CHAPTER III
MATERIALS AND MATHODS

Materials

The following materials were used as received:

- Acetonitrile HPLC grade (Fisher Scientific, UK)
- Amphotericin B (Lot No. 040513, Shanghai 21CEC Pharmaceutical, Ltd., China)
- Cremophor EL (Lot No. 7218832400, BASF, Germany)
- Cremophor RH40 (Lot No. 68-1498, Vita, Co., Ltd., Thailand)
- Cyclohexane AR grade (Fisher Scientific, UK)
- Dimethylsulfoxide AR grade (Fisher Scientific, UK)
- Disodium edentate (Lot no. 7727 KMTP, Malinckrodt chemical, USA)
- Di-sodium hydrogen ortho-phosphate dodecahydrate (Lot no. F2G096, APS Chemical Limited, Australia)
- Epikuron®200 (Lot no. 1-0-9036, Lucas Meyer GmBH, Germany)
- Fructose (Lot no. 47740, Fluka, Switzerland)
- Fungizone® (Lot No. A745, Bristol-Myers Squibb, France)
- Glycerin (Lot no. LG 1920, distributed by Srichand United dispensary Co., Ltd., Thailand)
- Glyceryl behenate (Compritol® ATO 888) (Lot No. 32535, Gattefosse', France)
- Glyceryl palmitostearate (Precirol® ATO 5) (Lot No. 28994, Gattefosse', France)
- Hydrochloric acid (Fisher Scientific, UK)
- Mannitol (Lot no. FIF 133, APS Chemical Limited, Australia)
- Methanol HPLC grade (Fisher Scientific, UK)
- Morpholinopropanesulfonic acid, MOPS (Lot no. 11/206, USB Corp., USA)
- Myrj 52 (Lot no. 205A0325, Uniqema Asia pacific, Malaysia)
- Myrj 59 (Lot no. 48181, Uniqema Asia pacific, Malaysia)

- N,N –Dimethylformamide AR grade (Fisher Scientific, UK)
- Phospholipon® 90H (Lot no. 70060, Nattermann Phospholipid GmBH, Germany)
- Poloxamer 188 (Lutrol® F 68) (Lot No. 60-0480, BASF, Germany)
- Poloxamer 407 (Lutrol® F 127) (Lot No. 52-0113, BASF, Germany)
- Polyethylene glycol 400 (Lutrol®E 400) (Lot no. 82-1765, BASF, Germany)
- Potassium chloride (Lot no. F1G253, APS Chemical Limited, Australia)
- Potassium dihydrogen ortho-phosphate (Lot no. F1F125, APS Chemical Limited; Australia)
- Propylene glycol (Lot no. 7879040502, distributed by Srichand United dispensary Co., Ltd., Thailand)
- Sodium chloride (Lot no. F2C273, APS Chemical Limited, Australia)
- Sodium hydroxide (Lot no. B464398 414, Merck, Germany)
- Sucrose (Lot no. F3D103, Ajax Finechem, New Zealand)
- Tween 20 (Lot No. 003555, distributed by Srichand United dispensary Co., Ltd., Thailand)
- Tween 80 (Lot No.103316, distributed by Srichand United dispensary Co., Ltd., Thailand)
- Yeast nitrogen base, YNB (Lot no. 0392-15-9, DisCo Laboratories, USA)

Equipment

- Analytical balance (Sartorius, A200S, Germany)
- Centrifuge (Model 5810, Eppendorf, Germany)
- Cryoscopic osmometer (model Osmomat® 030-D, Gonotec, Germany)
- Differential scanning calorimeter (Model DSC 822^e, Mettler Toledo, Germany)
- Fourier transform infrared spectrophotometer (Model 1760X, Perkin-Elmer, USA)
- Freeze dryer (Model FD-6-850MP0, Dura-Dry™, FTS System Inc., USA)
- H- Nuclear magnetic Resonance (Model JNM-A500, JEOL; Japan)

- Hematocytometer (Boeco, Germany)
- High performanace 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 (ITO Corporation, Japan)
  - C-18 Column (Hypersil BDS C18, 5 µm, 250x4.6 mmID Column)
- High pressure homogenizer (Model EmulsiFlex C5®, Avestin, Canada)
- Hot air oven (Model B7600, Mammert, USA)
- Hot stage microscope (Model FP 900, Mettler Toledo, Germany)
- Laser Diffractometer (Masterize 200, Marvern, UK)
- Magnetic stirrer (Variomay multipoint, Komet, Taiwan)
- Nanosizer (Nano ZS, Marvern Instrument, UK)
- Optical Microscope (Model ∈200, Nikon Eclipse, Japan)
- Photon Correlation spectrometer (Zatapals, Brookhavern Instrument, USA)
- pH meter (Model 210A$^+$, Thermo Orion, Germany)
- Scanning Electron Microscope (Model JSM-5410LV, JOEL, Japan)
- Shaking Incubator (Lab Tech, UK)
- Transmission electron microscope (Model JEM-1230, JEOL, Japan)
- Ultracentrifuge® (Model L 80, Beckman, USA)
- Ultrapure Water® equipped with filter system (Balson® , Balson Inc., USA), Boost pump, Option 3 water purifier, Maximum ultrapure water, and Reservoir (ELGA, USA)
- Ultrasonic bath (Transsonic digitals, Elma®, Germany)
- Ultrasonic processor (Vibracell®, Germany)
- UV visible spectrophotometer (Model UV-1601, Shimadzu, Japan)
- Vacuum filtration apparatus with sinter glass fiber No.3 (Waters, USA)
- Viscometer (Brookfield LVDV-II$^+$, USA)
- Water bath (ITS Co., Ltd., Thailand)
- X-Ray diffractometer (Model D8-discover, Bruker, Germany)

Glassware and Miscellaneous

- 0.22 cellulose acetate membrane filter (Waters, USA)
- 0.45 nylon membrane filter (Waters, USA)
- Aluminium foil (MMP Packaging, Thailand)
- Beaker (Pyrex, USA)
- Cylinder (Pyrex, USA)
- Disposable syringe and needle (Terumo, Thailand)
- Erlenmeyer flask (Pyrex, USA)
- Filter paper No. 1 (Whatmann, England)
- Funnel (Pyrex, USA)
- Graduating pipette (Witeg, Germany)
- Micropipette and disposable pipette tip (Socorex, Switzerland)
- Osmolality vessel (Gonotec, Germany)
- Parafilm (American National Can., USA)
- Polypropylene centrifuge tube (Corning. USA)
- Polycarbonate centrifuge tube (Nalge Company, USA)
- Screwed-cap tube (Pyrex, USA)
- Transfering pipette (Witeg, Germany)
- Vial type I glass with rubber cap and aluminium ring (Supplied by APPA Industrial Co.,Ltd., Thailand)

Methods

1. Formulation of solid lipid nanoparticles (SLN)

There are different approaches for the production of solid lipid dispersions (Mehnert and Mäder, 2001). In this study, solid lipid nanoparticles (SLN) prepared by warm microemulsion and high pressure homogenization methods were selected with regarding to the scaling up potential.

Preparation of drug-free SLN by warm microemulsion method (WME)

Solid lipid was melted approximately 5°C above the melting point. The ratio of surfactant:co-surfactant in preparations was 1:1. A mixture of water, co-surfactant and surfactant was heated to the same temperature and then was added under mild stirring to the lipid melt. An optically transparent system was formed. This hot microemulsion was then immediately dispersed in cold water (2-3°C) in a ratio 1:20 under mechanical stirring. The ingredients used in formulations were 10% glyceryl behenate (GB) and glyceryl palmitostearate (GP) as solid lipid with 15-35% of the ratio at 1:1 of surfactant:co-surfactant (tween 80 (Tw80), cremophor EL (CreEL), cremophor RH 40 (CreRH), glycerin (Gly), propylene glycol (PG), and polyethylene glycol 400 (PEG)).

Preparation of drug-free SLN by high pressure homogenization (HPH)

Solid lipid was melted approximately 5°C above the melting point of the lipid and dispersed in a surfactant solution previously heated to approximately the same temperature as the lipid melt. A hot pre-emulsion was formed by probe sonication. The pre-mix emulsion was passed through a high pressure homogenizer (Emulsiflex® C5, Avestin, Canada) for 5 cycles at 10,000 psi (Pichayakorn, 1999). The hot dispersion was allowed to cool down to room temperature and was stored at 4°C. The ingredients used in the formulations were 3% GB and GP as solid lipid with 1-5% of Tw80, Tween 20 (Tw20), CreEL, CreRH, poloxamer 188 (P118), poloxamer 407 (P407), myrj 52 (M52) and myrj 59 (M59) as stabilizer.

The effect of types of solid lipid, types and amount of surfactant, and types and amount of co-surfactant (in case of preparing by warm microemulsion method) were evaluated on the characteristics of SLN. The appearance of the drug-free SLN prepared by WME and HPH methods were observed visually i.e. color change, phase separation, coalescence, gel formation. The particle size, pH and osmolality were also evaluated. For physical stability, they were assessed after preparations for 1, 3 and 6 months under light protection at 4°C storage condition. The optimal concentration of surfactant in the formulations was selected for further study.

Formulation of amphotericin B (AmB) loaded SLN (AmB-SLN)

The drug free formulations that had good physical stability were chosen to load AmB. For both methods, 20 mg AmB solubilized in either 0.1 N sodium hydroxide (NaOH) or dimethylsulfoxide (DMSO) was added to the melt lipid and followed by the same procedure as in drug-free SLN.

Preparation of freeze dried SLN products

The AmB-SLN formulations prepared by both methods were lyophilized using Dura-Dry^{TM} lyophilizer. The bulking agents were added to AmB-SLN dispersions before freezing. Ten milliliters of each sample was rapidly precooled at -20°C, and lyophilized for 24 hr at a temperature range -50°C to -40°C and a vacuum of 500 mmHg. The resulting lyophilized products were reconstituted in distilled water for physical and chemical investigation.

Selection of bulking agents

Mannitol, fructose and sucrose and their mixtures as bulking agent in the concentration range of 7.5-12.5% were added to AmB-SLN dispersion before lyophilization. The optimum formulation which shown the good physical stability of AmB-SLN containing 10% GP with 20% of CreRH and Gly was selected to study as model formulation. The physical properties, speed of redispersion and the ratio of particle size on reconstituted AmB-SLN from freeze-dried to initial were observed.

Preparation yield

Preparation yield was calculated for the SLN obtained by the warm microemulsion and the high pressure homogenization technique following lyophilization. The following equation was used for calculation :

$$\% \text{ yield} = \frac{A}{B} \times 100 \qquad (1)$$

Where A = weight of particles obtained(g), and B = weight of solid material used in formulations (g)

Effect of lecithin incorporated with SLN formulations

The formulations of AmB-SLN that had appropriate physical and chemical stability were chosen to study. The AmB-SLN were prepared with the additional phospholipon®90H (PL) and a suitable surfactant in the ratio 1:2 for improving AmB solubility. The final formulation was designated AmB-SLN-L.

Effect of amount of drug loading on SLN and SLN-L formulations

The optimal formulations were selected to study the effect of drug loading. Two levels of AmB, 2.5% and 5% w/w of AmB calculated on total lipid were prepared under the same condition.

2. Formulation of AmB loaded nanostructured lipid carriers (NLC)

The formulations of AmB-SLN that had suitable physical and chemical stability were chosen to study. NLC was prepared exactly in the same manner as SLN dispersion (AmB-NLC) with only partially replacing 30% of solid lipid matrix by medium chain triglyceride oil (caprylic/capric triglycerides; MCT oil). Lecithin was incorporated to NLC formulation was designated AmB-NLC-L. The effect of oil and amount of drug loading were also investigated as the same procedure as AmB-SLN and AmB-SLN-L formulations.

3. Physicochemical characterizations of formulations

Physical characterization

Particle size analysis

Particle size analysis was performed by photon correlation spectroscopy, PCS (Brookhaven, UK) using an N4 Coulter at fixed angle of 90° and at temperature 25°C. A sample was dispersed in distilled water before use. A sample of dispersion was put in a quartz cuvette. The sample was then placed in the

instrument and allowed to be temperature equilibrium between sample and sample holder at 30°C. PCS yields the mean particle size (z value) and the polydispersity index (PI) as the measure of the width of the distribution. Each obtained value was the average of 3 measurements.

A laser diffraction particle sizer (Mastersize, Mavern, UK) was also used to determine the particle size of SLN. The sample was diluted with water at a suitable concentration. The correct amount was adjusted by observing the obscuration range. 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 diameter of particles of 50% volume percentile (d(v,0.5)) and uniformity were determined.

Zeta potential measurement

Zeta potential was measured by using a nanosizer (Malvern Instruments, UK). SLN Sample was diluted with distilled water and placed in an electric field by applying to the cell using zeta-cuvette. During the measurement, the temperature was controlled at 25°C. The zeta potential was automatically calculated and this processing was done by the software included within the system. Each sample was carried out in triplicate.

pH determination

The pH values of preparations were determined in triplicate at room temperature with Thermo Orion model 210 pH meter. The equipment was calibrated at pH 4 and 7 using Beckman standard buffer solutions.

Osmolality measurement

The osmolality of preparations was measured at room temperature using freezing point depression principle. Before the measurement of the osmolality of samples, the instrument had to be calibrated with water for injection and sodium

chloride for injection. The preparation volume of 50 µl was filled in a clean and dry measuring vessel by means of a 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.

Viscosity determination

The rheological measurements were performed with a viscometer (Brookfield LVDV-II^{+}, USA) equipped with a cone-and-plate. All measurements were carried out at a temperature 20 ± 0.1°C. The resulting shear stress was performed by increasing the shear rate from 0.5 to 100 s^{-1}. The relationship of shear stress of the sample as a function of shear rate was plotted.

Determination of drug content by HPLC method

Calibration curve of AmB

A stock solution was prepared by accurately weighing AmB reference standard (12.5 mg) into 50-ml volumetric flask and dissolving and diluting to volume with the mixture of DMSO:MeOH (1:999 v/v). The stock solution was diluted with the same solvent to a final concentration of 10 µg ml^{-1}. This solution was further diluted to give a range of AmB 0.8 – 8.0 µg ml^{-1}. Each solution was subjected to HPLC in triplicate. Peak areas were recorded for all the solutions. The equation was calculated from the relationship between peak area responses of AmB and their concentrations.

Extraction procedure

The extraction of the AmB performed in 50- ml centrifuge tubes with plastic caps. The contents of preparation were accurately pipeted , then mixed with 10 ml dimethylformamide (DMF) and 7.5 ml of cyclohexane and shaken thoroughly until

the AmB was dissolved (approximately 3 minutes). The sample was centrifuged for 10 min at 3000 rpm. The supernatant was eliminated, and 1 ml of the resulting solution was transferred into a 10-ml volumetric flask, and diluted to volume with DMSO:MeOH(1:999 v/v). A portion of the sample was filtered through 0.45 μm nylon filter membrane and subjected to analysis by HPLC (modified from Wilkinson et al., 1998).

For the determination of amount of drug in freeze-dried product, 200 μg of AmB calculated to the theoretical yield of freeze-dried SLN was redispersed in water under mechanical stirring. One-ml aliquot was withdrawn, immediately extracted and then analyzed by HPLC.

HPLC conditions

The determination of AmB by reverse phase HPLC was modified from the method described by Manosroi, et al (2004). The procedure was developed as follows:

Column :	Hypersil BDS C18, 5μm, 250x4.6 mmID
Mobile phase :	0.25 mM EDTA : Acetonitrile (63:37 % v/v) was prepared freshly and filtered through a 0.45 μm membrane filter. It was then degassed by sonication for 30 minutes
Flow rate :	1.0 ml/min
Detection wavelength :	403 nm
Injection volume :	20 μl
Temperature :	Ambient
Retention time :	4.20-4.40 minutes

Validation procedure

Specificity

Under the chromatographic condition, determination of AmB quantity was evaluated. Solvents, phosphate buffer, and all of the drug-free GP-SLN, GP-NLC formulations that had the same component as AmB loaded formulations were extracted. The resulting layer was properly diluted before determining by HPLC.

Precision

a) Within run precision

The within run precision was determined by analyzing three sets of five standard solutions of AmB in the same day. The coefficients of variation of the peak area responses (% CV) for each concentration were determined.

b) Between run precision

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

Accuracy and recovery

The recoveries of AmB from placebo were assessed by spiking placebo (SLN containing all the components except the drug) with AmB and following the extraction procedures described the earlier. Placebo was spiked in triplicate at five levels spanning 50-150% of the amount of AmB in dosage form. The average recovery and the coefficient of variance were calculated.

Linearity

Linearity was evaluated with various amounts of appropriately diluted stock standard solutions to form working solutions containing 0.4-8.0 μg/ml of AmB. For each concentration three measurements were performed and the calibration curves were obtained by plotting the peak area versus nominal concentration expressed in

μg/ml of AmB. The slope, intercept and correlation (r^2) of each calibration curve were determined.

System suitability

System suitability was evaluated by making 6 replicate injections of the standard and recording the peak responses. It was used to verify that the resolution and reproducibility of the chromatographic system were adequate for analysis to be done.

Determination of entrapment efficiency (%EE)

The content of AmB in loaded SLN was determined by ultracentrifugation. SLN dispersion of 7 ml was centrifuged at 60,000 rpm, 4°C for 6 hours (Zhang et al., 2000). The supernatant was saved for appropriate dilution and measured by HPLC. The triplicate observations were measured. Entrapment efficiency (%EE) could be achieved by the following equation.

$$\%EE = \frac{[D]t - [D]s}{[D]t} \times 100 \qquad (2)$$

Where [D]t = drug content of the resulting after extraction

[D]s = drug content of supernatant found

Morphology of AmB formulations

Transmission electron microscope (TEM) investigation

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

Cryo-Scanning electron microscope (Cryo-SEM) examination

The particle size and shape of SLN and NLC were observed by scanning electron microscope. The SEM observation method in this study was Cryo-SEM method. The sample was dropped into the hole of stub and the specimen stub was set on the specimen holder. The specimen holder was screwed with the specimen exchange rod. The specimen stub was then frozen in liquid nitrogen which made the sample kept below 0°C. The sample holder was set in the cryo-chamber through the air lock chamber. After the specimen was fractured with the built-in knife and was carried out sublimation (etching) by using etching heater about 1 minute, the sample was then transfered to cooling stage and observed particle shape and size.

Thermal analysis

Differential Scanning Calorimetry (DSC) analysis

The DSC analysis was used to investigate the crystalline structure of triglyceride, drug and freeze-dried solid lipid preparations. The instrument was calibrated with indium for melting point and heat of fusion. The sample was weighed about 2-5 mg into a crimped aluminium pan with 1 pinhole the empty pan was used as reference under nitrogen purge. DSC pattern was determined by using DSC822^e, Mettler Toledo with a heating rate of 10°C/min, in the temperature range from 25-250°C for all samples. The melting temperature (Mp) and enthalpy of transition (ΔH) were determined from the thermogram. The physical mixture of components in the preparations was similar to that of weight ratio in each formulation, P407 and PL were also subjected to the same thermal cycles.

Hot-stage microscopy (HSM) investigation

HSM analysis was conducted using a hot stage microscope (Mettler FP 900, Germany) assembled on a Nikon eclipse E200 microscope, Japan. Different types of samples (AmB-SLN, AmB-NLC, AmB-SLN-L and AmB-NLC-L) were observed under the microscope by using a scanning speed of 1°C/min. Changes in the

samples morphology (melting-crystallization) were recorded as a function of temperature. Captured images were analyzed using a digital camera.

Spectroscopic analysis

Fourier transform infrared spectroscopy (FT-IR) analysis

Fourier transform infrared spectroscopy (FT-IR), a high sensitivity of IR, was used to study the interaction between drug and other exipients. The changes of functional groups of triglyceride, drug, stabilizers and selected formulations after ultracentrifugation were acquired by mulling method. The samples were determined by FT-IR spectrometer (1760X Perkin Elmer) in the wave numbers of 750-4000 cm^{-1}.

Proton Nuclear Magnetic Resonance (^{1}H-NMR) analysis

Proton nuclear magnetic resonance (^{1}H-NMR) spectra of some components, AmB-SLN, AmB-NLC, AmB-SLN-L and AmB-NLC-L dispersions prepared in deuterated water or deuterated chloroform in case of MCT oil and GP were recorded on a JNM-A500 NMR spectrometer (JEOL, Japan) operating at 500 MHz. 2,2 Dimethyl-2-silapentan-5 sulfonate (DSS) was served as a reference for 0 ppm.

Spectroscopic studies – aggregate state of AmB

The commercial product, Fungizone® and AmB formulations were prepared in phosphate saline buffer (PBS), pH=7.4 while stock solutions of AmB were diluted with the aid of DMSO:MeOH (1:999 %v/v) prior to adjust with volume by PBS. Various levels of AmB were prepared by serial dilution at AmB concentrations of 2.0-12.0 μg/ml. UV/VIS spectra of AmB in solid lipid nanoparticles were recorded from 300 nm to 500 nm with UV-VIS spectrophotometer (Model UV-1601, Shimadzu, Japan). The ratio of the absorbance at 348 nm (peak I) and the absorbance at 409 nm (peak IV) of AmB was measured to assess the degree of aggregation of AmB in isotonic PBS, pH 7.4 (Aramwit et al., 2000; Moreno et al., 2001).

Powder X-ray Diffractometry (PXRD) analysis

Powder X-ray diffractometry was used to study the change of crystallinity of triglyceride, drug and exipients after preparation process (Jenning et al., 2000). The sample was made as fine as possible using an agate mortar and pestle. The proper amount of the sample was placed onto the acrylic plate containing rectangular window. After firmly pressed it down by using another piece of glass plate, any surplus of sample was removed. The sample plate stuffed with the sample was mounted onto the sample holder. X-ray diffractogram was scanned with the diffraction angle increasing from 3° to 45°, 2θ angle, with a step angle of 0.04° and count time of 1 second.

4. *In vitro* drug release

The *in vitro* drug release studies of SLN and NLC were carried out using modified Keshary-Chien diffusion apparatus consisting the donor and the receptor compartment. The donor chamber and the receptor compartment were separated with a 0.22 μm cellulose membrane. The cellulose membrane was cut 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 PBS, pH 7.4 for 6 hours.

PBS, pH 7.4, as 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. A 1-ml aliquot of receptor medium was withdrawn at 2, 4, 6, 8, 12, 16 and 24 interval hours and replaced immediately with an equal volume of fresh release medium. A portion of solution under test was determined by HPLC. The amount of drug release was calculated and corrected for the amount from calibration curve. The triplicate determinations of each sample were measured.

5. Biopharmaceutical characterizations of formulations

Hemolytic activity

Blood was collected from sheep and centrifuged for 10 minutes at 3,000 rpm. Supernatant and buffy coated were pipetted off. Red blood cells (RBCs) were washed 3 times and resuspended with isotonic phosphate buffer saline (PBS), pH = 7.4 to yield initial volume. The proper dilution for RBCs was counted with hematocytometer (Boeco, Germany). Various AmB formulations were added to the suspended RBCs and then the obtained suspensions were diluted with PBS to give final concentrations in the range of 1-40 μg/ml. AmB suspensions with different levels in either PBS were incubated in an incubater at 100 rpm, 37°C for 30 minutes and then placed in ice bath to stop hemolysis. The unlysed RBCs were removed by centrifugation at 3,000 rpm for 5 minutes, and the supernatant was analysed for hemoglobin by UV-Vis spectroscopy at 576 nm. The percentage of hemolysed RBC was determined by the following equation.

$$\% \text{ Hemolysis} = 100 \times (Abs - Abs_0) / (Abs_{100} - Abs_0) \quad (3)$$

Where Abs, Abs_0 and Abs_{100} were the absorbances for the sample, control with no AmB in PBS and control of distilled water, respectively.

AmB susceptibility testing

The standard method for antifungal susceptibility testing proposed by the NCCLS (proposed standard M27) was used for *in vitro* susceptibility test (Espinel-Ingroff, 1998). Four isolates fungal organisms (*Candida albicans, Cryptococcus neoformans, Aspergillus fumigatus and Penicillium marneffei*) were tested by broth macrodilution technique with YNB, pH 7.0 with morpholinopropanelsulfonic acid (MOPS) buffered medium. The isolated organisms were stored as a suspension in water at 4°C and were subcultured onto potato dextrose agar slants at 37°C for 10-15 days when the experiment was performed. The selected AmB formulations were dissolved in buffered medium; giving an AmB level of 0.03-16 μg/ml. AmB was

dissolved in DMSO and Fungizone® was reconstituted with 10 ml of sterile distilled water and finally both of them were diluted further to give the same concentration. AmB-free preparations were also used as a control. In addition, DMSO, AmB-free medium were included as a growth control. The temperature of incubation for *Penicillium maneffei* was 25°C while the others were incubated at 37°C. Minimum inhibitory concentrations (MICs) were read after 48 h of incubation. The MIC was defined as the lowest AmB concentration at which no growth could be seen. After MIC readings, tubes were agitated using a vortex. Then, 100 µl aliquots were removed from each growth-negative tube and were spread on SGA petridishs. The plates were incubated at 37°C (25°C for *Penicillium maneffei*) and the fungal colonies grown were counted after approximately 4-5 days of incubation. The minimum fugicidal concentrations (MFCs) were defined as the lowest drug concentration from which ≤ 3 colonies were visible on the agar plate (NCCLS, 1997; Franzot, et al., 1996; Pujol et al., 2000).

6. Stability studies

The AmB-SLN, AmB-NLC, AmB-SLN-L, AmB-NLC-L and some AmB-SLN freeze-dried formulations were observed for both physical and chemical stability studies under 4°C storage condition.

Physical stability studies were performed by analyzing macroscopic aspect under visual inspection and size measurement by PCS after 3-months storage. Chemical stability was evaluated on AmB loaded formulations by HPLC analyses at 0, 1, 2 and 3 months. Log (AmB remaining content, %) was plotted against time and the slopes (m) were calculated by linear regression. The slopes (m) were then substituted into the following equation for the determination of k values:

$$k = m \times 2.303 \quad (4)$$

Shelf life values (the time for 10% loss, t_{90}) were then calculated by the following equation:

$$t_{90} = 0.105 / k \quad (5)$$

7. Statistical analysis

Data were expressed as mean ± S.D. and statistically assessed by one-way analysis of variance (ANOVA). Differences between drug treated groups and the control group were evaluated by Student's *t*-test. $P<0.05$ was considered significant.