Chapter II

MATERIALS AND METHODS

Materials

1. Microorganisms

Following microorganisms* are used :

Staphylococcus aureus (ATCC No. 6538)

Pseudomonas aeruginosa (ATCC No. 9027)

Escherichia coli (ATCC No. 8739)

Candida albicans (ATCC No. 10231)

Aspergillus niger (ATCC No. 16404)

2. Apparatus and Instruments

Following glasswares** and instruments are necessary :

Test tubes and racks

Erlenmeyer flasks

Beakers

Graduated pipettes

Petri dishes

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* Available from American Type Culture Collection, 12031 Parklawn Drive, Rockville, Md. 20852

** All glasswares used are Pyrex® brand

Spectrophotometer (Eppendorf, West Germany)

Autoclave (Hirayama Mfg. Corp., Japan)

Hot air oven (Memmert, West Germany)

Incubaters (Memmert, West Germany)

Hot plate

3. Culture Media*

Culture media for the bacteria is Trypticase Soy Agar

Trypticase Soy Agar Medium**

(Soybean - Casein Digest Agar Medium)

Ingredients per liter :

Bacto - Tryptone	15 g
(Pancreatic Digest of Casein)	
Bacto Soytone	5 g
(Papaic Digest of Soybean Meal)	
Sodium Chloride	5 g
Bacto - Agar	15 g

To rehydrate, suspend 40 grams in 1 liter distilled or deionized water and heat to boiling to dissolve completely.

Sterilize in autoclave for 15 minutes at 15 pounds pressure (121 °C).

Final pH is 7.3 ± 0.2 at 25 °C.

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* Available from Difco Laboratories, Detroit, Michigan, USA.

Culture media for yeast and mold is Sabouraud Agar

Sabouraud Agar Medium**

Ingredients per liter :

Neopeptone, Difco	10 g
Bacto Dextrose	40 g
Bacto Agar	15 g

To rehydrate, suspend 65 grams in 1 liter distilled or deionized water and heat to boiling to dissolve completely.

Sterilize in autoclave for 15 minutes at 15 pounds pressure (121 $^{\circ}$ C). Avoid overheating which could cause a softer medium.

Final pH is 5.6 $\pm$ 0.2 at 25 $^{\circ}$ C.

4. Washing Solutions

Washing Solution A.

Sodium Chloride	0.9 g
Purified Water to	100.0 ml

Autoclave at 15 pounds pressure (121 $^{\circ}$ C) for 15 min.

Washing Solution B.

Polysorbate 80	0.1 g
Sodium Chloride	0.9 g
Purified Water to	100.0 ml

Autoclave at 15 pounds pressure (121 $^{\circ}$ C) for 15 min.

** Available from Difco Laboratories, Detroit, Michigan,USA.

5. Phosphate Buffer pH 7.2

Ingredients per liter :

Monobasic Potassium Phosphate 34 g

Sodium Hydroxide TS

Purified Water to 1,000 ml

Dissolve 34 g of monobasic potassium phosphate in about 500 ml of water contained in a 1000 - ml volumetric flask. Adjust to pH 7.2 ± 0.1 by the addition of sodium hydroxide TS (about 175 ml), add water to volume, and mix. Dispense and sterilize by autoclaving at 15 pounds pressure (121 °C) for 15 minutes. Store under refrigeration.

6. Diluting Fluid

Add 1 g of polysorbate 80 to 100 ml of the phosphate buffer pH 7.2. Dilute with water in the ratio of 1:800, and sterilize by autoclaving at 15 pounds pressure(121 °C) for 15 minutes.

7. Samples of Ophthalmic Solutions Used

7.1 Eye Drops with Antimicrobial Agents

7.1.1 Sulfacetamide Containing Eye Drops:

Sample No. 1 to 8

7.1.2 Chloramphenicol Containing Eye Drops :

Sample No. 9 to 15

7.1.3 Neomycin Containing Eye Drops :

Sample No. 16 to 20

7.1.4 Others :

Sample No. 21 to 23

7.2 Eye Lotions and Other Eye Drops

7.2.1 Boric Acid Eye Lotions :

Sample No. 24 to 29

7.2.2 Eye Lotions with Chlorobutanol :

Sample No. 30 to 34

Sample No. 38 to 41

7.2.3 Eye Lotions with Benzalkonium Chloride

Sample No. 35 to 37

7.2.4 Eye Lotions with Organic Mercurials :

Sample No. 42 to 44

7.2.5 Eye Lotions with Preservatives Combinations :

Sample No. 45 to 50

Formulas of Samples Used

Sample No. 1 :

Each ml contains 0.1 g of Sulfacetamide Sodium.

Sample No. 2 :

Sulfacetamide Sodium 10 % w/v in a buffer

Stabilized vehicle of Methylcellulose and Sodium Thiosulfate with 0.15 % of Chlorobutanol as a preservative.

Sample No. 3 :

Buffered isotonic aqueous solution of Sulfacetamide Sodium. 10 % (in dropper vial with guaranteed seal).

Sample No. 4 & 5 :

Each ml contains Sulfacetamide Sodium 0.1 g.

Sample No. 6 & 7 :

Each 100 ml contains N - Sulfanilylacetamide Sodium 20 g.

Sample No. 8 :

Each 100 ml contains 0.3 g of Sulfacetamide Sodium.

Sample No. 9 & 10 :

Each ml contains 5 mg of Chloramphenicol.

Sample No. 11 :

Each ml contains 1 mg of Chloramphenicol.

Sample No. 12 :

Each ml contains 4 mg of Chloramphenicol in isotonic aqueous solution.

Sample No.13, 14, 15 :

Each ml contains 2 mg of Chloramphenicol.

Sample No. 16 :

Each ml contains:

Dexamethasone Sodium Phosphate
Equivalent to Dexamethasone 21-Phosphate 1 mg

Neomycin Sulphate 5 mg
Equivalent to Neomycin base 3.5 mg

In a non-irritating buffered solution.

Sample No. 17 :

Each ml contains:

Dexamethasone 21 Phosphate Sodium 1 mg

Neomycin Sulphate 5 mg eq. to Neomycin 3.25 mg

Sample No. 18 :

Each ml contains:

Dexamethasone Phosphate	1 mg
Neomycin Sulphate	5 mg
Equivalent to Neomycin base	3.5 mg

Sample No. 19 :

Each ml contains:

Dexamethasone Sodium Phosphate equivalent to

Dexamethasone Phosphate	5 mg
Neomycin Sulphate	5 mg
Equivalent to Neomycin base	3.25 mg

in a non-irritating buffered solution.

Sample No. 20 :

Each ml contains:

Prednisolone-21-phosphate Sodium	4 mg
Neomycin Sulphate	4 mg
Equivalent to Neomycin base	2.6 mg

Sample No. 21 :

Each ml contains:

Bekanamycin Sulphate 5 mg potency

Sample No. 22 :

Each ml contains:

Oxytetracycline (as HCl)	5 mg
Hydrocortisone Acetate	15 mg

Sample No. 23 :

Each ml contains:

Gentamicin (as Sulphate)	3 mg

Sample No. 24 :

Each 100 ml contains:

Acid Boric	2.0 g
Sodium Borate	0.5 g
Zinc Sulphate	0.004 g
Distilled Water ad to	100 ml

Sample No. 25 :

Contains:

Boric Acid	2 %
Zinc Sulphate	0.017 %
Borneo Camphor	0.034 %

Sample No. 26 :

Each 100 ml contains 2.0 g of Boric Acid.

Sample No. 27 & 28 :

Each 100 ml contains 3.0 g of Boric Acid.

Sample No. 29:

Each 100 ml contains:

Zinc Sulphate	0.3 g
Boric Acid	0.3 g
Acriflavin	0.0025 g
Borneol Camphor Water to	100 ml

Sample No. 30 :

Each fl. oz. contains :

Chlorobutanol	65 mg
Zinc Sulphate	16.25 mg
Peppermint Water	10.8 ml
Boric Acid	650 mg

Sodium Chloride	16.25 mg
Berberine Sulphate	8.12 mg

Sample No. 31 :

Each ml contains:

Boric Acid	0. 2 g
Chlorobutanol	0.0033 g
Zinc Sulphate	0. 005 g

Sample No. 32 :

Each 100 ml contains :

Boric Acid	1.0 g
Sodium Borate	0.25 g
Chlorobutanol	0.05 g
Zinc Sulphate	0.004 g
Water to	100 ml

Sample No. 33 :

Each fl. oz. contains:

Zinc Sulphate	15 mg
Acriflavin solution 1:1000	0.6 ml
Boric Acid	600 mg
Chlorobutanol	60 mg

Sample No. 34 :

Each 100 ml contains:

Boric Acid	24.00 g
Zinc Sulphate	0.058 g
Chlorobutanol	0.240 g
Nitrofurazone	0.020 g

Sample No. 35 :

Each 100 ml contains:

Sodium Borate	0.02 g
Boric Acid	1.24 g
Sodium Chloride	0.29 g
Benzalkonium Chloride	2 mg
Boneo Camphor	5 mg
Peppermint Water	5 ml

Sample No. 36 :

Each 100 ml contains:

Boric Acid	1.24 g
Sodium Borate	0.02 g
Sodium Chloride	0.29 g
Benzalkonium Chloride	0.002 g

Sample No. 37 :

Each 100 ml contains:

Boric Acid	720 mg
Sodium Chloride	120 mg
Borax	720 mg
Benzalkonium Chloride	2 drops

Sample No. 38:

Each 100 ml contains:

Boric Acid	2 g
Sodium Borate	0.5 g
Salicylic Acid	25 mg
Chlorobutanol	20 mg
Distilled Water to	100 ml

Sample No. 39 :

Each 100 ml contains:

Distilled Witch Hazel	12.95 g
Boric Acid	2.00 g
Sodium Borate	0.50 g
Allantoin	0.05 g
Salicylic Acid	0.025 g
Chlorobutanol	0.02 g
Zinc Sulphate	0.004 g

Sample No. 40 :

Each 100 ml contains:

Hamamelis Water	1.2 ml
Boric Acid	1.9 g
Borax	0.50 g
Salicylic Acid	0.025 g
Chlorobutanol	0.02 g
Zinc Sulphate	0.004 g
Alcohol	1.75 %v/v
Distilled Water q.s.	100.00 ml

Sample No. 41 :

Each 100 ml contains:

Boric Acid	0.2 g
Zinc Sulphate	0.005 g
Chlorobutanol	0.003 g

Sample No. 42 :

Each 12 ml contains:

Taurine	60 mg
Borneol	6 mg
Camphor	6 mg
Boric Acid	120 mg
Menthol	2.4 mg
Sodium Citrate	78 mg
Sodium Ethylmercurithiosalicylate	0.24 mg
Naphazoline Hydrochloride	1.2 mg
Diphenhydramine Hydrochloride	2.4 mg
Fennel Oil	12 mg
Zinc Sulphate	12 mg

Sample No. 43 :

Each 100 ml contains:

Vitamin A	50,000 IU
Vitamin B_2 (Phosphate)	10 mg
Homosulfamine	3,000 mg
Boric Acid	1,300 mg
Borneol	1 mg
L-Menthol	2 mg
Thimerosal	3 mg

Sample No. 44 :

Each ml contains:

Ephedrine Hydrochloride	1 mg
Boric Acid	12.98 mg
Thimerosal	0.01 mg

Sample No. 45 :

Each 100 ml contains:

Naphazoline HCl	10 mg
Ephedrine HCl	50 mg
Procain HCl	80 mg
Thimerosal	3 mg
Zinc Sulphate	50 mg
Chlorobutanol	20 mg
Aminoethyl Sulfonic Acid	100 mg
Camphor	50 mg
Boric Acid	1,810 mg
Aethyl Parahydroxybenzoate	20 mg
Propyl Parahydroxybenzoate	10 mg

Sample No. 46 :

Each 100 ml contains:

Boric Acid	2 g
Sodium Borate	0.5 g
Chlorobutanol	20 mg
Zinc Sulphate	0.004 mg
Benzalkonium Chloride	0.002 mg
Distilled Water to	100 ml

Sample No. 47 :

Each 10 ml contains:

Zinc Sulphate	25 mg
Phenylephrine HCl	12 mg
Benzalkonium Chloride	0.4 mg
Chlorobutanol	15 mg

Sample No. 48 :

Each 100 ml contains:

Vitamin A (50,000 IU per ml)	0.6	ml
Zinc Lactate	30	mg
Boric Acid	2000	mg
Thimerosal	3	mg
Chlorobutanol	500	mg
Naphazoline HCl	3	mg
Borneol	10	mg

Sample No. 49 :

Each 100 ml contains:

Acid Boric	2.5	g
Zinc Sulphate	0.2	g
Methyl Paraben	0.0225	g
Propyl Paraben	0.011	g
Purified Water to	100	ml

Sample No. 50 :

Each 15 ml contains:

Chondroitin Sulphate	10	mg
Calcium Pantothenate	5	mg
Chlorpheniramine Maleate	1	mg
Thimerosal	0.20	mg
Boric Acid	200	mg
Chlorobutanol	5	mg

Methods

1. Preparation of the Inoculum

a. Subculture the standard strains of microorganisms used on agar slants, using each slant for each organism. The media and conditions of incubation for each organism are shown in Table 1, page 35.

b. Harvest the bacteria and yeast, using Washing Solution A to wash surface growth of each organism into each sterile test tube.

c. Harvest Aspergillus niger, using Washing Solution B to wash black spores from the slant surface into a sterile test tube. Filter through coarse sterile cotton to remove filaments of the fungi that may be included from harvesting. Centrifuge, if necessary, to concentrate the spore suspension obtained.

d. Dilute each suspension of microorganisms from b. and c.

e. Measure turbidity of each dilution from d., using suitable spectrophotometer, at the wavelength of 546 nm. Calibrate the concentrations of each with the turbidities by counting each organism. Use Trypticase Soy Agar Medium for counting the bacteria, and use Sabouraud Agar Medium for counting the yeast and mold. This calibration results will be used for adjusting concentrations of the organisms before use in the preservative effectiveness testing.

f. Adjust each organism to the microbial count of about 1×10^8 organisms per ml.

Use Washing Solution A for each step of dilution.

Table 1

Test Organisms

And the Requirements

Test Organisms	Culture Media	Incubation	
		Temp. (°C)	Time (Hrs.)
Staphylococcus aureus ATCC No. 6538 Pseudomonas aeruginosa ATCC No. 9027 Escherichia coli ATCC No. 8739	Trypticase Soy Agar Medium	30 - 35	18 - 24
Aspergillus niger ATCC No. 16404	Sabouraud Agar medium	20 - 25	7 Days
Candida albicans ATCC No. 10231	Sabouraud Agar medium	20 - 25	48 Hrs.

2. Test Procedure

a. Clean outer surface of five sample containers with 70% - alcohol or other appropiate antiseptics.

b. Open each container, avoid contamination.

c. Inoculate each container with the standardized organisms, using each organism for each container, not the mixed cultures. Use a ratio equivalent to 0.10 ml of inoculum to 20.0 ml of sample.

d. Mix the inoculated samples thoroughly.

e. Count for viable organisms from inoculated samples immediately after the inoculation and mixing, using plate method for counting.

f. Calculate the initial concentrations of each organism per ml of the sample.

g. Incubate the inoculated samples at the conditions prescribed by the manufacturers. If no storage temperature is specified use room temperature for incubation.

h. Sampling each inoculated sample again at time intervals of 7, 14, 21, and 28 days of incubation period, to check for viable count.

i. Calculate the percentage change in microbial concentration during the test if any growth appears. Record any changes observed from each container during the test period.

j. Repeat the test again from a. to i., using new five containers of the sample. So that, ten containers of each sample are required for "Testing Procedure".

3. Plate Method for Counting Viable Organisms

To count the viable organisms, sampling 1.0 ml of the test solutions and dilute with the "Diluting Fluid" to appropiate dilutions. Use 0.1 ml of the dilutions to spread on agar surface of the medium used for each strain of organisms. Duplicate the plate counting for each dilution. Incubate the agar plates with conditions shown in Table 1, page 35. Count the colonies formed on each agar surface. Average the duplicate plate counts.

In case of eye drops with antimicrobial agents, eliminate antimicrobial effects in sample solutions by filtering through the Millipore filter paper and wash the filter with three 10-ml portions of Diluting Fluid. Use this filter paper to make dilutions for counting viable organisms.

4. Interpretation of Results

A product sample may be considered to be effectively preserved if these three conditions are adopted:

a. The concentrations of viable bacteria are reduced to not more than 0.1 % of the initial concentrations within fourteen days.

b. The concentrations of viable yeast and mold remain at or below the initial concentrations during the first fourteen days.

c. There is no subsequent increase of each organism concentration during the remainder of the twenty eight days test period.

5. Testing for the Diluted Samples

If the sample in second group of ophthalmic solutions, eye lotions and eye drops without antimicrobial agents, meets the test, continue the test for diluted one:

Dilute the sample, uninoculated, to 1:2 and 1:10 dilutions. Use these dilutions as new samples and perform the test again as in the "Test Procedure".

To dilute the samples, use Solution A, the 0.9 % sterile sodium chloride solution.