CHAPTER 2
EXPERIMENTAL

2.1 Materials and equipments

2.1.1 Chemicals

All of the following chemicals were used as received.

- Amphotericin B (Fluka BioChemika, USA.)
- Fungizone® compose of 50 mg amphotericin B and 41 mg sodium desoxycholate with 20.2 mg sodium phosphates (Bristol-Myers Squibb. Donated by Bristol-Myers Squibb (Thailand) Ltd., Bangkok.)
- Hydrogenated soya phosphatidylcholine (Emulmetik 950®) (Lot no. 179716, Lucas Meyer. Donated by JJ-Degussa (T) Ltd., Bangkok.)
- Cholesterol (Sigma Chemical, USA.)
- Dicetyl phosphate (Sigma Chemical, USA.)
- Stearylamine (Sigma Chemical, USA)
- Potassium dihydrogen orthophosphate (BDH Laboratory Supplies, England.)
- Sodium hydroxide pellets (J.T. Baker Inc., USA.)
- Ethylenediaminetetraacetic acid disodium salt dihydrate (Fluka Chemika, USA.)
- Chloroform (Analytical reagent, Labscan Ltd., Ireland)
- Acetonitrile (HPLC grade, Labscan Ltd., Ireland)
- Methanol (HPLC grade, J.T. Baker Inc., USA.)
- Dimethyl sulfoxide (Merck-Schuchardt, Germany)

2.1.2 Skin : rat skin

- Full-thickness abdominal skin from the 200 to 250 g body weight of male Wistar Rats

2.1.3 Equipments

The equipments used in the experiment were as follows :

- Rota vapour (R-124 Büchi, Switzerland.)
- Analytical balance (Sartorius MC1, AC210S, Sartorius Co., Ltd., Germany.)
- HORIBA pH-Meter (HORIBA, Ltd., Kyoto, Japan.)
- Sonicator (Vibra cell, Sonic & Materials Inc., USA.)
- Centrifuge (Beckman Avanti™30 Centrifuge., USA.)
- Freeze Dryer (Model Lioalfa 10, Telstar, Spain.)
- Zeta-Meter System 3.0 (Zeta-Meter Inc., New York., USA.)
- High performance liquid chromatography (HPLC, HP1100, Vectra XM series 4, Hewlett Packard, USA.)
- Differential Scanning Calorimetry (Perkin Elmer DSC7, Perkin Elmer, Ltd., USA.)
- Transmission Electron Microscope (TEM1200S JEOL, JEOL Ltd., Japan.)
- Scanning Electron Microscope (SEM 840A JEOL, JEOL Ltd., Japan.)
- Gold coater (JFC-1100E Ion Sputtering device, JEOL Ltd., Japan.)
- Franz diffusion cell (Crown Bio Scientific, Inc., Somerville, NJ., USA.)
- Spectrophotometer (Milton Roy Spectronic 1001 plus, USA.)
- UV/VIS Spectrophotometer (Jasco Model 7800, Japan.)
- ELGA Ultra Pure Water (Elga Ltd., England.)

2.2 Methods

2.2.1 Preparation

2.2.1.1 Preparation of liposome dispersion samples

Liposome dispersion samples were prepared by a conventional chloroform film method together with sonification. The lipids used were the hydrogenated soya phosphatidylcholine (HSC), the cholesterol (CHL) and the charged lipids which were a positively charged lipid, stearylamine (SA) and a negatively charged lipid, dicetyl phosphate (DCP). Four different liposome formulations of HSC/CHL = 1:1, 7:2 ; HSC/CHL/SA = 7:2:1 and HSC/CHL/DCP = 7:2:1 in molar ratios with and without the entrapped amphotericin B (in the form of Fungizone® (FGZ)) were prepared. The concentration of amphotericin B (AmB) was 0.05 mg per mg of total lipid. For each lot, an amount of 40 ml of each liposome dispersion sample was performed. Table 2.1 showed the contents of eight different liposome formulations. Physical appearances and pH of different liposome formulations investigated by a pH meter were compared.

Table 2.1 : The contents (g) of eight different liposome dispersion samples (40 ml)

Compositions	1	2	3	4	5	6	7	8
HSC	0.1330	0.1750	0.1670	0.1600	0.1330	0.1750	0.1670	0.1600
CHL	0.0670	0.0250	0.0240	0.0230	0.0670	0.0250	0.0240	0.0230
SA	-	-	0.0090	-	-	-	0.0090	-
DCP	-	-	-	0.0170	-	-	-	0.0170
AmB	-	-	-	-	0.0100	0.0100	0.0100	0.0100
Molar Ratio	1:1	7:2	7:2:1	7:2:1	1:1	7:2	7:2:1	7:2:1
Expected Charges	none	none	posi-tive	nega-tive	none	none	posi-tive	nega-tive

The preparation method of liposome dispersion samples is as follows :

1. The lipid mixture was dissolved (with or without AmB) in chloroform (40 ml) in a 250 ml round-bottom flask. For the preparation of liposome with AmB, the lipid mixture and Fungizone® (AmB) were dissolved separately in 10 ml of chloroform and 30 ml of methanol respectively and the two solutions were then mixed before evaporation.
2. The solvent was evaporated at 65°C, 45 rpm, under vacuum at 400 mbar for 60 mins.
3. A thin film of lipid mixture (with or without AmB) was formed.
4. The film on the inner surface of the flask was further dried by placing in a desiccator with vacuum for 30 mins.
5. The film was flushed with the nitrogen gas for one min.
6. An amount of 40 ml phosphate buffer pH 7.4 was put into the film together with about 70 glass beads and the mixture was weighed and swelled by swirling in a water bath at 80°C, 190 rpm for 30 mins.
7. The dispersion was cooled to room temperature and the weight of the dispersion was adjusted to the weight before swelling.
8. The resulting liposome dispersion was sonicated with a microtip probe sonicator for 30 mins.
9. The dispersion was flushed with nitrogen gas for one min to remove dissolved oxygen.
10. The weight of the dispersion was adjusted to the initial weight before sonication.
11. The liposome formulations were kept in the refrigerator at 4 °C and protected from light for further experiment.
12. All liposome formulations were filtered through the Whatman filter paper No.42 before any tests to remove titanium and control the liposomal sizes.

2.2.1.2 Preparation of freeze dried liposome powder

The liposome samples were filtered through the Whatman filter paper No.42. An amount of 5 ml of each liposome sample was drawn and filled into vials with loosely covered. All vials were put into a freeze dryer with the conditions of prefreeze for four hours (at -25°C for 1.5 hours, -32°C for 1.5 hours, -36 °C for one hour). When the condenser was at -42°C, the primary drying vacuum pump was maximum at 0.35 mbar and minimum at 0.019 mbar of 10 hours and the shelf was heated at 25°C. The secondary drying with shelf heat was three hours

at 25°C to desorp the residue moisture. The freeze dried powder in vacuum seal in vials was finally obtained.

2.2.2 Physical properties study of liposome sample

2.2.2.1 Charges characterization of liposome formulations

The liposome samples (0.5 ml) dispersed in deionized water (15 ml) were filled in an eletrophoresis type GT-2 cell which had the molybdenum cylinder anode (+) and platinum rod cathod (-). Then, set "voltage setting" switch on "75" and "ocular micrometer" on "full". The liposomes were tracked and observed when they moved along the tracking line. If they move to the left, they are negatively charged, and to the right when they are positively charged. Mean and standard deviation of zeta potential were measured by tracking for 20-30 times. The experiments were performed in triplicate.

2.2.2.2 Particle size determination of liposome formulations

A. Scanning electron microscope (SEM)

The freeze dried liposome powder was picked up by the toothpick and gently spread on the adhesive copper tape which attached to the brass stubs. The sample surface on stubs was covered with gold. After coating, the stubs were examined with a scanning electron microscope. The photographs were taken and the contact prints were developed. The size measurement of liposome particles was performed by comparing the apparent size of the particles with a bar in the photograph. The mean and standard deviation were calculated from 100 particles of each photograph.

B. Transmission electron microscope (TEM)

The liposome dispersion was dropped on the parafilm. A formvar-coated grid which was coated with carbon was inserted horizontally into a drop of liposome on the parafilm for three mins. This grid was pulled out, blotted side of grid with filter paper and left for three mins until dry. Then, turn this grid with its face down floating on uranyl acetate for 10 mins and protected from light because uranyl acetate was unstable to light. After that, a grid was picked , blotted side with filter paper and left dry for three mins. This grid was then examined

with the transmission electron microscope. The photographs were taken and the contact were developed prints. The size measurement of liposome particles was performed by comparing the apparent size of the particles with a bar in the photograph. The lamellarity was also observed.

2.2.2.3 Determination of transition temperature and enthalpy of transition of liposome formulations by a differential scanning calorimeter (DSC)

An amount of 2 to 5 mg of the freeze dried liposome powder were put on the 40 µl-aluminium pan with cover. The pan was sealed and put in the sample holder. The reference holder was the 40 µl empty pan with cover. The temperature ranging from 30 to 250°C was used for the DSC with scanning rate of 5°C per min.

Hydrogenated soya phosphatidylcholine, cholesterol, stearylamine, dicetyl phosphate, amphotericin B and Fungizone® samples were also run with the same DSC conditions as those of the freeze dried liposome samples. All experiments were done in duplicate. The transition temperature and the enthalpy of transition were determined from the thermogram and the average values were calculated.

2.2.3 Analysis of amphotericin B

2.2.3.1 Determination of maximum absorption wavelength of amphotericin B

One mg of amphotericin B was first dissolved in 0.5 ml of dimethyl sulfoxide (DMSO) and 4.5 ml of methanol was then added and mixed until a clear solution was obtained. This made a 40-fold dilution solution with the final amphotericin B concentration of 5 µg/ml.

Five mg of Fungizone® was first dissolved in 10 ml of methanol until a clear solution was obtained. This made a 50- fold dilution solution with the final amphotericin B concentration of about 5 µg/ml.

A liposome sample (400 µl) was first dissolved in 600 µl of methanol until a clear solution was obtained. This made a 2- fold dilution solution with the final amphotericin B concentration of about 5 µg/ml.

All of the above prepared samples were scanned by using a spectrophotometer at wavelength from 500 to 200 nm with the speed of 2400 nm/min.

2.2.3.2 Determination and verification of HPLC conditions for the analysis of amphotericin B

The reverse phase column ODS-Hypersil C_{18}, 5 µm, 250 x 4 mm was used. The mobile phase was composed of acetonitrile and 2.5 mM disodium edetate in water with the mixing ratio of 45 per 55 v/v. The pumping rate was 1 ml/min and the UV detection was set at 382 nm.

Fifty µl of amphotericin B in methanol (0.5 µg/ml), Fungizone®in methanol (0.23 µg/ml), and methanol were injected to the HPLC. The peaks obtained were compared and verified.

Every sample was filtered through a 0.45 µm membrane filter before injecting to HPLC.

2.2.3.3 Preparation of the standard curve of amphotericin B

One mg of amphotericin B was dissolved in 1 ml of DMSO and adjusted to 10 ml with methanol. The concentration of this solution was 0.1 mg/ml. This solution was diluted to 10 µg/ml. The solutions containing amphotericin B was prepared from 10 µg/ml stock solution by dilution 0.02, 0.1, 0.5, 1.0, 1.5 µg/ml. Fifty µl of each solution were injected into HPLC using the above selected conditions. The experiment was done in duplicate. A standard curve was obtained with calculated correlation coefficient (r^2), intercept and slope which were used for further experiments.

2.2.3.4 Contents determination of amphotericin B in Fungizone®.

An amount of 27.7 mg of Fungizone® was dissolved in 50 ml of water and diluted to 1,000 folds. Fifty µl of this sample were injected into HPLC. The experiments were done in triplicate. The area under the peak (AUC) were compared with that from the calibration curve. The amount of amphotericin B and amphotericin B in Fungizone® were calculated by using the following equation :

Amphotericin B (mg) in Fungizone® (1 mg) = (XY) / 1000(Z)

Where : X = Concentration of amphotericin B(µg/ml)

Y = Dilution factor

Z = Initial weight of Fungizone®

2.2.4 Determination of the percentage of entrapment of amphotericin B in liposomes

The liposome formulations were filtered through the Whatman filter paper No.42. The 0.1 ml-filtrate was transferred to a 10ml-volumetric flask and adjusted to 10 ml with methanol. Then, one ml of this solution was transferred to the other 10ml-volumetric flask and adjusted to 10 ml with methanol. This solution was used for the analysis of the total amount of amphotericin B.

Another 1 ml-filtrate was put into an appendorf. They were then centrifuged at 50,000 g, 4°C for one hour. Then, a 900 µl-supernatant was drawn and put into a 10 ml- volumetric flask and adjusted to 10 ml with methanol. This solution was used for the analysis of the amount of the unentrapped amphotericin B.

The pellets collected from the centrifuged appendorf were resuspend with methanol and transferred to a 50 ml-volumetric flask and adjusted to 50 ml with methanol. Then, 1 ml of this solution was transferred to a 10ml-volumetric flask and adjusted to 10 ml with methanol. This solution was used for the analysis of the amount of the entrapped amphotericin B in liposomes.

An amount of 50 µl of each above sample was injected into HPLC. The AUC of each sample was determined and compared with those obtained from the standard curve and the amount of amphotericin B were calculated. The percentage of the entrapment of amphotericin B in liposomes was calculated according to the following equation :

The percentage of the entrapment of AmB in liposome = (A/C) x 100%

The percentage of the unentrapment of AmB = (B/C) x 100%

Where : A = The amount of the entrapped AmB

B = The amount of unentrapped AmB

C = The total amount of AmB

Each sample was prepared in duplicate and the experiments were done in triplicate.

2.2.5 Stability study of amphotericin B in liposome formulations comparing to Fungizone®solution and Fungizone®powder

2.2.5.1 Physical stability

Each liposome formulation was filtered through the Whatman filter paper No.42. Five ml of the filtrate were transferred to amber glasses. Fungizone®solution was prepared by dissolving 27.7 mg of Fungizone® in 50 ml of water and 5 ml of the solution were transferred to the amber glasses. An amount of about 40 mg of Fungizone®powder was filled into the amber glass. Three amber glasses per formulation were used. The first set of amber glass was kept in the refrigerator at 4±1°C. The second set was kept on shelf with ventilation of airflow at 30± 1°C. The third set was put in an incubator at 45±1°C.

At 90 days, The liposome sample, Fungizone®solution and Fungizone®powder were transferred into a clear glass tube for observing and comparing the sediment and color.

2.2.5.2 Chemical stability

The above samples were sampling at different time intervals of 0, 5, 20, 40 and 90 days.. For Fungizone®powder, 5 mg of the powder was sampling at the above time intervals, dissolved in 10 ml of water and 0.1 ml of the solution was used for further experiment. An amount of 0.1 ml of liposome and Fungizone®solution was sampling and diluted to 1,000 folds with methanol and 50 µl of the diluted solution were injected into the HPLC. The AUC 's were determined and the concentrations of amphotericin B at each sampling time were calculated from the standard curve.

2.2.6 Transdermal absorption of amphotericin B through the full-thickness of rat skin

2.2.6.1 Preparation of rat skin (Brain *et al.*, 1998)

Abdominal skin from the male Wistar rats with body weights in the range of 200 to 250 g was used. The skin was prepared by the following procedure :

1. The hair on abdominal area of rat skin was shaved off and left over night.
2. The rats were sacrificed and the abdominal skin was removed.
3. The subcutaneous fat from the removed skin was trimmed off.
4. The prepared skin was kept in a freezer at -40°C for the experiment.

2.2.6.2 The preparation of samples for loading to the vertical Franz diffusion cells

The samples selected for the transdermal experiment were 1:1 liposome AmB, 7:2 liposome AmB, 7:2:1(+) liposome AmB, 7:2:1(-) liposome AmB, amphotericin B solution in the mixtures of DMSO and methanol, amphotericin B suspension in phosphate buffer pH 7.4 and Fungizone® solution in deionized water. These samples were prepared as the followings :

The 1:1AmB, 7:2AmB, 7:2:1(+)AmB and 7:2:1(-)AmB liposome dispersion were filtered through the Whatman filter paper No.42. Amphotericin B solution in the mixtures of DMSO and methanol was prepared by dissolving 1 mg of amphotericin B in 0.5 ml of DMSO and adjusting it to 5 ml with methanol. Amphotericin B suspension in phosphate buffer pH 7.4 was prepared by dissolving 1 mg of amphotericin B in 5 ml of the buffer, vortexing the dispersion for 1 min and filtering through the Whatman filter paper No.42. Fungizone® solution in water was prepared by dissolving 0.0277 g of Fungizone® in 50 ml of deionized water.

The concentrations of the 1,000 folds dilute with methanol of all sample in the filtrates which was put in the donor chamber were analysed by HPLC.

2.2.6.3 The process of drug absorption test by a vertical Franz diffusion cells

The frozen skin was thawn at room temperature for 30 mins. The circulated water of the vertical Franze diffusion cell was set at 37±1°C. The receiver chamber was filled with 12 ml of ethanol/water (1:1). The skin was fixed in the diffusion cell with the dermal side in contact with the receiver medium and the epidermis side was facing with the donor chamber. The contact area was 1.77 cm^2. The cell was clamped and the receiver medium was stirred continuously by a magnetic bar for 30 mins. The two samples from section 2.2.6.2 were loaded in the donor chamber. The donor chamber and the sampling port were covered by a piece of parafilm. The cells were protected from light. At initial, the samples in donor side were diluted to 1,000 folds with methanol and assayed by HPLC. At initial and after 24 hours, the samples in the receiver chamber were withdrawn and the amphotericin B content was assayed by HPLC.

The experiment were done in duplicate. The control sample which were water, phosphate buffer pH 7.4 and DMSO/water mixture were also analyzed by HPLC.

2.2.6.4 Analysis of the remaining drug

At 24 hours, the amount of amphotericin B in the receiver chamber were assayed. One ml of the medium in the appendorf was centrifuged at 50,000 g, 4°C for 10 mins, an amount of 100 µl of the clear supernatant was injected to HPLC for amphotericin B content determination.

The amount of amphotericin B remaining in the donor chamber was analysed by withdrawing all the remaining donor and transferring to a 25 ml-volumetric flask. The used skin was washed with 2 ml of water for two times. The rinsed water was collected and put together with the above remaining donor solution. The remaining donor volume was adjusted by methanol to 25 ml. Dilutions were made when necessary. The solution was filtered and 50 µl of this solution was injected into HPLC in order to determine amphotericin B remaining in the donor chamber.

After rinsing, the skin was removed from the cell and swung in 100 ml of deionized water for two times. The rinsed water was discarded. The amount of amphotericin B within the skin were analysed in two parts which were in the stratum corneum and in the viable epidermis/dermis.

The amount of amphotericin B in the stratum corneum were determined by stripping the skin with a 3M Scotch Magic™ tape (1x1 cm) for one time of each tape (Plessis *et al.*, 1992). Nine tapes were used for each skin sample. These tapes were pooled in a 5 ml-vial filled with 5 ml of methanol. The vial was vortexed for 1 min, stood for 10 mins and vortexed again for 1 min. Then the solution was filtered through the membrane filter and 100 µl of the filtrate were injected into HPLC for amphotericin B contents determination.

The amount of amphotericin B in the viable epidermis and dermis were determined by cutting the skin into small pieces. These skin pieces were pooled in a 5 ml-vial and filled with 5 ml of methanol. The vial was vortexed for 1 min, stood for 10 mins and vortexed again for 1 min again. The solution was filtered through the membrane filter and 100 µl of the filtrate were injected into the HPLC for amphotericin B contents determination.