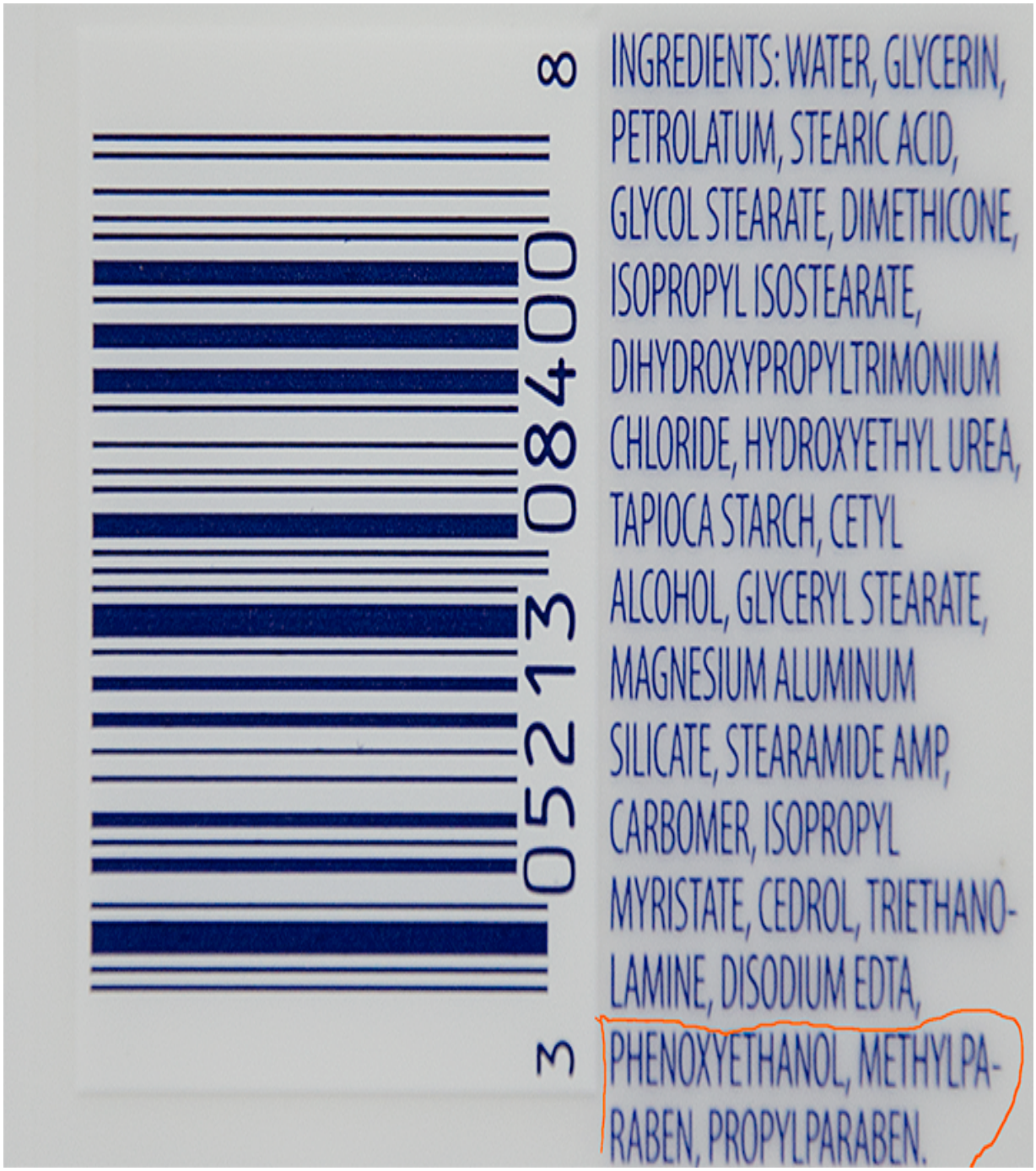
USP Antimicrobial Effectiveness Test and In-Use Tests

David A. Porter, PhD
President
Sense-Reflect-Act, LLC



USP CHAPTER 51

Chapter 51, Antimicrobial Effectiveness Testing of the United States Pharmacopeia, has been in existence for quite some time. In fact it was known previously as the preservative effectiveness test.

Along with considerable discussion pertaining to the USP test, we will also cover out of specification investigations when the AET test fails for a product under test, and we will briefly cover the use of the USP test for in-use studies.

Background

Purpose of USP <51>

- Provides tests to demonstrate effectiveness of antimicrobial protection
- Intended for use in original, unopened container
- Not meant to allow poor manufacturing technique
- Product configuration is also important to consumer safety

What is accomplished with this chapter is to examine antimicrobial effectiveness to ensure that the minimal required efficacy against mi□

croorganisms exists at levels of antimicrobial agents which are not in and of themselves toxic to humans.

Because the chapter is written such that original, unopened con□ tainers are required, it cannot be considered to be an in-use test directly.

Some less than scrupulous manufacturers might be inclined to use and antimicrobial preservative to make up for inadequate microbial con□ trol. You cannot correct for faulty GMP by adding such agents to correct for sloppiness!

A properly configured multiuse container should be designed to minimize the likelihood of microbial contamination. Most multiuse pharmaceutical products also require a preservative (antimicrobial agent).

How many?

There are hundreds of monographs in *USP* pertaining to sterility (chapter <71>) and microbial limits (chapters <61>, <62>) requirements.

Approximately how many monographs in *USP* refer to requirements related to *Antimicrobial Effectiveness Testing* (chapter <51>)?

This question is asking about how many USP monographs include a re□ quirements for <51> testing.

How many?

Zero!

The relevant chapter, <51> *Antimicrobial Effectiveness Testing*, is numbered below 1000. What does this customarily indicate about the chapter?

There used to be a number of USP monographs that included chapter <51> as part of the testing requirements. These have been weeded out over time.

Chapters with Numbers < 1000

Customarily, chapters numbered < 1000 have been considered to be enforceable given that they are referenced from within monographs, and *USP* monographs are recognized by law.

However, no monographs reference <51>. Where else in the *USP* might there be text referencing the chapter?

Chapter 51 of course has a number below 1000 which means, according to the USP, that it is an enforceable chapter. Enforceable chapters are made so because they are referenced from within USP monographs. Where else can you find text in the USP that would make a general chapter "enforceable"?

Many USP scientific liaisons are introduced to General Notices and Requirements by their supervisors. It is almost considered to be a rite of passage.

General Notices and Requirements

It's not exciting reading, but a tremendous amount useful information can be found in *General Notices and Requirements*. In this case, look in *General Notices and Requirements, Ingredients and Processes, Added Substances*

Preservatives

Additional useful information can be found in USP <1072> Disinfectants and antiseptics.

Classes of Preservatives Available

- Alcohols:

  Benzyl alcohol, Chlorbutol, Phenylethanol, Bronopol

- Aldehydes:

  Formaldehyde, Glutaraldehyde

- Biguanides:

  Chlorhexidine, PHMB

- Halogens:

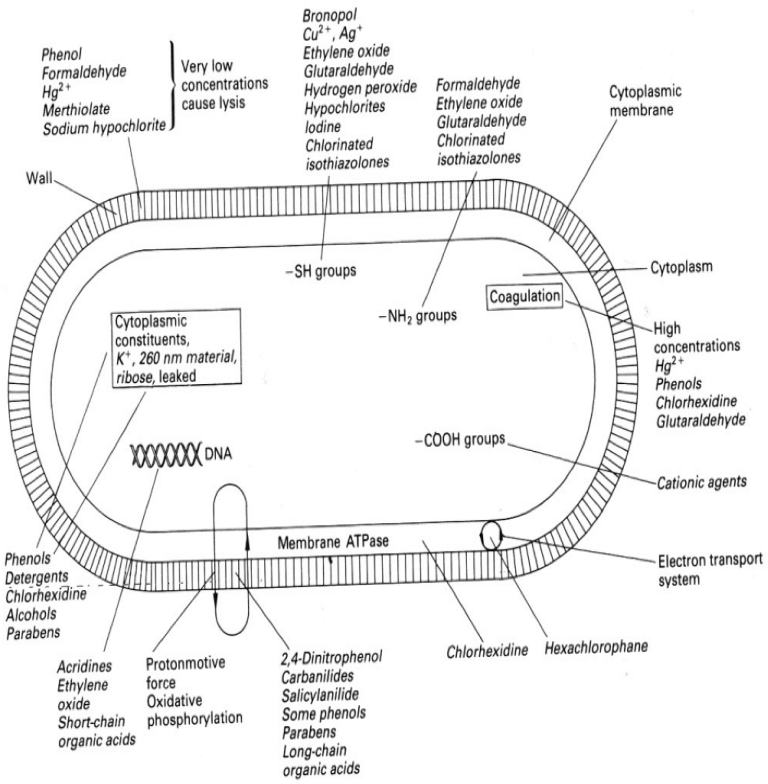
  Chlorine, Hypochlorite, Chloroform, Iodine

Classes of Preservatives Available

- Heavy Metals
  - Mercurials
- H_2O_2 and Peracid Compounds
- Phenols
- Surface-active agents (surfactants)
  - Anionic
  - Cationic
  - Ampholytic

Some ophthalmic products used to contain mercurials (e.g. thimerosal). These included a number of contact lens disinfectant products. To a large degree, these have been replaced with non-mercurial antimicrobial agents.

Sites of Action for Preservatives



From: Hugo, W.B. 1992. Mode of Action of Non-Antibiotic Antibacterial Agents. *IN* Pharmaceutical Microbiology, W.B. Hugo, and A.D. Russel (eds) pp 288-294.

Virtually every component of a microorganism can serve as a target for an antimicrobial agent.

Microbial Resistance to Preservatives

- Growth Rate a major factor - fast growing cells are less sensitive
- Biofilm formation
- Spores

In Jurassic Park, there was a statement from the chaos expert along the lines of “Life will find a way”. Indeed it will, and microorganisms have evolved, and will continue to evolve, strategies to deal with antimicrobial agents.

Biofilm is composed of adherent cells that are frequently embedded within a self-produced matrix of an extracellular matrix (typically slimy). Biofilms are generally protective, making the removal and destruction of microorganisms contained within a biofilm much more difficult.

Spores are much more resistant to antimicrobial agents.

Product Categories

What sorts of pharmacopeial articles might benefit from the inclusion of an antimicrobial preservative?

Antimicrobial preservatives are substances that are added to nonsterile dosage forms in order to protect them from microbiological growth or to protect them from microorganisms that are introduced by accident during usage.

Therefore, any nonsterile product might be suitable for including an antimicrobial agent.

Also, sterile, multiuse containers also generally require an antimicrobial agent. However, there have been some recent multiuse containers designed for sterile products. These are designed such that microbial contamination of the product within the container is prevented.

Product Categories

Category 1:

"Injections, other parenterals including

emulsions, otic products, sterile nasal products,

and ophthalmic products made with aqueous bases or vehicles."

Category 2:

"Topically used products made with aqueous bases or vehicles, nonsterile nasal products, and emulsions, including those applied to mucous membranes."

Categories 1 and 2 have more stringent requirements than categories 3 and 4. as you will see shortly. These categories are for oral products and liquid antacids. Note that none of the categories are for nonaqueous products. Why might nonaqueous vehicles be excluded?

Because microbial growth requires the presence of free water. Do you think this means life forms would be excluded from such products? What about spores?

Product Categories

Category 3:

"Oral products, other than antacids, made with aqueous bases or vehicles."

Category 4:

"Antacids made with an aqueous base."

A fair question is why are the aqueous antacid products included in the product category with the least stringent requirements for passage (as you will see later)?

It turns out that such products tent to inactivate the antimicrobial agent over time, and given that a USP test is applicable throughout shelf life, many such products would fail the test.

Preservative Amount

Why Include the "Minimum Amount"?

Antimicrobial preservatives are inherently toxic. If not so, they would not kill bacteria.

Antimicrobial agents wouldn't be very useful if they were not toxic to microorganisms. The risk, however, is that they might also has some toxicity to humans. Therefore what is accomplished with

this chapter is to examine antimicrobial effectiveness to ensure that the minimal required efficacy against microorganisms exists at levels of antimicrobial agents which are not in and of themselves toxic to humans.

There is no way of knowing what sorts of microorganisms could pose a threat to a product over time and in the myriad of possible locations it could be used in.

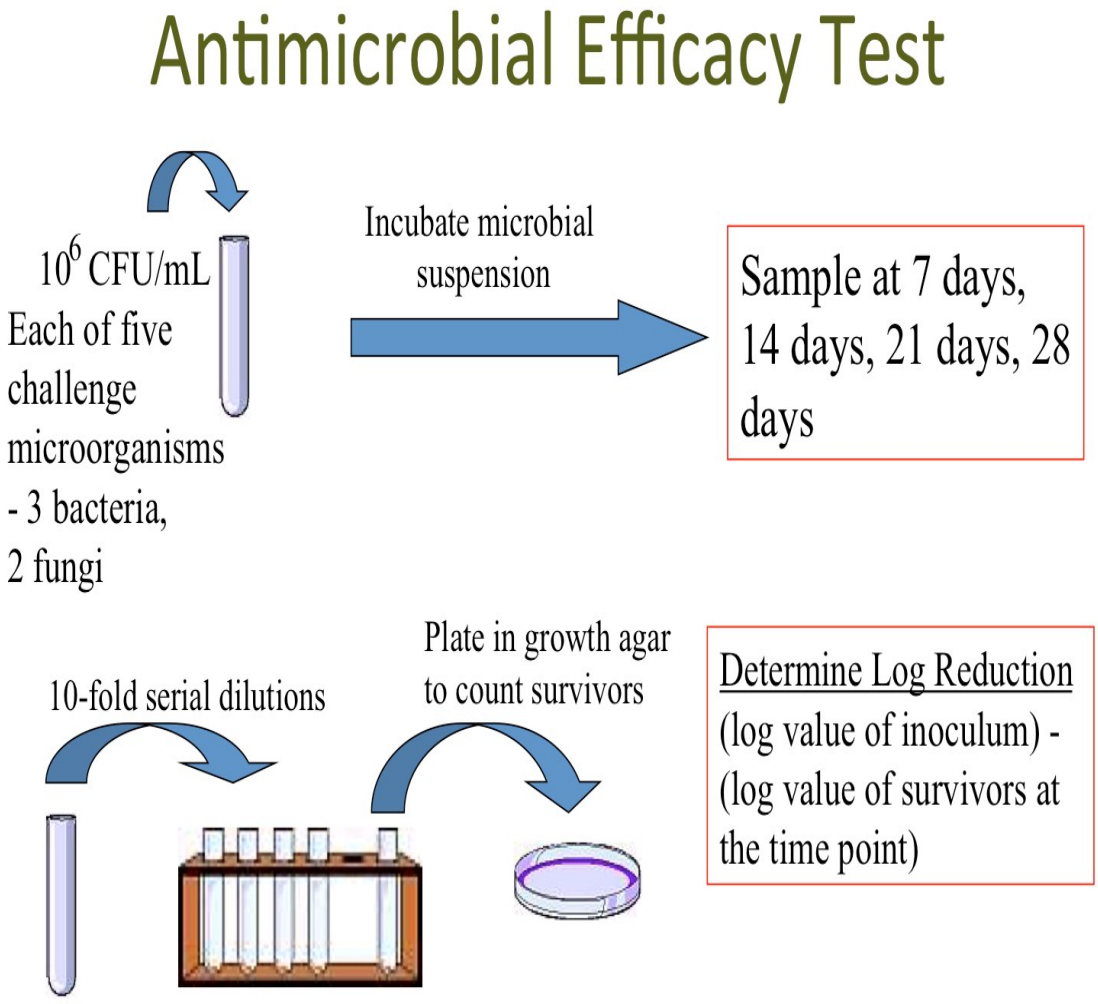
Microorganisms

- *Candida albicans* (yeast)
- *Aspergillus niger* (mold)
- *Escherichia coli* (Gram-negative rod)
- *Pseudomonas aeruginosa* (Gram-negative rod)
- *Staphylococcus aureus* (Gram-positive coccus)

Why is it important to include such a broad spectrum of microorganism types?

Therefore, a range of microorganisms is included spanning Gram positive and negatives, along with two fungal species.

Method Schematic



From time to time, people have considered adding all of the test species into the same container to be tested. This is not the proper approach because you then set up more of an experiment in

microbial population interactions than a straight forward demonstration of antimicrobial efficacy.

For products in Categories 1-3, 10^ 5-10^ 6 cfu/mL are required. Category 4 products require 10^ 3-10^ 4 cfu/mL. What does this suggest about the relative antimicrobial efficacy of liquid antacids?

Incubation is at room temperature.

Passage Criteria

Criteria*

Category	Organism	7 Days	14 Days	28 Days
1	Bacteria	1.0	3.0	NI
1	Fungi	NI	NI	NI
2	Bacteria	---	2.0	NI
2	Fungi	---	NI	NI
3	Bacteria	---	1.0	NI
3	Fungi	---	NI	NI
4	All	---	NI	NI

* Expressed as minimal $\log_{10}$ unit reduction from inoculum
NI – No Increase (within 0.5 log units)

Here are the criteria that must be satisfied for each product category to pass the test. Note that the requirements are less strin□

gent as you proceed from Category 1 down to Category 4. Note also that the requirements pertaining to fungi never exceed fungistasis.

Why is no more than fungistasis required? Remember that fungal cells are the same basic cell type as in humans (eukaryotic). While there are numerous substantial differences that can be taken advantage of between prokaryotic (bacterial) cell types and human cells, such differences are not as prominent between fungal and hu□ man cells.

About That 0.5 Log Difference

Log_{10} of 1,000 cfu/mL = 3.0 (at day 14)

" 'No increase' is defined as not more than 0.5 log_{10} unit higher than the previous value measured."

Does 1,500 cfu/mL (at day 28) meet the requirement?

[no increase calculator]

Why do you think it would be considered acceptable antimi□ crobial efficacy if you observed up to a half-log increase in the cfus? These types of microbial assays are notorious for having a high de□ gree of variability. Below a certain degree of difference, you cannot be relatively sure that the difference you measure is real.

Validation, And Why Bother?

AET – "Validation"

- Necessary to demonstrate adequate recovery of the challenge organism in the presence of residual product
- Instructions are provided in General Information Chapter <1227> "Validation of Microbial Recovery from Pharmacopeial Articles."

Remember that an official test method in USP, be it in a general chapter or a monograph, is considered validated. However, it is often necessary to demonstrate that your particular product is suit

able for use with the validated method. Given that USP microbio□ logical testing is growth-based, it is necessary to demonstrate that test microorganisms can grow in the presence of your product fol□ lowing sufficient neutralization of the antimicrobial agent.

When It's All Said and Done...

Nobody actually bothers with this microbiological determination of antimicrobial effectiveness once its been established. Once established, all that is necessary to do is determine the concentration of antimicrobial agent chemically from then on.

Right?

There was at least one circumstance where a polymeric antimicrobial agent was used in an ophthalmic product. The analytical chemistry department developed a test for the agent that showed the concentration of the agent was almost absolutely constant over time. However, over that same period of time, the chapter <51> test showed a steady decrease in efficacy. Which test was correct?

It turns out they were both correct. This polymeric agent agglomerates over time, creating what could be thought of a molecular snowballs. This meant that decreasing amounts of the agent were free to interact with the microorganisms. The analytical chemistry procedure first included a step that broke apart the snowballs. Because the polymeric molecules had not actually broken apart, they were indeed still all present, thus the steady concentrations.

OOS INVESTIGATIONS

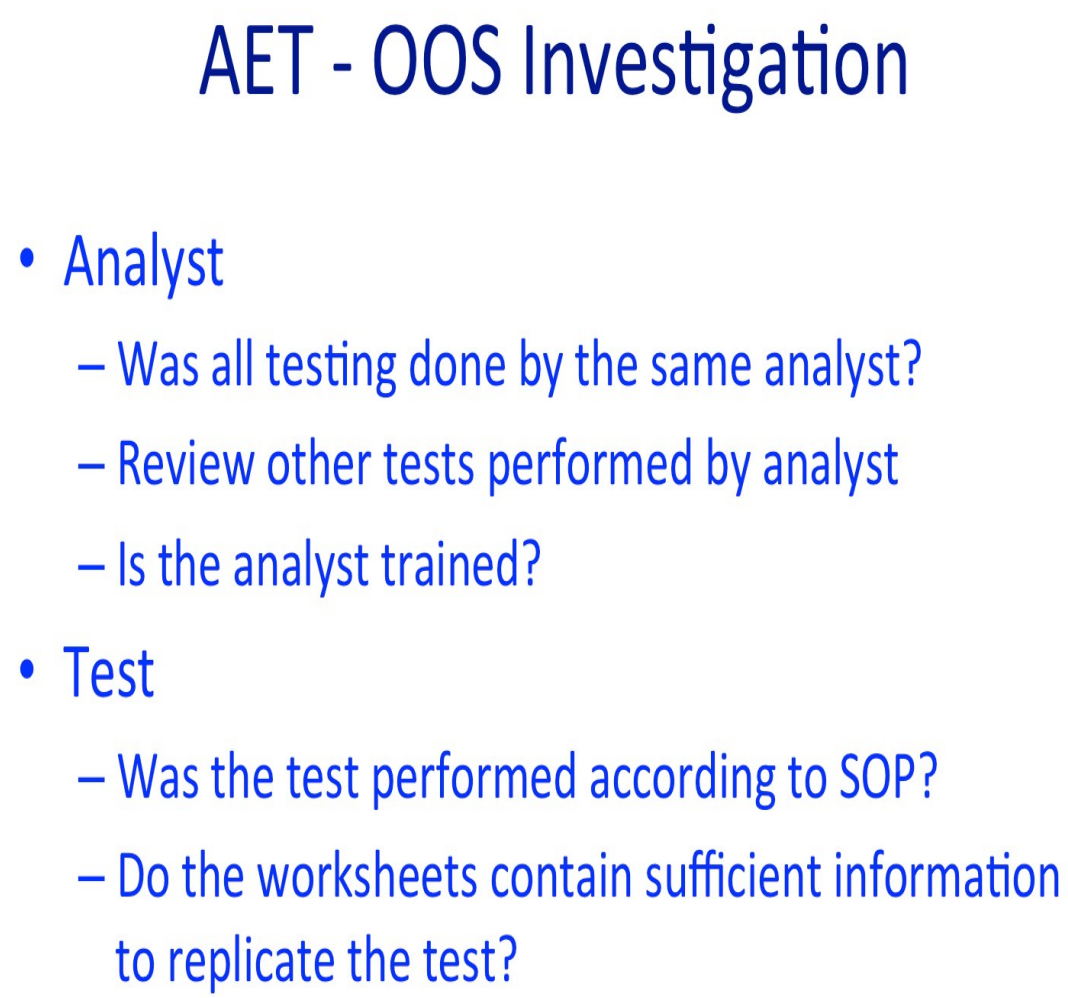
AET - OOS Investigation

"Validation"

- Was the recovery scheme adequately "validated"?
- Was the validated recovery scheme followed?
- Do the dilution series CFU show any sign of product inhibition?

Remember that this "validation" is typically more along the lines of demonstrating method suitability relative to chapter <51>.

If you need to develop an addition to the chapter method to bring about neutralization, that portion may need to be validated as per chapter <1227>.



The first bullet touches upon both method ruggedness and possible training issues.

The second bullet touches upon proper GMP documentation practices. If your documentation does not permit answering the questions, you need to improve your GMP documentation.

AET - OOS Investigation

Media

- Review Growth Promotion of Media
- Review Autoclave records of media preparation
- Review purity of any retains

Remember that USP microbiological testing is growth-based. If the media cannot support growth, the test results are invalid.

AET - OOS Investigation

Stock Cultures

- Were working cultures within 5 passages of the ATCC original?
- Preparation of Stock Cultures
  - Were the stock cultures maintained under the conditions described in USP <51>?
  - Were the stock cultures suspended in the correct buffer?
  - Were the stock cultures held too long?

The first bullet pertains to the notion that genetic drift can oc□ cur resulting in altered responsiveness to the antimicrobial agents. This notion has been the subject of some online debate.

The general concern again pertains to the ability of the mi□ croorganisms to grow.

AET - OOS Investigation

Dilutions & Plate Counts

- Were dilutions prepared correctly?
- Are dilution count averages reasonable when compared to neighboring count averages?
- Are replicate counts of an organism at the dilution with 70-130% of the average count for the organism?
- Were plates incubated as indicated in the procedure?
- Were all counts done at the correct time intervals?
- Is all math correct? Was $\log_{10}$ reduction calculated from measured inoculum?

Of particular interest in this list is the final bullet. Many peo□ ple have asked about what constitutes the initial concentration. It is important to note that it is from the inoculum prior to addition to the product. Some have thought it should come from a sample of product immediately after addition of the inoculum. This is ill-ad□ vised given the speed with which some antimicrobial agents can act.

AET - OOS Investigation

Equipment

- If inoculum is standardized by UV, is method and equipment validated and is calibration curve attached to data for analysis.
- If mechanical pipets were used, review calibration tolerances.
- If OOS is mold, was a hemacytometer used to obtain mold count? Is calculation correct?
- Are the incubators used on a cleaning and viable testing program? What does environmental data show?

These bullets pertain in general to good GMP, and environ□ mental monitoring is mentioned. How do you think environmental monitoring data can affect your investigation determination?

AET - OOS Investigation

Product

- Is the product is an ointment or cream?
- What do previous stability points show for log reduction?
- Is the product preserved?
- Does the Analytical Chemistry data support the adequacy of the preservative?

Remember the previous discussion pertaining to the interplay of stability, analytical chemistry and microbiology? Typically one would expect the analytical chemistry and microbiology results to support each other, but surprises can be lurking!

In-Use Testing

Remember!

- USP AET <51> is intended for use in original, unopened container, thus it is not designed to be a simulated in-use test

The chapter does provide for testing of product supplied in containers that are not amenable to the text. In such cases, five

sterile, capped bacteriological containers inert to the product may be used.

A Relevant Study

"Ability of Laboratory Methods To Predict In-Use Efficacy of Antimicrobial Preservatives in an Experimental Cosmetic"

J. K. FARRINGTON, 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

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1994, p. 4553-4558 Vol. 60, No. 12

This article directly addressed the possible use of a number of lab-based tests to evaluate in-use conditions.

Methods Compared

Six laboratory-based methods were compared with the results from an 8-week simulated in-use test:

- USP test
- BP test
- CTFA test
- Rapid screen test
- Sequential test
- FDA post-use test

Unlike chapters <61>, <62>, <71>, <85> and <1111>, the antimicrobial effectiveness test is not harmonized with the EP and JP.

This stems from the fact the the USP will not make a test official that would cause safely marketed products in the US to become misbranded/adulterated due to failure to meet the test requirements. Back in the 1990s, a lot of work went into attempting to

harmonize. The BP tests passage requirements were more stringent pertaining to fungal species, and certain US product could not meet the BP requirements. Thus, the test was never harmonized.

Test Materials

Nine formulations were prepared with different preservative strengths using:

- Methyl-paraben
- Propyl-paraben
- Quaternium 1

There are of course many more types of antimicrobial agents to choose from. Chapter <1072> is an excellent source of information

about the possible choices.

Summary of Results [1]

"In this study we found that laboratory microbiological tests can be used to predict the in-use efficacy of antimicrobial preservatives in products such as cosmetics and pharmaceuticals."

You would need to read the article for a discussion of what the "in-use conditions" were.

Summary of Results [2]

"Of all of the procedures used in this study, the RS test required the least time and the fewest materials."

The use of rapid microbiological methods has been the subject of considerable interest within industry and the compendia. Some of these methods are growth-based, others based upon biochemical capabilities, and still others based upon nucleic acid technologies.