REVISED GENERAL CHAPTER

2.2.2. Effectiveness of Antimicrobial Preservatives

NOTE—The test for effectiveness of antimicrobial preservatives shall be demonstrated during development of pharmaceutical preparation and during the commercial manufacturing. The test is not intended to be used for routine control purpose.

The efficacy of antimicrobial preservation of a pharmaceutical preparation on its own or, if necessary, with the addition of a suitable preservative has to be ascertained during the development of the product. The primary purpose of adding antimicrobial preservatives to dosage forms is to prevent adverse effects arising from contamination by microorganisms that may be introduced inadvertently during or subsequent to the manufacturing process. However, antimicrobial agents should not be used solely to reduce the viable microbial count as a substitute for good manufacturing procedures. There may be situations where a preservative system may have to be used to minimize proliferation of microorganisms in preparations that are not required to be sterile. It should be recognized that the presence of dead microorganisms or their metabolic by-products may cause adverse reactions in sensitized persons.

Any antimicrobial agent may show the protective properties of a preservative. However, for the protection of the consumer the concentration of the preservative shown to be effective in the final packaged product should be considerably below the concentrations of the preservative that may be toxic to human beings.

Antimicrobial efficacy test must be demonstrated for multiple dose parenteral, otic, nasal, ophthalmic, oral and topical products made with aqueous bases or vehicles, the effectiveness of any added preservatives, during the shelf-lives of the preparations to ensure that the antimicrobial activity has not been impaired by storage. The tests apply only to the product in the original, unopened container in which it was supplied by the manufacturer.

The test consists of challenging the preparation in its final container with a prescribed inoculum of suitable microorganisms, storing the inoculated product at a prescribed temperature, withdrawing samples from the container at specified intervals of time and counting the organisms in the samples removed. The preservative properties of the product are considered adequate if, in the conditions of the test, there is a significant fall or no increase in the number of microorganisms in the inoculated preparation after storage for the times and at the temperatures prescribed.

The organisms specified for use in the tests are intended to be representative of those that might be expected to be found in the environment in which the preparation is manufactured, stored and used. However, they should be supplemented by other strains or species, especially those likely to be found in the conditions under a particular product is made or used, or that might offer a particular challenge to the type of product being tested. Single strain challenges (rather than mixed cultures) should be used throughout.

Precautions. Challenge tests should be conducted under conditions that prevent accidental contamination of the product during the test but the precautions taken to prevent contamination should not affect the survival of organisms in the product being examined.

Test organisms. The following test organisms are used in the test.

Candida albicans ATCC 10231*

Aspergillus brasiliensis ATCC 16404

Escherichia coli ATCC 8739

Pseudomonas aeruginosa ATCC 9027

Staphylococcus aureus ATCC 6538

*Instead of *Candida albicans, Zygosaccharomyces rouxii* NCYC 381; IP 2021.92 may be used for oral preparations containing high concentration of sugar.

In order to prevent any phenotypic changes in the strains used, the organisms used in the test should not be more than 5 passages made from the original culture. One passage is defined as inoculation and growth of the organisms from existing culture to a fresh medium.

NOTE— All the media used in the tests should be tested for growth promotion as mentioned in Microbial Contamination in Nonsterile Products (2.2.9).

Preparation of inoculum. Grow each of the bacterial species separately in Casein soyabean digest agar and incubate them at 30° to 35° for 18 to 24 hours. Grow *Candida albicans* on Sabouraud dextrose agar and incubate at 20° to 25° for at least 48 hours. Grow *Aspergillus brasiliensis* on Sabouraud dextrose agar at 20° to 25° for 5-7 days. After incubation, harvest the growth and resuspend each of the organisms separately in sterile saline to obtain a microbial count of 1×10^8 CFU per ml. To suspend spores of *Aspergillus brasiliensis*, 0.05 per cent polysorbate 80 may be added to the saline. ~~Use suspension of these organisms within 2-4 hours. The suspension may be stored at 4° to 8° for a validated period of time.~~ Use bacterial and yeast suspension with in 2 hours, or within 24 hours if stored between 2° to 8°. A stable spore suspension stored in suitable preserving medium for validated period of time.

Remove immediately a suitable sample from each suspension and determine the number of CFU per ml in each suspension by pour plate method or filtration method. This value serves to determine the inoculum concentration and the baseline to use in the test.

Appropriateness of enumeration methods in presence of product

Prepare a 10^{-1} dilution by adding 1 ml of product (by volume) to 9 ml of sterile 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 per cent of this saline control count (averaged).

If the diluted product contains antimicrobial properties, then use the speciific inactivating agent may need to be incorporated into the diluents or the recovery media to neutralize the antimicrobial activity.

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 3log 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 3log unit reduction can be measured. Observed 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.

Procedure. If sufficient volume (at least 20 ml) of product is available in each container and the product container can be inoculated aseptically then the test can be conducted in five original containers of the product. If filled volume is less, or the container cannot be inoculated aseptically then transfer (at least 20 ml) the product in each of five suitable sterile containers. Inoculate each container with one of the prepared and standardized inoculum in such a way that after inoculation the final concentration of the organisms remains between 1×10^5 and 1×10^6 CFU per ml and the volume of the inoculum does not exceed 1 per cent of the volume of the product. The initial concentration of the viable organisms in each test preparation is estimated based on the concentration of the microorganisms in each of the standardized inoculum as determined by the pour plate method or membrane filtration method.

Incubate the inoculated containers at room temperature and protected from light. Remove a suitable sample from each container typically 1 ml and determine the viable count in duplicate by plate-count or membrane filtration method at 0 hour and at 7, 14, and 28 days subsequent to the inoculation. Record any changes observed in the appearance at these intervals. From the calculated concentration of CFU per ml present at the start of the test, calculate the percentage of reduction or log reduction in CFU per ml for each organism at the stated test intervals and express the changes in terms of percentage or log reduction of initial concentration. The log reduction is defined as the difference between the $\log_{10}$ unit value of the initial concentration of CFU per ml at the start of the test and the $\log_{10}$ unit value of CFU per ml of the survivors at that point.

Interpretation. The preservatives are considered to be effective if:

i) For parenteral, ophthalmic, sterile nasal and otic preparations: (a) the concentration of the viable bacteria are not more than 10 per cent or more than 1.0 log reduction of the initial concentration at 7 days and not more than 0.1 per cent or more than 3.0 log reduction of the initial concentration at 14 days and there is ~~a further decrease in count at 28 day~~ no increase from the 14 days count at 28 days. (b) there is no increase in yeast and mold count at 7, 14 and 28 days from the initial count.

ii) For topical preparations made with aqueous base, non-sterile nasal preparation and emulsions including those applied to mucous membrane: (a) the concentration of the viable bacteria are not more than 1 per cent or more than 2.0 log reduction of the initial

concentration at 14 days and ~~there is a further decrease in count at 28 day~~ no increase from the 14 days count at 28 days. (b) there is no increase in yeast and mold count at 14 and 28 days from the initial count.

iii) For oral preparations other than antacids made with aqueous base or vehicles: (a) the concentration of the viable bacteria are not more than 10 per cent or more than 1.0 log reduction of the initial concentration at 14 days and ~~there is a further decrease in count at 28 day.~~ no increase from the 14 days count at 28 days. (b) there is no increase in yeast and mold count at 14 and 28 days from the initial count.

iv) For oral preparations antacids made with aqueous base: there is no increase in bacteria, yeast and mold count at 14 and 28 days from the initial count.