CHAPTER III

MATERIALS AND METHODS

MATERIALS

1.SPAN 40® ,Sorbitan monopalmitate (The East Asiatic, batch No. 39770)

2.SPAN 60® ,Sorbitan monostearate (The East Asiatic, batch No. 40905)

3.SPAN 85® ,Sorbitan trioleate (Sigma, USA Lot No. 39H0149)

4.Cholesterol (Sigma, USA Lot No 50K5304 and Lot No 111H8488)

5.Solulan C-24® ,Poly(24)oxyethylene cholesteryl ether (Amerchol, USA Lot No 620303)

6.Chloroform AR grade (Lab-Scan Co.,Ltd Thailand)

7.Methanol AR grade (Lab-Scan Co.,Ltd Thailand)

8.Methanol HPLC grade (Lab-Scan Co.,Ltd Thailand)

9.Acetonitrile HPLC grade (Lab-Scan Co.,Ltd Thailand)

10.Isopropanol AR grade (Lab-Scan Co.,Ltd Thailand)

11.Isopropanol HPLC grade (Lab-Scan Co.,Ltd Thailand)

12.Retinyl palmitate 1.7million IU/g (BASF, Germany Lot No 90-0192)

13.All-trans retinol acetate 2.8million IU/g (Sigma, USA Lot No 70K0939)

14.Tween 80® ,Polysorbate 80(Fluka Biochemika, Switzerland Lot No 93780)

15.Arlasolve 200L® ,Polyoxyethylene(20)isohexadexyl ether (The East Asiatic Co.,Ltd, USA batch No 45413)

EQUIPMENTS

1.Analytical balance (BA2105, S/N 21203485, Sartorius Basic)

2.Rotary evaporator (Rotarvapor R-114 BUCHI)

3.High performance liquid chromatography

column :Inertsil ODS-3 5 μm

:column dimension 4.6*250mm

:serial No. 1GI86060

:material Lot No. TQ5-1625

liquid chromatography : LC-10ADvp, Shimadzu,Japan

UV-VIS detector : SPD-10Avp, Shimadzu,Japan

system controller : SCL-10Avp, Shimadzu,Japan

4.UV spectrophotometer (Spectronic 3000 array, Milton Roy, USA)

5.Modified Franz diffusion cell

6.Optical light microscope (BH-2, Olympus,) with an attached camera

(C-35 AD-4, Olympus, Japan)

7.Scanning electron microscope (JSM-5410LV, JEOL Co.,Ltd.,Japan)

8.Ion Sputter (SCD 040, BALZERS)

9.Critical point drier (Samdri 780, Tousimis, USA)

10.Mastersizer S long bed version 2.11 (Malvern Instrument, UK)

11.Ultracentrifugation (L-80, Beckman)

rotor type : 90Ti, Beckman

: serial No. 94u 773

tube : Lot No. 397310

:Oak Ridge Centrifuge tube, polypropylene copolymer

12.Hot air oven (BINDER)

13.Ultrasonic bath (T900/H, Elma, Germany)

14.Vortex mixer (Vortex-genie,model G560E, USA)

METHODS :

1. Methods of quantitative analysis of retinyl palmitate for entrapment studies

1.1 UV spectrophotometer

In order to determine the maximum absorption wavelength for both retinyl palmitate and retinyl acetate. Each compound was dissolved in the mixture of chloroform and methanol and then transferred to cells for UV detection. The blank solution was the mixture of chloroform and methanol. The concentrations of retinyl palmitate and retinyl acetate were 2 μg/ml.

1.2 HPLC assay for retinyl palmitate analysis

1.2.1 HPLC conditions

The high performance liquid chromatographic technique was used for analysis of retinyl palmitate. The system was composed of two pumps able to generate the variable flow of mobile phase, an adjustable wavelength UV detector, system controller and degasser. All of these were operated by the data station software. The

condition used for analyzing retinyl palmitate was initiated from the variation of many mobile phase types. It was found that acetonitrile and isopropanol were suitable mobile phase for retinyl palmitate analysis. The condition used was as follows:

Column : Inertsil ODS

Detector wavelength : 325 nm

Flow system : Binary gradient

Mobile phase : Acetonitrile and Isopropanol (1:1)

Total flow rate : 2 ml/min

1.2.2 Preparation of standard solutions

Both retinyl acetate and retinyl palmitate were conducted in a room illuminated with yellow light avoiding photodegradation of the compounds. Retinyl acetate was selected as an internal standard of retinyl palmitate because both retinyl palmitate and retinyl acetate has the similar physico-chemical properties that enabled them to use in the same condition of high performance liquid chromatography.

A stock solution of retinyl palmitate was prepared by dissolving 10.6 mg of retinyl palmitate in isopropanol. The solution volume was adjusted to 10 ml in a volumetric flask.

A stock solution of retinyl acetate was prepared by dissolving 10.7 mg of retinyl acetate in isopropanol. The solution volume was adjusted to 10 ml in a volumetric flask.

Standard solutions were prepared by firstly, individual pipetting 20, 40, 60 and 80 µl of retinyl palmitate stock solution into four 10 ml-volumetric flasks. Secondly, pipetting 10 µl of retinyl acetate into each of those volumetric flasks. The solutions were adjusted to volume with acetonitrile and isopropanol in the ratio of 1:1. The resulting solutions were 2, 4, 6 and 8 µg/ml of standard solutions of retinyl palmitate used for entrapment studies.

1.2.3 Validation for the quantitative determination of retinyl palmitate in niosomes by HPLC

The analytical method was validated to ensure its suitability, According to USP XXIII, the parameters for method validation include accuracy, precision, specificity and linearity (USP XXIII)

Accuracy

Accuracy of an analytical method is the closeness of the test result obtain with that method to the true value. Accuracy may often be expressed as the percentage of recovery on the assay of known, added amounts of analysis.

Procedure

The suspension of 33.33mM niosome suspensions were prepared by Span:cholesterol:Solulan C-24 (45:45:10) with 5 mg entrapped retinyl palmitate. The entrapped retinyl palmitate in niosomes was analyxed analog with the initial amount of retinyl palmitate added in niosome suspensions. The accuracy was then calculated in term of % recovery.

Precision

Precision of an analytical method is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogeneous sample. It is usually expressed as the percentage of the coefficient of variation (%CV) or the relative standard deviation (%RSD) of the results. Precision can also being determined as the reproducibility of the analytical method under normal operating circumstances.

Procedure

Each concentration was triplicately analyzed within the same day. Also the same pattern was analyzed in the different day for the others three days

(a) Within run precision

Retinyl palmitate solution was prepared with the range of concentration from 2 μg/ml to 8 μg/ml. The within run precision was accomplished by analysis of four

concentrations of retinyl palmitate solutions in the same day. Peak area ratios between retinyl palmitate and retinyl acetate were compared and the percentage of the coefficient of variation (%CV) for each group was determined.

(b) Between run precision

The same range of retinyl palmitate in the within run precision was used. The between run precision was accomplished by analysis of four concentrations of retinyl palmitate solutions in other different three days. Peak area ratios between retinyl palmitate and retinyl acetate were compared during these days and the percentage of the coefficient of variation was also determined.

Specificity

Specificity is the ability of the analytical method to specifically determine analytes accurately and specifically in the presence of foreign components. The specific the chromatographic conditions, the peak of other components in the sample must not interfere with the peak of retinyl palmitate.

Linearity

The linear relationship between retinyl palmitate concentration and the response from the analysis was determined. The linear range was expressed by analyzing the series of calibration solutions of retinyl palmitate according to the proposed HPLC condition.

1.2.4 Analysis of retinyl palmitate in niosome suspension.

Retinyl palmitate niosome suspensions were prepared by 300 μmole of the total lipid/surfactant with entrapped retinyl palmitate. The unentrapped retinyl palmitate was separated by ultracentrifugation at the speed of 50,000 rpm or 200,000Xg, 4 °C for 30 minutes. Both unentrapped and entrapped retinyl palmitate were then analyzed by HPLC.

1.2.5 HPLC condition for determination retinyl palmitate for permeation study.

The HPLC condition for retinyl palmitate analysis was similar to the previous condition. Only the flow rate of mobile phase was set into time program resulting in the better appearance of chromatogram.

The time program was set as follows:

Time (min)	Total flow rate of mobile phase (ml/min)
0.01	1
4.99	1
5.00	2
9.49	2
9.50	1

2.Preparation of retinyl palmitate niosomes

Multilamellar nonionic surfactant vesicles (niosomes) were prepared by a hand-shaking method (Baillie, 1985;Yoshioka, 1994). Span, cholesterol and solulan C-24 were the lipid/surfactant ingredient dissolved in the mixture of chloroform and methanol with the given molar ratio. Also the solution of retinyl palmitate in this mixture was added together. After that the resulting solution was put into 100 ml round bottom flask. The organic solvent was evaporated under reduced pressure at a temperature of approximately just above 60 °C using a rotary evaporator until a thin film on the flask wall. The excess organic solvent was then removed. The resulting film was dried completely. The dried lipid film was hydrated with distilled water by shaking with a shaker in a water bath at approximately 80 °C for 1 hour.

Also, the retinyl palmitate niosomes were prepared using the above procedure to investigate the effect of surfactants and cholesterol on drug entrapment.

3. Characterization of retinyl palmitate niosomes.

All three types of retinyl palmitate niosomes prepared by Span 40, Span 60 and Span 85 were typically prepared by using of 300 μmole total lipid/surfactant dispersed in distilled water. The mole ratio of total lipid/surfactant, Span:cholesterol:solulan

C-24 niosome suspensions, was 45:45:10, respectively.

They were normally characterized under optical microscopes and electron microscopes. However prepared multilamellar vesicles have noticeably large sizes that can not be characterized under transmission electron microscopy which suitable for no more than 1 μm of particle sizes.

3.1 Optical microscopy

Normally, the ordinary microscope is used for particle size measurement in the range of 0.2 μm to about 100 μm (Martin, 1969). Based on this study, the vesicle formation and its shape can be examined with an optical microscope with an attached camera. One drop of niosome suspensions was mounted on a slide and covered with cover slide and placed on the stage.

3.2 Scanning electron microscopy

The morphology of vesicles was investigated by scanning electron microscopy. Only one drop of niosome suspensions was in a cover slide and let the sample dry on the cover slide. Then the dry sample was prefixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.2 for two hours in the refrigerator.

After that, the dry sample was washed with phosphate buffer for three times, ten to fifteen minutes of each, followed by the post-fixed process. This sample was fixed with 1% osmium tetroxide in 0.1 M phosphate buffer at pH 7.2, washed with phosphate buffer and then washed with distilled water for two times, ten to fifteen minutes for each.

The dried and fixed sample was dehydrated through a graded series of alcohol series that was 30%, 50%, 70%, 90% and 100%, ten to fifteen minutes for each and three times for washing at only 100% alcohol.

The sample was dried in a critical point drier under CO_2, mounted on the stub under a stereomicroscope, and then it was coated with gold for four to five minutes with ion sputter, and finally it was observed under scanning electron microscopy.

3.3 Determination of particle size and particle size distribution

The particle size distribution can be examined by plotting the number or weight of particle against the particle size within a certain range This plotted data is called frequency distribution curve (Martin, 1969). Another method of representing the data is to plot the cumulative percentage under a particular size against the particle size (Martin, 1969).

The mastersizer is based on the principle of laser ensemble light scattering. The instrument is composed of lens 300 RF counting the size in the range of 0.05-878.7 μm. The light from a 2 mW Helium-Neon laser (633 nm wavelength) is used to form a analyser beam. The transmitter and receiver units mounted on an optical bed. The Fourier lens mount receiver and any particles introduced by the sample presentation modules present within it will scatter this laser light.

The unscattered light is brought and passes through the detector and out of the optical system. The total laser power passing out of the system in this way is monitored allowing the sample volume concentration to be determined and shown on computer (Scientific and Technological Research Equipment Center [STREC],:1-4)

4. Determination of retinyl palmitate entrapment efficiency.

4.1 Effect of surfactants and drug loading on entrapment efficiency.

33.33 mM niosome suspensions prepared by Span 40, Span 60 or Span 85:cholesterol:Solulan C-24 in the mole ratio of 45:45:10. The amount of retinyl palmitate added in niosome preparation was varied,i.e, 5 mg, 8 mg and 10 mg. The dry lipid film was hydrated with 9 ml-distilled water at 80 °C for 1 hour. All processes were conducted in a dark room illuminated with yellow light.

The amount and the percentage of retinyl palmitate entrapment was investigated and compared among those sets of preparation with the varied amount of retinyl palmitate for each of three preparations

For this amount of retinyl palmitate, it accounted for optimized drug loading. Unencapsulated retinyl palmitate can be seen by the yellow droplet located at the upper part of the centrifuged tube. Then both entrapped retinyl palmitate in pellets and unentrapped retinyl palmitate were analyzed by high performance liquid chromatography with suitable conditions. The quantitative retinyl palmitate analysis was shown in the percentage of entrapped retinyl palmitate.

4.1.1 Separation of entrapped retinyl palmitate niosomes.

In order to separate unentrapped retinyl palmitate from niosomes quickly and conveniently, ultracentrifugation method was chosen decidedly to accomplish this process. The ultracentrifuge tube containing 9 ml of retinyl palmitate niosome suspension was put in the rotor.

The maximum speed of the ultracentrifugation was 50,000 rpm at which the ultracentrifuged tube can tolerate.The temperaturee was set at 4 °C.

The ultracentrifugation process took just about 30 minutes that was the minimum period to separate them efficiently.

4.1.2 Quantitative analysis of retinyl palmitate in niosome pellets.

After separation, the pellet at the upper and lower part of tube was collected to analyse entrapped retinyl palmitate. The pellets were lysed by isopropanol to obtain the entrapped retinyl palmitate solution. This solution was used for analysis by HPLC. After that, this entrapped retinyl palmitate was pipetted into a 10 ml-volumetric flask with retinyl acetate, as an internal standard, and then adjust with mobile phase, acetonitrile and isopropanol.

4.1.3 Quantitative analysis of unentrapped retinyl palmitate.

After separation, the unentrapped retinyl palmitate was diluted with isopropanol in the tube and weighed the whole sample solution of free drug at the

same time.The analysis of unentrapped retinyl palmitate was achieved by pipetting with the small amount from this solution to a volumetric flask with retinyl acetate as its internal standard. The volume was adjusted to 10 ml with the mobile phase. The solution was analyzed by HPLC .

4.1.4 Calculation of the percentage of entrapment and encapsulation efficiency.

The percentage of retinyl palmitate entrapment of each preparation was determined from the following equation:

$$\% \text{ Entrapment} = \frac{\text{Amount of retinyl palmitate in the pellets}}{\text{Total amount of retinyl palmitate in niosomes}} \times 100 \qquad (6)$$

where total amount of retinyl palmitate in niosome suspension was expressed as the sum of amount of retinyl palmitate as a free drug and in the pellets.

Encapsulation efficiency was defined as the fraction of retinyl palmitate found in the niosome pellets and expressed as mole of retinyl palmitate per mole of total lipid material.

$$\text{Entrapment efficiency} = \frac{\text{mole of retinyl palmitate in pellets}}{\text{mole of total lipid/surfactant material}} \quad (7)$$

4.2 Effect of cholesterol on drug entrapment efficiency

Four sets of niosomes prepared by Span 40, 60 or 85: cholesterol: Solulan C-24 with mole ratio of 10:80:10, 45:45:10, 65:25:10 and 90:0:10 were studied for the effect of cholesterol on the drug entrapment efficiency. The excessive amount of retinyl palmitate was approximately 8 mg.

The dry lipid/surfactant film was hydrated with 9 ml-of distilled water at 80 °C for 1 hour. All processes were conducted in a dark room under yellow light.The percentage of entrapment efficiency of retinyl palmitate was investigated

5.Drug permeation study

The most useful apparatus for drug permeation studies was Franz diffusion cell. In vitro permeation measurements can be made with two chambers. Placing the dorsal region of the skin faces to the donor chamber containing niosome suspension.

In addition, some surfactants were added to the receptor fluid in order to increase the solubility of lipophilic drugs.

In permeation study, the solution in the receptor compartment is constantly removed and replaced with fresh receptor fluid to keep the concentration at a low level. This is referred to as "sink condition".

Four sets of three niosome preparations arising from Span 40, Span 60 and Span 85 were prepared by using of 300 μmole of total lipid that was Span: cholesterol: Solulan C-24.

Retinyl palmitate niosomes were prepared by 300 μmole of total lipid material with Span: cholesterol: Solulan C-24, 45:45:10. Also, retinyl palmitate was 5 mg added into the process of niosome preparation. Each of preparation employed 5 pieces of snake skin membranes.

5.1 The preparation of sample solution for the study of retinyl palmitate permeation

5.1.1 Franz diffusion cell

The Franz cells used in this study comprised of receptor chambers able to contain the fluid. The diameter of the Franz cell was 1.6 cm corresponding to an effectively permeable area of 2.01 cm^2. The receptor compartment was equipped with

a magnetic stirring bar and thermostated at 32 °C by circulating water through a jacket surrounding the cell body throughout the experiment.

5.1.2 Preparation and treatment of membrane

Shed snake skin was used as a skin membrane model providing a pure stratum corneum to study the ability of particular penetration enhancer. From this study, Cobra shed snake skin was used. It came from the snake farm at the Thai Red Cross Society. Only the single snake skin was used for this permeation study. A whole shed snake skin was cut into square then each piece of shed snake skin was soaked overnight in pure water prior to conducting the experiment. Then a piece of skin was clamped in place between the donor chamber and the receptor chamber of the cell.

5.1.3 The selection of receptor fluid

Retinyl palmitate was lipophilic compound. So it is necessary to add surfactants or other substances could increase the solubility of lipophilic compounds in the receptor compartment. Guenin (1995) studied the permeation of retinyl palmitate from vesicles through the skin. This study used 3% of polysorbate 80 as a surfactant added in the receptor fluid for permeation study of retinyl palmitate by hairless mouse skin and also stated that this surfactant may have a slight damaging effect. In addition, polyoxyethylene(20)isohexadecyl ether (Arlasolve 200) was used to increase the solubility of lipophilic compounds in receptor fluid corresponding to

the information on the application of polyethyleneglycol(20)oleyl ether may be effective in increasing partitioning of lipophilic compounds(Schaefer, 1999). Therefore, polysorbate 80 and Arlasolve 200 were decidedly chosen.

5.2 Method of quantitative analysis of retinyl palmitate for permeation studies

The method of retinyl palmitate analysis for permeation study was similar to that for entrapment studies. During the preliminary of permeation study, it was found that the concentration range of permeate retinyl palmitate collected at the receptor chamber was 0.02-0.14 μg/ml. Therefore, the retinyl palmitate standard solutions had the concentration between 0.02 μg/ml and 0.14 μg/ml.

5.2.1 Preparation of standard solutions

A stock solution of retinyl palmitate was prepared by dissolving retinyl palmitate 1 mg in isopropanol. The solution volume was adjusted to 25 ml in a volumetric flask.

A stock solution of retinyl acetate was prepared by dissolving retinyl acetate 10.8 mg in isopropanol. The solution volume was adjusted to 10 ml. This solution was pipetted for 1 ml and transfered to another volumetric flask as an applicable retinyl

acetate stock solution and then it was adjusted to 10 ml with isopropanol. Consequently, the concentration of retinyl acetate stock solution was 0.1 mg/ml

Standard solutions were prepared by firstly, adding the receptor fluid into four volumetric flasks. Secondly, pipetting 5,15,25 and 35 µl of retinyl palmitate stock solution into each individual of four 10 ml-volumetric flasks. Thirdly, pipetting 6 µl of retinyl acetate stock solution into each. The receptor fluid mixed with standard solutions accounted for the same condition for HPLC analysis.