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ORIGINAL ARTICLE

Preparation and evaluation of lyophilized liposome-encapsulated bufadienolides

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Abstract

Objective: The objective of this study was to prepare bufadienolides-loaded liposome (BU-lipo). *Methods*: The BU-lipo was prepared by a thin-film hydration method involving sonication and lyophilization procedures. The lyophilized BU-lipo was characterized with regard to the appearance and particle size by scanning electron microscopy, transmission electron microscopy, and photon correlation spectroscopy. The entrapment efficiency (EE) of BU-lipo was evaluated by the microdialysis technique. *Results*: In the optimal formulation, Lipoid E-80® and the mass ratio of cholesterol to lipid were fixed at 1.25% and 0.05. The media diameters of BU-lipo before and after lyophilization were about 100 nm, and the EEs of bufalin (B), cinobufagin (C), and resibufogenin (R) were 86.5%, 90.0%, and 92.1%, respectively. In the EE study, the probe recoveries of B, C, and R were 21.53±1.14%, 19.49±1.34%, and 20.19±1.25%, respectively, at a flow rate of 4 μL/min by the gain method. The EE of BU-lipo evaluated by microdialysis and ultrafiltration were equivalent. *Conclusion*: The lyophilized BU-lipo contained trehalose (10%) was stable up to 6 months in a desiccator under 2°C–8°C. The microdialysis technique has a wide application perspective in the investigation of the free-drug concentration of microcarrier systems.

Key words: *Bufadienolides; entrapment efficiency; liposomes; lyophilization; microdialysis*

Introduction

Liposomes are versatile drug delivery systems, as described by Bangham[1] and are formed by the self-assembly of amphiphilic lipid molecules in solution. Drugs with widely varying lipophilicities can be encapsulated in liposomes, either in the entrapped aqueous phase or at the bilayer interface. Many studies have demonstrated the efficacy and safety of liposomes[2–4]. They prevent extensive distribution in the body and are capable of carrying encapsulated drugs in a selective manner to target tissues, especially for drugs with a short therapeutic effect which require frequent dosing. Recently, liposomes have been used to stabilize hydrolytically susceptible drugs, reduce toxicity, deliver drugs to target organs, and enhance pharmacological effects[5–7].

The entrapment efficiency (EE) is one of the most important physicochemical characteristics of drug-loaded liposomes. Therefore, the determination of the EE is a critical part of the rational design of the formulation. Many methods have been used to evaluate the EE of liposomes, including the dialysis bag, gel filtration, ultracentrifugation, and ultrafiltration. Microdialysis is an in vivo technique that permits monitoring of unbound tissue concentrations of drugs and metabolites at specific sites in the body[8]. The in vitro microdialysis sampling has been used to determine the partition coefficient[9], protein binding of drugs[10,11], and release profile of pharmaceutical formulations[12,13]. In this work, the microdialysis technique has been used to determine the EE of bufadienolide-loaded liposome (BU-lipo). Factors that could interfere with the microdialysis probe recovery were investigated in detail.

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 Schneide*r or *Bufo bufo gargarzinas Gantor*). Toad venom has long been used as a therapeutic agent in China and other Asian countries, being given in small

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doses for antitumor activity, for the stimulation of myocardial contraction, to produce an anti-inflammatory effect, and for pain relief. The principal biologically active components of toad venom are bufadienolides, a class of C-24 steroids with a characteristic α-pyrone ring at C-17[14,15]. The major bufadienolides from toad venom include bufalin (B), cinobufagin (C), and resibufogenin (R), each showing significant cytotoxic activities against human liver and gastric cancer cells, with IC_{50} values of 10^{-2}–10^{-3} μmol/L[16–18]. Because of its anesthetic and antibiotic actions, toad venom is also used for the treatment of tonsillitis, sore throat furuncle, and palpitations[19]. The structures of the three bufadienolides are shown in Figure 1.

This article describes the development of a parenteral formulation with bufadienolides as active agents using liposomes to reduce cardiac toxicity[20] and increase treatment efficiency. The first aim of the study was to develop a stable liposomal suspension, which contains bufadienolides at the most suitable pH. Aggregation, fusion, phospholipid hydrolysis, and leakage of the encapsulated drugs may occur during long periods of storage in an aqueous medium. Thus, a freeze-dried product was developed to obtain long-term stability and provide the required quality after reconstitution. Determination of the EE of BU-lipo by microdialysis technology was another aim of this work.

Materials and methods

Bufadienolides were extracted from toad venom in the Department of Pharmaceutics, Shenyang Pharmaceutical University, PR China. Bufadienolides (purity 95%) mainly consisted of B, C, and R with a mass ratio of 2:3:5. The molecular weights of B, C, and R are 386.5, 442.5, and 384.5, respectively.

Lipoid E-80® (PC > 80%) and hydrogenated soy lecithin Lipoid S PC-3 (PC > 98%) were purchased from Lipoid (Ludwigshafen, Germany). Soybean phosphatidylcholine (SPC, Epikuron 200, PC > 92%) and hydrogenated soybean phosphatidylcholine (HSPC, Epikuron 200SH, PC > 92%) were a kind gift from Degussa (Freising, Germany). Cholesterol (chol) with 99% purity was purchased from Sigma (St. Louis, MO, USA) and used in this study. Trehalose dihydrate was obtained from Sinozyme Biotechnology Co., Ltd. (Nanning, China). Maltose, glucose, lactose, and sucrose were purchased from Tianjin Bodi Chemicals Co., Ltd. (Tianjin, China). All other chemicals were of analytical or chromatographic grade.

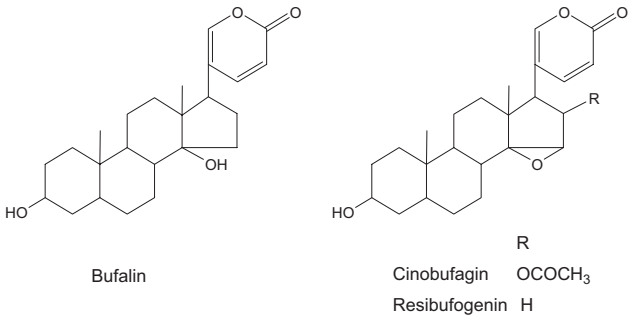

Figure 1. Chemical structures of three bufadienolides.

Preformulation investigation

Solubility study

The solubilities of B, C, and R in six different pH (3–8) buffer solutions (50 mM) and purified water were determined. The buffer solutions at pH 3 and 4 were disodium phosphate-citrate buffer solutions, the others were phosphate saline buffers (PBS). Excess amounts of bufadienolides were added to the appropriate volume of a variety of solutions or purified water in vials. The vials were then tightly closed under N_2 and shaken for 72 hours until equilibrium was reached at a temperature of 25°C (ZHWY-110X30; Zhicheng, Shanghai, China). Each sample was passed through a 0.45-μm membrane filter and was subjected to high-performance liquid chromatography (HPLC) analysis (see Section Assay of bufadienolides). The analyses were carried out in triplicate.

Effect of pH on stability

The stabilities of B, C, and R at various pH values at 80°C were evaluated. Bufadienolides solutions with different pH values (3–8) which were obtained from the solubility study were prepared to evaluate the optimum pH of B, C, and R. They were stored in a water bath at 80°C. Samples were withdrawn at 0, 0.25, 0.5, 1, 2, 4, 6, 8, 12, and 24 hours, and the concentrations of B, C, and R were determined by HPLC.

Determination of *n*-octanol/water partition coefficients

The partition coefficients of bufadienolides in *n*-octanol/PBS (pH 5–7, 50 mM) were measured by the shake-flask method. Aqueous phases with different pH values were mixed with an equal volume of *n*-octanol, shaken, and incubated in a water bath at 25°C for 48 hours. Then, bufadienolides were added to the system, and the system was shaken for 72 hours. The apparent partition coefficients were obtained from the ratio of the concentrations of B, C, and R in the octanol phase to those in the aqueous phase.

Preparation of bufadienolides-loaded liposomes

Thin-film and sonication. Multilamellar vesicles (MLVs) were obtained by dissolving appropriate amounts of

phosphatidylcholine, cholesterol, and bufadienolides in chloroform/methanol (2/1, v/v), and then the solvent was removed by evaporation (N-1001D; Eyela, Tokyo, Japan). The dry lipid film was purged with nitrogen gas for 1 minute and then placed in a vacuum for 12 hours to remove the organic solvent. Lipids and drugs were solubilized by addition of an appropriate volume of aqueous phase. The MLVs obtained were submitted to a probing sonication (Sonics & Material Vibra Cell, 400 W, 20 kHz) at 30% amplification in an ice bath for 3 minutes with a 3-second pulse-on period and a 1-second pulse-off period. When the formulation contained hydrogenated soybean phosphatidylcholine, the operational temperature was kept around 50°C.

The resulting liposomes were passed through a 0.22-μm sterile filter. The filtered formulation was transferred to vials and lyophilized using a lab freeze-dryer (FDU-1100; Eyela, Tokyo, Japan). The lyophilization cycle consisted of freezing at −70°C for 12 hours, primary drying at −35°C for 1 hour, then, the shelf temperature was raised to −25°C for 12 hours and secondary drying at 20°C for 4 hours. Finally, the vials were sealed with rubber caps.

Before the freeze-drying procedure, the filtered samples containing different cryoprotectants were frozen in a refrigerator at −20°C for 24 hours and thawed at room temperature prior to the evaluation of their thawed appearance and particle size distribution (PSD). Then the cryoprotectants with better effect were investigated in detail in the freeze-drying procedure.

Determination of particle size distribution and zeta potential

The PSD of the liposomes was assessed by photon correlation spectroscopy (PCS; dynamic light scattering, DLS) using a Nicomp™ 380 submicron particle sizer (Particle Sizing System, Santa Barbara, CA, USA) at 25°C. Prior to measurement, each sample was diluted with double-distilled water. It had been verified beforehand that dilution of the samples did not alter the size distributions obtained[21]. The zeta potential of the liposomes was determined using the Nicomp™ 380 with electrophoretic light scattering (ELS). Values reported are the mean of triplicate samples. Samples that were considered as completely aggregated were not measured.

Evaluating the entrapment efficiency of BU-lipo

Microdialysis equipment

The microdialysis system consisted of a microdialysis syringe pump (KD Scientific Company, Holliston, MA, USA) and a microdialysis probe. The dialysis probes were U-shaped and made of hollow cellulose fibers (DM-22, 200 μm i.d. and 220 μm o.d., Eicom Corp., Kyoto, Japan). The active region of the microdialysis probe was 10 mm in length with a molecular weight cut-off of 5000 Da. The perfusate was passed through a 0.22-μm nylon filter before use.

Effect of flow rate on probe recovery

The probe recovery was determined by immersing the microdialysis probes in the stirred bufadienolide solution. To evaluate the effect of flow rate on recovery, the probe was perfused with purified water at a rate of 2, 3, 4, 5, and 6 μL/min. Dialysate samples were collected at 20-minute intervals after a 30-minute equilibration period. It had been determined previously that 30 minutes was a suitable time to allow equilibration to occur. The experiments were performed at room temperature. The recoveries of B, C, and R were calculated as follows:

$$R_g = \frac{C_{dial}}{C_{med}} \times 100, \quad (1)$$

where R_g is the recovery by the gain method, C_{dial} is the drug concentration in the dialysate, and C_{med} is the drug concentration in the medium surrounding the dialysis probe.

Calibration

The probe recovery by the gain method was calculated as described above. The probe recovery by the loss method used a known concentration of the analyte as perfusate. The probe was perfused with bufadienolide solution, and the medium surrounding the dialysis probe was purified water. The recovery is calculated from Equation (2):

$$R_l = \frac{C_{perf} - C_{dial}}{C_{perf}} \times 100, \quad (2)$$

where R_l is the recovery by the loss method and C_{perf} is the drug concentration in the perfusate solution.

The liposomes may interfere with the probe recovery. In this case, to avoid the drug molecules being encapsulated in the bilayer of the blank liposomes, the recovery was estimated by the loss method. Blank liposomes were prepared to evaluate the influence of the nanostructure on the microdialysis process. The accuracy of the microdialysis sampling technique for determining the EE of BU-lipo was also validated.

Determination the EE of BU-lipo

The microdialysis probe was inserted into glass vials containing stirred BU-lipo at room temperature. The flow rate of dialysate was set at 4 μL/min. After a 30-minute equilibration period, the dialysate from the BU-lipo was

collected at 20-minute intervals and analyzed by HPLC. The concentrations of B, C, and R were also measured by HPLC after dissolution of the BU-lipo in a mixed solution of isopropanol and methanol (8:2, v/v). The EE was calculated from the following equation:

$$\mathrm{EE}\% = 1 - \frac{C_{\mathrm{dial}} / R}{C_{\mathrm{total}}} \times 100, \quad (3)$$

where C_{total} is the drug concentration in BU-lipo.

Assay of bufadienolides

The HPLC system (Jasco, Tokyo, Japan) consisted of an autosampler (AS-1555), four pumps (PU-1580), and a UV-VIS detector (UV-1575), interfaced with ChromPass software, as well as a C18 reversed-phase column (HiQ Sil, 250 × 4.5 mm, i.d. 4 μm; Kya Tech Corporation, Tokyo, Japan) and a precolumn (Fusion-RP 4 × 3.0 mm). The eluent was a 45:55 (v/v) mixture of acetonitrile : water (pH 3.2) containing 0.5% potassium dihydrogen phosphate and the flow rate was 1.0 mL/min. The UV detector was set at 296 nm and the column temperature was 40°C[22].

Transmission electron microscopy

Morphological observations of BU-lipo before and after lyophillization were carried out using an electronic transmission microscope (JEM-1200; Jeol, Tokyo, Japan) at 60 kV. The samples, after being diluted fivefold with purified water, were stained with 2% (w/v) phosphotungstic acid and placed on copper grids for examination.

Scanning electron microscopy

A LEO Supra 35 field emission scanning electron microscope was used to obtain SEM micrographs of the lyophilized product. 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.

Residual moisture

The residual moisture of the lyophilized substance was determined by thermogravimetric analysis (TGA) and Karl Fisher titration method. As far as TGA was concerned, samples (2.00–4.00 mg) were placed on the sample pan under nitrogen (TGA 50, Shimadzu Co., Kyoto, Japan). Each sample was equilibrated at 25°C and then data were collected using a heating rate of 10°C/min between 25°C and 200°C. The moisture content was determined as the weight loss between 50°C and 150°C. Fifty milligrams of powder was mixed with 2 mL of dry methanol and titrated with KFR-03 reagent (Sayfo Technology Co., Ltd., Tianjin, China) until the end point was reached in Karl Fisher titration method.

Stability study

The dilution stability of rehydrated BU-lipo in two diluting agents was studied. Briefly, BU-lipo was dispersed in 0.9% NaCl and 5% glucose. Their PSDs and zeta potentials were determined after 1, 2, 4, 6, and 8 hours.

The lyophilized BU-lipo was stored in a desiccator in the presence of dry silica gel under 2°C–8°C and ambient temperature for different periods of time. The appearance of the lyophilized products and the properties of the rehydrated BU-lipo such as pH, PSD, zeta potential, and EE were determined during storage.

Results and discussion

Preformulation investigation

Solubility study

Bufadienolides from toad venom, including B, C, and R, were poorly soluble in aqueous media. The ion strength of the buffer solutions used was 50 mM. As indicated in the Table 1, the solubilities of B, C, and R in aqueous media at different pH conditions were between 20 and 60 μg/mL. The solubility of B was about 40 μg/mL between pH 3 and 8, whereas that of C decreased with increasing pH. In the buffer (pH 3.0), the solubility of R was only 21.31 μg/mL. This may due to the different stabilities of B, C, and R at various pH values.

Effect of pH on stability

As shown in Figure 2, the optimum pH value for B was 6. C may be more stable at pH 4–5 than both B and R. A pH below 4 or above 6 would not be appropriate for maintaining the stability of C. When it came to R, the curve suggested that a suitable pH would be between pH 6 and 7. Bufadienolides are degradable and easily catalyzed by acid or base and this is due to their chemical structure. The three bufadienolides have an α-pyrone ring at C-17. It was interesting to note that C and R had a unique epoxy group in their structure compared with B. In addition, there was an acetoxy group at the C-16 position of C. The explanation for these observations could be the hydrolysis of the active structures. Figure 3 describes the degradation path of bufadienolides,

Table 1. The solubilities of bufadienolides in different media.

	Solubilities (μg/mL)		
Media	B	C	R
Purified water	43.01 ± 1.16	55.88 ± 1.26	48.34 ± 1.63
pH 3.0 buffer	37.17 ± 1.94	58.86 ± 1.94	21.31 ± 1.78
pH 4.0 buffer	34.49 ± 1.47	51.60 ± 1.56	40.68 ± 1.83
pH 5.0 buffer	42.76 ± 1.43	50.72 ± 1.52	46.39 ± 1.77
pH 6.0 buffer	37.81 ± 1.34	44.17 ± 2.15	51.59 ± 1.46
pH 7.0 buffer	36.84 ± 1.57	40.57 ± 2.37	50.78 ± 4.90
pH 8.0 buffer	42.01 ± 1.58	34.53 ± 2.27	42.07 ± 1.81

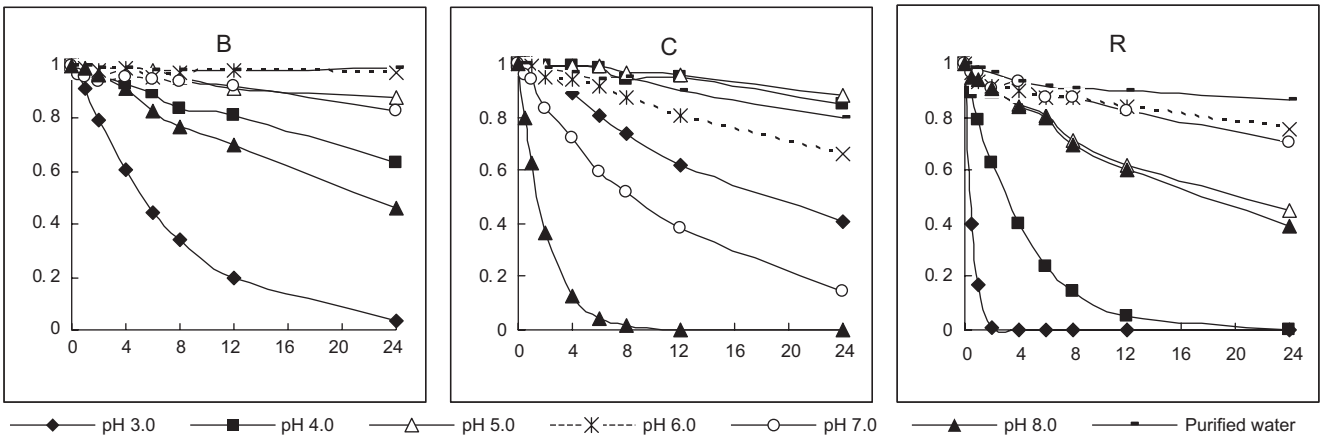

Figure 2. The stability of bufalin (B), cinobufagin (C), and resibufogenin (R) in various pH at 80°C (the reported data were of mean value, $n = 3$, the SD were not presented for describing the curve clearly).

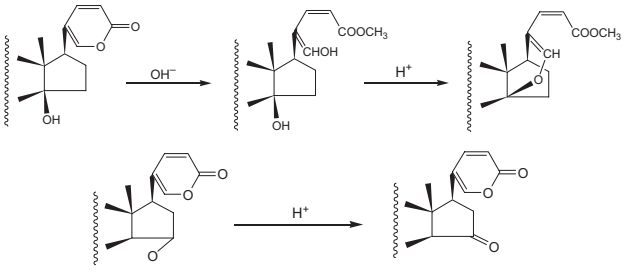

Figure 3. Degradation path of bufadienolides, induced by acid or base.

induced by acid or base[23,24]. In purified water (pH 6.0), all three bufadienolides were very stable. This phenomenon deserves further attention.

Based on the phospholipid hydrolysis, the optimal pH of phospholipids was approximately 6.5[25,26]. According to the effect of pH on the stability of bufadienolides, PBS (pH 6.0 or 6.5) and purified water (pH 6.0) as aqueous phase were selected for further investigation.

Determination of octanol/water partition coefficients

Following the pH study, the apparent partition coefficients of B, C, and R between the octanol and PBS were evaluated at different pH values (5–7) in this section. As shown in Table 2, the log *P* values of B, C, and R were all around 3 over the pH range 5–7, suggesting that they were pH-independent. These results were in agreement with the calculated value from SciFinder, that is, 3.42, 2.51, and 2.97 for B, C, and R, respectively. In addition to solubility, the affinity of the drug in the octanol phase is an essential characteristic for improving the encapsulation. In the literature[27], the EE of drug association within liposomes had been correlated with the partition coefficient, which reflects the lipophobicity or hydrophobicity of a compound. These log *P* data suggested that the amphiphilic structure of bufadienolides significantly facilitated their inclusion into liposomes.

Table 2. Apparent octanol/water partition coefficiencies of bufadienolides at different pH's.

Buffers with different pH	log *P*		
	B	C	R
5.0	3.45	2.99	2.95
5.5	3.30	2.92	2.85
6.0	3.35	2.95	2.88
6.5	3.31	2.76	2.72
7.0	3.08	2.76	2.73

Formulation development

It is known that MLVs can be obtained by thin-film evaporation and small unilamellar vesicles (SUVs) by sonication or extrusion[28]. The preformulation investigation indicated that the three bufadienolides were all lipophilic. Thus, passive loading methods were suitable for the preparation of BU-lipo. In this study, SUVs were prepared by thin-film hydration followed by sonication. Several formulations with different lipid compositions were prepared to develop optimum BU-lipo. Single-factor experiments were used to optimize the formulation.

Lipid composition

Liposomes consisting of different kinds of lipids were prepared in the aqueous medium of purified water (Table 3). The hydrogenated soybean phosphatidylcholine Epikuron 200SH and hydrogenated soy lecithin Lipoid S PC-3 yielded lower EE for B, C, and R. As for Lipoid E-80® and Epikuron 200, the EE of BU-lipo was markedly increased. Epikuron 200SH and Lipoid S PC-3 were not suitable for the BU-lipo formation and possibly because of the high rigidity of the lipid bilayers, bufadienolides could not be effectively loaded compared with the unsaturated phospholipid bilayer. Furthermore, the

Table 3. Effect of lipid composition on physical characterization of BU-lipo.

Lipid (1%, w/w)	Lipid epikuron 200SH	Lipid epikuron 200	Lipoid E-80®	Lipoid S PC-3
PSD (nm)	NA[a]	177.9 ± 98.0	91.0 ± 40.3	NA
D_{90} (nm)[b]	NA	309.5	156.2	NA
EEs of B, C, and R (%)	47.2, 51.9, and 54.7	84.4, 91.2, and 90.0	79.1, 86.1, and 88.7	61.0, 69.3, and 62.1

[a] NA, data were not available. The formulations were flocculation 12 hours after preparation.
[b] D_{90}, the D_{90} is a value on the Gaussian distribution such that 90% of the particles have a volume of this value or less.

formulations containing saturated lipids tended to flocculation more quickly than similar formulations containing unsaturated lipids (in less than 12 hours following storage at room temperature). Although the liposomes composed of Epikuron 200 resulted in the highest entrapment about 84.4%, 91.2%, and 90.0%, the PSD and D_{90} (177.9 ± 98.0 and 309.5) were larger than for the formulation containing Lipoid E-80®. So, Lipoid E-80® was chosen for the formulation of BU-lipo.

As shown in Figure 4, a higher Lipoid E-80® concentration increased the EE of BU-lipo. The study revealed that the liposome formulation would remain stable so long as the lipid-to-drug molar ratio was maintained at a value greater than 20 to 1. In addition, the experimental results showed that the higher the lipid concentration, the larger the PSD. This indicated that the particle size of BU-lipo was rather large and heterogeneous when the lipid concentration was too high. Considering the EE and PSD, a lipid concentration of 1.0% (w/w) was selected for further study.

Effect of cholesterol

For testing the effects of different amounts of cholesterol, BU-lipo was prepared by changing the ratio of cholesterol to lipid. Cholesterol was one of the common additives included in the formulation to prepare stable liposomes[29]. As indicated in Figure 5, the EE of BU-lipo decreased as the percentage of cholesterol in the formulation increased, provided that the concentration of lipid was fixed at 1.0% (w/w). According to the literature, cholesterol can reduce the partitioning of steroids into egg lecithin liposomes[30,31]. It was possible that the bufadienolides

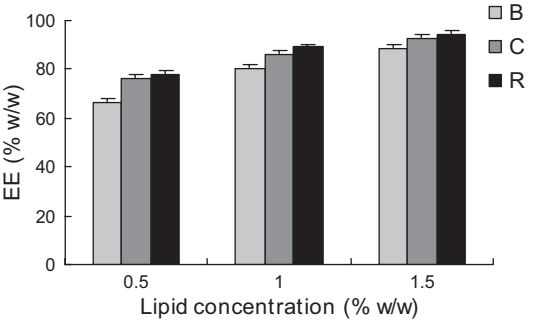

Figure 4. Effect of Lipoid E-80® concentration on the EE of BU-lipo.

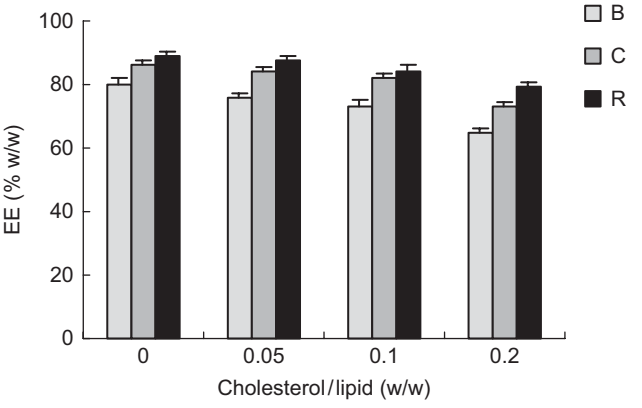

Figure 5. Influence of cholesterol concentration on the EE of BU-lipo.

molecules competed with cholesterol for the hydrophobic space in the lipid bilayer, and as this space was finite, there might be little or no space for steroids when the cholesterol content was high.

Cholesterol is known to effectively prevent drug leakage from liposomes. It is also an essential component of the BU-lipo formulation in this study. In the experiment, when we fixed the concentration of lipid and the ratio of cholesterol to lipid (w/w) at 1.0% and 0.05%, the EEs of B, C, and R were 75.6%, 84.1%, and 87.6%, respectively. As aforementioned, this may be induced by the finite hydrophobic space in the lipid bilayer and competition of cholesterol. The formulation failed to meet the requirements of the 2005 edition of the *China Pharmacopoeia* for the EE test of liposomes. Then a formulation with 1.25% (w/w) lipid and a mass ratio of cholesterol to lipid of 0.05 was investigated, the EEs of B, C, and R in the formulation were all above 80%. Thus, the formulation was chosen for further optimization.

Effect of aqueous media

Different aqueous media including purified water and PBS (pH at 6.0 and 6.5) were evaluated with the lipid and mass ratio of cholesterol to lipid fixed at 1.25% and 0.05%, respectively. There was no significant difference in the chemical stabilities of B, C, and R with regard to the three kinds of liposomes. Ion strength appeared to be more critical than pH to achieve a narrow PSD for these vesicles and increasing the ionic strength was also shown to

increase the observed PSD of BU-lipo (data not shown). When 50 mM PBS was used, the particle size of the liposomes was greater than that of purified water. Through a very different mechanism involving the extraction of ion pairs, this was probably due to the competition of water between electrolyte and lipid. Considering the stability of phospholipids, pH 6.5 was chosen for the BU-lipo formulation. A PBS (pH 6.5) with ion strength of 10 mM was employed for the formulation studies.

Effect of freeze-drying on BU-lipo

A freeze–thawing experiment was conducted to select the cryoprotectants (trehalose, maltose, glucose, sucrose, and lactose). The optimum concentration of each cryoprotectant is shown in Table 4. P_a and P_b represented the mean diameter of BU-lipo after and before freeze–thawing test. The mean diameter ratio (P_a/P_b) was calculated to evaluate the effect of the cryoprotectants. Without cryoprotectants, BU-lipo fully aggregated upon storage of the frozen samples for only one cycle at −20°C. In the presence of lactose (8%) and glucose (15%), the particle size of BU-lipo was significantly changed as the appearance of the reconstituted samples was not translucent without the Tyndall effect. As for trehalose, sucrose, and maltose at a concentration of 15%, the particle size of BU-lipos increased slightly and the reconstituted samples appeared translucent. Therefore, they were chosen for the lyophilization study.

Trehalose, sucrose, and maltose were added in increasing amounts (5%, 10%, and 15%) to determine the most suitable concentration. In terms of PSD, the concentration of 15% was normally sufficient in the freeze–thawing test. However, for freeze-drying, 10% seems to be the optimal concentration for the three kinds of cryoprotectants. Among them, the rehydrated BU-lipo containing maltose appeared to be translucent with light yellow opalescence rather than the original (before freeze-drying without maltose) light blue opalescence. Thus, sucrose and trehalose were selected as cryoprotectants for further study. Trehalose seems to be the optimal cryoprotectant for preventing the leakage of bufadienolides molecules from the lipid bilayer. Trehalose might form a glassy structure that could maintain individual vesicles in a state of low molecular mobility and reduce the surface tension of the vesicles during freeze-drying[32]. The particle size was changed least before and after lyophilization when 10% trehalose was used as the cryoprotectant, and this was 101.2 ± 46.5 nm after rehydrating. The SEM image of freeze-dried BU-lipo was used to illustrate the lamellar structure formed from trehalose (Figure 6). The lyophilized cakes were porous and friable. The properties include porosity, pore size, and the chemical composition of the cakes were very important, so the liposome could be well protected. The microstructure of the lyophilized cakes was not interfered by the vesicles. In the experiment, the freeze-dried products were all reconstituted with purified water.

Table 4. Effect of different cryoprotectants on the characteristics of BU-lipo in the freeze–thawing test.

Cryoprotectant	Thawed appearance[a]	P_a/P_b[b]
None	••	N/A
Lactose (8%)	••	N/A
Glucose (15%)	•	N/A
Sucrose (15%)	○	1.35
Trehalose (15%)	○	1.44
Maltose (15%)	○	1.30

[a]•• fully aggregated;
•slightly aggregated without a Tyndall effect;
○translucent with a Tyndall effect. [b]NA means the data could not be obtained because the thawed samples were aggregated.

The physical characterization of the optimal formulation

The results indicated that the concentrations of phosphatidylcholine, cholesterol, different media, and cryoprotectant had a greater effect on the physical characterization of BU-lipo than the other factors. The final BU-lipo appeared to be translucent with a PSD and zeta potential of 97.5 ± 43.7 nm and −8 to −4 mV, respectively. The quantity of B (0.050 mg/mL), C (0.075 mg/mL), and R (0.125 mg/mL) incorporated into the BU-lipo was 86.5%, 90.0%, and 92.1%. On addition of water, the lyophilized product spontaneously formed homogeneous submicron liposomes. The electron microscopic analysis revealed that the average diameter of the BU-lipo was approximately the same and they were roughly spherical in shape (Figure 7). The diameters of BU-lipo before freeze-drying and after rehydration ranged from 50 to 150 nm, which was in agreement with the PCS data.

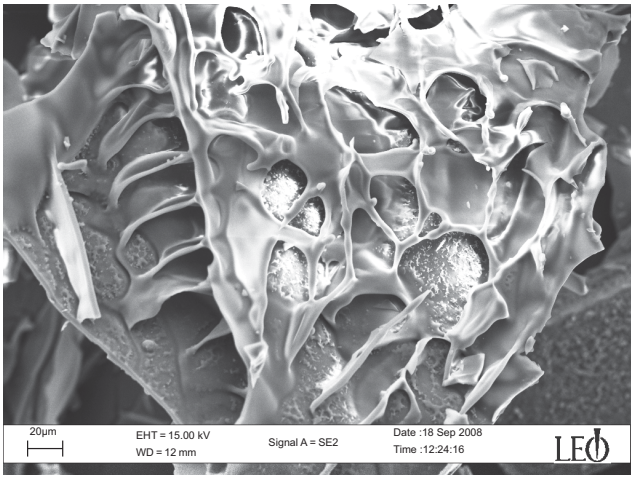

Figure 6. Scanning electron microscope (SEM) pictures of freeze-dried BU-lipo.

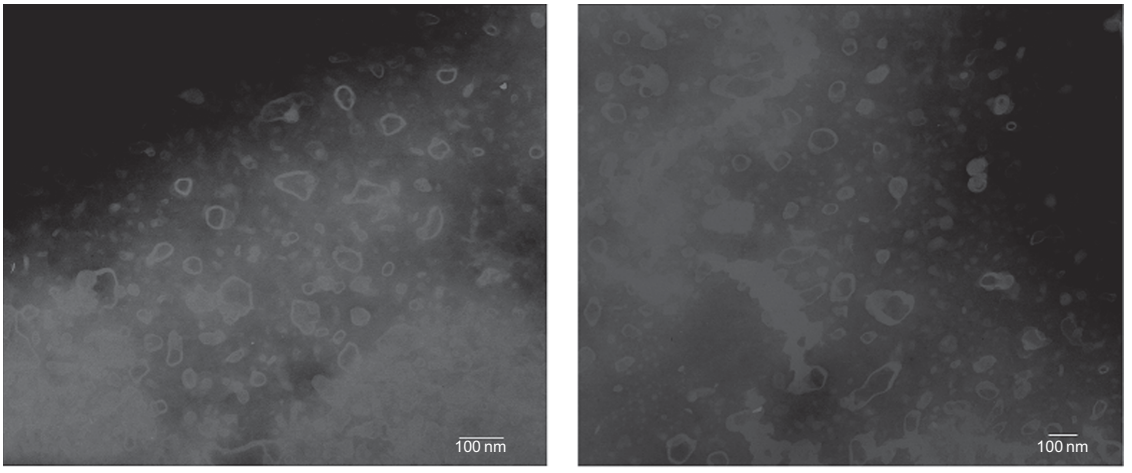

Figure 7. Transmission electron microscopy (TEM) photographs of BU-lipo before freeze-drying (a) and after rehydrated (b).

The residual moisture level immediately after freeze-drying of BU-lipo by the Karl Fisher method was 1.5%, which was not in agreement with a weight loss of 3.1% determined by TGA. The reason seemed that TGA, a type of testing method, was performed to determine the changes in sample's weight which related to the changes in temperature such as degradation and absorption moisture of materials. The karl fisher method was characterized by a better precision in the determination of low concentrations of water in samples. To keep the products at a low relative humidity, the dry cakes should be immediately sealed in the freeze dryer after freeze-drying and stored in a desiccator in the presence of dry silica gel. The osmolality of rehydrated BU-lipo containing 10% trehalose was 389 mosmol/kg. For an in vivo preparation, physiological osmolality (290 mosmol/kg) is recommended, and this could be achieved using 0.9% NaCl or 5% glucose.

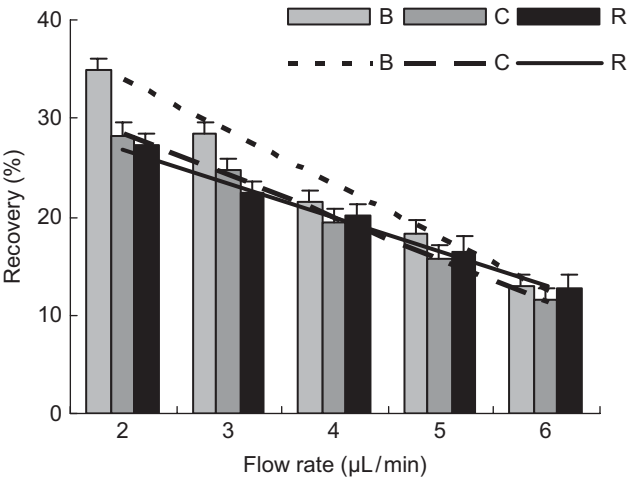

Figure 8. Probe recovery estimated by gain method at different perfusion flow rates for B, C, and R.

Microdialysis

In this study, the microdialysis technique was used to determine the EE of BU-lipo. Factors that could interfere with the probe recovery were investigated in detail. Probes must be calibrated systematically and the influence of the most relevant study variables on recovery must be tested under the experimental conditions to be used if reliable results are to be obtained.

The probe recovery depended on many factors. Of these, the perfusion flow rate was an important factor that defined the performance of a microdialysis probe and directly influenced the probe recovery. In accordance with previously reported data[10,33], a decrease in the recovery with increasing flow rate was found in our study and there was also a linear relationship between these two variables (Figure 8). The relationship between recovery (Y) and flow rate (X, μL/min) was such that $Y = -0.0542X + 0.394$ for B, $Y = -0.0.0424X + 0.326$ for C, and $Y = -0.0349X + 0.303$ for R. The R^2 of the regression for B, C, and R was 0.9863, 0.9974, and 0.9912, respectively. Because of the dialysate volume and relative recovery, a flow rate of 4 μL/min was chosen.

The probe recoveries of B, C, and R were also measured at low and high concentrations in water (Table 5). A previous study[34,35] showed that the effect of concentration on recovery depended on the flow rate. This experiment was necessary to allow the determination of drug entrapment using microdialysis[36]. Higher concentrations of B, C, and R were selected based on the solubility study. As shown in Table 5, there was no statistical difference between the recovery determined for the low and high drug concentrations at a flow rate of 4 μL/min ($P > 0.05$) and thus, the concentration had no effect on the recovery. The influence of the calculation method

Table 5. Probe recovery determined by gain and by loss methods for B, C, and R with different concentrations at flow rate of 4 μL/min ($n = 3$).

	B (μg/mL)		C (μg/mL)		R (μg/mL)	
	43.01	21.51	55.88	27.94	48.34	24.17
Recovery by gain (%)	21.58 ± 1.15	21.53 ± 0.89	19.48 ± 1.26	19.61 ± 1.13	21.47 ± 1.29	20.49 ± 1.01
Recovery by loss (%)	19.88 ± 1.24	20.06 ± 1.10	18.86 ± 1.16	19.74 ± 0.98	18.61 ± 1.33	20.40± 0.76

(by gain or loss) on the probe recovery was also evaluated. Table 5 shows the results of this comparative study. The calibrator loss (R_l) and analyte gain (R_g) were equal in this study.

The probe recovery was also determined using bufadienolides solution as the perfusion medium, and as the external medium, purified water and unloaded liposomes were used to confirm that the presence of these in the medium did not interfere with the drug diffusion. If the drug was added to the unloaded colloidal dispersions, it could adsorb onto the liposomes, decreasing the free drug concentration in the system, preventing estimation of the probe recovery[37]. This experiment was performed by the loss method. The probe recoveries of B, C, and R were 21.53 ± 0.89%, 19.61 ± 1.13%, and 20.49 ± 1.01% in purified water and in liposomes they were 19.36 ± 1.46%, 18.72 ± 1.53%, and 19.34 ± 1.49%, respectively. Thus, there was no significant difference among the results ($P > 0.05$). It was concluded that the nanostructures did not interfere with the probe recoveries of bufadienolides determined by microdialysis. Although the probe recoveries of B, C, and R were low, the high-performance liquid chromatography combined ultraviolet detection can provide a sensitive analysis of low concentrations of drugs in the dialysate. Thus, microdialysis is still a good method to determine entrapment efficacy.

In the freeze-drying study, the rehydrated BU-lipo contained different concentrations of trehalose and sucrose. The viscosity of system may affect the recovery of the probe and, consequently, with the EE of BU-lipo. Therefore, a series of bufadienolides solutions with different concentrations of cryoprotectants were prepared for measurement of the probe recovery. As shown in Table 6, the recovery decreased as the concentration of cryoprotectants increased. This showed that the probe recovery should be calibrated when measuring the EE of rehydrated BU-lipo containing different concentrations of cryoprotectants.

Table 6. Probe recovery determined by gain method for B, C, and R with different concentrations of cryoprotectants.

		Recovery (%)		
Cryoprotectants (%)		B	C	R
	10	16.25	15.16	17.02
Trehalose	15	13.43	12.87	14.98
	20	11.88	10.90	12.28
	10	18.04	16.36	17.97
Sucrose	15	16.10	15.03	17.03
	20	14.74	13.93	15.41

To test the accuracy of the microdialysis sampling technique for determining the EE of BU-lipo, we compared the microdialysis and ultrafiltration methods. The free compound was separated from the membrane-bound compound using a centrifugal filter device (Microcon YM-100, molecular weight cut-off 100,000, Millipore, Boston, MA, USA) at 5000 rpm for 10 minutes. The filtrate recoveries of B, C, and R were 85.77%, 83.70%, and 78.30%, respectively. For the same formulation of BU-lipo being studied, the EEs were 87.3 ± 1.8%, 92.0 ± 1.6%, and 93.1 ± 1.4% by the ultrafiltration method, and by the microdialysis technique they were 88.1 ± 1.5%, 91.8 ± 1.2%, and 92.1 ± 1.4%. In addition, this sampling method facilitated the EE study compared with conventional methods. Dialysis bag and gel filtration are laborious and time consuming. The ultracentrifugation method is based on the sedimentation velocity of vesicles. Because of the small size, BU-lipo will be still present in the aqueous phase. As far as ultrafiltration method is concerned, the high cost and low reproducibility are the hurdles for its wide application. In the dialysis, gel filtration, and ultracentrifugation process, some of drug molecules in the lipid bilayer may enter the aqueous phase. Because microdialysis does not change the fluid volume during the microdialysis sampling, the drug concentration remains constant. Therefore, the equilibrium of entrapped and untrapped drug is not disturbed by this technique. This study indicated microdialysis was a convenient, precise, and reproducible method for measuring free drugs of BU-lipo. The microdialysis technique has a wide application perspective in the investigation of the entrapment efficiencies of microcarrier systems, such as lipid microspheres, nanospheres, and solid lipid nanoparticles.

Stability test

As shown in Table 7, the PSD of BU-lipo was not statistically significantly affected by diluted with 0.9% NaCl and 5% glucose up to 8 hours ($P > 0.05$). But the PSD of BU-lipo diluted with NaCl was slightly larger. The EE decreased rapidly initially, probably because of the zeta potential changing markedly and the destruction of the layer of lipid bilayer which led to the leakage of the drug. These results suggest that the BU-lipo can be safely diluted with glucose solution during the therapy.

The long stability of the dried products was assessed by the determination of the liposomes, which were reconstituted from dried products for different periods

Table 7. Change in particle size distribution (PSD) of BU-lipo after diluted with different solutions.

	PSD (nm)					
	0	1 hour	2 hours	4 hours	6 hours	8 hours
0.9% NaCl	97.4 ± 44.5	98.5 ± 44.0	99.6 ± 44.3	101.4 ± 46.2	100.7 ± 48.3	101.5 ± 48.0
5% Glucose	98.0 ± 43.1	98.5 ± 43.5	98.9 ± 44.1	98.7 ± 44.7	99.1 ± 43.9	99.5 ± 44.7

Table 8. Stability data of lyophilized BU-lipo stored at 2–8°C and 25°C.

			Rehydrated BU-lipo			
Temperature (°C)	Time	Appearance	pH	PSD (nm)	Zeta potential (mV)	EEs of B, C, and R (%)
2–8	0 day	Intact cake	6.48	97.5 ± 43.7	−6.4	86.5, 90.0, and 92.1
	3 months	Intact cake	6.47	98.1 ± 43.1	−7.4	86.7, 89.4, and 91.7
	6 months	Intact cake	6.42	98.5 ± 44.0	−7.1	85.2. 88.0, and 91.0
25	0 day	Intact cake	6.51	97.8 ± 43.0	−5.4	86.2, 89.7, and 92.0
	3 months	Intact cake	6.45	98.7 ± 44.5	−6.0	84.7, 87.3, and 90.4
	6 months	cake with shrinkage and rugged	NA	NA	NA	NA

NA means the data could not be obtained because BU-lipo could not be completely reconsitituted.

of time. Our study showed that the properties of BU-lipo, such as pH, PSD, zeta potential, and EE, did not change for 6 months at 2°C–8°C and for at least 3 months at ambient temperature (Table 8).

Conclusion

This study clearly describes a new formulation of bufadienolides-loaded liposome with antitumor and analgesic properties, which consists of Lipoid E-80® 1.25%, cholesterol 0.06%. The BU-lipo has a particle size of 97.5 ± 43.7 nm, negative charge of −8 to −4 mV, and drug EE above 80%. In our study, bufadienolides were insoluble in water and the log *P* of B, C, and R were all around 3 showing no pH-dependence between the aqueous phase and the organic solvent. Freeze-drying technology was used for stabilizing the colloidal system. The optimum cryoprotectant for BU-lipo was trehalose (10%). The lyophilized BU-lipo powders were stable over a period up to 6 months. The EE of BU-lipo was evaluated using microdialysis technique. After calibration, the results indicated that a convenient and reproducible characterization of sampling free drug from various liposome formulations was readily obtainable by microdialysis. In conclusion, an intravenous delivery system of liposome for Traditional Chinese Medicines focusing on a better chemical stability of bufadienolides has been successfully developed and optimized.

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***Declaration of interest*:** The authors report no conflicts of interest.

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