CHAPTER III

MATERIALS AND METHODS

Materials

1. Propylthiouracil (supplied by Sriprasit Pharma Co., Ltd., Lot 9220747)
2. Phosphatidylcholine (Phospholipon®90 Nattermann Phospholipid GmbH, Cologne, Germany, Lot 770991)
3. α–Tocopherol (Approx. 95%, Sigma, USA, Lot 53H0444)
4. Stearylamine (Sigma, USA, Lot 45H3435)
5. Cholesterol (Sigma, USA, Lot 111H8488)
6. Dicetylphosphate (Sigma, USA, Lot 96H1069)
7. Dulbecco's Modified Eagle Medium (GibcoBRL, USA, Lot 1025102)
8. Calf serum (GibcoBRL, USA, Lot 1026097)
9. L-Glutamine (GibcoBRL, USA, Lot 1024284)
10. Antibiotic-Antimycotic (penicillin G, streptomycin sulphate, amphotericin B) (GibcoBRL, USA, Lot 1018186)
11. Sodium bicarbonate (GibcoBRL, USA, Lot 1019664)
12. HEPES (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) (Sigma, USA, Lot 127H5420)
13. Sodium hydroxide (Merck, Germany, Lot B870498625)
14. Sodium chloride (Fluka, Switzerland, Lot 71379)
15. Potassium dihydrogen phosphate (Merck, Germany, Lot K24331873808)
16. Sulphuric acid (Analar, England, Lot K2361283165112)
17. Ammonium molybdate (Fluka, Switzerland, Lot 232685)
18. Fiske-Subbarrow reducer (Sigma, USA, Lot 107H6249)
19. Hydrogen peroxide (Merck, Germany)
20. Dialysis membrane (Regenerated cellulose tubular membrane MWCO=12000-14000, Membrane Filtration Product, Inc., USA, Part# 1430-25)

21. Chloroform, AR grade (Merck, Germany, Lot 009K13659145)
22. Methanol, AR grade (JT Baker, USA, Lot N24B41)
23. Boric acid (Merck, Germany, Lot 805K01371765)
24. Citric acid (Merck, Germany, Lot K20379444349)
25. Dibasic sodium phosphate (Merck, Germany, Lot F997086532)
26. Potassium chloride (Merck, Germany, Lot 036TA915536)
27. Sterilization filtration membrane 0.22 μm (Supor® (polyethersulfone) membrane) (GelmanSciences, USA, Lot 81691 (25 mm), Lot 81244 (47 mm))
28. Trypsin-EDTA (GibcoBRL, USA, Lot 1025956)
29. Tissue culture flasks (Nunc, Denmark)
30. Multiwell plates (Costar, USA)

Equipment

1. Analytical balance (GMPH, Sartorius, Germany)
2. Analytical balance (UMT2, Mettler Toledo, Switzerland)
3. Rotary evaporator (RE120, Buchi, Switzerland)
4. Ultrasonic bath (T900/H, Elma, Germany)
5. Vortex mixer (G560E, Vortex-genie, USA)
6. Hot air oven (UL50, Memmert, Germany)
7. UV spectrophotometer (Model 7800, Jasco Corporation, Japan)
8. pH meter (Beckman, USA)
9. Modified Franz diffusion cells (Science Service, Thailand)
10. Light microscope (KHC, Olympus, Japan)
11. Ultracentrifuge (L80, Beckman, USA)
12. Refrigerated centrifuge (Sigma 2K15, B. Braun Biotech International, Germany)
13. Scanning Electron Microscope (JSM-5800LV, JEOL, Japan)
14. Shaking incubator (Innova 4230, New Brunswick Scientific, USA)
15. Hand-held extruder (LiposoFast™, AVESTIN, Canada)
16. Autoclave (HA-3D, Harayama Manufacturing Corporation, Japan)
17. Humidified carbondioxide incubator (Model 3164, Forma Scientific, USA)
18. Laminar air flow (HBB 24485, Holten, Denmark)
19. Inverted microscope (CK 2, Olympus, Japan)

Methods

1. Solubility of PTU

The aqueous solubility of PTU was experimentally determined since the different medium and temperature can affect the solubility. Solubilities of PTU in three different buffers: citrate buffer pH 5.5, HEPES buffer pH 7.4, and borate buffer pH 9.0 were determined by continuous shaking of excess amounts of PTU in each buffer using a shaking incubator. Since lecithin (phosphatidylcholine, PC), the major component of liposomes, is not stable at room temperature and liposomal preparations were to be kept refrigerated, the solubility studies were performed with temperature of the shaking incubator set at 4 °C (actual temperature 4-8 °C). The sample was removed at appropriate time intervals and was centrifuged for 10 minutes to separate drug crystals. The supernatant was appropriately diluted and analyzed by UV spectrophotometry at 275 nm.

2. Preparation of PTU-containing liposomes

Reverse phase evaporation vesicles (REVs) were prepared by modification of the method described by Szoka and Papakadjopoulous (Szoka and Pahadjopoulous, 1978). Briefly, PTU along with PC (either 10 mg or 100 mg) and α–tocopherol (0.1 mol%) were dissolved in 6 ml of chloroform in a 150-ml round bottom flask. One milliliter of saturated solution of PTU was added such that the organic to aqueous phase ratio was 6:1. The mixture was sonicated in an ultrasonic bath for 3 minutes at room temperature with care taken to prevent loss of the solvent from the system. The organic solvent was slowly removed at 35°C with a rotary evaporator until a viscous gel was formed. The flask was agitated vigorously on a vortex mixer so that the gel collapsed and transformed into suspension of liposomes.

All liposomal preparations were prepared under the same procedure described. Various compositions and conditions were used to study the effects of component and process variables on PTU entrapment, on release behavior, on water evaporation, on biological activity of PTU in cell culture, and on stability of PTU liposomes. Each experiment was performed in triplicate with three batches of liposomes.

2.1 Effects of preparation method, lipid concentration, and equilibrating time

The purpose of this experiment was to study the effect of lipid concentration, preparation method, and duration of storage on encapsulation efficiency of PTU liposomes. PC was used at concentrations of 10 mg/ml and 100 mg/ml, so that the effect of an order of magnitude difference in lipid concentration would be detected. Supawadee (1998) reported that since PTU does not have high solubility in lipid, hydration of the lipid phase with a large amount of water resulted in partitioning of the drug into the aqueous phase due to the partial solubility of the drug in water. To reduce the partitioning of the drug into the aqueous phase, hydration should be done with a saturated solution of PTU. In this study, three methods of preparation were used to further clarify the implication of this finding. The first method was that the drug was dissolved in chloroform along with PC and water was added to the organic phase (organic PTU method). In the second method, PC without PTU was dissolved in chloroform, and saturated solution of PTU in HEPES buffer pH 7.4 was added to the organic phase (aqueous PTU method). To study the partitioning behavior of PTU during the preparation process, the same total PTU amount of 1.1 mg/ml of the final preparation, which was the solubility of PTU in HEPES buffer pH 7.4 at 4 °C, was dissolved in either the lipid phase or the aqueous phase. The third method was used in an attempt to enhance the entrapment of the drug. In this method (organic-aqueous PTU method), PTU and PC were dissolved in chloroform and saturated solution of PTU in HEPES buffer pH 7.4 was added to the organic phase.

To determine the maximum amount of PTU to be used in the third method, the drug amount in the organic phase was varied from 0.2 mg to 2.0 mg with an increment of 0.2 mg. The resultant liposomal systems were kept refrigerated for 3 days. The system with highest drug loading (at which drug crystals were not observed at day 3 under optical microscope) was selected for the encapsulation efficiency study.

Encapsulation efficiencies were determined by the method described below. The assay was performed either immediately after preparation or after one week of storage in a refrigerator to study the effect of equilibrating time.

2.2 Effects of liposomal charge, pH, and cholesterol

In the following experiments, all liposomal preparations were prepared by the organic-aqueous PTU method, at a total lipid concentration of 100 mg/ml of the final preparation. The organic phase was mixed with saturated solution (at 4 °C) of PTU in three buffers: citrate buffer pH 5.5, HEPES buffer pH 7.4, and alkaline borate buffer pH 9.0 at the same ionic strength of 0.2. The maximum PTU amount in the organic phase of each system was determined individually as described in 2.1. Thus, the total content of PTU in the liposomal system depended on the lipid composition and the type of buffer used in each formulation. Liposomes prepared from PC and stearylamine (SA) at a weight ratio of 97:3 provided positive net charges, while those from PC and dicetylphosphate (DCP) at a weight ratio of 95:5 provided negative net charges. Liposomes were also prepared from PC with cholesterol (CH), PC with cholesterol and SA, or PC with cholesterol and DCP at a weight ratio of 70:30, 67:30:3, or 65:30:5, respectively. Encapsulation efficiencies were determined and compared between formulations.

3. Determination of PTU encapsulation efficiency

The liposomal suspension was separated into the supernatant containing the free drug and the pellet containing the entrapped drug by ultracentrifugation (see below). The PTU content in the liposomal pellet was assayed and used to calculate the encapsulation efficiency. The PTU in the supernatant was also assayed for the routine monitoring of percent analytical recovery.

3.1 Separation of liposomal pellet

An aliquot of liposomal suspension was centrifuged at 60,000 rpm at 4 °C for 5 hours in an ultracentrifuge. When necessary, saturated solution of PTU in an appropriate aqueous medium was added to the aliquot to aid the process of centrifugation. The PTU content in the supernatant and in the pellet was assayed according to the following assay protocol.

3.2 Quantitative analysis of PTU in liposomal pellet

The pellet separated from 1 ml of liposomal system was dissolved in methanol:chloroform (8:2) in a 10-ml volumetric flask, and the solution was adjusted to volume. One milliliter of this solution was transferred to and adjusted to volume with methanol in the second 10-ml volumetric flask. Then, two milliliters of this solution was further transferred to and adjusted to volume with methanol in another 10-ml volumetric flask. This final solution was assayed by UV spectrophotometry at 275 nm. The presence of lipid components did not seem to interfere with the assay (Appendix E).

3.3 Quantitative analysis of PTU in supernatant

The supernatant was appropriately diluted with methanol and analyzed by UV spectrophotometry at 275 nm.

3.4 Assay of phospholipids

The phosphorus content of phospholipid was determined by Bartlett assay (Bartlett, 1959). In this method, phospholipid phosphorus was acid hydrolyzed to inorganic phosphate and converted to phospho-molybdic acid by the addition of ammonium molybdate. The phospho-molybdic acid was reduced to a blue-colored compound by amino-naphthyl-sulphonic acid (Fiske-Subbarrow reducer). The intensity of the blue color was measured spectrophotometrically at 800 nm, and the concentration was determined from a calibration curve of phosphate standard solutions prepared from potassium phosphate. The phospholipids used for the preparation of liposomes contain one mole of phosphorus per mole of phospholipid.

3.4.1 Preparation of phosphate standard solutions

The anhydrous potassium dihydrogen phosphate was dried at 105 °C for 4 hours in a hot air oven. A stock solution of phosphate standard was prepared by accurately weighing 43.55 milligrams of dried anhydrous potassium phosphate into a 100-ml volumetric flask. The content in the flask was dissolved in double-distilled water and adjusted to volume. The final concentration of phosphorus was 3.2 μ mol/ml. Aliquots of phosphate stock solution (2, 3, 4, 5, 6, 7, and 8 ml, respectively) was transferred to seven 100-ml volumetric flasks. The solutions were adjusted to volume with double-distilled water so that the final concentrations of phosphorus were 0.064, 0.096, 0.128, 0.160, 0.192, 0.224 and μmol/ml, respectively.

3.4.2 Preparation of sample solutions

The liposomal suspension was diluted with double-distilled water to give a concentration of approximately 1 mg/ml of phospholipid before being subjected to further assay procedure.

3.4.3 Preparation of reagents

Ammonium molybdate-sulfonic acid reagent:

The solution was prepared by mixing of 5 ml of 5 M sulfuric acid with approximately 50 ml of double-distilled water and then adding of 0.44 g of ammonium molybdate to the acid solution. The solution was mixed until ammonium molybdate dissolved completely, and the volume of this solution was adjusted to 200 ml with double-distilled water.

1-Amino 2-naphthyl 4-sulfonic acid reagent:

The solution was prepared by weighing of 0.8 g of Fiske-Subbarrow reducer and then dissolving it in 5 ml of double-distilled water. This solution was freshly prepared on the day of use.

3.4.4 Bartlett Assay

The procedure for treating the samples were as follows:

Fifty microliters of diluted liposome suspension was added to empty test tubes. The sample was dried down and resuspended in 0.5 ml of double-distilled water. A calibration curve was set up by pipetting of 0.5 ml of the standard solutions into separate tubes, together with a blank (0.5 ml of distilled water). Each of the resuspended samples and the standard solutions was added with 0.4 ml of 5 M sulfuric acid and then incubated at 180-200 °C for an hour in a hot air oven preheated at 200 °C for 30 minutes. After the tubes were cooled down by standing them at room temperature, they were added with 0.1 ml of the freshly diluted hydrogen peroxide (10% v/v) and incubated at 180-200 °C for another 30 minutes in the hot air oven until colorless solutions were obtained. The solutions were cooled down to room temperature. Acid-molybdate solution (4.6 ml) was

added to each tube and the content of the tube was mixed well by vortexing. The solutions were added with 0.2 ml of Fiske-Subbarrow reducer and vortexed. The tubes were then covered and placed in a boiling water bath for 7 minutes. After the tubes were cooled down, the absorbances of the blue-colored solutions were measured at 800 nm against distilled water. The phosphorus content of liposomal suspensions was calculated as follows:

Phosphorus content = concentration x dilution factor x MW of lecithin
(μg/ml) (μmol phosphorus/ml)

3.5 Calculation of encapsulation efficiency

Encapsulation efficiency was defined as the fraction of PTU found in the liposomal pellet and expressed as millimole of drug per mole of lipid.

$$\text{Encapsulation efficiency} = \frac{\text{Amount of PTU in pellet (mg/ml) / MW of PTU}}{\text{Amount of phospholipid (mg/ml) / MW of phospholipid}}$$

When other lipids were present, the calculation was modified so that the encapsulation efficiency represented the amount of PTU per mole of total lipid.

4. Morphology of PTU liposomes

The morphology of the surface of liposomes were explored by a scanning electron microscopy procedure (Korakot, 1995). A few drops of liposomal suspension was smeared on a slide, followed by addition of phosphate buffer (pH 7.4) onto the smeared sample. After 5 minutes, the excess liquid was drained gradually. This step was repeated twice. Glutaraldehyde (2.5% w/v) was added to the sample and the excess liquid was gradually removed after 30 minutes. Then phosphate buffer was dropped onto the sample and left for 5 minutes. The excess liquid was drained. This step was repeated three times followed by staining with 1% (w/v) osmium tetroxide

for 30 minutes. After that, the sample was dehydrated with 30% (v/v) ethanol for 5 minutes and finally with 100% ethanol for 5 minutes. The dehydration step was repeated three times. The sample was dried using critical point dryer and finally coated with gold and examined with a scanning electron microscope.

5. Drug Release studies

Saturated aqueous solution of PTU in borate buffer pH 9.0 and four formulations of PTU-containing pellets of liposomes: 1) PC, pH 7.4, 2) PC/DCP, pH 7.4, 3) PC/SA, pH 9.0, 4) PC/CH/DCP, pH 7.4 were selected to study the release. The selected formulations were formulations with three different types of surface charge that gave the highest encapsulation efficiency within the same charge type. In addition, to investigate the influence of cholesterol on the release of PTU from liposomes, the release profile of the formulation which gave the highest encapsulation efficiency when cholesterol was present (PC/CH/DCP, pH 7.4) was also selected.

Modified Franz diffusion cells consisting of the donor and the receptor compartments were used to study in vitro release of PTU from different liposomal formulations. The internal diameter of the cells 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±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 13.44-14.50 ml (from calibration) of HEPES buffer pH 7.4 or borate buffer pH 9.0, according to the composition of PTU liposomes studied, as the release medium. The donor and the receptor compartments were separated by a dialysis membrane (cellulose tubular membrane, Cellu-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 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 contaminants. The membrane was then soaked in HEPES buffer pH 7.4 or borate buffer pH 9.0 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 (1.5 ml saturated PTU solution or liposomal pellets retrieved from 1.5 gram of the suspension) was carefully placed on the membrane surface of each cell and the cell was then covered completely and tightly with Parafilm®. Five milliliters of the receptor fluid was removed at appropriate time intervals and replaced with an equal volume of fresh buffer. The receptor fluid was diluted by transferring 2 ml to a 10-ml volumetric flask and adjusted to volume with the same buffer. The solution diluted with HEPES buffer pH 7.4 was measured by UV spectrophotometry at 275 nm while the solution diluted with borate buffer pH 9.0 was measured at 261 nm. The analysis was performed at different wavelengths because the maximum absorbance of released PTU in HEPES buffer pH 7.4 measured at 275 nm was shifted to 261 nm when the receptor fluid was borate buffer pH 9.0.

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 entrapped which was determined for each liposomal preparation.

The release studies for each liposomal formulation were performed in triplicate using three batches of each formulation.

6. Water Evaporation study

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 water evaporation profiles from liposomal systems, selected from the experiment 2.2 as in drug release studies. The profile of water evaporation from saturated solution of PTU was also studied for comparison.

Either liposomal pellets or saturated solution of PTU was packed and spread uniformly on aluminium crucibles with the thickness of 1.6 mm and the surface area of 0.216 cm^2 (normally used for Differential Scanning Calorimetry). These aluminium crucibles were then placed on a stainless steel plate with a water circulating system to control a constant temperature at 32 °C to simulate the human skin temperature. The weight loss of the samples was determined every 5 minutes with an analytical balance for up to 1.5 hours. All preparations were tested simultaneously in one experimental set-up to avoid the effect of relative humidity.

7. Biological activities of PTU liposomes

7.1 Preparation of tested solutions

PTU and caffeine were dissolved in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 8% (v/v) heat-inactivated calf serum (CS), 100 U/ml penicillin, 100 μg/ml streptomycin, 100 U/ml amphotericin B, and 2 mM glutamine (DMEM+) or in DMEM+ without CS. Saturated solution of PTU, at a concentration of 1.1 mg/ml, and the same concentration of caffeine solution were then sterilized by filtration through 0.22 μm sterile Supor®(polyethersulfone) membrane. A serial dilution of PTU solution were prepared in DMEM+ or in DMEM+ without serum (1.1 mg/ml to 1.1 μg/ml).

7.2 Preparation of liposomes

The PTU liposomes, hydrated with HEPES buffer pH 7.4, composed of various lipid compositions: PC/DCP, PC/DCP/CH, and PC/CH were prepared by the method described above. Each type of liposomes without PTU (empty liposomes) was also prepared to ensure that the effects of PTU liposomes on

proliferation of cells in culture were caused by PTU, not by lipid components of liposomes. The liposomal pellets harvested by centrifugation at 60,000 rpm at 4 °C for 5 hours were reconstituted with DMEM+ without CS. The liposomal pellets in DMEM+ without CS were extruded fifteen passages through a two-stacked 0.1 μ m polycarbonate membrane with a hand-held extruder. After extrusion, liposomes were then sterilized by filtration through 0.22 μm sterile membrane. The cell culture studies for liposomal formulations were performed in triplicate using the same batch of each formulation.

7.3 Cell culture experiments

7.3.1 Fibroblast cultures

BALB/c mouse 3T3 fibroblast cell line was routinely seeded in 25-cm^2 tissue culture flasks at a concentration of 2 X 10^5 cells in 5 ml DMEM+. For passage of the cells, fibroblasts were washed with 2 ml phosphate-buffered saline (PBS) and incubated for 2 minutes with 0.05% (w/v) trypsin-EDTA. Dispersed fibroblasts were reseeded in the 25-cm^2 flask for further passages (2 X 10^5 cells/ 5 ml DMEM+) and seeded in 6-well tissue culture plates for the experiments (5.5 X 10^4 cells/ 2 ml DMEM+ for one-day experiments or 3.8 X 10^3 cells/ 2 ml DMEM+ for three-day experiments). Cultures were maintained in a CO_2 incubator at 95% humidity, 5% CO_2, at 37 °C.

7.3.2 Assay of fibroblast proliferation

Dispersed fibroblasts were seeded in the multiwell plates for the experiments. Three days after seeding, at subconfluency of fibroblasts, the medium was removed, and the cells were re-incubated with 2 ml of tested samples using three wells for each sample. The effect of incubation time in the presence of the tested sample on cell proliferation was compared between 1 day and 3 days. In order to test the method used, the

comparison between the antiproliferative effect of caffeine (Schaffer and Touitou, 1991; Touitou et al., 1994) and PTU was performed using either 1.1 mg/ml PTU or caffeine.

Due to the inability of liposomes to retain entrapped substances when incubated with blood or plasma (Weiner, Martin, and Riaz, 1989) and due to possible alteration of the rate, extent, and mode of interaction of liposomes with cells which can occur in the presence of serum, the antiproliferative effects of PTU were compared using 1.1 μg/ml to 1.1 mg/ml PTU in the absence and presence of CS in DMEM+. From this study, the optimum PTU concentration of 0.11 mg/ml were selected for further studies of the antiproliferative effect of PTU.

The antiproliferative effect of either liposomal PTU or PTU in solution at the same amount of PTU was compared. PTU crystals was dissolved or liposomal pellets were reconstituted in DMEM+ without CS. Empty liposomes with the same composition of lipid were also tested.

Cultures were incubated in the incubator for an additional day or three days depending on the experiments. On the last day of the experiment, the medium was removed from the wells. The wells were washed with 1 ml PBS and incubated for 2 minutes with 1 ml trypsin-EDTA. Detached fibroblasts were retrieved and pelleted at 4,000 g for 10 minutes at 4 °C and then resuspended in 400 μl of DMEM+. Fifty microliters of the cell suspension was incubated with 50 μl of 0.4% trypan blue in saline. The viable cell number was determined under a light microscope using a hemacytometer. Results were given as the percent of ratio between viable cell numbers in compound-treated wells and viable cell numbers in control wells.

8. Physical stability of PTU liposomes

Four preparations of PTU liposomes: 1) PC, pH 7.4, 2) PC/DCP, pH 7.4, 3) PC/SA, pH 9.0, 4) PC/CH/DCP, pH 7.4 were prepared by the organic-aqueous PTU method. The physical stability was studied by monitoring aggregation, changes in color, presence of drug crystals, and changes in encapsulation efficiency of liposomal systems for up to 8 weeks. All preparations were kept in a refrigerator in the form of liposomal suspension as prepared without further separation. The experiments were performed in triplicate using three batches of each formula.

9. Statistical analysis

Statistically analysis of mean differences was performed on SPSS 7.5. The non-parametric tests, Kruskal-Wallis test or Mann-Whitney U test, were performed. However, the non-parametric test did not have sufficient power for multiple comparison. Whenever the statistically significant differences were detected with the Kruskal-Wallis test, the valid assumptions for ANOVA were justified using Kolmogorov-Smirnov normality test on pooled samples. If the normality test showed that the distribution of the data did not significantly deviate from normality, the ANOVA with SNK was used.