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Formulation and characterization of bufadienolides-loaded nanostructured lipid carriers

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Abstract

Objective: The aim of this study was to design bufadienolides-loaded nanostructured lipid carriers (BU-NLCs) to reduce the degradation of drugs in rat plasma. *Methods*: BU-NLCs were prepared by a modified melt-emulsification ultrasonic technique and then characterized by particle size distribution, zeta potential, entrapment efficiency, differential scanning calorimetry, and X-ray diffraction. *Results*: The optimal formulation consisted of glyceryl monostearate 1.8%, medium-chain triglyceride 0.75%, oleic acid 0.45%, Lipoid E-80® 1.5%, Pluronic F68 1.0%, and sodium deoxycholate 0.25%. The particle size distribution and the range of zeta potential of BU-NLCs were 104.1 ± 51.2 nm and −15 to −20 mV, respectively. The entrapment efficiencies of the bufadienolides were all above 85%. In the enzymolysis study, the chemical stability of cinobufagin (C) in BU-NLCs was enhanced by being encapsulated in particles of NLC and adjusting the pH of the surrounding environment to 7.0. The half-life of C was 17-fold longer than that in bufadienolides solution. The in vitro release showed that the release from BU-NLCs was slower than from bufadienolides solution and followed the Weibull equation. Differential scanning calorimetry and X-ray diffraction showed that BU-NLC was in an amorphous state after lyophilization. *Conclusion:* These results indicated that NLC could be developed as a carrier with improved drug plasma stability and offering controlled drug release.

Key words: *Bufadienolides; differential scanning calorimetry; enzymolysis; in vitro release; nanostructured lipid carriers; X-ray diffraction*

Introduction

Toad venom, a traditional Chinese medicine, is prepared from the dried white secretion of the auricular and skin glands of Chinese toads (*Bufo melanostictus Schneider* 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 doses for antitumor activity, stimulation of myocardial contraction, to produce an anti-inflammatory effect, and 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[1,2]. 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} to 10^{-3} μmol/L[3–5]. Because of its anesthetic and antibiotic actions, toad venom is also used for the treatment of tonsillitis, sore throat furuncle, and palpitations[6].

Among drug carriers nanostructured lipid carriers (NLCs), liposomes, and nanoemulsions are important candidates for use as intravenously injectable carriers that can be produced on an industrial scale. In our previous study, a formulation of bufadienolides-loaded liposome was prepared[7]. Although liposomes can be a useful vehicle for either hydrophilic or lipophilic drugs, the drug loading capacity of liposomes is less than that of NLCs[8]. In our study, the content of bufadienolides was 0.25 mg/mL in liposomes and 0.5 mg/mL in NLCs. The results of our previous study also suggested that liposomes were more easily captured by plasma protein. This characteristic of liposomes would make them unsuitable as a carrier for bufadienolides in vivo. In addition, the solid core of NLC, unlike the fluid core of

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liposomes, may protect the incorporated drugs from chemical degradation. NLCs represent as an improved generation of solid–lipid nanoparticles[8–10]. The particles of NLC consist of solid lipid and liquid lipid, which offer the advantage of biocompatibility, improved drug loading capacity, and release properties[11–13]. In addition, NLCs are less cytotoxic than polymeric nanoparticles[14]. Therefore, we decided to use NLC as the carrier and in this article a novel delivery system for bufadienolides with NLC was evaluated in detail.

The purpose of this research was to establish an effective therapeutic system for bufadienolides. The influence of lipid materials and surfactants in formulations was investigated in detail. Transmission electron microscopy (TEM) was employed to study the morphology of BU-NLCs. The BU-NLCs were characterized by particle size distribution (PSD), zeta potential, entrapment efficiency (EE), differential scanning calorimetry (DSC), and X-ray diffraction (XRD). The enzyme hydrolysis profiles of BU-NLCs and bufadienolides solution (BU-S) in different concentrations of rat plasma were determined. Release of drug from the BU-NLCs in vitro was also studied and compared with the diffusion of drug from BU-S in distilled water and normal saline solution, using the dialysis bag method[15].

Materials and methods

Materials

Bufadienolides were extracted from toad venom in the Department of Pharmaceutics, Shenyang Pharmaceutical University, PR China. Bufadienolides (purity 95%) mainly consisted of bufalin (B), cinobufagin (C), and resibufogenin (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.

The following materials were purchased or obtained from the sources in parentheses: glyceryl monostearate (GM) (Tianjin Bodi Chemicals Co., Ltd., Tianjin, China), Lipoid E-80®, oleic acid (OA), medium-chain triglyceride (MCT) (Lipoid KG, Ludwigshafen, Germany), Pluronic F68 (BASF AG, Ludwigshafen, Germany), sodium deoxycholate (SDC; Sigma-Aldrich, Steinheim, Germany), and Tween 80 (Shanghai Shenyu Medicine and Chemical Industry Company, Shanghai, China). Distilled water was used in all experiments, and all chemicals and reagents used were of analytical or chromatographic grade.

Preparation of formulations

Bufadienolides-loaded nanostructured lipid carriers (BU-NLCs) were prepared by a modified melt-emulsification and ultrasonic method. Briefly, GM, MCT, and OA were heated together in a water bath at 75°C. Then, the bufadienolides were dissolved in the melted lipid phase under stirring to form a lipid phase. Lipoid E-80®, Pluronic F68, SDC, and Tween 80 were dissolved in 80 mL distilled water at 75°C as aqueous phases. The aqueous phase was dispersed in the lipid phase under magnetic stirring at the same temperature. The obtained primary emulsion was ultrasonicated using probe sonication (Sonics & Material Vibra Cell, 750 W, 20 kHz) at 30% amplification for 10 minutes with a 3-second pulse-on period and a 1-second pulse-off period. The nanoemulsion (O/W) obtained was cooled in an ice bath to form NLC under magnetic stirring followed by dilution up to 100 mL with distilled water. Finally, the resultant NLCs were filtered through a 0.22-μm cellulose acetate membrane.

BU-S was prepared by dissolving bufadienolides in propylene glycol under ultrasonic. Then the propylene glycol solution was diluted to a concentration of 20% with distilled water. Finally, a 0.5 mg/mL BU-S for enzymolysis and diffusion test was obtained.

Lyophilization

The BU-NLC aqueous suspensions were frozen in a refrigerator at –70°C for 12 hours. Then the samples were lyophilized using a lab freeze-dryer (FDU-1100; Eyela, Tokyo, Japan). The freeze-drying was conducted as follows: primary drying at –35°C for 1 hour; then, the shelf temperature was raised to –25°C for 13 hours; secondary drying at 15°C for 5 hours. Finally, the vials were sealed with rubber caps.

Determination of particle size and zeta potential

The PSD and polydispersity index (PI) of the BU-NLCs were assessed by photon correlation spectroscopy (dynamic light scattering) 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[16]. The zeta potential of BU-NLCs was also determined using the Nicomp™ 380 with electrophoretic light scattering. 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. Samples that were considered as completely aggregated were not measured.

Drug content and entrapment efficiency

BU-NLCs (1 mL) were diluted with a 7- to 8-fold volume of ethanol in a 10-mL volumetric flask, and the solution

was shaken in a water bath at 60°C for 1 minute to dissolve the lipid materials. After cooling to room temperature with the precipitation of lipid materials, the volume of the solution was determined. The solution was filtered through a 0.45-μm membrane, the drug content in the supernatant was measured by high-performance liquid chromatography (HPLC). The HPLC system (Hitachi) consisted of an autosampler (L-7200), four pumps (L-7100), and a UV-VIS detector (L-7420), all interfaced with D-7000 HSM software along with 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. The UV detector was set at 296 nm with the column temperature at 40°C. The flow rate was 1.0 mL/min and 10-μL samples were injected.

The EE of BU-NLCs was evaluated by the microdialysis method[7,17]. The microdialysis probe was inserted into glass vials containing stirred BU-NLCs at room temperature. The flow rate of the dialysate was set at 4 μL/min. After a 30-minute equilibration period, the dialysate from the BU-NLC was collected at 20-minute intervals and analyzed by HPLC. The EE was calculated from the following equation:

$$EE\% = \left(1 - \frac{C_{\text{dial}} / R}{C_{\text{total}}}\right) \times 100,$$

where R is the recovery of drug by the gain method, C_{dial} is the drug concentration in the dialysate, and C_{total} is the drug concentration in BU-NLCs.

Transmission electron microscopy

The morphological observation of BU-NLCs was performed using an Electronic Transmission Microscope (JEM-1200; Jeol, Tokyo, Japan) at 60 kV. 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.

Thermal analysis

DSC analysis was employed using a differential scanning calorimeter (DSC-60WS; Shimadzu, Kyoto, Japan). For DSC measurement, a scan rate of 10°C/min was employed over the temperature range of 25–300°C under a nitrogen purge. The samples were placed directly in aluminum pans for analysis. GM; bufadienolides; the physical mixtures of GM, MCT, OA, and bufadienolides (GMOB); BU-NLCs (containing 0% and 10% sucrose); and 10% sucrose aqueous solution after lyophilization were subjected to the above thermal cycles. For GM (pre-tempering and post-tempering at 75°C for 30 minutes) and GMT (containing GM and MCT), the analysis was conducted over the range of 25–85–25°C at a scan rate of 5°C/min. The different GMTs were named according to the MCT content in the respective mixtures, that is, GMT (25) consisted of 75% GM and 25% MCT. The MCT percentage in the binary mixtures (GMTs) varied from 5% to 55%. Only the DSC thermograms of GMT (25) and GMT (45) are shown in Figure 1.

X-ray diffraction

XRD was performed using a type D/Max-2400 diffractometer (Rigaku Instrument, Tokyo, Japan). The samples were exposed to *CuKa* radiation under 56 kV and 182 mA over the 2-theta range from 3° to 45° in increments of 4°/min every 0.04°. Samples for DSC analysis (except GMTs) were chosen for XRD analysis.

Enzymolysis study

The enzyme hydrolysis of BU-NLCs and BU-S in different concentrations of rat plasma was determined as described below. Fresh rat plasma (0.25, 0.5, 1.25, and 2 mL) was mixed with BU-NLCs (0.25 mL) and different volumes of normal saline (2, 1.75, 1, and 0.25 mL) to make 2.5 mL. Thus, the plasma was diluted to various concentrations of 10%, 20%, 50%, and 80%. To prepare 100% concentration of plasma with bufadienolides in the same concentration, 12.5 μL bufadienolides methanol solution (10 mg/mL) was added into the 2.5 mL plasma. Each sample was incubated in a water bath at 37°C. Then, 200-μL samples were withdrawn at 0, 0.25, 0.5, 1, 2, 4, 6, 8, 12, and 24 hours, and the reactions were terminated by addition of 800 μL acetonitrile and then vortex-mixed for 10 minutes and centrifuged at 17233 × g for 10 minutes. The supernatant was collected and the content of bufadienolides was measured by HPLC as described above.

In vitro release study

The drug release from BU-NLCs was performed in distilled water (pH 6.12) and normal saline (pH 5.83) using the dialysis bag method. The dialysis bag with a cutoff of 12,000–14,000 Da could retain particles of NLC and allow drug transfer into the dissolution medium. The BU-NLCs (2 mL) mixed with human plasma (2 mL) or without human plasma were poured into the bag with the two ends fixed by clamps. The bags were then placed in a conical flask and 50 mL dissolution medium was added. The conical flasks were placed in a water bath

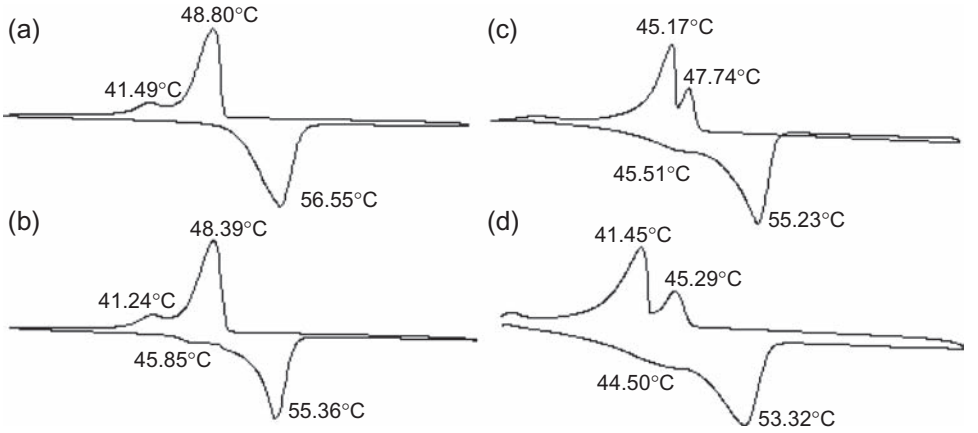

Figure 1. DSC thermograms: (a) GM before tempering; (b) GM after tempering; (c) GMT (25); (d) GMT (45).

(ZHWY-110X30; Zhicheng, Shanghai, China) and then shaken horizontally at 100 rpm and 37°C. At predetermined time intervals, the medium in the conical flask was completely removed by filtration for analysis and fresh dissolution medium was then added. The filtrate was analyzed by HPLC. A diffusion test of BU-S was also determined in the same condition as described above.

Result and discussion

Formulation development

NLCs have been prepared by a number of researchers using different methods[18–20]. In this study, BU-NLC was prepared by the melt-emulsification and ultrasonic technique with slight modification. The method is free of toxic organic solvents and is economical, simple, and reproducible. In our method, lipids materials and drugs were not dissolved in organic solvent. Bufadienolides were dissolved in the melted solid lipid and liquid lipid materials at approximately 10°C to 15°C above their melting point under stirring. This produced homogeneous dispersion of drug in the lipid. All the surfactants were added in the aqueous phase. We have screened many lipid materials including GM, Compritol 888 ATO, stearic acid, MCT, and OA. Lipoid E-80®, Pluronic F68, SDC, and Tween 80 were also screened as surfactants.

In the single-factor experiments, we found that Compritol 888 ATO and stearic acid were not suitable for the BU-NLC formation. The preparation containing stearic acid tended to flocculate more quickly than similar formulations containing Compritol 888 ATO and GM. In this study, the solubilities of bufadienolides were 123.62 and 294.99 mg/g in Compritol 888 ATO and GM, respectively. For Compritol 888 ATO, the operational temperature should be kept above 80°C and its emulsifying ability was inferior to that of GM. Thus, GM was selected for further study. A lipid concentration of 2% GM was selected for testing the effects of different surfactants. Table 1 shows the phenomena of the NLC

Table 1. Effect of different surfactants on the characteristics of BU–NLCs.

Surfactant[a]	Appearance	PSD (nm)	PI (nm)	Phase separation (25°C)
Epikuron 170	–	185.8 ± 108.1	0.339	2 days
Lipoid S-75®	–	194.7 ± 106.1	0.297	2 days
Lipoid E-80®	–	149.3 ± 79.5	0.282	1 week
Pluronic F68	+	114.3 ± 75.0	0.430	2 days
Tween 80	++	71.6 ± 45.8	0.412	1 day
Lipoid E-80® + Pluronic F68	+	142.9 ± 74.0	0.268	2 weeks
Lipoid E-80® + Tween 80	+	112.6 ± 72.7	0.417	1 week
Pluronic F68 + Tween 80	++	65.9 ± 39.8	0.365	1 week

[a]In all the formulations the concentration of GM was fixed at 2%. When single surfactant was used its concentration was 2%, whereas in the combination of surfactants each of them was 1%.–, light yellow opalescence; +, light translucence; ++, blue translucence.

Table 2. Ingredients in the formulations of BU-NLCs.

No.	GM (%)	MCT (%)	OA (%)	Lipoid E-80® (%)	Pluronic F68 (%)	SDC (%)
1	1	—	—	0.5	0.5	—
2	2	—	—	1	1	—
3	1.5	0.5	—	1	1	—
4	2.25	0.75	—	1	1	—
5	2.25	0.75	—	1	1.5	—
6	1.8	0.75	0.45	1	1.5	0.25
7	1.8	0.75	0.45	1.5	1	0.25
8	1.8	0.45	0.75	1	1.5	0.25

systems with different surfactants. The NLC formulations containing Lipoid E-80® achieved the smallest PSD in the three kinds of phospholipids. If nonionic surfactant Tween 80 was used alone and in combination with Lipoid E-80® and Pluronic F68, a relatively small mean diameter was obtained compared with those containing similar amounts of Pluronic F68 and Lipoid E-80®, although phase separation was easy. Lipoid E-80® combined with Pluronic F68 exhibited excellent stability. Thus, a formulation of 2% GM, 1% Lipoid E-80®, and 1% Pluronic F68 was chosen for the basic formulation.

Several formulations with different lipid compositions were prepared to develop an optimum BU-NLC. The representative formulations that we investigated are listed in Table 2. As shown in Table 3, when the GM was used as the sole lipid matrix (2% or 1%), the EE of B was below 80%. Then MCT (0.5%) was added as a liquid lipid material for its good solubility properties, and the EE of B increased to 79.3%. The addition of MCT combined with GM can increase the EE of B. The solid matrix with liquid domains could prevent drug leakage and show high solubility for lipophilic drugs. This may be because of the change in crystal order of the mixtures of lipid materials, which was discussed in detail in 'Differential scanning calorimetry' and 'X-ray diffraction' To achieve a higher EE, a formulation with a higher concentration of lipid matrix (including liquid lipid) and surfactants (Pluronic F68 and Lipoid E-80®) was prepared; the characteristics of the formulations are listed in Table 3. Considering the pH stability of bufadienolides[7], OA was added to the formulation of BU-NLCs to keep the pH of the surrounding environment at 7.0. Increasing the OA from 0.45% to 0.75% did not result in a significant reduction in pH. Thus, the OA concentration in this study was fixed at 0.45%. In addition, SDC was a key factor for improving the stability of NLC by decreasing the particle size. In contrast, its incorporation had no significant effect on the zeta potential of the NLC. This is different from the findings of a previous report[21]. This may be because different solid lipid materials were used in the formulation. In this study, a higher content of lipid materials (GM, MCT, and OA), up to 4% (w/w), was investigated, a higher EE of drug was not obtained, although more surfactants were needed to reduce the particle size. In the development of an optimum formulation, the short time stability (stored at 6 ± 2°C), EEs, and PI of the BU-NLCs are all very important. For an optimum BU-NLC system, the EEs of drugs should be above 80% and PI can range around 0.2–0.3. When the value is close or equal to 0.2, it indicates that the system is more stable. And the most important factor is to evaluate the short-time stability of the NLC system. We found that formulation 7 was more stable than other formulations. Considering these factors comprehensively, we can draw a conclusion that formulation 7 is the optimum formulation. Thus, the optimal formulation consisted of, according to the quality percentage, GM 1.8%, MCT 0.75%, OA 0.45%, Lipoid E-80® 1.5%, Pluronic F68 1.0%, and SDC 0.25%. The short-term stability study showed that it remained stable for 2 weeks at 6 ± 2°C.

Effect of lyophilization on BU-NLCs

Our preliminary studies revealed that the reconstitution of freeze-dried BU-NLC was impossible when the product was free of cryoprotectants. In this work, the final concentration of lipid materials (GM, MCT, and OA) in

Table 3. Characteristics of formulations 1–8.

No.	pH	PSD (nm)	PI	Zeta potential (mV)	EEs of B, C, and R (%)	Stored at 6 ± 2°C[a]
1	7.83	145.6±75.4	0.340	−10.17	69.8, 75.2, and 81.5	Within 2 days
2	7.90	142.9±74.0	0.268	−15.45	75.4, 84.7, and 87.6	Within 5 days
3	7.88	118.9±66.2	0.314	−12.01	79.3, 89.2, and 90.9	Within 5 days
4	7.55	164.1±90.6	0.305	−13.26	85.5, 92.3, and 93.7	Within 5 days
5	7.52	102.8±59.9	0.340	−12.98	81.2, 90.8, and 91.4	Within 3 days
6	6.95	92.0±48.5	0.278	−15.46	85.1, 92.4, and 93.5	Within 1 week
7	6.92	104.1±51.2	0.242	−16.95	87.4, 93.7, and 95.6	Within 2 weeks
8	6.76	91.0±45.2	0.245	−17.21	87.4, 93.7, and 95.6	Within 1 week

[a]Visible flake precipitate by bare eye occurred.

BU-NLCs was 3% (w/w). Various saccharides have been investigated with regard to their ability to stabilize BU-NLCs. We found that the final BU-NLCs had to be diluted before freeze-drying. The concentration of lipid materials should be below 0.3% (3 mg/mL). When the concentration of lipid matrix was more than 1%, the lyophilized cakes were not porous and could not be rehydrated readily. For the protection of the nanoparticles, the properties of the lyophilized cakes are very important. After 1:9 dilution with 11% of sucrose as a cryoprotectant, the final concentrations of lipid materials and sucrose were 0.3% and 10%. The aggregation of BU-NLCs could be prevented completely during the freeze-drying process. After freeze-drying, the products were porous and friable. The cakes could be reconstituted with purified water by manual shaking. The PSD and PI (114.5 ± 60.4 and 0.279) of BU-NLCs were slightly increased. These results indicate that sucrose was effective in preventing particle growth after freeze-drying[22]. BU-NLCs could be freeze-dried to improve the chemical and physical stability of these systems over an extended time period.

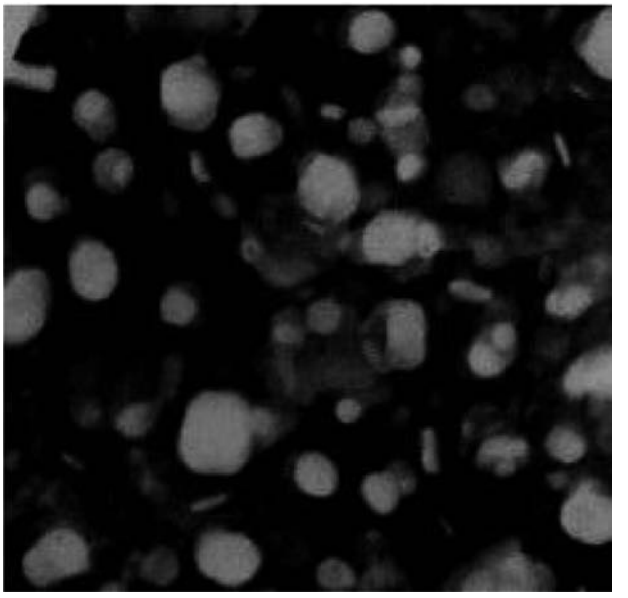

Figure 2. TEM photography of BU-NLCs (×58,000 magnification).

Physicochemical characteristics of the optimal formulation

In this study, we prepared a stable BU-NLC. The final BU-NLC (formulation 7) appeared to be translucent with a particle size of 104.1 ± 51.2 nm. The PSD expressed by the PI (0.242) was between 0.2 and 0.3, which was acceptable. As shown in Table 3, all systems had a zeta potential of about −10 to −20 mV. The zeta potentials of BU-NLCs were not dramatically affected by the combination ratio of Pluronic F68, Lipoid E-80®, and SDC. Although the range of zeta potential obtained was not high enough for effective sufficient electrostatic stabilization, Pluronic F68 provided additional steric stabilization of the particles[23,24]. We can expect combined electrostatic and steric stabilization of the BU-NLC formulation. The EEs of B, C, and R incorporated into the BU-NLCs were 87.4%, 93.7%, and 95.6%, respectively. The electron microscopic analysis revealed that the average diameter of the BU-NLC was approximately the same and they were roughly spherical in shape (Figure 2). The PSD of BU-NLCs ranged from 50 to 150 nm, which was in agreement with the photon correlation spectroscopy data. It was even possible to observe droplets of NLC undergoing deformation during the evaporation process.

Entrapment efficiency study

Microdialysis is an in vivo technique that permits monitoring of unbound tissue concentrations of drugs and metabolites at specific sites in the body[25]. Our previous studies[7,17] have indicated that the microdialysis technique is widely applicable for the investigation of the free-drug concentration of microcarrier systems. In this study, the EE of BU-NLCs was determined by microdialysis technology. Factors that could interfere with the probe recovery were investigated in detail in our previous work. The particles of NLC may interfere with the probe recovery. The probe recovery was also determined using BU-S as the perfusion medium, and as the external medium, purified water and blank NLCs were used to confirm that the presence of particles in the medium did not interfere with the drug diffusion. Microdialysis could be applied to the EE measurement of BU-NLCs.

Differential scanning calorimetry

As shown in Figure 1, the bulk GM after heating at 75°C for 30 minutes showed less ordered crystals with a broader peak width at 45.85°C. The DSC thermograms of physical mixtures of GMTs containing different concentrations of MCT were also investigated in this study. The melting point of GM was reduced and peak width was broadened on increasing the oil concentration. This indicated a massive crystal order disturbance of the NLC[26]. At a 25% oil load, the melting point was reduced by almost 1.32°C to 56.55°C. GM and MCT exhibited good miscibility in the solid state, and so phase separation could be excluded. An amorphous appearance was more marked in the carriers with MCT loads. In addition, the physical mixtures of GM and MCT had a sufficiently high melting point and could

prevent melting of the particles at body temperature. In our formulation, GMT (25) with a 25% content of MCT was enough to dissolve the bufadienolides. Therefore, in the case of BU-NLCs, it was concluded that the oil within the carrier was in a less ordered state compared with the SLN. The results showed an increase in drug EE of the NLC with the addition of MCT combined with GM.

Figure 3 shows an overview of the melting process of bufadienolides, GMOB, BU-NLCs (containing 0% and 10% sucrose), and 10% sucrose solution after lyophilization. The decreasing melting range could be correlated with impurities or less ordered crystals. The DSC thermogram confirms that the bufadienolides were not pure. The bufadienolides were in an amorphous state.

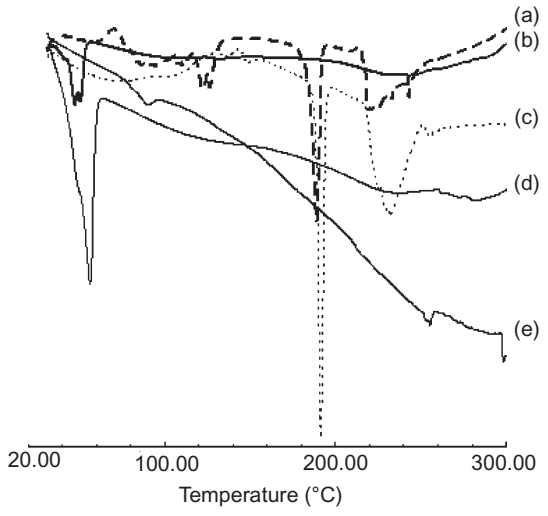

Figure 3. DSC thermograms: (a) 10% sucrose aqueous solution after lyophilization; (b) lyophilized BU-NLCs without sucrose; (c) lyophilized BU-NLCs containing 10% sucrose; (d) GMOB; (e) bufadienolides.

This phenomenon deserves further attention. For GMOB and lyophilized BU-NLCs without cryoprotectant, the melting process took place with a maximum at 56.23°C and 50.96°C, respectively. As for the 10% sucrose aqueous solution and BU-NLCs containing 10% sucrose after lyophilization, the melting peaks were at 190°C and 220°C, which were the diffraction peaks of lyophilized sucrose. These melting peaks were not detected in the lyophilized BU-NLCs without cryoprotectant. This suggests that the bufadienolides and the particles of BU-NLCs are presented in amorphous form in the cryoprotectant.

X-ray diffraction

The XRD data shown in Figure 4 were in good agreement with the results established by the DSC measurements. A typical amorphous state of the bufadienolides was apparent from the diffraction pattern. The diffraction pattern of lyophilized BU-NLCs without sucrose was markedly different from that of lyophilized BU-NLCs containing 10% sucrose. The diffraction pattern of the latter was similar to the 10% sucrose aqueous solution after lyophilization. The X-ray pattern in Figure 5 revealed that the bulk matrix (GM) was in good crystalline form and there were less ordered crystals in the GM after tempering, which was in agreement with the DSC results. The principal peak of bufadienolides was absent in GMOB. This result indicates that the drug was completely solubilized in the lipid materials of the BU-NLCs. The chemical nature of the lipid materials was important because lipids that form highly crystalline particles with a perfect lattice lead to drug expulsion. Lipids that are mixtures of different chain lengths form less perfect crystals with many imperfections, offering space to accommodate the drugs. This would contrib-

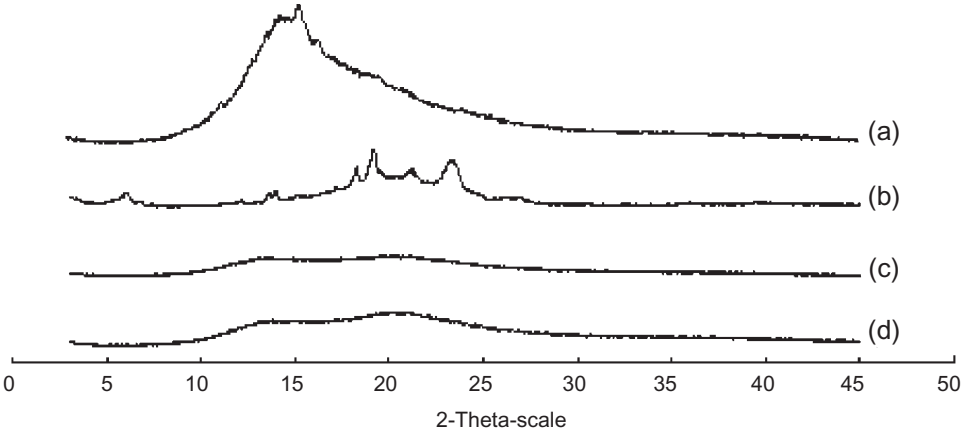

Figure 4. X-ray diffraction patterns: (a) bufadienolides; (b) lyophilized BU-NLCs without sucrose; (c) 10% sucrose aqueous solution after lyophilization; (d) lyophilized BU-NLCs containing 10% sucrose.

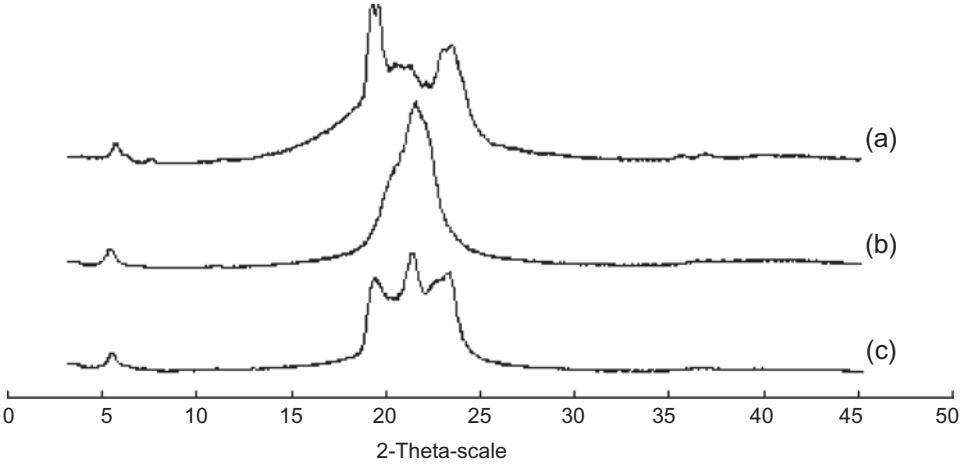

Figure 5. X-ray diffraction patterns: (a) GMOB; (b) GM after tempering; (c) GM before tempering.

ute to the higher drug loading capacity reflected in the EE of the drug.

Enzymolysis study

In the enzymolysis study, B and R were very stable in the presence of rat plasma at 37°C. They were undegraded in rat plasma at 37°C up to 24 hours, whereas C was readily degradable. As shown in Table 4, the enzyme kinetics of C in BU-S followed pseudo-first-order kinetics. The degradation of C was linear with the concentration of rat plasma. For C, there was a linear relationship between the rate constant (K) of enzymolysis and the concentration of rat plasma (C). The relationship was $K = 1.5742C$ and the R^2 of the regression was 0.9939. The stability of C in BU-NLCs was also investigated in this study. Figure 6 shows the residual percentages of C in BU-NLCs after incubation with different concentrations of rat plasma. The results show that C could be protected from degradation by the particles of BU-NLCs. When BU-NLC was incubated with a lower concentration of rat plasma, there was more C (89.54%, 74.63%, and 54.12% in 10%, 20%, and 50% rat plasma, respectively) left in the BU-NLC until 24 hours. For 80% rat plasma, the half-life of C in BU-NLC was 9.48 hours, which was 17-fold longer than that (0.55 hour) in BU-S.

Table 4. Enzymolysis rate constant and half-life of C in BU-S with different concentrations of rat plasma at 37°C.

Concentration of rat plasma (%)	k (hours^{-1})	Half-life (hours)
100	1.63	0.43
80	1.26	0.55
50	0.72	0.96
20	0.27	2.57
10	0.12	5.78

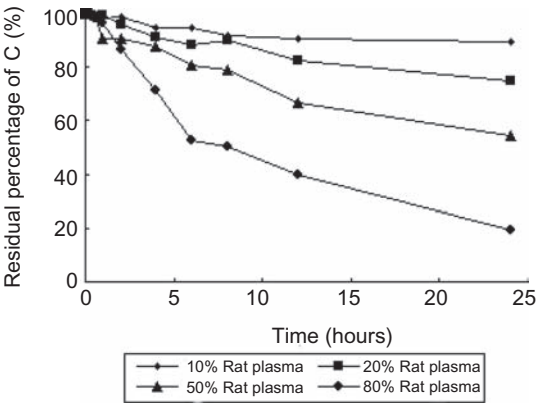

Figure 6. Residual percentage of C in BU-NLCs after incubation with different concentrations of rat plasma.

This may be because of the lipid materials of BU-NLCs, that is, mixtures of GM, MCT, and OA (GMOB, as shown in Figure 3 melting point about 50.96°C). The nature of the matrix might keep the structure of BU-NLCs rigid even in the presence of rat plasma. The solid lipid core of NLC could increase the chemical stability of incorporated drugs and protect them from degradation[27]. Therefore, the EE of the drug in the NLC should be ensured.

In vitro release study

In Figure 7a the diffusion test of bufadienolides from BU-S in normal saline was rapid. The release was fast and nearly 50% of the drug was released after 1 hour. After 4 hours, the accumulative release percentage was close to 90%. The release profile of bufadienolides from the BU-NLCs into normal saline up to 120 hours is shown in Figure 7b. For all release curves of B, C, and R, a biphasic drug release pattern was observed showing

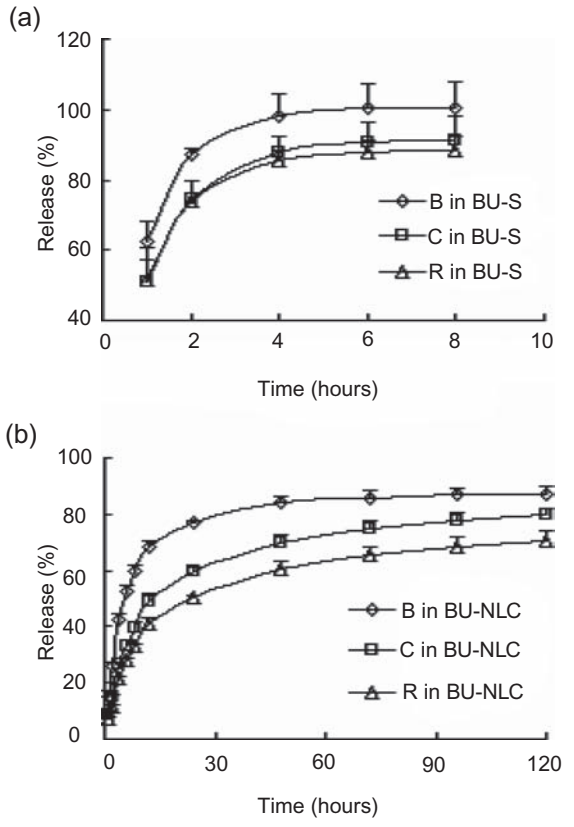

Figure 7. Diffusion test of BU-S (a) and in vitro release of B, C, and R from BU-NLCs (b) in normal saline.

that the drug release was rapid during the initial stage and then exhibited sustained release at a constant rate. The regression results for release of drug from BU-NLCs are listed in Table 5. The in vitro drug release fitted the Weibull distribution closely. This bufadienolides release pattern was probably related to the MCT distribution in nanoparticles. Most of the liquid lipid was located at the shell of the nanoparticles and there was little or no liquid lipid entrapped into the core during the cooling process from melted lipid droplet to solid nanoparticles[28,29]. Therefore, the NLC obtained at 75°C exhibited burst release during the initial 8 hours and sustained release subsequently. The release of bufadienolides from the NLC in distilled water was also investigated in our study. The release of drug was slightly slower than in normal saline. However, there was no significant difference between the release rates. We found that the release rate order of B, C, and R was as follows: B > C > R. The influence of rat plasma on drug release behavior was also investigated. We found that the presence of rat plasma did not markedly affect the release of drug from NLC (data not shown). Our results showed that BU-NLCs produced a sustained release of bufadienolides in vitro.

Conclusion

This study clearly describes a new formulation of BU-NLCs with antitumor and analgesic properties. A melt-emulsification and ultrasonic method was employed to prepare the BU-NLC with improved drug incorporation and release properties. Using DSC analysis and XRD, the drug was found to be in its amorphous state in the NLC particles. The enzymolysis study indicated that the BU-NLC increased the chemical stability of incorporated bufadienolides and protected C from degradation. The in vitro drug release behavior fitted the Weibull distribution closely and exhibited a biphasic drug release pattern. These results show that the NLC prepared in this study could be developed as a carrier. Future work will investigate the cytotoxicity of BU-NLCs in vitro and its fate after parenteral delivery in vivo.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

Table 5. Regressing results for release of B, C, and R from BU-NLCs.

Drug	First order $\ln(1-Q)$ versus t	Higuchi Q versus $t^{0.5}$	Viswanathan $-\ln(1-Q)$ versus $t^{0.65}$	Weibull $\ln[1/(1-Q)]$ versus $\ln t$
B	$y=-0.0149x-0.6693$	$y=0.0687x+0.301$	$y=0.0870x+0.4458$	$y=0.5018x-1.3960$
	$r=0.8789$	$r=0.8723$	$r=0.9338$	$r=0.9605$
C	$y=-0.0122x-0.3592$	$y=0.0685x+0.1482$	$y=0.0699x+0.1880$	$y=0.5786x-2.0863$
	$r=0.9345$	$r=0.9427$	$r=0.9733$	$r=0.9796$
R	$y=-0.0093x-0.2839$	$y=0.0621x+0.1106$	$y=0.0534x+0.1530$	$y=0.5706x-2.3104$
	$r=0.9331$	$r=0.9532$	$r=0.9723$	$r=0.9781$

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