CHARPTER III

EXPERIMENTAL

Materials

All materials employed in this study were obtained from commercial sources and as received.

- Acetronitrile HPLC grade (Fisher Scientific, UK)
- Asiatic acid (Batch No.AA0303610, Changzhou Natural Products Development Co., Ltd., China)
- Asiaticoside (Batch No.AS0303610, Changzhou Natural Products Development Co., Ltd., China)
- Dimethylsulfoxide AR grade (Fisher Scientific, UK)
- Dipotassium hydrogen ortho-phosphate (Lot no.F0B063 APS Chemical Limited, Australia)
- Disodium hydrogen ortho-phosphate dodecahydrate (Lot no.F2G069, APS Chemical Limited, Australia)
- Duulbecco's modified Eagle's medium (Lot no. 317807 GIBTHAI)
- Fetal bovine serum (Lot no. 41F7063K GIBTHAI)
- Glyceryl behenate (Compritol® ATO 888) (Lot no.102699, Gattefosse, France)
- Isopropyl alcohol (Srichand United Dispensary Co., Ltd Thailand)
- Lauroyl polyoxylglycerides (Gelucire® 44/14) (Lot no. 25638, Gattefosse, France)

- Lecithin 40 (Lot no. 1198941Biochemica Sigma-Aldrich)
- Methanol HPLC grade (Fisher Scientific, UK)
- Methyl ethyl ketone AR grade (Fisher Scientific, UK)
- Ortho phosphoric acid (Lot no. AA55024 Ajax Fnenchem Australia)
- Phosphate buffer saline (Lot no. 1380926 GIBTHAI)
- Phospholipon® 90H (Lot no. 70060, Natterman Phospholipid GmBH, Germany)
- Poloxamer 188 (Lutrol® F 68) (Lot no. 60-04, BASF, Germany)
- Potassium chloride (Lot no. F1G253, APS Chemical Limited, Australia)
- Potassium dihydrogen ortho-phosphate (Lot no. F1F125, APS Chemical Limited, Australia)
- Sodium Chloride (Lot no. F2C273, APS Chemical Limited, Australia)
- Thiazoyl blue tetrazolium bromide (sigma-Aldrich)
- Trypsin EDTA 0.25% (Lot no. 13199559 GIBTHAI)
- Tween 80 (Lot no. 507864, distributed Srichand United dispensary Co., Ltd., Thailand)

Equipment

- Analytical balance (Satorius, A200S, Germany)
- Cover slip
- Flow-cytometer
- Gas chromatography

- High performance liquid chromatography (HPLC) (Model SCL-10A VP, Shimadzu, Japan)
  - -Degasser (Model DGU-14A, Shimadzu, Japan)
  - -Pumb A, B liquid chromatography (Model LC-10AD, Shimadzu, Japan)
  - -Auto injector (Model SIL-10A, Shimadzu, Japan)
  - -Column oven (Model CTD-10AS, Shimadzu, Japan)
  - -UV-VIS detector (Model SPD-10A, Shimadzu, Japan)
  - -System controller (Model SCL-10A, Shimadzu, Japan)
- High pressure homogenizer (Model Emulsiflex C5®, Avestin, Canada
- High speed homogenizer
- Hood larminar flow
- Hot air oven (Model B7600, Mammert, USA)
- Incubator
- Invert micoscope
- Magnetic stirrer (Variomay multipoint, Komet, Taiwan)
- Micro plate reader
- Nanosizer (Nano ZS, Malvern, UK)
- pH meter (Model 210A+, Thermo Orion, Germany)
- Rotary evaporator
- Transmission electron microscope (Model JSM-5410LV, JOEL, Japan)
- Ultrasonic bath (Transsonic digitals, Elma®, Germany)

- Vacuum filtration apparatus with sinter glass fiber No.3 (Waters, USA)
- Water bath (ITS Co., Ltd., Thailand)

Glassware and Miscellaneous

- 0.22 cellulose acetate membrane filter (Waters, USA)
- 0.45 nylon membrane filter (Waters, USA)
- Aluminium foil (MMP Packing, Thailand)
- Beaker (Pyrex, USA)
- Cylinder (Pyrex, USA)
- Disposable syringe and needle (Terumo, Thailand)
- Micropipette and disposable pipette tip (Socorex, Switzerland)
- Osmolality vessel (Gonotec, Germany)
- Parafilm (American National Can., USA)
- Transfering pipette (Witeg, Germany)
- Volumetric flask (Pyrex, USA)

Methods

1. Formulations of solid lipid nanoparticles (SLN)

There are different approaches for the production of solid lipid dispersions. In this study, solid lipid nanoparticles (SLN) prepared by solvent emulsifition-diffusion and high pressure homogenization methods (HPH) were selected with regarding to the scaling up potential.

Formulation of solid lipid nanoparticle (SLN) by solvent emulsification diffusion method

SLN were prepared by solvent emulsification-diffusion method. It was noteworthy that all formulations of SLN in the present study were prepared in % w/v. The ingredients used in the formulations were 5% Gelucire 44/14(GC) and Compritol 888 ATO (GB) as solid lipids with 5-15% of Tween-80(T-80), Poloxamer 188 (P-188), Phospholipon 90 (PL-90) and Phospholipon 40 (PL-40) as stabilizers and are shown in Table 7.

In order to determine the solubility of each lipid in the solvent, a qualitative trial was performed. The partially water miscible solvent was saturated with water for five minutes. Approximately 150 mg of lipid were added to 3 ml of saturated solvent (5%w/v). The sample were sealed and stirred for 12 hours. The ability of the solvent to dissolve the lipid was considered when the content appeared transparent on visual observation. In case of solid lipid insolubility (GB), heating at 80 °C was required to achieve complete lipid dissolution.

Table 7. The composition of SLN prepared by solvent method

Chemicals	Concentration (%w/v)
Lipid	5
Stabilizer	5-15
Water for injection	to 100

Typically, 1000 mg (5%w/v) of lipid was dissolved in 20 ml of water saturated solvent. This organic phase (internal phase) was emulsified with 40 ml of solvent saturated aqueous solution containing 5-15 %w/v of stabilizer (dispersion medium) using a magnetic stirrer for 15 minutes. After the formation of an oil-in-water emulsion, 160 ml of water (dilution medium) were added to the system in order to allow solvent diffusion into continuous phase.

A high speed homogenizer was used to mix the emulsion at the speed of 12000 rpm for 5 minutes thus causing the aggregation of the lipid into nanoparticles. In case of GB, both phases were maintained at 80 °C and the diffusion step was performed at the same temperature. Finally, the solvent was eliminated by vacuum distillation at 40 °C and 70 mm Hg until 150 ml of final volume was obtained. A portion of each preparation was evaluated for the particle size, zeta potential, pH and osmolality.

The rest of preparations were sterilized by autoclaving at 121 °C, 15 minutes and were then allowed to stand at room temperature. The oil droplets solidified during cooling and formed SLN. The formulation after autoclaving was also determined for particles, zeta potential, pH and osmolality.

For drug loaded SLN, 750 mg of aciatic acid (AA) or asiaticoside (AS) was usually added into the organic phase prior to formulation of o/w emulsion.

Formulation of solid lipid nanoparticle (SLN) by HPH

For SLN prepared by hot homogenization method, a high speed, high pressure homogenizer was used to reduce particle size of emulsion. All formulations of SLN prepared by this method were listed in Table 8. The ingredients in each component was similar to SLN by solvent diffusion method but the concentration was in % w/w.

Table8. The composition of SLN prepared by HPH method

Chemicals	Concentration (%w/w)
Lipid	1
Stabilizer	1-5
Water for injection	to 100

The SLN were prepared by dissolving or dispersing stabilizer in aqueous phase. The required amount of aqueous phase and oil phase were separately heated to 80±1°C by using a water bath. The aqueous phase was then added to the oil phase. The high speed homogenizer was used to prepare coarse emulsion at the speed of 12000 rpm for 5 minutes. The coarse emulsion was then homogenized to produce fine emulsion using Emulsiflex® C-5 operating at 10000 psi for 5 cycles. The obtained homogenization was an o/w emulsion of melted lipid in the aqueous solution. Each preparation was evaluated similarly to solvent diffusion method. In AA or AS loaded SLN, 750 mg of AA or AS was usually added into the oil phase prior to formulation of o/w emulsion.

2. Physicochemical characterization of SLN

Determination of particle size and zeta potential

The AA, AS loaded SLN preparations were diluted with water with an appropriate concentration prior to the determination of particle size and zeta potential by Zetasizer® (model NanoZS, Malvern, UK). Triplicate observations of each sample were measured.

pH measurement

The pH of SLN was measured at room temperature using a pH meter. The equipment was calibrated at pH 4 and 7 using Beckman standard buffer solution before used. Each sample was performed in triplicate.

Osmolality measurement

The osmolality of SLN was measured at room temperature using freezing point depression principle. Before the osmolality measurement of the samples, the instrument had to be calibrated with water for injection. The SLN volume of 50 μl was filled in a clean and dry measuring vessel by means of pipette, avoiding the trapping of air bubbles. The measuring vessel was pushed on the measuring vessel holder to the upper limit and then let the holder down into the lower cooling system. The measuring result was automatically displayed as value for osmolality concentration in Osmol/kg. Each sample was measured in triplicate.

3. Morphology of SLN

Transmission electron microscope(TEM) investigation

The AA, AS loaded SLN formulations were diluted with distilled water. The samples were placed on a specimen mesh coated with colloidion film, being stained by 2% phosphotungstic acid solution, dried under room temperature and observed with JEM-1230 transmission electron microscope.

4. Effect of storage temperature

The suitable formulations exhibiting mean particle sizes in nanometer range, were kept at 45°C, 4°C and room temperature. The particle size, pH, zeta potential and osmolality were assessed after storage for 1 month and 3 months.

5. Stability testing

The stable SLN formulations showed white fluid dispersions after being sterilized, were also observed under accelerated condition (heating and cooling). The samples were stored at 4°C for 48 hours and 45°C for 48 hours for 6 cycles. The particle size, pH, osmolality and zeta potential were studied.

6. High Performance Liquid Chromatographic (HPLC)

HPLC conditions

Column:	Hypersil® BDS(C18) column(250x4.6mm) 5 μm (Thermohypersil, UK) equipped with guard column packed with BDS(C18), 5 μm set at an ambient temperature
Detector:	UV detector at 210 nm
Injection volume:	20 μl
Flow rate:	1 ml/min
Mobile phase:	Acetronitrile:Phosphate buffer (10mM K_2HPO_4) pH7.1=29:71

Mobile phase was filtrated through a membrane filter with a pore size of 0.45μm and degassed for at least 30 minutes prior to use.

Validation of HPLC method

The typical analytical parameters to be considered for assay validation are specificity, linearity, accuracy and precision.

Specificity

The specificity of the active constituent peak was determined by the resolution and tailing factor. The well resolved from the other peaks and symmetry of the peaks should be obtained. The standard solution of AA, AS in methanol at the concentration 400µg/ml was prepared and evaluated using chromatographic condition as describe above.

Linearity

Triplicate injections of solutions containing drug in various concentrations from 100 to 1000 µg/ml in methanol was prepared and analyzed. The linear equation of curve obtained by plotting the peak area at each level prepared versus the concentration of each standard was calculated using the least square method.

Precision

a) Within run precision

The within run precision was determined by analyzing three sets of five standard solutions of AA, AS in the same day. The coefficient of variation of the peak area response (%CV) for each concentration was determined.

b) Between run precision

The between run precision was determined by comparing each concentration of AA standard solutions prepared and injected on different days. The percentage coefficient of variation (%CV) of AA of peak area response from three sets of standard solutions on different days was calculated.

Accuracy and recovery

The recoveries of AA from placebo were assessed by spiking placebo (SLN containing all the components except the drug) with AA and following the extraction procedures described earlier. Placebo was spiked in triplicate at five level spanning

50-150% of the amount of AA in dosage form. The average recovery and the coefficient of variance were calculated.

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.

7. In vitro drug release

The in vitro drug release study of SLN were carried out using modified Keshary-Chein Diffusion Apparatus consisting of the donor and the receptor compartment. The donor chamber and the receptor compartment were separated with a 0.22 µm pore size cellulose membrane. The cellulose membrane was cut out into a circular shape with a diameter of 3 cm.

Before assembling the circular cellulose membrane onto the diffusion cell, the membrane was soaked in mixture of PBS (pH 7.4) and isopropyl alcohol (70:30) for 6 hours. Mixture of PBS (pH 7.4) and isopropyl alcohol (70:30), as the release medium in the receptor compartment and the membrane in Keshary-Chien diffusion cell 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 the study, and throughout the experiments.

After equilibration, 3 ml of AA, AS loaded SLN were carefully pipetted into the donor compartment, and the cell was then covered completely and tightly with Parafilm®. The study was operated continuously for 2 hours by magnetic stirring bar rotating at 300 rpm. Whole of release medium of receptor medium was withdrawn at 2 hours. The amount of drug release was calculated and corrected for the amount from calibration curve.

8. In vitro permeation study

Cell culture

The initial purpose of this experiment was to use HUVEC (human umbilical vein endothelial cell) to study permeation but HUVEC could not be expanded up to 80 % in T-25 flasks. This might be due to cell mutation or poor

Therefore, ECV-304 cells was used instead. ECV-304 cells were derived as a spontaneous transformant from human umbilical vein endothelial cells (HUVEC). Because the cells show endothelium-like properties such as producing endothelium specific Weibel-Palade bodies, endothelium-related antigens and angiotensin-coverting enzyme in addition to the HUVEC like morphology, this cell line has been assumed to be a convenient system for the study of vascular endothelial cells (Kobayashi et al 2004). Although recently genetic analyses suggest the ECV-304 cell line was derived from T24 bladder carcinoma cells, there are numerous reports on the use of the EVC-304 cell line as a model for human endothelium because this cell line exhibits many endothelial characteristics (Huang 2006).

ECV-304 Cells (human epithelial cell line from American Type Culture Collection) were cultured in Duulbecco's Modified Engle Medium (DMEM) medium supplement with 100 U/mlpenicillin, 100 mg/ml streptomycin and 10% fetal bovine serum (FBS). These cells were incubated in a humidified atmosphere of 5%CO_2 and 95% air at 37 °C.

Measurement of trans-epithelial electrical resistance

These cells were trypsinized and seeded on polystylene filters, 0.4 μm pore size, of 6 wells Transwell® at a density $2x10^6$cell/cm^2. The apical and basolateral compartment received 1.5 and 2.6 ml of cultural media, respectively. The cells were cultured for 12 days to obtain monolayer and the medium was changed every three days. The monolayer integrity was assessed by the measurement of trans-epithelial electrical resistance (TEER). The TEER of culturing cell monolayer was measured using an electrode (Millicell ERS meter, Millipore,

MA, USA.). The resistance values (in ohms) from the membranes were subtracted from the values for holding a ECV-304 cells monolayer to determine the resistance across the cell monolayer. Growth medium was removed and cell monolayer was washed with PBS for 3 times. Then 1.5 ml of concentration of 200 μg/ml of sample was added to the upper compartment. Five hundred microlites of PBS was kept in the lower compartment. The UV absorbance at 210 nm of sample from the lower compartment was measured.

Measurement of cell viability

The viability of ECV-304 cell was measured by 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT) assay. In brief, cells were seeded into 96-well plates at a density of 3000 cells/well for 24 hours, and the cells reached confluence.

Hundred microlites of AA, AS loaded SLN of different concentrations (2-fold series, in growing medium) were added and then mixed by gently shaking. The plates were incubated for 72 hours. Following addition of 50 μl of MTT solution (1mg/ml) to each well, the plates were incubated for 4 hours at 37°C.

After medium had been removed, the dye crystal was dissolved in 150 μl of dimethyl-sulfoxide (DMSO). Finally, the optical density (OD) of each well was immediately measured on ELISA micro-plate reader at 570 nm to represent cellular viability. The OD of formazan formed in control cell was taken as 100 % viability.

Flow-cytometry

To label AA, AS loaded SLN conjugates with FITC-labeled dextran (FD-4), AA, AS loaded SLN (50 μg/ml) were suspended in sodium bicarbonate buffer (0.05M Na_2Co_3 pH 9.5). ECV-304 cells were incubated for 2 hours at 37 °C. From each sample, 20000 cells were analyzed. The fluorescence intensity of control cells (incubation without nanoparticles) and test cells were compared with each other.

9. Residue of organic solvent

Residue of methyl-ethyl ketone after evaporating by vacuum distillation at 40°C and 70 mmHg was determined by headspace gas chromatography (model GC-7AG, Shimadzu, Japan) to confirm the complete evaporation process. These ml of AA, AS loaded SLN were+ transferred to vial, sealed, and heat to 50°C. One ml of methyl-ethyl ketone vapor was injected into 5% SE-30 column (3.3 mm×2 m) under the following condition.

Column temperature:	50 °C
Injection temperature:	180 °C
Detector temperature:	180 °C
Nitrogen gas flow rate:	20 ml/min

Triplicate data were calculated with respect to calibration curve of methyl-ethyl-ketone at various concentration.