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Application of aerosol solvent extraction system (ASES) process for preparation of liposomes in a dry and reconstitutable form

Sarinnate Kunastitchai [a,b], Lars Pichert [b], Narong Sarisuta [a], Bernd W. Müller [b,*]

[a] *Department of Manufacturing Pharmacy, Faculty of Pharmacy, Mahidol University, Sri-Ayudhya Road, Bangkok 10400, Thailand*
[b] *Department of Pharmaceutics and Biopharmaceutics, Christian Albrechts University Kiel, Gutenbergstraße 76, 24118 Kiel, Germany*

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Abstract

The aerosol solvent extraction system (ASES) process was applied to prepare liposomes in a dry and reconstitutable form. Dry ASES microparticles containing miconazole (MCZ) as a model drug were prepared by an optimized ASES process with various compositions of spraying solution containing phosphatidylcholine, cholesterol, and Poloxamer 407. The influence of such compositions and the pH of hydration medium on the physico-chemical properties of the produced microparticles were investigated before and after hydration. At optimized conditions, partially crystalline, spherical, and nonporous microparticles associated in aggregates varying from a few microns to 40 μm were produced with the residual content of methylene chloride and methanol lower than 30 and 86 ppm, respectively. The percentage of drug recovered in the produced microparticles was increased with an increase of the drug concentration in the spraying solution. The entrapment efficiency of hydrated MCZ microparticles was improved by increasing the pH of the hydration medium.

Keywords: Supercritical carbon dioxide; Aerosol solvent extraction system; ASES; Liposomes

1. Introduction

Conventional methods of liposome preparation are generally based on using a great amount of organic solvent(s), which are harmful to organisms and environment. For this reason, if liposomes are to be used as drug carrier, a complete removal of the solvents from the pharmaceutical preparations must be ensured. However, for large scale production, most of the methods used for laboratory-scale preparation are unlikely to be suitable with respect to process feasibility and the capability to remove the solvents effectively (Barenholz and Crommelin, 1994). Although freeze drying is most frequently used today as a possible tool in scaling up and improving the stability of liposomes (Vanleberghe and Handjani, 1978), it still exhibits some difficulties in terms of residual water content and chemical stability problems caused by the lyoprotectants used (van Winden and Crommelin, 1997). Furthermore, this technique itself consumes a larger amount of energy in the production process with considerably high cost due to the lyophilization step as compared to other techniques. For large scale preparation of liposomes, not only the desired product properties should be focused on, but efficiency and feasibility of the production process should be recognized as well (Barenholz and Crommelin, 1994).

Recently, supercritical carbon dioxide (SCD) has received much attention in the preparation of liposomes by using it either as an environment-friendly alternative solvent or as an anti-solvent (Frederiksen et al., 1997; Castor and Chu, 1998; Magnan et al., 2000; Badens et al., 2001; Perrut and Jung, 2001; Otake et al., 2001; Imura et al., 2002). As a solvent, the so-called supercritical reverse-phase evaporation (scRPE) method has been developed with the use of SCD concurrently with ethanol as cosolvent for the phospholipids before subsequently introducing aqueous systems and emulsion formation (Frederiksen et al., 1997; Otake et al., 2001; Imura et al., 2002). After the decompression process, a liposome suspension is obtained by a mechanism analogous to that of the conventional reverse-phase evaporation method. Nevertheless, since the system subjected to that depressurization step was a heterogeneous aqueous dispersion, some residual content of ethanol would certainly be

* Corresponding author. Tel.: +49 431 880 1333; fax: +49 431 880 1352.
E-mail address: bwmueller@pharmazie.uni-kiel.de (B.W. Müller).

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present in the product. Additionally, a certain amount of ethanol is commonly known to prevent liposome formation. In contrast to the solvent function of SCD, the aerosol solvent extraction system (ASES) process, using SCD as anti-solvent, has become an attractive approach in the preparation of liposomes (Magnan et al., 2000; Badens et al., 2001) because it diminishes organic solvents into exceptionally low residues and provides solid microparticles/liposomes instantaneously as in a single step of production (Bleich and Müller, 1996; Ruchatz et al., 1997; Thiering et al., 2001). This technique was first invented as a method to manufacture a sterile product which has a substance embedded in a biologically degredable carrier (Müller and Fischer, 1989). On the basis of this procedure, organic liquids which are sprayed through a nozzle into a bulk of SCD can rapidly be extracted, enabling fast and continuous precipitation of solutes out of the organic solution. After a relatively short period of drying with the circulating SCD, the residual solvent(s) can dramatically be removed from the precipitates produced (Ruchatz et al., 1997; Thiering et al., 2001). This is due to the fact that only the circulating supercritical gas phase carrying organic liquids is subject to pressure reduction during particle formation and drying stage. Transferring this technique to liposome preparation, the ASES process seems to be suitable for preparing pharmaceutical liposomes as dry and reconstitutable vesicles for large scale production. The direct production in a dry form can eliminate stability-associated problems which are encountered in the traditional aqueous dispersion of liposome preparation. Therefore, this study was focused on the optimization of processing conditions and liposome formulations for preparation of liposomes via the ASES process. The influence of critical process parameters and the effect of lipid and surfactant compositions on the physico-chemical properties of ASES-prepared liposomes either in dry form or in hydrated form were examined, using miconazole as a model drug.

2. Materials and methods

2.1. Materials

Miconazole base (MCZ) (GUFIC Biosciences, Gujarat, India) was chosen as a model drug. Phosphatidylcholine (PC) (Phospholipon® 90H; Rhône-Poulenc Rorer, Köln, Germany) and cholesterol (Chol) (Fluka Chemie GmbH, Buchs, Switzerland) were used as components of the lipid carrier. Poloxamer 407 (Polox) (Lutrol® F127, BASF, Ludwigshafen, Germany) was selected from the preliminary study to be employed as a nonionic surfactant. The reagents used in production and ordinary analysis – methylene chloride, methanol, and acetonitrile – were of analytical grade (Merck KG, Darmstadt, Germany). For residual solvent analysis, *N*,*N*-dimethylformamide (DMF), methylene chloride, and methanol were of residue analysis grade (>99.8% purity) (Merck, Darmstadt, Germany). Water was of double distilled quality. The utilized carbon dioxide was of high quality (99.97%) (Kohlensäurewerk Hannover EG, Laatzen, Germany).

Table 1
Experimental design for optimization

Temperature (°C)	Pressure (MPa)	Density (g/mL)
35	8.5	0.50
40	8.5	0.38
40	9.5	0.50
45	8.5	0.30
45	9.5	0.38
45	10.5	0.50
55	9.5	0.30
55	10.5	0.38

2.2. Optimization of processing conditions

The critical processing conditions to be basically optimized were temperature and pressure in order to adjust the extraction properties of SCD for microparticle production. The lower and upper limits of the operating temperature were given by the critical temperature of carbon dioxide (31 °C) and by the technical possibility of the used instrument (Sitec AG, Maur, Switzerland) (60 °C), respectively. Regarding the phase transition temperature of phosphatidylcholine (PC) (Phospholipon® 90H) (51 °C), which was the basic component of liposomes, the optimum conditions were investigated at below and above this value. As part of 3^2 factorial design, the pressure of the experimental runs, which is related to gas density, was varied within the range so that its density was less than 0.5 g mL^{-1} as shown in Table 1. Such set up conditions would avoid excessive solubilization of certain components including phosphatidylcholine in such supercritical fluid (Bleich et al., 1994; Badens et al., 2001; Steckel et al., 2004). For calculation of gas density, the extended gas equation previously described (Bleich et al., 1994) was used.

The spraying rate and carbon dioxide flow rate were held at moderate conditions of 6 mL min^{-1} and 6 kg h^{-1}, respectively, since these variables were demonstrated to have no direct effect on the extraction properties of supercritical gases in production of microparticles (Bleich et al., 1994). For optimization, a solution comprising 1% (w/w) of PC in the solvent mixture of methanol and methylene chloride (1:9, w/w) was prepared for spraying into the SCD at the above operating conditions. Methylene chloride was employed in this solvent system due to its considerably higher concentration limit of residual solvent (600 ppm) than that of chloroform (60 ppm) (USP 28, 2005). Additionally, both of solvents possess the relative chemical structure and polarity that can be used interchangeably for the ASES process.

The optimal concentrations for spraying through the nozzle system into the SCD were examined with a mixture of solutes containing miconazole (MCZ) and PC (1:1, w/w) as well as Poloxamer 407 (Polox) (Lutrol® F127) (0.2%, w/w, of spraying solution). Varying total concentrations of such mixtures were 7.7, 14.1, 16.7, and 18.9% (w/w).

2.3. Preparation of liposomes from dry ASES powder by reconstitution

Preparing liposomes via ASES process has to be performed in two steps. Firstly, microparticle production by means of ASES was carried out at optimized conditions to obtain microparticles consisting of drug–phospholipid aggregates. Secondly, microparticles were hydrated by an aqueous phosphate buffer and liposomal vesicles should be formed.

2.3.1. Microparticle production

The ASES process for microparticle production is described in detail elsewhere (Bleich et al., 1993). In brief, the solutions containing different MCZ levels (19 and 38%, w/w, based on total solutes) and varying ratios of PC and cholesterol (Chol) (10:0, 8:2, w/w) in the organic solvents (2:8, w/w, of methanol and methylene chloride) were prepared without and with the addition of Polox (10%, w/w, based on total solutes). The total concentration was employed in the range which had been optimized earlier. These solutions were sprayed by using a high-pressure pump (Gynkotek, Munich, Germany) through a nozzle (Schlick GmbH, Coburg, Germany) with a diameter of 0.4 mm and a spraying angle of 15° into SCD filled in the high pressure vessel. The solution feed rate and carbon dioxide flow rate were maintained at optimal conditions as used in the optimization step (6 mL min^{-1} and 6 kg h^{-1}, respectively). The particle formation under the adjusted extraction conditions occurred instantaneously when the organic solvents were miscible with the SCD and extracted. Subsequently, the precipitated particles were dried by washing with continuous flow of SCD to diminish the remaining organic solvents. This drying step was carried out within 3–4 h in which the organic solvents were drained out of the separator periodically. The expanded carbon dioxide was then condensed and returned into the CO_2-storage vessel. At the end of the process, after the supercritical gas was discharged from the high pressure column, solid microparticles were finally collected from the precipitation column and stored in a cool-dry place before further investigations.

2.3.2. Hydration of dry ASES powder

The dry microparticles were added to phosphate buffer (10 mg mL^{-1}) at different pH's (4.0 and 7.2) to allow the hydration of the microparticles. Complete hydration of the microparticles took place when being hydrated at 55 °C with gentle shaking. To remove unentrapped MCZ, the hydrated microparticle suspensions were isolated by means of centrifugation (Biofuge 17RS, Heraeus Sepatech GmbH, Osterode, Germany) at 7100–13,000 × *g* for 15–80 min (adjusted for each formulation) and redispersed with the aqueous phosphate buffer. This washing step was repeated several times until the drug concentration in the supernatant was lower than 3% of total drug in the preparation. Final products were kept at 4 °C prior to further investigations.

2.4. Characterization

2.4.1. Dry microparticle powder produced by the ASES process

2.4.1.1. Yield. The yield was determined by weighing the microparticles recovered in the precipitation chamber and calculating the percentage of yield with respect to the initial amount which had been added into the ASES processing system.

2.4.1.2. Particle size and morphology (scanning electron microscopy; SEM). Particle size and morphology of microparticles were observed under a scanning electron microscope (Model Philips XL20, Philips, Eindhoven, The Netherlands). Samples were fixed on an aluminium stub with conductive double-sided adhesive tape (Leit-Tabs, Plano GmbH, Wetzlar, Germany) and coated with gold in an argon atmosphere (50 Pa) at 50 mA for 50 s (Sputter Coater, Bal-Tec AG, Liechtenstein).

2.4.1.3. X-ray powder diffraction (XRPD) patterns. X-ray powder diffraction patterns were obtained in transmission technique using an X-ray diffractometer with a rotating Cu anode (Stoe and Cie GmbH, Darmstadt, Germany) operating at 1200 W. The Cu Kα1 radiation was generated at 30 mA and 40 kV and monochromatized by a graphite crystal. The powder was packed into a rotating sample holder between two films (PETP). Diffraction patterns recorded the X-ray intensity as a function of 2θ angle covering from 5.0° to 50.0°.

2.4.1.4. Drug content in microparticles. The drug content of the produced microparticles was analyzed by a validated high-performance liquid chromatography (HPLC) with 230-nm UV-detector. The HPLC system consisted of a Gynkotek High Precision Pump Model 300 (Gynkotek), a Kontron HPLC Autosampler 360, a Kontron HPLC Detector 430 (Kontron Instruments, Milano, Italy) and BDS Hypersil C8 column (5 μm; 250 mm × 4.6 mm; Thermohypersil, PA, USA). Peak integration was performed by a computer-controlled software (Data system 450, Kontron Instruments). A 10 mg sample was accurately weighed and dissolved in an appropriate amount of methanol. A mixture of 2.5% (w/v) of ammonium acetate solution and acetonitrile as well as methanol (2:3:5, v/v/v) was utilized as a mobile phase. Samples of 80 μL were injected at the mobile phase flow rate of 1.3 mL min^{-1}. The amount of drug was determined based on the standard curve of an external standard. All samples were analyzed in triplicate.

2.4.1.5. Residual solvents. A limit test for analysis of trace organic solvent was performed by means of static headspace gas chromatography (HS-GC) (Agilent Technologies 6890N Network GC, USA) using a DB-1 capillary column (30 m × 0.32 mm i.d. and 3.0-μm film thickness of 100% dimethylpolysiloxane) (J&W Scientific, Folsom, CA, USA). For determination of methylene chloride in the presence of methanol in the products, the experimental conditions used were modified from a validated method developed by Natishan and Wu (1998). A sample weight of 20 mg was dispersed in 1.0-mL bidistilled water together with 10 μL of DMF which was prepared in a

20-mL headspace vial. The GC temperature program was 35 °C isothermal for 8 min, then 25 °C min^{-1} to 200 °C. The injector temperature was held at 180 °C with a split ratio of 4:1 and flame ionization detector (FID) temperature of 250 °C. The headspace parameters were in static mode, 85 °C equilibration temperature, 15 min thermostating time, 2 min pressurization time, 0.05 min injection time, 125 °C loop temperature, and 135 °C transfer line temperature. Nitrogen carrier gas was employed with a column head pressure of 6.9 psi.

2.4.2. Hydrated microparticles

2.4.2.1. Particle size. The volume particle size and size distribution of hydrated microparticles were measured by laser diffraction using a Sympatec HELOS system (Sympatec GmbH, Clausthal-Zellerfeld, Germany) with a 20-mm lens for measuring range of 0.5–37.5 μm. The hydrated microparticle suspensions were sonicated for 30 s before measurements. All determinations were carried out in triplicate. The size distribution was characterized by the distribution parameters *d*10%, *d*50%, and *d*90%, and the distribution width expressed as 'span', which was calculated by the following equation:

$$\text{Span} = \frac{d90\% - d10\%}{d50\%} \quad (1)$$

In the case of very smaller size range found, determination of particle size and size distribution was achieved by photon correlation spectroscopy using a Malvern Autosizer 2C with Multi8 computing correlator type 7032 CN (Malvern Inc., Malvern, Great Britain), which detects the particle size in the range below 500 nm.

2.4.2.2. Entrapment efficiency. The amount of drug entrapped in hydrated microparticles was determined in the washed products. One milliliter of suspension was accurately pipetted and diluted with methanol in a series of dilutions to obtain an appropriate final concentration for analysis. The determination of drug content was performed using the HPLC system as already mentioned. All examinations were carried out in triplicate. The entrapment efficiency of the hydrated MCZ microparticles was calculated as follows:

$$\%\text{Entrapment efficiency} = \frac{\text{Amount of drug entrapped}}{\text{Amount of drug recoverd}} \times 100 \quad (2)$$

2.4.2.3. Drug leakage. The leakage of MCZ from hydrated microparticles under storage at 4 °C for 12 weeks was studied by using ultrafiltration membranes (Biomax-5; polyethersulfone membrane) at cut-offs of 5000 nominal molecular weight limit (NMWL) (Centricon Plus-20®, Millipore, USA). Five milliliters of hydrated microparticle suspension was accurately pipetted into an ultrafiltration tube and then centrifuged at 3000 × *g* for 45 min by the use of swinging-bucket centrifuge (Megafuge 1.0®, Heraeus Sepatech GmbH, Osterode, Germany). The amount of drug in the filtrate was analyzed by the HPLC system described above. The percentage of drug leakage from the hydrated microparticles was estimated according to the following equation:

$$\%\text{Leakage} = \frac{\text{Amount of drug leaked}}{\text{Amount of drug entrapped}} \times 100 \quad (3)$$

All measurements were carried out in triplicate.

2.4.2.4. Particle surface charge. The particle surface charge of the hydrated microparticles was estimated by means of a particle charge detector (PCD 03 pH, Mütek Analytic GmbH, Herrsching, Germany). A 1-mL sample was accurately pipetted into a teflon cylinder and the volume was adjusted by bidistilled water to be over the upper electrode. By van der Waals forces, the particles adsorbed on the teflon surface of the measuring cell and the piston located inside. When the piston was moved up and down with a frequency of 5 Hz, the potential was generated between the upper and lower electrodes. To determine the quantity of that potential, a polyelectrolyte of an opposite charge was titrated through the probe to neutralize the charge of particles. In the case of a negative charge, poly-(diallyldimethylammonium) chloride (PolyDADMAC) was used. For positively charged particles, sodium polyethylene sulfonate (Na-PES) was employed for the titration. All measurements were carried out in triplicate. The surface charge was calculated from the amount of titer solution with the following formula:

$$\text{Surface charge } (\sigma_0) = \frac{\text{Titer volume (mL)} \times 0.001\text{N} \times \text{Factor} \times 0.001 \times F(\text{C})}{\text{Sample volume (mL)}} \quad (4)$$

σ_0, the particle surface charge (C mL^{-1}); Titer volume, the volume of polyelectrolyte used for charge balance (mL); Factor, factor of titer solution (C mol^{-1}); *F*, Faraday constant (96,485 C); Sample volume, amount of sample pipetted into measuring cylinder (mL).

3. Results and discussion

3.1. Optimization of ASES conditions

3.1.1. Effect of temperature and pressure on the yield

The percentage of yield of microparticles in the precipitation chamber is one of the parameters indicating problems associated with extraction of the components used. The optimum conditions should lead to microparticle formation at an acceptable yield. Apart from partial extraction by SCD at a given condition, some loss of precipitates will inevitably occur since the particle separation is essentially a result of the gravitational force which is effective only for the large particles. Hence, during the sedimentation phase, some micronized particles precipitated could be lost with the circulating SCD. Additionally, when the experiment is performed by the laboratory-scale equipment, electrostatic phenomena can also limit the efficient recovery of the fine particles adhering to the vessel wall (Thiering et al., 2001; Bleich et al., 1994). As illustrated in Fig. 1, the suitable operating conditions for the phospholipid used should preferably be at the temperature below its phase transition temperature (<51 °C) and

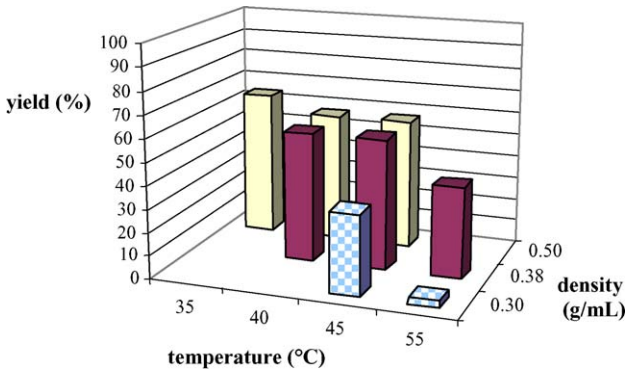

Fig. 1. Yield of PC collected from the precipitation chamber (percentage with respect to the initial amount being added into the ASES process).

the pressure at which the density of SCD is above 0.30 g mL^{-1} (ca. 57–64% yield). Production conditions above the phase transition temperature led to film formation at the column wall and thus to a reduction of the yield. If the same results are observed in terms of acceptable yields and microparticle appearances, the lower operating conditions (temperature and pressure) are more favorable. Therefore, the processing conditions for the following investigations and microparticle production were chosen at 35 °C and 8.0 MPa.

3.1.2. Effect of solute concentration

The SEM study of different solute concentrations (Fig. 2) demonstrated the appropriate concentration of solution which should be sprayed through a nozzle into the SCD. Generally the optimal concentration for spraying is limited by its apparent viscosity. Increasing concentration of solutes usually leads to larger and less spherical precipitates even though it results in a higher yield of the product. As the concentration of solute increased, the viscosity of the sprayed solution increased, acting as a stabilizing force on the jet. At high concentrations, the atomization forces were not sufficient to break up the liquid jet into droplets. The time scale for droplet formation was longer than the time scale for solute precipitation, resulting in fibers rather than in microparticles (Bodmeier et al., 1995). As a result, changes in the morphological properties of the microparticles will deter-

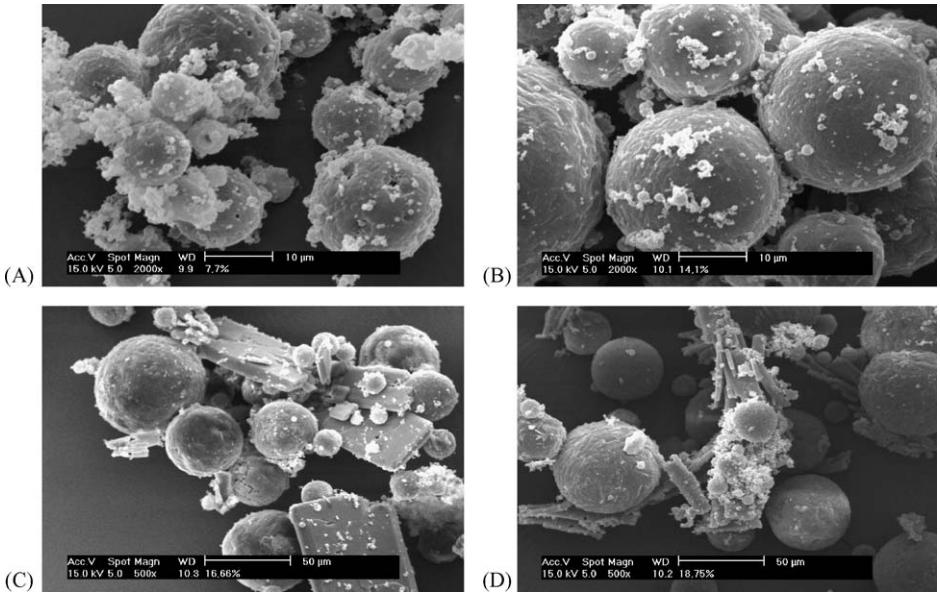

Fig. 2. SEM images of ASES-processed microparticles containing MCZ and PC (1:1, w/w) with addition of Polox (0.2%, w/w, of spraying solution) prepared at different total concentrations in the mixture of methanol and methylene chloride (2:8, w/w), at the chosen conditions of 35 °C and 8.0 MPa: (A) 7.7% (w/w), (B) 14.1% (w/w), (C) 16.7% (w/w), and (D) 18.9% (w/w).

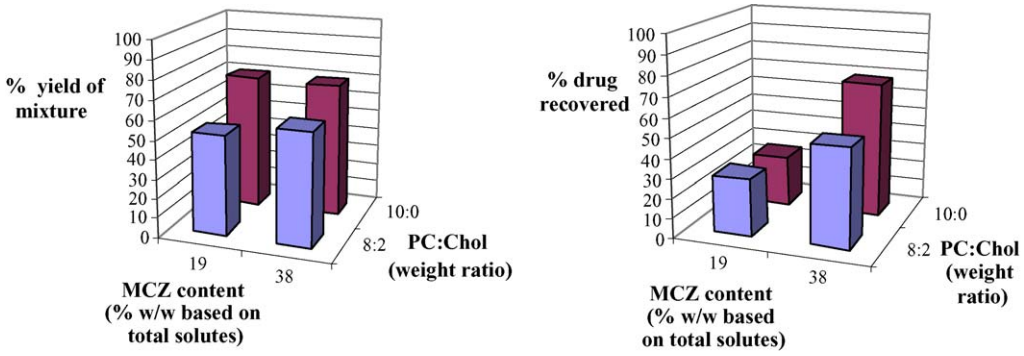

Fig. 3. Percentage of yield and drug recovered in dry ASES powder from spraying solutions containing various amounts of MCZ, PC, and Chol without Polox.

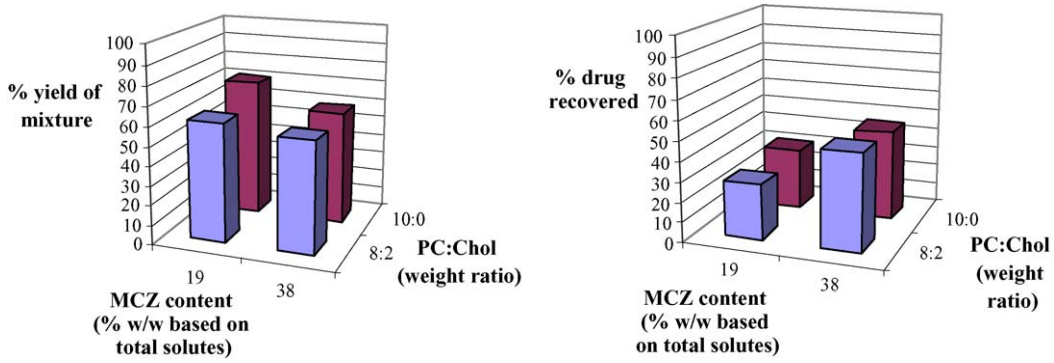

Fig. 4. Percentage of yield and drug recovered in dry ASES powder from spraying solutions containing various amounts of MCZ, PC, and Chol with Polox (10%, w/w, of total solutes).

mine the physical properties of the vesicles hydrated from them. In this study, raising concentrations of the spraying solution up to 16.7% (w/w) initiated cubic- and rod-like shape precipitation with some aggregation as shown in Fig. 2C and D. Thus, the proper concentration for the given mixture should be lower than this critical value (16.7%, w/w).

3.2. Preparation of dry ASES powder—effect of composition of spraying solution

As revealed in the optimization part, all preparations of dry ASES powder were produced via the ASES process at the conditions of $35 \pm 0.5\,^{\circ}C$ and 8.0 MPa with the solute concentration range between 8 and 15% (w/w), depending on the solubility of solute mixtures in the organic solvents used (2:8, w/w, of methanol and methylene chloride).

At these conditions, all compositions of spraying solution yielded microparticles in an acceptable range of about 50–70% (Figs. 3 and 4). As expected, solutions of higher MCZ level (38%, w/w) resulted in greater amounts of drug recovered from the ASES process, i.e. from 25–30 to 45–70%, as compared to those of lower MCZ level (19%, w/w), regardless of the other components involved. Polox addition seemed to have no significant effect on the percentage of drug recovered as was observed in the systems which contained Chol, but it imposed different effects in Chol-free systems depending on the level of MCZ. When adding Polox to a system with high MCZ-content, a lower amount of recovered drug was obtained (down from 69 to 45%) whereas in low MCZ-content system, a slight improvement of the percentage of drug recovered (from 25 to 31%) was found. These contradictions can be attributed to the difference in the surface area of sprayed droplets as reflected by the difference in the particle size distribution of dry ASES powder produced from spraying solutions with different compositions as shown in Fig. 5. The sprayed solution with high MCZ-content and 10% (w/w) Polox caused a tremendous reduction in droplet size due to the decrease in surface tension. This would probably enhance the partial extraction of the drug into SCD. Such behavior was also found to be the case for the addition of Chol, but with more aggregation of the produced microparticles (Fig. 5C).

Table 2
Residual content of methylene chloride and methanol in the ASES-produced microparticles with various compositions of spraying solution

Formulation no.	Compositions [MCZ (%)–(PC:Chol, w/w)–Polox (%)]	Residual solvents (ppm)	
		Methylene chloride	Methanol
A	19%–(10:0)–0%	<30	29
B	19%–(8:2)–0%	<30	15
C	19%–(10:0)–10%	<30	31
D	19%–(8:2)–10%	<30	50
E	38%–(10:0)–0%	<30	86
F	38%–(8:2)–0%	<30	23
G	38%–(10:0)–10%	<30	22
H	38%–(8:2)–10%	<30	42

Table 3
Particle size and %entrapment efficiency of MCZ microparticles hydrated at different pH's

Formulation no.	Compositions [MCZ (%)–(PC:Chol, w/w)–Polox (%)]	Particle size (μm) *d*50% (span)		%Entrapment efficiency[a]	
		pH 4.0	pH 7.2	pH 4.0	pH 7.2
A	19%–(10:0)–0%	2.7 (4.1)	5.3 (1.6)	34.7	75.1
B	19%–(8:2)–0%	7.7 (1.4)	4.8 (1.7)	11.8	97.8
C	19%–(10:0)–10%	3.2 (2.1)	3.7 (1.4)	16.2	60.3
D	19%–(8:2)–10%	6.7 (1.5)	9.4 (1.8)	10.2	58.9
E	38%–(10:0)–0%	N/D	4.7 (1.5)	10.6	68.2
F	38%–(8:2)–0%	3.2 (1.3)	3.6 (2.1)	12.0	82.3
G	38%–(10:0)–10%	3.1 (4.9)	4.4 (2.0)	10.1	80.7
H	38%–(8:2)–10%	3.2 (1.4)	6.1 (1.7)	4.0	100.2

N/D, not detectable by both light diffraction and light scattering techniques.
[a] Calculated based on total amount of drug recovered in the microparticles.

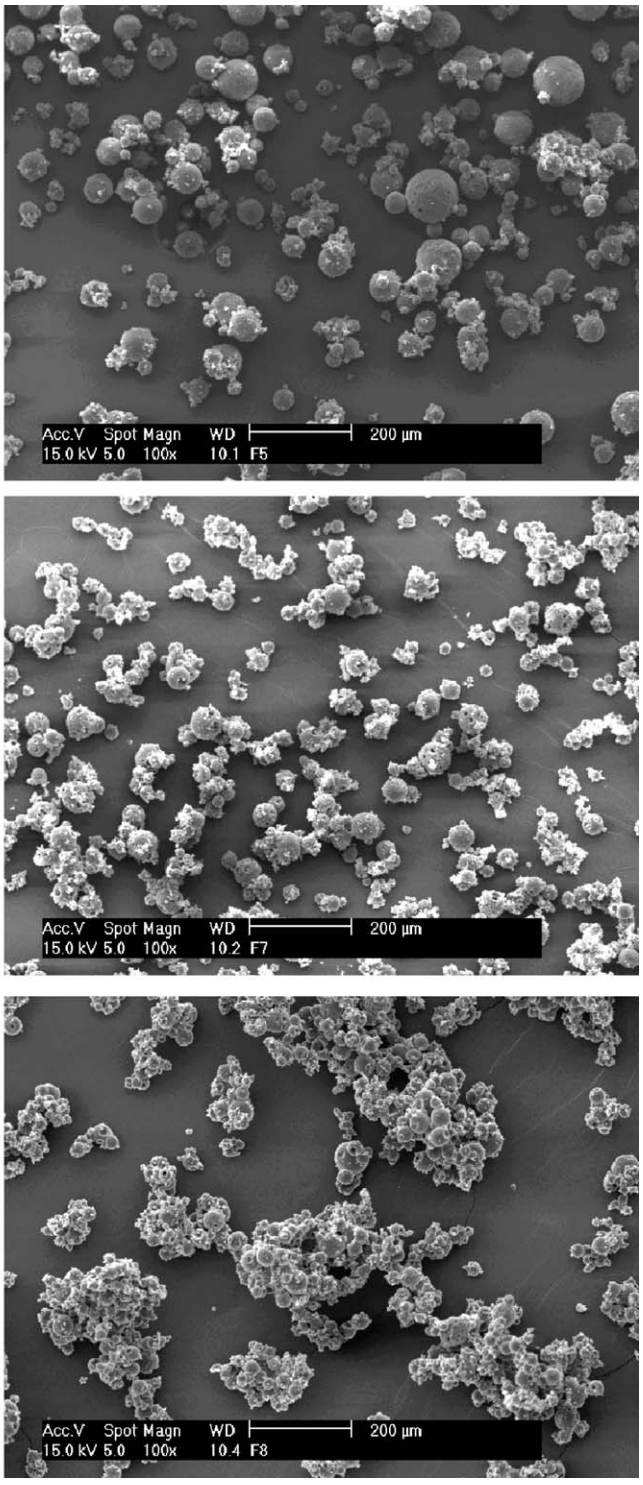

Fig. 5. SEM images of the ASES-produced microparticles with the various compositions of spraying solution [MCZ (%)–(PC:Chol, w/w)–Polox (%)]: (A) 38%–(10:0)–0%, (B) 38%–(10:0)–10%, and (C) 38%–(8:2)–10%.

An XRPD study on the ASES powder produced from various compositions of spraying solution (Fig. 6) revealed the partially crystalline state of the produced microparticles, with the exception of 38% MCZ, 8:2 (w/w) of PC to Chol without Polox (formulation F). However, as not determined by the main peaks of MCZ at 25.5°, 26.0°, 26.4°, 26.7°, and 27.4° 2θ in all cases, it can be concluded that MCZ was in the molecular distribution in the produced microparticles.

According to ICH guidance (1997) and USP 28 (2005), the limit residues of methylene chloride and methanol in the pharmaceutical products must not be more than 600 and 3000 ppm, respectively. In all cases, methylene chloride content was below the detectable limit of 30 ppm whereas methanol content was found to be lower than 86 ppm (Table 2). Accordingly, it can be concluded that residual content of the solvents used in the ASES process could dramatically be removed to an exceptionally low limit value.

3.3. Hydration of dry ASES powder—effect of pH of hydration medium

The particle size and entrapment efficiency of hydrated MCZ microparticles were clearly affected by the pH of the hydration medium as shown in Table 3. MCZ microparticles hydrated at pH 4.0 possessed a size range of 2.7–7.7 μm and an entrapment efficiency of 4–35% while those hydrated at pH 7.2 possessed larger particles (3.6–9.4 μm) with higher entrapment efficiencies (60–100%). The broad distribution width, indicated by 'span', of hydrated microparticles obtained from formulations A and G at pH 4.0 can be considered as the strong aggregation of some hydrated microparticles which were of incomplete vesicle formation. This also led to an inexact particle size determination of formulation E obtained by either light diffraction or light scattering techniques. The difference in the entrapment efficiency of microparticles hydrated at different pH's can be explained by a dissimilar degree of ionization of the drug. Low drug entrapment was attributed to the protonation of imidazole group of MCZ molecules at pH 4.0, causing molecular repulsion and an increased solubility in the aqueous phase during hydration. At low MCZ-content, the highest encapsulation efficiency was found in hydrated microparticles with 8:2 (w/w) of PC to Chol and hydrated at pH 7.2. At higher drug level, the maximum entrapment efficiency was observed with the same weight ratio of PC to Chol, however, containing 10% (w/w) Polox. Therefore, Polox appeared to play an important role in the hydrated

Table 4
Percent leakage of MCZ from hydrated microparticles produced via ASES process with various compositions of spraying solution and different pH's of hydration medium after storage at 4 °C for 12 weeks

Formulation no.	Compositions [MCZ (%)–(PC:Chol, w/w)–Polox (%)]	%Drug leakage[a]	
		pH 4.0	pH 7.2
A	19%–(10:0)–0%	N/D	N/D
B	19%–(8:2)–0%	0.33	N/D
C	19%–(10:0)–10%	2.03	N/D
D	19%–(8:2)–10%	N/D	N/D
E	38%–(10:0)–0%	N/D	N/D
F	38%–(8:2)–0%	0.87	N/D
G	38%–(10:0)–10%	2.70	N/D
H	38%–(8:2)–10%	N/D	N/D

N/D, not detectable.
[a] Calculated based on the amount of drug entrapped in the hydrated microparticles.

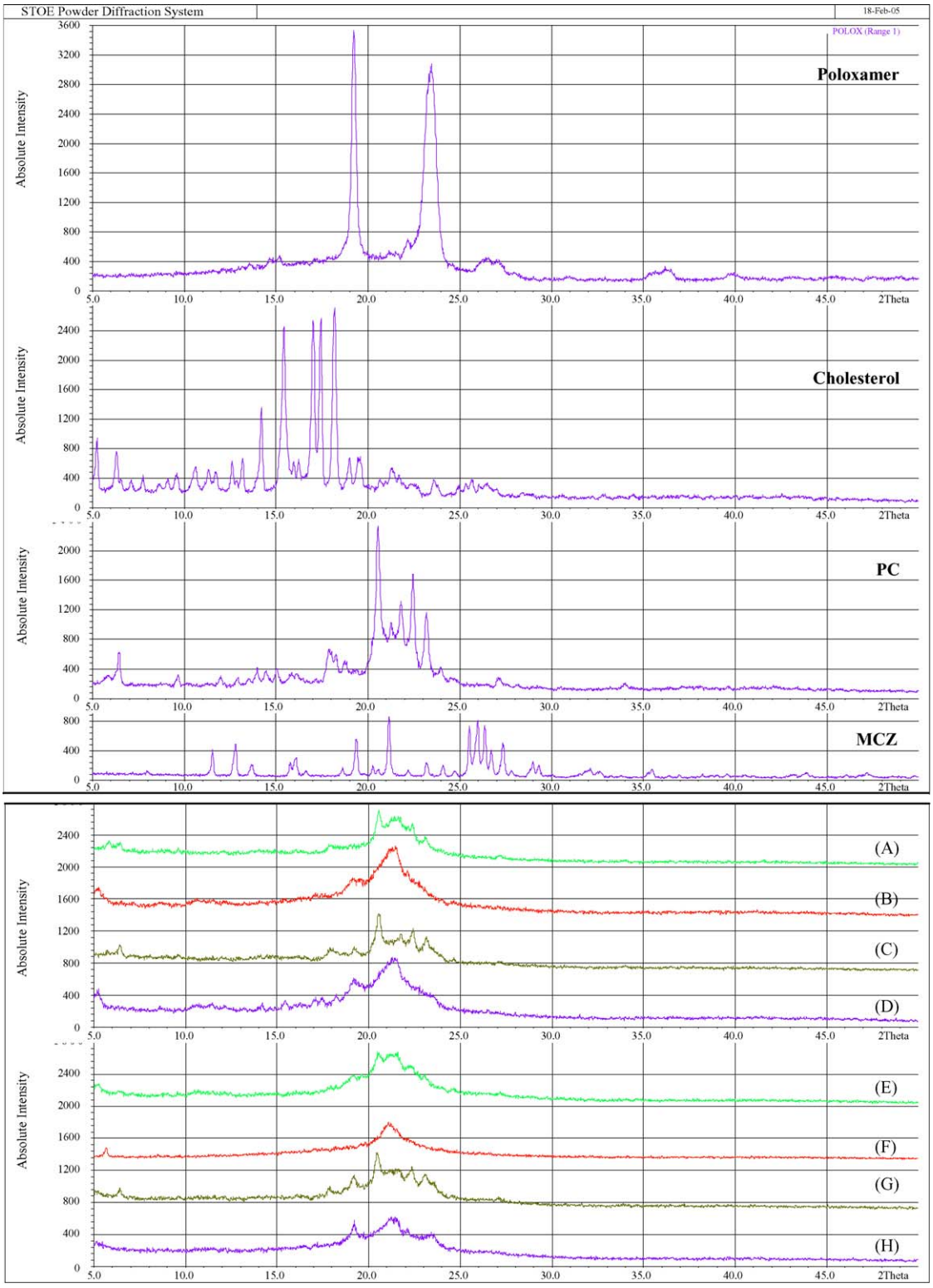

Fig. 6. XRPD patterns of pure substances and the ASES-produced microparticles with the various compositions of spraying solution [MCZ (%)–(PC:Chol, w/w)–Polox (%)]: (A) 19%–(10:0)–0%, (B) 19%–(8:2)–0%, (C) 19%–(10:0)–10%, (D) 19%–(8:2)–10%, (E) 38%–(10:0)–0%, (F) 38%–(8:2)–0%, (G) 38%–(10:0)–10%, and (H) 38%–(8:2)–10%.

microparticles containing high MCZ-content with respect to the higher encapsulation efficiency.

As shown in Table 4, there was an insignificant amount of drug released from the hydrated microparticles in all cases after storage at 4 °C for 12 weeks. This behavior could presumably be due to the high phase transition temperature of the PC used (Phospholipon® 90H) (51 °C), whose main fatty acids are composed of stearic acid (86%) and palmitic acid (14%) (data from

the company). Thus, these formulations may be suitable for drug delivery in biological systems.

The particle surface charges of the ASES-produced microparticles hydrated at pH 4.0 and 7.2 were detected as positive and negative charges, respectively, but with only very low potential. Therefore, the hydrated microparticles were obtained as nearly neutral particles.

4. Conclusion

The optimal supercritical gas conditions for preparation of liposomes in a dry state using ASES process must be primarily at the temperature below phase transition temperature of the phospholipid used and the pressure at which the moderate density of SCD (>0.30 to 0.50 g mL^{-1}) was achieved. The amount of drug recovered in the produced microparticles could significantly be improved by increasing the amount of drug being added at a constant total lipid content. The presence of either Chol or Polox could modify the physico-chemical properties of the produced microparticles and accordingly the liposomal vesicles. The drug entrapment efficiency as well as particle size of liposomes hydrated from the produced microparticles were found to increase with the increasing pH of hydration medium.

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