CHAPTER II

EXPERIMENTAL

MATERIALS

The following materials obtained from commercial source were used as received.

1. Chloramphenicol base (EGIS Phamaceuticals, Hungary, Batch no.548290991)
2. 2-hydroxypropyl-β-cyclodextrin (Aldrich Chemical, Germany, Lot no. 02709PZ, 04320EF)
3. Borax AR Grade (Merck, Lot no. 310K2147303)
4. Boric acid AR Grade (Merck, Lot no.547K1033265)
5. Phenylmercuric acetate AR Grade (Merck-Schuchardt, Lot no.3201427)
6. Propyl paraben (Supplied by Srijun, Lot no.VB19/1)
7. Methanol HPLC (J.T. Baker Inc., USA.)

APPARATUS

1. Analytical balance (Sartorius, Model A200s, Germany)
2. Top to bottom rotator
3. Corn wall syringe
4. Spectrophotometer (Bausch&Lomb, Spectronic-2000, U.S.A.)
5. Shell-freezer (Just-A-Tilt shell freezer, Model SF-4, U.S.A.)
6. Freeze-dryer (Dura-Dry, Model FD-6-85 DMPO, Japan)
7. FTIR Spectrophotometer (Perkin Elmer, Model 1760X, U.S.A.)
8. Differential thermal analysis (Shimadzu, Model DT-30, Japan)
9. X-ray diffractometer (Joel, Model JDX 8030, Japan)
10. pH meter (Pye, Model 292, England)
11. Viscometer (Haake, Model RV 20, Japan)
12. Osmometer (Osmomat, Model 030-DM, Germany)
13. Vortex mixer (Vortex Genie-2, Model G-560E, U.S.A.)
14. High Performance Liquid Chromatography
    Pump : Millton Roy, Model CM4000,U.S.A.
    UV Absorption detector : Milton Roy, Model SM 4000, U.S.A.
    Integrator : Milton Roy, Model 4100, U.S.A.
    Column : Bonclone 10 C_{18}, size 300x3.9 mm, U.S.A.
    Microsyringe : 100µl, Unimetrics, U.S.A.

METHODS

Determination of Maximum Absorption Wavelength of Chloramphenicol, Chloramphemicol : 2-HP-β-CD and 2-HP-β-CD

Preliminary study to determine the maximum absorption wavelength of chloramphenicol, chloramphenicol : 2-HP-β-CD were perfomed by UV absorption scanning at a wavelength in the range of 400 - 200 nm (The Bausch & Lomb, New York, USA.). It is necessary to find out the absorption of chloramphenicol, chloramphenicol : 2-HP-β-CD and 2-HP-β-CD to perform a suitable wavelength for UV absorption method in phase solubility study. A concentration of 25.0 μg/ml chloramphenicol in water and 2-HP-β- CD solution and 77.28 μg/ml of 2-HP-β-CD were prepared for this purpose. The absorption spectra of three solutions were evaluated to quantitate the amount of chloramphenicol.

Phase Solubility Analysis

1. Solubility determination

Solubility measurements were caried out and the stability constants (k_C) of the complex were determined according to the phase solubility method of Higuchi and Connors (1965). An exess amount of chloramphenicol (250 mg) was added to the screw-cap tubes containing aqueous solution of various concentrations of 2-hydroxypropyl-β-cyclodextrin. These tubes were continuously rotated in the top to bottom rotator at room temperature (27±1°C) and used fresh distilled water as a medium. After equilibrium was attained, approximate 24 hours, an aliquot was filtered through a 0.45 μm membrane filter. A 1 ml of aliquot of this clear solution was diluted with distilled water to make up suitable concentration. Concentration of dissolved drug was determined by UV-absorption spectrophotometer at 279 nm against distilled water as the blank.

The concentrations of soluble chloramphenicol were determined from standard curve and the molarity of soluble chloramphenicol in each solution was calculated in moles per litre.

2. Calibration curve of chloramphenicol

Chloramphenicol base 10 mg was accurately weighed (Satorious, Germany) and dissolved in distilled water. The solution was then adjusted to 100 ml with distilled water and used as stock solution.

The stock solution was individually pipetted 1, 2, 3, 4, 5, 7.5, and 10 ml into 50 ml volumetric flask and diluted to volume with distilled water. The final concentration of each solution was 2, 4, 6, 8, 10, 15, and 20 μg/ml, respectively.

The absorbance of known drug concentration was determined using UV absorption spectrophotometer in a 1-cm cell at 279 nm. Distilled water was used as a blank. Each concentration was determined in duplicate.

Preparation of Chloramphenicol : 2-HP-β-CD Solid Complexes

Following the phase solubility study, chloramphenicol solid complex was prepared by dissolving the appropriate amounts of chloramphenicol and 2-HP-β-CD in distilled water giving a molecular ratio of 1 : 2 (chloramphenicol : 2-HP-β-CD). The solution was agitated for 24 hours and then freeze-dried (Dura-Dry, model FD-6-85 DMPO, Japan).

Freeze-drying procedure

The chloramphenicol : HP-β-CD solution was filtered through a membrane filter (pore size 0.45 μm). Two millilitres of this solution was pipetted into 10 ml screw caped tubes and then placed onto a pair of moving belts in a shell-freezer (Just-A-Tilt, Model SF-4, U.S.A.) containing ethanol. The inclination of the reservoir was set to 5° for adjustment of tube angle and shell thickness uniformity. The temperature of ethanol reservoir was about -40° to -38°C. The rolling action between the tubes and the belts caused the refigerated ethanol to contact and ride with the outer wall of the tube while the chloramphenicol : 2-HP-β-CD solution froze to the inner wall in the form of a shell. The chloramphenicol : 2-HP-β-CD which was shell-frozen to its largest area would give a minimum thickness of the interior wall of a tube and would freeze-dry at the fastest and uniform rate.

The shell-frozen samples were dried in a vacuum using a Dura-Dry at a temperature -40°C and the pressure is about 2,500 - 1200 mT for 8 hours until there is no condensate on the outside of the tube and the tube temperature was conditioned at room temperature. Then, they were stored in a desiccator.

Detection of the Chloramphenicol : 2-HP-β-CD Inclusion Complex Formation in the Solid State

Inclusion complex formation of chloramphenicol was determined by the method described below :

1. Infrared spectrometry

Infrared spectra were examined by using a Fourier transform infrared spectrometer (Perkin Elmer, model 1760 X, USA.) and KBr disc.

2. Differential thermal analysis

DTA curves of chloramphenicol, 2-HP-β-CD and solid complex were obtained by using a thermal analyzer (model DT-30, Shimadzu, Japan) with a heating rate of 10°C/min in static air atmosphere.

3. Powder X-ray diffraction

X-ray diffractograms of samples were examined by the reflexion method with nickle-fittered CuKα radiation of Jeol diffractometer (model JDX 8030, Japan) operated in the ω-2θ scanning mode between 5° and 85°.

Preparation of Chloramphenicol Eye Drops

Reconstituted chloramphenicol powder for eye drops formulation in this study was modified from chloramphenicol eye drops BPC 1973 shown below :

Chloramphenicol	0.5	g
Phenylmercuric acetate	0.002	g
Borax	0.3	g
Boric acid	1.5	g
Purified water to	100	ml

Two formulations of reconstituted powder for eye drops of chloramphenicol containing 2-HP-β-CD were according to the composition as presented in Table 6.

Table 6 Composition of reconstituted powder for eye drops of chloramphenicol : 2-HP-β-CD.

Composition	Formula I (g)	Formula II (g)
Chloramphenicol } *	0.05	0.05
2-HP-β-CD } *	0.42	0.42
Borax	0.03	-
Boric acid	0.15	-
Phenylmercuric acetate	0.0002	-
vehicle for reconstition	Water for injection (10 ml)	Borax, Boric acid, Phenylmercuric acetate (10 ml)

* Chloramphenicol : 2-HP-β-CD inclusion complex

Formulation I was prepared by dissolving the amounts of chloramphenicol, 2-HP-β-CD, borax and boric acid in distilled water. The solution was agitated for 24 hours, sterilized by filtration through 0.45 μm membrane filter and then freeze dried by the method described above. The vehicle of this formulation was water for injection. The reconstituted powder (Formula I) for eye drops was reconstituted with water for injection before stability study.

In the case of Formula II, it was prepared by dissolving the amount of chloramphenicol and 2-HP-β-CD in distilled water. The solution was agitated for 24 hours, sterilized by filtration through 0.45 μm membrane filter and then freeze dried by the method described above. The vehicle of this formula was the solution of borax, boric acid and phenylmercuric acetate. The reconstituted powder (Formula II) was reconstituted with its vehicle before stability studying.

During stability study of Formula I and II, other two formulations were prepared for stability comparison.

a. Chloramphenicol : 2-HP-β-CD complex solution was prepared by the same amount of compositions and method without freeze drying as reconstituted powder for eye drops (Formula I).

b. Chloramphenicol eye drops BPC 1973 as shown above was prepared by dissolving boric acid, borax and phenylmercuric acetate in sufficient purified water with the aid of heat. The temperature of the solution was adjusted to 60°C and maintained at 60°C until chloramphenicol was added and dissolved. The sufficient purified water was added to produce the required volume. Then, the solution was sterilized by filtration (0.45 μm membrane filter).

Physical Properties Determinations

All formulations, reconstituted powder (Formula I and II) for eye drops after reconstitution with their vehicles, chloramphenicol : 2-HP-β-CD complex solution and chloramphenicol eye drops BPC 1973, were measured for their physical properties such as reconstitution time (reconstituted powder), clarity, viscosity, pH, and tonicity at room temperature. In the case of reconstituted powder for eye drops, they were also measured that physical properties at 45 °C and 75 % RH. The physical properties were measured before and during keeping for 4 months. The measurements were as follow :

1. Reconstitution time was recorded by shaking.

2.Viscosity was measured by Brookfield viscometer (HAAKE, model RV 20, Japan).

3. pH was measured by pH meter (Pye, model 292, England).

4. Tonicity was measured by osmometer (Osmomat, model 030-DM, Germany).

Stability Studies

1. Condition of stability study

Stability studies of chloramphenicol eye drops of Formula I and II were carried out in both powder form and solution. In the case of solution, the investigated preparations were reconstituted powder (Formula I and II) for eye drops after reconstitution with their vehicles, chloramphenicol : 2-HP-β-CD complex solution and chloramphenicol eye drops BPC 1973. In the case of solid state, the investigated preparations were reconstituted powder (Formula I and II) for eye drops.

2. Stability of reconstituted powder for eye drops, chloramphenicol complex solution and chloramphenicol eye drops BPC 1973 at 65 °, 55 °, 45 °, 37 °C and room temperature

The formulations were incubated at 65°, 55°, 45°, 37°C and room temperature (25°C) and sampling (n = 2) at determined times. The remained concentration of chloramphenicol and in each sample was immediately determined using condition which described in 4.

3. Solid state stability of reconstituted powder for eye drops of chloramphenicol

Formula I and II in powder forms were kept for 4 months in desiccator that had relative humidity (RH) controlled at 75 % by using saturated sodium chloride in a 45°C incubator. The remaining concentration of chloramphenicol solid complexes in each formula was determined using HPLC.

4. Assay procedure

The isocratic reverse-phase technique was used for quantitative analysis of chloramphenicol during stability testing. The condition of analysis was as follows :

Pump : Multi solvent delivery system, Milton Roy model CM 4000, Milton Roy, LDC division, Florida, USA.

UV absorption detector : Programmatic wavelength detector, Milton Roy model SM 4000, Milton Roy, LDC division, Florida, USA.

Integrator : Computer Integrator, Milton Roy model 4100, Milton Roy, LDC division, Florida, USA.

Microsyringe : 100 µl Unimetrics, Storewood, Illinois USA.

HPLC system was set to various parameter for analysis as follow :

Column : Phenomenex, Bondclone 10 C18 size 300 x 3.9 mm, USA.

Mobile Phase : methanol : H_2O (60 : 40)
Internal standard : propyl paraben
Injectable volume : 20 µl
Flow rate : 1 ml/min
Pressure : 2500 psi
Chart speed : 2 mm/min

The mobile phase was freshly prepared, consisted of mixture of 60 % methanol and 40 % distilled water. The mixture solution was filtered through 0.45 μm membrane filter and then degassed by sonication for 45 min prior to use.

5. Preparation of calibration curves of complex solution and chloramphenicol

The calibration curves of complex solution and chloramphenicol within the concentration range of 10-30 μg/ml was constructed. Standard solutions containing 10, 15, 20, 25 and 30 μg/ml of complex solution or chloramphenicol were prepared using propyl paraben (5 μg/ml) as internal standard in each dilution. A 20 μl standard solution were injected into HPLC and the calibration curves were made by plotting the ratio of peak areas under curve of complex solution or chloramphenicol and propyl paraben against the concentration of complex solution or chloramphenicol (μg/ml).

6. Determination for Arrhenius Equations

The linear regression analysis was used to determine the order of reaction rate. From these degradation curves, calculated the degradation rate constants (k) from slopes of each linear line.

The degradation rate constants (k) of each formula at 65°, 55°, 45°, 37°C and room temperature (25°C) were taken into natural logarithm (ln k). The ln k and reciprocal absolute temperature (1/T, $^{o}K^{-1}$) were plotted for Arrhenius plot. The simple linear regression was calcucuted. The Arrhenius equations were achieved. The Arrhenius plots were shown in Figure 23-26.

7. Calculation for Heat of Activation (Ea)

The slopes taken from Arrhenius plots were used in order to calculate the heat of activation (Ea).

8. Calculation of stability at room temperature (25°C) and 8°C

From Arrhenius equations, the natural logarithms of degradation rate constants (ln k) at 25°C and 8°C were calculated. Then the ln k_{25} and ln k_8 were converted to k_{25} and k_8, respectively (Appendix I).

Since the content of chloramphenicol eye drops BP 1993 was limited between 90-110% labelled amount. The shelf-life values of chloramphenicol and its complexes were also calculated according to this specification described in Appendix I.

In the same way, the apparent shelf-life values were calculated by using the degradation rate constants which was determined from the degradation curve at room temperature (25°C).

Antimicrobial Activity Test of Chloramphenicol and Complex by Agar Diffusion Method

The method employed was modified after the official method described in Code of Federal Regulation and the outlined in the USP XXII.

1. Reagents and apparatus

Plate : A 100 mm diameter flat-bottom petriplate.

Cylinder cups : Stainless steel cylinders having an outer diameter of 8 mm (±0.1 mm), an inner diameter of 6 mm (±0.1 mm), and a length of 10 mm (±0.1 mm).

Test organisms : The test organism was *Micrococcus luteus* ATCC 9431, maintained by culture on fresh sterile slants of antibiotic medium I (Difco) at 37°C for 24 hours.

Media : Medium I (Difco Laboratories, Detroit Michigan, U.S.A. Lot No. 0263-01-1)

Peptone	6.0 g
Pancreatic digest of casein	4.0 g
Yeast extract	3.0 g
Beef extract	1.5 g
Dextrose	1.0 g
Agar	15.0 g
Distilled water qs	1000.0 ml

Final pH 6.5 - 6.6 after sterilization

Sterile 0.9 % normal saline test solution :

These mentioned solutions should be prepared from sodium chloride AR grade and sterilized by autoclave at 15 lbs/inch^2 for 15 min.

Antibiotic assay solutions :

Solution I (1 % Potassium Phosphate bufter pH 6.0)

Dibasic potassium phosphate	2.0	g
Monobasic potassium phosphate	8.0	g
Distilled water qs.	1000.0	ml

Adjust with 18 N phosphoric acid or 10 N potassium hydroxide to yied a pH of 5.95 to 6.05 after sterilization.

2. Preparation of inoculated plates

Prepare the base layer by adding 21 ml melted agar to each petri dish. Distribute the agar evenly in each dish on a flat level surface. After the agar harden, place a cover on each plate.

To prepare the seed layer, the organism was washed from the agar slant with 3 ml of sterile normal saline solution. The suspension was standardized so that 1 : 10 dilution with sterile normal saline solution would give 10 % light transmittance at 580 nm. 1 ml of this inoculum was added to 100 ml of melted Antibiotic Medium I cooled to 45°-50°C. Swirl the flask to obtain a homogenous suspension, and added 4 ml of the inoculated media to each of the plates containing the base agar. Spread evenly over the agar surface, cover, and allow to harder on a flat,level surface for 15 min. After the agar has hardened place 6 cylinder cups on the agar surface by sterile forcep so that they are at approximately 60° intervals on a 2.8 centrimeter radius.

3. Preparation of standard solution

Chloramphenicol working standard of 98 % potency was used. 100 mg (do not dried) standard was dissolved in ethanol to make a stock solution containing 10,000 μg/ml. For the preparation of standard curve, stock solution was diluted with potassium phosphate buffer pH 6 to a final concentration of 32, 40, 50, 62.5 and 78.1 μg/ml.

4. Preparation of sample solution

The powder of chloramphenicol and complex should be prepared at 50 μg/ml (the mid-value in sterile phosphale buffer pH 6). These solutious should be freshly repared for the day of assay.

5. Procedure for assay

For the standard response line, at five levels concontrations (32, 40, 50, 62.5 and 78.1 μg/ml) used a total of 12 plates, three plates for each response line solution, expect the reference concentration solution, 50 μg/ml was included on each plate. On each set of three plates, filled three alternate cylinders with the reference concentration solution, 50 μg/ml and the other three cylinders with the concentrations of the response line. Thus there would be 36 reference concentration zones of inhibition and nine zones of inhibition for each of the four other concentration of the response line. For each sample tasted (chloramphenicol and complex solution) used three plates. Filled three alternate cylinders on each plate with 50 μg/ml standard reference concentration solution and the other three cylinders with the sample concentration solution.

6. Incubation

As soon as the cups were filled, the plates were incubated at 37°C for 24 hours.

7. Measurement of response

The diameter of inhibitory zones were measured with vernier callipers. Then, plotted curve for standard response curve, calculated the % relative potency of chloramphenicol and complex. The resulting data were shown in Table 31.

Irritability Test of Chloramphenicol and Chloramphenicol : 2-HP-β-CD Eye Drops in Rabbits.

These experiments were designed to study the irritability of chloramphenicol and chloramphenicol : 2-HP-β-CD eye drops using albino rabbits (n = 4) as test animal. Albino rabbits that weighed between 2 - 4 kg should be maintained to exclude sawdust, wood chips or other extraneous materials that might produce eye irritation. Both eyes of each animal in the test group should be examined before testing and only those animals without eye defects or irritation should be used.

An approximate volume of 0.05 ml (2 drops) of chloramphenicol and chloramphenicol : 2-HP-β-CD eye drops were instilled into conjunctival sacs

of rabbit eye by gently pulling the lower lid away from the eyeball to form a cup. The test solutions were dropped every 2 hours, 3 times a day (10.30 a.m. 12.30 and 14.30 p.m.) and 3 days a week (Monday, Thusday and Wednesday) for 6 weeks and compared to the effects of 0.9 % sodium chloride injection (n = 2).

An animal should be considered as exhibiting a positive reaction if the test solutions produced

- ulceration of the cornea
- opacity of the cornea
- inflammation of the iris
- swelling with partial eversion of the lids
- altered pupillary light reflex