CHAPTER III

MATERIALS AND METHODS

Materials

Brij® 52 (EAC Chemical, Lot no. 11930)
Brij® 72 (EAC Chemical, Lot no. 11935)
Brij® 76 (EAC Chemical, Lot no. 11936)
Brij® 93 (EAC Chemical, Lot no. 11941)
Brij® 97 (EAC Chemical, Lot no. 11948)
Chloroform (Lab-Scan Analytical Sciences, Thailand)
Cholesterol (Sigma, USA, Lot no. 58H5234)
Dialysis membrane (Regenerated cellulose tubular membrane MWCO=12000-14000, Membrane Filtration Product, Inc., USA, Part# 1430-25)
Dicetyl phosphate (Sigma, USA, Lot no. 96H1069)
Disodium hydrogen phosphate (Merck, Germany, Lot no F1327886434)
Glyceryl dilaurate (Sigma, USA, Lot no. 043K1311)
Glyceryl distearate (Stepan Company, USA, Lot no. 7067373)
Isopropanol (Lab-Scan Analytical Sciences, Thailand)
Methanol (Lab-Scan Analytical Sciences, Thailand)
Polycarbonate membrane (Northern Lipids, Canada, Lot no. 4281005)
Polyoxyethylene glycol-8-laurate (Stepan Company, USA, Lot no. 701586)
Potassium dihydrogen phosphate (Merck, Germany, Lot no A531473425)
Propylthiouracil (supplied by Sriprasit Pharma Co. Ltd., Lot no. 20030602)
Sodium chloride (Merck, Germany, Lot no K33800104441)
Sodium hydroxide (Merck, Germany, Lot no. B870498625)
Span® 20 (EAC Chemical, Lot no. 16790)
Span® 40 (EAC Chemical, Lot no. 11036)
Span® 60 (EAC Chemical, Lot no. 16794)

Span® 80 (EAC Chemical, Lot no. 11078)

Span® 83 (EAC Chemical, Lot no. 11049)

Sucrose laurate ester (Mitsubishi-Kagaku Foods corporation, Japan, Lot no. 3226111)

Tween® 20 (EAC Chemical, Lot no. 11704)

Tween® 40 (EAC Chemical, Lot no. 11693)

Tween® 60 (EAC Chemical, Lot no. 11708)

Tween® 80 (EAC Chemical, Lot no. 11743)

Tween® 85 (EAC Chemical, Lot no. 11707)

Equipment

Analytical balance (GMPH, Sartorius, Germany)

Analytical balance (UMTZ, Mettler Toledo, Switzerland)

Differential scanning calorimeter (DSC 822e, Mettler Toledo, Switzerland)

Disposable syringe nylon filter 13 mm, 0.45 μm (Chrom Tech, USA, Lot no F13-1045)

Dry bath incubator (Boekel Scientific, Japan)

Hand-held extruder (LiposoFast™, Avestin, Canada)

High Performance Liquid Chromatography System (HPLC) (Perkin Elmer, Germany) equipped with

- Automatic sample injector (SIL-10A, Shimadsu, Japan)
- Communication bus module (CBM-10A, Shimadsu, Japan)
- Liquid chromatography pump (LC-10AD, Shimadsu, Japan)
- UV-VIS Detector (SPD-10A, Shimadsu, Japan)
- Column (BDS Hypersil® C18, 5 μm, 300 x 4.6 mm,)
- Precolumn (μBondapack C18, 10μm, 125A0, Water Corporation, Ireland, Lot no. W2336B1)

Light microscope (KHC, Olympus, Japan)

Lipex™ Extruder (Northern Lipids, Canada)

Modified Franz diffusion cells (Science Service, Thailand)

Mastersizer 2000 (Malvern Instruments, UK)

pH meter (Beckman, USA)

Shaking water bath (Innova 4230, New Brunswick Scientific, USA)

Ultracentrifuge (L80, Beckman, USA)

Ultrasonic bath (TP 680 DH, Elma, Germany)

UV spectrophotometer (Model 7800, Jasco Corporation, Japan)

Vortex mixer (G 560 E, Vortex-genie, USA)

Methods

1. Solubility of PTU

The aqueous solubility of propylthiouracil (PTU) was experimentally determined since the medium and temperature can affect the solubility. Solubilities of PTU in water and phosphate buffer, pH 7.4, were determined by continuous shaking of excess amounts of PTU in each medium using shaking water bath at room temperature. The samples were removed at 1, 3, 5, 7, 9, and 15 days and were filtered through membrane filters (0.22 μm pore size) to separate drug crystals. The filtrate was appropriately diluted and analyzed by UV spectrophotometry at 275 nm.

2. Feasibility Study on Preparation of PTU Vesicles by Sonication Method

This experiment was done to determine feasibility of preparing vesicles by using a method devoid of organic solvent. The method used was modified from that of Baillie et al. (1985). Various groups of 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 cholesterol content was varied between 0-50% by weight with 10% increments and the groups of surfactant explored in this experiment were Tween® (20, 40, 60, 80, and 85), Span® (20, 40, 60, 80, and 83), Brij® (52, 72, 76, 93, and 97), sucrose laurate ester (L-595), glyceryl dilaurate (GDL) and glyceryl distearate (GDS). Either Solulan® C24 or dicetylphosphate (DCP) was used as a stabilizer.

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. The mixture was immediately sonicated at 70 °C for 5 mintues using an ultrasonic bath (Elma Transsonic Digital type TP 680 DH) at 140% power (40 kHz) and then vortexed for 1 mintues. The resultant preparation was left to cool down at room temperature. The product was checked under light microscope (at 400x or 1000x magnification) for completeness of vesicle formation and lipid remnants. As a routine, care was taken to detect any aggregation of vesicles, changes in color, or presence of drug crystals under the microscope. The experiment was done at least in triplicate before the formula was accepted as feasible for vesicle formation.

The aqueous phases used in this experiment were 1.1 mg/mL PTU in water and 1.2 mg/mL PTU in phosphate buffer, pH 7.4. These concentrations were at approximately 90% of saturation solubility of PTU in water and in phosphate buffer, pH 7.4, at ambient temperature.

3. Characterization of the PTU Vesicular Suspensions

The PTU vesicular suspensions were characterized for entrapment efficiency, size and size distribution, stability, elasticity, and phase transition.

3.1. Determination of PTU entrapment efficiency

After preparation, the vesicular suspension was left at room temperature overnight to allow complete annealing and equilibrating of PTU between the lipid and the aqueous phases. The suspension was then separated into the supernatant containing the free drug and the pellet containing the entrapped drug by ultracentrifugation (see below). The PTU content in the pellet was analyzed and used to calculate the entrapment efficiency (EE). PTU in the supernatant was also assayed for routine monitoring of total recovery. The UV spectrophotometric assay of PTU was carried out using the method

described in *Analytical profiles of drug substances* (Aboul-Enein, 1977). The analytical method was validated using guidelines in USP25 (The United States Pharmacopieal Convention, 2002). The validation results for accuracy, precision, linearity, and specificity are shown in Appendix C. The experiment was done in triplicate with pooled vesicles from at least three batches of each formulation.

3.1.1. Separation of pellets

Five milliliters of the aqueous phase used in the preparation method was added to an aliquot (1 mL) of vesicular suspension to aid the centrifugation process. The suspension prepared in water was then centrifuged at 60,000 rpm at 25 °C for 4 hours, but the suspension prepared in phosphate buffer was centrifuged at 65,000 rpm at 25 °C for 4-6 hours. The supernatant was carefully separated from the pellet. PTU contents in the pellet and the supernatant were determined.

3.1.2. Quantitative analysis of PTU in pellets

The pellet was dissolved in isopropanol in a 10 mL volumetric flask, and the solution was adjusted to volume. One milliliter of this solution was further diluted with isopropanol in a 25 mL volumetric flask. This final solution was assayed by UV spectrophotometry at 275 nm. The presence of surfactants and/or cholesterol in the pellets did not interfere with the assay.

3.1.3. Quantitative analysis of PTU in supernatants

An aliquot of supernatant was appropriately diluted with isopropanol and analyzed by UV spectrophotometry at 275 nm.

3.1.4. Calculation of entrapment efficiency

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

$$EE = \frac{\text{Amount of PTU in the pellet (mg/mL)}}{\text{Amount of total lipid (mg/mL)}}$$

3.2. Determination of size and size distribution

Size and size distribution of the vesicles was determined by laser diffraction technique (Mastersizer 2000, Malvern Instruments, UK). Size and size distribution were expressed as D [4, 3] as recommended by the manufacturer of the instrument. The experiment was done in triplicate with pooled samples.

3.3. Determination of phase transition temperature

The DSC thermogram was determined by differential scanning calorimeter (DSC822^{e}, Mettler Toledo, Switzerland) to detect phase transition temperature of the vesicles and melting point of PTU, pure surfactant, cholesterol, Solulan® C24 and surfactant mixture. An accurately weighed amount (6-10 mg) of pure compounds or surfactant mixtures was used. The scan rate was at 5 °C/min, over the temperature range of 0-200 °C under nitrogen atmosphere except for Span® 20 where the temperature range was -40-200 °C. To detect phase transition temperature of the vesicles, an accurately weighed amount 15-20 mg of the pellets was used. The scan rate was at 2 °C/min, over the temperature range of 0-60 °C under nitrogen atmosphere except for pellet prepared from Span® 20 where the temperature range was -40-60 °C. The measurement was done in triplicate with three batches of the vesicles.

3.4 Stability of PTU vesicular suspension

All preparations of PTU vesicular suspensions prepared by sonication method were studied for stability by monitoring aggregation, changes in color, presence of drug crystals, changes in entrapment efficiency, size and size distribution, and phase transition for up to 2 months. All preparations were kept at room temperature in the form of vesicular suspensions without further separation. The experiments were performed in triplicate with pooled samples.

3.5 Measurement of vesicle elasticity

Vesicle elasticity was determined using modified method of Honeywell-Nguyen et al. (2002) for the formulation of L595/PEG-8-L by measuring the size of the vesicles before and after extrusion through polycarbonate membrane with 50 nm pore diameter twice using a hand-held extruder (LiposoFast™, Avestin, Canada). The vesicles were extruded through polycarbonate membrane with 200 nm pore size to exclude the larger vesicle before extrusion through polycarbonate membrane with 50 nm pore size. The size was determined by photon correlation spectroscopy (ZetaPlus, Germany). The experiment was performed in triplicate.

4. Drug Release Studies

PTU solutions (at 90% saturation) in water and phosphate buffer, pH 7.4, and four formulations of PTU vesicular suspensions: 1) GDS:CHO:Brij® 76 (45:15:40 w/w in phosphate buffer, pH 7.4) 2) Span® 20:CHO:Solulan® C24 (57.5:37.5:5 w/w in water) 3) L-595:PEG-8-L (50:50 w/w in water) 4) Span® 40:CHO:Solulan® C24 (67.5:27.5:5 w/w in water) were selected to study the release. The choices of formulations were based on thermodynamic state, size, and PTU entrapment of the vesicles. GDS:CHO:Brij® 76 in phosphate buffer was selected as vesicles in gel state. Span® 20:CHO:Solulan® C24 in water was a representative of vesicles in liquid crystalline state. L-595:PEG-8-L in water was in elastic state. Span® 40:CHO:Solulan® C24 67.5:27.5:5 w/w in water was also

selected since this formulation gave the highest entrapment of all noisome formulations in this study. The solutions of PTU in water and in phosphate buffer at 90% saturation were also tested as references. Ultrapure® water or phosphate buffer, pH 7.4, was used as a receptor fluid, corresponding to the composition of PTU vesicles being tested.

Modified Franz diffusion cells were used to study in vitro release of PTU from different formulations. The internal diameter of the cells was 1.70 cm, corresponding to an effective permeable surface area of 2.27 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 °C by circulating water through a jacket surrounding the cell body throughout the experiments. The receptor compartment contained 14.14-14.55 mL (from calibration) of the release medium. The domor and the receptor compartment were separated by a dialysis membrane (cellulose tubular membrane, Cellu-Sep®) with a molecular cut-off of 12,000-14,000. The receptor fluid and the membrane were equilibrated to the desired temperature for 1 hour. After equilibration, 1.0 mL of the formulation was carefully placed on the membrane surface of each cell and the cell was then covered completely and tightly with Parafilm®. The receptor fluid 3.0 mL was removed at appropriate time intervals and replaced with an equal volume of prewarmed (37 °C) fresh medium. The receptor fluid was diluted at appropriate concentration and measured by UV spectrophotometry at 275 nm.

The percent of PTU released was calculated by the following equation:

$$\%PTU\ released = (A_t / A_0) \times 100 \qquad (8)$$

where A_t is the cumulative amount of PTU released at a particular time; A_0 is the initial amount of PTU in the formulation.

The release study for each formulation was done in quadruplicate.

5. Permeation Studies

The permeation of PTU from the formulations was studied utilizing modified Franz diffusion cell. The abdominal skin of a newborn pig was used as natural skin membrane (Schnook et al., 2001).

5.1 Preparation of newborn pig skin membrane

To prepare a full-thickness abdominal skin membrane, the skin was completely removed subcutaneous fat and extraneous tissue using forcepts and scissors. The separated skin was cleaned and bathed in purified water. Then it was wrapped in aluminum foil and stored in a freezer (-20 °C.) until used. The frozen skin was thawed and allowed to rehydrate by immersing in phosphate buffer saline, pH 7.4, (PBS) at room temperature for about one hour before used. The skin was cut to a circular shape with a diameter of about 3 cm.

5.2 Permeation study

The procedure of permeation study was the same as that of the release study in Section 4 except that the membrane used in this study was newborn pig skin and the receptor medium was PBS, pH 7.4. The excised pig skin was set in place with the stratum corneum facing the donor compartment and the dermal side facing the receptor compartment. The receptor fluid and the skin were equilibrated to the desired temperature for 30 mintues. After equilibration, 120 $\mu L/cm^2$ of PTU vesicular suspensions or control preparations (1.1 mg/mL of PTU in water and 1.2 mg/mL of PTU in phosphate buffer, pH 7.4 for preparations prepared using water and phosphate buffer, pH 7.4, as the aqueous phase, respectively) was carefully placed on the membrane surface of each cell and the system was run under non-occlusive condition. Samples (about 1.0 mL) were withdrawn from the receptor compartment at appropriate time intervals for up to 24 hours and replaced with an equal volume of pre-thermostated (37 °C) fresh PBS. Addition of fresh PBS to the receptor compartment was performed with

great care to avoid air trapping beneath the dermis. The samples were taken into 2.0 mL vial and kept in freezer until PTU concentrations were analyzed by HPLC method. The analytical method was validated using guidelines in USP25 (The United States Pharmacopieal Convention, 2002). The validation results for accuracy, precision, linearity, and specificity are shown in Appendix D. Each set of experiments was performed with at least four diffusion cells ($n \geq 4$).

The cumulative amount of PTU that permeated through the membrane after the nth sampling (Q_n) was calculated by Eq. (9) (Yu and Liao, 1996)

$$Q_n = C_nV + \sum_{i=1}^{n-1} VsCi \quad (9)$$

where C_i and C_n are the various measured concentrations from 1 to n, V is the volume of the solution in the receptor compartment and V_s is the sampling volume.

5.3 Skin retention of PTU

At the end of permeation study the skin surface and the donor cap were washed 3 times with methanol which was later assayed for PTU remaining in the donor compartment. The skin was then removed and blotted dry with filter paper. The methanolic solution containing PTU amount remaining in the donor compartment was appropriately diluted with methanol and analyzed by HPLC method. The skin was cut into small pieces and extracted with methanol (3 mL) by vortexing for 5 mintues, sonicating for 5 mintues, shaking at ambient temperature for 2 hours, and filtering through membrane filter 0.45 μm. Then the filtrate was analyzed for PTU accumulated in the skin, Q_s, by HPLC method.

5.4 Data treatment

For each membrane specimen, the cumulative permeated amount of PTU per diffusion area was plotted against time. The observed steady state flux (J_{ss}) was obtained from the slope. Permeability coefficient (P_s), was calculated using Eq. 10 (Hofland et al,. 1994)

$$P_s = J_{ss}/C_d \quad (10)$$

where C_d is the drug concentration in the donor compartment. The enhancement factor, EF of the formulation based on the permeability coefficient was defined as in Eq. 11.

$$EF = (P_s \text{ of the formulation})/(P_s \text{ of control}) \quad (11)$$

The enhancement factor of the formulation based on the PTU amount in the skin (Q_s) was defined as in Eq. 12.

$$EF \text{ of } Q_s = (Q_s \text{ of the formulation})/(Q_s \text{ of control}) \quad (12)$$

The enhancement factor of the formulation based on the cumulative amount of PTU in the receptor medium at 24 hours (Q_{24}) was defined as in Eq. 13.

$$EF \text{ of } Q_{24} = (Q_{24} \text{ of the formulation})/(Q_{24} \text{ of control}) \quad (13)$$

Relative flux (RF) was defined as in Eq 14

$$RF = (J_{ss} \text{ of the formulation})/(J_{ss} \text{ of control}) \quad (14)$$

5.5 Assay PTU by HPLC method

The concentration of PTU was determined by HPLC meyhod (Perkin Elmer, Germany). The HPLC conditions modified from USP 25 (The United States Pharmacopieal Convention, 2002) were as follows

Column	:	BDS Hypersil® C18, 5 µm, 300 x 4.6 mm
Precolumn	:	µBondapack C18, 10 µm, 125 A^0
Mobile phase	:	0.025 M phosphate buffer pH 4.6/acetonitrile 85/15 v/v
Injection volume	:	20 µL
Flow rate	:	1 mL/min
Detector	:	UV detector at 272 nm
Temperature	:	ambient
Run time	:	12 min
Internal standard	:	Theophylline 5.0 µg/mL

6. Effects of Formulation Factors on Permeation of PTU from Vesicular Suspensions

There are many formulation factors affecting drug permeation such as thermodynamic state (Bouwstra and Honeywell-Nguyen, 2002), size of vesicle (Verma et al., 2003), melting point of surfactant mixture (Waranuch et al., 1998), surfactant types, and the existence of vesicular structure (El Maghraby et al 2000a; Fang, Hong et al., 2001), etc. So this experiment was aimed to study the effects of some formulation factors on PTU permeation from the four formulas selected from Section 3 based on thermodynamic state, size, and PTU entrapment of the vesicles. These formulas were GDS:CHO:Brij® 76 45:15:40 w/w in phosphate buffer, pH 7.4, which represented the gel state; Span® 20:CHO:Solulan® C24 57.5:37.5:5 w/w in water which represented the liquid crystalline state; L-595:PEG-8-L 50:50 w/w in water which represented the elastic state; and Span® 40:CHO:Solulan® C24 67.5:27.5:5 w/w in water which was also in the gel state and gave the highest entrapment efficiency among the formulations studied.

6.1 Effect of thermodynamic state

To study the effect of thermodynamic state, the permeation of PTU from the three formulations were studied utilizing modified Franz diffusion cells as described in Section 5. The three formulations were Span® 40:CHO:Solulan® C24 67.5:27.5:5 w/w in water, Span® 20:CHO:Solulan® C24 57.5:37.5:5 w/w in water, and L-595:PEG-8-L 50:50 w/w in water. The permeation parameters such as flux (J_{ss}), permeability coefficient (P_s), cumulative permeated amount of PTU at 24 hours (Q_{24}), PTU accumulated in skin (Q_s), EF of P_s, Q_s, and Q_{24} were compared among the three formulations.

6.2 Effect of surfactant type

GDS:CHO:Brij® 76 45:15:40 w/w in phosphate buffer, pH 7.4, and Span® 40:CHO:Solulan® C24 67.5:27.5:5 w/w in water vesicles were selected to investigate the effect of surfactant type on PTU permeation because they were all in gel state and have approximately the same entrapment efficiency and size. The permeation of PTU from both formulas was studied utilizing modified Franz diffusion cell as described in Section 5 and the permeation parameters between both formulations were compared.

6.3 Effect of the existence of vesicular structure

The four vesicular suspensions selected from Section 3 were prepared using method described in Section 2. The permeation of PTU from the vesicular suspensions and solutions or physical mixtures of surfactants in 90% propylene glycol in water were studied using method described in Section 5. Permeation parameters of PTU from the vesicular suspensions were compared with those from solutions, or physical mixtures.

7. Elucidation of the Dominating Mechanisms of PTU Permeation from Vesicular Suspensions

7.1 Increased drug thermodynamic activity

The permeation of drug through/into the skin may be high if the formulations contained high drug entrapment efficiency. To elucidate the possible mechanism of PTU permeation, the correlation between entrapment efficiency and permeation parameters of PTU from the same vesicular suspensions was determined.

7.2 The "free drug" mechanism

If the mechanism of action of the vesicles is solely the free drug mechanism whereby the drug is released from the vesicles and independently permeates the skin. Thus, the rate limiting step of skin permeation is the release rate. To investigate this mechanism, the correlation between permeation parameters of PTU and in vitro release rate constants of corresponding formula was determined.

7.3 Penetration enhancement of the vesicles

In order to explore this mechanism, the effect of skin pretreatment with empty vesicles on PTU permeation from aqueous solution at 90% saturation was performed (Hofland et al., 1994) using modified Franz diffusion cells as described in Section 5. The empty vesicles (400 μL/cell) were carefully placed on the skin and the cell was then covered completely and tightly with Parafilm® (occlusive condition). At the end of 4 hours, the empty vesicles were removed from the skin and the skin was washed with water and dried with cotton bud. The permeation of PTU from aqueous solution at 90% saturation through the treated skin using the same experiment design as described in Section 5 was then performed. The control cells were treated with water or phosphate buffer, pH 7.4. EF of permeation of PTU, EF of PTU in the skin (EF of Q_s), EF of Q_{24} and relative flux (RF) were calculated from Eq. (15)-(18)

$$EF = \frac{P_s \text{ after pretreatment with empty vesicle}}{P_s \text{ after pretreatment with aqueous phase}} \quad (15)$$

$$EF \text{ of } Q_s = \frac{Q_s \text{ after pretreatment with empty vesicle}}{Q_s \text{ after pretreatment with aqueous phase}} \quad (16)$$

$$EF \text{ of } Q_{24} = \frac{Q_{24} \text{ after pretreatment with empty vesicle}}{Q_{24} \text{ after pretreatment with aqueous phase}} \quad (17)$$

$$RF = \frac{J_{ss} \text{ after pretreatment with empty vesicle}}{J_{ss} \text{ after pretreatment with aqueous phase}} \quad (18)$$

The permeation parameters of PTU from niosomes were compared with those from solution (90% saturation) after pretreatment.

7.4 Transepidermal osmotic gradient

Non-occlusive condition is essential to create a transepidermal osmotic gradient, which is believed to be the driving force for the transport of elastic vesicles into the skin (Cevc and Blume, 1992). The permeation of PTU from L595:PEG-8-L (elastic vesicles) and Span® 40:CHO:Solulan® C24 (gel state) vesicles were studied as described in Section 5 using both occlusive and non-occlusive conditions (Honeywell-Nguyen and Bouwstra, 2003). Permeation parameters of PTU obtained under occlusive and non-occlusive conditions were compared.

8. Statistical Analysis

Statistical analysis to compare treatment means was performed on SPSS version 11.5. The validity of assumptions for ANOVA was tested on pooled data by using Kolmogorov-Sminrov normality test. If the distribution of data did not significantly

deviate from normality, ANOVA, with Tukey's HSD or Dunnett 3T as a post hoc comparison, was used. The Student's t-test was used to compare two treatment means. The level of significance was chosen at 0.05 probability.