CHAPTER III

MATERIALS AND METHODS

Materials

1. Brij® 52 (EAC Chemical)
2. Brij® 76 (EAC Chemical)
3. Cholesterol (Sigma, USA, Lot no. 58H5234)
4. Disodium hydrogen orthophosphate (Univar, Australia, Lot no. FOJ067)
5. Ethanol, absolute, AR grade (Merck, Germany, Lot no. E5041)
6. Glacial acetic acid, AR grade (Lab scan, Ltd., Thailand, Lot no. 2789)
7. Glucose monohydrate (Merck, Germany, Lot no. GC002)
8. Isopropanol, AR grade (Lab Scan., Ltd., Thailand)
9. Methanol (Lab Scan., Ltd., Thailand)
10. Minoxidil (Trisama, Italy, Lot no. 00547)
11. Perchloric acid (Lab Scan., Ltd., Thailand, Lot no. 5724)
12. Potassium dihydrogen orthophosphaste (Univar, Australia, Lot no. F1F125)
13. Prednisolone base (a gift from V&S Chemi Group CO., Ltd., Thailand)
14. Propylene glycol (Merck, Germany, Lot no. 990807)
15. Sodium chloride, AR grade (Merck, Germany, Lot no. 28555404 049)
16. Sodium docusate (Sigma, USA)
17. Sodium dodecyl sulfate (Kao, Thailand, Lot no. 4170)
18. Solulan® C-24 (Amerchol, UK)
19. Span® 40 (EAC Chemical)
20. Span® 60 (EAC Chemical)
21. Sheep whole blood (Animal Husbandry Department, Faculty of Veterinary, Chulalongkorn University, Thailand)

Equipment

1. Analytical balance (Model AG285, Mettler Toledo)
2. Centrifuge (Model 4206, Centrifugette, ALC, Italy)
3. Dry bath incubator (Model 112001, Boekel Scientific, Japan)
4. High performance liquid chromatographic system equipped with:
    - Auto Injector (SIL-10, Shimadzu, Japan)
    - Communication bus module (CBM-10A, Shimadzu, Japan)
    - Liquid chromatograph pump (LC-10D, Shimadzu, Japan)
    - UV-VIS detector (SPD-10A, Shimadzu, Japan)
    - Column (Altima C18, 5µm, 4.6 x 150 nm, Lot no. 3153)
5. Light microscope (Axiovert, Zeiss, Germany)
6. Light microscope (KHC, Olympus, Japan)
7. Microcentrifuge (Hermle Z230 MA, Germany)
8. Micropipette (Gilson, France)
9. Microplate reader (Anthos htl, Anthos Labtec Instrument, Austria)
10. Orbital shaker (S05, Stuart Scientific, UK)
11. Modified Franz diffusion cells
12. pH meter (Model 420A, Orion, USA)
13. Suspension mixer (Model RSM5, Ratek Instrument. Pty. Ltd., Australia)
14. Ultracentrifuge (L 80, Beckman, USA)
15. Ultrasonic bath (TP 680 DH, Elma, Germany)
16. UV-visible spectrophotometer (UV-1601, Shimadzu, Japan)
17. Vortex mixer (Vortex Genies-2, Scientific Industries, Inc., USA)
18. UV cabinet (A. P. G. Engineering, Thailand)

Methods

1. Solubility of minoxidil

The aqueous solubility of minoxidil (MN) was experimentally determined. Saturation solubility of MN in water was determined at ambient temperature by continuous shaking of an excess amount of MN in tri-distilled water using a suspension mixer (Model RSM5, Ratek Instrument, Pty, Ltd., Australia). Samples were continuously rotated and aliquouts were taken at both 2 and 4 days to reassure that the equilibrium was reached. The aliquot portion was centrifuged at 5,000 rpm (Centrifugette 4206, ALC, Italy) to separate drug crystals. The MN content in the supernatant was assayed by UV spectrophotometric method at 288 nm.

2. Preparation of MN niosomes

Niosomes were prepared by a sonication method that was devoid of organic solvents. The method used was modified from that of Baillie et al. (1985). The non-ionic surfactants used to form niosomes in this study were Span® 40, Span® 60, Brij® 76 (Plookchit Chetratanont, 2002) and Brij® 52 (Wallach, 1990). Cholesterol (CHO) at various weight ratios and 5% by weight of Solulan® C24 were added to all preparations. The total lipid concentration (surfactant plus cholesterol plus Solulan® C24) used was 100 mg/ml. The ratios of surfactant to cholesterol (CHO) to Solulan® C24 were 67.5:27.5:5 for Span® 40, 57.5:37.5:5 for Span® 60, 67.5:27.5:5 for Brij® 52, and 47.5:47.5:5 for Brij® 76, respectively.

Each surfactant, CHO, and Solulan® C24 were accurately weighed in a 10-ml glass tube and melted in a dry bath incubator (Model 112001, Boekel Scientific, Japan) at 130 °C. The aqueous phase was 2.2 mg/ml MN solution in water. This concentration was selected since it was below 90% of the saturation solubility of MN in water at ambient temperature. Then the aqueous phase, which had previously been warmed and kept at 70 °C, was added to the melted mixture. As a standard practice, MN solution was protected from light by wrapping the container with aluminum foil at all times. The mixture was

immediately sonicated at 70 °C for 10 min, using an ultrasonic bath (TP 680 DH, Elma, Germany) at 100% power and then vortexed for 1 min. The resultant niosomal preparation was left to cool down at room temperature. The product was checked under light microscope for completeness of vesicle formation, lipid remnants, aggregation of niosome vesicles, and presence of drug crystals (at 400x magnification).

3. Characterization of MN niosomes

3.1 Determination of MN entrapment efficiency (Plookchit Chetratanont, 2002)

After preparation, the niosomal suspension was left at room temperature overnight to allow complete annealing and equilibrating of MN between the lipid bilayer and the aqueous phase. The product was protected from light by wrapping the container with aluminum foil. The suspension was then separated into the supernatant containing the free drug and the pellet containing the entrapped drug by centrifugation (see below). MN in the niosomal pellet was assayed and used to calculate the entrapment efficiency (EE). MN in the supernatant was also assayed for routine monitoring of total analytical recovery. The UV spectrophotrometric assay of MN was carried out using the method described in *Analytical profiles of drug substances* (Dennis, 1988). The analytical method was validated using guidelines in the USP27 (The United States Pharmacopieal Convention, 2004). The validation results for accuracy, precision, linearity, and specificity are shown in Appendix III.

3.1.1 Separation of niosomal pellets

Five milliliters of MN solution used in the preparation method was added to an aliquot (1 ml) of niosomal suspension to aid the centrifugation process. The suspension was then centrifuged at 60,000 rpm at 25 °C for 1.5 h in an ultracentrifuge (L80, Beckman, USA). The supernatant was carefully separated from the pellet. MN contents in the pellet and the supernatant were determined.

3.1.2 Quantitative analysis of MN in niosomal pellets

The niosomal pellet was dissolved with isopropanol in a 25 ml volumetric flask and the solution was adjusted to volume. One milliliter of this solution was further diluted with isopropanol to 10 ml in a volumetric flask. This final solution was assayed by UV spectrophotometric method at 288 nm.

3.1.3 Quantitative analysis of MN in supernatant

3.1.3.1 Quantitative analysis of MN concentration in supernatant

One milliliter of MN in supernatant was diluted with isopropanol in a 25 ml volumetric flask. One milliliter of this solution was further diluted with isopropanol in a 10 ml volumetric flask. This final solution was assayed by UV spectrophotometric method at 288 nm.

3.1.3.2 Quantitative analysis of total MN in the supernatant

The rest of the supernatant was collected and diluted with isopropanol in a 10 ml volumetric flask. One hundred microliters of this solution was further diluted with isopropanol in a 10 ml volumetric flask. This final solution was assayed by UV spectrophotometric method at 288 nm. Total MN in the supernatant was calculated as the sum of the results obtained from 3.1.3.1 and 3.1.3.2.

3.1.4 Calculation of the percentage of entrapment and entrapment efficiency

The percentage of MN entrapment of each preparation was determined from the following equation:

$$\% \text{ Entrapment} = \frac{\text{Amount of MN in pellet} \times 100}{\text{Total amount of MN in suspension}}$$

where the total amount of MN in niosomal suspension was expressed as the sum of the amount of MN in the supernatant and that in the pellets.

Entrapment efficiency was defined as the fraction of MN found in the niosomal pellets and expressed as microgram of MN in the pellet per milligram of total lipid (Uchegbu and Vyas, 1998).

$$\text{Entrapment efficiency} = \frac{\text{Amount of MN in pellets (µg)}}{\text{Amount of total lipid (mg)}}$$

3.2 Optical microscopy

In this study, the feasibility of vesicle formation and vesicle shape, as well as overall quality of the preparation, were monitored with an optical microscope (KHC, Olympus®, Japan) at 400x magnification as a routine practice.

3.3 Polarized light microscopy

Polarizes light microscopy can be used to verify existence of vesicles in the preparation (Manosroi et al., 2003). A drop of the sample was placed on a glass slide and examined between two crossed-polarizing filters under a light microscope (Axiovert 135®). The polarized light photomicrographs were recorded using a digital camera.

4. Physical stability of MN niosomes

Physical stability of MN niosomes was monitored using the conditions under which the niosomes were protected from light. Four batches of each MN niosomal formulation were prepared as described under Section 2 and were pooled and thoroughly mixed with a vortex mixer. The pooled suspension was distributed into 10 ml glass tubes, with one ml of the suspension in each tube. The tubes were tightly sealed with closures and wrapped with paraffin film. MN niosomes were protected from light by wrapping the container with aluminum foil. The stability of MN niosomes was monitored under ambient conditions for three months. The temperature and relative humidity were recorded daily with a thermometer and a digital hygrometer. Three tubes of each

formulation were randomly taken at times 0, 1, 2, and 3 months. The physical stability was studied by monitoring for niosome aggregation, changes in color, presence of drug crystals, and changes in entrapment efficiency of niosomal systems.

5. Drug release study (Rattana Rattanatraiphop, 2000)

Four batches of MN niosomes were prepared and pooled together. Four 1-ml aliquots of the pooled suspension were used for the release study for each formulation. The aqueous solution of MN in water was also studied for comparison.

Modified Franz diffusion cells consisting of the donor and the receptor compartments were used to study in vitro release of minoxidil from different niosomal formulations and MN solution. The internal diameter of the cell ranged from 1.60-1.71 cm, corresponding to an effective permeable surface area of 2.01-2.30 cm^2. The receptor compartment was equipped with a magnetic stirring bar rotating at 600 rpm and the temperature was kept constant at 37 °C by circulating water through a jacket surrounding the cell body throughout the experiments. The receptor compartment contained 13.77-15.08 ml (from calibration) of water as the release medium. A dialysis membrane (cellulose tubular membrane, Cellu-Sep®) with a molecular weight cut-off of 12,000-14,000 separated the donor and the receptor compartments. The membrane was cut into a circular shape, with a diameter of 3 cm, and soaked in tri-distilled water overnight before use. Before being mounted onto a diffusion cell, the membrane was rinsed with boiling water to wash off any soluble contaminants. The membrane was then soaked in water for at least 15 minutes and clamped in place between the donor and the receptor compartments of the cell. The receptor fluid and the membrane in Franz diffusion cell were equilibrated to the desired temperature for at least 30 min. After equilibration, the samples (1 ml of MN niosomes or MN solution) were carefully placed on the membrane surface of each cell and the cell was then covered completely and tightly with Parafilm®. The entire glass cells were wrapped with aluminum foil to protect the samples from light. Two milliliters of the receptor fluid was removed at appropriate time intervals and replaced with an equal volume of fresh medium. The sample taken was diluted as

appropriate and the final solution was assayed by UV spectrophotometric method at 288 nm. The fraction of minoxidil release was calculated by the following equation:

$$\% \text{ minoxidil release} = (A_t/A_0) \times 100$$

where A_t is the cumulative amount of minoxidil released at a particular time; A_0 is the total MN in the donor compartment at time 0.

6. Chemical stability of MN niosomes

The pooled suspension from four batches of MN niosomes was used for each formulation. The aqueous solution of MN in water was also studied for comparison. Aliquots of MN niosomes and MN solution (2.2 mg/ml) were stored in 30 well-closed 2-ml glass vials per formulation. These vials were wrapped with aluminum foil and stored in a UV cabinet at ambient temperature. These samples also served as the control treatment for photodegradation study in Section 7. Samples were randomly taken at time intervals of 0, 10, 20, 30, 40, 50, 60, 70, 80, and 90 days. The amount of minoxidil in the samples was determined quantitatively by HPLC. The analytical method was validated using guidelines in the USP27 (The United States Pharmacopieal Convention, 2004). The validation results for accuracy, precision, linearity, and specificity are shown in Appendix IV.

6.1 HPLC conditions for minoxidil analysis

The high performance liquid chromatographic technique was used for quantitative analysis of minoxidil. The system consisted of a constant flow pump, a variable wavelength UV detector, an integrator, and a fixed volume sample injector with a 20-microliter loop. The conditions used for analyzing minoxidil by HPLC method was modified from USP 27 and are presented as follows:

Column : Altima C18, 5μm, 4.6 x 150 nm

Mobile phase : methanol, water, and glacial acetic acid (700:300:10), 3.0 g/l of docusate sodium, adjusted with perchloric acid to a pH of 3.0

Flow rate	:	1 ml/min
Injection volume:		20 microliters
Run time	:	20 min
Detector	:	UV detector
Wavelength	:	254 nm
Temperature	:	ambient
Internal standard:		prednisolone base

6.2 Preparation of internal standard solutions

A stock solution of prednisolone base was prepared by accurately weighing 10.00 mg prednisolone base in a 100-ml volumetric flask. The drug was dissolved and the solution was adjusted to volume with the mobile phase. The final concentration of prednisolone base was 100 μg/ml.

6.3 Preparation of standard solutions

An accurate amount of 10.00 mg MN was placed in a 10-ml volumetric flask. The drug was dissolved and the solution adjusted to volume with water. This stock solution had a final concentration of 1 mg/ml. An aliquot of 20 μl of the stock solution was transferred to a 10-ml volumetric flask and diluted to volume with the mobile phase. Then, 1.0 ml of this diluted solution was transferred into a 10-ml volumetric flask and 1.0 ml of 100 μg/ml prednisolone stock solution was added. The solution was adjusted to volume with the mobile phase to the final concentration of 0.2 μg/ml. Certain volumes of 40.0, 80.0, 120.0, 160.0, and 200.0 μl of the drug stock solution and 1.0 ml of the internal standard stock solution were transferred into 10-ml volumetric flasks. The solution was adjusted to volume with the mobile phase, resulting in the final concentrations of 4.0, 8.0, 12.0, 16.0, and 20.0 μg/ml of MN, respectively. The concentration of the internal

standard was 10 μg/ml. A calibration curve of minoxidil was plotted between its concentrations and peak area ratios.

6.4 Determination of MN in niosomal formulations

MN entrapment efficiency of each formulation was determined as described under Section 3. The samples were kept in the containers wrapped in aluminum foil in a UV cabinet equipped with a turntable for 0, 10, 20, 30, 40, 50, 60, 70, 80, and 90 days. The amounts of MN in the niosomal pellets and in the supernatant were determined by HPLC.

6.5 Determination of MN in aqueous solution

Samples were diluted by transferring 1 ml of the solution to a 10-ml volumetric flask and the solution was adjusted to volume with water. The solution was diluted with the mobile phase and assayed by HPLC at 254 nm.

7. Photodegradation study under UV irradiation

Vesicular structures have been proposed as systems capable of improving the stability of photosensitive drugs. This experiment focused on how the incorporation of MN into niosomal suspension and solution would affect stability of the drug upon exposure to UV radiation.

The degradation of MN was studied using a UV cabinet equipped with UVA and UVB lamps at $1.0 \pm 0.1 \times 10^{-3}$ w/cm^2. Pooled MN niosomes was thoroughly mixed with a vortex mixer before being dispensed into 2-ml glass vials. The process used was the same as that described under Section 2. Aliquots (1 ml) of MN niosomes and MN solution (2.2 mg/ml) were stored in 60 well-closed 2-ml glass vials per formulation. The MN solution or MN niosomal suspension was maintained at ambient temperature and exposed to UV radiation at a fixed distance for 90 days. Samples (three vials for each formulation) were taken every 10 days for 3 months. MN contents in niosomal suspensions and solution were determined by HPLC. The results were compared with those of the control samples,

which was protected from light, previously described under Section 6. Rate constant and order of reaction of MN degradation in solution were estimated.

8. Estimation of the irritation potential of MN niosomes (Pape, 2003)

MN niosomal suspension was aimed for treating scalp hair loss. As a topical product, its potential to cause severe irritation to mucous membranes should be estimated before efficacy studies in animals or humans will be carried out.

8.1 Preparation of Phosphate Buffered Isotonic Saline (PBS), pH 7.4

PBS, pH 7.4, was prepared according to the formula that follows:

Na_2HPO_4	22.2	mmol/l
KH_2PO_4	5.6	mmol/l
NaCl	123.3	mmol/l
Glucose	10.0	mmol/l

The vehicle used was ultrapure water (ELGA, UK). The buffer was stored at 4 °C and used within one week.

8.2 Preparation of red blood cells (RBC)

Blood samples (10 g) were weighed and centrifuged at 4,000 rpm for 12 minutes (Centrifugette 4206, ALC, Italy). The supernatant was carefully separated from the RBC. The RBC was washed for four times with PBS, pH 7.4. This washing procedure removed the bulk of the white cells, any traces of plasma, and the buffy coat. Red blood cells were counted with a hemacytometer. The RBC suspension was appropriately diluted to contain about 8×10^9 cells/ml.

8.3 Hemolysis study

8.3.1 Preparation of standard tenside solutions

A stock solution of sodium dodecyl sulfate (SDS) was prepared by accurately weighing 10 mg of SDS into a 10-ml volumetric flask. SDS was dissolved and the solution was adjusted to volume with PBS. This solution had a final concentration of 1 mg/ml. The following volumes of the stock solution were pipetted into the reaction vials: 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 µl. Each vial was filled up to 975 µl with PBS. An aliquot (25 µl) of RBC suspension containing about 8 $x10^9$ cell/ml was added into the reaction vials and the vials were shaken for 30 seconds. The mixture was incubated for 10 minutes on an orbital shaker (S05, Stuart Scientific, UK S05, Stuart Scientific, UK) at 150 rpm at room temperature. The incubation period was terminated by rapid, high-speed (15,000 rpm) centrifugation for 1 minute in a microcentrifuge (Hermle Z230 MA, Germany). This removed intact cells and debris from the medium. After centrifugation, aliquots of the supernatant (100 µl) were immediately separated and placed into a 96-well plate and the absorbance monitored photometrically at 570 nm against the blank using a microplate reader (Anthos htl, Anthos Labtec Instrucment, Austria). The blank consisted of the sample diluted in PBS without RBC.

8.3.2 Preparation of negative and positive controls (0% and 100% hemolysis)

Spontaneous hemolysis was monitored by adding 25 µl of RBC suspension to 975 µl of PBS. This gave the zero hemolysis value. Another aliquot (25 µl) of RBC suspension was added to 975 µl of distilled water to give the 100% hemolysis value. The procedure was similar to that described under Section 8.3.1.

8.3.3 Preparation of test samples

8.3.3.1 MN niosomes and MN solutions

MN niosomes were prepared as described under Section 2. MN solutions were prepared in water and in a solvent mixture used as the vehicle for the commercial solution, Rogaine®, (60% ethanol. 20% propylene glycol, and 20% water). The procedure

was the same as that described under Section 8.3.1. However, since niosomal vesicles were much smaller than RBC and could introduce interference to the assay due to turbidity, the samples containing niosomes were centrifuged at 60,000 rpm at 25 °C for 1.5 hr in an ultracentrifuge after the intact RBC and cell debris had been removed.

8.3.3.2 Blank niosomes, solvents, and surfactants

Blank niosomes, water, and the solvent mixture described above were prepared. Dispersions of surfactants and lipids used in niosomes were also studied for irritation potentials. The concentrations used were as follows: 67.5 mg of Span® 40, 57.5 mg of Span® 60, 67.5 mg of Brij® 52, 47.5 mg of Brij® 76, 5 mg of Solulan® C24, or 47.5 mg of cholesterol in 1 ml water. These amounts were corresponding to those present in niosomal formulations. Degree of RBC hemolysis was determined as described under Section 8.3.1.

All hemolysis experiments were done in triplicate. Graphs were constructed by plotting the OD at 570 nm against the concentration of the test substance. The concentrations of the test substances that caused 50% hemolysis (H_{50}) were compared to assess the irritation potential with that of SDS, which was used as the standard tenside.

9. Statistical analysis

Kolmogorov-Smirnov normality test on pooled samples was used to justify the valid assumptions for parametric tests. If the normality test showed that the distribution of data did not significantly deviate from normality, analysis of variance (ANOVA), with either Tukey's HSD or Dunnett or Dunnett's T3 test as a post hoc comparison, was used. The level of significance was chosen at the 0.05 probability.