

CHAPTER III

EXPERIMENTAL METHODOLOGY

Materials

All chemicals were of analytical or pharmaceutical grade and were used as received.

Minoxidil USP (lot No. 00141), Transo Pharm., Germany

β -cyclodextrin (β -CD), MW. 1135 (lot No. 23102), Rama Production Co., Ltd.

Hydroxypropyl- β -cyclodextrin (HP- β -CD) Average MS = 0.6, MW. 1380 (lot No. 04811 BG), Aldrich Company, Inc., USA

Prednisolone (lot No. not known), Upjohn, USA

Propylene glycol AR grade, Farmitalia Carlo Erba, Italy

Disodium Edetate (EDTA sodium), Merck, Germany

Ethanol USP.

Methanol HPLC grade, Mallinckrodt, Germany

Triethylamine, Merck-Schuchardt, Germany

Glacial acetic acid HPLC grade, J.T.Baker Inc., USA

Disodium hydrogen phosphate anhydrous AR grade, Merck, Germany

Potassium dihydrogenphosphate AR grade, Merck, Germany

Sodium chloride AR grade, Merck, Germany

Regaine[®] (batch No. HDS 20, Mfd. 25/8/1994, Exp. 25/8/1996), Olic (Thailand) Ltd.

Membranes

Newborn pig skin was kindly donated from a local farm in Nakorn Prathom province.

Equipments

Modified Franz diffusion cells, FDC-400 Crown Glass Company, Inc., USA

UV spectrophotometer, Model 7800, Jasco Corporation, Japan

pH meter, Model 420A, Orion, USA

Analytical balance, Sartorius GMPH, Germany

Top to Bottom Rotating shaker, thermostatically controlled

Ultrasonic bath Model 3210E-MTH, Branon, USA

Hot air oven, Memmert^R, Germany

High Performance Liquid Chromatography (HPLC) equipped with

a tunable absorbance detector, Model 484, Serial No. 484-PRA 902,
Waters, USA

a 20 microlitre injector, Rheodyne

a constant flow pump, Waters 510 HPLC pump, Millipore, USA

an integrator, Waters 745B Data Module, Serial No. 7BE /400678,
USA

an autoinjector, Waters 712 WISP, Serial No. 712-007617, Millipore,
USA

a Spherisorb HPLC cartridge column phase separation S5 ODS2 (250
x 4.6 mm) 5 μ m, Phase Separation Inc, USA

Methods

1. Solubility studies

Solubility measurements were carried out according to method of Higuchi and Connors (1965). Minoxidil in ten times excess its solubility was added into a series of screw capped tubes. Various amounts of β -CD or HP- β -CD were added, then 5 ml of water was added to each tube. These tubes were sealed and rotated in the Top to Bottom Rotator at 30°C for 2 days. After solubility equilibrium was attained, an aliquot volume was centrifuged and filtered through Sartorius 0.45 μ m membrane filter and then was diluted with distilled water to make up a suitable concentration. An analysis was done with spectrophotometer at 229 nm against distilled water as the blank.

The concentrations in molarity of soluble minoxidil were determined from standard curve. Phase solubility diagram was constructed, stoichiometric ratio and apparent formation constant (K_c) were calculated.

From the phase solubility diagram, the appropriate concentration of β -CD or HP- β -CD was selected to use in the preparation of minoxidil-CDs solid complexes and in the formulations.

2. Preparation of minoxidil-CDs solid complexes

The solid complex of minoxidil with β -CD was prepared by mixing appropriate amounts of minoxidil and β -CD in water which were derived from the descending portion of the phase solubility diagram (Figure 7). That was, minoxidil 0.3139 g (0.15 M) and β -CD 1.7025 g (0.15 M) were added into 10 ml of purified water, and stirred by magnetic stirrer at room temperature for 2 days. The complex,

which precipitated as white microcrystalline powder, was filtered and dried at room temperature for 2 days.

The complex of minoxidil and HP- β -CD was prepared by using the same condition. Minoxidil 0.3139 g (0.15 mol/l) and HP- β -CD 2.0700 g (0.15 mol/l) were dissolved in 10 ml of purified water. In case of minoxidil-HP- β -CD complex, there was no powder precipitated out of the solution after 2 days. Therefore, the freeze-drying technique was used to obtain the complex powder.

3. Investigation of the minoxidil-CDs complexes

3.1 Differential scanning calorimetry (DSC)

The DSC technique was used to investigate melting points of minoxidil, β -CD, HP- β -CD, the complexes and the physical mixtures of minoxidil with both CDs. The powder of each compound was accurately weighed and put into the equipment using following conditions.

Sample size	=	7-14 mg
Heating rate	=	20°C per minute
Atmosphere	=	N ₂ , 15 ml per minute

3.2 Infrared spectrophotometry

The IR spectra of minoxidil, β -CD, HP- β -CD and the complexes of minoxidil with both CDs were performed utilizing potassium bromide (KBr) disc method.

4. Formulation of minoxidil solutions

From the solubility studies, β -CD 0.1 %w/v or HP- β -CD 5 %w/v was used as the beginning concentration in the solutions containing minoxidil 2 %w/v. Propylene glycol, ethanol USP and purified water were used as cosolvents. The suitable proportion of these cosolvents was estimated by adding various proportions of solvent components with 2% minoxidil in screw capped tubes and storing the mixtures at room temperature for 48 hours and in a refrigerator 48 hours. The formulations selected must give clear solution.

5. Effects of CDs on minoxidil solutions

To study the effects of CDs on minoxidil solutions, β -CD and HP- β -CD were added in solutions. Amounts of cosolvents used in the formulation were obtained from the above study. In case of β -CD, the concentrations were varied in a short range due to its limited solubility from 0, 0.1, 0.4 and 0.7 %w/v. On the other hand, HP- β -CD could be added in higher concentrations from 0, 5.0, 10.0, 15.0 and 20.0 %w/v.

5.1 Stability studies

All solutions were filled and sealed in 2-ml amber glass ampoules and kept at room temperature and in a hot air oven at 70°C. At appropriate time intervals, three ampoules of each formulation were sampled for analysis of minoxidil remaining by HPLC method. The curves of minoxidil concentration versus time were plotted by first order approach and then appropriate rate constants were determined from the plots.

5.2 In vitro permeation studies

5.2.1 Experimental conditions

Steady state fluxes of minoxidil through newborn pig skin were determined using the following conditions.

Donor solutions : minoxidil solutions containing various concentrations of β -CD or HP- β -CD or the commercial solution (Regaine[®])

Receiving solutions : Isotonic phosphate buffer pH 7.4 which consisted of

Monobasic potassium phosphate	0.190 g
Disodium hydrogen phosphate	0.810 g
Sodium chloride	0.411 g
Distilled water to	100 ml

Membranes : Newborn pig skin

Permeation cells : Modified Franz diffusion cells with an internal diameter of 1.30-1.59 cm corresponding to an effective permeable area of 1.33-1.99 cm². Receptor volumes were varied from 12.39-12.75 ml.

5.2.2 Preparation and treatment of membranes

Excised full thickness skin was used for permeation studies. A piece of abdominal skin of a newborn pig was carefully excised. Subcutaneous fat and other extraneous tissues adhering to the dermis were completely removed and trimmed using a forcep and scissors. The skin was cleaned and bathed in a receiving solution. The skin was then cut into a circular shape with a diameter of about 3 cm,

wrapped in an aluminium foil and stored in a freezer until used. The frozen skin was thawed and allowed to rehydrate by immersing in the receiving solution overnight in a refrigerator and then at room temperature for 15 minutes before use. Its thickness was measured at 12 different points using a Teclock micrometer. An average of the 12 values were used for subsequent calculations.

5.2.3 Permeation experiments

The excised newborn pig skin was mounted and clamped in place between the donor and the receptor compartments of a permeation cell with the stratum corneum facing the donor compartment. The receptor fluid was filtered under vacuum prior to use. The receptor compartment was continually stirred with a magnetic stirring bar rotating at 600 ± 5 rpm with synchronous and maintained at 37°C by circulated water which was pumped through a jacket surrounding the cell body throughout the permeation studies.

The membranes and the receptor fluid were equilibrated and warmed for 15 minutes. After equilibration, a 200 microlitre portion of minoxidil solution was applied on top of the membranes. Each donor solution was run in triplicates.

At appropriate time intervals, 6 ml of receiving solution was drawn using a syringe fitted with a long needle through the sampling side arm of receptor cell. The volume of the receptor compartment was immediately replaced with an equal volume of fresh receiving solution and the run was continued.

At the completion of each experiment, all minoxidil concentrations in the receiving solution withdrawn were determined by HPLC method. The permeation amount was calculated by multiplying the drug concentration by the receptor volume. For each membrane, the cumulative

permeation amount per unit area was plotted versus times; the slope and the intercept of the steady portion were derived by the regression analysis. The observed steady state flux (J_{ss}) was the slope which was also expressed as a normalized flux ($J_{ss} \times h / \bar{h}$), where h is the membrane thickness and \bar{h} is the average thickness of all membrane used in the studies.

6. HPLC assay

6.1 HPLC conditions for minoxidil analysis

The high pressure liquid chromatography technique was used for analysis of minoxidil. The system consisted of a constant flow pump, a variable wavelength UV detector, an integrator and a volume sample injector with a 20 microlitre loop. The conditions used for analyzing minoxidil by HPLC method are presented as follows

Column	: Spherisorb S5 ODS2 (250 x 4.6 mm) 5 μ m
Mobile phase	: a mixture of 60 % v/v methanol, 1 % v/v glacial acetic acid, 0.1 % v/v triethylamine and purified water to 100 % v/v, adjusted to pH 3 with perchloric acid.
Detection wavelength	: 254 nm
Flow rate	: 1 ml/min
Attenuation	: 4
Chart speed	: 0.1 cm/min
Injection volume	: 20 microlitres
Internal standard	: prednisolone 0.48 mcg/ml
Retention time of minoxidil	: 4.65-4.93 min

Retention time of : 8.38-9.13 min
prednisolone
Standard solutions : 0.020, 0.060, 0.100, 0.200, 0.400, 0.600,
0.800, 1.000 mcg/ml

6.2 Preparation of standard solutions

Stock solution of Prednisolone :

A stock solution of prednisolone was prepared by accurately weighing 100 mg of prednisolone into a 50-ml volumetric flask. Methanol was added to dissolve and adjust to volume. An aliquot of 0.6 ml was pipetted and transferred into a 50-ml volumetric flask and was further adjusted to volume by the mobile phase so that the final concentration was 24.0 mcg/ml

Stock solution of minoxidil :

A stock solution of minoxidil was prepared as described. One-hundred milligrams of minoxidil was accurately weighed into a 25-ml volumetric flask. Methanol was added to dissolve and adjust to volume. An aliquot of 0.5 ml was pipetted and transferred into a 10-ml volumetric flask. The mobile phase was added to volume, and then 0.5 ml of this diluted solution was pipetted and transferred into a 50-ml volumetric flask and adjusted to volume with the mobile phase. The final concentration of this standard solution was 2.0 mcg/ml.

Standard solutions :

The stock solution of minoxidil was pipetted (0.1, 0.3, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 ml) and transferred into eight 10-ml volumetric flasks, respectively. Two-hundred microliters of the prednisolone stock solution was added into these 8

volumetric flasks. The solutions were adjusted to volume with mobile phase so that the concentrations of the standard solution were 0.020, 0.060, 0.100, 0.200, 0.400, 0.600, 0.800 and 1.000 mcg/ml, respectively.

6.3 Preparation of sample solutions for HPLC analysis

6.3.1 Stability studies

The previous stored ampoules in hot air oven at 70°C were placed at room temperature until the solutions were cool. Seventy-five microlitres of the drug solution from each ampoule stored either at room temperature or at 70°C were pipetted using a micropipet and then transferred into a 25-ml volumetric flask, adjusted to volume with the mobile phase. Once more, 75 microlitres of the diluted sample solution was pipetted and transferred into a 10-ml volumetric flask containing 200 microlitres of 24.0 mcg/ml internal standard. The solution was adjusted to volume with the mobile phase and ready for HPLC analysis.

6.3.2 In vitro permeation studies

The receiver solution drawn from the receptor compartment at individual time intervals was diluted to an appropriate concentration in a 10-ml volumetric flask containing 200 microlitres of 24.0 mcg/ml internal standard. The mobile phase was added to volume and this solution was ready for HPLC analysis.