CHAPTER III

MATERIALS AND METHODS

Materials

1. Propylthiouracil (A gift from Sriprasit Pharma Co., Ltd, Lot 9220747)
2. Triamcinolone acetonide (Supplied by T.O. Chemicals, Lot 2198NMO 0089720)
3. Phosphatidylcholine (Phospholipon®90 Nattermann Phospholipid GmbH, Cologne, Germany, Lot 770991)
4. α-Tocopherol (Approx. 95%, Sigma, USA Lot 53H0444)
5. Urea (Supplied by S. Tong Chemicals Co., Ltd)
6. Sodium Chloride (Farmitaria Carlo Erba, Italy Code 479687)
7. Trehalose (Sigma, USA Lot 87H3810)
8. Triethanolamine (Fluka, Switzerland Lot 30911881)
9. Oleic acid (Aldrich, USA Lot 305704)
10. Brij®72 (A gift from EAC Chemicals Co., Ltd)
11. Brij®721 (A gift from EAC Chemicals Co., Ltd)
12. Arlamol®E (A gift from EAC Chemicalsn Co., Ltd)
13. Isopropyl myristate (Supplied by Vannarat Co., Ltd)
14. Chloroform, AR grade (BDH Laboratory Supplies, England K 23408941)
15. Isopropanol, AR grade (BDH Laboratory Supplies, England K)

16. Dialysis membrane (Regenerated cellulose tubular membrane, Membrane filtration product, Inc, USA Part # 1430-25 MWCO 12,000-14,000)
17. Sodium dodecyl sulfate (Sigma, USA Lot 126H1009)
18. Decanol (Sigma, USA Lot 126H0159)
19. Arlatone®2121 (A gift from EAC Chemicals Co., Ltd)
20. Monopotassium Phosphate (Merck, Germany Lot A776073 412)
21. Disodium Phosphate (Merck, Germany Lot F 127283 628)

Equipment

1. Analytical balance (Sartorius GMPH, Germany)
2. Rotary evaporator (RE 120, Buchi, Swizerland)
3. Ultrasonic bath (T900/H, Elma, Germany)
4. Vortex mixer (Vortex-genie, Model G 560 E, USA)
5. Hot air oven (UL 50, Memmert, Germany)
6. UV spectrophotometer (Model 7800, Jasco Corporation, Japan)
7. Modified Franz diffusion cells (Crown Glan Company, Inc, USA)
8. Differential Scanning Calorimetry (Perkin-Elmer DSC 7 Instrument)
9. pH meter (Beckman, USA)
10. Ultracentrifuge (L80, Beckman, USA)
11. Polarized Light Microscope (KHC, Olympus, Japan)

Methods

1. Preparation of Liquid Crystalline Systems Using Various Surfactants and Lecithin

Amphiphiles, including several surfactants and lecithin, were prepared into liquid crystalline systems. Since lecithin would not melt to a molecular dispersion before starting to degrade, a special method of hydration was required. Various compositions were used for each amphiphile, and partial phase diagrams were constructed. Surfactant selected were Brij®72, Brij®721, Arlatone®2121, triethanolamine oleate, and sodium dodecyl sulfate (SDS).

1.1 Liquid Crystals from Surfactants

Mixtures containing different concentrations of each surfactant were prepared by accurately weighed the surfactant and distilled water in a 10-ml screw-capped test tube. The total weight of each preparation was one gram. The mixture wes heated and allowed to melt in a water bath at 80^0C for ten minutes and then vigorously mixed at room temperature with a vortex mixer at maximum speed for ten minutes. The liquid crystalline systems prepared from sodium dodecyl sulfate, decanol and water were not carried out in a water bath; instead, they were mixed at room temperature with a vortex mixer at maximum speed for ten minutes to minimize possible evaporation of decanol and to avoid hydrolysis of SDS at elevated temperatures. The cap of each test tube was then wrapped completely and tightly with

Parafilm®. The system was examined under a polarized light microscope after 7 days of storage at room temperature to allow the system to reach equilibrium. Systems which exhibited liquid crystalline structures were selected for further investigation.

1.2 Liquid Crystals from Lecithin

1.2.1 The liquid crystalline system in the form of emulsion

The liquid crystalline systems in the form of emulsion were prepared by varying ratios of lecithin to water (at 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, and 90:10 ratios of lecithin to water) to find the ratio that yielded the liquid crystalline system. All preparations were prepared on a weight by wight (w/w) basis, and the total weight of each preparation was one gram. As mentioned earlier, melting method could not be applied to systems with lecithin, and a different method of hydration must be used. Lecithin was dissolved in 2 ml of chloroform in a test tube, and chloroform was slowly evaporated using rotary evaporator at 45^0C for 4 hours under reduced pressure. Then, distilled water was added, and the mixture was blended by a combination of alternate heating to 45^0C and vortex mixing for 20 minutes.

1.2.2 The liquid crystalline system in the form of vesicles or liposomes

The liquid crystalline system in the form of vesicles or liposomes was prepared under the similar process described above. For lecithin and other

the system with low lecithin content in this study, liposomal suspension was obtained. To prepare a homogeneous preparation with low lecithin content, a thin film of lecithin was deposited on the surface of a round bottom flask to facilitate lipid hydration. The proper amount of lecithin that yielded a smooth film on the flask surface was from trial and error. In brief, lecithin (581.2 mg) was dissolved in 15 ml of chloroform in a 1000-ml round bottom flask, and the chloroform was slowly evaporated using rotary evaporator at 45^0C for 5 hours under reduced pressure to deposit a thin film of dry lipid on the wall of the flask. Then, 7.5 ml of distilled water which was heated up to the same temperature was added to the dried film to make a suspension with final lipid concentration of 100 μmol/ml. The flask was vigorously shaken and then left attached to a rotary evaporator at 45^0C for 1 hour to allow complete hydration and annealing of the vesicles. Liposomes were too small in size to give accurate identification by polarized light microscopy.

2. Characterization of the Liquid Crystalline Systems

Systems which exhibited strong birefringence under a cross-polarized light microscope were selected to further investigation. Different phases of liquid crystals were identified from the pattern seen under the microscope as described by Rosevear (1954). Microscopic interactions within the system was evaluated from DSC thermograms.

2.1 Polarized Light Microscopy

The samples prepared as described above were examined for diffferent phases of the liquid crystalline system under polarized light microscope after 7 days and 2 months of storage. The samples prepared from surfactants were stored at room temperature, but those prepared from lecithin were stored in a refrigerator since it is known that lecithin is unstable at room temperature. A small amount of each sample was transferred from the sample test tube onto a glass slide and was covered immediately with a coverslip. The appearance of the sample was observed between crossed polarizers. The liquid crystalline phases were identified according to the textures described by Rosevear (1954). Photographs were taken with a camera attached to the microscope when liquid crystalline phases were observed.

2.2 Differential Scanning Calorimetry (DSC)

The DSC measurements were performed with a Perkin-Elmer DSC 7 instrument. The heating rate was 10^0C /min. Each scan used the starting temperature of -80^0C and the final temperature of 80^0C. The samples were stored for 3 days and 2 months before analysis, and those selected to be analyzed by DSC composed of Brij®72:water (40:60), Brij®72:Arlamol®E:water (15:10:75), triethanolamine:oleic acid:water (10:50:40, 25:30:45, 15:50:45, and 15:20:65), SDS:decanol:water (5:10:85 and 20:30:50), lecithin:water (40:60) and pellets of liposomes (from a 100 μmol lipid/ml suspension). The pellets of liposomes were prepared by centrifugation of liposomal suspension at 50,000 x g and 4^0C for 5 hours using an ultracentrifuge. The supernatant was discarded, and the pellets were characterized by DSC.

3. Effects of Additives on the Formation and the Structure of Liquid Crystalline Systems

Effects of additives on the formation and the structure of liquid crystalline systems were studied using trehalose, urea, sodium chloride, and α-tocopherol as model molecules. These molecules, having different structures and are usually found as additives in many preparations, serve as either a cryoprotectant, a hydrating agent, a modifier of ionic strength, or an antioxidant. The liquid crystalline systems studied were selected from phase diagrams so that different phases of liquid crystals were included. Selection was also based on the good stability after 2 months and the criterion of the lowest possible concentration of surfactant that still formed liquid crystals. Some systems that displayed similar phases but were formed by different compositions were also studied. These systems were Brij®72 Arlamol®E:water (15:10:75 and 20:10:70), triethanolamine:oleic acid:water (10:50:40, 25:30:45, and 15:50:45), SDS:decanol: water (5:10:85 and 20:30:50), and lecithin:water (40:60). The liposomal system was also selected since it is a commonly used delivery system for topical products with the unique structure of vesicular bilayer. The effects of these added substances on the liquid crystalline systems were studied by varying the amounts of sodium chloride, trehalose and α-tocopherol from 1 to 3, 5, 10, and 15% w/w while urea concentration was fixed at 10% w/w. Sodium chloride, urea, and trehalose were first dissolved in the water phase whereas α-tocopherol was incorporated directly into the mixture. The products were characterized, as previously described, by polarized light microscopy and differential scanning calorimetry after 3 days and 2 months of storage.

4. Assessment of the Possibilities of Using Liquid Crystalline Systems as Drug Delivery Systems

The liquid crystalline systems subjected to this evaluation were the same as those in part 3 of the methods. The experiments in 4.1-4.3 were performed in triplicate using three different batches for each system. The study in 4.4 was not replicated due to the descriptive nature of the method used.

4.1 Ability to Solubilize Propylthiouracil (PTU) and Triamcinolone Acetonide (TA)

Molecular structures and physicochemical properties of PTU and TA are shown in Appendix F.

4.1.1 Liquid Crystalline Systems in the Form of Emulsion

The saturation solubility of each drug in the liquid crystalline system was studied by varying concentrations of drug added into the system from 0.1 to 1% w/w with an increment of 0.1%. The saturation solubility of each drug in the systems was determined by polarizing microscopy. The highest drug concentration at which drug crystals were not observed after 3 days was considered to represent the saturation solubility of the drug in that system. For surfactant systems, the drug was incorporated into liquid crystalline by mixing it with other components in the systems and melting using the same protocol as described in 1.1. For the systems produced

from lecithin, the drug was dissolved in chloroform along with lecithin and the sample was further processed as described under 1.2.1.

4.1.2 Liquid Crystalline Systems in the Form of Liposomal Suspension

In the case of liposomal suspension, the study was carried out by varying concentration of drugs in the system from 5 to 30 mg with an increment of 5 mg. The drugs were first dissolved in chloroform along with lecithin. Drug-containing liposomes were then prepared under the same procedure described under 1.2.2. In the case of PTU liposomes, two methods of hydration were used in an attempt to enhance the entrapment of the drug. Since PTU does not have high solubility in the lipid phase, it is probable that hydration of the lipid phase with a large amount of water may result in partitioning of the drug into the aqueous phase due to partial solubility of the drug in water. To study this possibility, distilled water and a solution of 0.8 mg/ml PTU in distilled water were used as two media of hydration. The saturation solubility of the drug in the liposomal bilayer was determined by optical microscopy. The highest drug concentration at which drug crystals were not observed after 3 days was considered to represent the saturation solubility of the drug in the system. Since only the fraction of drug entrapped in the bilayer was the drug molecules that actually resided in the liquid crystalline system, PTU encapsulation in the lipid bilayer of liposomes was also determined. On the other hand, this process could not be done with other liquid crystalline systems where water contents were much lower and where all the water in the system was interconnected. PTU encapsulation in liposomal pellets was determine as follows:

a) Separation of Liposomal pellets

An aliquot (1 ml) of liposome suspension was separated into the supernatant containing the free drug and the pellet containing the entrapped drug using ultracentrifugation at 50,000xg and 4^0C for 5 hours. The PTU content in the liposomal pellets was assayed according to the following assay protocol. PTU in the supernatant was also assayed to monitor the recovery of the procedure.

b) Quantitative Analysis of PTU in Liposomal Pellets

The pellets were dissolved in isopropanol in a 10-ml volumetric flask, and the solution was adjusted to volume. One milliliter of this solution was transferred and adjusted to volume in another 10-ml volumetric flask. This final solution was assayed by UV spectrophotometry at λ = 275 nm.

c) *Quantitative Analysis of PTU in the Supernatant*

The supernatant was diluted with isopropanol and adjusted to volume in a 10-ml volumetric flask. One milliliter of this solution was transferred to the second 10-ml volumetric flask and adjusted to volume. Then, 2 ml of this solution was transferred to the third 10-ml volumetric flask and adjusted to volume. This final solution was subjected to UV spectrophotometric analysis at 275 nm.

d) *Calculation of Percent Entrapment*

The percent entrapment was determined by the following equation:

$$\% \text{ Entrapment} = \frac{\text{Amount of PTU in the pellet}}{\text{Total amount of PTU in the liposomal suspension}}$$

Due to the minimal incorporation of triamcinolone acetonide into liquid crystalline systems studied including the liposomal system, the drug was not further quantitatively assayed and was not used for the release study.

Saturation solubility of PTU in distilled water at room temperature was also determined (See Appendix F).

4.2 Water Evaporation Rates from Liquid Crystalline Systems

Since the ability of a dosage form to keep the stratum corneum hydrated is known to enhance percutaneous delivery of many drugs. The following experiments were set up to compare rates of water evaporation from the liquid crystalline systems. Rates of water evaporation from a nonionic cream base and bulk water were also studied for comparison. The nonionic cream base was prepared according to the Ministry of Public Health Hospital Formulary. The formula of the cream base is displayed in Appendix K.

Samples of liquid crystalline systems, a nonionic cream base, and bulk water were packed and spread uniformly on pans with the thickness of 3 mm and the surface area of 2.21 cm^2. The pans were then placed on a controlled-temperature warm plate set at 32^0C in ambient atmosphere. The weight loss of the samples was

determined every 5 minutes with an analytical balance for up to 1.5 hours. The temperature and the humidity of the ambient atmosphere were recorded periodically throughout the experiment using a hygrothermometer.

4.3 Drug Release from Liquid Crystalline Systems

The in vitro drug release studies were carried out using Franz diffusion cells consisting of the donor and the receptor compartments (see Figure 13). The internal diameter of each cell was 1.5 cm, corresponding to an effective permeable surface area of 1.77 cm^2. The receptor compartment was equipped with a magnetic stirring bar rotating at 600±5 rpm and the temperature was kept constant at 37^0C by circulating water through a jacket surrounding the cell body throughout the experiments. The receptor compartment contained 11.43-12.77 ml of Sorensen phosphate buffer pH 6.5 as the release medium. The donor and the receptor compartments were separated by a dialysis membrane (cellulose tubular membrane, Cell-Sep®) with a molecular weight cut-off of 12,000-14,000. The membrane was soaked in deionized water overnight before use. The membrane was cut into a circular shape with a diameter of 3 cm. Before mounting the circular cellulose membrane onto a diffusion cell, the membrane was rinsed with boiling water to wash off any soluble cantaminants. The membrane was then soaked in the Sorensen buffer pH 6.5 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 cells were equilibrated to the desired temperature for 2 hours. After equilibration, the sample was carefully placed on the membrane surface of each cell, and the cell was

then covered completely and tightly with Parafilm®. Ten milliliters of the receptor fluid was removed at appropriate time intervals and replaced with an equal volume of fresh buffer. PTU contents in the receptor fluid were determined by UV spectroscopy at λ = 274.2 nm.

The PTU contents in the receptor fluid from Brij®72:Arlamol®E:water (15:20:65) and SDS:decanol:water (20:30:50) were measured by UV spectrophotometry determined without dilution, but PTU contents in the receptor fluid from triethanolamine:oleic acid:water (10:50:40) was diluted by taking 5 ml of the receptor fluid to a 10-ml volumetric flask and adjusted the solution to volume. Samples from triethanolamine:oleic acid:water (15:50:35), lecithin:water (40:60), and liposomal system were diluted by taking 2 ml of the receptor fluid to a 10-ml volumetric flask and adjusting to volume. Sorensen phosphate buffer was used as the diluent.

The released amount was calculated by multiplying the drug concentration by the receptor volume. The fraction of PTU released was calculated by the following equation:

$$\% \text{ PTU released} = (A_t / A_0) \times 100$$

where A_t is the cumulative released amount of PTU at a particular time; A_0 is the initial amount of PTU solubilized in the system as determined under 4.1.

The drug-loaded liquid crystalline systems selected to study the release were as follows:

- 0.2% PTU in Brij®72:Arlamol®E:water (15:10:75)
- 0.6% PTU in triethanolamine:oleic acid:water
  (15:50:35 and 10:50: 40)
- 0.2% PTU in SDS:decanol:water (20:30:50)

- 0.2% and 0.5% PTU in nonionic cream base

- 1.5 mg/ml of PTU aqueous solution

Drug concentration in each system was at its saturation solubility to ensure that the thermodynamic activity of the drug was the same in all systems.

PTU was solubilized in all systems except for the nonionic cream base where PTU existed as solid crytsals.

The standard calibration curve was prepared for each analysis of PTU. A blank system of similar composition but without the incorporation of PTU was also prepared for each drug-loaded liquid crystalline system and was similarly processed. The results obtained from the blank system were used for background correction. Preparation of the calibration curve and validation of analysis of PTU by UV spectrophotometry are in Appendix I and Appendix H, respectively.

4.4 Stability of the Drug-Containing Liquid Crystalline Systems

The stability of the drug-containing liquid crystalline systems was studied by qualitative comparison between liquid crystalline systems stored for 3 days and for 2 months. The comparison was based upon physical appearances, patterns observed by polarized light microscopy, and thermograms acquired from differential scanning calorimetry.