Chapter 2

MATERIALS, APPARATUS AND METHODS

I. Materials

Materials used in the experiments of entired project were:

Preservatives:

Phenol B.P.	May & Baker Ltd., Dagenham, England
Chlorocresol B.P.	Wright Layman and Umney Ltd., Southwark, London, S.E. 1
Sorbic acid	The British Drug House Ltd., Poole, England
Methyl hydroxybenzoate B.P.	Rhodia Ltd., France
Propyl hydroxybenzoate B.P.	Rhodia Ltd., France
Benzyl alcohol	May & Baker Ltd., Dagenham, England
Phenylethyl alcohol	S.B. Penic and Company New York, Chicago, U.S.A.
Phenylmercuric nitrate B.P.	The British Drug House Ltd., Poole, England

Thiomersal	Keck's formerly Barnes-Hind 430 Post Street, Sanfrancisco, U.S.A.
Benzalkonium chloride	Imperial Chemical Industries Ltd., Macclesfield Cheshire, Gt. Britian
Propylene glycol	May & Baker Ltd., Dagenham, England
EDTA disodium	May & Baker Ltd., Dagenham, England
Salicylamide	The British Drug House Ltd. Poole, England

Other materials:

Arachis oil	Aladin, Wan Fah Long Co. Ltd. Bangkok, Thailand
Liquid paraffin	May & Baker Ltd., Dagenham, England
Sodium chloride	Mallinckrodt Chemical Works, St. Louis, New York, Montreal
Tween 80 (Polyoxyethylene 20 sorbitan monooleate)	Croda Inc., 51 Madison Avenue, Suite 0109, New York 10010

The testing organisms

1. *Escherichia coli* ATCC 10536 obtained from Dumex Co. Ltd., Charoen Nakorn Road, Bangkok, Thailand.

2. *Staphylococcus aureus* ATCC 1538-P obtained from Dumex Co. Ltd., Charoen Nakorn Road, Bangkok, Thailand.

3. *Pseudomonas aeruginosa* obtained from Department of Clinical Microbiology, Faculty of Medical Technology, Mahidol University.

4. *Aspergillus niger* obtained from Applied Scientific Research Corporation of Thailand.

The first three bacterial organisms are maintained in this laboratory by cultivating on nutrient agar slant medium and were subcultured once a week. *Aspergillus niger* was cultivated on Sabouraud agar slant medium and subcultured twice a month. After growth, all of these cultures are kept in $4^{o}C$ refrigerator.

II. Apparatus

1. Spencer Haemacytometer U.S. Pat No. 2,660,091

2. Klett Summerson Photoelectric Colorimeter, model 800-3, serial 26116.

3. Ultraviolet Spectrophotometer, double beam, Pye Unicam SP 1800.

4. Millipore filter, HAWP 047 00,25 ea. HAO 45 μ

5. Clay Adams safeguard centrifuge, Base CT 1004/D.

6. Dialyzer tubing, No. 4465-2, diameter inflated 5/8 inch, width flat, approximate 1 inch. Arthur H. Thomas Company.

III. Methods

1. Determination of Minimal Inhibitory Concentration (MIC)

The procedure employed for determination of the MIC of the preservatives was derived from Cook (21).

The initial ranging for estimation of dilutions was carried out by using a ten fold dilution of the preservatives with each test organism. The experiment was then repeated using a narrower range of dilutions of the preservatives, each experiment was done duplicately for the average value, the controls of uninoculated tubes were also set up. The technique was performed as follows:

1.1 Aseptically prepared various concentration of each preservative in 5 ml nutrient broth.

1.2 Added with a sterile pipette, one drop (approximately 0.02 ml) of a 24 hours standardized culture of the test organism as the inoculum per tube.

1.3 The tubes were incubated at 37°C, 24 hours for bacterial cultures and one week at room temperature for the fungus, and examined for growth by observing turbidity.

1.4 Set up controls of uninoculated tube for each experiment with the same incubation period and condition of the inoculated tubes.

Preparation and Standardization of Inoculum

For bacterial cultures. Inoculated the organism to be tested on the nutrient agar slant, incubated at 37°C for 24 hours, and transferred to a nutrient broth, incubated at 37°C for 24 hours. Determination of the amount of bacterial cell was performed by using Spencer Haemacytometer U.S. Pat. No. 2,660,091, then diluted the broth culture with sterile fresh nutrient broth to obtain 10^6 cells per ml.

For *Aspergillus niger*. Inoculated the fungus on sabouraud agar slant, incubated at room temperature for 4-5 days or until the black spores were formed, washed the spores from the slant culture with sterile nutrient broth and diluted to the absorbance value of 75 on Klett Summerson Photoelectric colorimeter, model 800-3, serial 26116.

2. Determination of Bactericidal Activity Concentration

The modified method of Rideal-Walker test (23) was used to determine the bactericidal activity concentration of all the preservatives in this project.

Preparation and standardization of inoculum

Prepare the broth culture of testing organisms and standardized as the method mentioned in the MIC procedure .

The experiment was carried out as follows:

2.1 Made up 5 concentrations of 5 ml portions of each preservative to be tested in distilled water.

2.2 Added each tube, with a sterile pipette, 5 drops (approximately 0.1 ml) of the 24 hours standardized broth culture of the test organism and shook the mixtures. The culture suspension should be added to the tubes of preservative in succession at definite interval of 1 minute.

2.3 After 5, 10 and 15 minutes respectively, transferred a loopful from each mixture to tubes of 5 ml sterile nutrient broth with proper intervals.

2.4 Incubated the broth tubes at 37°C for 24 hours for bacterial cultures and one week at room temperature for the fungus, observed the result using turbidity as the growth criteria.

2.5 Each test was carried out duplicately.

2.6 Recorded the data and figured out the tube which indicated the highest dilution of preservative that permitted growth in 5 minutes but inhibited growth within 10 minutes as the bactericidal activity concentration for that preservative.

3. Determination of Oil-Water Partition Coefficients

The protocol was set up as follows:

3.1 Prepared 3 concentrations of each preservative to be tested with distilled water to the final volume of 40 ml.

3.2 Transferred the above aqueous solution of preservative to a separatory funnel, then added with 40 ml liquid paraffin (specific gravity 0.87-0.89).

3.3 Shook throughly and set aside for 24 hours with occasionally shaking. Repeated with 2 another concentrations of each preservative.

3.4 Separated the mixture and determined the aqueous phase for preservative content by using a spectrophotometric method (Unicam SP 1800 Ultraviolet Spectrophotometer).

3.5 Calculated the partition coefficients for each of the three concentrations of preservative using equation (3) as mentioned before in the Literature Review section.

3.6 Repeated the experiment using arachis oil in place of liquid paraffin.

Note: The aqueous phase separated from liquid paraffin and arachis oil must be clarified by membrane filter, (Millipore filter, HAWP 047 00, 25 ea. HAO 45μ) reject the first 10 ml.

The wavelength which gave maximum absorption used to determine the aqueous phase of preservatives by Ultraviolet Spectrophotometer was as follows:

Phenol	270 mμ
Chlorocresol	280 mμ
Sorbic acid	254 mμ

Methyl hydroxybenzoate	255 mμ
Propyl hydroxybenzoate	255 mμ
Benzyl alcohol	256 mμ
Phenylethyl alcohol	258 mμ
Phenylmercuric nitrate	257 mμ
Thiomersal	198 mμ
Benzalkonium chloride	261.5 mμ
Propylene glycol	192 mμ
EDTA disodium	192 mμ
Salicylamide	214 mμ

4. Absorption of Preservatives by Bacteria

The measurement of absorption of preservatives was determined using Pseudomonas aeruginosa as the test organism.

Preparation and standardization of inoculum

The inoculum was prepared by inoculating the organism on nutrient agar slant, incubated at 37°C for 24 hours. The slant culture was washed with sterile normal saline (0.9%). The bacterial cell content was determined by using haemacytometer and the bacterial suspension was adjusted to contain 1×10^9 cells per ml.

The experiment was carried out as follows:

4,1 Took 2 ml of the Pseudomonas aeruginosa suspension which contain 1×10^9 cells per ml.

4.2 To it, added 2 ml of a solution of the allocated preservative at a concentration of 40 µg per ml, thus the reaction mixture contained 5×10^8 organism per ml and preservative at a concentration of 20 µg per ml.

4.3 Set aside for 10 minutes after which the absorption should be complete.

4.4 Centrifuged for 10 minutes at 4000 rpm (Clay Adams Safeguard Centrifuge, Base CT 1004/D) to separate the cells from the supernatant, and clarified by filtration through a membrane filter.'

4.5 Assayed for preservative remained in the supernatant using a spectrophotometric method.

4.6 Studied the effect of other material on absorption of preservatives by repeat the experiment in the presence of 5% v/v propylene glycol. Propylene glycol was selected in this study because it was sometimes included in pharmaceuticals at this concentration.

4.7 Studied influence of concentration of preservative on absorption by repeating the above experiment with 4 other series of each preservative concentrations.

5. Interaction between Preservatives and Emulsifying agent

Equilibrium dialysis method by Patel and Kostenbauder (47) was derived to set up the method of assessing the binding between preservatives and nonionic surface active agents

(Tween 80) in this project.

The method depends on the fact that if a mixture of preservative and nonionic substance is placed on one side of a suitable membrane and water on the other side, preservative will diffuse through the membrane into the water compartment until equilibrium is established.

The experiment was carried out as follows:

5.1 Prepared a 20 ml mixture of the preservative to be tested with 5 concentrations of Tween 80 (0.5, 1.0, 2.0, 3.0 and 4.0%) respectively.

5.2 The dialyzer tubing* was used for each concentration, the size of each tubing is uniform.

5.3 Into each tubing, placed the preservative-Tween 80 mixture, and then tightly closed.

5.4 Each tubing was placed in a measuring cylinder containing 20 ml distilled water, stoppered tightly and occasionally agigated for 5 days.

5.5 At equilibrium, determined the concentration of preservative in the water compartment by spectrophotometric method.

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*Dialyzer tubing-Arthur H. Thomas Company, Philadelphia, U.S.A.

Note: The amount of preservative bound to the membrane is often very small, for the sake of simplicity it was being ignored in the current experiments.