AAPS PharmSciTech, Vol. 12, No. 1, March 2011 (© 2011)
DOI: 10.1208/s12249-010-9575-9

Review Article

Theme: Sterile Products: Advances and Challenges in Formulation, Manufacturing, Devices and Regulatory Aspects
Guest Editors: Lavinia Lewis, Jim Agalloco, Bill Lambert, Russell Madsen, and Mark Staples

Comparison of Compendial Antimicrobial Effectiveness Tests: A Review

Cheryl L. Moser[1] and Brian K. Meyer[1,2]

Received 9 August 2010; accepted 16 December 2010; published online 8 January 2011

Abstract. The antimicrobial effectiveness or preservative effectiveness test is described in the tripartite compendia for sterile parenteral multi-dose formulated products. The execution of the test is essentially harmonized with respect to inoculum preparation and test execution but not the acceptance criteria. This article describes how a single test can be performed that procedurally satisfies all of the compendia and their acceptance criteria.

KEY WORDS: antimicrobial effectiveness; pharmacopeia; preservative effectiveness.

INTRODUCTION

Antimicrobial preservatives are added to sterile multi-dose parenteral products for the purpose of inhibiting and/or killing the growth of microorganisms that may have been inadvertently introduced during multiple withdrawals of the product from its container. Common antimicrobial preservatives that are utilized in parenteral products include phenol, m-cresol, benzyl alcohol, cholorobutanol, phenoxyethanol, methyl, and proplyparaben, and in some cases, thimerosal (1–3). The ability of these antimicrobial preservatives to inhibit or kill microorganisms in parenteral formulations is evaluated using antimicrobial effectiveness tests (AETs).

The antimicrobial effectiveness test, also known as the preservative effectiveness test, is a compendial test performed during formulation development and stability testing of a parenteral drug product intended as a multi-dose product. The test procedures and acceptance criteria are described in the three major compendia. The procedures are the *United States Pharmacopeia* (USP) <51> Antimicrobial Effectiveness Testing, the *European Pharmacopeia* (Ph. Eur. or EP) 5.1.3 Efficacy of Antimicrobial Preservation, and the *Japanese Pharmacopeia* (JP) 19, Preservative Effectiveness Tests (4–6). These compendial chapters are essentially harmonized with respect to how the test is performed; however, there are minor differences with respect to selection of challenge organisms, test intervals, and the acceptance criteria. In addition to the compendia, there are other references describing how to evaluate the efficacy of antimicrobial preservatives (7). The article presented here will review each of the preservative effectiveness tests in the three Pharmacopeia and demonstrate how they can be applied to sterile multi-dose parenteral drug products.

THE DRUG PRODUCT

During the development of a multi-dose parenteral product, formulation scientists and microbiologists must make a decision as to which preservative and what concentration will be utilized in the drug formulation. Interactions of the preservative with the drug product must be considered as well as with the container and closure (2,3). The preservative must also remain effective, not just "present" or measureable, in the formulated product throughout its shelf life at the labeled storage conditions. Historical data from other marketed products can be useful in choosing the appropriate preservative and concentration for the product (2,3). It is also possible that the drug substance and pharmaceutical ingredients or excipients may possess some intrinsic antimicrobial activity that will add to or enhance the antimicrobial effectiveness of the formulated drug product, and help to minimize the amount of preservative that needs to be added. Furthermore, the physicochemical attributes of the product such as extremes in pH or osmotonicity may have antimicrobial properties.

A major consideration for selecting an antimicrobial preservative for a parenteral formulation is the "use period" or storage conditions and time after the initial product withdrawal. Some multi-use parenteral formulations, due to chemical or microbial stability, must be used within a 24-h period whereas others may remain stored for up to 1 week at 2–8 C following the initial use (1). The Ph. Eur. requires testing of antimicrobial activity at 6 and 24 h after the microbial challenge. This activity ensures that any microorganisms inadvertently added to the product are killed prior to repeat administration. However, the USP tests are designed to evaluate antimicrobial activity after 7 days.

[1] Merck & Co., 770 Sumneytown Pike, PO Box 4, West Point, Pennsylvania 19486, USA.
[2] To whom correspondence should be addressed. (e-mail: brian_meyer@merck.com)

Table I. Strains from Major Culture Collection Recommended for the Test (4–6)

Organism	ATCC (USP/EP/JP)	NCIMB (EP)	CIP (EP)	NCTC (EP)	NCPF (EP)	IP (EP)	IMI (EP)	NBRC (JP)
S. aureus	6538	9518	4.83	10788	–	–	–	13276
P. aeruginosa	9027	8626	82.118	–	–	–	–	13275
E. coli	8739	8545	53.126					3972
C. albicans	10231	–	–	–	3179	48.72	–	1594
A. brasiliensis	16404	–	–	–	–	1431.83	149007	9455

ATCC American Type Culture Collection (USA)
NCIMB National Collection of Industrial, Marine and Food Bacteria (Scotland)
CIP Collection de l'Institut Pasteur (France)
NCTC National Collection of Type Cultures (UK)
NCPF National Collection of Pathogenic Fungi
IP Institute Pasteur (France)
IMI CABI Genetic Resource Collection (UK)

Once a preservative has been chosen and the final formulation of the drug product has been established, the preservative levels in the drug product are chemically assayed at stability time intervals to assure that the preservative remains at effective concentrations in the drug product over the shelf life. It is also a regulatory requirement to measure the efficacy of the preservatives using the preservative effectiveness tests on the drug product in its final container through expiry. To establish the lower effective shelf life specifications, the product is formulated at 100%, 75%, and 50% of the labeled preservative concentration and its effectiveness at these concentrations confirmed using the AET (4–6). Based on these findings, future marketed product stability testing may be conducted using the chemical assay and not the microbiological challenge test.

The compendial chapters divide the types of products to be tested into categories such as sterile multi-dose preparations, topical products, non-sterile oral products, etc. This review article focuses on the sterile multi-dose parenteral drug products that are category 1 products in USP <51>, 5.1.3.1 products in Ph. Eur., and 1 in JP (4–6).

Summary of Test

The AET is performed by spiking a panel of challenge microorganisms (representing Gram-positive cocci, Gram-negative bacilli, yeast, and mold) individually into the product and determining the log reduction of organisms at prescribed time intervals to quantitatively evaluate the effectiveness of the antimicrobial preservative to prevent microbial proliferation and/or kill the organisms (4–6).

Preparation of Challenge Microorganisms

The inocula of challenge microorganisms are typically prepared from fresh, recently grown stock cultures. Each compendia recommends the use of fresh cultures as this assures that log phase cells are used to challenge the product. There are subtle differences between the compendia with respect to how these fresh cultures are to be prepared, but none of the differences are scientifically significant with respect to growing healthy challenge organisms.

The USP and JP list the use of five challenge organisms for the AET of sterile multi-dose parenterals (4,6). These organisms are *Staphylococcus aureus* (Gram-positive coccus), *Pseudomonas aeruginosa* (Gram-negative bacillus), *Escherichia coli* (Gram-negative bacillus), *Candida albicans* (yeast), and *Aspergillus brasiliensis* (mold). The Ph. Eur. specifies the same organisms, but does not make E. coli mandatory for sterile multi-dose parenterals and does recommend it as appropriate for oral liquids (5). Each compendia also lists the source strains for each organism from major strain culture

Table II. Recommended Challenge Organism Inocula Preparation (4–6)

Cultures	Growth Media	Incubation temperature	Duration until harvest	Harvesting fluid
Bacteria *S. aureus* *P. aeruginosa* *E. coli*	Soybean-Casein Digest	30–35 C	USP: 18–24 h EP: 18–24 h JP: 18–24 h	0.9% Saline
Yeast *C. albicans*	Sabouraud Dextrose (JP: also states glucose-peptone agar and potato dextrose agar can be used)	20–25 C	USP: 44–52 h EP: 48 h JP: 40–48 h	0.9% Saline
Mold *A. brasiliensis*	Sabouraud Dextrose (JP: also states glucose-peptone agar and potato dextrose agar can be used)	20–25 C	USP: 6–10 days or until good sporulation EP: 1 week or until good sporulation JP: 1 week or until good sporulation	0.9% Saline with 0.05% polysorbate 80 to disperse spores

Table III. Recommended Inoculum Preparation (4–6)

Cultures	Harvest fluid	Standardization level	Duration of use	Storage
Bacteria	USP: Saline	USP: 1×10^8 CFU/ml	USP: within 2 h or	USP: 24 h
S. aureus	EP: Saline	EP: 10^8 CFU/ml	refrigerate	EP: no mention
P. aeruginosa	JP: Saline or 0.1% Peptone water	JP: 10^8 CFU/ml		JP: 24 h
E. coli				
Yeast	USP: Saline	USP: 1×10^8 CFU/ml	EP: use immediately	USP: 24 h
C. albicans	EP: Saline	EP: 10^8 CFU/ml		EP: no mention
	JP: Saline or 0.1% Peptone water	JP: 10^8 CFU/ml	JP: within 2 h or refrigerate	JP: 24 h
Mold	USP: Saline with 0.05% PS80	USP: 1×10^8 CFU/ml		USP: 7 days
A. brasiliensis	EP: Saline with 0.05% PS80	EP: 10^8 CFU/ml		EP: no mention
	JP: Saline with 0.05% PS80 or 0.1% Peptone water	JP: 10^8 CFU/ml		JP: no mention
A. brasiliensis				

collections (Table I; 4–6). These strains are considered (8). However, if the American Type Culture Collection (ATCC) source is used, compliance with all three compendia is assured.

Multiple subculturing and the number of passages of the stock cultures become an important parameter to control for this test, as continuously propagating cells could lead to changes in phenotypic expression, especially antimicrobial susceptibility. For that reason, both the USP and JP recommend using recently grown cells that are no more than five passages from the stock cells (4,6). The Ph. Eur. does not specifically state the number of passages, but does state to keep the cell passages to a minimum (5). A passage is understood as the transfer of organisms from an established culture to fresh medium. The USP is the only compendia to mention the use of frozen and stored "stock" cultures prepared from source strains such as ATCC (4). The use of stock frozen cultures or purchased standardized inocula is often the practice for laboratories especially during formulation development. Both the USP and JP also mention using both broth cultures and solid media cultures to prepare cells while the EP does not specifically mention broth cultures (4–6). Solid media-derived cells are easier to harvest and standardize as one has to harvest by centrifugation and wash the broth-derived cells to remove the growth media.

Parameters for microbial growth conditions, times, and temperatures, as well as the recommended media are defined in each compendium. The purpose of these parameters is to grow healthy viable cells for challenging the preservative in the product. There are minor differences between compendia, none of which are significant. A single preparation strategy can be employed that satisfies all requirements (see Table II). For all compendia, bacteria are grown at 30–35°C for 18–24 h on Soybean-Casein Digest medium. This period of time assures the cells to be viable and growing in the log phase, minimizing the amount of dead cells harvested and standardizing the response to antimicrobial agents. Yeast is grown at 20–25°C which is a suboptimal temperature and requires a longer period of time. The USP states 44–52 h, the Ph. Eur. states 48 h, and the JP states 40–48 h (4–6). Anytime around 48 h is appropriate but should not exceed 52 h (4–6). Molds are grown "until good sporulation is obtained" (4–6). It takes approximately 6–7 days to observe a copious lawn of black *A. brasiliensis* spores on solid media. Since mold spores are used for the microbial challenge, attempts are made to harvest as many spores as possible. The spore cells can be harvested anytime between 6 and 10 days depending upon visual observation of the culture (4–6). The culture medium for growing yeast and mold is usually Sabouraud Dextrose Agar although other media can be used as specified by the JP (4–6).

Table IV. USP Criteria for Tested Microorganisms (4)

For category 1 (sterile parenteral) products	
Bacteria	Not less than 1.0 log reduction from the initial calculated count at 7 days, not less than 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.

HARVESTING THE CELLS

Each culture is "harvested" after it has been grown for the appropriate amount of time. After harvesting, the cell suspensions are then "standardized" to provide an inoculum of approximately 10^8 colony forming units (CFU)/ml (4–6). The USP states to standardize to approximately 1×10^8 CFU/ml, while the EP and JP are less specific and state the standardization to be to approximately 10^8 CFU/ml (see Table III) (4–6). For this test, there is no significant difference. All three compendia state that the cultures should be grown on solid media and, except for *A. brasiliensis*, harvested using sterile saline (0.9% saline) (4–6). Harvesting cells from solid media is performed by adding some diluent (saline) to the media and scraping the cells from the surface with a sterile tool. The cells are then diluted to the approximate 10^8 CFU/ml level using the same diluent (i.e., sterile saline) (4–6). Usually spectrophotometric measurements are used to standardize to the use level with

Table V. Incubation Conditions (4–6)

Cultures	Temperature	Duration USP	Duration EP	Duration JP
Bacteria *S. aureus* *P. aeruginosa* *E. coli*	30–35°C	3–5 days	3–5 days (refers to the microbial limits test)	≤3 days (refers to the microbial limits test)
Yeast *C. albicans*	20–25°	3–5 days	5–7 days (refers to the microbial limits test)	≤5 days (refers to the microbial limits test)
Mold	20–25°	3–7 days	5–7 days (refers to the microbial limits test)	≤5 days (refers to the microbial limits test)

turbidity readings (percentage of transmittance or absorbance). When harvesting *A. brasiliensis* mold spores, the diluent contains 0.05% polysorbate 80 that is added to help to disperse the spore cells and prevent clumping (4–6). The JP also mentions that 0.1% peptone water can be used in place of sterile saline (6). The use of sterile saline *vs.* the 0.1% peptone water has no impact on the preservative effectiveness test.

Once the cells have been harvested and standardized, the cells should be used within the specified amount of time to assure a healthy viable cell challenge to the product. Two objectives are to be met during the test. The first objective is to enumerate the cells for a baseline CFU number against which the product preservative effectiveness is measured. The other objective is to spike the healthy cells into the product for the actual AET. The USP and JP state to use the cells within 2 h or refrigerate for up to 24 h (4,6). The EP states to use the cells immediately and mentions no storage conditions (5). The USP states that the harvested *A. brasiliensis* spores can be stored at 2–8°C for up to 7 days (4). The EP and JP are silent with respect to *A. brasiliensis* spore storage (5,6). Once the cells are harvested and standardized, all enumeration testing and product spiking should occur within an 8 h period of time and the cells should be stored at 2–8°C when not in use. *A. brasiliensis* spores have been successfully stored at refrigerated temperatures for 7 days without losing viability.

ENUMERATING THE CELLS

A zero time baseline enumeration result for the entire challenge cell cultures used for spiking product must be established. The organisms are diluted to the level where they are theoretically within the countable ranges. This is performed by making dilutions (usually tenfold) of each organism in diluting fluid (i.e., sterile saline) and using the pour plate technique to quantitatively establish the enumeration of the working stock culture in terms of colony forming units per milliliter. The organisms are diluted from the 10^8 CFU/ml stock to a countable number (25–250 CFU for bacteria and yeast, 8–80 CFU for mold; 4–6). A 1-ml aliquot of the diluted organism is added to a standard 100×15-mm Petri plate. Media tempered to approximately 45°C is then added to the plate (see Table IV). Laboratories may perform variations of this enumeration method, for example, spread plates or membrane filtration methods, but the result would be the same. The temperature of incubation and duration of microbial growth on the Petri plates for each organism is defined in each compendia and differs only slightly in wording (see Table V; 4–6). Organisms must be grown sufficiently to be counted visually as colonies. ATP bioluminescence, flow cytometry, or other rapid methods have been used that will detect colonies before they can be counted by the naked eye. In general, growth for longer periods of time is of no detriment as colonies will not "disappear." However, mold may need to be "precounted" 1–2 days early as these colonies tend to sporulate and spread thus making colony differentiation difficult if the plate is overgrown. Each colony forming unit is theoretically derived from one cell.

Even though small differences exist between the different compendia in the wording for the duration of plate incubation, good scientific practice is to incubate the plates long enough for all cells to have propagated into colonies that can be counted by the naked eye. As mentioned before, rapid microbiological methods have been developed and may be used to detect cells before they can be seen with the naked eye.

PERFORMING THE ANTIMICROBIAL EFFECTIVENESS TEST

All compendia prefer that the test be executed on the product in its marketed container. The rationale is that the final container test best represents "real world" contamina-

Table VI. EP Criteria for Tested Microorganisms (5)

		Log reduction				
		6 h	24 h	7 day	14 day	28 day
Bacteria	A	2	3	–	–	NR
	B	–	1	3	–	NI
Fungi	A	–	–	2	–	NI
	B	–	–	–	1	NI

NR no recovery, *NI* no increase, *A* recommended, *B* mandatory

Table VII. JP Criteria for Tested Microorganisms (6)

For category 1A (sterile parenteral) products	
Bacteria	14 days: reduction of 0.1% of inoculum count or less 28 days: same or less than level after 14 days
Yeast and Molds	14 days: same or less than level after 14 days 28 days: same or less than level after 14 days

tion. Often this is neither possible nor practical. Enough material must be present in the final container to allow one to remove aliquots at the time intervals for enumeration. For this reason, all of the compendia do allow for product to be removed from its final container, pooled, and placed into a suitable vessel for testing (4–6). The amount of inoculum or standardized challenge organism added to challenge the preserved product should not be a volume that dilutes out the product and its preservatives changing their concentrations. The volume of inoculum should be negligible in comparison to the total volume of the test product. All compendia state that this volume should not exceed 1% of the total volume of the product to be tested and should result in a 10^5 to 10^6 CFU/ml product challenge (4–6). All compendia require that the challenged contaminated product be stored at 20–25°C (ambient room temperature) for the duration of the test period irrespective of the storage conditions of the product. Aliquots of contaminated product are to be removed at specified time intervals for enumeration (4–6). The Ph. Eur. and JP also state that the challenged product containers be protected from light during this time, but the USP is silent on this parameter (4–6). The USP and JP also mention recording obvious signs of microbial contamination and proliferation such as changes in color, odor, and appearance while the EP is silent on this parameter (4–6). In practice, most microbiologists store the test material in a 20–25°C incubator for the duration of the test.

TEST INTERVALS AND ACCEPTANCE CRITERIA

The acceptance criteria for the product type determine the time intervals at which the samples are enumerated for log reduction over the initial time zero inoculum levels. The three compendia are not harmonized with respect to acceptance criteria in terms of challenge reduction and the significance level that is expressed (4–6). However, one test can be executed that satisfies all time intervals and compendial acceptance criteria. The major difference for sterile multi-dose formulations is that the Ph. Eur. has 6- and 24-h time interval criteria while the USP and JP do not have criteria until days 7, 14, and 28 (4–6). Given these different criteria, enumerations performed at 6 h, 24 h, 7 days, 14 days, and 28 days after the initial microbial challenge satisfy all compendial requirements (4–6). These plate counts are converted to $\log_{10}$ and compared to the time zero enumeration performed on the saline control inoculum levels (4–6).

The Ph. Eur. (see Table VI) has the most strict acceptance criteria in that it requires a log reduction at 6 and 24 h for "Criteria A" preservative effectiveness (5). These criteria are difficult to achieve with many preservative systems. Often, the level of preservative added to achieve these results has detrimental effects on the product and/or is at toxic levels. The Ph. Eur. also has "Criteria B" that is considered mandatory by EU regulatory agencies and is more achievable (5). By 24 h, the preservative is expected to achieve at least a 1 log reduction and prevent proliferation throughout the 28 day period (5). The USP (see Table IV) does not have criteria for acceptance until day 7, and the JP (see Table VII) begins criteria at day 014 (4,6). The USP also defines "no increase" in proliferation as not more than 0.5 log higher than the previous measured level that corresponds to the expected plate count variability (4). Quality sterile multi-dose preparations should have a preservative that can rapidly prevent cell proliferation and destroy any microorganisms inadvertently introduced by multiple withdrawals of product throughout the product's use period. The USP chapter is an official test method and although AET is not a product release specification, the FDA requires products to meet the log reduction requirements in the chapter.

CONCLUSION

The antimicrobial effectiveness or preservative effectiveness test is described in USP <51>, EP 5.1.3 and JP 19 for sterile parenteral multi-dose formulated products (4–6). The execution of the test is essentially harmonized with respect to inoculum preparation and test execution. Harmonization has not been achieved with respect to acceptance criteria. One test can be performed that satisfies all of the compendia and their acceptance criteria.

ACKNOWLEDGMENTS

We thank Anthony Cundell for a critically reviewing this manuscript.

REFERENCES

1. Physicians' Desk Reference. 64th ed. Montvale, NJ: Medical Economics; 2010.
2. Meyer BK, Ni X, Hu B, Shi L. Antimicrobial preservative use in parenteral products: past and present. J Pharm Sci. 2007; 96:3155–67.
3. Meyer BK, Shi L. Antimicrobial preservative use in parenteral products: an overview. Eur J Parent Pharm Sci. 2009;14:115–7.
4. United States Pharmacopeia. USP <51>. Antimicrobial effectiveness testing. Rockville, MD.
5. European Pharmacopeia. EP <5.1.3>Efficacy of antimicrobial preservatives.
6. Japanese Pharmacopeia. JP <19>Preservative effectiveness tests.
7. Akers MJ, Boand AV, Binkley DA. Preformulation method for parenteral preservative efficacy evaluation. J Pharm Sci. 1984;73 (7):903–5.
8. Cundell, AM, Chatellier, S, Schumann, P, Lilischkis, R. Equivalence of Quality Control Strains of Microorganisms Used in the Compendial Microbiological Tests: Are National Culture Collection Strains Identical? PDA J Pharm Sci Tech. 2009.