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The properties of bufadienolides-loaded nano-emulsion and submicro-emulsion during lyophilization

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Abstract

The aim of this study was to prepare two types of emulsions, bufadienolides-loaded nano-emulsion (BU-NE) and submicro-emulsion (BU-SE) which were separately prepared by ultrasonic-high-pressure homogenization (UHPH) and high-pressure homogenization (HPH) methods, and to try to stabilize the colloid systems by lyophilization. The lyoprotective effects of cryoprotectant carbohydrates during the freeze-thawing and freeze-drying cycles on the emulsions were investigated in detail. The lyophilized products were characterized with regard to their appearance and particle size by transmission electron microscopy (TEM), scanning electron microscopy (SEM), photon correlation spectroscopy (PCS) and zeta potential. The median diameter, polydispersity index (PI) and zeta potential of BU-NE and BU-SE were 43.5 nm versus 161.4 nm, 0.100 versus 0.143 and −19.7 to −26.2 mV versus −29.4 to −35.3 mV, respectively. With the same drug content (0.28 mg mL^{-1}) BU-SE exhibited a higher entrapment efficiency than BU-NE. The optimum cryoprotectant for BU-NE and BU-SE was maltose (20%) and trehalose (20%), respectively. The median diameters (95.7 and 168.5 nm) of the rehydrated BU-NE and BU-SE were slightly increased. For both of them, the bufadienolides entrapment efficiency was reduced whereas the drug content was not. The lyophilized BU-NE and BU-SE powders were stable over a period up to 3 months with no change in visual appearance, reconstitution ability, particle size distribution and drug concentration. This shows that freeze-drying could be a promising method to stabilize the emulsions.

Keywords: Bufadienolides; Nano-emulsion; Submicro-emulsion; Cryoprotectant; Lyophilization

1. Introduction

Over the last few decades, emulsions (Seki et al., 2004), liposomes (Maurer et al., 2001) and nanoparticles (De Chasteigner et al., 1996) have been attracting increasing attention as drug delivery systems for poorly water-soluble drugs because of their drug targeting effect and therapeutic benefit (Juliano, 1988). Commercially available formulations are still limited in number compared with other oral dosage forms. However, aggregation, fusion, phospholipid hydrolysis and leakage of the encapsulated drugs may occur during a long period of storage in an aqueous medium.

Freeze-drying appears as one of the most suitable methods to stabilize and improve the handling of colloidal systems (Seki et al., 1997; Franks, 1998), and this consists of nucleation, freezing and a subsequent sublimation process. The separation of water from a solution or a dispersion of given concentration in the form of a solid phase, ice, and the subsequent removal of water by vacuum sublimation, leaves the solutes or substrates in their anhydrous or almost anhydrous states. On the other hand, colloidal carriers are not easily lyophilized, due to several forms of damage, including drug leakage, fusion, and lateral phase separation. This fact has been discussed in many papers (Van Winden and Crommelin, 1999; Komatsu et al., 2001; Wang et al., 2006) mainly with regard to liposomes. The addition of sugars as cryoprotectants has been demonstrated to be effective in maintaining the initial formulation characteristics (Miyajima, 1997).

Toad venom, a traditional Chinese medicine, is prepared from the dried white secretion of the auricular glands and the skin glands of Chinese toads (*Bufo melanostictus Schneider* or *Bufo bufo gargarzinas Gantor*). The principal biological active components of toad venom are bufadienolides, a class of C-

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24 steriods with a characteristic α-pyrone ring at C-17 (Krenn and Kopp, 1998). The major bufadienolides from toad venom include bufalin (B), cinobufagin (C), and resibufogenin (R). Recently, bufadienolides have been shown to exert potent antitumor and analgesic activity (Hashimoto et al., 1997; Efferth et al., 2002; Yeh et al., 2003). In previous studies, bufadienolides were shown to be poorly water-soluble and exhibit pH-dependent distribution between aqueous phase and organic solvent, which make them excellent candidates for emulsions. In the present work, two kinds of o/w emulsions—nano-emulsions (NE) and submicro-emulsions (SE) were prepared by different methods. The drug was incorporated into the lipid phase or lecithin layer at the oil–water interface and the incorporated drug was sequestered on direct contacting with body fluids and tissues (Singh and Ravin, 1986).

In this study, the suitability of several cryoprotectant carbohydrates (trehalose, maltose, glucose, mannitol, sucrose, lactose and fructose) has been investigated to preserve the structure of NE and SE in comparison with an established freeze-drying process (Saleki-Gerhardt and Zografi, 1994). The particle size distribution (PSD) and zeta potential of samples both before and after lyophilization were assessed by photon correlation spectroscopy (PCS) and electrophoretic light scattering (ELS). The transmission electron microscope (TEM) photos of the emulsions before and after lyophilization and the scanning electron microscope (SEM) micrographs of the lyophilized products were also described.

2. Materials and methods

2.1. Materials

Bufadienolides were extracted from toad venom by staff at the Department of Pharmaceutics, Shenyang Pharmaceutical University, China. The bufadienolide mixture mainly consisted of bufalin (B), cinobufagin (C) and resibufogenin (R) in a ratio of 7:9:11.

Lipoid E-80® was purchased from Lipoid (Ludwigshafen, Germany). The Lipoid E-80®, according to manufacturer's specifications, consisted of 80% phosphatidylcholine, 8% phosphatidylethanolamine, 3.6% nonpolar lipids and about 2% sphingomyelin. Soybean oil was obtained from Tieling BeiYa Pharmaceutical Company (Liaoning, China). Pluronic F68 was obtained from BASF AG (Ludwigshafen, Germany). Sodium oleate, glycerin and Tween-80 were obtained from Dongshang Co., Ltd. (Shanghai, China), Suichang Glycerin Company (Zhejiang, China), Shenyu Medicine and Chemical Industry Company (Shanghai, China), respectively. Trehalose dihydrate was purchased from Sinozyme Biotechnology Co., Ltd. (Nanning, China). Mannitol, maltose, glucose, lactose, fructose and sucrose were purchased from Tianjin Bodi Chemicals Co., Ltd. (Tianjin, China). All other chemicals were of analytical or chromatographic grade.

2.2. Preparation of bufadienolides-loaded nano-emulsion (BU-NE)

BU-NE was prepared by an ultrasonic-high-pressure homogenization (UHPH) method. Bufadienolides were dissolved in soybean oil at 75 °C with magnetic stirring for 30 min, then the oil phase was filtered through a 0.45 μm membrane. Lipoid E-80®, Pluronic F68, Tween-80, glycerin and sodium oleate were dispersed in distilled water under the same conditions as the oil phase. Then, the oil phase was added dropwise to the water phase and stirred magnetically. A coarse emulsion was prepared by high shear mixing (FJ-200, Shanghai sample model factory, Shanghai, China) at 10,000 rpm for 10 min followed by ultrasonication at an intensity of 40 W, at 4 °C for 60 min using a high intensity ultrasonic processor (ultra-cell 750 W, Sonics Materials Inc., USA). Then, the volume was adjusted to 100 mL with double-distilled water and the pH was adjusted to 6.8 with 0.1 mol L^{-1} HCl. High-pressure homogenization was performed using a homogenization apparatus (NS10012K; Niro Soavi, Italy) at 40 °C applying 120 MPa for 8 cycles. BU-NE was sterilized by filtration through a 0.22 μm membrane and then transferred to vials, and stored at 4 °C. The drug-free NE was produced by the same procedure without drug incorporation.

2.3. Preparation of bufadienolides-loaded submicro-emulsion (BU-SE)

BU-SE was prepared using the high-pressure homogenization (HPH) method. The coarse emulsion was prepared by the same procedure as BU-NE. The differences were as follows: Lipoid E-80® was added to the filtered BU-loaded oil phase; the final BU-SE was obtained by passing the coarse emulsion directly through a high-pressure homogenizer without sonication; The homogenization conditions were 40 °C, and 70 MPa for 8 cycles; BU-SE in vials was sterilized on a water bath at 100 °C for 30 min and also stored at 4 °C. The basic formulae of BU-NE and BU-SE used in this study are shown in Table 1.

2.4. Freeze-thawing procedure

The BU-NE and BU-SE samples were diluted with high concentration of cryoprotectant solutions. Different cryoprotectants were added in increasing amounts to assess the optimum concentration (Table 2). Then samples containing cryoprotectant with

Table 1
The basic formulae of BU-NE and BU-SE

	Bufadienolides (mg mL^{-1})	Soybean oil (mg mL^{-1})	Lipoid E-80® (mg mL^{-1})	Pluronic F68 (mg mL^{-1})	Tween-80 (mg mL^{-1})	Sodium oleate (mg mL^{-1})	Glycerin (mg mL^{-1})
BU-NE	0.28	20	12	5	2	1	22.5
BU-SE	0.28	100	12	0	2	1	22.5

Table 2
List of cryoprotectants and the concentrations used in the freeze-thawing and freeze-drying of BU-NE and BU-SE

Cryoprotectant	Freeze-thawing[a] (concentration%)	Freeze-drying (concentration%)	
		BU-NE	BU-SE
Maltose	5, 10, 20	10, 15, 20	10, 15, 20
Glucose	5, 10, 20	10, 15, 20	10, 15, 20
Sucrose	5, 10, 20	10, 15, 20	10, 15, 20
Trehalose	5, 10, 20	10, 15, 20	10, 15, 20
Mannitol	5, 10		5, 10
Lactose	4, 8		4, 8
Fructose	5, 10, 20		10, 15, 20

[a] The data of the freeze-thawing test were for both BU-NE and BU-SE.

different concentrations were obtained. The samples were frozen in a refrigerator at −20 °C for 24 h and then thawed at room temperature prior to the evaluation of their visual appearance and particle size distribution. All the samples were investigated after undergoing 5 cycles.

2.5. Freeze-drying procedure

The cryoprotectants used in the freeze-drying procedure were chosen following the freeze-thawing studies. The NE and SE solutions were diluted with the cryoprotectant solutions to give different concentrations of cryoprotectants (Table 2). The lyophilization was carried out in a lab freeze-dryer (FDU-1100, EYELA, Japan). The samples were freeze-dried in 10 mL semi-stoppered vials with the height of the samples being lower than 1.0 cm (2 mL). The freeze-drying technology was as follows: freezing at −70 °C for 12 h in a super-cold refrigerator; primary drying at −35 °C for 4 h; then, the shelf temperature was raised to −20 °C for 13 h; secondary drying at 20 °C for 5 h. The chamber pressure was maintained at 13.1 Pa and the temperature of the cold trap was −50.1 °C during the whole freeze-drying procedure. Finally, the vials were sealed with rubber caps and stored at 4 °C until analysis.

2.6. Bufadienolides assay

The high-performance liquid chromatography (HPLC) system (Jasco) consisted of an autosampler (AS-1555), four pumps (PU-1580), and a UV-VIS detector (UV-1575), all interfaced with ChromPass software along with a C18 reversed-phase column (HiQ Sil, 250 mm × 4.5 mm, i.d. 4 μm; KYA TECH Corporation; Japan) and a precolumn (Fusion-RP 4 mm × 3.0 mm). For the analysis of bufadienolides, the eluent was a 45:55 (v/v) mixture of acetonitrile:water (pH 3.2) containing 0.5% potassium dihydrogen phosphate. The UV detector was set at 296 nm with the column temperature was 40 °C. The flow rate was 1.0 mL min^{-1} and 20 μL samples were injected.

BU-NE and BU-SE were subjected to ultracentrifugation (Hitachi CS120GXL Micro Ultracentrifuge, Japan) at 46,000 rpm for 4 h at 4 °C. Polyallomer tubes were used and their bottoms were pricked after centrifugation with a syringe needle to collect the aqueous phase. Creamed oil and pelleted materials could then be collected without further washing steps. The concentrations of bufadianolides in the aqueous phases were measured by HPLC.

2.7. Determination of particle size and zeta potential

The median diameter and polydispersity index (PI) of samples both before and after freeze-drying were assessed by photon correlation spectroscopy (PCS, dynamic light scattering, DLS) using a Nicomp™ 380 submicron particle sizer (Particle Sizing System, Santa Barbara, USA) at 25 °C. Each sample was diluted 1:5000 immediately before measurement with doubly distilled water. It has been verified that the dilution of samples did not alter the size distributions obtained (Müller et al., 2004). The cumulative distribution of the particle size was obtained at the same time with intensity-weighting, volume-weighting and number-weighting while the value of the PI showed the width of the distribution. The particle size analysis data were evaluated using the volume-weighting pattern and these were of median diameter.

The zeta potential of the emulsion particles was determined using the Nicomp™ 380 by electrophoretic light scattering (ELS). The ELS technique was based on the scattering of light from particles that move in liquid under the influence of an applied electric field. The value of the mean zeta potential was obtained from the electrophoretic mobility μ which was computed from the measured Doppler shift $\Delta\nu$, for a given applied electric field strength E of 15 V/cm. Values reported were the mean value for triplicate samples. Samples that were considered as fully aggregated were not measured.

2.8. Reconstitution of freeze-dried products

After freeze-drying, the products can be reconstituted by addition of purified water. The rehydration was observed visually as the vials were gently agitated by hand, leading to the formation of the aqueous liquid BU-NE and BU-SE for further characterization.

2.9. Transmission electron microscopy (TEM)

The morphological observations of BU-NE and BU-SE before and after lyophilization were performed using an Electronic Transmission Microscope (JEM-100CX/II, JEOL Ltd., Japan). The samples, after being diluted with purified water, were placed over a copper grid coated with carbon film and air-dried. Then they were stained with 2% phosphotungstic acid. Finally, the samples were air dried prior to placing them in the TEM instrument for analysis.

2.10. Scanning electron microscopy (SEM)

A Shimadzu SSX-500 field emission scanning electron microscope was used to obtain SEM micrographs of the lyophilized products, Samples were fixed on an SEM-stub using conductive double-sided tape and then made electrically conductive by coating in a vacuum with a thin layer of gold/palladium. An accelerating voltage of 15 kV was used.

2.11. Residual moisture

The residual moisture of the lyophilized substance was determined by thermogravimetric analysis (TGA) using TGA 50 (Shimadzu Co., Japan). Samples (2.00–4.00 mg) were placed on the sample pan under nitrogen. Each sample was equilibrated at 25 °C and then data were collected using a heating rate of 10 °C min^{-1} between 25 and 200 °C. The moisture content was determined as the weight loss between 50 and 150 °C. The amounts of residual moisture in all lyophilized cakes were determined.

3. Result and discussion

3.1. Physicochemical characteristics of BU-NE and BU-SE

In the present work, NE and SE were colloidal particles of vegetable oils and lecithin for intravenous administration. The amount of drug (0.28 mg mL^{-1}) did not change after sterilization by filtration or autoclaving in BU-NE or BU-SE. The entrapment efficiencies of bufalin (B), cinobufagin (C) and resibufogenin (R) incorporated into SE were 81.5% (w/w), 90% (w/w) and 92.0% (w/w), separately. For BU-NE, the corresponding values were only 62.2% (w/w) for B, 68.7% (w/w) for C, and R 71.5% (w/w). Due to the differences in formula and preparation processes of NE and SE, the major physical difference between them was the particle size. Thus, BU-NE appeared to be translucent, while BU-SE appeared milky. The PSD, PI and zeta potential of BU-NE and BU-SE were 43.5 ± 13.8 nm versus 161.4 ± 73.3 nm, 0.100 versus 0.143 and −19.7 to −26.2 mV versus −29.4 to −35.3 mV, respectively. NE had outstanding advantages: great stability and higher vascular permeability at target sites by diffusion due to its very small particle size distribution, approximately 20–100 nm (Fig. 1). Furthermore, the system was considered to be appropriate to avoid renal excretion and nonspecific capture by the reticuloendothelial system (Seki et al., 1994; Fukui et al., 2003). Surprisingly, although the small size of these drug carriers was likely to maximize their targeting ability, only a few studies have addressed the issue of the small particle size effect.

In a preliminary study, the factors in the formulation and preparation process influencing the PSD of NE and SE were investigated in detail. The basic formulae of BU-NE and BU-SE are shown in Table 1. It is generally known that the mean particle size depends on the ratio of oil to emulsifier, and it is necessary to increase the ratio of the emulsifier to prepare smaller particles (Ishii et al., 1990). A sub-100 nm BU-NE could be obtained by soybean oil (2%) as the oil phase, Lipoid E-80®/Pluronic F68 (1.2/0.5%) as emulsifiers, and Tween-80/sodium oleate (0.2/0.1%) as coemulsifiers. The mixed interfacial film of Lipoid E-80® and Pluronic F68 has been proved to be strong enough to prevent coalescence of oil droplets (Elbaz et al., 1993). Lipoid E-80® was able to dissolve in the hot oil, but could not dissolve in the water. The amount (2%) of soybean oil was so small that Lipoid E-80® could not completely dissolve in the oil phase at 75 °C. So in the preparation of BU-NE, Lipoid E-80® was dispersed in the water phase uniformly. The small particle size of BU-NE was achieved using a sonication process (at an intensity of 40 W, 4 °C for 60 min) combined with high homogenization. The process involved shear, impact and cavitation forces, thus, the emulsifiers, Lipoid E-80® and Pluronic F68 could form the interfacial layer of the emulsion which stays around the oil droplets. In the BU-SE formula, the soybean oil content was 10% and the emulsifier was Lipoid E-80® (1.2%). In the preparation process, the Lipoid E-80® could all dissolve in the soybean oil. The homogenization condition was 70 MPa for BU-SE which was different from BU-NE (120 MPa). During this process the soybean oil droplets were stabilized by the interfacial layer which was composed of Lipoid E-80® (Roland et al., 2003). In addition, sub-100 nm particles had the advantage of being able to undergo sterilization by membrane filtration (Konan et al., 2002). However BU-SE was sterilized on a water bath at 100 °C for 30 min, which could increase the instability of the system.

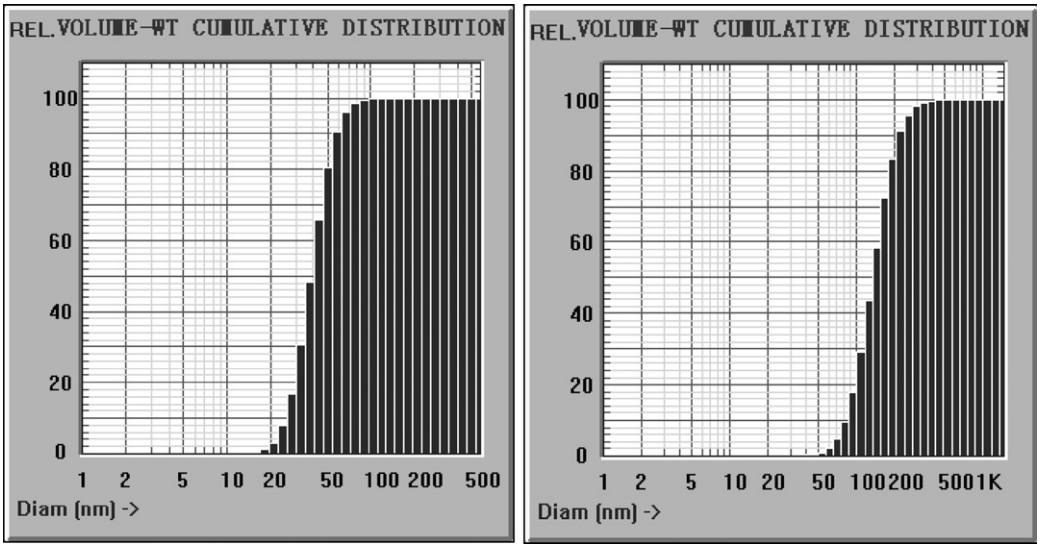

Fig. 1. Pictures of BU-NE (left) and BU-SE (right) with particle size distribution of 43.5 ± 13.8 and 161.4 ± 73.3 nm, respectively.

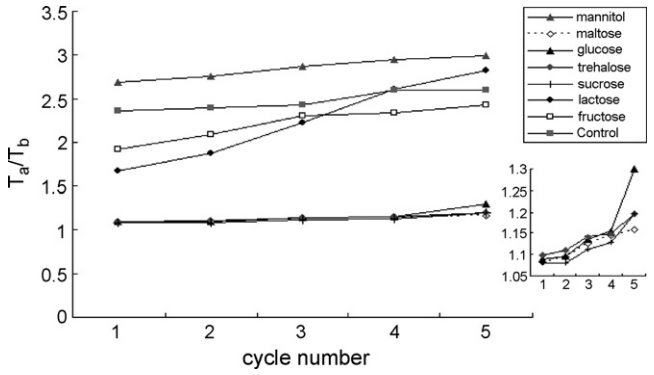

Fig. 2. The change of particle size of BU-NE in freeze-thawing test (the concentration of each cryoprotectant in the figure, i.e. the most suitable concentration, was as follows: 10% mannitol, 20% maltose, 20% glucose, 20% trehalose, 20% sucrose, 8% lactose, 20% fructose).

3.2. Effect of freeze-thawing on BU-NE and BU-SE

To avoid too lengthy freeze-drying process, a freeze-thawing experiment was conducted to select the cryoprotectants. The cryoprotectants were added in increasing amounts to determine the most suitable concentration (Table 2).

As shown in Fig. 2, T_b and T_a represent the median diameter of the BU-NE before and after freeze-thawing. The median diameter of BU-NE after freeze-thawing increased significantly in comparison with the initial particle size (T_a/T_b). BU-NE without cryoprotectant fully aggregated upon storage of the frozen samples for 5 cycles at −20 °C. In the presence of sucrose, maltose, trehalose and glucose, only moderate particle aggregations were observed. The concentration of them between 10 and 20% proved to be suitable. Fructose and lactose were ineffective in protecting BU-NE during the freeze-thawing experiment as the appearance of the reconstituted samples was not translucent without the Tyndall effect. Mannitol produced serious particle damage and, thus the appearance of the BU-NE changed with macroscopic and irreversible aggregation. Similar levels of aggregations were observed in all mannitol-containing samples following five freeze-thawing cycles. No variations in the PSD of the BU-SE were observed for all sugars tested during freeze-thawing. In the absence of a cryoprotectant the particle size was also not significant changed. The increase in the mean particle size and the polydispersity index (PI) were negligible when thawing the frozen BU-SE (data not shown).

Therefore sucrose, maltose, trehalose and glucose were chosen for the lyophilization study of BU-NE. For BU-SE, all the cryoprotectants were investigated during the lyophilization process. The freeze-thawing experiment showed that lyophilization technology might be suitable for the production of dried BU-NE and BU-SE.

3.3. Effect of freeze-drying on BU-NE and BU-SE

3.3.1. Methods of adding cryoprotectants

In the pretest, three methods (A, B and C) were compared when adding the cryoprotectant to the BU-NE or BU-SE solution. (A) Adding the cryoprotectant to the water phase of BU-NE or BU-SE in the preparation process. This will increase the viscosity of the system with the increasing PI being observed. The conformation of the particles may be changed in the system. (B) Dissolving equal solid sugars directly into the final BU-NE or BU-SE solution. However, some pooly water-soluble sugars, such as mannitol and lactose, needed ultrasound, which would increase the particle size and induce leakage of the drug. (C) The BU-NE or BU-SE solution was diluted with a high concentration of cryoprotectant solution. When using this method, cryoprotectants showed better protective effect, and it was much easier to perform this in the laboratory. So, method (C) was chosen. The sample pH was 6.8–7.0 in the case of using sugars as cryoprotectants.

Table 3
Effect of different cryoprotectants on the characteristics of freeze-dried BU-NE and BU-SE

Cryoprotectant[a]	Appearance[b]		Rehydrated appearance[c]		Reconstitution velocity[d]	
	NE	SE	NE	SE	NE	SE
None	−−	−−	○○	○○	3	3
Trehalose (20%)	++	++	●	●●	1	1
Glucose (15%)	−	−	○	○	2	2
Mannitol (15%)	++	++	○○	○○	1	1
Maltose (20%)	++	++	●●	●	1	1
Lactose (8%)	+	+	○	○	2	2
Sucrose (20%)	−	−	●●	●	2	2
Fructose (15%)	−	−	○	○	2	2

[a] The optimum concentration of each cryoprotectant.
[b] ++: Best, brittle and snow-like, smooth full; +: better, a little shrinkage and rugged; −: bad, collapse, rather porous; −−: worse, serious shrinkage to slice stick on the bottom.
[c] ●●: Similar to the original sample; ●: similar to the original sample with a Tyndall effect; ○: not translucent for NE or not opalescent for SE without a Tyndall effect; ○○: macroscopic and irreversible aggregation.
[d] 1, 2 and 3 represent the minutes required for rehydration with manual shaking.

3.3.2. Redispersibility of freeze-dried BU-NE and BU-SE

After freeze-drying, BU-NE and BU-SE were first evaluated with regard to the appearance of the lyophilized products and the reconstituted solution (Table 3), with the reconstituted methods and rehydrated media being of secondary consideration.

Redispersion of the lyophilized products was studied by manual shaking, vortexing and sonication in a water bath. There was a significant increase in particle size when the products were reconstituted by sonication in a water bath and this was a less convenient method for clinical application. The PCS measurement showed that there was no difference between the PSD of BU-NE or BU-SE obtained by manual shaking and vortexing. The manual shaking method, which was rapid and easy to perform, was used in our test.

The velocities of reconstitution were also different in the presence of different cryoprotectants (Table 3). Trehalose and maltose formulations exerted a rapid hydration velocity (less than 1 min), the lyophilized products possessed excellent features of loose and could be very easily and rapidly rehydrated. Sucrose and glucose formulae dissolved in 2 min but then dramatically collapsed. It was difficult to reconstitute the mannitol

formula uniformly. Although the freeze-dried cakes appeared good, the particles were completely aggregated.

For clinical use, reconstitution was carried out in different media including purified water, 0.9% NaCl and 5.4% glucose. The particle size distribution and zeta potential were determined after 0.5, 1, 2, 4, 8 and 12 h. The average particle size and zeta potential of BU-NE and BU-SE were not statistically significantly affected by the three media up to 12 h. In the experiment, the freeze-dried products were all reconstituted with purified water.

3.3.3. Effect of cryoprotectants during lyophilization

In present work, various saccharides have been investigated with regard to their ability to stabilize BU-NE and BU-SE. The appearances of lyophilized BU-NE and BU-SE before and after reconstitution were evaluated and compared with the reference unfrozen samples that were initially free of aggregates and displayed a Tyndall effect (Table 3). PSD measurements were also carried out but only for those formulations with a Tyndall effect. As shown in Fig. 3, S_b and S_a, P_b and P_a represented the median diameter and the polydispersity index of the emulsions

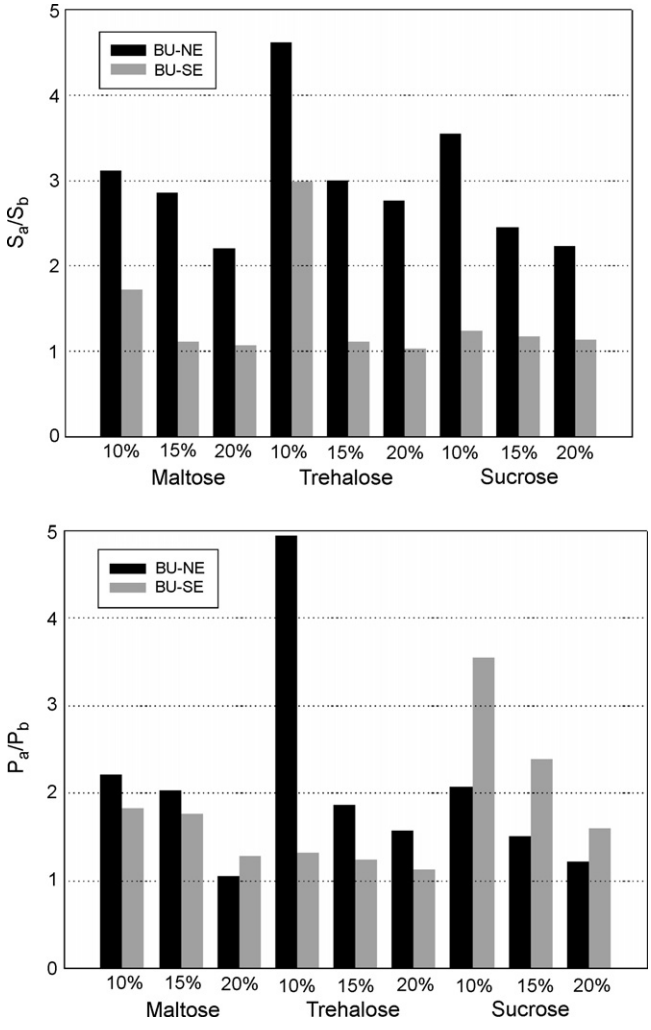

Fig. 3. The changes of particle size and PI for BU-NE and BU-SE during freeze-drying, separately.

before and after freeze-drying. The final-to-initial median diameter ratio (S_a/S_b) and polydispersity index ratio (P_a/P_b) were calculated to evaluate the effect of the cryoprotectants.

The results for BU-NE are shown in Fig. 3. Addition of maltose (20%), sucrose (20%) and trehalose (20%) was found to prevent permanent particle aggregation, while glucose was not effective in the freeze-drying process. As the S_a/S_b index shows, sucrose (2.23) and maltose (2.20) were more effective than trehalose (2.76) in preventing the particles of BU-NE from aggregating during lyophilization, but the freeze-dried BU-NE containing sucrose appeared highly collapsed after freeze-drying, This may be due to the fact that the product temperature during sublimation was well above the collapse temperature of the sample or partly as a consequence of the relatively high T_g of trehalose and maltose in the pure state compared with the other disaccharides (MacFarlane et al., 2002). According to the appearance and PSD of the lyophilized BU-NE, the protective efficiency of the cryoprotectants used alone was evaluated using maltose, trehalose and sucrose. These excipients also proved to be the most efficient at the same concentration (20%). Paradoxically, particle aggregation increased at higher cryoprotectant concentrations during lyophilization. Samples containing trehalose/sucrose and maltose/sucrose mixtures with varying compositions did not produce a better cryoprotective effect.

As for the BU-SE, freeze-drying gave somewhat different results (See Fig. 3). Substantial aggregates were observed after freeze-drying in the absence of cryoprotectant. The effect of different cryoprotectants (glucose, fructose, mannitol, sucrose, lactose, trehalose and maltose) on BU-SE has been evaluated. The presence of a certain amount of amorphous disaccharides during freeze-drying was found to be a critical factor for rehydrated BU-SE. Trehalose and maltose have been shown to act as good cryoprotectants, allowing the reconstitution of BU-SE. For trehalose and maltose formulations, acceptable particle sizes for intravenous injection were obtained, and their PSD values were only slightly increased from that of the original BU-SE. Their S_a/S_b and P_a/P_b indexes were 1.04 versus 1.07 and 1.12 versus 1.27, respectively. The initial BU-SE appeared milky. The effect of cryoprotectants in the different formulae was difficult to compare visually. From PCS data, the polydispersity index (PI) needs close attention. The PI of the original BU-SE was 0.143. Although the S_a/S_b indexes of rehydrated BU-SE containing sucrose (10, 15 and 20%) were below 1.5, the P_a/P_b indexes were all above 1.6, which means that the PI values of rehydrated BU-SE were all above 0.228. The PI was zero for monodisperse particles, whereas parenteral fat emulsions are typically in the range 0.10–0.20 (Müller et al., 1991). Thus, the formulation containing sucrose was not uniform. The rehydrated BU-SE containing mannitol, glucose, fructose, and lactose showed no Tyndall effect. These sugars were ineffective in preserving BU-SE during freeze-drying.

As shown in Fig. 3, the optimum cryoprotectant for BU-NE and BU-SE was maltose (mBU-NE) and trehalose (tBU-SE), separately. The S_a/S_b indexes of mBU-NE (20% maltose) and tBU-SE (20% trehalose) were about 2.20 and 1.04. This indicated that the change of particle size of BU-NE was larger than

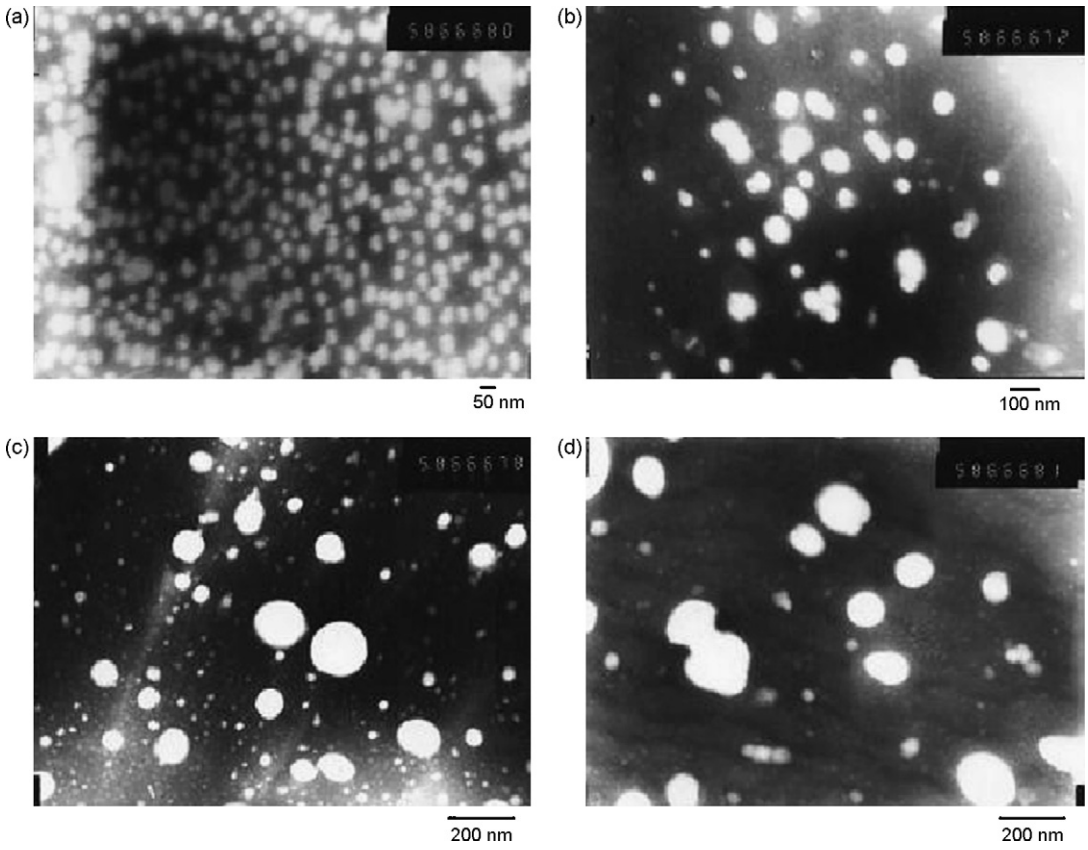

Fig. 4. Transmission electron microscopy (TEM) photographs of (a) BU-NE before freeze-drying, (b) rehydrated mBU-NE, (c) BU-SE before freeze-drying and (d) rehydrated tBU-SE (58000× magnification).

that of BU-SE during lyophilization. The effect of maltose in BU-NE may not be as effective as that of trehalose in BU-SE, but the P_a/P_b index of mBU-NE was quite small than the other formulae. The PI of the rehydrated mBU-NE was 0.105, which meant that the system was still very stable. Consequently, mBU-NE and tBU-SE were chosen for further study.

3.4. TEM investigation

Fig. 4 shows the shapes of nano-emulsion and submicro-emulsion before and after lyophilization. It is even possible to observe the droplets of emulsion that have been deformed during the evaporation process. The diameters of BU-NE and rehydrated mBU-NE ranged from 20 to 100 nm and 50 to 150 nm, while the diameters of BU-SE and rehydrated tBU-SE were both in the range from 50 to 300 nm, which were in agreement with the PCS data. The freeze-dried mBU-NE and tBU-SE for TEM investigated were stored in a desiccator in the presence of dry silica-gel at 25 °C for 3 months. Both of the results of PCS and TEM indicated that the freeze-dried samples stable without great increase in particle size.

3.5. SEM investigation

Saccharides, such as maltose and trehalose, are cryoprotectants known to protect membranes from damage during freeze-drying (Crowe et al., 1987). As can be readily seen from Fig. 5, freeze-dried maltose solution formed a reticulum matrix which was not affected by the addition of BU-NE during freeze-drying. The trehalose solution formed a lamellar structure. The particles of BU-SE also did not affect the microstructure of the support. The properties of the porous, friable lyophilized cakes played an important role in the rehydration of mBU-NE and tBU-SE. These properties include the porosity, pore size and distribution, and the chemical composition of the cakes, so the particles of BU-NE and BU-SE could be well protected by the cryoprotectants.

Several mechanisms have been proposed to explain these protective effects. In the water-replacement hypothesis (Sum et al., 2003), hydrated water molecules on the head group of lecithin were replaced by saccharide molecules, which protected against aggregation and fusion of particles during the lyophilization process. In the vitrification model (Abdelwahed et al., 2006), the glassy state of the cryoprotectants, characterized by maintaining individual particles in a state of low molecular mobility and by reducing the surface tension of the particles during freeze-drying, could be the factor responsible for the stability of mBU-NE and tBU-SE. Maltose and trehalose (Komatsu et al., 2001) formed a glassy structure during drying under suitable conditions when the sensitive particles were embedded.

Most physical changes in lyophilized BU-NE and BU-SE (including stickiness and structural collapse, or shrinkage) resulted from the sharp reduction in viscosity that occurs above their glass transition temperature (T_g) (Lodato et al., 1999). The

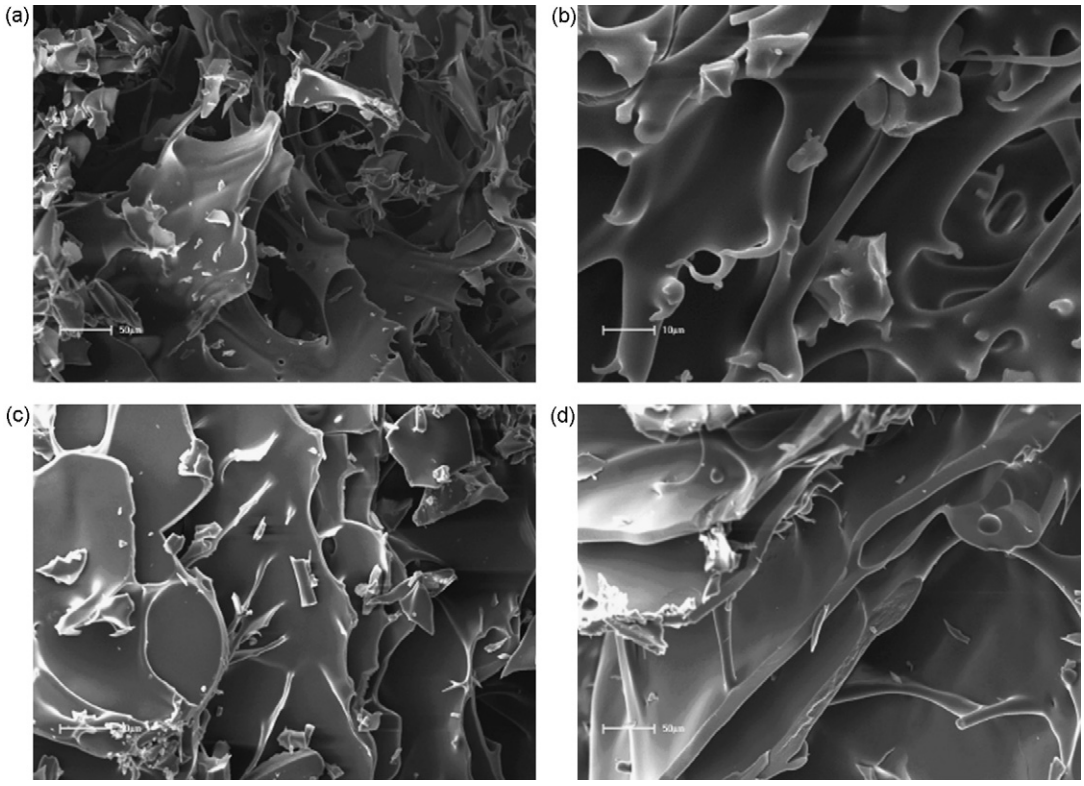

Fig. 5. Scanning electron microscope (SEM) pictures of (a) freeze-dried maltose solution, (b) freeze-dried mBU-NE, (c) freeze-dried trehalose solution and (d) freeze-dried tBU-SE.

T_g was dependent on the moisture content of the systems. Usually, the T_g of pure sugar was higher than its solution. In the process of vitrification involving water removal by desiccation and/or sublimation of ice, vitrification of the sample occurred when T_g rose above the drying temperature. Then, the sugar molecules may prevent disruption of the bilayer by maintaining the phospholipid in a fluid-like state to avoid passage through the gel to the liquid-crystalline phase transition (Koster et al., 2000; Wolkersa et al., 2004). These may also be reasonable explanations for the effect of the cryoprotectants on the size of BU-NE which has a similar structure to that of freeze-dried liposomes.

3.6. Residual moisture

The residual moisture levels immediately after freeze-drying were 5.1 and 4.2% (w/w) for mBU-NE and tBU-SE, respectively. This residual moisture was partly related to the unfrozen water trapped in the sugar matrix during the sublimation drying step. The freeze-drying conditions (temperature, time and vacuum) were also a key factor. In order to keep the products under low relative humidity, the dry cakes should be immediately sealed in the freeze dryer after freeze-drying, thereby avoiding particulate and bacterial contamination.

Both freeze-dried mBU-NE and tBU-SE were stored in a desiccator in the presence of dry silica-gel at 25 °C during the test. Their short-term stability was also studied during storage at 4, 25 and 37 °C in the presence of dry silica-gel. The lyophilized products were stable at 4 or 25 °C in terms of the bufadienolides content, entrapment efficiency, PSD and appearance over a period of more than 3 months, whereas these properties changed significantly at 37 °C.

4. Conclusion

This study clearly illustrates a new strategy for a drug delivery system and an application for the intravenous injection of bufadienolides. BU-NE and BU-SE with a suitable PSD could be obtained even after freeze-drying. The freeze-drying test showed that the optimum cryoprotectants for BU-NE and BU-SE were maltose and trehalose at the same concentration (20%). The particle size and appearance before and after freeze-drying changed slightly in both emulsions. The TEM photos of the rehydrated emulsions and the SEM micrographs of the lyophilized products have also been described. The lyophilized mBU-NE and tBU-SE powders were stable over a period of up to 3 months with no change in appearance, reconstitution ability, particle size, drug concentration and entrapment efficiency. Consequently, freeze-drying could be a promising method to stabilize these emulsions.

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