CHAPTER III

EXPERIMENTAL METHODOLOGY

Materials

All chemicals were analytical or pharmaceutical grade and they were used as received.

1. Ibuprofen (Lot no. 770771), Boots Company, UK.
2. Soybean phosphatidylcholine (Phospholipon®90, Lot no. 70991), Nattermann Phospholipid GmbH, Cologne, Germany
3. Cholesterol (Lot no. 111H8488), Sigma Chemical Co. Ltd., USA.
4. Stearylamine (Lot no. 53H34651), Sigma Chemical Co Ltd., USA.
5. (±)-α-Tocopherol (Lot no. 53H0444), Sigma Chemical Co. Ltd., USA.
6. Chloroform, AR grade (Lot no. K23924045), BDH, USA.
7. Isopropyl alcohol, AR grade (Lot no. K43629), J. T. Baker, Germany
8. Methanol, HPLC grade (Lot no. 3041KPDE), Mallinckrodt, USA.
9. Potassium dihydrogen phosphate, AR grade (Lot no. 304A707573), Merck, Germany
10. Sodium chloride, AR grade (Lot no. 004K13445704), Merck, Germany
11. di-Sodium hydrogen phosphate anhydrous, AR grade, (Lot no. 550F971786), Merck, Germany
12. Sterile water for injection (Lot no. 005440), GPO, Thailand

Equipments

1. Analytical balance, Model 1605, Satorious GmbH, Germany
2. Rotary evaporator, Model WB 2000, Heidolph Elektro GmbH, Germany
3. Vortex mixer (Genie 2), Model G-605E, Scientific Industry, USA.
4. pH meter, Model 420A, Orion, USA.
5. Double beam UV-Visible Spectrophotometer, Model UV 160A, Shimadzu, Japan
6. Optical microscope (Olympus®), Model 200/00, Tokyo, Japan
7. Particle size laser scattering analyzer (Mastersizer® version 2.11), Malvern, UK.
8. Scanning Electron Microscope (JEOL®), Model JSM 5410 LV, Japan.
9. Transmission Electron Microscope (JEOL®), Model JEM-200CX, Japan
10. Ultracentrifuge (Beckman®), Model L-80, Germany
11. Refrigerator (Kelvinator®), USA.

Methods

1. Study of quantitative analysis of ibuprofen

1.1 High performance liquid chromatographic (HPLC) method

In preliminary study, high performance liquid chromatographic technique was used for analysis of ibuprofen. The system consisted of a constant flow pump, a variable wavelength UV detector, an integrator and a volume sample

injection with a 20 microliter loop. The conditions used for analyzing ibuprofen by HPLC method modified from Rao et al (1992) were presented as follows:

Column	: μBondapak C18 (300 x 3.9 mm), 10 μm
Mobile phase	: a mixture of 70% v/v methanol and 1 ml of 85% of phosphoric acid
Detection wavelength	: 220 nm
Flow rate	: 1.5 ml/min
Injection volume	: 20 μl
Internal standard	: benzophenone 2 μg/ml
Retention time of ibuprofen	: 7.59-7.83 min
Retention time of benzophenone	: 4.85-4.96 min
Standard solution	: 0.5, 1, 2, 3, 4, and 5 μg/ml

1.2 UV spectrophotometric assay

UV spectrophotometric assay was studied after there were many problems in HPLC assay.

1.2.1 Selection of blank solvent

Blank solvent for UV spectrometric assay was phosphate buffer saline pH 7.4. Ibuprofen was dissolved in phosphate buffer saline pH 7.4 in various concentrations and carried to study for the maximum absorption wavelength in the further section.

1.2.2 Study of maximum absorption wavelength of ibuprofen

The maximum absorption wavelength of ibuprofen in phosphate buffer saline pH 7.4 was studied by scanning the absorption spectra of the solutions in a 1-cm cuvette cell with double beam UV/visible spectrophotometer using phosphate buffer saline pH 7.4 as a blank in the reference cell.

1.2.3 Preparation of standard solutions

Stock solution of ibuprofen :

A stock solution of ibuprofen was prepared by accurately weighing 50 mg of ibuprofen into a 100 ml volumetric flask. Phosphate buffer saline pH 7.4 was added to dissolve and adjust to volume. Thus, the final concentration of this solution was 500 μg/ml.

Standard solutions :

The stock solution of ibuprofen was pipetted (200, 400, 600, 800, and 1,000 μl) and transferred into five 25 ml volumetric flasks, respectively. The solutions were adjusted to volume with phosphate buffer saline pH 7.4 so that the concentrations of the standard solutions were 4, 8, 12, 16 and 20 μg/ml, respectively.

1.2.4 Calibration curve of ibuprofen in phosphate buffer saline pH 7.4

The absorbance of each standard concentration solution prepared as above conditions was measured by UV spectrophotometer at the maximum absorption wavelength using phosphate buffer saline pH 7.4 as a blank solvent. The relationship between absorbance and concentration was determined using linear regression analysis.

2. Preparation of liposomes using mechanical dispersion method

Empty liposomes were prepared using mechanical dispersion method modified from the method of Bangham, Standish, and Watkins (1965). Briefly, dissolved soybean lecithin in chloroform in a round bottom flask. Then, the solution was evaporated using rotary evaporator under reduced pressure until the thin film deposited on the wall of the round bottom flask. The sterile water was added to hydrate the film and vortexed vigorously. The final product was obtained as a milky suspension.

2.1 Determination of suitable size of round bottom flask for preparing liposomes

Various sizes of round bottom flasks of 50, 100, 250, 500 and 1,000 ml were used to prepare 3 ml of empty liposomes. The suitable size was selected at a point that the thin film with the most amounts of soybean lecithin used could be obtained.

2.2 Determination of suitable amount of chloroform and suitable temperature and time for removing chloroform for preparing the thin film

The various amounts of chloroform of 15, 20, 25 and 30 ml were used to dissolve soybean lecithin for preparing 3 ml of liposomal preparation. The temperature used for evaporating chloroform was varied as, 30, 35, 40 and 45°C. The time to remove chloroform, 1, 2, 3 and 4 hours were determined. The point of selection was the shortest time that the thin film which chloroform odor no longer existed could be obtained.

2.3 Determination of suitable amount of soybean lecithin for preparing liposomes

The various amounts of soybean lecithin of 22.7 to 136.4 μmol/ml were selected to make the thin film. The largest amount of soybean lecithin that appropiate thin film could be prepared was selected.

2.4 Determination of suitable temperature for preparing liposomes

The suitable amount of soybean lecithin in section 2.3 was dissolved in the suitable amount of chloroform from section 2.2 in a suitable size of round bottom flask from section 2.1. Then, the solution was evaporated by using a rotary evaporator under reduced pressure until the thin film deposited on the wall of the flask. After that the 3 ml of sterile water was added. The various temperatures of 30, 35, 40 and 45°C was determined for hydrating the thin film and a homogenous milky suspension formed. In this case, the appropriate temperature that various compositions of lipid could prepared was determined.

2.5 Preparation of empty liposomes

Empty liposomes were prepared using the above conditions. Liposomes formed was confirmed by optical microscopy.

3. Preparation of ibuprofen liposomes

Seven batches of ibuprofen liposomes were prepared by dissolving ibuprofen and lipid that was soybean lecithin in chloroform using the conditions determined in section 2 which resulted in 3 ml in each batch. Non-encapsulated ibuprofen was separated from preparation by using ultracentrifugation as described in section 8.

4. Study of the effects of the soybean lecithin to drug molar ratios on the preparation of ibuprofen liposomes

The various molar ratios of soybean lecithin to drug of 1:0.047, 1:0.096, 1:0.144, 1:0.170 and 1:0.191 were used to prepare ibuprofen liposomes using the method and conditions in section 3 while the total lipid was constant at the value determined in section 2.3. The 3 ml of ibuprofen liposomes were obtained in each batch. The amounts of encapsulated drug and particle size of liposomes prepared were determined as described in section 8. Ibuprofen liposomal preparation with the highest percentage drug entrapment without drug crystal was selected for the further study in section 5 and stability study in section 9.

In addition, two batches of corresponding empty liposomal preparations were prepared. Their average absorbance was used to subtract from the absorbance of unencapsulated ibuprofen in liposomal preparations in the UV spectrometric analysis.

5. Study of the effects of the soybean lecithin to cholesterol molar ratios on the preparation of ibuprofen liposomes

The various molar ratios of soybean lecithin to cholesterol of 9:1, 8:2, 7:3, 6:4 and 5:5 were used to prepare ibuprofen liposomes while the total lipid was constant at the value determined in section 2.3 and the constant appropriate molar ratio of soybean lecithin to drug determined in section 4. Soybean lecithin, cholesterol and ibuprofen were accurately weighed and dissolved in chloroform in the appropriate sized round bottom flask. Ibuprofen liposomes was prepared under conditions in section 3. The amounts of encapsulated drug and particle size of liposomes prepared were determined as described in section 8. Ibuprofen liposomal preparation with the highest percentage drug entrapment without drug crystal was selected for the further study in section 6 and stability study in section 9.

In addition, two batches of corresponding empty liposomal preparations were prepared. Their average absorbance was used to subtract from the absorbance of unencapsulated ibuprofen in liposomal preparations in the UV spectrometric analysis.

6. Study of the effects of the amount of stearylamine on the preparation of ibuprofen liposomes

The various amount of stearylamine of 20, 15, and 10 mole% were added to the appropriate lipid solution from section 5 to prepare ibuprofen liposomes using conditions established in section 3. The amounts of encapsulated drug and particle size of liposomes prepared were determined as described in section 8. Ibuprofen liposomal preparation with the highest percentage drug entrapment without drug crystal was selected for the further study in section 7 and stability study in section 9.

In addition, two batches of corresponding empty liposomal preparations were prepared. Their average absorbance was used to subtract from the absorbance of unencapsulated ibuprofen in liposomal preparations in the UV spectrometric analysis.

7. Study of the effects of the amount of (±)-α-tocopherol on the preparation of ibuprofen liposomes

The various amounts of (±)-α-tocopherol of 1, 0.5, and 0.1% were added to the appropriate lipid solution from section 6 to prepare ibuprofen liposomes using conditions established in section 3. The amounts of encapsulated drug and particle size of liposomes prepared were determined as described in section 8. Ibuprofen liposomal preparation with the highest percentage drug entrapment without drug crystal was selected for stability study in section 9.

In addition, two batches of corresponding empty liposomal preparations were prepared. Their average absorbance was used to subtract from the absorbance of unencapsulated ibuprofen in liposomal preparations in the UV spectrometric analysis.

All of selected preparations in section 4, 5, 6 and 7 were further studied on stability study in section 9.

8. Study of the physicochemical properties of ibuprofen liposomes

8.1 Quantitation of drug entrapment

8.1.1 Separation of non-encapsulated ibuprofen from liposomal preparation

Whole amount of ibuprofen liposomes prepared was filled in the ultracentrifuge tube and 7 ml of sterile water was added. The conditions for centrifugation were at 4°C, 50,000 rpm for 5 hours. After that, supernatant liquid containing free ibuprofen was collected for further analysis by UV spectrophotometer.

8.1.2 Analysis of ibuprofen by UV spectrophotometer

In each analysis, the calibration curve of ibuprofen in phosphate buffer saline pH 7.4 was freshly prepared following the conditions described in section 1.2.4. The entrapment efficiency of ibuprofen in several preparations was determined using UV spectrophotometer. The supernatant of free drug obtained from the centrifugation in section 8.1.1 was diluted with phosphate buffer saline pH 7.4 and adjusted to 25 ml. The absorbance of each solution was measured at the maximum absorption wavelength determined from section 1.2.2 by using phosphate buffer saline pH 7.4 as a blank solvent. Then, the concentration of ibuprofen was calculated from the regression equation of the calibration curve of known ibuprofen in phosphate buffer pH 7.4. The average absorbance of supernatant liquid of corresponding empty liposomal preparations was used to subtract from the absorbance of free drug, unencapsulated ibuprofen, before calculation.

The percentage encapsulated ibuprofen in ibuprofen liposomes was calculated using the following equation:

$$\% \text{ Ibuprofen entrapment} = \frac{\text{Amount of total drug} - \text{Amount of free drug}}{\text{Amount of total drug}} \times 100$$

8.2 Determination of liposomes size and size distribution

Liposomes size and size distribution were determined by particle size laser scattering analysis using a Mastersizer® version 2.11.

8.3 Electron microscopy

8.3.1 Scanning electron microscopy (SEM)

The morphology of liposomes prepared was examined by scanning electron microscope. The SEM photomicrographs were recorded by JEOL® model JSM 5410 LV, Japan. The samples were treated following Figure 7.

8.3.2 Transmission electron microscopy (TEM)

The appearance of lamellar structures of liposomes prepared was examined by negative staining transmission electron microscopy and recorded by JEOL® model JEM 200CX, Japan. Liposomes samples were treated as follows: a drop of liposomal suspension was placed on a 300 mesh copper grid and allowed 5 minutes to attach to the grid, the excess liquid suspension was removed with the aid of

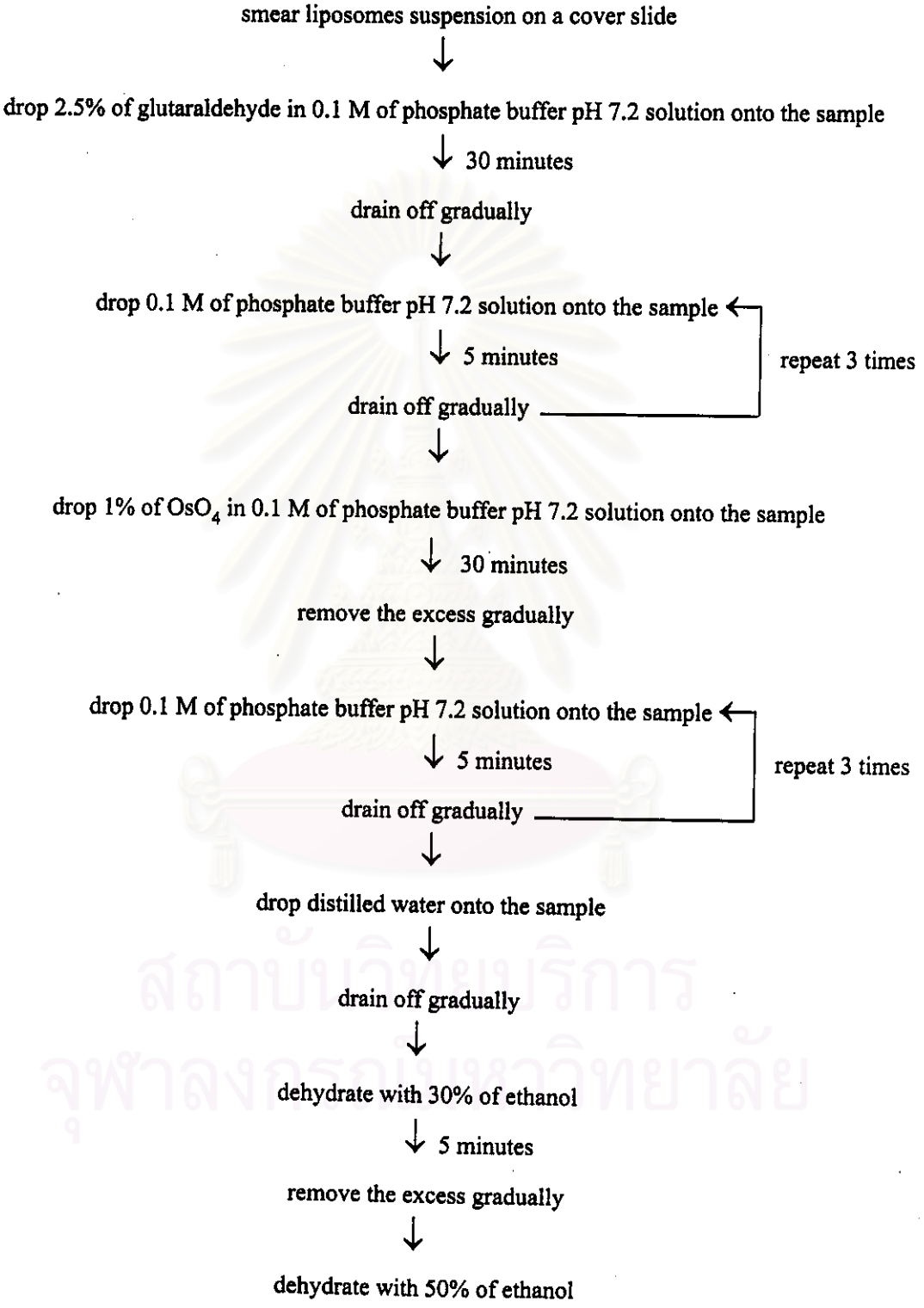

Figure 7 Diagram of the procedures for treating the liposomes suspension for scanning electron microscopy

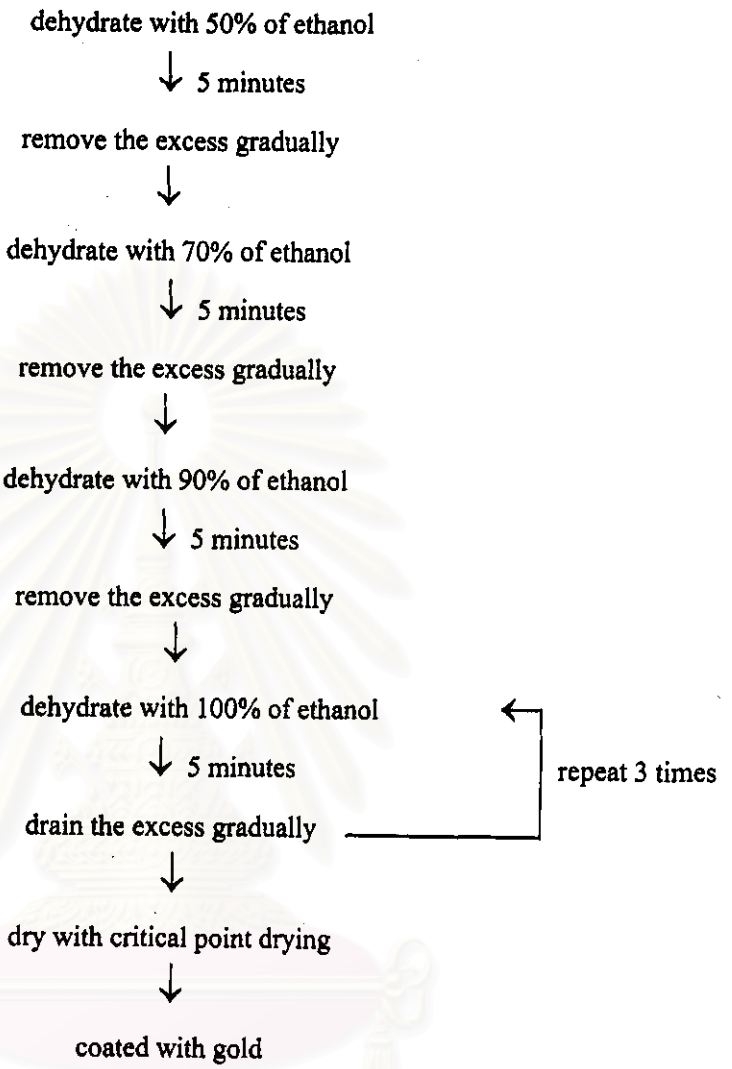

Figure 7 (Continued) Diagram of the procedures for treating the liposomes suspension for scanning electron microscopy

filter paper. Then, 1% of phosphotungsitic acid was used to stain the sampled on the grid and allowed to air-dry for approximate 15 minutes.

9. Study of the stability of ibuprofen liposomes

The stability of Ibuprofen liposomes selected with the highest percentage drug entrapment from section 4, 5, 6 and 7 was studied. Physicochemical properties

of liposomal preparations which freshly prepared and after storage at 4°C for 1 month were investigated. The properties compared were the percentage drug entrapment, size and size distribution and the morphology of ibuprofen liposomes.

10. Statistical analysis

Statistical analysis of the difference in the percentage drug entrapment and particle size of ibuprofen liposomes which were freshly prepared or between freshly prepared preparations and ones kept at 4°C for 1 month were studied using analysis of variance and Duncan's new multiple range test. The P-value of 0.05 was used as the level of significance.