

## Development of the Antimicrobial Effectiveness Test as USP Chapter <51>

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**ABSTRACT:** The antimicrobial effectiveness test first appeared as a USP General Chapter in the 18<sup>th</sup> revision, official September 1, 1970. This chapter, at the beginning, was designed to evaluate the performance of antimicrobials added to inhibit the growth of microorganisms that might be introduced during or subsequent to the manufacturing process. As Good Manufacturing Practices (GMPs) became a governing principal in pharmaceutical manufacturing, the purpose of the test was refined to focus on activity of the preservative system as a protection against inadvertent contamination during storage and usage of the product. This article will review the history of the antimicrobial test; its function, technique, and the background discussions that resulted in the changes from the test that appeared in USP XVIII to that of the current USP 25.

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### Introduction

The antimicrobial effectiveness test (AET) is designed to provide a laboratory test that gauges the level of biological activity possessed by the preservative system of a pharmaceutical product. It is not meant to be a simulation of a real-world situation, nor is it meant as a guarantor that a preservative system that meets its requirements will never allow a contaminant to grow in the product. It was originally designed, and remains to this day, an assay that a careful laboratory can reproducibly perform and one that will yield comparable results among a variety of laboratories. The value of those results in estimating the performance of the preserved product in the field is a subject of significant debate. Before looking at this controversy, however, let's look to the genesis of today's AET.

### USP XVIII - The Original Test

The first appearance of this chapter was in the 18<sup>th</sup> edition of the USP in 1970 (1), and is closely related to the one suggested in 1967 to USP by the Biological Section of the Pharmaceutical Manufacturer's Association (2). It is of interest to note that there were other potential preservative tests being used at this time.

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The stated purpose of the chapter "Antimicrobial Agents-Effectiveness" was "to demonstrate, in parenteral and ophthalmic products, the level of any added antimicrobial agent(s), the presence of which is declared on the label of the product concerned." The introduction to the assay also cautions that the tests apply only to products in the original container and that if a specific inactivator of the preservative is available, a suitable amount should be added to the agar plating medium.

### Challenge Organisms

The test organisms specified were to be tested separately. This method differed from the method supported by Squibb and Abbott Laboratories which used a test with a mixed population of 21 different organisms and assayed for survivors over a 10 week period (3). The USP method used the five species individually which was subsequently shown to be a better indicator of preservative effectiveness (4) than challenging with a mixed culture. Although the species are familiar to today's practitioners, they are not the same strain in all cases:

*Candida albicans* ATCC 10231  
*Aspergillus niger* ATCC 16404  
*Escherichia coli* ATCC 4352  
*Pseudomonas aeruginosa* ATCC 9027  
*Staphylococcus aureus* ATCC 6538

These microorganisms were based on the recommendations of a Committee of the Biological Section of the Pharmaceutical Manufacturer's Association, which

**Table 1. Preparation of inocula per USP XVIII.**

Microorganism	Incubation Temperature	Incubation Time	Wash Solution
Bacterial Cultures	37°C	18 – 24 hours	Sterile Saline TS
<i>C. albicans</i>	25°C	48 hours	Sterile Saline TS
<i>A. niger</i>	25°C	1 week	Sterile Saline TS containing 0.05% polysorbate 80

prepared a draft proposal in 1967. Interestingly, the original list of candidates was much longer and consisted of several groups:

- Group 1 – Vegetative bacteria or yeast from standard sources
  - Candida albicans* ATCC 10231
  - Staphylococcus aureus* ATCC 6538
  - Escherichia coli* ATCC 4352
  - Pseudomonas aeruginosa* ATCC 14502
- Group 2 – Special organisms isolated from products or the manufacturing environment
- Group 3 – Bacterial or mold spore-formers
  - Bacillus subtilis* ATCC 6633
  - Aspergillus niger* ATCC 16404

This committee concluded that the types of test organisms should be those that were found to contaminate the product—either through use or introduced with the raw materials. This seems strange to us today, as the AET is now well established as a referee test and so must be suitable for use with no prior knowledge of the product. At the time the test was first introduced however, there were no monographs that made explicit references to the chapter. A requirement for the testing contained in the chapter could be inferred from text in the “Added Substances of General Notices” requiring that an added substance such as a preservative not exceed the amount necessary to provide its intended effect. It was not a mandatory test. In fact, it was not until publication of the First Supplement to USP XXII (official Jan 1, 1990) (5) that a monograph for a preserved product specifically stated that it must meet the requirements of “<51> Antimicrobial Preservatives–Effectiveness” (reviewed in 6).

#### *Media*

The user was instructed to use a suitable agar media for initial cultivation of the microorganisms. The only specific media mentioned was Soybean-Casein Digest media which had been shown to be effective in micro-

bial recovery (7). Interestingly, the media composition was referenced to the Microbial Limits Tests chapter, a practice that continues to this day.

#### *Preparation of Inoculum*

The practitioner was instructed to grow the inoculum on the surface of a suitable agar plate from a recently grown stock culture. The cells were harvested using the solutions shown below and suspended to result in a microbial count of “about 100 million microorganisms per mL.” Conditions are described in Table 1.

The contemporary practitioner will note with interest that the original instructions were to determine the number of CFU/mL in each solution, and then use this to determine the size of the inoculum to use in the test (Table 1). Further, if the standardized solutions were not used promptly, the suspensions were to be stored under refrigeration (defined as not above –45°F).

#### *Procedure*

This original procedure stated that the product was to be transferred to five tubes of 20 mL each, and then inoculated with 0.1 mL of the appropriate microbial stock (inoculum at a concentration of approximately 50 million CFU per mL) to yield a final suspension of between 125,000 and 500,000 organisms per mL. These tubes were to be held at 30° – 32°C during the test. The inoculated product was to be examined “at suitable times, making not less than two observations, 7 days apart, at any time not later than 28 days subsequent to adding the inoculum” The investigator was to record any changes observed in the appearance of the sample, and make a plate count of the number of viable microorganisms present. These counts were then converted to a percentage change from the inoculum.

#### *Interpretation*

The preservative system was defined as effective if there was “no significant increase in the number of *Candida*

*albicans* or *Aspergillus niger* organisms, and if the number of viable vegetative microorganisms is reduced to not more than 0.1 percent of the initial number and remains below that level for a 7-day period within the 28-day period.” These criteria are so confusing as to be almost unusable, and the next version includes many revisions to the text to make both the procedure and the criteria more comprehensible.

It is interesting to read some of the early commentaries on this test (2, 4, 8). Practitioners were already concerned with questions of how to make the test more reliable, less variable, the physiological state of the challenge organisms, and the test’s predictive power. These concerns are continually being addressed as the revision process proceeds.

### USP XIX - Clarification

The response to the original chapter indicated a need for much more clarity in the procedure. This redefinition began with the title, which changed from “Antimicrobial Agents – Effectiveness” to “Antimicrobial Preservatives – Effectiveness” to prevent confusion about the chapter’s impact on antibiotic test methods. The introduction to the chapter also includes much more detail, describing antimicrobials as “substances added to dosage forms to protect them from microbial contamination...used primarily in multi-dose containers to inhibit the growth of microorganisms that may be introduced inadvertently during or subsequent to the manufacturing process” (9). The USP goes on to caution that “antimicrobial agents should not be used solely to reduce the viable microbial count as a substitute for good manufacturing practice.” The chapter further notes “. . . all useful antimicrobial agents are toxic substances. For maximum protection of the consumer, the concentration of the preservative shown to be effective in the final packaged product should be considerably below the concentration of the preservative that may be toxic to human beings.”

This is far more information and guidance than what had originally appeared in this chapter and sets the stage for a fundamental conflict in the structure of this chapter. According to the *USP General Notices* in USP 25 (para10, p4) there are three different categories of General Chapters:

“Each general chapter is assigned a number that appears in brackets adjacent to the chapter name

(e.g., <621> Chromatography). General chapters that include general *requirements* for tests and assays are numbered from <1> to <999>, chapters that are *informational* are numbered from <1000> to <1999>, and chapters relating to *nutritional supplements* are numbered from <2000> to <2999>.”

The type of information introduced into this chapter by the 1975 revision underscores the status of the test as a control test to be performed by the manufacturer. As mentioned above, it would not be until 1990 that a preserved product would be required to meet the criteria of this test. However, this text, or text very much like it, persisted in subsequent revisions to the present day.

### Test Organisms

The test organisms specified in 1975 did not change from the original test, with the exception of *E. coli* ATCC 4352, which upon examination turned out to be *Klebsiella pneumoniae*. The reference strain of *E. coli* for the AET became ATCC 8739. A new allowance was added to provide for the inclusion of other organisms that may be introduced during the use of the product. However, no information was provided on how the testing laboratory was to choose these challenge organisms.

### Media

Instruction was provided on the media used for recovery of organisms from the test in the section “Preparation of Inoculum.” This recovery was to be performed on the same media used to grow the inoculum, and if a neutralizer for the antimicrobial was known, then this neutralizer was to be included in the solid agar media.

### Preparation of Inoculum

Several significant changes occurred in this section. The incubation temperatures were changed from a specific temperature to a 5° range, and the concentration of CFU/mL in the inocula was significantly increased (see Table 2).

These more detailed instructions stated that if the standardized solutions were not used promptly, the suspensions were to be monitored by the plate-count method and could be used until a drop-off in viability was observed (presumably several days after the test

**Table 2. Preparation of inocula per USP XVIII vs. USP XIX.**

Microorganism	Incubation Temperature		Inoculum CFU/mL	
	1970	1975	1970	1975
Bacterial Cultures	37°C	30° – 35°C	About 50 million	About 100 million
<i>C. albicans</i>	25°C	20° – 25°C		
<i>A. niger</i>	25°C	20° – 25°C		

**Table 3. Summary of USP criteria through revisions.\***

	Inoculum (CFU)	Criteria				Comments
		7 Day	14 Day	21 Day	28 Day	
USP XVIII (1970)	125,000-500,000	Take "...not less than two observations, not less than 7 days apart at any time not later than 28 days subsequent to adding the inoculum. . . . An agent is adequate . . . if the number of viable vegetative microorganisms is reduced to not more than 0.1 percent of the initial number and remains below that level for a 7-day period within the 28-day test period."				This original test was fundamentally sound, but the criteria were very difficult to interpret.
USP XIX (1975)	100,000 – 1,000,000	--	0.1% Survival	NI	NI	These criteria were introduced for clarity. Although testing was required at Day 7 there was no criterion at that time point.
USP 24 (2000) Category 1A Category 1B Category 1C Category 2	1 x 10 <sup>5</sup> - 1 x 10 <sup>6</sup>	1.0** -- -- NI	3.0 2.0 1.0 NI	-- -- -- NI	NI NI NI NI	The motive for all changes in criteria was the international harmonization effort. (see text) Anhydrous medications included as "Category 2"
USP 25 (2002) Category 1-3	Criteria same as categories 1A, 1B, and 1C, respectively					Anhydrous medications deleted to improve harmonization with <i>Ph. Eur.</i> Antacids were removed as a class from Category 1C and given a unique category based on market and regulatory input.
Category 4	1 x 10 <sup>3</sup> - 1 x 10 <sup>4</sup>	NI	NI	NI	NI	

\* The USP test has required stasis for *Aspergillus niger* and *Candida albicans* since its inception. The criteria listed in this table are only for the bacterial challenge organisms.

\*\* All subsequent criteria are in terms of log<sub>10</sub> unit reduction from the measured inoculum.

using those inocula). The provision for refrigeration of the stock cultures was deleted from this revision.

Instruction was provided on how to select the media used for recovery of organisms from the test. This recovery was to be performed on the same media used to grow the inoculum, and if a neutralizer for the antimicrobial was known, then this neutralizer was to be included in the solid agar media.

*Procedure*

This revision included a significant change in the procedure. Where the original procedure clearly stated that the test solution should be transferred to test tubes prior to inoculation, this version states a strong preference for conducting the test with the solution in the original container – even to the point of providing instruction

on how to enter the container aseptically with a needle to inoculate and to sample the product. The inoculum volume was to be equivalent to a ratio of 0.10 mL of inoculum (inoculum concentration of “about 100 million CFU per mL”) to 20 mL of sample, so that the final concentration of microorganisms in the test is between “100,000 and 1,000,000 microorganisms per mL” (see Table 3). The inoculated samples were then stored at the storage temperature specified on the label or at 20°–25°C if no storage temperature was specified. This point is worth exploring. The intent of stipulating the label storage temperature was to test the antimicrobial efficacy of the formulation under conditions similar to those of its intended storage conditions. This change in temperature (from USP XVIII to XIX) had the potential to dramatically affect the measured efficacy of the products as a decrease in temperature usually has the affect of reducing the potency of a preservative (11). The test

samples were examined at 7, 14, 21, and 28 days for surviving microorganisms. This section of the chapter most dramatically shows the push for additional clarity in the revision.

### Interpretation

This section was completely rewritten to improve the clarity, and account for the specific test intervals described in the procedure. The preservative system was defined as effective if “(a) the concentrations of viable bacteria are reduced to not more than 0.1% of the initial concentrations by the fourteenth day; (b) the concentrations of viable yeasts and molds remain at or below original levels during the first 14 days; and (c) the concentration of each test organism remains at or below these designated levels during the remainder of the test period.” These criteria, established in 1975, remain fundamentally unchanged to this day.

### USP XX, XXI & XXII – A Period of Calm

The 15 years from 1975 through 1990 saw little change in the chapter. USP XX (1980 - 12), USP XXI (1985-13) and USP XXII (1990-14) were published with text nearly identical to that which first appeared in 1975. One change that did occur was to reverse the decision on incubating the test samples at the label condition. The reference to storage temperatures specified on labels was simplified to “incubate the inoculated containers or tubes at 20° to 25°[C]” (initially proposed in 1982 (15) and finalized in USP XXI (13)). The only other change occurred in USP XXII where a provision was made for the inocula to be grown in liquid media rather than requiring growth on solid media. As an aside, 1980 was the first year that the

USP chapters carried numbers, and so the official title of the chapter changed from “Antimicrobial Preservatives – Effectiveness” to “<51> Antimicrobial Preservatives – Effectiveness” in USP XX.

There were several suggestions for change during these years in the published literature. Orth (16, 17, 18, 19) recommended the use of D-values to establish preservative efficacy, despite the fact that many chemical systems do not yield linear kill slopes (20, 21). The FDA was also developing an antimicrobial efficacy test for use with contact lens solutions (22). In addition, there were suggestions that the container closure system may have much to do with an adequately preserved product (23). Finally, the problem of testing anhydrous ointments was receiving some attention (24).

In summary, although there was little activity by USP on the topic of antimicrobial effectiveness, a good amount of thought was being directed at the topic. A good review of the contemporary thinking can be found in a 1989 review article by Cooper (25). The main points are questions of harmonization with the *British Pharmacopeia*, variability, validation of microbial recovery, testing of ointments, and the criteria for passage.

### USP 23, 24, & 25 - Attempts to Reduce Variability

Several proposals were made in the period of 1990 through the present with the goal of reducing the reputed level of inter-laboratory variability in the test (summarized in Table 4). The use of the Phenol Coefficient as a method to determine the suitability of the challenge organisms was proposed in 1992 (26). This test was intended to be used to qualify the stock cultures, provid-

**Table 4. Changes proposed to reduce variability.\***

Change	Rationale	Disposition
Phenol coefficient to validate stock cultures	Reduce variability in inoculum	Proposal Rejected
Biocide qualification of stock cultures	Reduce variability in inoculum	Proposal Rejected
Restrict number of passages to 5 from original ATCC	Reduce variability in inoculum	Official
Greater detail in media and incubation conditions for inoculum prep.	Reduce variability in inoculum	Official
Requirement that inoculum be prepared fresh	Reduce variability in inoculum	24 hours was defined as “fresh” to allow different shifts in the same facility to use the same inoculum for testing
Change in criteria from one significant figure to two significant figures	Reduce variability in interpretation of results	Official

\* See text for details

ing documentation that the resistance of the challenge organisms was not changing with time. Due to severe concerns over the adequacy and appropriateness of this method, the Subcommittee proposed several changes designed to qualify the stock cultures used in the assay (27, 28), the first of which was proposal for an Antimicrobial Resistance Suitability Test in 1995. This test was designed to address the shortcomings of the Phenol Coefficient. The challenge organisms would be qualified using several common preservative agents, rather than just the single agent phenol. This qualifying test was not well received either. On the basis of comments and recommendations made at the USP Microbiology Open Conference in 1996<sup>1</sup>, the Microbiology Subcommittee (MCB) resubmitted the previously proposed revision of this general test chapter with substantive changes. The new proposals included the deletion of the *Stock Culture Antimicrobial Resistance Suitability* section, the requirement for a 21-day sampling interval, and the requirement to use microorganisms that have been isolated from the environment. In addition, a new requirement was added to ensure that all stock cultures used were within five passages from the original ATCC stock. This requirement, a component of the Sterility Test since USP XXI (13), was included in an attempt to establish control over the organisms used in the test.

Another change in inoculum handling dealt with the age of the inoculum suspension. Recall that in the original test the inoculum suspensions were to be used promptly, or held under refrigeration until use (1). The next revision (9) stated that if the suspensions were not used promptly, then the viability should be monitored. This 1996 proposal recommended changing the holding times to not more than 24 hours for bacteria and yeast, and not more than 7 days for fungal spores (28). It was in this proposal, made at the end of 1996, that media for growth of the challenge organisms was specified and finally stated bacteria were to be grown on Soy Casein Digest Media while the fungi were to be grown under different conditions on Sabouraud Dextrose Media. In addition, the text was changed to clarify that the inoculum suspensions were to be standardized using a spectrophotometer, and the numbers confirmed by plate count. This method had been shown, at least for yeast, to provide a reproducible concentration of cells in the inoculum (29). It must be noted, however, that this proposal remains controversial (30).

Other changes in this revision did not deal expressly with reducing variability. These included renaming some of the product categories – Category “1D” for antacids ap-

peared as Category 1C for oral products. After lengthy debate over the peculiar requirements of liquid antacids, it was decided that, if special requirements were indeed necessary for this product class, these requirements were to be included in the specific antacid monograph. The MCB Subcommittee planned to develop an informational chapter on the Antimicrobial Effectiveness Test, which would deal with a number of issues raised at the January 1996 Open Conference.

This proposal generated a great deal of discussion in the pharmaceutical community, and was the subject of more discussion at the 1996 Interpharmacoepial Conference<sup>2</sup>. An *In-process Revision* was published (31) clarifying the requirement that multi-dose products must fulfill the criteria in the chapter (thus finalizing the status of <51> as a referee test).

The criteria for passage were modified as well. The criteria for passage had been expressed in percent survival (for example, not more than 0.1% survivors after 14 days), and then as log reduction (see discussion on harmonization below). There was confusion about the interpretation of this; however, as many practitioners looked to the *General Notices* discussion on significant figures and decided that a “3 log reduction” was satisfied by data demonstrating at least a 2.5 log reduction. This was not the intent of the subcommittee and so the criteria were amended to two significant figures (*i.e.*, “3.0 log reduction”) to eliminate this source of variability in data interpretation.

Final editorial changes were presented early in 1997 (31). This version was approved by the United States Pharmacopeial Convention and published in the *Eighth Supplement* to USP 23 – NF 18 (p. 1681) effective May 15, 1998 (32). At this point it seemed that the obvious steps had been taken on the part of the Pharmacopeia to clarify those factors that would reasonably be expected to contribute to variability in the test outcome.

### **USP 23, 24 & 25 - Trying to Harmonize Internationally**

The desire to harmonize at least the *European Pharmacopoeia* (*Ph. Eur.*) and the USP versions of this test was well established by the early 1990s (33, 34). However, after the pair of meetings in Sanibel Harbor and in Barcelona on the topic, there was some confusion in the field about the status of the harmonization efforts for both the AET and the Sterility Test. A review of the status of this effort was published in 1997 (35) as a *Stimuli to the Revision Process*. At that time, the

test had reached a point where most of the contentious issues had been analyzed, discussed, and considered. International face-to-face meetings of the pharmacopeial experts along with Open Conferences have resulted in advances in harmonization. However, the criteria for antimicrobial effectiveness were outstanding among the issues that were not harmonized.

Several new concerns were raised at the 1998 USP Open Conference on Microbiology<sup>3</sup>. Among these was the need to delete the requirements for antimicrobial effectiveness testing of products with a nonaqueous base or vehicle. The deletion of this requirement would improve harmonization with the *European and Japanese Pharmacopoeias*. Therefore, a proposed revision was published in 1999 (36) with this change. This became official with the publication of USP 25, in January of 2002 (10) (this volume is alternately referred to as USP 2002).

There have been several substantive changes in the official USP AET test from 1995 to the present that have been directly linked to the harmonization effort (sum-

marized in Table 5). There are three main areas where USP has attempted to improve harmonization with the *Ph. Eur.* – criteria, inoculum, and product categories. The first is a change in the manner that the criteria are expressed. Prior to the 8<sup>th</sup> Supplement, the criteria for bacteria were expressed as “per cent reduction.” The *Ph. Eur.* suggested that this did not accurately convey the level of precision available to the microbiology laboratory, and so these reductions should be expressed in terms of their log<sub>10</sub> values. The *Ph. Eur.* also insisted that 14 days was too long to wait for the first evidence of activity, urging 6 hour, 24 hour, and 7 day criteria (see 37 for rationale). USP added a 7 day time point but could not add criteria at 6 and 24 hour as no information existed as to the performance of currently marketed products at these time points. Finally, *Ph. Eur.* suggested that the term “No Increase” was too stringent, and that an increase of 0.5 log<sub>10</sub> units should be allowed to account for variability (38), a position supported by independent research (39). USP had previously suggested a factor of 150% to address this issue (26), but accepted the *Ph. Eur.* recommendation. *Ph. Eur.* later changed this

**Table 5. Changes in USP to promote harmonization with *Ph. Eur.*\***

<b>Change</b>	<b>Rationale</b>	<b>Disposition</b>
Change in criteria from % reduction to log reduction	More accurately expresses level of precision in results	USP Adopted <i>Ph. Eur.</i> suggestion
Requirement that inoculum be prepared fresh	Reduce variability in inoculum	USP settled on 24 hours to take shifts into account, <i>Ph. Eur.</i> at 8 hours
Addition of 7 day criterion	<i>Ph. Eur.</i> insistence on need for short time points	Concern over products on market prevented 6 hr and 24 hour time-points
Inoculum in <i>Ph. Eur.</i> is 1%, 0.5% in USP	Compromise	USP widened inoculum range to include the <i>Ph. Eur.</i> preference
Product Categories	Different routes of administration have different risks	USP adopted product categories
Non-sterile Otic and Nasal products should not be in Parenteral category	Sterility not required	USP changed categories to reflect non-sterile attributes of products
Non-aqueous category is unnecessary	Low water activity prevents growth of microorganisms, therefore no need to test	USP removed this product category from testing requirements at <i>Ph. Eur.</i> recommendation
Variability in counting should allow 0.5 log units as “no increase”	This recommendation by <i>Ph. Eur.</i> was later changed on part of <i>Ph. Eur.</i> to 0.3 log	USP rejected own suggestion of 150% and adopted original European suggestion of 0.5 log. Unlikely to change again to 0.3 log <sub>10</sub> unit definition of variability

\* See text for details

recommendation to 0.3 log<sub>10</sub> units without explanation (40), and this difference is now a point of disagreement between the pharmacopeia.

A second point bears some discussion. The *Ph. Eur.* AET contains a “zero” time point. The intent of this time point is to validate the test for its ability to recover organisms in the presence of the preserved product (41). However, in practice it is found that strongly preserved formulations immediately reduce the viable microorganisms recoverable from the suspension, and so this provision was viewed as ill-advised by the USP. The concern is a valid one, however, and has been addressed by USP with the introduction of a new guidance chapter titled “<1227> Validation of Microbial Recovery from Pharmaceutical Articles.”

Harmonization efforts continued with changes in the preparation of the inoculum. *Ph. Eur.* suggested that the inocula should be prepared fresh, defined as not more than eight hours old at time of use. USP agreed that the wording in effect since 1980 should be changed (in no small part to reduce variability of the test), but felt that 24 hours was acceptable and allowed for different shifts in the same laboratory to use the same inoculum preparation. Although it has been suggested that growth in liquid media might be more reproducible (42), the pharmacopeias agreed to allow growth on solid media or in liquid, so long as the culture was fresh. Another point of harmonization was in the volume of the inoculum used. The USP has always used 0.5% (0.1 mL into

20 mL of product). However, *Ph. Eur.* insisted that 1% inoculum was fine. In the end, the USP provided a range of 0.5 – 1% for the inoculum in the product sample as a compromise.

Disagreements existed over product categories. Product categories were introduced by *Ph. Eur.*, which suggested that product categories should have different criteria, and this was adopted by USP (see 37 for rationale). Later, it was suggested that otic products should not be classed with the parenterals, and this was changed. Finally, *Ph. Eur.* objected to applying this test to non-aqueous products as the low water activity would prevent microbial growth. Although there was a significant amount of discussion on the merits of this point (43, 44), this change was finally recommended in 1999 (36) and adopted in USP 2002 (10).

A major impediment, and perhaps the only significant obstacle remaining given the USP flexibility in changing all other aspects of the test, is the criteria. The *Ph. Eur.* has two sets of criteria for each product category – a target (the “A” criteria) and an acceptable level (the “B” criteria). The preservation efficacy at the level of the “B” criteria is acceptable only if there are strong reasons why the “A” criteria cannot be met. Both sets of criteria are more potent than the USP criteria (see Table 6 for example of criteria for Category 1 products). This difference seems to be a fundamental point of contention. Initially there seemed to be hope that the “B” criteria could be modified to accommodate existing

**Table 6. Criteria for AET passage (Category “1”).**

	Inoculum (CFU)	Log <sub>10</sub> Reduction					
		6 Hr	24 Hr	7 Day	14 Day	21 Day	28 Day
USP: Bact.	10 <sup>5</sup> -10 <sup>6</sup>			1.0	3.0	--	NI*
EP-A: Bact.	10 <sup>6</sup>	2	3	--	--	--	NR**
EP-B: Bact.	10 <sup>6</sup>	--	1	3	--	--	NI
USP: Yeast	10 <sup>5</sup> -10 <sup>6</sup>			NI	NI	NI	NI
EP-A: Yeast	10 <sup>6</sup>			2	--	--	NI
EP-B: Yeast	10 <sup>6</sup>			--	1	--	NI
USP: Mold	10 <sup>5</sup> -10 <sup>6</sup>			NI	NI	NI	NI
EP-A: Mold	10 <sup>6</sup>			2	--	--	NI
EP-B: Mold	10 <sup>6</sup>			--	1	--	NI

\* NI - No increase  
 \*\* NR – No recovery

products (41, p 89). What was particularly interesting was that this suggestion was voiced by the *Ph. Eur.* expert on AET, whose studies had been the most influential in Europe in establishing the justification for the existing European criteria (45, 46). However, this hope evaporated as the European position shifted to state that the existing “B” criteria were the absolute minimum required for safe products (a good review of the situation in 1993, and an eloquent presentation of this argument, can be found in 47). The USP saw the situation somewhat differently. As the USP criteria have been in effect for over thirty years, both the manufacturers and the regulatory agencies have extensive experience with products meeting those criteria and they simply saw no problem that needed to be corrected. The US position has literature support. Recent studies have shown the adequacy of the USP test (48), and even studies showing some level of contamination in preserved products have indicated that the microorganisms are not of clinical significance (49, 50). Finally, it must also be remembered that the AET is now a referee test and that all preserved products must meet this criteria. If the criteria were made significantly more restrictive (e.g., adoption of the *Ph. Eur.* “A” or “B” criteria), then many products with established histories of safety on the marketplace would be subject to recall for failure to meet this new USP criteria (i.e., the *Ph. Eur.* criteria). In addition to the cost of reformulating existing products with long histories of safe and effective service, the increase in preservative required to meet the more stringent requirements could well increase the toxicity of the formulations. The USP could not agree to this step and so the harmonization process stalled.

In summary then, the USP has implemented fundamental changes in criteria, inocula, and structure of the test since 1993 in an effort to achieve harmony with the *Ph. Eur.* To date, *Ph. Eur.* is virtually unchanged from its original draft published in 1991 (51), and only recently proposed to change a diluent as a “first step” towards harmonization (52). Given the current situation, it seems unlikely that the AET test will be harmonized, or that any further changes in USP chapter “<51> Antimicrobial Effectiveness Testing” will occur in the near future.

## Other Antimicrobial Effectiveness Tests

### <52> *Antimicrobial Effectiveness Testing for Vaccines*

The current general chapter <51> *Antimicrobial Effectiveness Testing* applies to vaccines in multi-use containers. Significant concern was expressed to the USP by the *Ph. Eur.* that, because of their nature and composition, most vaccines could not fulfill the requirements criteria proposed by the *Ph. Eur.* At the request of interested parties, USP developed a “stand-alone” chapter designed for the testing and evaluation of vaccines and is offering it as a point of departure for international harmonization discussions. This proposed chapter <52> appeared in the May – June 1998 issue of *PF* (53). No further developments for the proposed chapter <52> are planned as *Ph. Eur.* is developing different criteria of effectiveness that would apply to vaccines. This proposal was canceled in the Jan. – Feb. 2001 issue of *PF* (54).

### *Simulated In-use Antimicrobial Effectiveness Testing*

The concern over the in-use characteristics of preservative systems has led to an interesting situation in Europe where the Quality Working Party of the CPMP (Committee for Proprietary Medicinal Products) and the CVMP (Veterinary Products) have issued several notes for guidance on the need to do additional testing on multiple-use products. A review of the early situation stressed the point that both the *Ph. Eur.* and USP AET tests are designed to demonstrate “adequate protection from adverse effects that may arise from microbial contamination or proliferation during storage or use of the preparation” (55). The concern on in-use stability is a prudent one in situations where a volatile component of the preparation can be affected by opening of the container. However, many preparations are very stable to exposure to oxygen, and are packaged in oxygen permeable containers that provide years of stability data during development of the product.

The EMEA (European Agency for the Evaluation of Medicinal Products) has published a variety of “Notes for Guidance” on in-use stability testing of products<sup>4</sup>. These tests are primarily geared to demonstrate the

chemical stability of the preparation, but there are also multiple recommendations to perform microbial monitoring of product dispensed as a patient would (56-60). It is unclear that there is any real value gained in terms of safety to the patient from this additional microbiological testing on all products.

### Summary

The AET, as currently described in USP 25, will probably remain in its present form for many years. The past 10 years have seen a great deal of change in the test in attempts to reduce variability and to harmonize with the *Ph. Eur.* This work is at a stopping point, and until the situation changes no further significant revisions in the AET should be expected.

### References

1. The United States Pharmacopeial Convention, Inc., *The United States Pharmacopeia, 18<sup>th</sup> Revision*, (1970), Rockville, MD., p. 845.
2. Leitz, M., (1972), "Critique of U.S.P. microbiological test," *Bull Parenter Drug Assoc.*, **26** (5), 212-216.
3. Rdzok, E. J, W. E. Grundy, F. J. Kirchmeyer, and J. C. Sylvester, (1955), "Determining the efficacy of preservatives in pharmaceutical products," *J Amer Pharm. Assoc., Sci. Ed.*, **44**, 613-1616.
4. Yablonski, J. I., (1972), "Fundamental concepts of preservation," *Bull Parenter Drug Assoc.*, **26** (5), 220-227.
5. The United States Pharmacopeial Convention, Inc., (effective January 1, 1990), "Ranitidine Oral Solution," *First Supplement to The United States Pharmacopeia 22/National Formulary 17*, Rockville, MD., p 2167.
6. Cooper, M. S., (1994), "Some issues associated with international harmonization of the preservative effectiveness test," *Pharmacoepial Forum*, **20** (6), 8589-8592.

### Footnotes

1. "USP Open Conference on Microbiological Compendial Issues" held at Sanibel Harbour, Fort Myers, Florida in January of 1996.
2. "Harmonization of the Sterility Test and the Antimicrobial Efficacy Test" was held in Barcelona, Spain in February 1996.
3. "USP Open Conference on Microbiology in the 21<sup>st</sup> Century" held in New Orleans, LA in May 1998.
4. The EMEA maintains a web site at <http://www.emea.eu.int/>. This site is the best source for current EMEA or CPMP guidance documents.
7. Leavitt, J. M., I. J. Naidorf, and P. Shugaesky, (1955), "The undetected anaerobe in endodontics; a sensitive medium for detection of both aerobes and anaerobes," *The NY J Dentist*, **25**, 377-382.
8. Kellog, D. S., (1972), "Preservative testing as applied to quality control systems," *Bull Parenter Drug Assoc.*, **26** (5), 216-220.
9. The United States Pharmacopeial Convention, Inc., (effective July 1, 1975), *The United States Pharmacopeia 19<sup>th</sup> Revision*, Rockville, MD., p. 587.
10. The United States Pharmacopeial Convention, Inc., (effective January 1, 2002), *The United States Pharmacopeia 25<sup>th</sup> Revision*, Rockville, MD.
11. Tan, T. G. and R.C. Wilson, (1980), "The efficacy of antimicrobial agents in Parenteral Products assessed by the USP XIX method compared to the USP XVIII method," *J. Parenteral Drug Assoc.*, **34** (6), 431-435.
12. The United States Pharmacopeial Convention, Inc., (effective July 1, 1980), *The United States Pharmacopeia 20<sup>th</sup> Revision*, Rockville, MD., p. 873.
13. The United States Pharmacopeial Convention, Inc., (effective January 1, 1985), *The United States Pharmacopeia 21<sup>st</sup> Revision* Rockville, MD. p. 1151.

14. The United States Pharmacopeial Convention, Inc., (effective January 1, 1990), *The United States Pharmacopeia 22<sup>nd</sup> Revision*, Rockville, MD, p. 1478.
15. *Pharmacopeial Forum*, **8** (3), 2058, May-June 1982.
16. Orth, D. S., (1979), "Linear regression method for rapid determination of cosmetic preservative efficacy," *J. Soc. Cosmet. Chem.*, **30**, 321-332.
17. Orth, D. S., C. M. Lutes, S. R. Milstein, and J. J. Allinger, (1987), "Determination of shampoo preservative stability and apparent activation energies by the linear regression method of preservative efficacy testing," *J. Soc. Cosmet. Chem.*, **38**, 307-319.
18. Orth, D. S., C. M. Lutes, and D. K. Smith, (1989), "Effect of culture conditions and method of inoculum preparation on the kinetics of bacterial death during preservative efficacy testing," *J. Soc. Cosmet. Chem.*, **40**, 193-203.
19. Orth, D. S., (1980), "Establishing cosmetic preservative efficacy by use of d-values," *J. Soc. Cosmet. Chem.*, **31**, 165-172.
20. Davies, D. J. et al. (1976), "Design of a standard protocol for the challenge testing of antimicrobial preservative solutions," *J. Pharm Pharmacol Suppl.*, **28**, 49.
21. Sutton, S. V. W., R. J. Franco, D. A. Porter, M. F. Mowrey-McKee, S. C. Busschaert, J. Hamberger, and D. W. Proud, (1991), "D-value determinations are an inappropriate measure of disinfecting activity of common contact lens disinfecting solutions," *Appl. Environ. Microbiol.*, **57**, 2021-2026.
22. Houlby, R. D., (1980), "An alternate approach for preservative testing of ophthalmic multiple-dose products," *J. Parenter Drug Assoc.*, **34** (4), 272-276.
23. Brannan, D. K. and J. C. Dille, (1990), "Type of closure prevents microbial contamination of cosmetics during consumer use," *Appl Environ Microbiol.*, **56** (5), 1476-1479.
24. Abshire, R. L. and B. A. Schelech, (1982), "A method for evaluating the effectiveness of preservative systems in water-immiscible ointments," *J. Parent. Sci. Technol.*, **36**, 216-221.
25. Cooper, M. S., (1989), "Preservative efficacy: compendial and regulatory issues," *J. Parent. Sci. Technol.*, **43**, 187-189.
26. *Pharmacopeial Forum*, **18** (2), 3048, Mar-Apr 1992.
27. *Pharmacopeial Forum*, **21** (4), 1040, July-Aug 1995.
28. *Pharmacopeial Forum*, **22** (6), 3212, Nov-Dec 1996.
29. Pfaller, M. A., L. Burmeister, M. S. Barlett, and M. G. Rinaldi, (1988), "Multicenter evaluation of four methods of yeast inoculum preparation," *J. Clin Microbiol.*, **26** (4), 1437-1441.
30. Muth, H. and W. Casey, (2000), "The effects of antimicrobial preservatives on organisms derived from fresh versus frozen cultures," *Pharmacopeial Forum*, **26** (2), 519.
31. *Pharmacopeial Forum*, **23** (2), 3806, Mar-Apr 1997.
32. The United States Pharmacopeial Convention, Inc., (effective May 15, 1998), *Eighth Supplement to The United States Pharmacopeia 23/National Formulary 18*, Rockville, MD, p. 1681.
33. The United States Pharmacopeial Convention, Inc., (1990), "Proceedings from the November 1990 Open Conference on Microbiology and Sterilization Issues," *USP*, Rockville, MD.
34. Cooper, M. S., (1994), "Some issues associated with international harmonization of the preservative effectiveness test," *Pharmacopeial Forum*, **20** (6), 8589-8592.
35. Dabbah, R. and J. E. Knapp, (1997), "Harmonization of microbiological methods – A status report," *Pharmacopeial Forum*, **23** (6), 5334.
36. *Pharmacopeial Forum*, **25** (1), 7513, Jan-Feb 1999.

37. Wallhäusser, K.-H., (1973), "Antimicrobial preservatives in Europe: Experience with preservatives used in pharmaceuticals and cosmetics," *Develop. Biol. Standard*, **24**, 9-28.
38. *PharmEuropa*, **5** (4), 355-358, 1993.
39. Hodges, N. A., S. P. Denyer, G. W. Hanlon, and J. P. Reynolds, (1996), "Preservative efficacy tests in formulated nasal products: Reproducibility and factors affecting preservative efficacy," *J. Pharm Pharmacol.*, **48**, 1237-1242.
40. *PharmEuropa*, **6** (4), 387-388, 1994.
41. *PharmEuropa*, Special Issue, October 1996, p. 89.
42. Gilbert, P., M. R. W. Brown, and J. W. Costerton, (1987), "Inocula for antimicrobial sensitivity testing: A critical review," *J. Antimicrob Chemother.*, **20**, 147-154.
43. Cooper, M. S., (1998), *The Microbiological Update*, **16** (9).
44. Cooper, M. S., (1999), *The Microbiological Update*, **17** (7).
45. Davison, A. L., W. L. Hooper, D. F. Spooner, J. A. Farwell, and R. Baird, (1991), "The validity of the criteria of pharmacopeial preservative efficacy tests – A pilot study," *Pharm J.*, **246**, 555-557.
46. Spooner, D. F. and A. L. Davison, (1993), "The validity of the criteria for pharmacopeial antimicrobial preservative efficacy tests," *Pharm J.*, **251**, 602-605.
47. Matthews, B. R., (1993), "Preservative efficacy testing," *Reg Affairs J.*, pp. 455–461.
48. Farrington, J. K., E. L. Martz, S. J. Wells, C. C. Ennis, J. Holder, J. W. Levchuk, K. E. Avis, P. S. Hoffman, A. D. Hitchins, and J. M. Madden, (1994), "Ability of laboratory methods to predict in-use efficacy of antimicrobial preservatives in experimental cosmetic," *Appl Environ Microbiol.*, **60** (12), 4553-4558.
49. Livingstone, D. J., G. W. Hanlon, and S. Dyke, (1998), "Evaluation of an extended period of use for preserved eye drops in hospital practice," *Br. J. Ophthalmol.*, **82**, 473-475.
50. Kim, G. E., K. D. Fern, and J. A. Perrigin, (1997), "Sterility of ophthalmic drugs dispensed from spray bottles," *Optometry and Vision Sci.*, **74** (10), 865-867.
51. *PharmEuropa*, **3** (2), 241-244, 1991.
52. *PharmEuropa*, **13** (3), 597-599, 2001.
53. *Pharmacopeial Forum*, **24** (3), 6269, May-June 1998.
54. *Pharmacopeial Forum*, **27** (1), 2034, Jan-Feb 2001.
55. Sutton, S. V. W., B. Matthews, and D. Dunn, (1998), "In-use shelf-life testing – What data are required and when?" *Regulatory Affairs Journal*, **9**, 728-733.
56. CPMP/QWP/2934/99 "Note for guidance on in-use stability testing of human medicinal products." Effective September 2001.
57. CPMP/QWP/159/96, corr "Note for guidance on maximum shelf-life of sterile products for human use after first opening or following reconstitution," effective July 1998.
58. CPMP/CVMP/QWP/115/95, "Note for guidance on inclusion of antioxidants and antimicrobial preservatives in medicinal products," effective January 1998.
59. EMEA/CVMP/127/95. "Note for guidance: In-use stability testing of veterinary medicinal products," effective September 1996.
60. EMEA/CVMP/424/01, "Note for guidance on in-use stability testing of veterinary medicinal products (excluding immunological veterinary medicinal products)," effective September 2002.