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Research Article

Formulation, preparation, and stability of intravenous bufadienolides-loaded lipid microspheres

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The purpose of this study was to develop a new formulation of intravenous lipid microspheres for bufadienolides (bufadienolides-loaded lipid microspheres, BU-LM) and investigate the stability of BU-LM after dilution and long-term storage. Photon correlation spectroscopy, electrophoretic light scattering, and light microscopy were used to evaluate the physical characteristics of BU-LM. The chemical properties of bufadienolides were measured by high-performance liquid chromatography. The optimum BU-LM, consisting of 10% MCT, 3.0% Lipoid S75, 0.4% F68, 0.05% sodium oleate and 2.5% glycerol, was prepared by emulsification with a high-speed shear mixing at 10 000 rpm for 12 min and a high-pressure homogenization at 800 bar for 8 cycles at 30°C. The BU-LM at pH 6.48, with the particle size distribution of 175.2 ± 57.3 nm, ζ-potential of −32.10 mV, entrapment efficiency of 86.9%, 91.7%, 92.9%, and contents of 20.8%, 31.3%, 50.4% for B, C, R, respectively, was stable after sterilization in a 121°C rotating autoclave water steam bath for 10 min. Moreover, BU-LM had sufficient physicochemical stability after dilution with 5% glucose injection and long-term storage at 4 ± 2°C for 18 months. BU-LM has a great potential for clinical applications as a new drug delivery system for bufadienolides.

Practical applications: Bufadienolides, mainly composed of bufalin (B), cinobufagin (C), and resibufogenin (R) have poor aqueous solubility and are chemically unstable. Currently the main marketable products of bufadienolides are solution injections, which cause serious venous irritation and in this formulation, the drug is not protected from degradation. Lipid microspheres, with the unique properties of a low cost, low toxicity, good storage stability, easy to scale up production, sustained release and targeting effect, incorporate the drugs into the interior oil phase and the oil-water interfacial film; thus a direct contact of the drug with body fluids and tissues is prevented, which increases the stability of the drugs and minimizes the possible side effects.

Keywords: Bufadienolides / Formulation / Lipid microsphere / Preparation / Stability

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1 Introduction

Chan Su (toad venom), the traditional Chinese medicine, is obtained from the postauricular and skin glands of Chinese toads. It has been used as a cardiotonic, diuretic, anodyne and hemostatic agent [1] in China, Japan and other Asian countries for centuries, and over the last decade, it has gained considerable favor in the United States and elsewhere [2].

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Abbreviations: B, bufalin; **C,** cinobufagin; **R,** resibufogenin; **HPLC,** high-performance liquid chromatography; **BU-LM,** bufadienolides-loaded lipid microsphere; **BU-S,** bufadienolides-loaded aqueous solution; **EE,** entrapment efficiency; **DDW,** double-distilled water; **MCT,** medium-chain triglyceride; **LCT,** long-chain triglyceride; **SO,** sodium oleate; **PSD,** particle size distribution; **PC,** phosphatidylcholine; **RT,** room temperature

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The major effective components, generally called bufadienolides, have a novel steroidal A/B *cis* and C/D *cis* structure with an α-pyrone ring at C-17 and a β-hydroxyl at C-3 position, which exhibit a range of biological activities, such as cardiotic, blood pressure stimulating, respiration, and antineoplastic activities. Of the bufadienolides, bufalin (B), cinobufagin (C), and resibufogenin (R), shown in Fig. 1, are three typical bufadienolides and have the promising prospect as antineoplastic agents [3]. However, they have poor aqueous solubility and chemical stability. In current, commercially marketable products of bufadienolides are mainly Toad Venom Injection and Huachansu Injection, but their usages are limited by two problems. On one hand, the intravenous aqueous solution causes venous irritation, which results in serious pain in some patients. On the other hand, none of these can effectively reduce the degradation of bufadienolides. Therefore, in order to ensure both high solubility and stability of the drug, it is of great importance to develop a new dosage form for intravenous administration of bufadienolides.

Over the last few decades, lipid microspheres (LMs), liposomes and nanoparticles have been attracting increasing attention as drug delivery systems for poorly water-soluble drugs because of their drug targeting effect and therapeutic benefit. However, liposomes are relatively unstable, also the enormous cost and complexity associated with the manufacturing process often limit their commercial viability. Similar problems exist in nanoparticles. And besides, great toxicity of the polymer in nanoparticles cannot be avoided. Nevertheless, it is distinctly different that LMs, also called lipid emulsions or microemulsions, have been considered promising parenteral drug delivery systems, attributable to the unique properties of low cost, low toxicity, good storage stability and easy large-scale production [3]. LMs can also enhance the solubility and stability of the drugs to obtain sustained release and targeting by incorporating the drugs into the interior oil phase and the oil-water interfacial film [4]. In addition, LMs can prevent direct contact of the drug with body fluids and tissues to minimize the possible side effects.

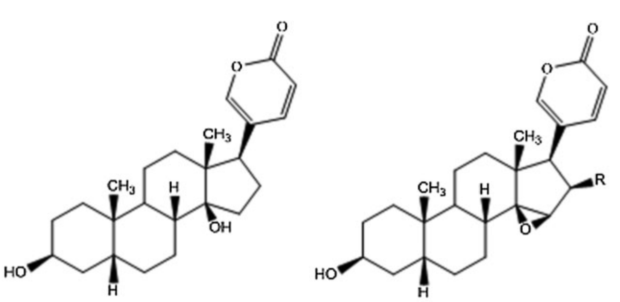

Figure 1. Chemical structures of three kinds of bufadienolides.

All these excellent characteristics may make the LMs be an attractive carrier for bufadienolides. Thus, in our study, BU-LM were developed and investigated. Firstly, the formulation and preparation procedures of BU-LM were systemically studied, then the stability of BU-LM after long-term storage at 4 ± 2°C over 18 months and dilution with 0.9% sodium chloride injection and 5% glucose injection for 4 h were investigated in detail.

2 Materials and methods

2.1 Materials

Bufadienolides were extracted from toad venom in the Department of Pharmaceutics, Shenyang Pharmaceutical University, P. R. China. Bufadienolides (purity 95% above) mainly consist 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.

The following materials were purchased or obtained from the sources in parentheses: Lipoid S75®, Lipoid E80®, sodium oleate (SO) and medium-chain triglycerides (MCT) (Lipoid KG, Ludwigshafen, Germany), PL-100M (Q.P. Co., Tokyo, Japan), long-chain triglyceride (LCT) (Tieling Beiya Pharmaceutical Co., Liaoning, China), Tween80 for parenteral use (Shenyu Medicine and Chemical Industry Ltd. Co., Shanghai, China), Poloxamer188 (F-68) (BASF AG, Ludwigshafen, Germany), and glycerin (Suichang Glycerin Plant, Zhejiang, China). Double-distilled water (DDW) was used in all experiments and all other chemicals and reagents used were of analytical or chromatographic grade.

2.2 Methods

2.2.1 Preparation of BU-LM

The BU-LM with a concentration of 0.5 mg/mL was prepared by high-pressure homogenization. Firstly, 3.0% (w/v) Lipoid S75 was dispersed in 10% (w/v) MCT at 78 ± 2°C in a constant temperature water bath (HH.SY11-Ni, Hangfeng Apparatus and Instrument Co., Beijing, China) until dissolved, then the bufadienolides were added and stirred for another 20 min to prepare the oil phase. The aqueous phase, consisting of 2.5% (w/v) glycerol, 0.4% (w/v) F-68 and 0.05% (w/v) SO, was also heated at 70 ± 2°C in another water bath until uniformly dissolved. Then, the oil phase was added slowly to the aqueous phase with high-speed shear mixing (ULTRA RURRAX®IKA® T18 basic, Germany) at 10 000 rpm to obtain the coarse emulsion. The high-speed shear mixing process was carried out for 12 min with a 3-min high speed-shear mixing-on period and a 1-min-off period. Thirdly, the volume was adjusted with DDW, and the pH with 0.1 mol/L HCl to 6.5. After that, the coarse emulsion was subjected to high-pressure homogenization (Niro Soavi NS 10012K homogenizer, Via M. da Erba, 29/A-43100

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PARMA, Italy) at 800 bar for eight cycles. The temperature of the entire homogenization process was controlled at 30°C using an ice-water bath. Finally, the BU-LMs were sealed in vials, and sterilized in a 121°C rotating autoclave water steam bath for 10 min.

2.2.2 Investigations of BU-LM

2.2.2.1 Formulation investigations of BU-LM

The composition and ratio of the oil phase (LCT and MCT), the types and amounts of the emulsifiers (soybean lecithin Lipoid S75, egg yolk lecithin Lipoid E80, and PL-100M) and co-emulsifiers (Tween80, F68 and SO), the addition of the osmotic agent (glycerol) were employed to investigate the influence of different compositions of formulation on the physical and chemical stability of BU-LM. The BU-LMs were prepared according to Section 2.2.1 with one condition changed while the others constant.

2.2.2.2 Preparation investigations of BU-LM

To prepare BU-LM with the optimized physicochemical properties, the processes of high-speed shear mixing, high-pressure homogenization, thermal sterilization, temperature, and pH were also investigated in details. The BU-LMs were prepared according to Section 2.2.1 with one condition changed while the others constant.

2.2.3 Characteristics of BU-LM

2.2.3.1 Particle size distribution, zeta potential and pH

The mean particle size and SD of BU-LM, used to evaluate the particle size distribution (PSD) and polydispersity index (PI) of BU-LM, were determined by photon correlation spectroscopy (PCS; dynamic light scattering, DLS) using a Nicomp^{TM} 380 Particle Sizing system (Zeta Potential/Particle Sizer Nicomp^{TM} 380ZLS, Santa Barbara, California, USA). The system was sensitive to particles with size ranging from 5 nm to 3 μm. The BU-LM sample was diluted 1:5000 with DDW immediately before measurement at 25°C. The Nicomp and Gaussian distributions of particle size were obtained at the same time with intensity weighting, volume-weighting, and number-weighting. The particle size analysis in this study was all evaluated using the intensity-weighting Gaussian distribution pattern unless otherwise specified. In addition, the microscopic assessment was carried out using a Motic DMBA450 microscope (MoticChina Group Co. Ltd., Beijing, China). The emulsion samples were analyzed without being diluted using an oil immersion microscope with 1,000-fold magnification; typically, 20 microscopic fields were analyzed for the detection of the number of large droplets exceeding 5 μm.

The pH of BU-LM was measured using a pH meter (Leici®, Shanghai Precision Science Instrument Ltd., Shanghai, China) fitted with a microelectrode at 25 ± 2°C.

2.2.3.2 Entrapment efficiency (EE) of bufadienolides in BU-LM

Ultracentrifugation method was used to determine the EE of BU-LM by measuring the concentrations of B, C, and R in the dispersion phase. Firstly, the sample of BU-LM was put in the polyallomer tube and ultracentrifugated at 4°C using a CS120GXL ultracentrifuge (HITACHI Co. Japan) at 50 000 rpm for 1.5 h. Then, the bottom of the tube was pricked with a syringe needle to collect the aqueous phase. Lastly, the amounts of B, C, and R in the separated aqueous phase were measured by high-performance liquid chromatography (HPLC), and the EEs were calculated according to the following equation.

$$\mathrm{EE}\,(\%) = \frac{C_{\mathrm{total}}V_{\mathrm{total}} - C_{\mathrm{water}}V_{\mathrm{water}}}{C_{\mathrm{total}}V_{\mathrm{total}}} \times 100$$

where C_{total} and V_{total} were the concentration and the volume of the drug in the whole BU-LM, and the C_{water} and V_{water} were those in the water phase, respectively.

2.2.4 High-performance liquid chromatography (HPLC) analysis

A RP HPLC analytical method was employed for the analysis of BU-LM. 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 an HiQ sil C18 column (250 × 4.5 mm, i.d. 4 μm; KYA TECH Corporation, Tokyo, Japan) and a precolumn (Fusion-RP 4 × 3.0 mm). The mobile phase was a 45:55 (v/v) mixture of acetonitrile:-water (pH 3.2) containing 0.5% potassium dihydrogen phosphate at a flow rate of 1.0 mL/min. The wavelength was 296 nm, the injection volume was 10 μL and the column temperature was 40°C. The sample of BU-LM was diluted with isopropanol and passed through a 0.22-μm micropore membrane filter before injection into the column.

2.2.5 Stability study

2.2.5.1 Dilution stability of BU-LM

The physical stabilities of BU-LM in 5% glucose (Glu) and 0.9% sodium chloride (NaCl) injections were investigated. The PSD and 99% cumulative distribution were determined at 0, 0.5, 1, 2, 3, and 4 h after being diluted to 5-fold and 10-fold with 5% Glu or 0.9% NaCl injection at room temperature (RT), respectively.

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2.2.5.2 Long-term stability of BU-LM

The long-term stability assessment of BU-LM was monitored over 18 months at 4 ± 2°C. At pre-determined time intervals, samples were removed and allowed to reach the RT. Their physicochemical properties were evaluated by the physical appearance, PSD, ζ-Potential, pH, EE and drug remaining. All the studies were performed in triplicate.

3 Results and discussion

3.1 Formulation investigations of BU-LM

3.1.1 Oil phase composition and ratio of BU-LM

The oil phase composition in emulsions plays an important role in emulsion formulations. Normally, LCT and MCT are employed mainly as the oil phase in fat emulsions intended for total parenteral nutrition [5]. Hence, the 10% (w/v) oil phases of LCT, MCT and their mixture at ratio of 5%:5% were employed to investigate the effect of different oil phases on physicochemical stability of BU-LM, including the physical appearance, PSD and EE. According to Table 1, oil droplets were observed in the Formulations with LCT after sterilization, and with the increase of LCT from 5 to 10%, the oil droplets became more and larger. The explanation for this phenomenon was that the longer the hydrocarbon chain length of the dispersed oil phase, the greater the interfacial tension against the continuous aqueous phase, the more unstable the emulsion is [6]. When the oil phase was pure 10% MCT in Formulation 3, BU-LM displayed good physicochemical characteristics, with PSD of 166.6 ± 61.8 nm and EEs of 85.4, 91.6, and 93.9% for B, C and R, respectively. The high EEs might be due to the higher polarity of MCT. MCT was approximately 100 times more water-soluble than LCT and so, it had an increased drug uptake capability. Besides, the Formulation 3 had sufficient physical stability even stored at 60°C for 5 days. It had been reported that MCTs might reduce the toxicity associated with pure LCT-based lipid emulsions and provide more stable all-in-one admixtures, also they could reduce the high amount of linoleic acid, balance the fatty acid pattern and adjust the viscosity of the oil phase [7–9]. Taken together, pure MCT was selected as the oil phase in the end. However, as MCT ratio decreased from 10 to 5% in Formulation 4, the mean particle size of BU-LM became remarkably smaller while the SD of PSD almost unchanged, which led to a wider size distribution and resulted in the instability of BU-LM. In general, emulsions with the mean particle size of about 200 nm tended to be the most physically stable. Too small particle existing in the BU-LM with a very large interfacial surface had a rapid Brownian motion, which easily coalesced to big particle and led to aggregation after placed for a long time. Besides, Formulation 4 with the EE decreased 5% underwent emulsion breakage after stored at 60°C for 5 days, which might be because that 5% MCT was insufficient to encapsulate the drug effectively and form a strong oil film to remain the long-term stability of BU-LM. Hence, a 10% (w/v) MCT as the oil phase in BU-LM was employed.

3.1.2 The type and amount of lecithin as emulsifier in BU-LM

Emulsifiers are another essential component of lipid emulsions. It is well known that the emulsification depends on the emulsifiers used, and the emulsification process significantly influences the stability of the emulsion [10]. Though various emulsifiers on the market are concerned, lecithin should always be the first choice. The natural lecithins, including egg yolk lecithin and soybean lecithin, can be totally biodegraded and metabolized, so they are regarded as well-tolerated and non-toxic compounds with excellent biocompatibility, and suitable for long-term use and high-dose infusion. Lipoid S75 (phosphatidylcholine, PC 72%) as soybean lecithin, Lipoid E80 (PC 82%) and PL-100M (PC 80%) as egg yolk lecithin, were employed to investigate the influence of different types of lecithins on the properties of BU-LM. The results of our study in Table 2 showed that BU-LM prepared with egg yolk lecithin could not undergo the autoclaving sterilization, because the visible oil droplets or emulsion breakage were observed for the BU-LM with PL-100M or Lipoid E80, respectively. However, under the same condition, BU-LM with soybean lecithin Lipoid S75 exhibited a good physical appearance and higher ζ-potential of

Table 1. The influence of oil phase composition and ratio on the properties of BU-LM

Formulation	LCT:MCT	PSD (nm)	PI	EE (%)			Physical appearance	
				B	C	R	After autoclaving	After stored at 60°C for 5 days
1	10:0	ND	ND	ND	ND	ND	Bulk oil droplets	Bulk oil droplets
2	5:5	ND	ND	ND	ND	ND	Visible oil droplets	Bulk oil droplets
3	0:10	166.6 ± 61.8	0.138	85.4	91.6	93.9	Good	Good
4	0:5	137.2 ± 59.3	0.187	80.5	86.2	87.5	Good	Emulsion breaking

ND, not determined.

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Table 2. The influence of the type of lecithin on the properties of BU-LM

	3.0% (w/v) Lecithin	Before autoclaving				After autoclaving			
Formulation	Lecithin type	ζ-Potential (mV)	PSD (nm)	PI	Physical appearance	ζ-Potential (mV)	PSD (nm)	PI	Physical appearance
5	Lipoid S75	−29.15	177.0 ± 69.8	0.156	Good	−28.76	164.6 ± 70.8	0.185	Good
6	PL-100M	−21.04	164.5 ± 57.9	0.124	Good	ND	ND	ND	Visible oil droplets
7	Lipoid E80	−17.81	192.4 ± 96.2	0.250	Good	ND	ND	ND	Emulsion breaking

ND, not determined.

−28.76 mV. This could be due to the emulsification of different types of lecithins. Hansrani [11] and Yamaguchi et al. [12] had reported that the stability of lipid emulsions was influenced by the purity of the lecithin, and certain "impurities" of lecithin were necessary to form stable emulsions. In natural lecithin, the unesterified phosphatidic acid (PA) is present to a much lower extent than PC and PE (phosphatidylethanolamine), but it takes an important role in the stability of the emulsions since it can contribute to more negative ζ-potentials and, thus, increase the heat-stress or storage stability of the emulsions. Consequently, soybean lecithin containing more PA and some other ionic components than egg yolk lecithin exhibited excellent emulsifying properties [13]. Therefore, soybean lecithin of Lipoid S75 was selected as the main emulsifier in this paper.

In the following, different amounts of Lipoid S75 were also investigated. As shown in Table 3, oil droplets floating on the Formulation 8 and 9 were observed. This was because 1.20 or 1.5% (w/v) lecithin was not enough to emulsify the 10% oil phase in BU-LM. The BU-LM exhibited a good physical appearance after thermal sterilization when the amount of lecithin was up to 2.5% (w/v) and 3.0% (w/v), but the former was still insufficient to ensure the stability of BU-LM since flocculation, which was a significant indicator of the coalescence, was observed after storage at 25°C for only a week. In addition, Table 3 also suggested that the EE became higher with the increased lecithin, indicating that only 3% Lipoid S75 could encapsulate the drug entirely. Consequently, adding 3.0% (w/v) Lipoid S75 produced an emulsion with good physicochemical stability following heat-stress or storage, also PSD (165.5 ± 66.1 nm) and high EEs (all above 85%) could confirm its safety for intravenous administration. Hence, 3.0% (w/v) Lipoid S75 was chosen as the optimum amount of lecithin.

3.1.3 The co-emulsifiers of Tween80, F68, sodium oleate and their amounts

A single emulsifier of Lipoid S75 was insufficient to maintain the stability of the emulsion. The co-emulsifiers could help further emulsify the oil phase and form a tight complex interfacial film between the water phase and the oil phase to keep the emulsion stable. However, many of the non-ionic co-surfactants had led to various toxic reactions, thus their selection and incorporation should be carefully studied.

Usages of Tween80, F68 and sodium oleate for parenteral were investigated in this study. The result in Table 4 showed that the formulations with Tween80 could not undergo the autoclaving sterilization and visible oil drops were observed. This might be due to the low cloud point of Tween80. It had been established previously that emulsion formulations, containing an emulsifier with a high cloud point, were more resistant to coalescence during the autoclaving process, while formulations with a low cloud point, precipitated at the sterilization temperature leading to a breakdown of the film around the oil droplets and the coalescence of the

Table 3. The influence of the amount of Lipoid S75 on the properties of BU-LM

		After autoclaving					After storage at 25°C for a week
Formulation	Lipoid S75 (%, w/v)	PSD (nm)	PI	ζ Potential (mV)	Physical appearance	EE of B, C and R (%)	Physical appearance
8	1.2	ND	ND	ND	Bulk oil droplets	ND	Flocculation
9	1.5	ND	ND	ND	Visible oil droplets	ND	Flocculation
10	2.5	183.7 ± 63.5	0.119	−26.20	Good	71.8,80.5, 84.6	Flocculation
11	3.0	165.5 ± 66.1	0.160	−27.80	Good	86.1,91.9, 92.8	Good

ND, not determined.

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Table 4. The influence of the amount of F-68 and Tween-80 on the properties of BU-LM

			Before autoclaving			After autoclaving			
Formulation	F-68 (%, w/v)	Tween-80 (w/v)	PSD (nm)	PI	Physical appearance	PSD (nm)	PI	EE of B, C and R (%)	Physical appearance
12	0.0	0.2	165.7 ± 64.8	0.153	Good	ND	ND	ND	Visible oil droplets
13	0.2	0.2	156.4 ± 54.7	0.122	Good	ND	ND	ND	Visible oil droplets
14	0.4	0.2	153.3 ± 73.6	0.231	Good	ND	ND	ND	Visible oil droplets
15	0.2	0.0	179.2 ± 59.7	0.111	Good	170.6 ± 64.0	0.141	80.2,88.3,90.6	Good
16	0.4	0.0	174.5 ± 55.3	0.100	Good	166.3 ± 57.8	0.121	85.4,91.6,93.9	Good

ND, not determined.

droplets [14]. In addition, Jumaa et al. [15] reported that LM with only Tween80 as an emulsifier showed a remarkable erythrocyte membrane damage, but the formulations using only S75 and F68 did not. Thus, Tween80, which had a low cloud point and remarkable hemolytic activity but no extra advantages, was excluded, and F68 was selected as the co-emulsifier.

The addition of F68 was of great importance to emulsify the oil phase. Table 4 exhibited that the Formulation 15 with 0.2% of F68 had a good physical appearance and satisfactory PSD of 170.6 ± 64.0 nm after autoclaving, but the EEs were not all above 85%. This might be because that the F68 was insufficient to encapsulate the drug entirely. In Formulation 16, the good physicochemical property of BU-LM with PSD of 166.3 ± 57.8 nm and EE of 85.4%, 91.6%, 93.9% for B, C, R after autoclaving sterilization, respectively, indicated that 0.4% of F68 was essential and sufficient in BU-LM. Tadros and Vincent [16] pointed out that emulsion stability was dependent on the structure of the surfactant. F68, as a block copolymer and water-soluble non-ionic emulsifier, had a strong emulsifying effect. It could stabilize the newly created interface immediately and within only one homogenization cycle maximum dispersion had been already achieved [17]. Besides, the combination of emulsifier of Lipoid S75 and co-emulsifier of F-68 could form a close-packed mixed film to confer improved stability [10]. Therefore, the amount of F68 was fixed to be 0.4% (w/v).

Sodium oleate, a water-soluble co-emulsifier, was adsorbed or incorporated by the oil-water interfacial film to give a negative charge to the emulsion surface and enhance the electrostatic repulsion between emulsion droplets. It could not only stabilize the emulsion against coalescence, but could also decrease the granularity of emulsion particles. In addition, SO had a certain buffering capacity to reduce the pH change to a minimum [18]. Moreover, the interfacial film composed with lecithin-F68-SO was strong enough to remain the stability of the emulsion [19]. Hence, a small amount of SO was often used in commercial parenteral emulsions (Lipofundin®MCT, Abbolipid®, Schiwalipid® and others) to obtain stable formulations during the autoclaving process [20]. According to the Formulations from 17 to 19 in Table 5, it was obvious that the incorporation of 0.05% SO made an important contribution to increase the absolute value of the ζ-Potential and keep pH value nearly unchanged before and after autoclaving. However, SO was not endogenous, and the less used, the better. Therefore, 0.05% (w/v) SO should be added to prepare BU-LM.

3.1.4 The addition of osmotic agent

The aqueous phase should be augmented by the incorporation of ionic or osmotic agents, antioxidants, buffers and preservativesas as required. Isotonic adjustment (to 280–300 mOsm/kg) was important for large-volume parenterals such as the injectable fat emulsions because emulsified oil

Table 5. The effect of the addition of sodium oleate on the properties of BU-LM

		Before autoclaving				After autoclaving			
Formulation	Sodium oleate (%, w/v)	PH	ζ-Potential (mV)	PSD	PI	PH	ζ-Potential (mV)	PSD	PI
17	0	6.52	−17.55	183.7 ± 62.5	0.116	6.00	−17.67	175.5 ± 65.2	0.138
18	0.05	6.51	−25.06	174.8 ± 65.9	0.142	6.46	−26.44	168.2 ± 66.3	0.155
19	0.1	6.45	−27.64	172.3 ± 69.3	0.162	6.34	−27.95	161.0 ± 65.1	0.163

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exerted no osmotic effect. Glycerol with the ability of tonicity was preferred by the manufacturers of commercial soybean oil emulsion [Intralipid®, Soyacal® (Alpha Therapeutic, CA, USA), Travamulsion® (Baxter-Travenol,Il, USA)] and a commercial safflower oil emulsion (Liposyn®, Abbott, North Chicago, IL, USA). In addition to its contribution to tonicity, glycerol had been also shown to reduce the globule size and improve the creaming stability of o/w emulsions [21]. In our study, 2.5% (w/v) glycerin was used to adjust the osmotic pressure.

3.2 Preparation investigations of BU-LM

3.2.1 Effect of coarse emulsion "dispersion" preparation

To prepare an O/W emulsion, the oil phase was added to the aqueous phase when both dissolved, accompanied by the emulsification with a high-speed shear mixing at an optimum velocity. Our preliminary experiment suggested that too rapid addition induced many visible oil droplets separating from the bulk, which might be due to the incomplete dispersion of the oil into the aqueous phase. Also, too slow addition needed more time for high-speed shearing, which produced more extra heat, causing more rapid Brownian motion and leading to aggregation of the emulsion particles. To disperse the oil phase in the water phase sufficiently and avoid producing extra heat, the oil phase should be added to the aqueous phase within 2 min when to prepare a 100-mL BU-LM.

During the high-speed shear mixing process, the interface between the oil phase and the water phase was deformed to an extent to form a stable and homogeneous emulsion by breaking large liquid drops into smaller drops. The most significant method to decrease the drop diameter depended on the high-shearing intensity and the mixing time. Verbich et al. [22] reported that the radii of the droplets in the emulsion decreased with increasing stirring speed and emulsifying time. On one hand, the lowest speed of high-speed shearing mixer was 6000 rpm, insufficient for emulsion system because the appearance of the mixture could not become uniform even mixed for a long time, whereas a shearing speed higher than 10 000 rpm was not required since it caused energy consumption and easily led to emulsion breaking away from the oil-water interface. On the other hand, we controlled the high-speed shear mixing process for 4, 8, 12, and16 min respectively, with a 3-min high-speed shear mixing-on period and a 1-min-off period to optimize the mixing time. The obtained coarse emulsion was directly observed by microscope without dilution. The results exhibited that the number of the large droplets exceeding 5 μm per microscopic field decreased rapidly in the first minutes and then reached a plateau after 12 min. It was reported that the emulsifier became more effective with increasing mixing time, but too long mixing time could cause the emulsifier to drop out from the oil-water interface and as a result of this, the effectiveness of emulsifier decreased, leading to an instability of the emulsion [23]. Therefore, high-speed shear mixing with a shearing speed 10 000 rpm for 12 min including a 3-min rest period was selected to prepare the coarse emulsion "dispersion".

3.2.2 Homogenization pressure and cycles

Once the coarse emulsion was formed, it was necessary to reduce the globule size by homogenization because the coarse emulsion was unstable when placed at the RT. A two-stage high-pressure homogenization, combining procession of shear, turbulence and cavitation forces that could result in the energy-efficient production of consistently fine droplets with a narrow size distribution of approximately 40–200 nm, was used extensively to prepare emulsions. It had been reported that the most important homogenization parameters for controlling droplet size were homogenization pressure, number of cycles and temperature [24, 25]. An ice-water bath was used to avoid producing extra heat that could disrupt droplets over-processed and keep the temperature about 30°C during the homogenization process. Besides, seven different homogenization pressures and twelve homogenization cycles were involved to investigate and optimize the homogenization condition. As shown in Fig. 2A, the mean particle size and SD values reduced as the homogenization pressure increased from 400 to 800 bar, but above 800 bar, the values almost did not change and had a slight increase now and then. Likewise, when the homogenization cycle was up to eight, excess cycles did not obviously decrease the mean particle size and SD values anymore (Fig. 2B). This was because when homogenization pressure and cycles increased to a certain extent, the forces of hole force, shear force and collision force between particles were enhanced, resulting in too much energy in the system and producing some very limited coalescence, which led to an increase in the PSD [26]. Consequently, 800 bar for 8 cycles was chosen as the optimum homogenization parameters.

3.2.3 Temperature

The temperature was another important factor to form a stable emulsion. On one hand, a relative high temperature was needed to dissolve phospholipids and drugs sufficiently. It had been reported that a relatively stable emulsion with smaller particle size could be prepared only if the oil phase and aqueous phase were dissolved at the phospholipids' phase inversion temperature of 70–80°C [27]. Also, a high temperature could decrease the viscosity of the oil phase, leading to a less shearing power to emulsify the oil-water mixture. Therefore, 78 and 70°C were controlled to dissolve or disperse the oil phase and aqueous phase, respectively. On the other hand, a relative low temperature was required during the process of high-pressure homogenization to avoid producing extra heat. Chen and Tao [23] reported that too

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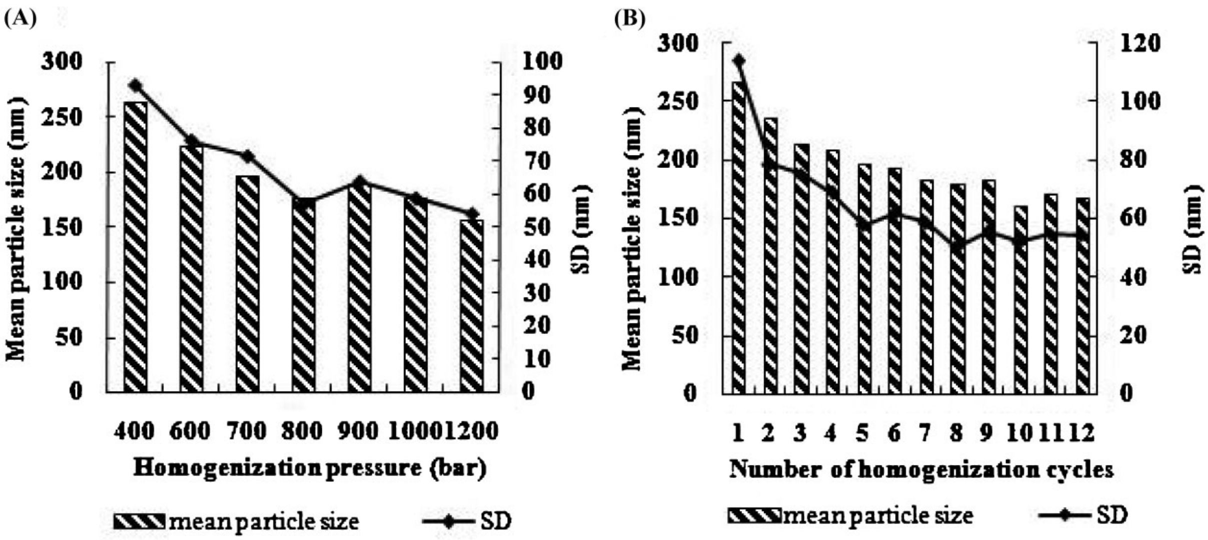

Figure 2. Influence of homogenization pressure (A) and homogenization cycles (B) on PSD of BU-LM.

high temperature would cause more rapid Brownian motion, leading to aggregation of some emulsion particles, and the most stable emulsion was produced at 30 °C. Temperature often had an indirect effect on emulsification as a result of altering the interfacial tension and adsorption of the emulsifier. Usually, the interfacial tension decreased with increasing temperature and a higher temperature was favorable for emulsification. However, an abnormal increase in temperature needed to be avoided because it tended to coagulate the particles, causing a deterioration of the emulsions. In addition, the surfactant, which was loosely adsorbed at the oil-water interface would be separated out from the emulsion to increase the probability of collision and coalescence, thus destabilizing the emulsions. Hence, in our study, ice-water bath was used in the high-pressure homogenization processes to keep the emulsifying temperature at 30°C.

3.2.4 pH

A suitable pH value of intravenous administration of lipid microsphere is from 4.0 to 9.0 since the pH value of human blood is 7.4, and the optimum pH range of the finished emulsion is 6.0–7.0 [28]. Besides, the final pH value of the emulsion should also ensure the slowest degradation of the drug, soybean lecithin, and triglycerides simultaneously. Bufadienolides are easily degradable and catalyzed by acids, bases or enzymes and, C with the most active structures was the most unstable of the three bufadienolides [29]. In the previous study, the detailed degradation processes of B, C, and R in bufadienolides-loaded solution (BU-S) and BU-LM over a range of pH values (4.0–8.0) were investigated. The results demonstrated that the degradation pattern of bufadienolides in BU-LM and BU-S both followed pseudo-first-order kinetics; C was far more unstable than B and R, and the optimum pH value for BU-LM was 6.5 which also minimized the hydrolysis of soybean lecithin; BU-LM could significantly increase the chemical stability of bufadienolides by incorporating the drug into the interior oil phase and the oil-water interfacial film and, C could be protected from degradation to the greatest extent. Consequently, the pH of BU-LM should keep 6.5 before and after sterilization [30].

3.2.5 Thermal sterilization

Sterilization was achieved by autoclaving. It was reported that the thermal sterilization process might be a re-emulsifying procedure or an irreversible redistribution of emulsifier compounds within the oil and aqueous compartments [31]. During the thermal sterilization process, F_0 value, which was used to express the time required to kill a particular organism under specified conditions, should be ensured above 8. Hence, to optimize the thermal sterile conditions, several thermal sterile methods were selected and used, including rotating autoclave in a 115°C water steam bath for 30 min (I), rotating autoclave in a 115°C water steam bath for 45 min (II), rotating autoclave in a 121°C water steam bath for 10 min (III), and rotating autoclave in a 121°C water steam bath for 15 min (IV). Table 6 showed the study results.

According to Table 6, all these sterilization methods provided a good physical appearance without oil droplets or phase separation, demonstrating that the BU-LM could undergo thermal steam autoclaving. However, the results also indicated that longer thermal sterilization could cause hydrolysis of lecithin, produce FFA and cause larger ζ-potential but slower pH value and contents. It is well known that when the sterilization effects are similar, the method with higher sterilization temperature and shorter sterilization time

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Table 6. The effect of different thermal sterilization conditions on the characteristics of BU-LM

						Bufadienolide content (%)			EE(%)		
Sterilization conditions	PSD (nm)	PI	pH	ζ-Potential (mV)	Physical appearance	B	C	R	B	C	R
Before sterilization	175.3 ± 56.6	0.104	6.52	−27.65	Good	21.80	33.12	54.50	86.61	91.88	93.07
Method I	172.3 ± 50.9	0.087	6.43	−26.44	Good	21.61	32.39	53.35	86.24	91.67	93.34
Method II	173.1 ± 56.3	0.106	6.35	−25.21	Good	20.99	32.00	52.60	85.98	91.53	92.87
Method III	169.7 ± 61.4	0.131	6.50	−27.58	Good	21.73	32.99	54.26	86.91	92.08	93.25
Method IV	164.6 ± 57.3	0.121	6.44	−27.14	Good	21.54	32.68	53.71	86.35	91.79	92.89

is better for maintaining the stability of drug on the account of the lower activation energy. Therefore, a 121°C rotating autoclave water steam bath for 10 min was the most suitable thermal method for BU-LM.

3.3 Dilution stability of BU-LM

Injectable drugs often needed to be diluted in clinical application to meet different requirements of the patients. 0.9% sodium chloride injection (NaCl) and 5% glucose injection (Glu) were chosen as the main dilution solvents, because both of them were very commonly used infusion fluids to prevent blood vessel irritation [32]. Lipid microspheres were different from the ordinary injection solutions because of their thermodynamic and dynamic instability, so dilution would play an important role in the stability of LMs. In this study, BU-LM was diluted to 5-fold and 10-fold with 5% Glu or 0.9% NaCl injection to investigate the stability of BU-LM at different time intervals, and the result was shown in Fig. 3.

BU-LM exhibited uniform milky appearance but different PSD after dilution with Glu and NaCl injections. Figure 3A and B showed the mean particle size and SD values of BU-LM after 5-fold or 10-fold dilution with NaCl and Glu injections, respectively. It was worth noticing that after dilution with 5% Glu injection for 4 h, the mean diameter and SD were basically unchanged whatever dilution times, whereas the mean diameter increased gradually and reached a maximum at 4 h, also the SD had an increasing tendency after dilution with 0.9% NaCl injection. All of these indicated that BU-LM was unstable when diluted with 0.9% NaCl injection, which might be due to the high ionic strength of the NaCl solution. The positive charges of Na^+, H^+ and others existed in NaCl injection could be absorbed by the emulsion droplets with negative charges to decrease the ζ-Potential of the emulsion surface, so the electrostatic repulsion between particles decreased and the small particles easily gathered into big particles, leading to the instability of the emulsion in the end. In addition, the positive charges could form electric bridge function between emulsion particles, which was a direct reason for the aggregation of the emulsion droplets. Besides, Fig. 3 displayed that the dilution times almost unaffected the mean diameter and SD under a fixed

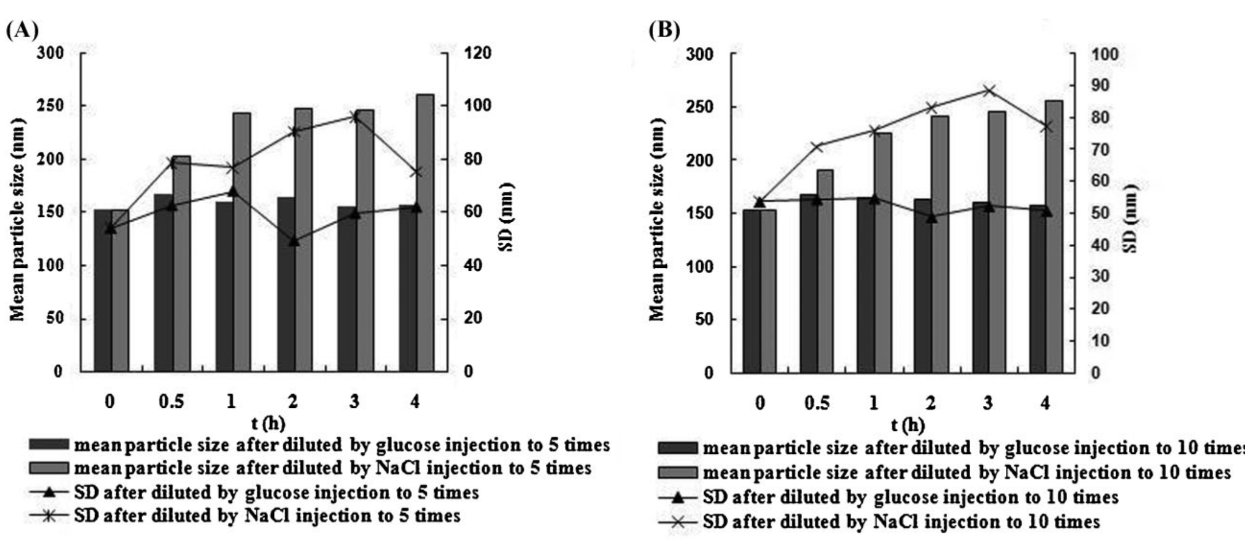

Figure 3. Influence of dilution on PSD of BU-LM.

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Table 7. Characterization of BU-LM after a 121°C rotating steam sterilization for 10 min and storage at 4 ± 2 °C for 18 months (n = 3)

Characterization of BU-LM	Physical appearance	PSD	PI	pH	ζ-Potential (mV)	Bufadienolide content (%)			Sum of the bufadienolide content (%)	EE(%)		
						B	C	R		B	C	R
After autoclaving	Good	175.2 ± 57.3	0.107	6.48 ± 0.02	−32.10 ± 1.04	20.8 ± 0.3	31.3 ± 0.4	50.4 ± 0.4	102.5 ± 0.9	86.9 ± 0.4	91.7 ± 0.5	92.9 ± 0.2
Storage at 4 ± 2°C for 18 months	Good	179.8 ± 59.9	0.111	6.20 ± 0.03	−28.99 ± 1.36	20.3 ± 0.3	30.7 ± 0.2	49.7 ± 0.1	100.7 ± 0.6	86.1 ± 0.5	90.9 ± 0.4	92.1 ± 0.4

dilution medium, for example 5 times or 10 times. It is, therefore, concluded that BU-LM should be diluted with 5% Glu injection, if necessary, to ensure the stability and safety of the emulsion.

3.4 Long-term stability of BU-LM

The long-term stability investigation was carried out for 18 months. The results in Table 7 showed that the parameters which were applied to evaluate the physicochemical stability of BU-LM did not significantly change during 18-month storage at 4 ± 2°C, indicating the excellent physical and chemical stability of BU-LM. Therefore, it could be concluded that BU-LM was an excellent intravenous administration system, which could undergo the real storage conditions for 18 months.

4 Conclusions

In conclusion, a new formulation of intravenous lipid microspheres for bufadienolides was developed in this study to overcome the problems associated with the poor solubility and the chemical instability of bufadienolides. High-speed shear mixing, high-pressure homogenization and thermal sterilization were used for the preparation of BU-LM; PCS, ELS, HPLC, and light microscopy were used for evaluation of BU-LM in vitro by determining essential parameters, such as PSD, ζ-potential, contents and EEs of B, C, and R. The factors influencing the physicochemical stability of BU-LM, such as the oil phase composition and ratio, the type and amount of lecithin, the choice of the co-emulsifier, the addition of osmotic agent, pH, temperature, homogenization and sterilization conditions were discussed thoroughly in this paper. Based on the systematic investigations, the optimum BU-LM was composed of 10% (w/v) MCT, 3.0% (w/v) soybean lecithin, 0.4% (w/v) F68, 0.05% (w/v) sodium oleate, and 2.5% (w/v) glycerol. The oil phase dissolved at 78°C was added slowly to the aqueous phase which was dispersed at 70°C, accompanied by the emulsification with a high-speed shear mixing at 10 000 rpm for 12 min including a 3-min high-speed shear mixing-on period and a 1-min-off period. Then the coarse emulsion was adjusted to pH 6.5, subjected to high-pressure homogenization at 800 bar for 8 cycles at 30°C and sterilized in a 121°C rotating autoclave water steam bath for 10 min. The results obtained proved that BU-LM had a sufficient physicochemical stability after dilution with 5% glucose injection and long-term storage at 4 ± 2°C for 18 months, respectively. Therefore, LM a potentially promising parenteral drug delivery system for bufadienolides.

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