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 18th 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.

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 18th 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

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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
C. albicans	25°C	48 hours	Sterile Saline TS
A. niger	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 subtilus 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 microbial 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*

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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

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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
C. albicans	25°C	20° – 25°C		
A. niger	25°C	20° – 25°C		

Table 3. Summary of USP criteria through revisions.*

	Inoculum (CFU)	Criteria				
		7 Day	14 Day	21 Day	28 Day	Comments
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	1×10^5 - 1×10^6	1.0**	3.0	--	NI	The motive for all changes in criteria was the international harmonization effort. (see text) Anhydrous medications included as "Category 2"
Category 1B		--	2.0	--	NI	
Category 1C		--	1.0	--	NI	
Category 2		NI	NI	NI	NI	
USP 25 (2002) Category 1-3	Criteria same as categories 1A, 1B, and 1C, respectively					Anhydrous medications deleted to improve harmonization with *Ph. Eur.* Antacids were removed as a class from Category 1C and given a unique category based on market and regulatory input.
Category 4	1×10^3 - 1×10^4	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 $\log_{10}$ 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

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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 20º to 25º[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

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