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ORIGINAL ARTICLE

A lyophilized etoposide submicron emulsion with a high drug loading for intravenous injection: preparation, evaluation, and pharmacokinetics in rats

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Abstract

Objective: To develop a submicron emulsion for etoposide with a high drug loading capacity using a drug–phospholipid complex combined with drug freeze-drying techniques. *Methods*: An etoposide–phospholipid complex (EPC) was prepared and its structure was confirmed by X-ray diffraction and differential scanning calorimetry analysis. A freeze-drying technique was used to produce lyophilized etoposide emulsions (LEPE), and LEPE was investigated with regard to their appearance, particle size, and zeta potential. The pharmacokinetic study in vivo was determined by the UPLC/MS/MS system. *Results*: It showed that EPC significantly improved the liposolubility of etoposide, indicating a high drug loading intravenous emulsion could be easily prepared by EPC. Moreover, the obtained loading of etoposide in the submicron emulsion was 3.0 mg/mL, which was three times higher than that of the previous liquid emulsions. The optimum cryoprotectant was trehalose (15%) in freeze-drying test. The median diameter, polydispersity index, and zeta potential of the optimum formulation of LEPE were 226.1 ± 5.1 nm, 0.107 ± 0.011, and −36.20 ± 1.13 mV, respectively. In addition, these parameters had no significant change during 6 months storage at 4 ± 2°C. The main pharmacokinetic parameters exhibited no significant differences between LEPE and etoposide commercial solution except for area under the concentration–time curve and clearance. *Conclusions*: The stable etoposide emulsion with a high drug loading was successfully prepared, indicating the amount of excipients such as the oil phase and emulsifiers significantly decreased following administration of the same dose of drug, effectively reducing the metabolism by patients while increasing their compliance. Therefore, LEPE has a great potential for clinical applications.

Key words: *Emulsion; etoposide–phospholipid complex; freeze-drying; high drug loading; pharmacokinetics*

Introduction

Etoposide (also commonly known as VP-16) is a semisynthetic epipodophyllotoxin derivative, which has a wide antitumor spectrum of activity[1,2], and is especially active in small-cell lung cancer and testicular carcinoma[3–5]. Because of its poor aqueous solubility and chemical instability[6,7], the optimum method of administering etoposide has not been fully established. Poorly soluble drugs are often a challenging problem as far as the preparation of successful drug formulations are concerned, especially when the drugs are poorly soluble in both aqueous and nonaqueous media. The only currently commercially marketable dosage form for injection is a nonaqueous IV parenteral solution such as the etoposide injection from Gensia Sicor Pharmaceuticals, Inc. (Irvine, CA, USA), which is a solution of 20 mg/mL and each milliliter contains: 20 mg etoposide, 2 mg citric acid, 30 mg benzyl alcohol, 80 mg polysorbate 80, 650 mg polyethylene glycol 300, and 30.5% (v/v) alcohol. Etoposide injection must be diluted before its use with 0.9% sodium chloride injection. If solutions are prepared at concentrations above 0.4 mg/mL, precipitation may occur (USP31). The usual dose of etoposide injection is 50–100 mg/m^2/day, days 1–5. Hence, a large volume of etoposide solution needs to be administered each day, and the injection should be by rapid infusion to avoid precipitation and reduce patient

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discomfort. However, it is reported that hypotension can occur following rapid IV administration. Moreover, other adverse side effects, such as anaphylaxis and bronchospasm, have also been reported mainly due to the use of vehicles such as solubilizers in the formulation[8,9]. Therefore, it is of great importance to develop new dosage forms for etoposide with a high drug loading capacity to improve patient tolerance and reduce the adverse reactions.

Lipid emulsions are a promising alternative vehicle for parenteral drug administration due to their advantages in terms of industrial production, stability, and cost effectiveness relative to other drug carriers[10,11]. The lipid emulsions can keep the drug from coming into direct contact with tissues and body fluids and reduce the opportunity of drug precipitation during administration by IV injection[12–14], which can be avoided to minimize the possible side effects compared with systems based on organic solvents, pH adjustment, and surface-active agents. Moreover, increased organic targeting or reduced drug hydrolysis by incorporation of drugs into emulsions has been reported in many publications[15,16]. Etoposide parenteral emulsion was shown to effectively improve the chemical stability of the drug in aqueous media in our previous study[17]. Furthermore, an increased anticancer activity and brain targeting were shown by etoposide-encapsulated parenteral emulsions[18]. However, the drug loading capacity of an etoposide parenteral emulsion is limited to only 1.0 mg etoposide per milliliter of emulsion because of the low solubility of etoposide in oil phases. This is the main reason that the number of drug-loaded emulsions for IV injection on the market is very limited. Therefore, the development of emulsions for etoposide with a high drug loading seems to be a feasible strategy.

In this study, a new kind of etoposide o/w emulsion loaded with an etoposide–phospholipid complex (EPC) was examined. A high liposolubility of etoposide was obtained by the EPC, and the characteristics of this complex were systemically investigated. The freeze-drying technique was used to improve the physicochemical stability of the emulsion during long-term storage. Furthermore, the physicochemical stability and pharmacokinetics of the formulation were investigated in detail.

Materials and methods

Materials and animals

Soybean lecithin (EPIKURON 170, PC72%) was purchased from Degussa Food Ingredients (Hamburg, Germany). Soybean oil [long-chain triglyceride (LCT)] and medium-chain triglyceride (MCT) were purchased from Tieling Beiya Foods Ltd. (Tieling, China). Glycerin was obtained from Suichang Glycerin Company (Zhejiang, China). Tween-80 was provided by Shenyu Medicine and Chemical Industry Company (Shanghai, China). Trehalose dihydrate was purchased from Sinozyme Biotechnology Co., Ltd. (Nanning, China), while mannitol, maltose, glucose, and sucrose were purchased from Tianjin Bodi Chemicals Co., Ltd. (Tianjin, China). Etoposide was purchased from Shanghai Pudong Pharma Ltd. (Shanghai, China), and teniposide, the internal standard, was kindly provided by Jiangsu Yabang Technology Ltd. (Jiangsu, China). Etoposide injection (EPS) was purchased from Jiangsu Hengrui Medicine Co., Ltd. (Jiangsu, China). All other chemicals and reagents were of analytical or chromatographic grade. The laboratory animals in this study were purchased from the Animal Center of Shenyang Pharmaceutical University (Shenyang, China).

Methods

Preparation of the EPC

The complex was prepared by mixing etoposide and phospholipids in a suitable ratio. The required amount of etoposide and phospholipids for a 100 mL emulsion were placed in a 50 mL round bottom flask and 20 mL tetrahydrofuran was added. The mixture was agitated at room temperature for 3 hours and the solution was evaporated to obtain the dried EPC. Then, the solvent was evaporated and the mixture was vacuum-desiccated overnight to make sure that the residual tetrahydrofuran was lower than 0.072% (USP31).

Investigations of the EPC

X-ray diffraction analysis. X-ray diffraction analysis was performed using a type D/Max-2400 diffractometer (Rigaku Instrument, Tokyo, Japan). The samples were exposed to CuKα radiation under 56 kV and 182 mA over a 2-theta range from 3° to 45° in increments of 4° per minute every 0.04°. Etoposide, the physical mixtures of etoposide and soybean lecithin, and EPC were all subjected to X-ray diffraction analysis.

Differential scanning calorimetry analysis. Differential scanning calorimetry (DSC) analysis was carried out using a differential scanning calorimeter (DSC-60WS, Shimadzu, Japan). For DSC measurement, a scan rate of 10°C/min was employed over the temperature range of 20–200°C under a nitrogen purge. The samples were placed directly in aluminum pans for analysis. Samples for X-ray diffraction analysis were subjected to the above thermal cycles.

Solubility of etoposide, physical mixtures of etoposide, soybean lecithin, and EPC in MCT and LCT

Excess etoposide powder, the physical mixture of etoposide and soybean lecithin, and EPC were predissolved in MCT and LCT under magnetic stirring (DF-101S, YUHUA, YingXiaYuHua Instrument Co., Gongyi, China) at 80°C

for 3 hours in order to allow the soybean lecithin to blend sufficiently with the oil. Then, the oversaturated solutions were placed in a shaking air bath (HZQ-C, Dongming Medical Instrument Co., Harbin, China) at 25°C and 100 rpm for 72 hours to ensure solubility equilibrium. Finally, the different samples were centrifuged and the supernatants were analyzed by high-performance liquid chromatography (HPLC) in Drug Analysis section.

Preparation of etoposide emulsions

EPC containing etoposide (0.3 g) and soybean lecithin (1.8 g) was dispersed in MCT (10 g) and the system was heated to 80°C on a water bath. Glycerol (2.5 g) and Tween-80 (0.2 g) were dissolved in water and added to the oil phase at the same temperature. A coarse emulsion was prepared by high shear mixing (ULTRA RURRAX® IKA® T18 basic, Staufen, Germany) at 10,000 rpm for 5 minutes followed by high-pressure homogenization using a Niro Soavi NS10012k homogenization apparatus (Niro Soavi, Parma, Italy). The preparation was carried out at 800 bar and 8 homogenization cycles. Finally, after the volume was adjusted to 100 mL with double-distilled water and the pH was adjusted to 5–6 with 0.1 mol/L NaOH, the emulsion was sterilized by passage through a 0.22 μm nylon filter, and packaged in sterile glass bottles[19].

Freeze-drying procedure

Samples of the etoposide emulsion (EPE) were diluted with the cryoprotectant solutions to different concentrations of cryoprotectants[20]. The lyophilization was carried out in a laboratory freeze-dryer (FDU-1100, EYELA, Tokyo, Japan). The samples were freeze-dried in 10 mL semistoppered vials with the height of the samples being kept lower than 1.0 cm (2 mL). The freeze-drying technology was as follows: freezing at −73°C for 12 hours in a super-cold refrigerator; primary drying at −35°C for 4 hours; then, the shelf temperature was raised to −20°C for 13 hours; secondary drying at 20°C for 5 hours. The chamber pressure was maintained at 13.0 Pa and the temperature of the cold trap was −50°C during the whole freeze-drying procedure. Finally, the vials were sealed with rubber caps and stored at 4°C until analysis.

Reconstitution of lyophilized etoposide emulsions

Lyophilized etoposide emulsions (LEPE) can be reconstituted by addition of water for injection after freeze-drying. Then, the rehydration was observed visually as the vials were gently agitated by hand, and the formation of the aqueous liquid emulsions was examined for further characterization.

Characteristics of EPE and LEPE

Particle size and zeta potential. The median diameter (MD) and polydispersity index (PI) of samples were assessed by photon correlation spectroscopy (dynamic light scattering) using a Nicomp™ 380 submicron particle sizer (Particle Sizing System, Santa Barbara, CA, USA). The system covered a range from 5 nm to approximately 3 μm. Each emulsion sample was diluted 1:5000 with double-distilled water immediately before the measurement. The Guassian and Nicomp distributions of particle size were obtained at the same time with intensity-weighting (z-average), volume-weighting, and number-weighting while the value of the PI showed the width of the distribution. The characterization parameters of photon correlation spectroscopy diameters of 50%, 90%, 95%, and 99% were also calculated.

The Nicomp™ 380 was also used to measure the zeta potential by electrophoretic light scattering. Before measurement, double-distilled water used to dilute emulsion samples, which were adjusted to the same pH value using 0.01 mol/L HCl or NaOH solutions and then each emulsion sample was diluted 1:50 with the water.

Light microscopy studies for detection of etoposide crystals. Light microscopy was performed using a Motic DMBA450 microscope (Motic China Group Co., Ltd., Beijing, China), and the magnification selected was 400-fold. Emulsions were analyzed undiluted and, typically, 20 microscopic fields were analyzed for the detection of any remaining drug crystals.

Drug analysis

A reverse phase HPLC analytic method was used for drug analysis[17]. A phenyl column (5 μm, 4.6 × 250 mm) (Spherigel) was employed. The mobile phase used was acetonitrile–water (30:70) containing 0.3% sodium acetate (pH 4.0) with a flow rate of 1.0 mL/min and the UV detector was set at 254 nm.

Pharmacokinetic study

Rats weighting 200 ± 20 g were divided into two equal groups of six rats per group. Each group of rats received either rehydrated LEPE or EPS at a dose of 10 mg/kg via the caudal vein. The EPS was diluted with 0.9% sodium chloride for injection to make the concentration equal to that of rehydrated LEPE. At designed times (5, 15, 30, 45 minutes, 1, 2, 4, 6, and 8 hours), 0.3 mL blood samples were collected from the retro-orbital sinus, transferred to heparinized tubes, and then immediately centrifuged at 1735 × *g* for 15 minutes to obtain plasma samples. The plasma samples were stored at −70°C until analysis. The pharmacokinetic study in this paper conformed to the National Institutes of Health Guide for Care and Use of Laboratory animals.

Sample disposal. For the analysis, 10 μL teniposide methanol solution (8 μg/mL) was used as an internal standard and added to 100 μL plasma. The mixture was vortexed for 1 minute in a Liquid Fast Mixer (YKH-3, Liaoxi Medical Apparatus and Instrument Factory, Liaoning, China), and then extracted with 4 mL methyl

tert-butyl ether by vortexing for 10 minutes. After centrifugation at 1735 × *g* for 10 minutes, the supernatant was transferred to a clean tube and evaporated to dryness in a centrifugal concentrator at 40°C (Labconco Corp., Kansas, MO, USA). The residue was reconstituted in 800 µL methanol and a 5 µL sample was injected into the UPLC-ESI-MS/MS.

Sample determination by UPLC-ESI-MS/MS. Chromatography was performed on an ACQUITY™ UPLC system (Waters Corp., Milford, MA, USA) with an autosampler maintained at 4°C. The separation was carried out on an ACQUITY UPLC™ BEH C18 column (50 mm × 2.1 mm i.d., 1.7 µm; Waters Corp., Milford, MA, USA). The column temperature was maintained at 35°C and the determination was carried out by gradient elution using (A) acetonitrile and (B) water (containing 0.1% formic acid) as the mobile phase at a flow rate of 0.2 mL/min. The gradient conditions of the mobile phase were as follows: A was changed linearly from initially 70% to 30% during the first 1.0 minute, and then held for 1.2 minutes. After 2.2 minutes, the composition was reset to the initial composition and a 0.8-minute re-equilibration time was allowed. The injection volume was 5 µL using the partial loop mode. A Waters ACQUITY™ TQD triple-quadrupole tandem mass spectrometer (Waters Corp., Manchester, UK) with an electrospray ionization interface was connected to the UPLC system. The optimal electrospray ionization source parameters were as follows: capillary 3.1 kV, cone voltage 30 V, extractor 3.0 V, and Radio Frequency (RF) 0.3 V. The source and desolvation temperature was set at 100°C and 400°C, respectively. Nitrogen was used as the desolvation gas (450 L/h) and cone gas (50 L/h). For collision-induced dissociation, argon was used as the collision gas at a flow rate of 0.2 mL/min. The multiple reaction monitoring mode was used for quantification. The fragmentation transitions for the multiple reaction monitoring (MRM) were *m*/*z* 589.1→228.9 amu for etoposide, and *m*/*z* 656.9→382.9 amu for the internal standard (IS), with a scan time of 0.02 second per transition. All data collected in centroid mode were acquired using MassLynx™ NT4.1 software (Waters Corp., Milford, MA, USA). Post-acquisition quantitative analyses were performed using a QuanLynx™ program (Waters Corp., Milford, MA, USA).

The validation of the analytical method for etoposide under the selected conditions showed that the chosen method was precise and accurate with a linear response of 10–2.0 × 10^4 ng/mL. The lower limit of quantification was 10 ng/mL. The method also showed acceptable precision and accuracy. The intra-day variation at three concentrations (20, 200, 16,000 ng/mL) was less than 16.8%, 8.5%, and 9.5%, and the inter-day variation was 13.6%, 8.1%, and 7.1%. These values were within the limits specified for inter- and intra-accuracy and precision. The etoposide relative recovery from plasma at three concentrations (20, 200, 16,000 ng/mL) was 71.0%, 78.6%, and 74.2%, respectively.

Result and discussion

Investigations of the EPC

Ratio of etoposide to phospholipid

The conception of a drug–phospholipid complex was first put forward by Gabetta over 30 years ago[21]. Many researchers have reported that a complex of a drug and phospholipid could effectively improve the liposolubility of the drug[22,23]. Because of the poor liposolubility of etoposide, a probe-type sonicator, SONICS vibra cell™ (Sonic & Material, Inc., Newtown, CT, USA) was needed to resolve enough etoposide in the oil solution[17], however, the amount of etoposide loaded in the emulsion was limited to 1 mg/mL. To overcome this problem, the EPC was used to increase the liposolubility of etoposide in this study.

As reported in the published literatures, 1.8% (g/mL) soybean lecithin was used to achieve excellent emulsifier effect[17,18]. So, the amount of soybean lecithin was fixed to 1.8% in this study. As shown in Table 1, the ratio of etoposide to soybean lecithin in EPC to ensure that etoposide is dissolved well in both 10% (w/v) LCT and 10% (w/v) MCT was 0.3:1.8 (w/w). However, when the etoposide–phospholipid ratio was increased to 0.5:1.8 (w/w), the EPC did not completely dissolved in LCT after 3-hour agitation at 80°C, while it can well dissolve in MCT. So, it can be concluded that the liposolubility of etoposide was significantly improved by the EPC, especially in MCT media. The formation of EPC needs further investigations using X-ray diffraction and DSC.

X-ray diffraction analysis

X-ray diffraction is the standard technique for studying the crystalline or amorphous nature of drugs and it is always used to confirm the loss of drug crystallinity. As shown in Figure 1, etoposide has several distinct crystalline peaks at 2-theta angles between 5° and 35°, indicating that etoposide is present in the crystalline state, while no diffraction peak was detected after 8° in the

Table 1. Dissolution of etoposide–phospholipid complex with different ratios in 10% (w/v) LCT and 10% (w/v) MCT under magnetic stirring at 80°C.

Etoposide and soybean lecithin ratios (g/g)	Dissolution status (dissolution time)	
	10% (w/v) LCT	10% (w/v) MCT
0.2:1.8	Settled solution (1 hour)	Settled solution (0.5 hour)
0.3:1.8	Settled solution (2 hours)	Settled solution (1 hour)
0.5:1.8	Unsettled solution (3 hours)	Settled solution (2 hours)

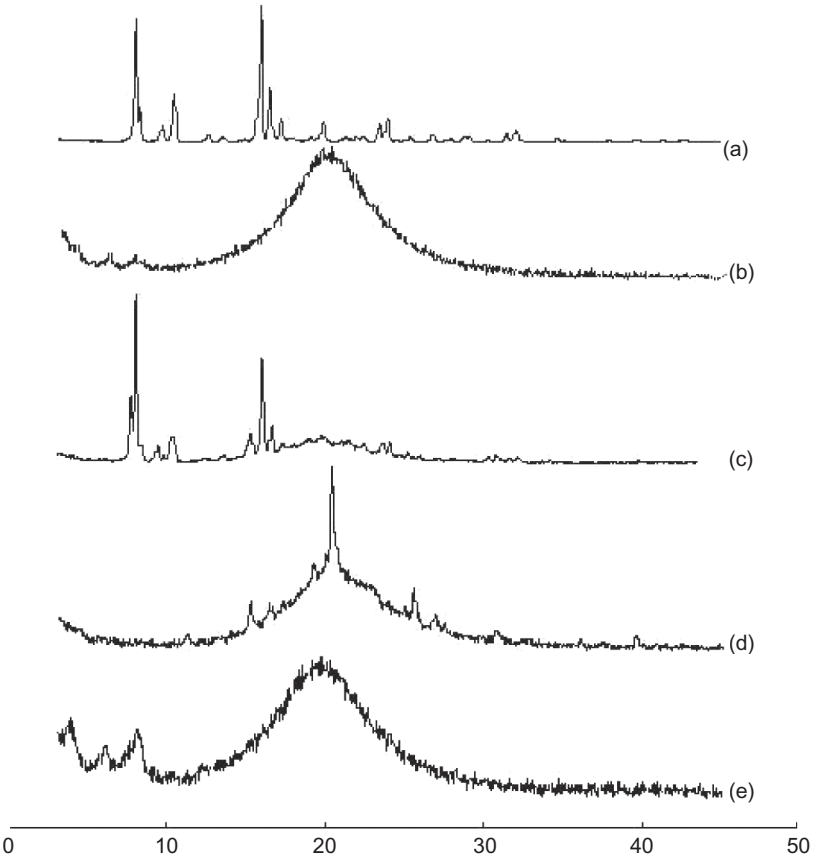

Figure 1. X-ray diffraction of etoposide (a), phospholipid (b), the physical mixture of etoposide and phospholipid at a ratio of 0.3:1.8 (c), the etoposide–phospholipid complex at a ratio of 0.5:1.8 (d), and 0.3:1.8 (e).

diffraction patterns of the phospholipid. The diffraction patterns of the physical mixtures showed that the peaks of etoposide were still present, suggesting that the crystallinity of etoposide did not change in the physical mixture. The diffraction patterns of EPC (0.5:1.8) showed that few crystalline peaks were present, and these became much smaller. However, when the ratio was 0.3:1.8, the crystalline peaks disappeared, suggesting that etoposide was in an amorphous state in the EPC. This phenomenon indicated that the phospholipids might not be enough to surround each etoposide molecule in the phospholipid complex (0.5:1.8). This explains why the phospholipid complex at 0.5:1.8 did not dissolve properly in LCT after 3-hour agitation at 80°C. Therefore, an EPC ratio of 0.3:1.8 was selected for further study.

Differential scanning calorimetry analysis

Figure 2 shows the DSC thermograms of etoposide, phospholipid, physical mixture, and EPC. The thermogram of etoposide showed the presence of endothermal peaks at 182.05°C. The phospholipid exhibited some small sharp endothermal peaks between 172°C and 180°C, suggesting that the formation of these peaks was due to thermal movements of the polar part of the phospholipid molecule[24]. The physical mixture of etoposide and phospholipids exhibited two peaks, one at 170.85°C and the other at 184.45°C. The former had the same onset temperature (172.51°C) as etoposide and the latter had the same onset temperature (182.05°C) as the phospholipids. In other words, the endothermal peaks of the physical mixture are a combination of the characteristic peaks of etoposide and phospholipids. This phenomenon indicated that the physical mixture did not affect the nature of the drug and phospholipids. The DSC of the EPC exhibited a single peak (146.95°C), which differed from the peak of etoposide or phospholipid, and the endothermal peaks of drug and phospholipid both disappeared. Moreover, the phase transition temperature was lower than the temperatures of phospholipids and etoposide. There might be some interactions between etoposide and phospholipids in EPC, such as the formation of hydrogen bonds and van der Waals forces. The interaction of etoposide with the polar part of phospholipid molecules allows the long hydrocarbon tail of phospholipids to turn freely and 'envelop' the polar head of phospholipids containing the etoposide molecule[23]. Therefore, the peaks of phospholipids and etoposide disappear and the phase transition temperature is lowered.

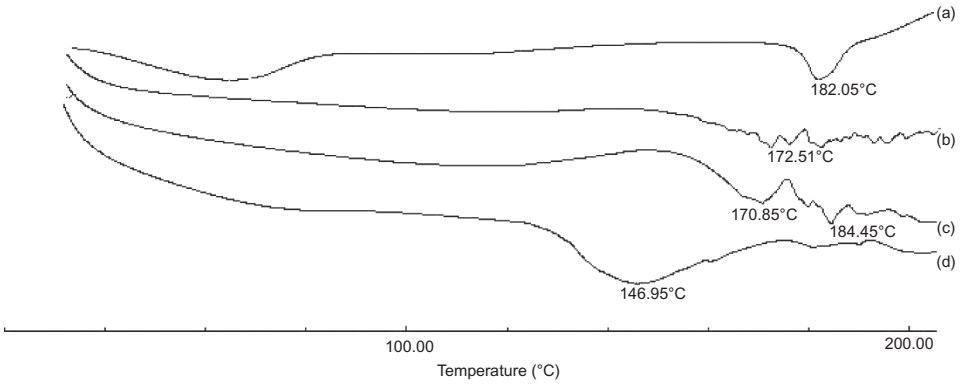

Figure 2. DSC thermograms showing the peak transition onset temperature of etoposide (a), phospholipid (b), the physical mixture of etoposide and phospholipid (0.3:1.8) (c), and the etoposide–phospholipid complex (0.3:1.8) (d).

Solubility of etoposide, the physical mixtures of etoposide, phospholipid, and EPC in MCT and LCT

As shown in Table 2, the solubility of etoposide powder dissolved in LCT and MCT was only 0.16 and 3.17 mg/g, which showed the poor liposolubility of etoposide. The solubility of the physical mixtures of etoposide and phospholipid improved as phospholipid enhances solubilization to a certain extent and the solubility of the EPC was markedly increased. The solubility in LCT is 2.5-fold higher than that of the physical mixture, whereas the solubility in MCT is nearly 15-fold higher than that of physical mixture. Even in this case, the phospholipid complex dissolved in MCT still did not reach saturation. However, the viscosity of its oil solution was markedly increased so that the oil solution was not very fluid at room temperature. These results clearly demonstrate that the liposolubility of etoposide was effectively improved by the EPC, especially in MCT media.

Characteristics of EPE prepared using the EPC

About 100 mL of EPE was prepared with EPC containing etoposide (0.3 g) and soybean lecithin (1.8 g). It means that the loading of etoposide in the submicron emulsion was 3.0 mg/mL, which was three times the loading compared with the previous liquid emulsions[17]. MCT (10%, w/v) was used as the oil phase because of the good solubility of the EPC in MCT. The other ingredients were all similar to those used in a previous study[17]. Unfortunately, etoposide crystals formed within 10 days in the emulsions kept at 4 ± 2°C (Figure 3a). Although the EPC significantly improved the liposolubility of etoposide, etoposide crystals were still detected in emulsions after 10 days of storage. The reason for this might be as follows: it is well known that the formation of drug–phospholipid complex is based on the action of hydrogen bonds and van der Waals forces between drug and phospholipids[23,24]. Water appeared to be an important factor inducing the observed crystallization and the presence of water in the emulsion might slowly interrupt the above interaction between etoposide and phospholipids. Also, the release of etoposide into the aqueous phase is likely an interim step in the crystallization process, since crystals were always observed in the aqueous phase[25].

Freeze-drying was used to solve the crystallization problem in this study. Although the mechanism of the protective effect remains unclear, it has been reported that the particles could be well protected by saccharides[26]. A water-replacement hypothesis has been proposed in which the hydrated water molecules on the head group of lecithin were replaced by saccharide molecules, which would protect against aggregation and fusion of the particles during the lyophilization process[27,28]. Also, saccharide would not affect the hydrogen bonds and van der Waals forces between drug and phospholipids. Therefore, this technique could effectively improve the stability of EPE since the aqueous phase was removed.

Table 2. Solubility of etoposide, the physical mixtures of etoposide, phospholipid, and etoposide–phospholipid complex in LCT and MCT.

	Solubility in LCT (mg/g)	Solubility in MCT (mg/g)
Etoposide powder	0.16	3.17
Physical mixture	7.61	6.34
Phospholipid complex	18.79	88.93[a]

[a]Do not reach saturation.

Effect of freeze-drying on EPE

Methods of adding cryoprotectants

There are several published methods for adding cryoprotectants before freeze-drying[29]. In the first method,

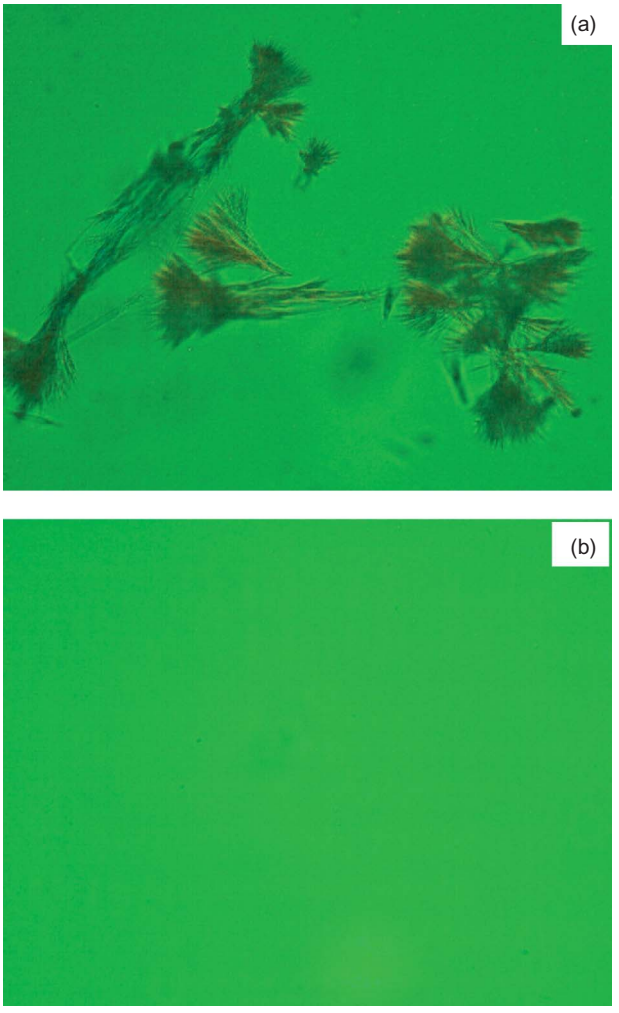

Figure 3. Photomicrographs of etoposide emulsion (3.0 mg/mL). (a) Original liquid emulsion, 10 days after production (4 ± 2°C), (b) freeze-dried emulsion 6 months after production (4 ± 2°C) and 24 hours after reconstitution at room temperature.

the emulsions diluted with a high concentration of cryoprotectant solution, since it was much easier to select different cryoprotectants in the laboratory. In the second method, adding the cryoprotectants to the water phase during the preparation process was easy under practical manufacturing conditions. The lyophilized emulsions prepared by the above two methods were compared in a pretest, and the results showed that there were no differences in their physical and chemical properties. Therefore, the first method was used to select different cryoprotectants and the second method was used to prepare the final LEPE in this study.

Effect of cryoprotectants during lyophilization

No solid emulsions could be obtained without adding cryoprotectants during the freeze-drying process. Therefore, the cryoprotectant has to be added in order to obtain a solid, reconstitutible emulsion. Maltose, glucose, sucrose, trehalose, and mannitol were used as cryoprotectants to prepare a solid, reconstitutible emulsion in this study and the concentrations, involving 10% and 15% of the cryoprotectants, were investigated. LEPE were evaluated by examining the appearance of the lyophilized products and the reconstituted emulsion, and the velocities of reconstitution (Table 3). The freeze-dried cakes appeared brittle and smooth when mannitol was used as a cryoprotectant, but the particles were completely aggregated. The redispersed emulsions with 10% glucose were not opalescent without a Tyndall effect and this condition did not change when the concentration was increased to 15% and, inversely, the reconstitution rate was reduced. Hence, it was difficult to prepare solid emulsions using mannitol and glucose as cryoprotectants. Luckily, the appearance of LEPE using maltose, sucrose, and trehalose was fairly good in all cases. Particle size distribution measurements were carried out for further evaluation of the above three saccharides. The MD and PI of the emulsions before and after freeze-drying are shown in Figure 4. Compared with the control group, there was a significant increase in particle size when the concentration of cryoprotectants was 10%, indicating that this concentration was ineffective in preserving EPE during freeze-drying. As shown in Figure 4, these cryoprotectants proved to be the most efficient at a higher concentration (15%). The PI of the formulation containing 15% maltose was above 0.5. Müller has reported that the PI of parenteral fat emulsions was typically in the range

Table 3. Effect of different cryoprotectants on the characteristics of lyophilized etoposide emulsions.

Cryoprotectant		Appearance[a]	Rehydrated appearance[b]	Reconstitution velocity[c]
None		−−	☆☆	3
Maltose	10%	+	★	1
	15%	++	★★	1
Glucose	10%	−	☆	1
	15%	−	☆	2
Sucrose	10%	−	★	2
	15%	+	★★	2
Trehalose	10%	++	★★	1
	15%	++	★★	1
Mannitol	10%	++	☆☆	1
	15%	++	☆☆	1

[a]++: Best, brittle, and snow-like, smooth full; +: better, a little shrinkage and rugged; −: bad, collapse, rather porous; −−: worse, serious shrinkage to slice stick on the bottom.
[b]★★: Similar to the original sample; ★: similar to the original sample with a Tyndall effect; ☆: not opalescent without a Tyndall effect; ☆☆: macroscopic and irreversible aggregation.
[c]1, 2, and 3 represent the minutes required for rehydration with manual shaking.

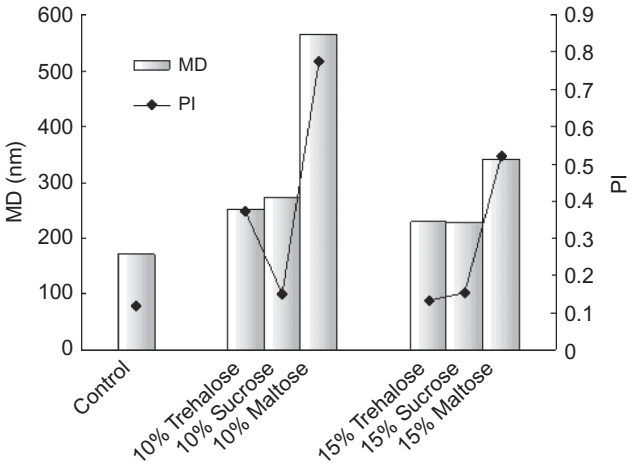

Figure 4. Mean particle size (MD) and polydispersity index (PI) of the reconstituted etoposide emulsions after freeze-drying in the presence of different cryoprotectants at concentrations of 10% and 15%.

0.10–0.20[30]. Thus, the formulation containing 15% maltose was not uniform. The MD and PI of sEPE (15% sucrose) and tEPE (15% trehalose) were 227.5 nm versus 228.8 nm and 0.154 versus 0.132, respectively, which means that the system was stabilized by using the above two saccharides as cryoprotectants. However, the reconstitution rates were different for trehalose and sucrose (Table 3). The lyophilized products using trehalose could be very easily and rapidly rehydrated (less than 1 minute) and were very loose, whereas the sucrose formulation needed to be dissolved with gentle shaking for 2 minutes. Therefore, the optimum cryoprotectant for EPE was trehalose.

Characteristics and stability of LEPE

The resultant emulsions were prepared by adding the cryoprotectants to the aqueous phase during the preparation process, and the other operational parameters were the same as in Preparation of Etoposide Emulsions section. The mean diameter of rehydrated LEPE given by the In-Wt Gaussian distribution was 228.8 nm and the PI was 0.132. The cumulative results of particles were also provided by the Gaussian distribution. The results showed that 99% of the particles were smaller than 489.2 nm and, of these 90% were less than 341.0 nm. The zeta potential was –36.74 mV and the formulation contained 2.994 mg/mL etoposide. A 6-month stability investigation was carried out. No etoposide crystals were detected in the freeze-dried emulsions 6 months (4 ± 2°C) after production and 24 hours after reconstitution at room temperature (Figure 3b), and this reconstituted emulsion could be stable over a week. However, while the commercially marketable etoposide injection was diluted to the same concentration, etoposide crystals were detected only within 2 hours. Therefore, compared to the marketable etoposide injection, the lyophilized emulsions in this study effectively delayed the precipitation. As shown in Table 4, the parameters that were used to evaluate the physicochemical stability of LEPE did not significantly change during 6 months storage at 4 ± 2°C, confirming the excellent physical and chemical stability of LEPE. Therefore, it can be concluded that the most suitable method is to use an EPC combined with freeze-drying to prepare the LEPE, which has three times the loading compared with the previous liquid emulsions[17] without a crystallization problem. Furthermore, while delivering the same amount of etoposide, this formulation effectively reduces the volume of emulsion for IV administration, thereby improving patient compliance.

Pharmacokinetic studies

The pharmacokinetic studies were carried out to compare LEPE and EPS by determining the drug concentration in rat plasma up to 8 hours after IV administration. The mean plasma concentration–time profiles of LEPE and EPS are shown in Figure 5. Analysis was carried out using a statistical package (DAS) version 2.0 software (Mathematical Pharmacology Professional Committee of China, Shanghai, China). The Student's independent samples *t*-test was used to analyze the pharmacokinetic results and it was expressed as one-way *P*-value. When the difference between two groups yielded a value for $P < 0.05$, it was considered statistically significant. All statistical analyses were carried out using the statistical package for social sciences (SPSS, version 11.5). The data on both the preparations fitted a two-compartment model with a weighting factor of 1/cc. As shown in Figure 5, the concentrations of etoposide in the plasma declined significantly and were higher for LEPE than

Table 4. Characterization of rehydrated LEPE during 6 months investigation at 4 ± 2°C (mean ± SD, $n = 3$).

Time (months)	Size (nm)	PI	Zata potential (mV)	Drug content (%)
0	226.1 ± 5.1	0.107 ± 0.011	–36.20 ± 1.13	99.81 ± 0.12
1	225.3 ± 6.7	0.122 ± 0.020	–35.54 ± 1.52	99.68 ± 0.16
2	224.1 ± 4.9	0.130 ± 0.024	–33.57 ± 2.69	99.25 ± 0.17
3	224.7 ± 0.9	0.124 ± 0.021	–38.94 ± 7.70	99.31 ± 0.07
6	225.8 ± 3.3	0.127 ± 0.015	–36.39 ± 0.85	99.17 ± 0.12

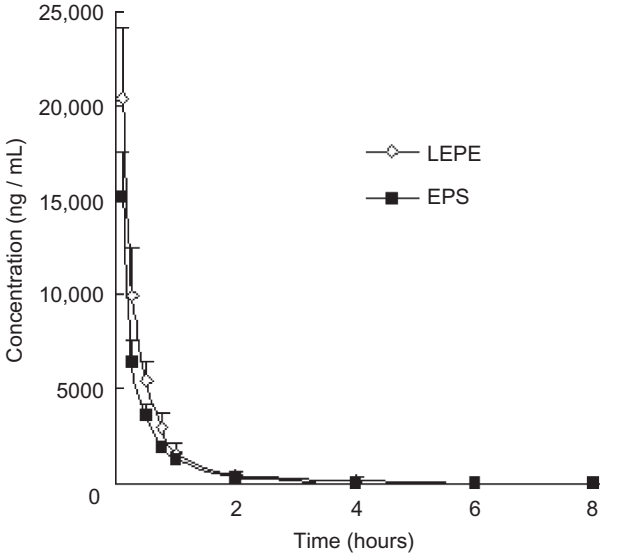

Figure 5. Etoposide plasma concentration versus time after IV administration (10 mg/kg) of rehydrated LEPE and etoposide commercial solution (EPS) in rats ($n = 6$).

EPS at all time points, especially at the initial time points. Most of the pharmacokinetic parameters (Table 5) exhibited no statistically significant differences ($n = 6$, $P > 0.05$) except for area under the concentration–time curve (AUC) and clearance (CL) also calculated by DAS. It was found that LEPE exhibited a 1.46-fold increase in AUC when compared with the solution. Moreover, the CL after administration of LEPE was 1.44 times lower than that of EPS. The increased AUC and decreased CL of LEPE are likely explained by the fact that a drug-loaded emulsion usually exhibits a slow release because the loaded drug needs time to transfer from the oil phase to the aqueous phase. When the drug is incorporated in the lipid core of the emulsions, it can reduce the penetration of drug into the tissues, and produce a higher plasma concentration, consequently leading to an increased AUC and decreased CL for most of the emulsions. Patlolla and Vobalaboina[18] have reported that the AUC after administration of an EPE (1.0 mg/mL) was 2.19-fold higher than that of the solution, whereas the CL was 2.33 times lower than that of etoposide solution. Therefore, rehydrated LEPE (3.0 mg/mL) in this study produces a lower AUC together with a higher CL compared with the EPE in the published literature[18]. This suggests that the drug would be released more rapidly into the blood when etoposide was loaded in emulsions and its availability would be increased by the penetration of etoposide into tissues. Furthermore, the content of etoposide in LEPE in this study was three times higher than the previously achieved loading of the liquid emulsion, so the amount of excipients such as the oil phase and emulsifiers significantly decreased following administration of the same dose of drug, effectively reducing the metabolism by patients while increasing their compliance. Therefore, LEPE has a great potential for clinical applications.

Table 5. Pharmacokinetic parameters after IV administration of rehydrated LEPE and EPS at a dose of 10 mg/kg (mean ± SD, $n = 6$).

Parameter	Unit	LEPE	EPS
AUC*	ng/mL h	9640.260 ± 2028.548	6604.536 ± 821.440
AUMC		5346.835 ± 2139.018	3197.631 ± 462.344
MRT	h	0.538 ± 0.129	0.484 ± 0.024
VRT	h^2	0.622 ± 0.364	0.41 ± 0.062
$t_{1/2}$	h	0.716 ± 0.252	0.603 ± 0.126
CL*	L/h/kg	1.065 ± 0.212	1.531 ± 0.190
Vss	L/kg	1.065 ± 0.310	1.303 ± 0.119

$t_{1/2}$, half-life; AUC, area under the concentration–time curve; AUMC, area under the cross product of the time and plasma concentration–time curve; MRT, mean residence time; VRT, variance of mean residence time; CL, clearance; Vss, steady state apparent volume of distribution.
*$P < 0.05$.

Conclusion

In conclusion, a new drug delivery system for hydrophobic and instable drugs is proposed. Combination of a drug–phospholipid complex and freeze-drying techniques were developed to prepare a stable EPE with a high drug loading. The liposolubility of etoposide was effectively improved by the EPC and the characteristics of the EPC were confirmed by X-ray diffraction and DSC analysis. EPE with a suitable particle size distribution and appearance could be obtained even after freeze-drying. The freeze-drying investigation showed that the optimum cryoprotectant was trehalose (15%). The physicochemical stability of LEPE did not significantly change during 6 months storage at 4 ± 2°C, confirming the excellent stability of LEPE. The in vivo pharmacokinetic study using UPLC/MS/MS indicated that there were no significant differences in the main parameters of LEPE and EPS, except for the AUC and CL. Further research is needed to examine the potential of producing LEPE on an industrial scale and to investigate its clinical applications.

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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.

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