitability of the Counting Method in the Presence of Product

Prepare a 10^{-1} dilution by adding 1 mL of product (by volume) to 9 mL of saline or other neutralizing diluent. Continue this dilution scheme to 10^{-2} and 10^{-3} dilution levels. Add an appropriate number of challenge organisms to each tube of diluted product, mix, and then plate a suitable volume from each dilution to yield less than 250 cfu/plate for bacteria and yeast (ideally between 25 and 250 cfu) or less than 80 cfu/plate for *A. brasiliensis* (ideally between 8 and 80 cfu). This plating should be performed minimally in duplicate (although a greater number of replicates can be useful to minimize variability in the plate count estimate). A positive control for this procedure is to introduce the same inocula into saline, and transfer similar volumes of saline to agar plates. A suitable recovery scheme is the one that provides at least 50% of this saline control count (averaged).

If the diluted product exhibits antimicrobial properties, specific neutralizers may need to be incorporated into the diluents or the recovery media. See *Validation of Microbial Recovery from Pharmacopeial Articles ⟨1227⟩* for more information.

The ability of the procedure to measure preservative efficacy may be compromised if the method suitability requires significant dilution (10^{-2} or 10^{-3}) as this will affect the measured recovery (e.g., it may be difficult to measure a 3 log unit reduction for a 10^5–10^6 inoculum). If no suitable neutralizing agent or method is found and method suitability requires significant dilution, a higher level of inoculum (e.g., 10^7–10^8) may be used so that a 3 log unit reduction can be measured. Reported recovery cannot be less than 1 cfu/plate on average (or 100 cfu/mL if 1 mL is plated in duplicate at the 10^{-2} dilution).

Membrane filtration may be used to filter larger volumes of dilutions to overcome this difficulty or to assist in the neutralization of antimicrobial properties.

Testing of Products

PRODUCT CATEGORIES

For the purpose of testing, compendial articles have been divided into four categories (see *Table 1*). The criteria of antimicrobial effectiveness for these products are a function of the route of administration. It is expected that formulations containing preservatives will meet minimal efficacy standards, whether packaged as multidoses or unit doses.

Table 1. Compendial Product Categories

Category	Product Description
1	Injections; other parenterals including emulsions, otic products, sterile nasal products, and ophthalmic products made with aqueous bases or vehicles
2	Topically used products made with aqueous bases or vehicles; nonsterile nasal products and emulsions, including those applied to mucous membranes
3	Oral products other than antacids, made with aqueous bases or vehicles
4	Antacids made with an aqueous base

PROCEDURE

The procedure can be conducted either in five original containers if a sufficient volume of product is available in each container and if the product container can be entered aseptically (i.e., needle and syringe through an elastomeric rubber stopper), or in five sterile, capped bacteriological containers [inert relative to the antimicrobial agent(s)] of suitable size into which a sufficient volume of product has been transferred. Inoculate each container with one of the prepared and standardized inocula, and mix. The volume of the suspension inoculum used is between 0.5% and 1.0% of the volume of the product to minimize potential effects on the product. The concentration of test microorganisms that is added to the product (*Category 1, 2,* or *3*) is such that the final concentration of the test preparation after inoculation is between 1×10^5 and 1×10^6 cfu/mL of the product. For *Category 4* products (antacids), the final concentration of the test preparation after inoculation is between 1×10^3 and 1×10^4 cfu/mL of the product.

The initial concentration of viable microorganisms in each test preparation is estimated based on the concentration of microorganisms in each of the standardized inocula as determined by the plate-count method. Incubate the inoculated containers at 22.5 ± 2.5°. Sample each container at the appropriate intervals (specified in *Table 3*). Record any changes observed in appearance at these intervals. Determine, by the plate-count procedure, the number of cfu present in each test preparation for the applicable intervals (see *General Procedures* in *Microbial Enumeration Tests ⟨61⟩*). Plate counts will be conducted using a minimum of duplicate plates, with the cfu averaged before determination of deduced cfu/mL. If membrane filtration is used, duplicate membrane filters will be used for each estimate. Using the calculated concentrations of cfu/mL present at the start of the test, calculate the change in $\log_{10}$ values of the concentration of cfu/mL for each microorganism at the applicable test intervals, and express the changes in concentration in terms of log reductions. The log reduction is defined as the difference between the $\log_{10}$ unit value of the starting concentration of cfu/mL in the suspension and the $\log_{10}$ unit value of cfu/mL of the survivors at that time point.

Table 2. Culture Conditions for Inoculum Preparation

Organism	Suitable Medium	Incubation Temperature	Inoculum Incubation Time	Microbial Recovery Incubation Time
Escherichia coli (ATCC No. 8739)	Soybean–Casein Digest Broth; Soybean–Casein Digest Agar	32.5 ± 2.5°	18–24 h	3–5 days
Pseudomonas aeruginosa (ATCC No. 9027)	Soybean–Casein Digest Broth; Soybean–Casein Digest Agar	32.5 ± 2.5°	18–24 h	3–5 days
Staphylococcus aureus (ATCC No. 6538)	Soybean–Casein Digest Broth; Soybean–Casein Digest Agar	32.5 ± 2.5°	18–24 h	3–5 days
Candida albicans (ATCC No. 10231)	Sabouraud Dextrose Agar; Sabouraud Dextrose Broth	22.5 ± 2.5°	44–52 h	3–5 days
Aspergillus brasiliensis (ATCC No. 16404)	Sabouraud Dextrose Agar; Sabouraud Dextrose Broth	22.5 ± 2.5°	6–10 days	3–7 days

Criteria for Antimicrobial Effectiveness

The requirements for antimicrobial effectiveness are met if the criteria specified in *Table 3* are met (see *General Notices, 7. Test Results*). "No increase" in counts is defined as NMT 0.5 $\log_{10}$ unit more than the value to which it is compared.

Table 3. Criteria for Tested Microorganisms

For Category 1 Products	
Bacteria	NLT 1.0 log reduction from the initial calculated count at 7 days, NLT 3.0 log reduction from the initial count at 14 days, and no increase from the 14 days' count at 28 days

Yeast and molds	No increase from the initial calculated count at 7, 14, and 28 days
For Category 2 Products	
Bacteria	NLT 2.0 log reduction from the initial count at 14 days, and no increase from the 14 days' count at 28 days
Yeast and molds	No increase from the initial calculated count at 14 and 28 days
For Category 3 Products	
Bacteria	NLT 1.0 log reduction from the initial count at 14 days, and no increase from the 14 days' count at 28 days
Yeast and molds	No increase from the initial calculated count at 14 and 28 days
For Category 4 Products	
Bacteria, yeast, and molds	No increase from the initial calculated count at 14 and 28 days

[1] Available from American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209 (http://www.atcc.org).

Auxiliary Information - Please check for your question in the FAQs before contacting USP.

Topic/Question	Contact	Expert Committee
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