CHAPTER III

MATERIALS AND METHODS

Materials

The following materials were obtained from commercial sources and used as received.

1. Diazepam (Lot No. R1-44/00585, The Government Pharmaceutical Organization, Thailand)
2. Soybean oil (Lot No. 99H0081, Sigma Chemical, USA)
3. Tween 20 (Lot & Control No. 003555, Distributed from Srichand United Dispensary Co., Ltd., Thailand)
4. Tween 80 (Lot No. 807870, Distributed from B.L.Hua & Co., Ltd., Thailand)
5. Glycerin (Lot & Control No. 8-99, Distributed from Srichand United Dispensary Co., Ltd., Thailand)
6. Propylene glycol (Lot & Control No. PL 70/611, Distributed from Srichand United Dispensary Co., Ltd., Thailand)
7. Polyethylene glycol 400 (Lot No. R3-43/00102, The Government Pharmaceutical Organization, Thailand)
8. Water for injection (The Government Pharmaceutical Organization, Thailand)
9. Diazepam injection (Lot No. J440456, The Government Pharmaceutical Organization, Thailand)
10. Furosemide (Lot No. R3-44/00816, The Government Pharmaceutical Organization, Thailand)
11. Potassium dihydrogen phosphate (Batch No. 0071508, Fisher Scientific, UK)
12. Sodium hydroxide pellets (Lot No. 7708, Mallinckrodt, Mexico)
13. Methanol AR grade (Batch No. 01 04 1072, Labscan Asia Co., Ltd., Thailand)
14. Methanol HPLC grade (Batch No. 01 07 0118, Labscan Asia Co., Ltd., Thailand)
15. Isopropyl alcohol HPLC grade (Batch No. 01 03 0168, Labscan Asia Co., Ltd., Thailand)

16. Ultrapure water® equipped with filter system (Balson®, Balson Inc., USA)
17. Nitrogen gas (Supplied by Praxair Co., Ltd., Thailand)
18. Standard buffer solution (Merck, Germany)

Equipment

1. Analytical balance (Model PB 8001, Mettler Toledo, Switzerland)
2. Hot air oven (Model 110, Mammert, USA)
3. Water bath (Model TBVS01, Hetomix and DT Hetotherm, Heto, Denmark)
4. Autoclave (Model 69120 EP, Tuttnauer, USA)
5. pH meter (Model 420A, Orion, USA)
6. UV visible spectrophotometer (Model UV-1601, Shimadzu, Japan)
7. High-performance liquid chromatography (HPLC) instrument equipped with the following
    - Liquid chromatograph pump (LC-10AD, Shimadzu, Japan)
    - UV-VIS detector (SPD-10A, Shimadzu, Japan)
    - Recorder (C-R6A Chromatopac, Shimadzu, Japan)
    - Microsyringe 100 µl (SGE, Australia)
    - C-18 Column (250 x 4.6 mm, 5 µ, Hypersil® BDS, England)
8. Vacuum filtration apparatus with sintered glass fiber No.3 (Waters, USA)
9. Rheology viscometer (Model RI:2:M-H, Rheology (International) Shannon Ltd., Ireland)
10. Refractometer (Model RE40, Mettler Toledo, Switzerland)
11. Transmission Electron Microscopy (Model JEM-200 CX, Jeol®, Japan)
12. Magnetic stirrer (Model 31516 VIRIOMAG® POLY15, H+P Labortechnik GmbH, Germany)
13. Magnetic Stirrer (Heidolph, Germany)
14. Modified Franz Diffusion Cell

Glassware and Miscellaneous

1. 0.45 μm membrane filter (Waters, USA)
2. Dialysis membrane (Lot No. 10B040530, molecular weight cut off 12,000 Dalton, Sigma Chemical, USA)
3. Vial type I glass with rubber cap and aluminum ring (Supplied by APPA Industries Co., Ltd., Thailand)
4. Beaker (Pyrex, USA)
5. Cylinder (Pyrex, USA)
6. Transferring pipette (HBG, Western Germany)
7. Disposable syringe and needle (Terumo, Thailand)
8. Aluminum foil (MMP Packaging, Thailand)
9. Parafilm (American National Can., USA)
10. Buret (Witeg, Germany)

Methods

1. Formulation of microemulsion and construction of phase diagrams

The formulation of microemulsion system composed of tween 20 or tween 80 as surfactant, glycerin, propylene glycol or polyethylene glycol 400 as cosurfactant, soybean oil and water for injection were prepared. Initially, the mixtures of surfactant and cosurfactant were prepared at four weight ratios of tween 20:glycerin, tween 20:propylene glycol, tween 20:polyethylene glycol 400, tween 80:glycerin, tween 80:propylene glycol and tween 80:polyethylene glycol 400 at 1:1.5, 1:1, 1:0.7 and 1:0.5. Required amount of soybean oil was added to the mixture to obtain 15 grams of surfactants:oil weight ratios of 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8 and 1:9. The amount of surfactant, cosurfactant, and oil for surfactant:cosurfactant systems are listed in Tables 2-5. The mixture was mixed thoroughly until homogeneous dispersion was obtained. Then, each mixture was titrated with water for injection. The end point was detected whether the clear solution became turbid or vice versa.

Table 2 Composition of ingredients in oil dispersion of 1:1.5 surfactant:cosurfactant.

Weight ratio of surfactants:oil	Amount (g)		
	Surfactant[a]	Cosurfactant[b]	Soybean oil
9:1	5.40	8.10	1.50
8:2	4.80	7.20	3.00
7:3	4.20	6.30	4.50
6:4	3.60	5.40	6.00
5:5	3.00	4.50	7.50
4:6	2.40	3.60	9.00
3:7	1.80	2.70	10.50
2:8	1.20	1.80	12.00
1:9	0.60	0.90	13.50

[a] = Tween 80 or Tween 20

[b] = Glycerin or Propylene glycol or Polyethylene glycol 400

Table 3 Composition of ingredients in oil dispersion of 1:1 surfactant:cosurfactant.

Weight ratio of surfactants:oil	Amount (g)		
	Surfactant[a]	Cosurfactant[b]	Soybean oil
9:1	6.75	6.75	1.50
8:2	6.00	6.00	3.00
7:3	5.25	5.25	4.50
6:4	4.50	4.50	6.00
5:5	3.75	3.75	7.50
4:6	3.00	3.00	9.00
3:7	2.25	2.25	10.50
2:8	1.50	1.50	12.00
1:9	0.75	0.75	13.50

[a] = Tween 80 or Tween 20

[b] = Glycerin or Propylene glycol or Polyethylene glycol 400

Table 4 Composition of ingredients in oil dispersion of 1:0.7 surfactant:cosurfactant.

Weight ratio of surfactants:oil	Amount (g)		
	Surfactant[a]	Cosurfactant[b]	Soybean oil
9:1	7.94	5.56	1.50
8:2	7.06	4.94	3.00
7:3	6.18	4.32	4.50
6:4	5.29	3.71	6.00
5:5	4.41	3.09	7.50
4:6	3.53	2.47	9.00
3:7	2.65	1.85	10.50
2:8	1.76	1.24	12.00
1:9	0.88	0.62	13.50

[a] = Tween 80 or Tween 20

[b] = Glycerin or Propylene glycol or Polyethylene glycol 400

Table 5 Composition of ingredients in oil dispersion of 1:0.5 surfactant:cosurfactant.

Weight ratio of surfactants:oil	Amount (g)		
	Surfactant[a]	Cosurfactant[b]	Soybean oil
9:1	9.00	4.50	1.50
8:2	8.00	4.00	3.00
7:3	7.00	3.50	4.50
6:4	6.00	3.00	6.00
5:5	5.00	2.50	7.50
4:6	4.00	2.00	9.00
3:7	3.00	1.50	10.50
2:8	2.00	1.00	12.00
1:9	1.00	0.50	13.50

[a] = Tween 80 or Tween 20

[b] = Glycerin or Propylene glycol or Polyethylene glycol 400

The existence of the microemulsion was monitored by the corresponding pseudo-ternary phase diagram with the mixture of the surfactant and cosurfactant on the top of the phase diagram, and the water phase and the oil phase on the bottom left and right corners, respectively. The microemulsion phase was identified as the shade area in the phase diagram where clear, transparent and non-birefringent property was obtained based on visual inspection and polarized light microscopy. The boundary of microemulsion region was determined using the end point from the water titration and confirmed by polarized light microscopy. Due to toxicity and irritation of surfactant at high concentration, the use of surfactant in parenteral injection was limited. Therefore, the microemulsion systems using suitable and allowable amount of surfactant (Kibbe, 2000; Powell et al., 1998) were selected for further study.

2. Preparation of drug-free and drug-loaded microemulsion

To study the effect of type and amount of surfactants and amount of oil on the microemulsion system and limitation of surfactants in parenteral dosage form, microemulsions containing low amount of surfactants from selected microemulsion system were prepared. In drug-free microemulsion, the mixture of oil, surfactant, cosurfactant, and water was mixed until clear and homogeneous. For drug-loaded microemulsion, the amount of diazepam in microemulsion was 5 mg/ml and 10 mg/ml. Diazepam was dissolved in the internal phase (oil) and added with the mixture of surfactant and cosurfactant. Then, the mixture was transferred to the external phase (water) and thoroughly mixed until uniformity was obtained. The obtained microemulsion, with or without drug was filled into 20 ml and 50 ml vials, purged with nitrogen gas for a few seconds before sealing with rubber caps and aluminum rings. The 20 ml vials were for the study of microemulsion type, non-birefringent property, particle size, and pH before autoclaving. The 50 ml vials were sterilized by autoclaving at 121°C, 15 psi for 15 minutes (British Pharmacopoeia Commission, 1993) and were then stood at room temperature for 24 hours prior to further study of pH, viscosity, refractive index, particle size, *in vitro* drug diffusion and stability.

3. Physicochemical characterization

3.1 Determination of microemulsion type

Two different tests were used to determine the type of microemulsions. They were the dilution test and the dye solubility test. The first method, the dilution test, involved the phase separation of the microemulsion after its dilution with soybean oil or water. In this study, microemulsions was diluted with water or oil. Each component was weighed about 2 grams and mixed together. If water was easily dispersed in the external phase, the microemulsion was o/w type. On the other hand, if soybean oil was dispersible in the external phase, the microemulsion was w/o type. The second method, the dye solubility test, was performed by adding a water- or oil-soluble dye to microemulsion. The intense staining of the external phase after the addition of a water-soluble dye (tartrazine) or an oil-soluble dye, (D&C red No.17), indicated an o/w microemulsion or w/o microemulsion, respectively.

3.2 Non-birefringent Property

A microscope with polarized len was employed to examine the preparations and the boundary of microemulsion in the phase diagram at room temperature and to verify the non-birefringent property of microemulsion. A drop of sample was placed between a coverslip and a glass slide and then examined under polarized light by turning polarized len at 90°. The sample that appeared dark would exhibit non-birefringent property. Thus, it would be classified as microemulsion. The sample not showing non-birefringent property would be classified as liquid crystal (Alany, 2001; Schmalfuβ, 1997).

3.3 Refractive Index

For measuring refractive index, a refractometer was used. The instrument was calibrated with deionized water at room temperature before used. The sample was dropped on the prism for 60 seconds before measuring. For measuring, the light source was a light emitting diode whose beam passed through a polarizing filter and

various lenses before it passed through the sapphire prism and encountered the sample. The reflected light was led via a lens to the optical sensor, which the boundary between the dark and light areas represented the critical angle needed to calculated the refractive index. And the refractive index was printed on printer. The triplicate determinations of each sample were measured.

3.4 Viscosity

The viscosity of microemulsion was monitored by a viscometer at room temperature. The instrument was calibrated with tween 80 before used. Each sample was measured using ASTM spindle No.6 for 30 seconds. Triplicate observations of each sample were measured.

3.5 pH

The pH of microemulsions both before and after autoclaving was measured at room temperature using a pH meter. The equipment was calibrated at pH 4 and 7 using standard buffer solution before used.

3.6 Particle size determination

The mean particle diameter of microemulsions with or without diazepam was measured at room temperature by transmission electron microscopy (TEM) following negative staining. A drop of sample was placed on formvar coated copper grid (size 400 mesh) for 15 minutes. Excess sample was adsorbed with filter paper. The sample on grid was then stained with 1% phosphotungstic acid solution for 30 seconds. Excess solution was adsorbed. When the sample was dried, pictures were taken at various magnifications. The particle diameter of each sample both before and after autoclaving was measured from pictures of 300 particles/sample by a program computer. Then, the average particle size of each sample was calculated.

Prior to use, the computerized program was validated. This program and calibrated equipment was performed on three groups of particles. Each group composed of 50 particles. The measurement of particle diameter was based on

Martin's diameter which was the length of a line that bisected the particle image and the line may be drawn in any direction but should be in the same direction for all particles measured (Martin et al., 1993). This program measured the distance between two horizontal on opposite sides of the particle by clicking mouse of computer at the left side and then to the right side of particle, after that the diameter was shown. For calibrated equipment measurement, a calibrated digital vernier was used to measure the diameter of particle. This digital vernier was calibrated by gauge block set which the results of calibration as shown in Appendix C. Similar to a computerized measurement, the calibrated equipment measurement determined the distance between two horizontals on opposite sides of the particles. The results that obtained from computerized program and calibrated digital vernier were tested statistical significant difference by 2-tailed paired-sample T test.

3.7 *In vitro* drug diffusion

In vitro drug diffusion studies of microemulsions were carried out using modified Franz diffusion cell (Gasco, Pattarino et al., 1990; Rhee et al., 2000) as shown in Figure 6. The inner diameter of diffusion cell was 1.70 cm. The diffusion apparatus consisted of donor and receptor compartment. The receptor compartment contained the mixture of 80%v/v phosphate buffer solution pH 7.4 and 20%v/v propylene glycol as release medium and it was calibrated prior to use.

The donor chamber and the receptor compartment were separated by dialysis membrane that had molecular weight cut-off of 12,000 dalton. Before putting up the dialysis membrane onto a diffusion cell, the membrane was soaked in deionized water for 12 hours, and was then rinsed with boiling water to wash off any soluble contaminants. The membrane was then soaked in the release medium and clamped between the donor and the receptor compartments of the cell.

The diffusion medium in the receptor compartment and the membrane in modified Franz diffusion cells were allowed to equilibrate and maintained at temperature of 37± 0.5°C by circulating water through a jacket surround the cell body 30 minutes before studying, and throughout the experiments. After equilibration, 1.0

ml sample as carefully pipetted into the sample compartment, and the cell was covered completely and tightly with Parafilm®. The study was operated continuously for 48 hours by a magnetic stirring bar rotating at 750 rpm. A 10-ml aliquot of receptor medium was withdrawn at appropriate time intervals and replaced immediately with an equal volume of fresh release medium. A portion of solution under test was diluted and then was determined for the amount of drug diffused. The amount of drug diffused was then calculated from calibration curve and corrected for the amount previously withdrawn for assay. The triplicate determination of each sample was measured. Furthermore, diazepam injection was tested for drug diffusion through dialysis membrane for comparison between drug diffusion profiles of solution and o/w microemulsion.

Five models of diffusion kinetics: Zero order model, Higuchi model, Cube root model, Power expression model, and Weibull model were used to elucidate the drug diffusion model. (Costa and Lobo, 2001; Siepmann and Peppas, 2001). The highest coefficient of determination (R^2) was accepted as a model of drug diffusion.

The equations for elucidating the dissolution model were as follows

Zero order model : $Q_t = Q_0 + kt$ (3)

Higuchi model : $Q_t = kt^{1/2}$ (4)

Cube root model : $Q_o^{1/3} - Q_t^{1/3} = kt$ (5)

Power expression model : $Q_t / Q_\infty = kt^n$ (6)

or $\ln Q_t / Q_\infty = \ln k + n \ln t$ (7)

Weibull model : $\log[-\ln(1-Q_t/Q_0)] = b \log t - \log a$ (8)

where Q_t was the amount of drug diffusion at time t;

Q_0 was the initial amount of drug (most times, $Q_0 = 0$);

Q_∞ was the amount of drug penetrated at infinite time (which should be equal to the drug incorporated within the pharmaceutical dosage form at time $t = 0$)

k was the correlation constant; and

n was the power of expression model

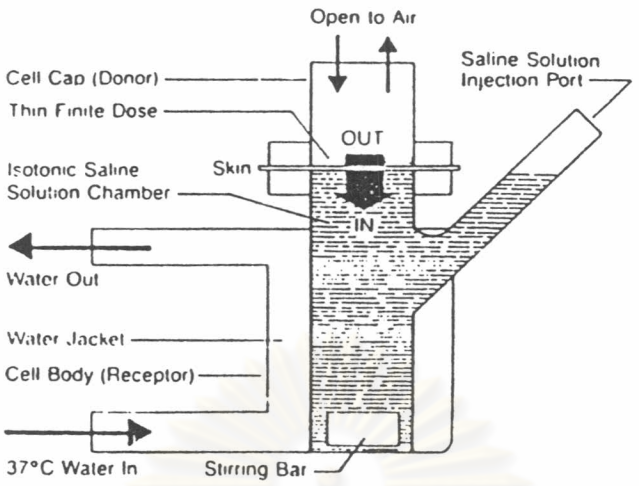

Figure 6 Schematic diagram of the apparatus of the *in vitro* diffusion studies.

3.8 Stability Testing

The microemulsion formulations after being sterilized were also observed under accelerated conditions (heating and cooling cycle) by storing the sample at 4°C for 48 hours and 45°C for 48 hours for 6 cycles (Reiger, 1986). The content of diazepam, pH, particle size, and refractive index of microemulsions were studied. And the sign of instability was also visually investigated.

4. Method for quantitative analysis of drug

4.1 UV-visible assay for diazepam analysis

4.1.1 Calibration curve of diazepam in the mixture of 80% pH 7.4 phosphate buffer solution and 20% propylene glycol

The calibration curve of diazepam in the mixture of 80% v/v phosphate buffer solution pH 7.4 and 20% v/v propylene glycol as release medium was performed to calculate the amount of drug dissolved in drug release testing. Diazepam of 50 mg was accurately weighed into 50 ml volumetric flask. Diazepam was completely dissolved with methanol AR grade. The stock solution was accurately diluted with release medium to the concentration of 2, 3, 4, 5, 6 µg/ml, respectively. The absorbance of standard solutions was performed using a UV visible spectrophotometer at 231 nm. The relationship of diazepam concentration and absorbance was fitted using linear regression.

4.2 HPLC assay for diazepam analysis

The high-pressure liquid chromatography with ultraviolet detector was used to determine the amount of drug in the microemulsion formulations.

Validation characteristics

4.2.1 Specificity

Under the chromatographic condition used, the peak of diazepam had to be completely separated from the peaks of other components in the sample. Diazepam, tween 20, tween 80, glycerin, propylene glycol, polyethylene glycol 400, soybean oil and furosemide were determined.

4.2.2 Accuracy

Three sets of the standard solutions of diazepam having concentrations of 5-25 µg/ml were prepared and injected. The percentage of analytical recovery of each standard solution was calculated.

4.2.3 Precision

a) Within run precision

The within run precision was determined by analyzing three sets of the five standard solutions of diazepam in the same day. Peak area ratios of diazepam to furosemide were compared and the percentage coefficient of variation (% CV) for each concentration was determined

b) Between run precision

The between run precision was determined by comparing each concentration of diazepam standard solutions prepared and injected on different days. The percentage coefficient (% CV) of diazepam to furosemide peak area ratios from three sets of standard solutions having the same concentration was determined.

4.2.4 Linearity

Linearity was determined by calculating a regression line by method of least squares of peak area ratios of diazepam to furosemide and concentrations of diazepam in sample. The slope, intercept and coefficient of variation were performed.

System suitability

System suitability tests were used to verify that the resolution and reproducibility of the chromatographic system were adequate for analysis to be done.

4.2.5 Resolution

The resolution was a function of column efficiency and was specified to ensure that diazepam was resolved from furosemide, respectively. The resolution, R, was determined by the following equation.

$$R = \frac{2\,(t_2 - t_1)}{W_2 + W_1}$$

in which t_2 and t_1 were the retention times of diazepam and furosemide. W_2 and W_1 were the corresponding widths at the bases of the peaks obtained by extrapolating the relatively straight sides of the peak to the baseline, as shown in Figure 7.

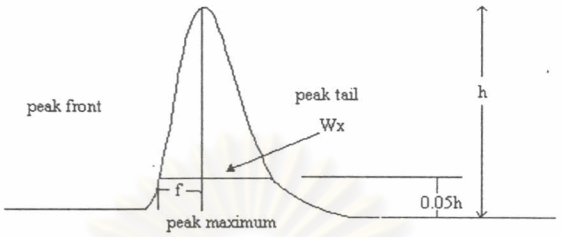

Figure 7 Asymmetrical chromatographic peak.

4.2.6 Tailing factor

Tailing factor was performed by collecting data from injection standard curve. This test was determined by the following equation.

$$T = \frac{W_x}{2f}$$

in which W_x was the width of peak of diazepam or furosemide at 5 % height, f was the distance from the peak maximum to the leading edge of the peak, the distance being measured at a point 5% of the peak height from the baseline as Figure 7.

4.2.7 Calibration curve of diazepam ranging 5-25 µg/ml

Furosemide as an internal standard, of 25 mg was accurately weighed into 100 ml volumetric flask. Furosemide was completely dissolved with mobile phase. A 10 ml of this stock solution was diluted with mobile phase to 50 µg/ml. Diazepam of 25 mg was accurately weighed into 50 ml volumetric flask. Diazepam was completely dissolved with mobile phase. Stock solution of diazepam was diluted to 5, 10, 15, 20

and 25 μg/ml, respectively. And furosemide's stock solution was mixed to 5 μg/ml into each concentration of diazeapm, mobile phase was then added to adjust volume. The equation was calculated from the relationship between peak area ratios of diazepam to furosemide and diazepam concentration.

HPLC conditions

The procedure was developed as follows

Column	: Hypersil BDS® (250 x 4.6 mm, 5 μ)
Mobile phase	: 40% isopropyl alcohol : 50 % methanol : 10 % H_2O was freshly prepared and filtered through a 0.45 μm membrane filter, and was then degassed by sonication about 30 minutes.
Flow rate	: 0.8 ml/minute
Detector wavelength	: 320 nm
Injection volume	: 70 μl
Internal standard	: Furosemide
Attenuation	: 16