# REVIEWS

# Antimicrobial Preservative Use in Parenteral Products: Past and Present

**BRIAN K. MEYER, ALEX NI, BINGHUA HU, LI SHI**

Biologics and Vaccines, Pharmaceutical Research and Development, Merck Research Laboratories, West Point, Pennsylvania 19486

*Received 16 October 2006; revised 13 January 2007; accepted 12 February 2007*

*Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/jps.20976*

**ABSTRACT:** The following review provides a comprehensive summary of antimicrobial preservatives that are commonly used in licensed parenteral products to date. The information reviewed includes the general properties of the preservatives, the doses and frequency of their use, the classes of the preserved products (peptide, protein, vaccine, and small molecule products), the interactions with other formulation components, and the criteria commonly used for their selection in parental product formulations. It was revealed that phenol and benzyl alcohol are the two most common antimicrobial preservatives used in peptide and protein products, while phenoxyethanol is the most frequently used preservative in vaccines. Benzyl alcohol or a combination of methylparaben and propylparaben are generally found in small molecule parenteral formulations. The key criteria for antimicrobial preservative selection are the preservative's dose, antimicrobial functionality, and effect on the active ingredient. Additionally, the use of spectroscopic techniques (circular dicroism (CD) and fluorescence) and differential scanning calorimetry (DSC) were identified as common techniques used in evaluating an antimicrobial preservative for its impact on the conformational stability of peptide, protein, and vaccine antigens. The future use of preservatives is also discussed, including antimicrobial agents such as peptides, and regulatory requirements for antimicrobial effectiveness testing. © 2007 Wiley Liss, Inc. and the American Pharmacists Association J Pharm Sci 96:3155 3167, 2007

**Keywords:** parenteral; formulation; antimicrobial preservatives; benzyl alcohol; phenol; phenoxyethanol; methylparaben; propylparaben; thimerosal

## INTRODUCTION

There are currently over 350 parenteral products on the market worldwide.[1] Approximately one-third of these products are in multi-dose formulations. The specific challenge of developing a multi-dose product is the need for an antimicrobial preservative.[2] These formulations can inhibit the growth of microorganisms that may be inadvertently introduced into the containers during product withdrawal. Multi-dose formulations containing preservatives offer several advantages over single dose containers, including: (1) product wastage is minimized because different size doses may be obtained from the same container, (2) doses may be obtained from the container over a period of time without the concern for microbial growth, and (3) packaging is minimized because multiple doses are supplied in a single vial.

Alex Ni's present address is Centocor R&D Inc., 145 King of Prussia Road, Radnor, PA 19087, USA

*Correspondence to*: Li Shi (Telephone: +1 215 652 8856; Fax: +1 215 652 5299; E mail: li_shi@merck.com)

Journal of Pharmaceutical Sciences, Vol. 96, 3155–3167 (2007)
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FDA-CBER-2022-908-0014069

The information summarized in this review is based on the most recent texts and literature sources that describe the characteristics and uses of preservatives in parenteral products. In addition to the available literature, the Handbook of Pharmaceutical Excipients, the Handbook of Food, Drug, and Cosmetic Excipients, the Guide to Microbiological Control in Pharmaceuticals, and the Physician's Desk Reference (PDR) are the major sources used for this review.[3–6] Preservatives discussed in this review can be found in the FDA Inactive Ingredients Guide (IIG), the licensed parenteral and non-parenteral medicines in the UK, and the Canadian List of Acceptable Non-medicinal Ingredients.

## FUNCTIONALITY OF ANTIMICROBIAL PRESERVATIVES USED IN PARENTERAL PRODUCTS

The most commonly used eight antimicrobial preservatives in licensed parenteral products at the present are listed in Table 1.[6,7] They are benzyl alcohol, chlorobutanol, m-cresol, methylparaben, phenol, phenoxyethanol, propylparaben, and thimerosal. Table 1 lists the chemical structure, typical in-use concentration in parenteral formulations, antimicrobial activity expressed as the minimal inhibitory concentration (MIC), and optimum pH for antimicrobial activity.[3–5] In general, preservatives are used in a relatively low dose varying from 0.002 to 1%, although in some parenteral formulations, preservatives are used in a dose beyond 1% (Tabs. 1–4). This is due to the fact that some of the preservatives show a minimum inhibitory concentration (MIC) of lower than or equal to 5000 μg/mL or 0.5% (Tab. 1).

There are also many other commonly used preservatives including benzalkonium chloride, benzethonium chloride, and phenyl mercuric nitrate. These preservatives are not included in this review due to their absence in parenteral products listed in the PDR.[6] Additionally, preservatives used in the diluent for lyophilized parenteral products are also not covered.

### Peptide and Protein Parenteral Products

There are over 145 peptide and protein-based drugs listed in the 2006 PDR, which include monoclonal antibody formulations.[6] Of these products, 34 contain antimicrobial preservatives (Tab. 2).[6] The two most commonly used preservatives are phenol and benzyl alcohol, which are used in over half of these products (11 and 12 products, respectively). The next most commonly used preservative is m-cresol, with a total of nine products. Chlorobutanol and thimerosal are each present in 3 of these products (Tab. 2).

Phenol concentrations used in peptide and protein-based products range from 0.0715 to 0.5% (Tab. 2).[6] Phenol, in the class of phenolic compounds (Tab. 1), is bacteriostatic when present in 1% (w/v) solutions and has activity against mycobacteria, fungi, and viruses.[1,3] The solubility of phenol in water is 1 in 15 (w/w) at 20°C.[3] Aqueous solutions of phenol are stable, can be sterilized by dry heat or autoclaving, and should be maintained in containers that are protected from light. Phenol is incompatible with albumin and gelatin, which will result in precipitates possibly due to phenol-induced denaturation of these molecules. There is a low likelihood of adverse reactions from phenol in parenteral products due to the low concentrations used in these products.[3] However, infusion of large doses of phenol should not exceed 50 mg in a 10-h period.[3,8]

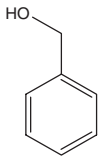
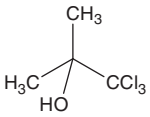
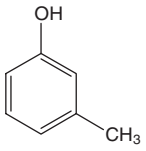
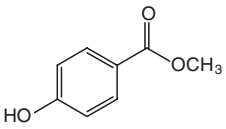
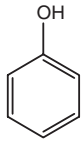
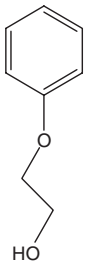
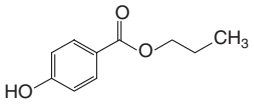
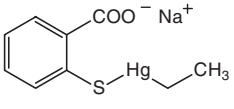
It has been reported that monoclonal antibody formulations containing phenol result in soluble and insoluble protein aggregates.[9] Although phenol is used in many peptide and protein-based drugs, there are several reports of interactions between this preservative and protein formulations. The interaction between phenolics and proteins was initially studied in plant enzymes.[10] Research performed in mammalian systems in the past 10 years found that phenol causes aggregation of human growth hormone (HGH) after freeze drying.[11,12] It is known that phenols interact with proteins and peptides by forming hydrogen bonds with the carbonyl group of these molecules.[13] Additionally, the oxidation of phenols will result in formation of quinines.[13]

Benzyl alcohol concentrations used in peptide and protein multi-dose products range from 0.9 to 1.1%.[6] Benzyl alcohol is an aromatic, primary alcohol (Tab. 1) and is effective against most Gram-positive bacteria, yeast, and mold, but is less effective against Gram-negative bacteria (Tab. 1).[3,4] Its solubility in water is 1 in 25 (w/w) at 25°C.[3] The optimum antimicrobial activity occurs at pH less than 5 and is less active above pH 8.[3] It may be stored in glass or metal containers or in polypropylene containers coated with Teflon or other inert fluorinated polymers.[3]



FDA-CBER-2022-908-0014070

**Table 1.** Chemical Structures and Antimicrobial Activity of Preservatives Commonly Used in Parenteral Biological and Pharmaceutical Products[1,2,3,7]

| Preservative Name and Typical In-Use Concentration[5] | Chemical Structure[7] | Antimicrobial Activity (Minimum Inhibitory Concentration (MIC), μg/mL)[3] | | | | |
|---|---|---|---|---|---|---|
| | | Gram Positive Bacteria (*S. aureus*) | Gram Negative Bacteria (*E. coli*/*P. aeruginosa*) | Yeast (*C. albicans*) | Mold (*A. niger*) | Optimal pH[3] |
| Benzyl alcohol 1% |  | 25 | 2000/2000 | 2500 | 5000 | <5 |
| Chlorobutanol 0.3 0.5% |  | 650[a] | 1000[a] | 2500[a] | 5000[a] | <5.5 |
| m-cresol 0.3% |  | Not specified | Not specified | Not specified | Not specified | <9 |
| Methylparaben 0.2% |  | 2000 | 1000/4000 | 2000 | 1000 | 4 8 |
| Phenol 0.25 5% |  | Not specified | Not specified | Not specified | Not specified | <9 |
| Phenoxyethanol 1% |  | 8500 | 3600/3200 | 5400 | 3300 | <7 |
| Propylparaben 0.2% |  | 500 | (100 500)/(>1000) | 250 | 200 500 | 4 8 |
| Thimerosal 0.002 0.01% |  | 0.2 | 4/8 | 32 | 128 | 7 8 |

[a]MIC values are not specific for S. aureus, E. coli, P. aeruginosa, C. albicans, or A. Niger.



FDA-CBER-2022-908-0014071

**Table 2.** Use of Preservatives in Peptide and Protein Drug Formulations Listed in the PDR[6]

| Generic Product Name | Product Brand Name | Manufacturer | Preservative | Preservative Concentration |
|---|---|---|---|---|
| Antivenin (Micrurus fulvius) (Equine Origin) | ANTIVENIN | Wyeth-Ayerst | Phenol | 0.25% |
| | | | Thimerosal | 0.005% |
| Antivenin (Crotalidae) (Equine Origin) | ANTIVENIN Polyvalent | Wyeth-Ayerst | Phenol | 0.25% |
| | | | Thimerosal | 0.005% |
| Antivenin (Latrodectus mactans) | ANTIVENIN (Black widow spider Antivenin) | Merck | Thimerosal | 1:10,000 |
| Calcitonin-salmon injection, synthetic | MIACALCIN® | Novartis | Phenol | 0.5% |
| Desmopressin acetate | DESMOPRESSIN Acetate | Ferring | Chlorobutanol | 0.5% |
| Desmopressin acetate | DDAVP® | Sanofi-Aventis | Chlorobutanol | 0.5% |
| Etanercept | ENBREL® | Amgen | Benzyl Alcohol | 0.9% |
| Epoetin alfa (recombinant) | EPOGEN® | Amgen | Benzyl alcohol | 1.0% |
| Epoetin alfa (recombinant) | PROCRIT® | Ortho Biotech | Benzyl alcohol | 1.0% |
| Follitropin alfa injection | GONAL® | Serono | Chlorobutanol | 0.3% |
| | | | Benzyl alcohol | 0.9% |
| Insulin aspart (recombinant) | NOVOLOG® | Novo Nordisk | m-Cresol | 0.172% |
| | | | Phenol | 0.15% |
| 70% insulin aspart protamine suspension 30% insulin aspart injection (rDNA origin) | NOVOLOG® MIX 70/30 | Novo Nordisk | m-Cresol | 0.172% |
| | | | Phenol | 0.15% |
| Insulin glargine [rDNA origin] injection | LANTUS® | Aventis | m-Cresol | 0.27% |
| Insulin glulisine (rDNA origin) injection | APIDRA™ | Aventis Pasteur | m-Cresol | 0.315% |
| Regular U-500 (Concentrated) (insulin human injection USP [rDNA] origin) | HUMULIN® R | Eli Lilly | m-Cresol | 0.25% |
| Insulin Lispro Injection (rDNA origin) | HUMALOG® | Eli Lilly | m-Cresol | 0.315% |
| 75% Insulin Lispro Protamine Suspension and 25% Insulin Lispro Injection (rDNA origin) | HUMALOG® MIX 75/25™ | Eli Lilly | m-Cresol | 0.176% |
| | | | Phenol | 0.0715 % |
| Interferon alfa-n3 (human leukocyte derived) | ALFERON N INJECTION® | Hemispherx Biopharma Inc. | Phenol | 0.3% |
| Interferon alfa-2b, recombinant | INTRON® A | Schering | Benzyl alcohol | 0.9% |
| Interferon alfa-2a, recombinant | REFERON®-A | Roche | Benzyl alcohol | 1.0% |
| Leuprolide acetate injection | LUPRON® | TAP Pharmaceuticals | Benzyl alcohol | 0.9% |
| Octreotide acetate injection | SANDOSTATIN® | Novartis | Phenol | 0.5% |
| peginterferon alfa-2a | PEGASYS® | Roche | Benzyl alcohol | 1% |
| Rho (D) Immune Globulin (human) | MICRhoGAM® | Ortho Clinical | Thimerosal | 0.003% |
| Rho (D) Immune Globulin (human) | RhoGAM® | Ortho Clinical | Thimerosal | 0.003% |
| Sargramostim (recombinant) | LEUKINE® | Berlex | Benzyl alcohol | 1.1% |
| Somatropin (rDNA origin) for injection in a two-chamber cartridge | GENOTROPIN® | Pharmacia/Upjohn | m-Cresol | 0.3% |



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| | | | | |
|---|---|---|---|---|
| Somatropin (rDNA origin) for injection vials and cartridges for use with the HumatroPen™ Injection Device | HUMATROPE® | Eli Lilly | m-Cresol | 0.3% |
| Somatropin | NORDITROPIN® CARTRIDGE | Novo Nordisk | Phenol | 0.3% |
| Somatropin (rDNA origin) injection | NUTROPIN® | Genentech | Benzyl alcohol | 0.9% |
| Somatropin (rDNA origin) injection | NUTROPIN AQ® | Genentech | Phenol | 0.25% |
| Somatropin | SAIZEN® | Serono | Benzyl alcohol | 0.9% |
| Trastuzumab | HERCEPTIN® | Genentech | Benzyl alcohol | 1.1% |
| Tuberculin purified protein derivative, diluted (stabilized solution) diagnostic antigen | APLISOL® | King | Phenol | 0.35% |

Teflon coatings of other surfaces, such as butyl rubber, are also effective against adsorption of benzyl alcohol.[3,14] The World Health Organization (WHO)-suggested intake limit of the benzyl/benzoic moiety daily is 5 mg/kg.[4,15]

It has been reported that benzyl alcohol causes aggregation of recombinant human interferon-γ (rhIFN-γ), recombinant human granulocyte colony stimulating factor (rhGCSF), recombinant human interleukin-1 receptor antagonist (rhIL-1ra), and monoclonal antibody formulations.[9,13,16,17] It is common knowledge that most protein aggregation is associated with protein conformational denaturation or instability. Benzyl alcohol has been demonstrated to bind to and accelerate aggregation of partially unfolded proteins.[17] It was hypothesized that precipitation of rhIFN-γ is due to loosening of the protein tertiary structure which in turn pre-disposes the protein to aggregate.[13] Minimization of preservative dose and use of acetate as the buffer have led to a stable multiple-dose formulation of rhIFN-γ.[13] It was determined using differential scanning calorimetry (DSC) studies that rhIL-1ra was the least stable in 0.9% benzyl alcohol when compared to 0.1% m-cresol and 0.065% phenol-containing formulations.[18] In studies with a monoclonal antibody formulation, a concentration of 1% benzyl alcohol resulted in cloudiness and the formation of soluble aggregates.[9] Concentrations of benzyl alcohol greater than 2% in the same monoclonal antibody formulation resulted in precipitation of the protein.[9]

The next most commonly used preservative in peptide and protein multi-dose formulations is m-cresol, a phenolic compound, with a frequency of use in nine products. This compound is active against Gram-positive bacteria, but less active against Gram negative bacteria, yeasts, and mold, and is inactive against sprores.[3] Its solubility in water at 20°C is 1 in 50 (w/w).[3] It has the highest antimicrobial activity in acidic conditions and should be stored in a closed vessel, in the absence of light, and in a cool, dry location. A low concentration of m-cresol in parenteral products may not induce any adverse events. Reports of adverse reactions are linked to the bulk or solutions containing 50% m-cresol.[3]

## Vaccines

There are significantly fewer vaccine products than peptide/protein products that contain preservatives as specified in the PDR.[6] Table 3 shows



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**Table 3.** Use of Preservatives in Vaccine Formulations Listed in the PDR[6]

| Generic Product Name | Product Brand Name | Manufacturer | Preservative | Preservative Concentration |
|---|---|---|---|---|
| Diphtheria and tetanus toxoids and acellular pertussis adsorbed | DAPTACEL® | Sanofi Pasteur | Phenoxyethanol | 0.6% |
| Diphtheria and tetanus toxoids and acellular pertussis adsorbed, hepatitis B (recombinant) and inactivated poliovirus vaccine combined | PEDIARIX™ | GlaxoSmithKline | Phenoxyethanol<br>Thimerosal | 0.5% < 12.5 ng mercury[a] per 0.5 mL dose |
| Diphtheria and tetanus toxoids and acellular pertussis vaccine adsorbed | INFANRIX® | GlaxoSmithKline | Phenoxyethanol | 0.5% (2.5 mg in 0.5 mL) |
| Hepatitis A vaccine, inactivated | HAVRIX® | GlaxoSmithKline | Phenoxyetahnol | 0.5% |
| Hepatitis A inactivated and hepatitis B (recombinant) vaccine | TWINRIX® | GlaxoSmithKline | Phenoxyetahnol | 0.5 % |
| Hepatitis B vaccine (recombinant) | ENGERIX-B® | GlaxoSmithKline | Thimerosal | <0.5 mcg mercury[a] (0.5 mL pediatric and 1.0 mL adult dose) |
| Pneumococcal vaccine polyvalent | PNEUMOVAX®23 | Merck | Phenol | 0.25% |

[a]Indicates ethyl mercury.

the preservatives used in a total of seven vaccine products. Among those preservatives, phenoxyethanol is used at a range of 0.5–0.6% in five out of the seven vaccines. One of the vaccines contains thimerosal, which contains less than 12.5 nanograms of ethyl mercury per 0.5 mL dose, and another contains phenol at a concentration of 0.25% (Tab. 3).

Phenoxyethanol is a phenolic derivative and is effective against Gram negative organisms such as *P. aeruginosa* (Tab. 1).[3] Its antimicrobial activity is enhanced when combined with parabens.[3,19,20] Phenoxyethanol's solubility in water is 1 in 43 (w/w).[3] Aqueous solutions are stable and can be autoclaved.[3] The usage of phenoxyethanol at 10% (v/v) on the skin in animal studies did not result in adverse events.[3,21]

Thimerosal is an anionic organic mercurial crystalline compound (Tab. 1). Since the 1930s, it has been widely used as a preservative in a number of biological and drug products, including many vaccines, to help prevent potentially life-threatening contamination with harmful microbes. Its solubility in water is 1 to 1 (w/w) and is bacteriostatic and fungistatic at a pH greater than 7, but is ineffective against spore-forming organisms.[3] Aqueous solutions of thimerosal are stable at room temperature and may be autoclaved.[3] Thimerosal powder or sealed solutions should be stored in a cool, dry location.[3] Containers should be protected from light and protected from exposure to copper and other metals.[3] Hypersensitivity has been reported for thimerosal.[3,4]

## Small Molecule Pharmaceutical Products

Approximately 2.5 % of small molecule parenteral pharmaceutical products contain preservatives in their formulations as listed in Table 4. The two most commonly used preservatives in these products are benzyl alcohol or a combination of methylparaben and propylparaben (Tab. 4). In almost all products, the concentration of benzyl alcohol used is within the range of 0.5–2.0% and the molar ratio of methylparaben to propylparaben is between 7.5:1 and 9:1 and (Tab. 4).[6] Chlorobutanol and phenol have also been used in one or three products, respectively (Tab. 4). Among the eight most frequently used preservatives (Tab. 1), parabens and chlorobutanol are used only in small molecule drug products (Tab. 4) with the exception of a peptide product, desmopressin acetate (Tab. 2). While the total amount of



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**Table 4.** Use of Preservatives in Small Molecule Drug Formulations Listed in the PDR[6]

| Generic Product Name | Product Brand Name | Manufacturer | Preservative | Preservative Concentration |
|---|---|---|---|---|
| Apomorphine hydrochloride injection | APOKYN™ | Mylan Bertek | Benzyl alcohol | 0.5% |
| Enalaprilat | VASOTEC I.V. | Merck | Benzyl alcohol | 0.9% |
| Enoxaparin sodium injection | LOVENOX® | Sanofi-Aventis | Benzyl alcohol | 1.5% |
| Intravenous (conjugated estrogens, USP) for injection | PREMARIN® | Wyeth-Ayerst | Benzyl alcohol | 2% |
| Famotidine | PEPCID® Multidose | Merck | Benzyl alcohol | 1.9% |
| Fulvestrant injection | FASLODEX® | AstraZeneca | Benzyl alcohol | Not indicated |
| Haloperidol | HALDOL® Decanoate | OrthoMcNeil | Benzyl alcohol | 1.2% |
| Hydrocortisone sodium phosphate | Hydrocortone Phosphate Injection | Merck | Methlyparaben<br>Propylparaben | 0.15%<br>0.02% |
| Hydromorphone hydrochloride | DILAUDID® | Abbott | Methlyparaben<br>Propylparaben | 0.18%<br>0.02% |
| Metaraminol bitartrate | Aramine® Injection | Merck | Methlyparaben<br>Propylparaben | 0.15%<br>0.02% |
| Nalbuphine hydrochloride | NUBAIN® | Endopharmaceuticals | Methlyparaben<br>Propylparaben | 0.2% of a 9:1 mixture |
| Methylprednisolone acetate injectable suspension, USP | DEPO-MEDROL® | Pharmacia/Upjohn | Benzyl alcohol | 0.9% |
| Ondansetron hydrochloride | ZOFRAN® | GlxoSmithKline | Methlyparaben<br>Propylparaben | 0.12%<br>0.015% |
| Penicillin G benzanthine suspension | BICILLIN® L-A | King | Methlyparaben<br>Propylparaben | 0.1%<br>0.1% |
| Phytonadione | AQUAMEPHYTON® Injection | Merck | Benzyl alcohol | 0.9% |
| Sodium ferric gluconate complex in sucrose injection | FERRLECIT® | Watson | Benzyl alcohol | 0.9% |
| Testosterone Enanthate Injection USP, Multiple Dose Vial | DELATESTRYL® | Savient Pharmaceuticals Inc. | Chlorobutanol | 0.5% |
| Medroxyprogesterone acetate injectable suspension, USP | Depo-Provera® | Pharmacia/Upjohn | Methlyparaben<br>Propylparaben | 0.137%<br>0.015% |
| Medroxyprogesterone acetate and estradiol cypionate injectable suspension | LUNELLE™ | Pharmacia/Upjohn | Methlyparaben<br>Propylparaben | 0.09%<br>0.01% |
| Flumazenil injection | ROMAZICON® | Roche Laboratories | Methlyparaben<br>Propylparaben | 0.18%<br>0.02% |
| Dolasetron mesylate injection | ANZEMET® | Sanofi Aventis | Phenol | 0.5% |
| Cimetidine hydrochloride injection | TAGAMET® | GlaxoSmithKline | Phenol | 0.5% |
| Streptomycin sulfate, USP | STREPTOMYCIN SULFATE | Pfizer | Phenol | 0.25% |

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parabens used are no more than 0.2%, the concentration of chlorobutanol used in small molecule parenteral products is 0.5% (Tab. 4). It is bacteriostatic against Gram-positive and negative organisms, and has some activity against yeasts and fungi (Tab. 1).[3,4] The fact that chlorobutanol is used mainly in small molecule products is possibly due to the observation that it is only stable at pH 3 in aqueous solutions, and increasing the pH results in its instability and decrease in its antibacterial activity.[3,4] Such an acidic pH environment is good for some small molecule compounds but not for any protein, peptide, or vaccine product. Solutions of chlorobutanol should be stored in tightly sealed containers due to its volatility and maintained at a temperature of 8–15°C.[3] It is not compatible with plastic or rubber stoppers due to adsorption.[4,22–26] Reactions have been reported following administration of parenteral drugs containing chlorobutanol include Type IV delayed hypersensitivity and Type I anaphylaxis.[4]

The concentrations of methylparaben and propylparaben used in small-molecule pharmaceuticals are in a range from 0.09 to 0.18% and 0.01 to 0.2%, respectively (Tab. 4). Parabens are benzoic acid esters (Tab. 1) and the solubility of methylparaben and propylparaben in water is 1 in 400 (w/w) at 25°C, and 1 in 2500 at 20°C, respectively.[3,27] Due to inherent low solubilities, paraben sodium salts are frequently utilized in the final dosage forms.[3] Parabens have a broad spectrum of antimicrobial activity at a pH range of 4–8, but are more effective against yeasts and molds when compared to bacteria.[3] Antimicrobial activity is normally enhanced when combinations of parabens are used with excipients such as propylene glycol, phenylethyl alcohol, and edetic acid.[27–29] Aqueous solutions of methylparaben and propylparaben are stable at a pH range of 3–6 and these solutions can be autoclaved.[3,30] Methylparaben and propylparaben will be hydrolyzed at pH greater than 8 or higher.[3,31] Methylparaben can react with sugars and sugar alcohols.[3,32] Adsorption of the parabens may occur in some plastic containers.[3,33] Hypersensitivity has been reported with the use of parabens.[3,5]

The presence of polyoxyethylene sorbitan fatty acid esters (Polysorbates 20, 60, and 80) can impact the antimicrobial activity of all of the preservatives reviewed. The inactivation of the preservatives may occur due to precipitation of the preservative or micellization.[3,5,34,35] For example, the antimicrobial activity of the parabens will be reduced by Polysorbate-80 (PS-80) due to micellization.[34,35] It was determined using an equilibrium dialysis technique that the parabens were bound to distinct loci in the polysorbate micelle.[36] Precipitation of Polysorbates 20, 60, and 80 will occur in the presence of phenols (m-cresol and phenol).[3] Antimicrobial activity of benzyl alcohol will be reduced in the presence of PS-80.[3]

## ANTIMICROBIAL PRESERVATIVE SELECTION

### Antimicrobial Effectiveness (AME) Testing

Multi-dose products are required to pass tests designed to challenge the ability of the preservative to inhibit or kill microorganisms that may be inadvertently introduced into the vial or container. Guidance for performing these tests is provided in the United States Pharmacopoeia (USP) <51> and the European Pharmacopoeia (EP).[37,38] These tests consist of inoculating $10^5$–$10^6$ CFU/mL microorganisms (e.g., bacteria and fungi) per container at time zero, and evaluating the log reduction over time. The criterion used for passing these tests is described in Table 5. The EP A criteria is the most stringent of these tests, which requires no less than a 2 log reduction after the first 6 h, and no less than a 3 log reduction after the first 24 h following introduction of organisms. EP A criteria are more difficult to meet because the amount of preservative required to kill microorganisms within the first 24 h must be balanced with compatibility with the active ingredients and toxicity of the agents. EP B and USP criteria also require some bactericidal activity, however, they are generally viewed as being bacteriostatic.

Recently, The World Health Organization (WHO) issued a guidance document which describes a procedure in which the product is challenged multiple times over the duration of the AME test with microorganisms at $10^3$ organisms per inoculation.[39] This procedure differs from the USP and EP compendial tests which challenge the container with $10^6$ organisms only at the initiation of the study.

### Compatibility Testing

The impact of a preservative on the active ingredient in addition to AME testing are two of the factors that should be considered during preservative screening and selection.[40] The following sections describe specific methods that



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**Table 5.** USP and EP Requirements for Antimicrobial Effectiveness Testing[37,38]

| Time | USP | EP A | EP B |
|---|---|---|---|
| Requirements for bacterial log reduction | | | |
| 6 h | Not required | 2 | Not required |
| 24 h | Not required | 3 | 1 |
| 7 d | 1 | No recovery | 3 |
| 14 d | 3 | No recovery | No increase |
| 28 d | No increase | No recovery | No increase |
| Requirements for fungal log reduction | | | |
| 7 d | No increase | 2 | Not required |
| 14 d | No increase | No increase | 1 |
| 28 d | No increase | No increase | No increase |

may be used to determine the impact of an antimicrobial preservative on the active ingredient.

### Peptides and Proteins

Prior to 2002, few articles were published that described the problems of compatibility between biopharmaceutical products and preservatives, and the results of these studies have been summarized.[41] In one study, it was determined that benzyl alcohol resulted in the aggregation of rhIFN-γ.[13] In the past several years, additional studies have been conducted to further evaluate interactions between preservatives and proteins.

At least five different proteins have been studied in the presence of preservatives, including a monoclonal antibody, rhIFN-γ, rhGCSF, HGH, and rhIL-1ra.[9,12,13,16,17] These proteins were introduced in a previous section of this review. Both analytical and biophysical methods were used to evaluate the impact of preservatives on these proteins. One common technique to study the impact on the protein is to incubate the protein (control) or protein-preservative combination under accelerated temperatures followed by analysis using analytical or biophysical methods.

Analytical methods used to study the effect of preservatives include size-exclusion high performance liquid chromatography (SEC-HPLC) and hydrogen–deuterium (H–D) exchange. SEC-HPLC has been utilized to detect the amount of monomer (monoclonal antibody, rhGCSF, rhIL-1ra) following incubation with preservatives under accelerated temperatures.[9,16,17] In these studies, the presence of monomer indicated protein that did not aggregate in the presence of different preservatives. H–D exchange using infrared spectroscopy was used to demonstrate that benzyl alcohol induced the partial unfolding of rhIL-1ra and rhGCSF.[16,17]

Spectroscopy has also been used extensively to study protein-preservative interactions. Methods used include near-UV circular dicroism (CD), Fourier transform infrared (FTIR), and ANS-fluorescence spectroscopy. Near-UV CD spectroscopy was used to evaluate the impact of benzyl alcohol on rhGCSF, and rhIL-1ra.[16,17] For example, the ellipticity was decreased for rhGCSF incubated in the presence of 0.9% benzyl alcohol, and this effect was enhanced at 37°C when compared to samples incubated at 25°C.[16] This decrease indicated a change in the asymmetric environment of tyrosine and tryptophan residues in the presence of benzyl alcohol.[16] FTIR spectroscopy was utilized by taking the second-derivative IR spectra of rhGCSF in the presence of 0.9% benzyl alcohol and in the presence of sucrose.[16] A decrease in the native α-helix and concomitant increase in the non-native intermolecular β-sheet peak was observed in these studies, which is indicative of aggregation.[16] ANS-fluorescence spectroscopy has been used to study the impact of preservatives on protein structure. ANS is a dye that interacts with hydrophobic residues of partially unfolded proteins and fluoresces.[42] It was determined that 2% benzyl alcohol induced partial unfolding of rhIL-1ra as observed by increased ANS fluorescence.[17]

Biophysical methods, including dynamic light scattering (DLS) and DSC have also been used to evaluate the effects of preservatives on proteins. DLS was used to evaluate aggregation of rhIFN-γ over time that was induced by benzyl alcohol.[13] It was determined that increasing amounts of benzyl alcohol resulted in decreasing $T_m$ values for IL-1 receptor, and the thermal transition was irreversible.[13]



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The impact of excipients, such as sucrose, or different buffers with different preservatives, have also be measured using these techniques.[13,16,17] Additionally, biological activity assays are also useful in determining if a preservative adversely affects the function of the protein.

### Vaccines

The number of publications documenting methods to screen preservatives for use in vaccines is not as high as those for protein therapeutics. However, many of the methods used for determining the compatibility of preservatives with proteins is applicable to vaccines, since most vaccines contain proteins as the active ingredient. The effects of preservatives on adjuvants should also be considered when evaluating preservative-containing vaccine formulations.

### Small Molecule Pharmaceutical Products

The most commonly used antimicrobial preservative in small molecule pharmaceutical products is methylparaben and propylparaben (Tab. 4). Methods have been developed to detect both the active pharmaceutical ingredient and preservative in the formulation. For example, solid-phase extraction and HPLC analysis (SPE/HPLC) was used to determine the amount of methylparaben and propylparaben in an oxytetracycline injectable suspension.[43] Another assay using reverse-phase HPLC (RP-HPLC) was used to simultaneously detect the presence of medroxyprogesterone acetate (MPA) and parabens in the bulk drug and injectable suspension.[44] Following exposure of the formulation to acidic and alkaline hydrolysis, as well as oxidation conditions, the RP-HPLC assay was able to resolve the methylparaben, propylparaben, degradation products, and megestrol acetate (main related substance).[44]

In summary, the techniques described can be used to study the impact that a preservative may have on a protein, vaccine, or small molecule pharmaceutical formulation. The use of analytical and/or biophysical studies in conjunction with AME testing will aid in determining the appropriate preservative for preservative-containing formulations.

## HISTORICAL REVIEW OF PRESERVATIVE USE

A historical review was performed to determine the frequency of use of different preservatives in parenteral products in the past decade. Table 6 shows the general distribution of the eight commonly used preservatives among three types of parenteral products, including peptides/proteins, vaccines, and small molecule products, and the frequency of the preservative used in parenteral product formulations. The frequency results shown in Table 6 were based on data selected from 1996, 2001, and 2006.[6,45] Data on preservative-containing products prior to 1996 have also been reviewed.[40] The results indicate an overall decrease in preservative use in parenteral products over the past decade. Preservatives were used in almost 300 parenteral products in 1996 while less than 100 products contain preservatives today (Tab. 6). However, a slight increase occurred in the use of two preservatives, m-cresol and phenoxyethanol.

Chlorobutanol, methylparaben, and propylparaben were not used in biological formulations with

**Table 6.** Number and Type of Parental Product Containing Preservatives in the Past 10 Years

| Preservative | Product Type | | | Frequency of Use[6,45] | | |
|---|---|---|---|---|---|---|
| | Peptide/Protein | Vaccines | Small Molecule | 1996[45] | 2001[5] | 2006[5] |
| Chlorobutanol | Present (2 products) | | Present | 17 | 13 | 3 |
| Methylparaben | | | Present | 50 | 40 | 9 |
| Propylparaben | | | Present | 40 | 33 | 9 |
| Benzyl alcohol | Present | | Present | 74 | 69 | 19 |
| Phenol | Present | Present | Present | 48 | 30 | 15 |
| Thimerosal | Present | Present | | 46 | 20 | 6 |
| m-cresol | Present | | | 3 | 7 | 11 |
| Phenoxyethanol | | Present | | 3 | 4 | 5 |
| Total | | | | 281 | 216 | 77 |



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the exception of desmopressin acetate, which is a peptide product (Tab. 6). It is not clear why these molecules have been used almost exclusively in small molecule products, although parabens are known to be less soluble than other preservatives in water and are sensitive to pH.[3] Benzyl alcohol and phenol were used in both biological and small molecule products (Tab. 6). The preservatives m-cresol, phenoxyethanol, and thimerosal are used in only peptide, protein, and vaccine products rather than small molecule products (Tab. 6).

## FUTURE USE OF PRESERVATIVES

In addition to the small molecule preservatives discussed in this review, larger molecules, such as peptides, have been identified as having antimicrobial activity. There are over 600 different types of cationic peptides that have been identified that kill microbial pathogens.[46] These peptides have an overall net positive charge and contain approximately 50% hydrophobic residues.[46] The hydrophobic residues allow the peptides to fold into an amphipathic form during the interaction with the cell membrane.[47] To date, these molecules have not been used in commercially available products.[6] The future use of these peptides remains to be determined.

## SUMMARY

In summary, antimicrobial preservatives are used in a significant number of parenteral products, including peptide, protein, vaccine, and small molecule formulations. There are eight preservatives most commonly used in these parenteral product formulations. The overall dominated concentration range of the preservatives used is between 0.002% and 1% although in a few cases, the preservative concentration used is beyond 1%. It was also demonstrated that various analytical and biophysical assays may be used to monitor the impact of a preservative on the active ingredient, whether it be a protein or small molecule pharmaceutical.

It was noted that the overall use of preservatives in parenteral products, however, has declined in the past decade. Although the total number of products containing preservatives has decreased from ~300 to less than 100 in the past 10 years, antimicrobial preservatives continue to play an important role in multi-dose pharmaceuticals.

## ACKNOWLEDGMENTS

Cheryl Moser is acknowledged for useful discussions regarding antimicrobial effectiveness testing.

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FDA-CBER-2022-908-0014081