TABLE OF CONTENTS

• Preservatives
• Purpose for Using Preservatives in Formulation
• Types of Preservatives
• Preservative Ideals
• Formulation Factors Affecting the Antimicrobial Activity of Preservatives
• Manufacturing Conditions Can Have an Affect on Preservatives
• What is Antimicrobial Effectiveness Test
• Antimicrobial Effectiveness Test Method General Procedure
A chemical agent that will either kill or inhibit growth of microorganism

- Commonly used in food, cosmetic, and pharmaceutical industries to prevent microbial growth from contaminating finished products.
- For products packaged in multi-dose containers, to inhibit growth of microorganism that might be introduced from repeatedly withdrawing doses.
- To protect product from inadvertent contamination by consumer during use.
**Purpose for Using Preservatives in Formulations**

- **Prevent the Development of Adverse Risks:**
  - **Finished Product:**
    - Malodor
    - Viscosity Changes
    - Discoloration
    - Presence of Visible Microbial Growth
  - **Consumer:**
    - Eyes – an infection could lead to blindness
    - Development of skin infections if the consumer has open sores or cuts.
    - Death for those consumers that are either immunocompromised or has a pre-existing condition.
Types of Preservatives

- Acids – Benzoic acid, sorbic acid
- Alcohols – Ethyl, Isopropyl, Chlorbutol, Bronopol
- Biguanids – Chlorhexidine, polyhexamethylene biguanide
- Halogen – Hyprochlorite, povidone-iodine, chloroform, chlorphenexin
- Organic mercurial – Mercury, silver, thimerosal
- Aldehyde – Formaldehyde, glutaraldehyde
- Parabens – Methylparaben, Ethylparaben
- Phenolic – Cresols, chlorcresol, bisphenol, phenoxyethanol, benzyl alcohol
- Quaternary ammonium-compound – cetrimide, benzalkonium chloride
**Preservative Ideals**

- Board spectrum of activity
- Effective over wide pH range
- Stable to light & elevated temperature for expected shelf of product
- Soluble in formulation at the required concentration
- No effect over color, odor, rheological property of formulation
- Compatible with formulation component and packaging
- Non toxic at in used concentration
- Inexpensive and readily available
- Approved by appropriate regulatory agencies
FORMULATION FACTORS AFFECTING
THE ANTIMICROBIAL ACTIVITY OF
PRESERVATIVES

- Water Activity
  - USP<1112> Application of Water Activity Determination to Non-Sterile Pharmaceutical Products
- pH
- Solubility of Preservatives
- Compatibility with Other Raw Ingredients
Microbial Metabolism and Growth

- Need a source of available water and nutrients.
- By having a reduction in the amount of available water in a formulation, microorganisms will be affected by having a longer generation time or reduce metabolic activity.
- Water is necessary for microbial growth to occur.
- Microorganisms will only proliferate in the water phase of a product formulation.
- To prevent microorganisms from growing, a preservative has to be present in the aqueous phase of a product formulation.
## General Water Activity Values Required for Microbial Growth

<table>
<thead>
<tr>
<th>Water Activity Value</th>
<th>Type of Microorganisms Capable of Proliferation</th>
<th>Antimicrobial Spectrum of a Preservative for Inclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.96 to 0.99</td>
<td>Gram-positive and Gram-negative bacteria (e.g. <em>Ps. Species</em>), mold and yeasts</td>
<td>Preservative system needs to have a broad spectrum of antimicrobial activity (e.g. Gram-negative and Gram-positive bacteria, yeast and mold)</td>
</tr>
<tr>
<td>0.90 to 0.95</td>
<td>Several Gram-negative and most Gram-positive bacteria (e.g. <em>Enterobacter aerogenes, Escherichia coli, Bacillus</em> species), mold and yeasts</td>
<td></td>
</tr>
</tbody>
</table>
# General Water Activity Values Required for Microbial Growth

<table>
<thead>
<tr>
<th>Water Activity Value</th>
<th>Type of Microorganisms Capable of Proliferation</th>
<th>Antimicrobial Spectrum of a Preservative for Inclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.80 to 0.89</td>
<td>Gram-positive bacteria (e.g. <em>S. aureus</em>), mold and yeast</td>
<td>Preservative system needs to be active against Gram-positive bacteria, yeast and mold</td>
</tr>
<tr>
<td>0.70 to 0.79</td>
<td>Halophilic bacteria, mold and yeasts</td>
<td>Preservative system needs to be active against yeast and mold</td>
</tr>
<tr>
<td>Below 0.6</td>
<td>None</td>
<td>Inclusion of a preservative system may not be necessary</td>
</tr>
</tbody>
</table>
**pH Microbiological Affects**

- **Bacteria** – Optimum pH for growth is between 5.5 and 8.5.
- **Fungi (Yeasts and Mold)** – Optimum pH for growth is between 4.0 and 6.0.
- For product formulations with a pH less than 4.0 or greater than 10.0, microorganisms are not able to proliferate or survive in a formulation due to:
  - Metabolic injury to microbial cells
  - Cellular stress by which microorganism expend a greater amount of energy to maintain intracellular pH. After energy has been used up, microbial cells will die.
  - The function of many microbial cellular enzymes is dependent on the maintenance of proper intracellular pH.
Some raw ingredients can be:

- **Microbial Nutrients**
  - Botanical Extracts, Carbohydrates, Proteins, Amino Acids, Emulsifiers, Lipids, Gums and Vitamins

- **Preservative Inactivators**
  - Polysorbate (Tween), Lecithin, Cellulose derivatives, Gelatin

- **Preservative Absorbers**
  - Bentonite, Calamine, Carbonates, Silicon dioxide, Zinc oxide, Talc, and some color pigments

- **Preservative Potentiators**
  - Propylene Glycol, EDTA, Antioxidants, Ethanol, Pentylene Glycol, Essential Oils Fragrances
Manufacturing Conditions Can Have an Affect on Preservatives

- Raw ingredient order of addition.
- pH of the formulation at the time of preservative addition.
- Temperature during processing.
- Packaging affects on Preservatives.
Mold Contamination
Mold Contamination
Mold Contamination
WHAT IS THE ANTIMICROBIAL EFFECTIVENESS TEST?

• A microbial challenge test that determines the antimicrobial effectiveness of a preservative system added in a formulation will work as expected over time.
• Used during formulation development and in stability program.
• Compendial Test
• Not truly harmonized around the world
MICROBIAL CHALLENGE TEST METHODS

• Pharmacopeia Challenge Test Methods
  o USP<51> Antimicrobial Effectiveness Test
    • The first appearance of this chapter was in the USP XVIII in 1970. It was not a mandatory test until publication of the First Supplement to USP XXII (official Jan 1, 1990) that a monograph for a preserved product specifically stated that it must meet the requirement of USP<51> Antimicrobial Preservatives-Effectiveness.
  o EP 5.1.3 Efficacy of Antimicrobial Preservation

• Other Challenge Test Methods
  o CTFA M-3 Determination of Preservative Adequacy of Water Miscible Cosmetics
  o CTFA M-4 Method for Preservative Testing of Eye Area Cosmetics
  o ASTM E640-78 Standard Test method for Preservatives in Water Containing Cosmetics
MICROBIAL CHALLENGE TEST METHODS

• Other Challenge Test Methods
  o AOAC 998.10 Preservative Challenge Efficacy Test of Non-Eye Area Water Miscible Products
  o ISO 11930 Efficacy Test and Evaluation of Preservation of a Cosmetic Product
DIFFERENCES BETWEEN THE VARIOUS TYPES OF PRESERVATIVE CHALLENGE TEST METHODS

• Types of Challenge Test Microorganisms
• Inoculum Levels
• Mix Culture verses Pure Culture Inoculums
• Sampling Time-Points After Inoculation
• Acceptance Criteria
<table>
<thead>
<tr>
<th>Type</th>
<th>Microorganism (ATCC Number)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>USP</strong></td>
</tr>
<tr>
<td>Gram-Positive Cocci</td>
<td>\textit{Staphylococcus aureus} (6538)</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Fermentative Gram-Negative Bacilli</td>
<td>\textit{Escherichia coli} (8739)</td>
</tr>
<tr>
<td></td>
<td><em>\textit{E. coli} is used for all oral preparation and \textit{Zygosaccharomyces rouxii} for oral preparations containing a high concentration of sugar</em></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Type</td>
<td>Microorganism (ATCC Number)</td>
</tr>
<tr>
<td>-------------------------------------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>Non-Fermentative Gram-Negative Bacilli</td>
<td></td>
</tr>
<tr>
<td>USP</td>
<td>EP</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa (9027)</td>
<td>Pseudomonas aeruginosa (9027)</td>
</tr>
<tr>
<td>Burkhodera cepacia (25416)</td>
<td></td>
</tr>
<tr>
<td>Pseudomonas flourescens (13525)</td>
<td></td>
</tr>
<tr>
<td>Pseudomonas putida (31483)</td>
<td></td>
</tr>
<tr>
<td>Yeast</td>
<td></td>
</tr>
<tr>
<td>Candida albicans (10231)</td>
<td>Candida albicans (10231)</td>
</tr>
<tr>
<td>Mold</td>
<td></td>
</tr>
<tr>
<td>Aspergillus brasiliensis (16404)</td>
<td>Aspergillus brasiliensis (16404)</td>
</tr>
</tbody>
</table>
## Challenge Testing Parameters - Topical Product Formulations

<table>
<thead>
<tr>
<th>Challenge Test Formulation</th>
<th>Inoculum Level in Product (CFU/gram)</th>
<th>Testing Intervals (Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacteria</strong></td>
<td><strong>Yeast and Mold</strong></td>
<td></td>
</tr>
<tr>
<td>USP</td>
<td>$1.0 \times 10^5 - 1.0 \times 10^6$</td>
<td>$1.0 \times 10^5 - 1.0 \times 10^6$</td>
</tr>
<tr>
<td>EP</td>
<td>$1.0 \times 10^5 - 1.0 \times 10^6$</td>
<td>$1.0 \times 10^5 - 1.0 \times 10^6$</td>
</tr>
<tr>
<td>JP</td>
<td>$1.0 \times 10^5 - 1.0 \times 10^6$</td>
<td>$1.0 \times 10^5 - 1.0 \times 10^6$</td>
</tr>
<tr>
<td>CTFA</td>
<td>$1.0 \times 10^6$</td>
<td>$1.0 \times 10^5$</td>
</tr>
<tr>
<td>ASTM</td>
<td>$1.0 \times 10^6$</td>
<td>$1.0 \times 10^5$</td>
</tr>
<tr>
<td>ISO</td>
<td>$1.0 \times 10^5 - 1.0 \times 10^6$</td>
<td>$1.0 \times 10^4 - 1.0 \times 10^5$</td>
</tr>
<tr>
<td>AOAC</td>
<td>$1.0 \times 10^6 - 9.9 \times 10^6$</td>
<td>$1.0 \times 10^5 - 1.0 \times 10^6$</td>
</tr>
</tbody>
</table>
### Challenge Acceptance Criteria (Log$_{10}$ Reduction)

<table>
<thead>
<tr>
<th>Challenge Test Method</th>
<th>Challenge Acceptance Criteria (Log$_{10}$ Reduction)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 2</td>
</tr>
<tr>
<td></td>
<td>Bacteria</td>
</tr>
<tr>
<td>USP</td>
<td>NT</td>
</tr>
<tr>
<td>EP</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>B</td>
</tr>
</tbody>
</table>

**NT** = Not Tested  
**NI** = No Increase  

**Criteria A:** The recommended efficacy to be achieved  
**Criteria B:** In Justified cases where the A criteria cannot be attained, for example for reasons of an increased risk of adverse reactions, the B criteria must be satisfied
# Various Challenge Testing Acceptance Criteria for Topical Product Formulation

**Challenge Acceptance Criteria (Log$_{10}$ Reduction)**

<table>
<thead>
<tr>
<th>Challenge Test Method</th>
<th>Challenge Acceptance Criteria (Log$_{10}$ Reduction)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 2</td>
</tr>
<tr>
<td></td>
<td>Bacteria</td>
</tr>
<tr>
<td>CTFA</td>
<td>----</td>
</tr>
<tr>
<td>ISO</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>B</td>
</tr>
</tbody>
</table>

**Criteria A**: The recommended efficacy to be achieved

**Criteria B**: In Justified cases where the A criteria cannot be attained, for example for reasons of an increased risk of adverse reactions, the B criteria must be satisfied.

NT = Not Tested

NI = No Increase
**PET TESTING FLOW DIAGRAM**

**Test Product**

- **Total Bioburden Count**
  - **Preparation of Inoculum**
    - Grow Test Culture in a Suitable Liquid Medium with the Grown Stock Culture
    - Incubate Cultures Bacteria at 30-35°C, 18-24 hrs
    - Yeast at 20-25°C, 44-52 hrs
    - Mold at 20-25°C, 6-10 days
    - Harvest Culture by Centrifugation
    - Resuspend Culture with Sufficient Suspending Fluid to obtain a microbial count of about $10^8$ CFU/mL.
    - Verification of Inoculum by Plate Count
  - Place 20gm of Product in Sterile Container
  - Inoculate Product with Prepared Inocula (Volume of Suspension Used is Between 0.5% and 1.0% of the Volume of Product)
  - Incubate the Inoculated Container at 20-25°C
  - Sample each Container at the Appropriate Intervals and Perform Plate Count
  - Remove 1 gm or mL of Test Samples
  - Perform 10-fold Serial Dilutions
  - Incubate Plate @Specified Time and Temperature
  - Plate Dilutions to Determine Number of Survivors
  - Read/Record CFU Counts
  - Calculate the Log Reduction
- **CTFA**
- **USP**
- **EP**
- **Other**
Antimicrobial Effectiveness Test Method General Procedure

• Prepare the cultures to be used. Demonstrate that the inocula have the right levels of microorganisms.
• The culture must be freshly prepared.
• Inoculate the products individually with either pure or mixed microbial culture suspensions.
  o Pure microbial cultures will yield specific data on each test microorganism employed in the challenge study.
  o Mixed culture inocula may serve to simulate real world conditions during use.
    • It is recommend that closely related types of microorganisms such as Gram-positive bacteria, Gram-negative fermentative bacilli, Gram-negative non-fermentative bacilli, and yeasts and molds be pooled into separate distinct groups.
Antimicrobial Effectiveness Test Method General Procedure

- Antagonism between different types of organisms may occur due to differences in growth factors and nutritional requirements.
- A rapidly growing organism may impede the detection of a more slowly growing organism.
- Competition for growth factors or production of inhibitory by products and other factors may result in antagonism between different types of microorganisms.
  - Single inoculation or Rechallenge
    - A rechallenge is consisting of a second inoculation may be considered if more information is desired, to determine if a formulation is marginally preserved

- Perform inoculum recovery to assure the original inoculation level and to estimate the concentration of organisms in the challenge products.
- Store products, protected from light at 20-25°C
**Antimicrobial Effectiveness Test Method General Procedure**

- At each sampling/test time, remove aliquots and perform plate counts
  - Perform 10-fold serial dilutions and plate dilution
- Determine number of survivors. Calculate the log reduction.
Bioburden of the Test Sample

• Microbial bioburden should be performed prior to performing the antimicrobial effectiveness test
  o To verify that the level and type of microorganisms in the test sample will not interfere with the recovery of the interpretation of the challenge test data.
  o An initially high microbial bioburden in the test sample before inoculation with microorganisms could compromise the preservation system.
Method Validation

• Must be able to show inactivation of the preservative by demonstrating recovery of organisms in presence of the preservative.

• Inactivation may be done by
  o Use Neutralizer – chemical Inhibition
  o Dilution

• The neutralizer (inactivating agent) must have the following properties:
  o Not have inhibitory effects on the microorganisms
  o Should completely overcome the activity of the preservative
  o If it inactivates the preservative by combining with it the resultant product must not be toxic to the microorganisms.
Method Validation

• The following must be shown:
  o Neutralizer efficacy – The neutralizer effectiveness demonstrated in inhibiting the antimicrobial properties of the product
  o Neutralizer Toxicity – The neutralizer is not, itself, toxic to the microorganisms.
  o The challenge colony forming unit (CFU) should not be less than 70% of the viable count
SOURCE OF VARIABILITY

• Laboratory Expertise
  o has a widespread experience and knowledge in various methodologies for Preservative Effectiveness Test
  o Is well aware of the changes in the compendia
  o Has all the proper controls in place
  o Utilizes SOP and Protocols
  o Has Registration and/or Certification with recognized regulatory body

• Environmental Controls
  o Incubation temperature and duration
  o Inoculum preparation
  o Product bioburden level
CONCLUSION

- Formulation of products with preservative requires consideration of multiple factors.
- Preservative selection needs to balance stability, pH range, AET requirements, safety.
- Preservatives has multiple sources of variability, requires careful planning to design the experiment.
- Antimicrobial Effectiveness Test is critical part of development of products.
- When contracting out, you need understand the experience and capabilities of the contract laboratory.
Recent Information
FDA Bans Triclosan and Triclocarban from Over-The-Counter (OTC) Antibacterial Washes

- The rule only addresses products that are intended for use with water, and are rinse off after use.
  - Does not affect consumer hand sanitizers or wipes, or antibacterial products used in health care settings.
- The rule applies to consumer antiseptic wash products containing one or more of 19 specific active ingredients.
- Including the most commonly used ingredients – Triclosan and Triclocarban.
FDA Bans Triclosan and Triclocarban from Over-The-Counter (OTC) Antibacterial Washes

- These ingredients were not demonstrated safe for long term daily use and more effective than plain soap
- FDA has deferred rule making for one year on three additional ingredients used in consumer wash products:
  - Benzalkonium chloride, benzethonium chloride and chloroxylenol (PCMX)
BIBLIOGRAPHY


BIBLIOGRAPHY


