CHAPTER III

MATERIALS AND METHODS

Materials

A. Chemicals

1. Acetic Acid, AR grade (Merck, Germany, lot no. K14090663)
2. Acetonitrile HPLC grade (Labscan Asia, Thailand, lot no.06050219)
3. Agar (Gibco BRL, UK, lot no 20H8524B)
4. Chitosan, 95%Deacetylation, MW 110,000 Da (Seafresh Chitosan Lab Company Limited, Thailand)
5. di-Sodium Hydrogen Orthophosphate Anhydrous (APS Finechem, Australia, lot no. F2F136)
6. Doxycycline Hyclate (Donated from Siam Bhaesach Co., Ltd., Thailand, lot no. D-041013)
7. Glyceryl Monooleate(Donated from Cognis Thai Ltd., lot no.GR60414301)
8. Isopropanol, AR grade (Merck, Germany, lot no. K32632434346)
9. Mueller Hinton Broth (Becton Dickinson, USA, lot no 4104193)
10. Perchloric Acid (Merck, Germany, lot no)
11. Prednisolone (Donated from V & S Chemi group Co., Ltd. Bangkok, lot no. 198)
12. Sesame Oil (Chee Seng Oil Factory (PTE) Ltd., Singapore, lot no. HC 5092)
13. Sodium Chloride, AR grade (Merck, Germany, lot no.K32104204324)
14. Sodium Dihydrogen Orthophosphate (APS Finechem, Australia, lot no. Z9G017A)
15. Sodium Hydroxide, AR grade (Mallinckrodt, Germany, lot no)
16. Sodium Tripolyphosphate (Donated from FA Unity Co., Ltd., Thailand)
17. Span® 85 (EAC. Thailand, lot no.52246)
18. Sunflower Oil (Thanakorn Vegetable)

B. Accessories

1. Syringe needle guage no. 23 (Nipro Corporation, Japan, lot no.2010-04)
2. Disposable syringe filter nylon 13 mm, 0.45 µm (Chrom Tech, USA)
3. Whatman filter paper No.1. 150 mm (Whatman International Ltd., England, lot no.E1479621)

C. Microbes

1. *Staphylococcus aureus* ATCC 6538P (Donated from Department of Microbiology, Faculty of Pharmaceutical science, Chulalongkorn University, Thailand)

D. Eqiupments

1. Analytical Balance(Model AX105, Mettler Toledo, Switzerland)
2. Autoclave (Model HA-3D, Hirayama, Japan)
3. Centrifuge (Model 4206, ALC, Italy)
4. Differential Scanning Calorimeter (DSC822e, Mettler Toledo, Switzerland)
5. Digital Camera (Coolpix 5400, Nikon, Japan)
6. Lyophilizer (Dura-Dry II MP, FTS System, Inc., USA)
7. High Performance Liquid Chromatography
   - Auto Injector (SIL-10A, Shimadzu, Japan)
   - Communications bus module (CBM-10A, Shimadzu, Japan)
   - Liquid chromatograph pump (LC-10AD, Shimadzu, Japan)
   - UV-VIS detector (SPD-10A, Shimadzu, Japan)
   - Column (Mightysil RP-18 GP, 5µm, 150 x 4.6 mm, Kanto Chemical Co., Inc., Japan, lot no. 8043012)
   - Precolumn (µBondapack C18, 10 µm, 125 A°, Water Corporation, Ireland, lot no. W2334B1)
8. Hot Air Oven (Model B40, Memert, Germany)
9. Incubator Chamber (Model B40, Memmert, Germany)
10. Modified Franz Diffusion Cells (Crown Glass Company, USA)
11. pH Meter (Model 420A, Orion, USA)

12. Refrigerated Incubator (FOC 225I, VELP Scientifica, Italy)
13. Simple paddle (Eurostar digital, Germany)
14. Ultrasonicator (Crest Ultrasonics, Malaysia)
15. UV-visible spectrophotometer (UV-1601, Shimadzu, Japan)
16. Vacuum Pump (CB169 Vacuum system, Buchi, Switzerland)
17. Vernier Caliper (Macoh, China)
18. Vortex Mixer (Vortex Ginies-2, Scientific Industries, Inc., USA)

Methods

A. Doxycycline Hyclate Loaded Chitosan Microspheres

1. Preparation of Doxycycline Hyclate Loaded Chitosan Microspheres

The chitosan microspheres were prepared by emulsification and ionotropic gelation method. The dispersed phase was prepared by dissolving varied concentration of 2%, 3% and 4% w/v chitosan in 2% v/v aqueous acetic acid solution and left to stand for 24 hours or until the solution was clear, The chitosan solution with equivalent amount to 0.5 g chitosan was then mixed with varied concentrations of percentages by weight of doxycycline hyclate of 10, 30 and 75% by weight of chitosan. The continuous phase consisted of 150 g of sunflower oil containing 5% of Span® 85 as the emulsifier.

The dispersed phase was added to the continuous phase under agitation to form a w/o emulsion. After a specified emulsification time (20 min), 10 ml of sodium tripolyphosphate (STPP) solution with varied concentrations of 5-15% w/v was added. Stirring was continued for an hour after the addition of STPP. The microspheres were removed by filtering the solution through filter paper No.1, washed with 50 ml isopropyl alcohol, and followed by 100 ml distilled water, prior to lyophilization.

2. Morphology and Particle Size

The morphology of the microspheres was observed by scanning electron microscopy (SEM). The sample was coated with gold by ion sputtering under a high vacuum and high voltage. The coated samples were then examined under SEM.

The particle size of the microspheres was measured using a laser light diffraction (Mastersizer®, Malvern, UK). The microspheres were uniformly dispersed in liquid paraffin prior to the measurement.

3. The Percentage Yield of Doxycycline Hyclate Loaded Chitosan Microspheres

The chitosan microspheres obtained from the lyophilization were collected and weighed. The percentage yield of doxycycline hyclate loaded chitosan microspheres was determined from equation (1).

$$\% \text{ Yield} = \frac{\text{Wt. of dried microspheres (g)} \times 100}{\text{Theoretical Wt. of microspheres (g)}} \qquad (1)$$

Where,

-Theoretical Wt. of microspheres (g) = Wt. of drug load (g) + Wt. of chitosan (g) + Wt. of STPP (g)

4. Determination of Drug Content of Chitosan Microspheres

4.1 Encapsulation Efficiency

Encapsulation efficiency was determined by accurately weighing 20 mg of doxycycline hyclate loaded microspheres into a 10 ml test tube. To extract doxycycline hyclate from the microspheres, 5 ml of 1 N hydrochloric acid was added and mixed for 24 hours, centrifuged at 5000 rpm for 10 min, and poured the clear portion into a 25 ml volumetric flask. Then repeated the extraction again with the same volume of 1 N hydrochloric acid, sonicated for 15 min, centrifuged and combined the clear portion with the previously obtained solution. The solution was adjusted to volume with 1 N hydrochloric acid. The solvent 1 N HCl was chosen to extract doxycycline hyclate from

the chitosan microspheres due to the ability to dissolve the entire amounts of doxycycline hyclate. The process was repeated until steady amount of doxycycline hyclate was obtained. The amount of drug loaded was determined by spectrophotometer at 268 nm. All the experiments were carried out in triplicate. The loading efficiency was determined from the equation (2)

$$\text{Loading efficiency (\%)} = [Wa/Wt] \times 100 \qquad (2)$$

Where Wa is the actual doxycycline hyclate content and Wt is theoretical doxycycline hyclate content.

4.2 Assay of Encapsulation Efficiency

In the determination of drug content of chitosan microspheres, UV spectrophotometric method was used for quantitation of doxycyline because the method was convenient and rapid.

4.2.1 Standard Solutions for UV Spectrophotometric Method

An accurately weighed amount 3 mg of doxycycline hyclate was transferred to a 50 ml volumetric flask. Hydrochloric acid 1N was used to dissolve and adjust to volume. This solution had a final concentration of 60 μg/ml.

Standard solutions of doxycycline hyclate were prepared by pipetting 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4 ml of doxycycline hyclate stock solutions into 10 ml volumetric flasks, diluted and adjusted to volume with the same solvent. The final concentrations of the solution were 3, 6, 9, 12, 15, 18, 21 and 24 μg/ml, respectively.

All of standard solutions were analyzed spectrophotometrically at 268 nm. hydrochloric acid 1N were used as a blank. The standard curve was plotted between concentration and absorbance.

4.2.2 Validation of UV Spectrophotometric Method

The analytical parameters used for the assay validation were specificity, linearity, accuracy and precision.

4.2.2.1 Specificity

Under the condition selected for in vitro release study, the absorbance of doxycycline hyclate must not be interfered by the absorbance of other components in the sample.

4.2.2.2 Linearity

Three sets of seven standard solutions of doxycycline hyclate ranging from 3 to 24 μg/ml were prepared and analyzed. Linear regression analysis of the absorbances versus their concentrations was performed. The linearity was determined from the coefficient of determination (R^2).

4.2.2.3 Accuracy

The accuracy of an analytical method is the closeness of test result obtained by that method to the true value. The accuracy of the method was determined from the percentage of recovery. Five sets of three concentrations at 10.5, 16.5 and 22.5 μg/ml were prepared and analyzed. The percentage of recovery of each concentration was calculated from the ratio of inversely estimated concentration to known concentration multiplied by 100.

4.2.2.4 Precision

a) Within Run Precision

The within run precision was determined by analyzing five sets of three concentrations at 14, 22 and 30 μg/ml in the same day. The percent coefficient of variation (%CV) of each concentration was determined.

b) Between Run Precision

The between run precision was determined by analyzing five sets of three concentrations at 10.5, 16.5 and 22.5 μg/ml on five different days. The percent coefficient of variation (%CV) of doxycycline hyclate of each concentration was determined.

Acceptance criteria:

For accuracy, the percentage of recovery should be within 98-102% of each nominal concentration, whereas the percent coefficient of variation for both within run and between run precision should be less than 2%.(USP 27, 2004)

B. Experimental Design and Optimization of Doxycycline Hyclate Loaded Chitosan Microsphere Formulation

A Box-Behnken experimental design was employed to statistically optimise the formulation parameters of doxycycline hyclate microsphere preparation for controlled drug release. Response surface methodologies, such as the Box-Behnken and Central Composite Designs, model curvature in the response function were considered. The Box-Behnken design was specifically selected since it required fewer treatment combinations than a Central Composite Designs in cases involving three or four factors. The Box-Behnken design was also rotable and contained statistical "missing corners" which might be useful when the experimenter was trying to avoid combined factor extremes. This property prevents a potential loss of data in those cases.

Generation and evaluation of the statistical experimental design were performed with Design Expert Software Version 7.0.3 (USA). The studied factors were doxycycline hyclate concentration (DC), sodium tripolyphosphate concentration (STPPC) and chitosan concentration (CC). The response variables were considered to be the percentage encapsulation efficiency and percentage yield.

A design matrix comprising of 16 experimental runs was constructed. An interactive second order polynomial model was utilized to evaluate the response variable as shower in equation (3).

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_1X_1 + b_5X_2X_2 + b_6X_3X_3 + b_7X_1X_2 + b_8X_1X_3 + b_9X_2X_3 \quad (3)$$

where b_0-b_9 are the regression coefficients, X_1-X_3 are the factors studied and Y is the measured response associated with each factor level combination.

Preliminary studies provided a setting of the levels for each formulation variable. Table 3 summarises the factors and their levels and Table 4 summarises the design matrix with the experimental runs, factor levels and combinations.

Table 3 Independent variables: factors and their levels for the Box-Behnken design

Factors	Level		
	-1	0	1
X_1: Doxycycline hyclate concentration(DC)	10	30	75
X_2: Chitosan concentration(CC)	2	3	4
X_3: Sodium Tripolyphosphate concentration(STPPC)	5	10	15

Table 4 Experimental runs and factor combinations for the Box-Behnken design

Experiment	Factor combinations at different levels		
	DC (% w/w)[+]	CC (% w/w)	STPPC (% w/v)
1	75	2	10
2	30	2	5
3	30	2	15
4	10	2	10
5	75	3	5
6*	30	3	10
7*	30	3	10
8	75	3	15
9	10	3	5
10*	30	3	10
11*	30	3	10
12	10	3	15
13	30	4	15
14	30	4	5
15	75	4	10
16	10	4	10

* = The center points of the design

[+] = Based on chitosan weight

C. Characterization of Selected Doxycycline Hyclate Loaded Chitosan Microspheres

From the section B, the selection of the formulations of doxycycline hyclate loaded chitosan microspheres was carried out. An optimized formulation obtained from the program with high desirability of responses was selected into the further studies. The other two formulations (formulations 11 and 15) were selected additionally due to their observed high encapsulation efficiency.

1. The Release of Doxycycline Hyclate from Chitosan Microspheres

1.1 *In vitro* Release Study

The in vitro released study was performed using 15 ml conical tubes containing 10 ml PBS pH 6.8 release medium at 37°C and shaken continuously at 70 rpm using a shaker water bath (Memmert, Germany) to ensure thorough mixing. The release medium was equilibrated to the desired temperature for 1 hr before the release study. After equilibration, microspheres approximate weight equivalent to doxycycline hyclate 10 mg of sample was accurately weighed and placed into the release medium (Bromberg et al., 2001).

Samples of 5 ml were withdrawn at certain time intervals at 1, 2, 3, 4, 6, 8, 12, 24, 48, 72, 96 and 120 hr. The sample medium was removed by using a syringe and replaced with the same amount of medium to keep the constant volume during the experiment. Doxycycline hyclate concentrations of all sample solutions taken were analyzed by using UV spectrophotometer at 268 nm.

Figure 12 Water bath (Memmert model WB22) with stainless rack

The amount of drug released was calculated by multiplying the drug concentration with medium volume. The percentage of drug release was calculated by the following equation:

$$\% \text{ drug release} = (A_t / A_0) \times 100 \quad (4)$$

where A_t is cumulative released amount of drug at a particular time; A_0 is the initial amount of drug.

1.2 Assay of *In vitro* Doxycycline Hyclate Release

The determination of amount of doxycycline hyclate release was performed by UV spectrophotometry due to rapidity and convenience.

1.2.1 Standard Solutions for UV Spectrophotometric Method

An accurately weighed amount 4 mg of doxycycline hyclate was transferred to a 50 ml volumetric flask. Phosphate buffer saline pH 6.8 was used to dissolve and adjust to volume. This solution had a final concentration of 80 μg/ml.

Standard solutions of doxycycline hyclate were prepared by pipetting 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4 ml of doxycycline hyclate stock solutions into 10 ml volumetric flasks, diluted and adjusted to volume with the same solvent. The final concentrations of the solution were 4, 8, 12, 16, 20, 24, 28 and 32 μg/ml respectively.

All of standard solutions were analyzed spectrophotometrically at 268 nm. Phosphate buffer saline pH 6.8 were used as a blank. The standard curve was plotted between concentration and absorbance.

1.2.2 Validation of UV Spectrophotometric Method

The analytical parameters used for the assay validation were specificity, linearity, accuracy and precision.

1.2.2.1 Specificity

Under the condition selected for in vitro release study, the absorbance of doxycycline hyclate must not be interfered by the absorbance of other components in the sample.

1.2.2.2 Linearity

Three sets of seven standard solution of doxycycline hyclate ranging from 4 to 32 µg/ml were prepared and analyzed. Linear regression analysis of the absorbances versus their concentrations was performed. The linearity was determined from the coefficient of determination (R^2).

1.2.2.3 Accuracy

The accuracy of an analytical method is the closeness of test result obtained by that method to the true value. The accuracy of the method was determined from the percentage of recovery. Five sets of three concentrations at 14, 22 and 30 µg/ml were prepared and analyzed. The percentage of recovery of each concentration was calculated from the ratio of inversely estimated concentration to known concentration multiplied by 100.

1.2.2.4 Precision

a) Within Run Precision

The within run precision was determined by analyzing five sets of three concentrations at 14, 22 and 30 µg/ml in the same day. The percent coefficient of variation (%CV) of each concentration was determined.

b) Between Run Precision

The between run precision was determined by analyzing five sets of three concentrations at 14, 22 and 30 µg/ml on five different days. The percent coefficient of variation (%CV) of doxycycline hyclate of each concentration was determined

Acceptance criteria:

For accuracy, the percentage of recovery should be within 98-102% 0f each nominal concentration, whereas the percent coefficient of variation for both within run and between run precision should be less than 2% (USP 27, 2004).

2. Differential Scanning Calorimetric (DSC) Method

The DSC thermogram of samples was determined by using differential scanning calorimeter (DSC822^e, Mettler Toledo, Switzerland). A highly sensitive ceramic sensor in DSC instrument was used to measure the difference between the heat flows to the sample and reference crucibles. The standard type of pan for DSC measurement was standard 40 μl aluminum crucible. An accurately weighed amount of 3-5 mg of the sample was placed in the aluminum crucible. Then the aluminum crucible was sealed with the lid under the pressure of the plunger by using crucible sealing press. After sealing, The aluminum crucible was placed on a DSC sensor in the furnace. Samples were heated from 0 to 300 °C at a heating rate of 10°C/min. All tests were performed under a nitrogen atmosphere of 2 ml/min.

Figure 13 Standard 40 μl aluminum crucible

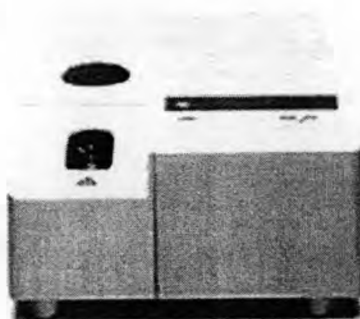

Figure 14 Differential scanning calorimeter (DSC822^e, Mettler Toledo, Switzerland)

3. Powder X-ray Diffractometry

Powder X-ray diffractograms were carried out by using powder X-ray diffractometer (Bruker AXS model D8 Discover, Germany) with Cu-K_α radiation as the source of X-rays. The measurement conditions were as follows: voltage of 40 kV, current of 40 mA, scanning speed of 6°/min in the 2θ angle range of 7-45°.

4. Stability Study of Doxycycline Hyclate Loaded Chitosan Microspheres

4.1 Analysis of Doxycycline Hyclate by High Performance Liquid Chromatographic (HPLC) Method

In the stability study, HPLC method was used for quantitation of doxycycline hyclate because of specificity and high sensitivity. The condition of HPLC analysis for remaining doxycycline hyclate in formulations was modified from Skúlason et al. (2003).

4.1.1 Chromatographic Condition

The HPLC conditions for the analysis of doxycycline hyclate remaining were as follows:

Column	:	Mightysil RP-18 GP, 5μm, 150 x 4.6 mm
Mobile Phase	:	Acetonitrile : H_2O : $HClO_4$ of 26:74:0.2, pH 2.5
Injection volume	:	20 μl
Flow rate	:	1.5 ml/min
Detector	:	UV detector 268 nm
Temperature	:	ambient
Run time	:	15 min
Internal standard	:	Prednisolone

The mobile phase was prepared by using acetonitrile, water and perchloric acid with the ratio of 26:74:0.25 v/v. The solution was thoroughly mixed, filtered through 0.45 μm membrane filter and then degassed by sonication for 30 min prior to use.

4.1.2 Standard Solutions for HPLC Method

From the preliminary study, prednisolone was chosen to be the internal standard. A stock solution of internal standard was prepared by accurately weighing of 24 mg of prednisolone in a 50 ml volumetric flask. Methanol was added to dissolve the internal standard and the solution was adjusted to the final volume by mobile phase. The final concentration of prednisolone was 0.48 mg/ml

A stock solution of doxycycline hyclate was prepared by accurately weighing 50 mg of doxycycline hyclate in a 50 ml volumetric flask. Mobile phase was used to dissolve the drug and adjusted the final volume. This stock solution had a final concentration of 1 mg/ml

Standard solutions of doxycycline hyclate were prepared by pipetting 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 ml of the doxycycline hyclate stock solution in to 10 ml volumetric flasks, respectively. Then 1000 μl of the prednisolone stock solution was added into each of these volumetric flasks. The solutions were adjusted to volume with mobile phase so that the concentrations of doxycycline hyclate in the standard solutions were 20, 30, 40, 50, 60 and 70 μg/ml, respectively, and that of prednisolone was 48 μg/ml. Three sets of standard solutions were prepared for each HPLC run.

As a result, the standard curve of doxycycline hyclate between concentration and peak area ratio was plotted.

4.1.3 Validation of HPLC Method

The analytical parameters used for validation of the HPLC method were specificity, linearity, accuracy and precision.

4.1.3.1 Specificity

Under the chromatographic conditions used, the peak of doxycycline hyclate must be completely separated from and not be interfered by the peak of other components in the sample.

4.1.3.2 Linearity

Three sets of six standard solutions were prepared and analyzed. Linear regression analysis of the peak area ratios versus their concentrations was performed. The linearity was determined from the coefficient of determination (R^2).

4.1.3.3 Accuracy

The accuracy of an analytical method is the closeness of test result obtained by that method to the true value. The accuracy of the method was determined from the percentage of recovery. Five sets of three concentrations at 35, 45 and 55 μg/ml were prepared and analyzed. The percentage of recovery of each concentration was calculated from the ratio of inversely estimated concentration to known concentration multiplied by 100.

4.1.3.4 Precision

a.) Within Run Precision

The within run precision was determined by analyzing five sets of three concentrations at 35, 45 and 55 μg/ml in the same day. Peak area ratios of doxycycline hyclate to prednisolone were calculated and the percent coefficient of variation (%CV) of each concentration was determined.

b.) Between Run Precision

The between run precision was determined by analyzing five sets of three concentrations at 35, 45 and 55 μg/ml on five different days. The percent coefficient of variation (%CV) of doxycycline hyclate of each concentration was determined.

Acceptance criteria:

For accuracy, the percentage of recovery should be within 98-102% of each nominal concentration, whereas the percent coefficient of variation for both within run and between run precision should be less than 2%.

4.1.4 Sample Solutions for HPLC Method

Samples of doxycycline hyclate loaded microspheres were prepared by accurately weighing 25 mg of the microspheres into a 10 ml test tube. The amount of 5 ml of 1 N hydrochloric acid was added to extract doxycycline hyclate in the microspheres with mixing for 24 hours, centrifuged at 5000 rpm for 10 min, poured the clear solution into a 10 ml volumetric flask. Then repeat the extraction again with the same volume of 1 N hydrochloric acid, sonicated for 15 min, centrifuged and combined the extract. The solutions were adjusted to volume with 1 N hydrochloric acid. Then 1.0 ml of the above solution was transferred into a 10 ml volumetric flask and 1.0 ml of prednisolone stock solution was added into this flask. The solution were adjusted to volume with mobile phase to give the sample solution of 50 µg/ml of doxycycline hyclate and 48 µg/ml of prednisolone, respectively, and then injected into the HPLC column The peak area ratio of doxycycline hyclate and prednisolone was calculated and the concentration of remaining doxycycline hyclate was determined from the daily prepared standard curve.

4.2 Accelerated Stability Studies

The formulation of chitosan microspheres was kept in amber-glass vials at controlled temperatures of 40, 50, 60 and 70°C with $\pm$ 1°C deviation. Three samples were withdrawn at appropriate time intervals. The analysis of remaining doxycycline hyclate in the sample was carried out by the HPLC method (Al-Gohary and Kassas, 2000).

The observed degradation rate constants (k_{obs}) of doxycycline hyclate were determined. The ln k_{obs} and reciprocal absolute temperature (1/T) were plotted according to the Arrhenius equation.

From the arrhenius equation, extrapolated degradation rate constant ($K_{extrapolated}$) and extrapolated shelf-lives values ($t_{90,observed}$) at 30°C were determined.

D. Preparation of Monoglyceride-Based Drug Delivery System

1. Monoglyceride-Based Drug Delivery System

Monoglyceride-based drug delivery system was prepared from the mixture of monoglyceride and triglycerides and water. Triglyceride used in this study was sesame oil due to the ability to improve the flowability of the system (Tan, 2004).

Monoglyceride-based drug delivery systems were prepared on a weight by weight (w/w) basis by weighing the three components in screw-capped tubes and vigorously mixed using a vortex mixer for 5 min. The samples were heated at 45$\pm$0.5°C and occasionally mixed for 30 min. The samples were stored at room temperature for 2 days before further studies (Tan, 2004).

The ternary phase diagram consisted of triglyceride, monoglyceride and water was constructed. The visual appearance and structure of the ternary mixtures were determined and specified into the phase diagram.

2. Characterization of Monoglyceride-Based Drug Delivery System

2.1 Determination of physicochemical properties

The physicochemical properties of monoglyceride-based drug delivery system before incorporation of doxycycline hyclate loaded microspheres were determined as following:

2.1.1 Physical Appearances

The physical appearances of the formulations such as color, clarity and phase separation were visually observed.

2.1.2 Viscosity Measurement

The viscosity of formulations was determined by using the Cone and Plate Viscometer (Brookfield viscometer, Scientific Industries, USA). The determination of the viscosity was performed by applying about 0.5 ml of sample to the lower plate of the

viscometer. According to the guideline, the cone CP-40 was selected in the experiment. The measurements were performed in triplicate.

2.1.3 Polarized Light Microscopy

A drop of sample was placed on a glass slide and examined for the liquid crystalline phases under the polarized light microscope (Eclipse E200, Nikon, Japan). Anisotropic liquid crystalline phases, such as hexagonal, lamella and reversed hexagonal phases, are optical by birefringent.

3. Determination of Injectability through the Syringe Needle

The injectability through the syringe needle guage no. 23 was performed to ensure that the formulation could be administered by syringe into a periodontal pocket. The low viscous formulations were tested for the flowability through the syringe needle guage no. 23. When they are on contact with water of gingival fluid, the formulation was in situ transform into a liquid crystalline state and change to a semi-solid at the injection sites.

4. *In vitro* Liquid Crystalline Phase Formation Study

This study was performed to select the formulation from the ternary phase diagram which showed a short setting time to transform into liquid crystalline phases upon dilution with water. The study was modified from Scherlund et.al.(2001). The test was conducted within microcentrifuge tube at the temperature 37$\pm$0.5°C using water bath. The water-free formulation of 0.5 ml was added into 1 ml of water. Then the liquid crystal formation was detected by observing the viscosity change and examining under the polarized light microscope.

5. Preparation of Monoglyceride-Based drug Delivery System Containing Doxycycline Hyclate Loaded Chitosan Microspheres

The samples that could form reverse hexagonal phases and exhibited short duration time to form liquid crystalline state upon contact with water was selected to be incorporated with doxycycline hyclate loaded chitosan microspheres. Drug loaded

chitosan microspheres containing doxycycline hyclate equivalent weight to 15 mg were incorporated in glyceryl monooleate-based drug delivery system weight about 150 mg and vigorous shaked by vortext mixer.

6. *In vitro* Release Study of the Doxycycline Hyclate Loaded Chitosan Microspheres in Glyceryl Monooleate-Based Drug Delivery System

6.1 *In vitro* Release Study

The *in vitro* release study was performed using modified Franz diffusion cell which consisted of donor and receiver compartments. The cellophane membrane was placed between two compartments of modified Franz diffusion cell. The membrane was soaked in the medium for 24 hrs before use. The receiving compartment contained 14 ml of phosphate buffer saline buffer pH 6.8 which was maintained at 37±0.5°C by a circulating water jacket. The receptor fluid and membrane were equilibrated to the desired temperature for 1 hr before the release study. After equilibration, 150 mg of the sample was carefully placed into the donor compartment using a micropipette and 1 ml of PBS pH 6.8 was also drop into donor compartment then covered with paraffin to prevent evaporation. The receptor fluid was continuously mixed by magnetic stirring bar at 600 rpm throughout the time of release study. Any air bubbles formed under the preparation had necessarily been removed before the experiment was started. Three specimens of each formulation were examined.

Samples of 5 ml were taken from receiver medium at certain time intervals of 2, 4, 6, 8, 12, 24, 48, 72, 96 and 120 hr via the sampling port of the diffusion cell. The receiver medium was removed by using a syringe and the receiver compartment was replaced with the same amount of medium to keep the constant volume during the experiment. All receiver solutions taken were analyzed for doxycycline hyclate concentration in the medium by using UV spectrophotometer at 268 nm.

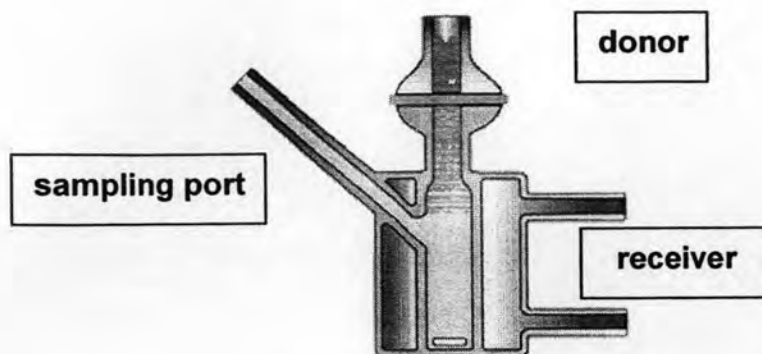

Figure 15 Schematic illustration of the two compartments of Franz diffusion cell

The amount of drug release was calculated by multiplying the drug concentration with the receiver volume. The percentage of drug release was calculated by the equation (5).

$$\%\text{drug release} = (A_t / A_0) \times 100 \quad (5)$$

where A_t is the cumulative released amount of drug at a particular time; A_0 is the initial amount of drug.

6.2 Assay of *In vitro* Release

The determination of amount of doxycycline hyclate released was performed by UV spectrophotometry due to rapidity and convenience. The preparation of standard solutions and validation method were followed as Topic C 1.2.

7. Differential Scanning Calorimetric (DSC) Method

The DSC thermograms were determined by using differential scanning calorimeter. An accurately weighed amount of 3-5 mg of the sample was placed in an aluminum pan and pierced lid. The run was performed at a heating rate of 10°C/min, in the temperature range from -20 to 250 °C under a nitrogen gas atmosphere of 2 ml/min.

8. Determination of *In vitro* Antimicrobial Activity of Glyceryl Monooleate-Based Drug Delivery System Containing Doxycycline Hyclate Loaded Chitosan Microspheres

Staphylococcus aureus is one of the isolates bacteria found in periodontitis patients (Parthasarathy et al., 2002). The activity of the formulations against *Staphylococcus aureus* was evaluated using agar diffusion method. The efficacy was determined by the diameter of inhibition zone. The principle of agar diffusion method was dependent upon the inhibition of the growth of bacteria on the surface of an incubated agar plate, by the antimicrobial agent that diffuses into the surrounding medium.

Agar medium was prepared by boiling Mueller Hinton Broth 6.3 g and agar 4.5 g, respectively, in 300 ml of distilled water until the medium completely dissolved. Afterthat the sterilization was performed by autoclaving at 121 ± 0.5 °C for 15 min. The medium was left overnight to check sterility before use. Each agar plate was prepared by pouring 20 ml of the melting medium at 45-50 °C to Petri dish with a diameter of 10 cm and allowed to solidify at room temperature. The total count agar plates were examined for the viable count of the test organisms.

The inoculum was prepared by picking the isolated colonies of *Staphylococcus aureus* ATCC 6538P into normal saline solution, then the turbidity was compared equivalent to the McFarland No.0.5 standard (1.5×10^8 Cell/ml). If the suspension was too dense, the inoculum was diluted with additional sterile normal saline solution.

A sterile swab was dipped into the suspension and rotated the swab several times with a firm pressure on the inside wall of the tube above the fluid level to remove excess inoculum from the swab. The inoculum was swabbed over the entire agar surface in three different directions to ensure an even distribution of the inoculum. Then 6 mm diameter cylindrical cups were placed on agar about 4-6 cups/plate. Each formulation was carefully filled into the cup, and performed in triplicate. Then the plates were incubated at 37 ± 0.5 °C for 18-24 hr. After incubation, the diameters of the inhibition zones were measured by a Vernier caliper. To verify the effect of the glyceryl monooleate based drug delivery systems on the antimicrobial activity, the sample of

corresponding doxycycline hyclate loaded chitosan microspheres was also studied. The blank glyceryl monooleate-based drug delivery system and phosphate buffer saline pH 6.8 were used as negative controls.