Chapter I

INTRODUCTION

Preservatives are included in pharmaceutical preparations when it is necessary to combat the effects of contaminating microorganisms which may be inherent in ingredients or introduced during production processes or during use by the patient. Products with water are particularly susceptible to attack by microorganisms and if growth is not checked, adverse changes in the nature of the preparation may result. Some preparations containing raw materials in which microorganisms are capable of multiplying, particularly those intended for oral and topical administration and those which employ aqueous vehicles, preservatives are included. All products intended for parenteral and ophthalmic administration, for introduction into body cavity as irrigations, or for application to wounds must be specially prepared and sterilized. If presented in multiple dose containers, an effective antimicrobial preservative must be included in the formula.

Antimicrobial activity of preservatives in pharmaceutical products may be greatly reduced by many factors such as pH of the product, partition coefficient in disperse systems, association of preservatives with nonionic surface active agent, absorption of preservatives by microorganisms, and adsorption of preservatives by

containers and closures (1,2,3,4,5, 6,7,8,9, 10). Blackburn et al studied about using chlorbutol as a preservative in ophthalmic preparations and plastic containers were used. They found the concentration of chlorbutol in such preparations to be lower than the amount stated on the labels. This may be because of degeneration of chlorbutol during sterilization processes and during time the preparations were stocked for marketing (4).

The type of preservatives used in a product and the concentration needed to prevent microbial growth is determined by preservative efficacy testing (11). Preservative testing is extremely important to consumer acceptance of the product for the following reasons :

1. Use of too little preservative will result in microbial growth, which may alter product attributes and which may render the product injurious to the user.

2. Use of too much preservative may cause irritation, which causes consumer dissatisfaction.

3. Use of too much preservative increases cost of the product which is passed on to the consumer.

Although preservatives are included, some products or some lots of products are overgrowth with microorganisms. Ophthalmic solutions are also sometimes found to be nonsterile. Effectiveness of preservatives used must be checked for the information. For this project, the purpose is to check preservative effectiveness of the ophthalmic solutions used in Thailand by using the challenge test.

The method used will give more reasonable results for effectiveness evaluation of finished products than the other methods used in the past which factors about containers and closures are not involved in the tests (11, 12, 13).

Literature Survey

Sources of Microbial Contamination

1. Raw Materials

Water occupies an important role as vehicle in pharmaceutical preparations (14). In order to remove organic and inorganic substances found in natural water supplies, the water used usually goes through ion exchange treatment or distillation. But this purified water is easily contaminated with various microorganisms (15) and it has been reported that contamination in products was frequently due to water-borne bacteria. Most of bacteria found in deionized water were *Pseudomonas spp.*. Some strains were able to grow rapidly to a level of about 10^6/ml in deionized water. Although they can be killed at 60° C in 5 minutes, the crude enzyme remained active even after heating at 80° C for 10 minutes. In addition to polysorbate 20, the crude enzyme was found to decompose the other types of ester surface active agents.

Raw materials used in the manufacture of solutions, emulsions, and suspensions are excellent growth media for bacteria. Substances such as gums, dispersing agents, surfactants, sugars, and flavors can be the carriers of bacteria which contaminate the product.

2. Equipments

Bacteria grow well in the nooks and crevices of pharmaceutical equipment (and in the simple equipment used in the dispensary). Such equipment should be thoroughly cleaned prior to use.

3. Environment and Man

Environment and personnel can contribute to product contamination. Hands and hair are the most important carriers of contaminants. General cleanliness is thus vital. Head coverings must be used by those involved in process of manufacturing, and face masks should be used by those individuals suffering from colds, coughs, hay fever, and other allergic manifestation.

Commonly Effects of Contaminants

1. Pathogenesis

Pathogenic and potentialy harmful microorganisms associated with pharmaceutical preparations include Salmonella, certain species of Pseudomonas (including Pseudomonas aeruginosa), Staphylococcus aureus, and Escherichia coli.

2. Spoiling

A major source of spoilage contamination is water used in production processes. Pseudomonas, Xanthomonas, Achromobacterium, Escherichia, Aerobacter, and Flavobacterium spp. are generally known to proliferate in potable, distilled, and deionized waters.

Staphylococcus, Streptococcus, Bacillus spp., yeasts, and fungi, particularly Aspergillus spp. and Penicillium spp., frequently contaminate pharmaceuticals and may result in spoilage of products, separation of emulsions and suspensions, changing in odor, color, taste and stability of products.

Controling of Microbial Contaminations

1. Physical Methods

1.1 Pasteurization

1.2 Sterilization : dry heat or moist heat sterilization

1.3 Low moisture content

1.4 Unfavorable pH

1.5 Low temperature

1.6 Minimum nutrient content

1.7 High osmotic pressure

2. Chemical Methods

Preservatives are used in many kinds of products. They play an essential role in protecting the safety of cosmetics in which very easily contaminated by microorganisms from consumers and also from environment (16, 17). Pavanetto et al (18) studied about talcum used in Italy and found that it was contaminated by bacteria up to 40,000 per gram of talcum and fungi over 2,000 per gram of talcum. The presence of microorganisms in a cosmetic product, at the time of introduction into interstate commerce, would be an adulteration. In the context of toiletry and cosmetic products, pathogenic groups may

include Pseudomonas aeruginosa, Salmonella sp., Escherichia coli and Staphylococcus aureus. Pseudomonas aeruginosa is particularly resistant to many microbial agents and probably represents one of the greatest challenges to preservatives (16). Seung Ho and Foye found that rhodamine and its derivatives have antimicrobial activity to bacteria, fungi, and some parasites and can be used as preservatives to Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Aspergillus niger, and Candida albicans.

In pharmaceutical preparations, preservatives are also used widely. They are listed in USP XX as antifungal and antimicrobial preservatives.

Antifungal preservatives :

- Benzoic acid
- Butylparaben
- Ethylparaben
- Methylparaben
- Propylparaben
- Sodium benzoate
- Sodium propionate

Antimicrobial preservatives :

- Benzalkonium chloride solution
- Benzalkonium chloride
- Benzethonium chloride
- Benzyl alcohol
- Cetylpyridinium chloride
- Chlorobutanol

Phenol

Phenylethyl alcohol

Phenylmercuric nitrate

Thimerosal

Preservatives in Pharmaceutical Preparations

Preservatives are used in multiple dose containers to inhibit growth of microorganisms that may be introduced inadvertently during or subsequent to the manufacturing process or during use by patients. They should not be used solely to reduce viable microbial count as a substitute for good manufacturing practice.

Antimicrobial agents may have preservative properties but also toxic properties to human being. For maximum protection of the consumer, the effective concentration of preservatives in final products should be considerably below the toxic concentrations.

A suitable antimicrobial preservative or mixture of them should posess as many as possible the following properties :

1. Broad Spectrum. It should have wide activity against organisms which have been found to be present in and are undesirable in pharmaceutical preparations, especially the virulent-resistant strains of Pseudomonas aeruginosa. The activity may be bacteriostatic or fungistatic but preferably to be bactericidal or fungicidal.

2. Continuing Activity. The activity should remain under normal conditions of autoclaving, storage, and contamination during use and preferably under less favorable circumstances.

3. Rapid Action. After contaminated during use, the product should be resterilized quickly by the preservatives.

4. Sensitivity. It should be effective in low concentration within wide range of pH and temperature.

5. Nonallergic and Nonsensitizing. It should not have any allergenic or sensitizing tendencies.

6. Nontoxic and Nonirritating. Some preparations may be used frequently, especially ophthalmic solutions. Preservatives used should be nontoxic and nonirritating to ocular tissues in the concentrations and frequencies used and should specifically produce no damage to the corneal epithelium.

7. Compatibility. It should not alter pH and tonicity of system and should be chemically and pharmacologically compatible with other ingredients in the preparation.

8. Stability. It should be chemically stable and undergo discoloration.

9. Inert and Nonvolatile. It should not affect color, odor, and taste of the preparations.

10. Inactivation. It should be inactivation and thus, not interfere in analytical control of the preparation.

11. Solubility. It should be readily soluble in appropiate vehicles.

12. Cost. It should not be too expensive.

Searching for the best antimicrobial preservatives, it is found that no single antimicrobial preservative or mixture of them meets all of the above conditions (19). From theoretical considera-

tion and experimentation, sometimes more than one agent of preservatives are used in pharmaceutical preparations. The reasons for the use of preservative combinations were discussed (14). These are some reasons :

1. The spectrum of activity can be increased.

2. The toxicological hazard can be reduced by using lower concentrations of component preservatives.

3. The development of microbial resistance to one preservative alone may be prevented.

4. The response may exceed prediction from the separate preservative action or from any concentration of one preservative alone.

5. Convenience in use of smaller preservative amounts or economic savings may result.

Factors Affecting Preservative Activities (9, 14, 20, 21, 22, 23)

1. pH, Temperature and Storage Times.

The pH tolerance limit for microorganisms is between pH 2 to 11 and ideal preservative should be effective and stable over pH range of the preparations. It should also be stable at temperatures likely to be encountered during the manufacture and storage life of the product.

For benzoic, dehydroacetic, salicylic and sorbic acids, only undissociated molecules are active against microorganisms and activity is lost with increasing pH. The parabens do not depend on pH like that but show greater activity on acid side of neutrality.

Volatility is a disadvantage of chlorocresol, chloroxylenol and formaldehyde, affecting its activity to be lower at normal storage temperatures.

2. Effect of Oil/Water Partition coefficient

Aqueous solutions are easy to preserve. It is more complicated for cream or emulsion since only a proportion of total amount of preservative is available in the aqueous phase where it is required. The ideal preservative should therefore have a low oil/water partition coefficient.

3. Compatibility with Other Ingredients or Preservatives

Interaction between these ingredients may result in higher or lower activity of preservatives. Tromp, et al (22, 23) studied about preservatives used in ophthalmic preparations. It was found that preservative effect of benzalkonium chloride to Pseudomonas aeruginosa was lower by hydroxypropylmethylcellulose because 7 per cent of 0.5 per cent w/v benzalkonium chloride used was combined with hydroxypropylmethylcellulose. Phenylmercuric nitrate or benzalkonium chloride may show preservative activity more rapidly and with lower concentration if phenethyl alcohol is added. Disodium edetate and phenethyl alcohol can also enhance activity of chlorhexidine acetate and chlorocresol.

Some preservatives, such as sodium benzoate, sorbic acid, methyl and propyl esters of p-hydroxybenzoic acid, are in common use. Many of them, including the parabens, substituted phenols, and

quaternary ammonium compounds are inactivated to some extent when the ratio of nonionics to preservative exceeds certain critical values (14). The parabens in present of polysorbate 80 will lost its activity because of the complex compound formation between them, leaving low concentration of free parabens for acting as preservative. This complex formation is not commom to eliminate by substitution with other substances (20).

4. Compatible with Packaging

Container and closure may have some interaction with preservatives in pharmaceutical preparations(14). The ideal preservative must not be lost by passage through, or binding to, the packaging material. Interaction between preservatives in products and chemicals from containers or closures may happen and lower the activity of such preservatives. Benzyl alcohol may be adsorbed by some rubber closure but this problem may be solved by using butyl rubber closure.

5. Effect of Microorganism Contamination

Microorganisms, such as some species of fungi and Pseudomonas, can destroy the preservative. In this case, if conditions allow the microorganisms to multiply, the product will be spoilage and that organisms may cause pathogenicity. It was found that the parabens using in ophthalmic solution may be used by some fungi and some species of Pseudomonas.

Preservatives in Ophthalmic Preparations

Antimicrobial preservatives are required, except when contraindicated, even if the ophthalmic solution is sterilized by other means such as autoclaving or bacterial filtration. "The use of these chemicals does not alter the need to use very clean and careful technique in making and packaging ophthalmic solutions."(24)

Preservatives are contraindicated for ophthalmic solutions used for intraocular(intracameral) use because most of them are irritating to the anterior chamber lining of the eye. Preparations for use with conjunctival sac of injured eyes are also in this contraindication. These ophthalmic preparations are recommended to be packaged in sterile single-dose containers, then any kind of antimicrobial preservatives is needed.

Types of Antimicrobial Preservatives Used in Ophthalmic Preparations

1. Quaternary Ammonium Germicides

The most commonly used antimicrobial agent in ophthalmic solutions is benzalkonium chloride, often in combination, especially with edetate disodium. It is widely compatible with ophthalmic ingredients but not compatible with anionic drugs, salicylates, nitrates, or nonionic surfactants in high concentration. The concentration of 1:10,000 is most widely used, but the greater concentration may be used, such as 1:3,000, for the special purpose of enhancing penetration of a drug. A 1:1,000 solution is very irritating to human conjunctiva, and produces edema and desquamation.

Other quaternaries used in ophthalmic solutions are 0.025 % benzethonium chloride, 0.02 % cetylpyridinium chloride, and myristyl-gamma-picolinium chloride.

2. Substituted Alcohols and Phenols

Chlorobutanol is effective against both gram-positive and gram-negative microorganisms, including Pseudomonas aeruginosa and some fungi. It is slow acting and broadly compatible with other ingredients. The concentration used is 0.5 %. Ophthalmic solutions containing chlorbutanol should be buffered between pH 5.0 and 5.5 because normally it is decomposed rapidly at high temperature and slowly at room temperature in unbuffered solutions that are neutral or alkaline originally, yielding hydrochloric acid as hydrolysate product.

A combination of chlorobutanol with phenylethyl alcohol is more effective against Pseudomonas aeruginosa, Staphylococcus aureus, and Proteus vulgaris. Other substituted alcohols and phenols used is 0.5 % phenylethyl alcohol, but is very inferior against Pseudomonas aeruginosa.

3. Organic Mercurials

Instead of benzalkonium chloride, phenylmercuric nitrate or acetate in 0.002 % concentration can be used for salicylates and nitrates containing preparations and in solutions of physostigmine and epinephrine salts that contain 0.1 % of sodium sulfite.

Phenylmercuric compounds are slow bactericidal action and

produce sensitization reactions. Ion of phenylmercuric is compatible with halides and forms precipitate with them. Against Pseudomonas aeruginosa, a combination of phenylmercuric nitrate, thimerosal, and sodium metabisulfite, at an acidic pH seems to be effective.

Other organomercurial compound used is thimerosal (merthiolate) which has bacteriostatic and antifungal activity and is used in 0.005 to 0.02 % concentrations in ophthalmic preparations.

4. Parahydroxybenzoic Acid Esters

Methylparaben and propylparaben mixtures may be used in concentration of 0.1 to 0.2 % of methylparaben and 0.04 % of propylparaben. They are not sufficient bacteriostatic agents and also cause ocular irritation.

5. Polymyxin B Sulfate

It is useful against Pseudomonas but the organisms may have resistance to it. Therefore, it is reserved for the treatment of known Pseudomonas aeruginosa infections. Combinations of 0.01 % of benzalkonium chloride and 100 USP Units per ml of polymyxin B sulfate may effective against most resistant strains of Pseudomonas and is nonirritating to eye tissues.

Test Methods of Preservative Efficiency

Most ophthalmic preparations provide an environment for bacterial or fungal growth, unless they are adequately preserved. Common pathogenic groups that may contaminate to the pharmaceutical

products and have to be aware of are Pseudomonas aeruginosa, Salmonella spp., Escherichia coli, and Staphylococcus aureus. Pseudomonas aeruginosa is particularly resistant to many antimicrobial agents and represents one of the greatest challenges to the preservative. Each preservative may posess high antimicrobial activity in some preparations but not in others (16).

As a result, a number of tests have been proposed for measuring the efficacy of preservatives. These differ significantly from the tests which are used in making an initial choice for the preservative assessment. Thus, minimum inhibitory concentration tests (agar diffusion and tube dilution test) and killing dilution tests are quite acceptable for initial selection of preservative systems (25), but they may have little bearing on the activity of preservative displays in the final formulation (12, 26, 27, 28). The final assessment of preservative activity can only come from a study of the way in which an inoculum is reduced when added to the preserved product in the final package (11, 13, 28). This method, the challenge test, is now used widely for testing efficiency of preservatives in pharmaceutical and cosmetic products.

Principle of the method is contaminating the preparation, in its final container if possible, with suitable microorganisms, storing the contaminated preparations in prescribed conditions, and sampling for eneumeration at specific time intervals. Effectiveness of the preservative is interpreted from degrees of lowering in the viable microorganisms of the inoculated samples.

Microorganisms Used

1. Staphylococcus aureus

Staphylococcus aureus is gram-positive cocci. Growth is best under aerobic conditions, but it is facultatively anaerobe; the optimum temperature for growth is from 30° to 37 °C. It is nonmotile and nonspore - forming. A few strains of Staphylococcus aureus form capsules. The organism has ability to produce many extracellular substances such as exotoxin, leukocidin, enterotoxin, coagulase, hyaluronidase, staphylokinase,etc.

Staphylococcus aureus is often found colonizing the nasopharynx. It is commonly found also on the skin but is thought to be contaminants derived from nasal secretions. Pathogenicities from some strains of Staphylococcus aureus are abscess, supperative infections, staphylococcal pneumonia, scolded skin syndrome, toxic shock syndrome, and food poisoning from its enterotoxin. Normally Staphylococcus aureus strains are highly sensitive to most of antibiotics but some strains are resistant to some antimicrobial agents such as penicillins, amikacin, kanamycin, flucloxacillin, and tobramycin. The resistance may be caused by its penicillinase production, or by plasmids transfering from resistant strains of microorganisms (29, 30, 31, 32).

2. Pseudomonas aeruginosa

Pseudomonas aeruginosa is the predominant cause of pseudomonal infections in human. Pseudomonas are strictly aerobic, gram

negative bacilli. Pseudomonas aeruginosa is actively motile, with single polar flagellum. Mucoid strains contain a polysaccharide capsule or glycocalyx.

Pseudomonas aeruginosa grows well on all common culture mediums and most rapidly at temperatures from 30° to 37° C. In common with other pseudomonads, a large number of organic substances are utilized as carbon sources. Pseudomonas aeruginosa resists to many antibacterial agents (5, 33, 34, 35) and is increasingly very important infectious organism for man (36, 37).

Primary infections with Pseudomonas arise in the respiratory tract; urinary bladder; ears; and in burns, wounds, and surgical sites. Pseudomonal pneumonia, often bilateral, is a serious disease. Physical signs, such as cough and sputum, are usually modest, while fever is generally high. Mortality in pneumonia is often marked, approaching 80 per cent in most studies. The frequency of septicemia is high, except in cystic fibrosis patients. Septicemia is also the most serious sequel to other localized infections, with high mortality (32).

3. Escherichia coli

Escherichia coli is gram negative bacilli, grows luxuriantly on ordinary nutrient medium and may be cultivated in the simplest of synthetic medium, i.e., those with an inorganic nitrogen source and glucose. Temperature for growth is in range between 10° to 46° C but 37 °C is the optimum temperature.

Escherichia are common flora of human intestinal tract, and are the predominant members of aerobic bacteria of the bowel. Some strains may be isolated also from infectious urinary tract, septicemia, and endocarditis. Its endotoxin may cause diarrheal disease; and its plasmid transfering is the problem of antibiotic resistance in some microorganisms (32, 38, 39). Several kinds of Escherichia coli infections are urinary tract infections, septicemic infections with sometimes meningitis, diarrheal disease, and nasocomial infection.

4. Aspergillus niger

Aspergillus niger is a black mold, always seen on damp bread, bacon, or organic materials. It is widely distributed. With every breath, we may inhale some conidia of the organism. Most of the time, these conidia are exposed to us without any injury. Under certain conditions in some individuals, this organism may provoke injury to man in one or two ways : allergic response to the presence of its conidia or, much more rarely, invasion of pulmonary or other tissues. The symptoms of aspergillosis is sometimes mistaken by some doctors to be tuberculosis (32).

Because of its widely distribution, Aspergillus niger is always being the representative fungi to be studied for antimicrobial agents efficacy in many kinds of products, such as in woods, optical instruments, food, drugs, and cosmetic products (28, 40).

5. Candida albicans

Candida albicans is the pathogenic yeast. Reproduction is by pinched blastoconidia. The organism may form pseudomycelium or true mycelium, but not form capsules.

The organism is dimorphism. Under faverable conditions of growth in the presence of fermentable carbohydrate, it grows as a budding yeast. Without fermentable carbohydrate and with semi - anaerobic conditions and/or a high nitrogen content, it elongates, forming pseudomycelium and mycelium with blastoconidia and chlamydoconidia (32, 41).

Candida albicans which is responsible for most infectious processes, is endogenous in man. It is part of the normal flora of buccal cavity, large intestine, and vagina. If normal flora are in imbalance, as in overdose of antibiotics or local physiological change, the organism begins to proliferate at a rapid rate and then establishes an infection. Most commonly, it is associated with intertriginous dermatophyte like infection, as well as onychomycosis, foot infection, vaginitis, and thrush. It may also be involved in bronchitis, pneumonitis, and rarely meningitis, endocarditis, and systemic involvement (32).