CHAPTER III

MATERIALS AND METHODS

Materials

1. Lactic acid (Approx. 98%, Sigma, USA Lot 106H0722)
2. Phosphatidylcholine (from fresh egg yolk, typeXIE, Sigma, USA)
3. Stearylamine (Sigma, USA Lot 45H3435)
4. Phosphatidylglycerol (Sigma, USA Lot 96H8404)
5. Cholesterol (Sigma, USA Lot 111H8488)
6. α-Tocopherol (Approx. 95%, Sigma, USA Lot 53H0444)
7. Chloroform, AR grade (BDH Laboratory Supplies, England K23408941 642)
8. Absolute methanol, AR grade (BDH Laboratory Supplies, England K22540270 604)
9. Methanol HPLC grade (Lab-Scan Co., Ltd Ireland)
10. Isopropanol HPLC grade (Lab-Scan Co., Ltd Ireland)
11. Phosphoric acid (Merck, Germany)
12. MES acid (2-(N-Morpholino)ethanesulfonic acid) (Sigma, USA Lot 114 H57281)
13. Sodium hydroxide (BDH Laboratory Supplies, England 191294D036)
14. Sodium chloride (Farmitalia Carlo Erba, Italy Cod 479687)
15. Sodium dihydrogen phosphate (Merck, Germany Lot A768946 407)
16. Disodium hydrogen phosphate (Merck, Germany Lot F997086 532)
17. Dialysis membrane (Regenerated cellulose tubular membrane, Membrane Filtration Product, Inc, USA Part#1430-25 MWCO=12000-14000)
18. Potassium dihydrogen phosphate (Merck, Germany A768946 407)
19. Sulfuric acid (Merck, Germany K23612831 651)
20. Ammonium molybdate (Fluka, Swizerland Lot 232685)

21. Fiske-Subbarrow reducer (Sigma, USA Lot 084H78212)
22. Hydrogen peroxide (Merck, Germany)

Equipment

1. Analytical balance (Sartorius GMPH, Germany)
2. Rotary Evaporator (RE120, Buchi, Swizerland)
3. Ultrasonic bath (T900/H, Elma, Germany)
4. Vortex mixer (Vortex-genie, Model G560E, USA)
5. Hot air oven (UL50, Memmert, Germany)
6. High Performance Liquid Chromatography (HPLC) instrument equipped with the following:
   - a tunable absorbance detector (Model 484, Waters, USA)
   - a constant flow pump (Model 600E, Waters, USA)
   - an integrator (Model 746,Waters, USA)
   - an autoinjector (Model 712 WISP, Waters, USA)
   - a C8 reverse phase chromatography column (Spherisorb ODS2, 250x4.6 mm, 5 micron, Phase Separations, USA)
7. UV Spectrophotometer (Model 7800, Jasco Corporation, Japan)
8. pH meter (Beckman, USA)
9. Modified Franz diffussion cells (Crown Glass Company, Inc, USA)
10. Refrigerated centrifuge (Model CR20B3, Hitachi, Japan)
11. Ultracentrifuge (L80, Beckman, USA)
12. Light Microscope (KHC, Olympus, Japan)
13. Transmission Electron Microscope (Model JEM-200CX, JOEL®, Japan)
14. Scanning Electron Microscope (Model JSM-5410LV, JOEL®, Japan)

Methods

1. Methods of quantitative analysis of lactic acid and phospholipids

1.1 HPLC assay for lactic acid analysis

1.1.1 HPLC conditions

The high pressure liquid chromatography technique was used for analysis of lactic acid. The system consisted of a constant flow pump, a variable wavelength UV detector, an integrator and an autoinjector. The conditions used for analyzing lactic acid by HPLC method are adapted from Handbook of Food analysis by HPLC (Gomis, 1992) and presented as follows:

Column	:	Spherisorb C8 ODS2 (250 x 4.6 mm), 5 micron
Mobile phase	:	20 mM Phosphoric acid
Detector wavelength	:	210 nm
Flow rate	:	1 ml/min
Attenuation	:	32
Chart speed	:	0.25 cm/min
Injection volume	:	50 microliters
Internal standard	:	tyrosine 4 mcg/ml
Retention time	:	lactic acid, 4.24-4.28 min
		tyrosine, 11.88-12.45 min

1.1.2 Preparation of standard solutions

A stock solution of internal standard was prepared by completely dissolving 5.0 mg of tyrosine in 20 mM Phosphoric acid in a 25-ml volumetric flask. The solution was adjusted to volume, giving the final concentration of 200 mcg/ml.

A stock solution of lactic acid was prepared by dissolving 25.0 mg of lactic acid in 20 mM Phosphoric acid. The solution volume was adjusted to 25 ml in a volumetric flask.

Standard solutions were prepared by pipetting 0.2, 0.5, 1, 2, 3 and 5 ml of lactic acid stock solution and tranferring each aliquot to each one of six 10-ml volumetric flasks. Two-hundred microliters of the tyrosine stock solution was added into each of these volumetric flasks. The solutions were adjusted to volume with 20 mM Phosphoric acid so that the concentrations of the standard solution were 20, 50, 100, 200, 300 and 500 mcg/ml, respectively.

1.1.3 Validation for the quantitative determination of lactic acid in liposomes by HPLC

The parameters essential to ensure the acceptability of the performance of an analytical method are accuracy, precision, sensitivity, specificity, and linearity (USPXXI).

1.1.3.1 Accuracy

a) Analysis of lactic acid in solution

Lactic acid solutions were prepared at 100, 300, and 500 mcg/ml. Three sets of these solutions were prepared. Each individual sample was analyzed by HPLC. Percent analytical recovery of each sample was calculated.

b) Analysis of lactic acid in liposome suspension

Three lots of lactic acid liposomes were prepared at each of the following lactic acid concentrations:10, 20, and 60 mg/ml. The free drug was seperated from the pellet fractions by high-speed centrifugation (18,000 rpm at 4°C

for 2 hours). The free drug samples were analyzed by HPLC as such, while lactic acid in the pellets was extracted from lipid composition before injection to HPLC. The extraction was performed by dissolving the pellet in 2 ml of chloroform:isopropanol (1:4). Phase seperation was induced by addition of 5 ml of distilled water. The mixture was then vortexed for 10 sec and centrifuged at 5000xg for 5 min. The aliquot of the upper phase was analyzed for lactic acid. The amount of lactic acid entrapped in liposomes was calculated accordingly.Total amount of lactic acid was expressed as the sum of amount of lactic acid in the supernatant and in the pellet fractions. Percent analytical recovery of each sample was calculated.

c) Recovery of lactic acid after seperation of lactic acid liposomes from the free drug

Separation of lactic acid liposomes from free drug was done by preparing a preparation of liposome suspensions containing lactic acid at concentration of 10 mg/ml. Three one ml aliquots of the liposome suspensions was seperately pipetted and centrifuged at 31,000xg at 4° C for 2 hours. Lactic acid contents in the supernatant and in the pellet were analyzed. Percent analytical recovery was calculated from the total amount the total amount of lactic acid found in the supernatant and the pellet by comparing with the actual amount of lactic acid used to prepare liposome suspensions.

1.1.3.2 Precision

a) Within run precision

The within run precision was determined by analyzing of three sets of the calibration curve in the same day. Peak area ratios of lactic acid to tyrosine were compared and the percent coefficient of variation (%CV) for each concentration was determined.

b) Between run precision

The between run precision was determined by comparing each concentration of three sets of the calibration curve prepared on different days. Peak area ratios for the three standard curves injected on different days were determined and %CV of each concentration was calculated .

1.1.3.3 Specificity

Under the chromatographic conditions selected, the peaks of other components in the sample must not interfere with the peak of lactic acid. Liposome suspensions were seperated into pellets and supernatant by high-speed centrifugation (18,000 at 4°C for 2 hours). The sample solutions of lactic acid extracted from liposomal pellets and of the free drug in the supernatant were injected. Chromatograms were evaluated by comparing with those of the standard solutions of lactic acid.

1.1.3.4 Linearity

Seven lactic acid standard solutions in the concentration range of 20-500 mcg/ml were prepared and analyzed. Linear regression analysis of the peak area ratios versus their concentrations was performed.

1.2 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. The phospholipids used for the preparation of liposomes contain one mole of phosphorus per mole of phospholipid.

1.2.1 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 of 43.55 milligrams of dried anhydrous potassium phosphate in 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 μmol/ml. Aliquots of phosphate stock solution (2, 3, 4, 5, 6 and 7 ml, respectively) were transferred to six 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 and 0.224 μmol/ml, respectively.

1.2.2 Preparation of sample solutions

The liposome 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.

1.2.3 Preparation of reagents

Ammonium molybdate-sulfonic acid reagent :

The solution was prepared by mixing of 5 ml of 5 M sulphuric acid with approximately 50 ml of distilled water and 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 distilled water.

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

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

1.2.4 Bartlett Assay (Bartlett, 1959)

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 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 sulphuric acid and then incubated at 180-200°C for an hour in a hot air oven pre-heated at 200°C for 30 min. 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%) and incubated at 180-200°C for another 30 min 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 min. 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 liposome suspensions was calculated as follow:

Phosphorus content = concentration x dilution factor x MW of lecithin (1)
(μg/ml) (μmol phosphorus/ml)

2. Preparation of lactic acid - containing liposomes

Reverse phase evaporation vesicles (REVs) were prepared by the modified method described by Szoka and Papahadjopoulous (Szoka and Pahadjopoulous, 1978). Briefly, twenty milligrams of egg phosphatidylcholine (EPC) was dissolved in 12-ml of chloroform:methanol (1:1) in a 500 ml pear-shaped flask. Two milliliters of 10 mg/ml of lactic acid in water was added such that the organic to aqueous phase ratio was 6:1. The mixture was sonicated for 5 min at room temperature . 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 vortex mixer so that the gel was collapsed and tranformed into suspension of liposomes.

All liposomes were prepared under the same procedure described. Various components and conditions were used in preparation of lactic acid liposomes to study the effects of component and process variables on lactic acid entrapment, on release behavior, and on stability of lactic acid liposomes. The experiments were performed in triplicate with three batches of liposomes.

2.1 Preparation of lactic acid liposomes for studying the effects of total lipid concentration, lactic acid concentration, liposomal charge, pH, ionic strength, and cholesterol on entrapment

2.1.1 Effect of total concentration of lipid on lactic acid entrapment

Liposomes were prepared by varying the amount of EPC from 10, 20, 30, and 40 mg/ml. A fixed lactic acid concentration of 10 mg/ml in 0.05 M Mes buffer pH 5 (μ=0.1) was used as the aqueous phase. Percent entrapments and encapsulation efficiencies were determined and compared.

2.1.2 Effect of lactic acid concentration on lactic acid entrapment

Liposomes with various concentrations of lactic acid (10, 20, 40, 60, 80, and 100 mg/ml) were prepared in 0.05 M Mes buffer (pH 5, μ=0.1). Each liposome preparation used a fixed EPC concentration of 10 mg/ml. Encapsulation efficiencies of lactic acid were determined and compared.

2.1.3 Effect of liposomal charge, pH, and ionic strength on lactic acid entrapment

All liposome preparations had a total lipid concentration of 10 mg/ml. Liposomes were prepared from EPC and a charged amphiphile in a molar ratio of 9:1. Stearylamine was used for providing positive charges while phosphatidyl glycerol was used for negative ones. The concentration of lactic acid in the aqueous phase was 10 mg/ml and pH was adjusted to 3, 4, and 5 at various ionic strengths (0.1, 0.3, and 0.5). Encapsulation efficiencies of lactic acid were determined and compared.

2.1.4 Effect of cholesterol on lactic acid entrapment

From the preparation that gave the highest lactic acid entrapment in the experiment 2.1.3, cholesterol (50 mol%) was added to the lipid composition. Liposomes were prepared from EPC with cholesterol or EPC with cholesterol and a charged amphiphile in a molar ratio of 1:1 or 4.5:4.5:1 with a total lipid concentration of 10mg/ml, respectively. The concentration of lactic acid was 10 mg/ml.

Encapsulation efficiencies of lactic acid were determined and compared between formulations with and without cholesterol.

2.2 Preparation of lactic acid liposomes for studying the effects of liposomal composition on stability of liposomes

α-Tocopherol (0.1 mol%) was added to each one of the four compositions of lactic acid liposomes: 1) EPC, 2) EPC and stearylamine, 3) EPC and cholesterol, and 4) EPC, cholesterol and stearylamine. The concentration of lactic acid was 10 mg/ml. Lactic acid liposomes were prepared in the same molar lipid ratio as in 2.1.4. Encapsulation efficiencies of the eight preparations (with and without α-tocopherol) were determined immediately after preparation and after one week of storage.

3. Characterization of lactic acid liposomes

Lactic acid liposomes prepared from EPC by reverse phase evaporation method at pH 2.5 and μ=0.1 were characterized under electron microscopes.

3.1 Transmission Electron Microscopy

The procedure for negative staining of a liposome preparation sample was as follows. A drop of liposome suspensions was applied to a grid covered with a thick formvar flim. After leaving for 5 minutes to allow adsorption of liposomes to the grid, the excess was removed by filter paper. Phosphotungstic acid (1%) was dropped onto the grid. Then the grid was air-dried for approximately 10 minutes and examined under a transmission electron microscope.

3.2 Scanning Electron Microscopy

The morphology of the surface and the size of liposomes were explored by a scanning electron microscopy procedure (Korakot, 1995). A few drops of liposome 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%) 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% osmium tetroxide for 30 minutes . After that, the sample was dehydrated with 30% 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.

4. Determination of lactic acid encapsulation efficiency of REVs

The liposome suspension was seperated into the supernatant containing the free drug and the pellet containing the entrapped drug by high-speed centrifugation. The lactic acid content in the liposome pellet was assayed and used to calculate the encapsulation efficiency. The lactic acid in the supernatant was also assayed for the routine monitoring of percent analytical recovery.

4.1 Separation of entrapped lactic acid liposomes

An aliquot (1 ml) of liposome suspension was centrifuged at 31,000 x g and 4°C for 2 hours. The amounts of lactic acid in the supernatant and in the pellet were determined.

4.2 Quantitive analysis of lactic acid in liposome pellets

The liposomes containing entrapped lactic acid was dissolved in 2 ml of chloroform:isopropanol (1:4), then 5 ml of distilled water was added. The mixture was shaken with vortexed-mixer for 10 sec and then, centrifuged at 5,000 x g for 5 min to separate the two phases. The aqueous upper phase was analyzed for lactic acid by HPLC method. Three millilitres of the aqueous upper phase from each preparation was pipetted and transferred into a 10-ml volumetric flask containing 200 microlitres of 200 mcg/ml internal standard. The solution was adjusted to volume with the mobile phase and ready for HPLC analysis.

4.3 Quantitative analysis of lactic acid in the supernatant

The supernatant was diluted with distilled water and adjusted to volume in a 10-ml volumetric flask. Two ml of the solution was transfered to a 10-ml volumetric flask containing 200 microlitres of 200 mcg/ml internal standard. The solution was adjusted to volume with 20 mM Phosphoric acid and was ready for HPLC analysis.

4.4 Assay of Phospholipids

Phosphorus content of lipid was determined by the method of Barlett as described in the experiment 1.2.

4.5 Calculation of percent entrapment and encapsulation efficiency

The percent entrapment of each of the three aliquots was determined from the following equation:

$$\%\text{Entrapment} = \frac{\text{Amount of lactic acid in the pellets}}{\text{Total amount of lactic acid in liposome suspensions}} \quad (2)$$

where Total amount of lactic acid in liposome suspensions is expressed as the sum of amount of lactic acid in the free drug and the pellets

Encapsulation efficiency was defined as the fraction of lactic acid found in the liposome pellet and expressed as mole of drug per mole of lipid.

$$\text{Encapsulation efficiency} = \frac{\text{Amount of lactic acid in pellet (mg/ml)/ MW of lactic acid}}{\text{Amount of phospholipid (mg/ml) / MW of phospholipid}} \quad (3)$$

When other lipids were present, the calculation was modified so that the encapsulation efficiency represented the amount of lactic acid entrapped per mol of total lipid.

5. Drug Release studies

Modified Franz diffusion cells were used to study *in vitro* release of lactic acid from different liposomal formulations.

5.1 Preparation of diffusion cells

The Franz cells used in this study had receiver compartment volumes varied from 11.50-12.05 ml and an internal diameter of 1.5 cm corresponding to an effective permeable area of 1.77 cm^2. The receptor compartment was equipped with a magnetic stirring bar rotating at 600 ± 5 rpm and thermostated at either 4 °C or 37 °C by circulating water through a jacket surrounding the cell body throughout the experiments.

5.2 Preparation and treatment of membranes

Cellulose tubular membrane (Cellu-sep®) with a molecular weight cutoff of 12000-14000 which was previously soaked in purified water overnight, was used for release studies. The membrane was cut into a circular shape with a diameter of 3 cm. Before being mounted in a diffusion cell, the circular cellulose membrane was soaked in the phosphate buffer that was used as the medium in the receptor for at least 15 minutes . Then, the membrane was clamped in place between the donor and the receptor compartments of the cell.

5.3 *In vitro* release studies of a diffusion cell

Seven batches (2 ml each) of each liposome formulation were prepared and pooled together. This amount of liposome preparation was sufficient for conducting the release experiment in triplicate. One millilitre of the pooled lactic acid liposomes was taken and assayed for the initial amount of lactic acid entrapped as in 4.1-4.2. Four milliliters of lactic acid liposomes was centrifuged in an ultracentrifuge at 200,000xg at 4°C for 2 hours. The supernatant was discarded, and the pellet was redispersed with 1 ml of isoosmotic phosphate buffer pH 4 or 5 depending on the liposome preparation. The receptor fluid (the corresponding iso-osmotic phosphate buffer) in Franz diffusion cells was equilibrated to the desired temperature for 15 minutes. After equilibration, the redispersed lactic acid liposome suspension (1 ml) was carefully placed on the membrane surface of one of the diffusion cells. The cell was then covered completely and tightly with Parafilm®. Two ml of the receptor fluid was removed at appropriate time intervals and replaced with an equal volume of fresh buffer. Lactic acid contents in the receptor fluid samples were determined by HPLC method. Briefly, the receptor fluid was transferred into a vial containing 100 microliters of 200 mcg/ml internal standard and was ready for HPLC analysis. The injection volume of HPLC was adjusted to 80 microliters for an appropriate detection.

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

$$\%\text{Lactic acid released} = (A_t / A_o) \times 100 \quad (4)$$

where A_t is the cumulative released amount of lactic acid at various time; A_o is the initial amount of lactic acid entrapped which was determined for each pooled liposomal sample.

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

6. Effect of liposomal composition on stability of liposomes

Encapsulation efficiencies of liposome suspensions immediately after preparation and after one week of storage in a refrigerator were used to evaluate the effects of liposomal composition on stability of liposomes. Liposomal compositions were as described in 2.2.

A aliquot (1.5 ml) of each lactic acid liposomal suspension was filled in 2 ml glass vials wrapped in aluminum foil to prevent light exposure and kept in a refrigerator since lecithin (which is major component of liposomes) is unstable when stored at room temperature or exposed to light. The vial was capped and sealed with Parafilm® to prevent water evaporation. At appropriate time intervals, one milliliter of liposome suspension was sampled for analysis of lactic acid remaining in the vesicles. The sample was centrifuged at 31,000 X g at 4°C for 2 hours. The pellet was analyzed for lactic acid by HPLC as described in the experiment 4.2. One hundred microliters of the same liposome suspension was assayed for phosphorus

content by the Bartlett assay. The encapsulation efficiency after the storage was calculated and compared with that analyzed immediately after liposome preparation.

The experiments were performed in triplicate using three batches of each formula.