CHAPTER III

MATERIALS AND METHODS

Materials

1. Acetic acid (Merck, Germany, Lot no. A420746)
2. Boric acid (Merck, Germany, Lot no. 547K1033265)
3. Cholesterol (Sigma, USA, Lot no. 58H5234)
4. Dicetylphosphate (Sigma, USA, Lot no. 96H1069)
5. Minoxidil (Trisama, Italy, Lot no. 1101)
6. Polyoxyl-10 stearyl ether (EAC Chemical, Lot no. 11936)
7. Potassium chloride (Merck, Germany, Lot no. 036TA915536)
8. Sodium acetate (Fluka, Switzerland, Lot no. 711503)
9. Sodium borate (Fluka, Switzerland, Lot no. 714539)
10. Sodium hydroxide (Merck, Germany, Lot no. B870498625)
11. Span®20 (EAC Chemical, Lot no. 16790)
12. Span®40 (EAC Chemical, Lot no. 11036)
13. Span®60 (EAC Chemical, Lot no. 16794)
14. Span®80 (EAC Chemical, Lot no. 11078)
15. Span®85 (EAC Chemical, Lot no. 110 49)
16. Tween®20 (EAC Chemical, Lot no. 11704)
17. Tween®80 (EAC Chemical, Lot no. 11743)

Equipment

1. Analytical balance (GMPH, Sartorius, Germany)
2. Analytical balance (UMTZ, Mettier Toledo, Switzerland)
3. Dry Bath Incubator (Boekel Scientific, Japan)
4. Light microscope (KHC, Olympus, Japan)
5. pH meter (Beckman, USA)
6. Shaking water bath (Innova 4230, New Brunswick Scientific, USA)
7. Ultracentrifuge (L 80, Beckman, USA)

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

8. Ultrasonic bath (TP 680 DH, Elma, Germany)
9. UV spectrophotometer (Model 7800, Jasco Corporation, Japan)
10. Vortex mixer (G 560 E, Vortex-genie, USA)

Methods

1. Solubility of minoxidil

The aqueous solubility of minoxidil (MN) was experimentally determined in various solvents. Solubility of MN in water was determined by continuous shaking of excess amounts of MN in tri-distilled water using a shaking bath at ambient temperature. Samples were removed at appropriate time intervals and were passed through membrane filters (0.22 μm pore size) to separate drug crystals. The MN solution was appropriate diluted and assayed by UV spectrophotometry at 280 nm. The same procedure was used to determine MN solubility in 15% propylene glycol (PG), in 30% PG, in 50 mM acetate buffer pH 4.6, and in 50 mM borate buffer pH 7.0. The buffer solutions were prepared according to *USP24* (The United States Pharmacopieal Convention, 2000).

2. Feasibility study on preparation of MN niosomes by sonication method

This experiment was done to determine feasibility of preparing niosomes with a method devoid of organic solvents. The method used was modified from that of Baillie et al. (1986). Various non-ionic surfactants previously reported to be vesicle formers, regardless of method of preparation, were explored. The total lipid (a surfactant with or without cholesterol) concentration used was 100 mg/mL. The ratio of surfactant to cholesterol was varied, starting with 1:1 for Span® and Tween® and 85:15 for polyoxyl-10-stearyl ether (POE-10). The surfactants explored in this experiment were Tween®20, Tween®80, Span®20, Span®40, Span®60, Span®80, Span®85, and POE-10.

A surfactant or a mixture of surfactant and cholesterol was accurately weighed and melted in a 10 mL glass tube in a dry bath incubator at 130°C. The aqueous phase, which had previously been warmed and kept at 70°C, was then added to the melted mixture. MN solution had been protected from light by wrapping the container with

aluminum foil. The mixture was immediately sonicated at 70°C for 5 minutes (min) using ultrasonic bath (Elma Transsonic Digitals type TP 680 DH) at 100% power (40 kHz) 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 and lipid remnants. All of the preparations were regularly monitored for physical stability. As a routine, care was taken to detect any aggregation of niosome vesicles, changes in color, or presence of drug crystals under the microscope (at 400x magnification). The experiment was done in triplicate before the formula was accepted as feasible for niosome formation.

The aqueous phase used in this experiment was 2.2 mg/mL MN in water. This concentration was below the saturation solubility of MN in water at ambient temperature. Since MN was incorporated into the aqueous phase, drug loading occurred at the same time that niosome vesicles were formed. Thus, this process was further referred to as "in-process loading" method.

3. Determination of MN entrapment efficiency

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 ultracentrifugation (see below). The MN content 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 percent analytical recovery. The UV spectrophotometric 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 *USP24* (The United States Pharmacopieal Convention, 2000). The validation results for accuracy, precision, linearity, and specificity are shown in Appendix C.

3.1. Separation of niosomal pellets

Five milliliters of the aqueous medium 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 50,000 rpm at 25°C for 1.5 h in an ultracentrifuge. The supernatant was carefully separated from the pellet. MN contents in the pellet and the supernatant were determined.

3.2. Quantitative analysis of MN in niosome pellets

The niosome pellet was dissolved in isopropanol in a 25 mL volumetric flask, and the solution was adjusted to volume. One milliliter of this solution was further diluted with isopropanol in a 10 mL volumetric flask. This final solution was assayed by UV spectrophotometry at 280 nm. The presence of surfactants and/or CHO in the pellets did not interfere with the assay (see Figures 4-17 in Appendix D).

3.3. Quantitative analysis of MN in supernatant

An aliquot of the supernatant was appropriately diluted with isopropanol and analyzed by UV spectrophotometry at 280 nm.

3.4. Calculation of entrapment efficiency

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

$$\text{Entrapment efficiency} = \frac{\text{Amount of MN in the pellet (mg/mL)}}{\text{Amount of total lipid (mg/mL)}}$$

4. Effects of equilibrating time on MN entrapment in niosomes

Since MN has marginal partition coefficient, partitioning of MN from the aqueous phase where the drug was initially incorporated into the niosome bilayer was expected. According to the literature, the product was usually kept overnight before EE was determined. However, with different compositions and methods of preparation, the time required for equilibration might be different. The purpose of this experiment was to study the effect of the time lapse on partitioning of the drug between the lipid bilayer and the aqueous phase.

Three niosomal formulations selected from Section 2 as feasible formulas were studied. These were Span®40:CHO (70:30), Span®60:CHO (60:40), and POE-10:CHO (50:50). The aqueous phase was 2.2 mg/mL MN in water.

Eight milliliters of MN niosomes was prepared in a 10 mL screw capped test tube by the in-process loading method. The process used was the same as that described in Section 2. The resultant niosome preparation was kept in the test tube and protected from light. An aliquot of 1 mL was taken at each time point after the suspension was well mixed on a vortex mixer. MN entrapment efficiency of each formulation was determined as described in Section 3 after the products were kept at room temperature for 1, 3, 5, 7, and 10 days. During these time lapses, MN partitioning was expected to take place and finally reach equilibrium. The experiments were performed in triplicate, using three batches of each formula.

5. Effects of formulation factors on niosome formation and MN entrapment in niosomes

The three feasible formulas selected from Section 2 were further studied. These formulations were Span®40:CHO (70:30), Span®60:CHO (60:40), and POE-10:CHO (50:50). In the feasibility study in Section 2, the type of surfactant and the ratio of surfactant:CHO were varied. This present experiment was aimed at other factors that might be further modified for MN niosomes. The compositions of MN niosome vesicles from the feasibility study were used. The advantages of such modification could be cost effectiveness, time reduction, and increase in EE. These factors were total lipid concentration, addition of a stabilizer, and modification of the aqueous phase. Their effects on niosome formation and drug entrapment were studied. All of the experiments were performed in triplicate, using three batches of each formula.

5.1. Effects of total lipid concentration

One milliliter of MN niosomes was prepared in a 10 mL screw-capped test tube by the in-process loading method described in Section 2. The total lipid concentrations were 50, 100, and 200 mg/mL. Vesicle formation was checked under a light microscope. The preparation was left overnight at room temperature. EE of the preparation was determined according to Section 3.

5.2. Effects of stabilizer

To study the effect of stabilizer commonly used in niosome preparations, 5% by weight of either dicetylphosphate (DCP) or Solulan®C24 was added to the preparation.

The total lipid concentration (surfactant plus CHO plus a stabilizer) was kept at 100 mg/mL. The ratios of surfactant to CHO to the stabilizer were 67.5:27.5:5 for Span®40, 57.5:37.5:5 for Span®60, and 47.5:47.5:5 for POE-10, respectively. The aqueous phase was 2.2 mg/mL MN solution in water. One milliliter of MN niosomes was prepared, and the EE was determined as described in Section 3. Controls were the corresponding preparations without a stabilizer.

5.3. Effects of modification of the aqueous phase

Since MN is slightly soluble in water, concentration of the drug in the aqueous phase was expected to affect EE of niosomes. This study was designed to elucidate effects of a commonly used co-solvent, propylene glycol (PG), and pH of the aqueous phase on niosome formation and drug entrapment

5.3.1. Effects of propylene glycol

Niosomal suspensions were prepared in a 10 mL screw-capped test tube by the in-process loading method described in Section 2. The total lipid concentration was 100 mg/mL. The aqueous phase was either MN solution in 15% PG or in 30% PG. The concentration of MN used was at 90% saturation of the drug in each solvent. The control was the niosomal suspension prepared with 2.2 mg/mL MN in water as the aqueous phase. Feasibility of niosome formation and MN entrapment were determined.

5.3.2. Effects of pH of the aqueous phase

MN is a weak base. The pH of the aqueous medium might affect drug solubility and, thus, the EE. Niosomal suspensions were prepared in a 10 mL screw-capped test tube by the in-process loading method described in Section 2. The total lipid concentration was 100 mg/mL. The aqueous phase was either MN solution in 50 mM acetate buffer pH 4.6 or 50 mM borate buffer pH 7.0. The concentration of MN used was at 90% saturation of the drug in each solvent. The control was the niosomal suspension prepared with 2.2 mg/mL MN in water as the aqueous phase. The feasibility of niosome formation and MN EE were determined.

6. Effects of preparation method on MN entrapment in niosomes

In the in-process loading method, the drug must be exposed to relatively high temperature during the preparation process. If the drug could be loaded after the

niosome vesicles had been formed, exposure to high temperature might be avoided. This process should be beneficial to drugs with low thermal stability. If no driving force other than the concentration gradient is applied, the process can be referred to as a passive loading method. This method should be plausible for drugs with proper partition coefficients. MN should be a good model for such drugs. This experiment was to compare the EE of MN niosomes prepared by the in-process loading and the passive loading methods.

6.1. Preparation of MN niosomes by in-process loading method

Eight milliliters of MN niosomes was prepared in a 10 mL screw-capped test tube by the in-process loading method described in Section 2. The compositions of niosomes were Span®40:CHO (70:30), Span®60:CHO (60:40), and POE-10:CHO (50:50). The total lipid concentration was 50 mg/mL, and the aqueous phase was 1.1 mg/mL MN solution in water. The total lipid concentration and the MN concentration were reduced from the previous experiments to make it comparable to the conditions used in the passive loading method below. The product was kept protected from light at room temperature overnight before the first sample was taken. An aliquot (1 mL) of the suspension was taken after the preparation was well mixed on a vortex mixer. The sample was processed as described in Section 3 to determine the EE. Samples were taken again at 3, 5, 7, and 10 days after preparation, and the EE was determined. The physical appearance of the niosomal suspension was observed and recorded before each sampling.

6.2. Preparation of MN niosomes by passive loading method

Blank niosomal preparations were prepared using the same compositions as in Section 6.1. The total lipid concentration was 100 mg/mL, and the aqueous phase was tri-distilled water. Four milliliters of each formula was prepared in a 10 mL screw-capped test tube. The product was carefully transferred and mixed with 4 mL of 2.2 mg/mL MN solution in water in a 10 mL flask. Thus, the final lipid concentration was 50 mg/mL, and the resultant MN concentration was 1.1 mg/mL. The flask was covered with Parafilm® and kept protected from light. The mixture was constantly mixed on a magnetic stirrer. Samples were taken the next morning and again at 3, 5, 7, and 10 days after

preparation, and the EE was determined. The physical appearance of the niosomal suspension was observed and recorded before each sampling.

The EE of the two methods were compared. The experiments were performed in triplicate, using three batches of each formula.

7. **Statistical analysis**

Statistical analysis to compare treatment means was performed on SPSS version 9. The non-parametric Mann-Whitney U test was used to compare two treatment means since the sample size was small. When more than two treatment means were compared, the validity of assumptions for the analysis of variance (ANOVA) was tested on pooled data, using Kolmogorov-Sminrov normality test. If the distribution of data did not significantly deviate from normality, ANOVA, with Tukey's HSD test as a post hoc comparison, was used. The level of significance was chosen at 0.05 probability.

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย