CHAPTER III
MATERIALS AND METHODS

Materials

The following materials were obtained from commercial sources and used as received. Deionized water (from Ultrapure Water®) which filtered through a 0.22-μm membrane filter was used throughout these experiments.

1.Active ingredients

1.1 Diltiazem hydrochloride USP23 (Siam Pharmaceutical Co., Ltd., Bangkok, Batch No. 0690798)

1.2 Ibuprofen BP1988 (Shasun Chemicals (M) Ltd., Singapore, Lot No. L9012197)

1.3 Piroxicam USP1990 (S. Tong Chemicals Co., Ltd., Bangkok, Lot No. 931052)

1.4 Theophylline anhydrous (B. L. Hua & Co., Ltd., Bangkok, Lot No. 951203)

2.Lipids

2.1 Soybean oil (Thai Vegetable Oil, Co., Ltd., Bangkok, Mfg. Date 17/8/39)

2.2 Stearic acid (Srichand United Dispensary Co., Ltd., Bangkok, Lot No. ACL25)

2.3 Trimyristin (C-14 triglyceride) (Fluka Chemika, Switzerland, Lot No. RA15826)

purity ≥ 97% trimyristin assay by gas chromatography

2.4 Tripalmitin (C-16 triglyceride) (Fluka Chemika, Switzerland, Lot No. RA10197)

purity ~ 95% tripalmitin assay by gas chromatography

~ 55% tripalmitin assay by thin layer chromatography

2.5 Tristearin (C-18 triglyceride) (Fluka Chemika, Switzerland, Lot No. RA3477)

purity ~ 65% tristearin mixed with 30% tripalmitin assay by gas chromatography

3.Stabilizers

3.1 Egg lecithin (Merck, Germany, Lot No. 244G465231)

3.2 Poloxamer 407 (Lutrol® F127) (BASF, Germany, Lot No. 67-0754)

3.3 Tween 80 (Polyoxyethylene sorbitan mono-oleate) (Srichand United Dispensary Co., Ltd., Bangkok, Lot No. TGD15)

4.Analytical substances

4.1 Dialysis membrane (Sigma, USA, Lot No. 28H0141)

4.2 Mefenamic acid (Sigma, USA, Lot No. 29F0571)

4.3 Methanol HPLC grade (BDH laboratory Supplies, England, Lot No. L848602)

4.4 Monobasic potassium phosphate (Ajax Chemicals, Australia, Lot No. 612612)

4.5 Ortho-phosphoric acid 85% (Ajax Chemicals, Australia, Lot No. 504431)

4.6 Potassium bromide (Fluka Chemika, Switzerland, Lot No. G07029)

4.7 Sodium chloride BP1973 (Merck, Germany, Lot No. SEH04/33)

4.8 Sodium hydroxide (Mallinckrodt, Mexico, Lot No. 7708MVHT)

Equipments

1. Analytical balance (Model A200S, Satorius analytic, Germany)
2. Autoclave (Hirayama Mfg. Corp., Japan)
3. Balance (Model PB3002, Mettler toledo, Switzerland)
4. Cryoscopic osmometer (Model Osmomat® O30-D, Gonotec, Germany)
5. Differential scanning calorimeter (Model DSC7, Perkin Elmer, Germany)
6. Fourier transform infrared spectrophotometer (FT-IR Spectrometer®) (Model Spectrum 2000, Perkin Elmer, Germany)
7. High-performance liquid chromatography (HPLC) instruments equipped with the following:
   - a constant flow pump (Model 600E, Waters, USA)
   - a tunable absorbance detector (Model 484, Waters, USA)
   - an auto-injector (Model 712 WISP, Waters, USA)
   - an integrator (Model 746 Data Module, Waters, USA)
   - C18 column (Spherisorb® S10 ODS2 4.6 x 250 mm, Waters, USA)
8. High pressure homogenizer (Model EmulsiFlex® C5, Avestin, Canada)
9. High speed homogenizer (Model D-7801, Ystral, Germany)
10. Magnetic stirrer (Model MR3001, Heidolph, Germany)
11. Modified Keshary-Chien diffusion apparatus
12. Particle size analyzer (Mastersizer® S long bed Ver.2.11, Malvern Instruments Ltd., Malvern, UK)
13. pH meter (Model Φ50pH, Beckman, USA)
14. Scanning electron microscopy (Model JSM-5410LV, JOEL®, Japan
15. Shaker (Model TBVS Hetomix® and DT Hetotherm®, Heto, Denmark)
16. Transsonic digital (Ultrasound ELMA® Model T900, Elma, Germany)
17. Ultracentrifuge® (Model L80, Beckman, USA)
18. Ultrapure Water® equipped with filter system (Balston®, Balston Inc., USA), Boost pump, Option 3 water purifier, Maximum ultrapure water, and Reservoir (ELGA, USA)
19. UV/visible recording spectrophotometer (Model UV-Vis 160A, Shimadsu, Japan)

20. Viscometer, Haake Rotovisco® RV20 equipped with Haake Rheo controller® RC20 and computerized system (Haake Mess-Thchnik GmbH, West Germany)
21. X-ray diffraction spectroscopy (JDX-3530 Diffractometer system, JOEM, Tokyo, Japan)
22. Zeta meter® 3.0+ (Model ZM 3UG, Zeta Meter Inc., USA)

Methods

1.Preliminary study

In this study, SLN were prepared by hot melt homogenization method. EmulsiFlex® C5 was used to reduce the particle size of the fat emulsion in the micro- or nanometer size range. In preliminary study, the efficiency of EmulsiFlex® C5 was investigated. The influence of pressure and number of cycle of homogenization on the particle size of fat emulsion was examined. The following formula was the example of the emulsion used in this preliminary study:

R_x	soybean oil		7.5	g
	poloxamer 407		3.0	g
	deionized water	qs	150.0	g.

The emulsion was produced by dissolving poloxamer 407 in aqueous phase. The aqueous and oily phases were separately heated to 75°C. The oily phase was added into the aqueous phase and homogenized for 5, 10, 20, and 30 minutes at 75°C using a high speed homogenizer at 4,080 rpm. Oil droplets on the surface of emulsions were investigated to select the appropriate mixing time. The primary emulsion was then passed through a high pressure homogenizer at a pressure between 4000–12000 psi and between 1–10 homogenization cycles.

To investigate the efficiency of EmulsiFlex® C5, 100 µl of the emulsion was diluted with 50 ml of water, and the percentage of transmittance was measured at 480 nm by spectrophotometry (Apte and Turco, 1992). This detected beam was in the blue region of the spectrum which the intensity was high (Ingle and Crouch, 1988). The statistical analysis was undertaken using the ANOVA test with a SPSS® software programme to evaluate the optimum pressure and number of cycle of homogenization.

2. Preparation of SLN

The SLN was produced by the conditions similar to the preliminary study. The oily phase was added into the aqueous phase and homogenized by a high speed homogenizer at 4,080 rpm using the appropriate mixing time. The coarse emulsion was passed through a high pressure homogenizer. The number of homogenization cycle and homogenization pressure were chosen from the preliminary study. The same conditions were used in all preparations. The obtained homogenization product was an o/w emulsion of melted lipid in the aqueous surfactant solution. This emulsion was divided into 10-ml and 100-ml vials, and was then sealed with rubber and aluminum cap. The preparations were sterilized by autoclaving using standard condition (15 minutes at 121°C, 15 psi) (British Pharmacopoeia Commission, 1993) and were then allowed to stand at room temperature. The oil droplets solidified during cooling and formed SLN.

To study the influence of types and amounts of stabilizer, lipid, and drug on the characteristics of SLN, 1–5 %w/w egg lecithin (EL), tween 80 (T80) and poloxamer 407 (P407) were used as stabilizers in the preparations of SLN. Trimyristin (TM), tripalmitin (TP), tristearin (TS), and stearic acid (SA) of 3-7% were used as the lipids. And three groups of drug that have different solubility properties were loaded into SLN. Diltiazem hydrochloride was a model of very water soluble drug, theophylline was a model of slightly water soluble drug, piroxicam and ibuprofen were models of water insoluble drugs. Their physicochemical properties were investigated.

To study the effect of pH of the dispersion medium on the entrapment efficiency, pH 7 phosphate buffer was used instead of water for preparing the preparations. After autoclaved, the percentage of drug entrapment was investigated.

Method to prepare pH 7 phosphate buffer

A 50 ml portion of 0.2 M monobasic potassium phosphate solution was pipetted into 200-ml volumetric flask. Then, 29.1 ml of 0.2 M sodium hydroxide was mixed and adjusted to volume with water.

3. Physicochemical characterizations of SLN

After preparation and sterilization, the preparations were cooled to room temperature. Then their physicochemical characteristics were studied within 5 days for all preparations.

3.1 size determination

A laser particle sizer (with 300RF mm range lens, 2.40 mm beam length) was used to determine the particle size of SLN. The sample was diluted with water. Particle size distribution was analyzed by the curve plotted between particle diameter versus percentage volume of particles. Cumulative frequency of volume diameter was calculated, and the surface weighted mean diameter (d(4,3)), the volume weighted mean diameter (d(3,2)), diameter of particles of 10%, 50%, and 90% volume percentile (d(v,0.1), d(v,0.5), d(v,0.9), respectively) were determined. Percentage of the large particles was calculated for considering the injection possibility of preparations.

3.2 Zeta potential

The zeta potential of dispersions of SLN was determined by microelectrophoresis using the Zeta meter® system 3.0+ with a fused quartz and teflon electrophoresis cell equipped with a cylindrical molybdenum anode and

platinum rod cathode. A 200-µl sample was diluted in 50-ml water. Before beginning to track the particles, the specific conductance of each sample was measured for selecting an appropriate voltage, which did not cause thermal overtone. About 100 particles were tracked for each sample. The zeta potential was automatically calculated by the Zeta meter® system 3.0+. These results were all normalized with respect to zeta potential of –49 millivolts for Minusil® standard solution. The measurements were made at room temperature (25°C) (Quintanar-Guerrero et al., 1998; Muslin Limpanasitthikul, 1991).

3.3 Osmolality

The osmolality of dispersions of SLN was determined using freezing point depression method. Osmomat® O30-D was calibrated using deionized water before used. The triplicate observations of each sample were measured.

3.4 pH

The pH of dispersions of SLN was measured at room temperature using a pH meter. The equipment was calibrated at pH 4 and 7 using Fisher standard buffer before used.

3.5 Viscosity

Haake viscometer® was used to determine the viscosity of dispersions of SLN at room temperature. The sample was loaded and the shear rate was increased from 0 s^{-1} to 1000 s^{-1} in two minutes. The shear rate was maintained at 1000 s^{-1} for one minute. The last step was two minutes to decrease the shear rate back to 0 s^{-1}. The complete cycle took five minutes to shear the sample. Shear stress values from 50 different points of triplicate observations were used to plot a rheogram of each sample. The viscosity at shear rate of 1000 s^{-1} was used to compare in each preparation.

3.6 Scanning electron microscopy

Particle shape and size of SLN were studied by a scanning electron microscope. The specimen was obtained by cryo-fixation technique. Dispersion of SLN was cryo-fixed under standard conditions, using Balzer-type specimen support plates immersed in liquid nitrogen (-140°C) (Quintanar-Guerrero et al., 1998). The sample was immediately observed by the scanning electron microscope under the temperature of –140°C by circulating liquid nitrogen through a jacket surrounding the instrument.

3.7 Infrared Spectroscopy.

Fourier transform infrared spectrophotometry (FT-IR) was used to study the change in the functional groups of waxes, drugs, and solid lipid products after centrifugation and drying in desiccator by observing the positions and intensities of IR spectra. This method was used to identify the entrapment of drugs into solid lipid particles and their compatibility.

The IR spectra of waxes, drugs, and stabilizers were examined using the potassium bromide disc method, except tween 80 that used mulling method. The sample was diluted with potassium bromide and ground with mortar and pestle. The mixture powder was then pressed to obtain a transparent disc of sample on the window. The sample disc was determined by FT-IR Spectrometer (Model Spectrum 2000, Perkin Elmer, Germany) in the wavenumbers of 400-4000 cm^{-1}. For tween 80, the sample was smeared on the sodium chloride disc and determined in the same process.

3.8 Differential Scanning Calorimetry.

Differential scanning calorimetry was used to determine the thermograms of waxes, drugs, and solid lipid products after centrifugation and drying in desiccator. The differences in thermal energy patterns between the original

substances and their products were evaluated. This method was used to study interaction between two or more components after preparing.

About 5-10 mg of each sample was accurately weighed into the DSC pan. Then it was crimped with the hermetically sealed pan and was placed in the equipment beside the reference pan made by the same method except without powder. The thermal runs were controlled at a heating rate of 10°C per minute and in the range of –30 °C to 250°C using liquid nitrogen to cool in lower room temperature step.

3.9 Powder X-ray Diffractometry.

Powder X-ray diffractometry was used to determine the diffraction angles of the substances which showed crystallinity and interplannar spacing of the crystal plans. This method was used to study the change of crystallinity of waxes and drugs after preparing which could explain some physicochemical properties of SLN.

The crystallinity of waxes ,drugs, and solid lipid products after centrifugation and drying in desiccator was examined by powder X-ray diffractometer. Each sample powder was packed into a thin rectangular quartz slide. After firmly packed by the other cover slide, the sample-packed slide was exposed to the X-ray beam in the X-ray diffraction chamber. The X-ray diffraction pattern was recorded at the speed of 2.4° per minute from 3° to 60° 2-θ angle.

3.10 In vitro drug release

The *in vitro* drug release studies of SLN were carried out using modified Keshary-Chien diffusion apparatus consisting of the donor and the receptor compartments (see Figure 7) (Banakar, 1992b; Huang, 1987). The internal diameter of each cell was 1.5 cm, corresponding to an effective permeable surface area of 1.77

cm^2. The receptor compartment contained 11.98-15.04 ml of 0.9% sodium chloride solution as release medium.

The donor chamber and the receptor compartment were separated with a dialysis membrane that had a molecular weight cut-off of 12,000–14,000 dalton. The dialysis membrane was cut into a circular shape with a diameter of 3 cm. Before putting up the circular dialysis membrane onto a diffusion cell, the membrane was soaked in deionized water 12 hours, and was then rinsed with boiling water to wash off any water soluble contaminants. The membrane was then soaked in the release medium and clamped between the donor and the receptor compartments of the cell.

The release medium in the receptor compartment and the membrane in Keshary-Chien diffusion cells were allowed to equilibrate and maintain at temperature of 37±0.5°C by circulating water through a jacket surrounding the cell body 1 hour before studying, and throughout the experiments. After equilibration, 1-ml sample was carefully pipetted into the sample compartment, and the cell was then covered completely and tightly with Parafilm®. The study was operated continuously for 24 hours by a magnetic stirring bar rotating at 750 rpm (except ibuprofen preparation that was operated for 168 hours). 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 the solution under test was diluted and then was determined for the amount of drug dissolved. The amount of drug released was then calculated from calibration curve and corrected for the amount previously withdrawn for assay. The triplicate determinations of each of sample were measured.

Saturated solution of each drug was initially tested for drug diffusion through dialysis membrane for comparison between drug release profiles of solution and dispersions containing SLN. The supernatant of each preparation was also assayed for drug release in compensating the release of drug outside the lipid particles.

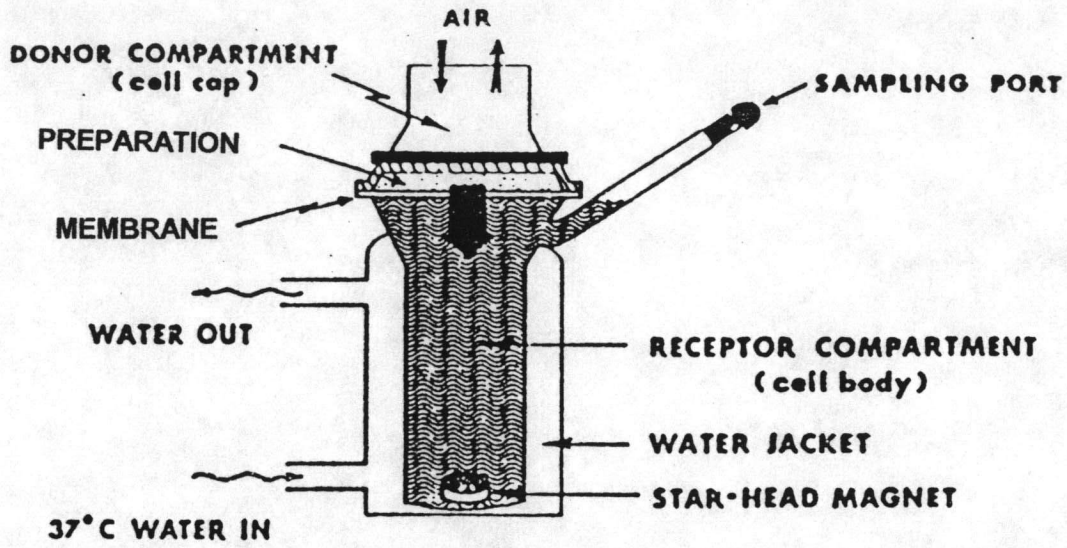

Figure 7. A schematic illustration of Keshary-Chien diffusion apparatus (from Banakar, 1992b: 335).

3.8 The entrapment efficiency

The amount of drug encapsulated per unit volume of SLN was determined after centrifugation of the SLN to separate the solid lipid from the aqueous medium. This was carried out using the Ultracentrifuge® at 60,000 rpm for 6 hours. The entrapment efficiency of the drug was determined indirectly by measuring the concentration of the free drug remaining in the supernatant after centrifugation (Almeida et al., 1997). The triplicate observations of each of sample were measured.

The entrapment efficiency was calculated by the following equation:

$$\%entrapment = \frac{([D]_{total} - [D]_{water})}{[D]_{total}} \times 100 \qquad (2)$$

Where: $[D]_{total}$ = theoretical drug content per 1 ml of preparation

$[D]_{water}$ = practical drug content per 0.95 ml of supernatant (minus 5%w/w of lipid in preparation, assuming density of lipid was similar to that of water).

3.12 Physical stability

The preparations of SLN after autoclaving were kept at room temperature and protected from light. Their particle sizes were determined for 6 months and 12 months after preparing. The flocculation, coalescence, aggregation or separation was evaluated.

4. Reproducibility

The best preparation that had good properties and possibility to develop into the long acting parenteral dosage forms was chosen to study the reproducibility. This preparation was prepared 3 times by the same condition. Their physicochemical properties were investigated.

Methods for quantitative analysis of drugs

1. UV-Visible assay for diltiazem hydrochloride analysis

1.1 Preparation of calibration curve of diltiazem hydrochloride in water and 0.9% sodium chloride solution.

Standard diltiazem hydrochloride of 200 mg was accurately weighed into 100-ml volumetric flask. The drug was dissolved and adjusted to volume with water (or 0.9% sodium chloride solution). This stock standard solution was appropriately diluted with the same medium to obtain the final standard solutions which had the concentrations of 2, 4, 6, 8, 10, 12, and 16 μg/ml, respectively.

The absorbance of the standard solutions was determined by the UV/visible spectrophotometer at 237 nm, which was the λ_{max} of diltiazem hydrochloride of both mediums. The relationship between absorbances and concentrations of diltiazem hydrochloride was fitted using linear regression analysis. The calibration curve was done in triplicate.

1.2 Analysis of diltiazem hydrochloride content in solution

A portion of the solution under drug solubility testing was appropriately diluted with the same medium and was then determined by the UV/visible spectrophotometer at 237 nm. The amount of drug solubility was then calculated from calibration curve and multiplied with dilution factors.

A portion of the solution under drug release testing was diluted appropriately with 0.9% sodium chloride solution and was then determined by the UV/ visible spectrophotometer at 237 nm. The amount of drug released was then calculated from calibration curve, multiplied with dilution factors, and corrected for the amount previously withdrawn for assay.

A supernatant of diltiazem hydrochloride SLN was diluted appropriately with water and was then determined by the UV/visible spectrophoto meter at 237 nm. The amount of drug in the supernatant was then calculated from calibration curve and multiplied with dilution factors.

2. UV-Visible assay for theophylline analysis

2.1 Preparation of calibration curve of theophylline in water and 0.9% sodium chloride solution.

Standard theophylline of 200 mg was accurately weighed into 100-ml volumetric flask. The drug was dissolved and adjusted to volume with water (or 0.9% sodium chloride solution). This stock standard solution was appropriately

diluted with the same medium to obtain the final standard solutions which had the concentrations of 2, 4, 6, 8, 10, 12, and 16 μg/ml, respectively.

The absorbance of the standard solutions was determined by the UV/ visible spectrophotometer at 272 nm, which was the λ_{max} of theophylline of both mediums. The relationship between absorbances and concentrations of theophylline was fitted using linear regression analysis. The calibration curve was done in triplicate.

2.2 Analysis of theophylline content in solution

A portion of the solution under drug solubility testing was appropriately diluted with the same medium and was then determined by the UV/visible spectrophotometer at 272 nm. The amount of drug solubility was then calculated from calibration curve and multiplied with dilution factors.

A portion of the solution under drug release testing was diluted appropriately with 0.9% sodium chloride solution and was then determined by the UV/ visible spectrophotometer at 272 nm. The amount of drug released was then calculated from calibration curve, multiplied with dilution factors, and corrected for the amount previously withdrawn for assay.

A supernatant of theophylline SLN was diluted appropriately with water and was then determined by the UV/visible spectrophotometer at 272 nm. The amount of drug in the supernatant was then calculated from calibration curve and multiplied with dilution factors.

3. UV-Visible assay for piroxicam analysis

3.1 Preparation of calibration curve of piroxicam in water and 0.9% sodium chloride solution.

Standard piroxicam of 200 mg was accurately weighed into 100-ml volumetric flask. The drug was dissolved with methanol. This stock standard solution was appropriately diluted with water (or 0.9% sodium chloride solution) to obtain the final standard solutions which had the concentrations of 2, 4, 6, 8, 10, 12, 16, and 20 μg/ml, respectively.

The absorbance of the standard solutions was determined by the UV/ visible spectrophotometer at 360 nm, which was the λ_{max} of piroxicam of both mediums. The relationship between absorbances and concentrations of piroxicam was fitted using linear regression analysis. The calibration curve was done in triplicate.

3.2 Analysis of piroxicam content in solution

A portion of the solution under drug solubility testing was appropriately diluted with the same medium and was then determined by the UV/visible spectrophotometer at 360 nm. The amount of drug solubility was then calculated from calibration curve and multiplied with dilution factors.

4. HPLC assay for ibuprofen analysis

The high-pressure liquid chromatography with ultraviolet detection technique was used for analysis of ibuprofen. This method could detect ibuprofen quantity below 1 μg/ml (Albert et al., 1984).

4.1 HPLC conditions

The determination of ibuprofen by reverse phase HPLC assay with UV detection was modified from the method described by Wannapa Thamasucharit (1988: 22-24). The procedure was developed as follows:

Column	: Waters Spherisorb® S10 ODS2 (4.6 x 250 mm)	
Mobile phase	: Methanol : 0.1% ortho-phosphoric acid (75:25) was prepared freshly and filtered through a 0.45 µm membrane filter. It was then degassed by sonication for about 1 hour prior to use.	
Flow rate	: 1.5 ml/minute	
Detector wavelength	: 220 nm	
Injection volume	: 100 µl	
Internal standard	: mefenamic acid	
Temperature	: ambient	
Pressure	: 1300-1500 psi	
Attenuation	: 64	
Chart speed	: 0.25 cm/minute	
Retention time	: ibuprofen	6.29 – 6.37 minutes
	mefenamic acid	9.33 – 9.52 minutes

4.2 Validation for the quantitative determination of ibuprofen

The typical analytical parameters that should be considered in the validation of the quantitative determination of ibuprofen by HPLC method were as follows: (United States Pharmacopeial Convention, 1995)

4.2.1 Specificity

The specificity of the HPLC method used to determine ibuprofen quantity was evaluated by comparing the chromatograms of ibuprofen and

the peaks of other components in the SLN systems. The blank preparation that had the same component as ibuprofen SLN was prepared and separated by ultracentrifuge, and the supernatant was determined. The peaks of other components in the sample must not interfere with the peak of ibuprofen.

4.2.2 Precision

4.2.2.1 Within run precision

The within run precision was evaluated by analyzing peak area ratios of drug and internal standard of three repetitions of each concentration determined in the same day. The mean, standard deviation (SD) and percentage coefficient of variation (%CV) of each concentration were determined.

4.2.2.2 Between run precision

The between run precision was evaluated by analyzing peak area ratios of drug and internal standard of three repetitions of each concentration injected on different days. The mean, standard deviation and percentage coefficient of variation of each concentration were determined.

4.2.3 Accuracy

The accuracy of ibuprofen assay was evaluated by analyzing percent recoveries of each concentration of ibuprofen solution. Percent recovery of each injection was calculated by comparing the concentration fitted from a calibration curve with the known concentration.

4.2.4 Linearity

The linearity was evaluated by plotting the standard curve between the peak area ratios of ibuprofen to internal standard and the

concentrations of ibuprofen. Linear regression analysis was performed. The equation and the coefficient of determination (R^2) were calculated.

4.3 Preparation of standard solutions

A stock solution of internal standard was prepared by completely dissolving 150 mg of mefenamic acid in a 50-ml volumetric flask and diluted to volume with methanol. A 1 ml aliquot of this solution was diluted to 500 ml with methanol and used as the internal standard solution.

About 250 mg of ibuprofen was accurately weighed into a 50-ml volumetric flask, the drug was dissolved and diluted with methanol. Nine appropriate dilutions were then made with 0.9% sodium chloride solution to obtain standard solutions with various concentrations ranging from 0.5 to 25 μg/ml.

A 2 ml portion of standard or sample solution was mixed with 1 ml of the internal standard solution. This solution was assayed by HPLC method. The ratio of the peak area of ibuprofen to that of the internal standard was used to calculated the ibuprofen concentration.

4.4 Analysis of ibuprofen content in solution

A portion of the solution under drug solubility testing was appropriately diluted with the same medium and was then determined by HPLC at 220 nm. The amount of drug solubility was calculated from calibration curve and multiplied with dilution factors.

A portion of the solution under drug release testing and a supernatant of ibuprofen SLN were appropriately diluted with 0.9% sodium chloride solution and water, respectively, and was then determined by HPLC at 220 nm. The amount of drug released was calculated from calibration curve, multiplied with dilution factors, and corrected for the amount previously withdrawn for assay.