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Preparation and Characterization of 10-Hydroxycamptothecin Loaded Nanostructured Lipid Carriers

Kai Liu, Jin Sun, and Yongjun Wang
Department of Biopharmaceutics, School of Pharmacy, Shenyang Pharmaceutical University, Shenyang, People's Republic of China

Yuan He
School of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University, Shenyang, People's Republic of China

Kun Gao and Zhonggui He
Department of Biopharmaceutics, School of Pharmacy, Shenyang Pharmaceutical University, Shenyang, People's Republic of China

Two types of 10-hydroxycamptothecin (10-HCPT) loaded nanostructured lipid carriers (NLC-F 68 and NLC-Brij 35) intended for use as the alternative formulation of 10-HCPT for parenteral administration were prepared using an emulsification-ultrasonication method and fully characterized from physicochemical and in vitro release standpoint. The particle size was measured by laser diffraction, being 108 nm and 126 nm for NLC-F 68 and NLC-Brij 35, respectively. Zeta potentials of two NLCs were 28.5 mV and 32.1 mV analyzed by photon correlation spectroscopy. The incorporated efficiency was more than 85%. It is observed that NLCs are homogeneous and spherical in shape by transmission electron microscopy. Differential scanning calorimetry analysis of NLCs showed that 10-HCPT was dispersed within NLC in an amorphous state. The combination of trehalose and mannitol as cryoprotectant was most suitable for HCPT-NLC lyophilization. The in vitro release behavior for two types of NLC was similar and displayed biphasic drug release pattern with rapid release at the initial stage and prolonged release afterwards. These results suggest that NLC could be exploited as a carrier of 10-HCPT with high incorporation efficiency and controlled release and that NLC may serve as the alternative delivery system for parenteral administration of 10-HCPT.

Keywords 10-hydroxycamptothecin; nanostructured lipid carriers; incorporation efficiency; lyophilization; in vitro release

Address correspondence to Zhonggui He, Mailbox 59#, Department of Biopharmaceutics, School of Pharmacy, Shenyang Pharmaceutical University, 103 Wenhua Road, Shenyang 110016, P.R. China. E-mail: hezhonggui@gmail.com

INTRODUCTION

Camptothecin (CPT) and its derivatives including 7-ethyl-10-hydroxycamptothecin (SN-38), 10-hydroxycamptothecin (10-HCPT), 9-nitro-camptothecin, topotecan, irinotecan and so on, are an important class of antitumor agent (Wall et al., 1966). They inhibit the DNA topoisomerase I of tumors and suppress the proliferation of cancer cells to elicit antitumor effect (Carbonero & Supko, 2002; Jaxel et al., 1989; O'Leary & Muggia, 1998). Unfortunately, poor solubility in water and in physiologically acceptable organic solvents presents a serious barrier to the practical use of CPTs. Camptothecin analogs share α-hydroxylactone moiety in their structures. The α-hydroxylactone ring plays an important role in the biological activity (Potmesil, 1994). However, the lactone exists in a pH-dependent equilibrium with an open carboxylate form, and the lactone ring is stable at pH 7 or lower and opens rapidly and completely to carboxylate form under alkaline condition (Figure 1). CPTs are usually used in the form of either the toxic and less active water-soluble carboxylate salt or prodrugs with increased solubility. Nevertheless the prodrugs also have some limitations, such as a compromise in drug potency, variability, and unpredictability of metabolism in vivo. Therefore, developing suitable delivery systems for CPTs is of great significance in completely liberating the potential of the CPT family drugs. Many attempts were made to develop high performance delivery systems for the insoluble lactone-formed CPTs (Cortesi et al., 1997; Ertl, Platzer, Wirth, & Gabor, 1999; Hatefi & Amsden, 2002; Shenderova, Burke, & Schwendeman, 1997; Tong, Wang, & D'Souza, 2003; Yang & Zhu, 2002; C. Zhang et al., 2007; Zhang et al., 2004;

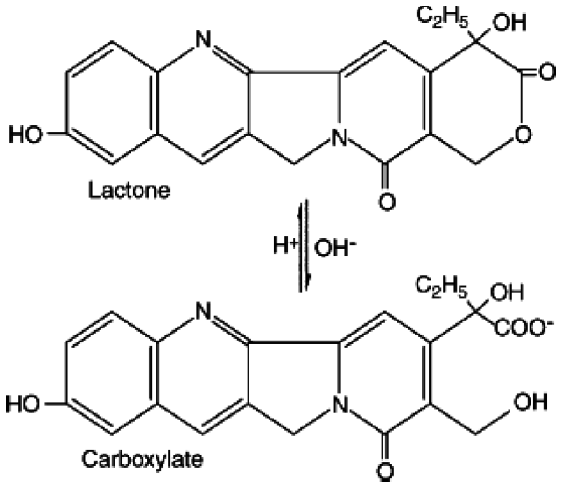

FIGURE 1. The structure of 10-HCPT and equilibrium reaction between the lactone form and the ring-opened carboxylate form.

L. Y. Zhang et al., 2007), such as polymer microspheres, polymeric nanoparticles, liposomes and microemulsions.

Solid lipid nanoparticles (SLN) have attracted increasing attention as a potential drug delivery carrier owing to their advantages, such as the possibility of simple and large scale production and low toxicity (Arica-Yegin, Benoit, & Lamprecht, 2006; Liu, Zhu, Du, & Qin, 2005; Müller et al., 1995; Müller, Madar, & Gohla, 2000). However, there are some potential limitations associated with SLN, including limited drug loading capacity, drug expulsion during storage, and so forth (Müller, Radtke, & Wissing, 2002a). Nanostructured lipid carriers (NLC) composed of a solid lipid matrix with a certain content of liquid lipid are a new generation of solid lipid nanoparticles (Joshi & Patravale, 2006; Müller et al., 2002a). Liquid lipids solubilize drugs to a much higher extent than solid lipids (Pouton, 2000). In a preferred scenario, the liquid lipids form droplets within the solid lipid particles matrix (Garcia-Fuentes, Aolores, & Torres, 2005). According to this model, the NLC nanoparticles would provide a high incorporation capacity (due to the liquid lipid) and control of drug release (due to the encapsulating solid lipid) (Müller, Radtke, & Wissing, 2002b). Therefore, we think that 10-HCPT loaded NLC, with most of 10-HCPT being the active lactone form, will be a promising drug delivery system.

As we know, nanoparticles modified by the surfactants with PEG chain such as Brij, Myrij, and PEGylated phospholipids could prolong the drug release time to achieve the long circulating purpose (Hung, Fang, Liao, & Fang, 2007; Lee, Lim, & Kim, 2007). In this study, we selected Brij 35 to modify the surface of nanoparticles and compared it with commonly used surfactant Lutrol F 68 (poloxamer 188), which is one of few emulsifiers that can be used in parenteral injection and has high emulsification effect and less toxicity. Compritol 888 ATO and Miglyol 812 were used in the formulations, which were solid lipid and liquid lipid, respectively. They are both physiological compatible lipids and usually are used together to prepare nanostructured lipid carriers in many reports. Then we prepared two types of HCPT-loaded NLCs with high encapsulation efficiency by emulsification-ultrasonication method, and optimized the freeze-drying conditions. The percentage of lactone formed at different pH values was determined, and the results indicated that the NLC can effectively protect 10-HCPT in its active form.

MATERIALS AND METHODS

Materials

10-Hydroxycamptothecin (10-HCPT, 99% purity) was obtained by Huangshi Fy Pharmaceutical CO. Ltd. (China). Oleic acid was purchased from Yixing Chemical reagent CO. Ltd. (China). Polysorbate 80 (Tween 80) was donated by Croda (U.K.). Compritol 888 ATO (INCI: tribehenin, USP: glyceryl behenate, GB) is a mixture of approximately 15% mono-, 50% di- and 35% triglycerides of behenic acid (C22) while fatty acids other than behenic acid account for less than 20%. Its melting point ranges from 69 to 74°C. It was a gift from Gattefosse´ (D-Weil am Rhein). Miglyol 812 (DAC: oleum neutrale; CTFA: caprylic/capric triglyceride, medium chain triglycerides; MCT) was provided by Caelo (DHilden). Lutrol F 68 (poloxamer 188, a polyoxyethylene-polyoxypropylene polymer) was donated by BASF (D-Ludwigshafen). All other materials were purchased from Sigma (Germany).

Preparation of 10-HCPT Lipid Nanoparticles

HCPT-loaded nanoparticles were prepared by emulsification-ultrasonication method. Briefly, 5 mg 10-HCPT, 1% (w/w) Compritol, 1% (w/w) Miglyol 812, 2% (w/w) oleic acid, and 2.5% (w/w) Tween 80 were heated together in a water bath at 80°C, then 2 ml of anhydrous ethanol was added into the hot lipid phase to dissolve the 10-HCPT, and ethanol was removed at 80°C. 0.5% (w/w) deoxycholic acid sodium salt, 1% (w/w) poloxamer 188, or Brij 35 were dissolved in 80 ml of distilled water at 85°C as aqueous phases. The aqueous phase was dispersed in the lipid phase under magnetic stir conditions at the same temperature. The obtained primary emulsion was ultrasonified using a probe sonicator (250 W, Ultrasonic Cell Pulverizer, JY88-II, Xinzhi Scientific Instrument Institute, China) for 15 min. The obtained nanoemulsion (O/W) was cooled down in an ice bath to form NLC and diluted up to 100 ml with distilled water and gained two types of HCPT-NLCs (NLC with poloxamer 188 and NLC with Brij 35). Finally, the resultant aqueous suspension was filtered through a 0.22 μm cellulose acetate filter membrane.

Measurement of Particle Size and Zeta Potential

The mean particle size of the NLCs was determined by Laser diffractometry (LD) using a LS 230 instrument (Beckmann-Coulter Electronics, Germany). The NLCs were

diluted with water for injection to give an intensity of 300 Hz as recommended by the manufacturer. The zeta potential was measured using a Zetasizer 3000 (Malvern, UK) with the NLCs diluted in water for injection according to the manufacturer's manual.

Transmission Electron Microscopy (TEM)

The morphology of the 10-HCPT-NLC aqueous suspension was examined by TEM (H-600, Hitachi, Japan). The samples were stained with 2% (w/v) phosphotungstic acid for 30 s and placed on copper grids with films for viewing.

Differential Scanning Calorimetry (DSC)

DSC analysis was performed using a TA-60 WS Thermal Analyzer (Shimaszu, Japan). For DSC measurement, Compritol, 10-HCPT, physical mixture of Compritol and 10-HCPT, and lyophilized 10-HCPT loaded NLC were weighed into an aluminum pan, which was then sealed with a pinhole-pierced cover. Heating curves were recorded at a scan rate of 10°C/min from 30 to 300°C.

Determination of Incorporation Efficiency of 10-HCPT

A HPLC method was established to determine the concentration of 10-HCPT. Chromatographic condition was as follows: column: Spherisorb ODS C18 (250 mm × 4.6 mm, 5 μm); mobile phase: methanol-0.01 mol/L PBS buffer (pH 5.0) (60:40, v/v); flow rate: 1.0 mL/min; injection volume: 20 μL; column temperature: 35°C; and detection wavelength: 384 nm.

For determination of incorporated 10-HCPT in NLCs, 0.5 ml of 10-HCPT-loaded NLCs was mixed with 0.5 ml of 0.1 mol/L HCL and 25 ml of methanol, then incubated at 80°C for 5 min, sonicated for 10 min, and then cooled down at ice bath for 10 min. The cold suspension was filtered through a 0.45 μm cellulose acetate filter membrane, and the clear filtrate was injected into the HPLC system.

For determination of incorporation efficiency of 10-HCPT, NLCs (0.5 ml) were applied to a micro-G-50 Sephadex column (8 × 50 mm), and eluted with pH 5.0 PBS buffer through centrifugation at 1000 rpm. NLC-containing fractions (2 ml) were collected and the amount of 10-HCPT was determined for each fraction. The cumulative amount of 10-HCPT was calculated from the NLC-containing fractions containing. Incorporation efficiency was defined as the percentage of 10-HCPT from the NLC-containing fractions compared to the initial loading amount.

Determination of the Percentage of Lactone Form at Different pH Values

For determination of the lactone form of 10-HCPT, 2 mg of 10-HCPT was dissolved in 100 ml of 0.1 mol/L NaOH, and 5 ml of this 10-HCPT solution was diluted to 100 ml with PBS buffer at different pH values (3.0, 5.0, 6.0, 7.0, 8.0). The contents of the lactone form at different pH were determined. The content at pH 3.0 was defined as W_0, and the contents at other pH were defined as W_L. The percentage of the lactone form (E) was calculated from Eqs. (1).

$$E = W_L / W_0 \times 100\% \quad (1)$$

For determination of the lactone form of HCPT-NLC, 10 ml of HCPT-NLC suspension was adjusted to different pH (5.0, 6.0, 7.0, 8.0), and the contents of the lactone form at different pH (W_L) were determined. The total content of 10-HCPT (W_0) was measured after acidified by 0.1 mol/L HCL. The percentage of the lactone form (E) was calculated from Eqs. (1).

Lyophilization

The HCPT-NLCs aqueous suspensions were lyophilized using a Gamma 2–20 apparatus (Christ, Osterode A. H., Germany). The different volume cryoprotectant solutions were added to the NLC dispersions and 2 ml of the suspension was placed in 10 ml glass vials. The NLC were pre-frozen in the refrigeratory (−75°C) for 12 h and subsequently lyophilized at a temperature of −25°C for 24 h, followed by a secondary drying phase of 12 h at 20°C.

In Vitro Release Study

In vitro release study was conducted within 12 h after the preparation of NLC. The two types of NLCs (5 ml), respectively mixed with human plasma (1 ml) and without human plasma, were transferred to a dialysis tube (molecular weight cutoff 10,000 Da), and the tube was introduced into 400 ml of PBS buffer (pH 5.0) and stirred at 37°C. At predetermined time intervals, 2 ml of sample was taken and replaced with the same amount of fresh medium. The amount of 10-HCPT released from the NLC was measured by HPLC.

RESULTS AND DISCUSSION

Preparation of NLC

The methods to prepare NLC are diverse. In this study, we have developed an economical, simple, and reproducible method, and it was free of toxic organic solvent during NLC preparation—iemulsification followed by ultrasonication at above the melting point of the lipid. The compositions of two formulations are listed in Table 1. During preparation, we screened many solubilizers including Tween 20, 80, 85, Span 20, 60, 80, Cremorphor EL, Labrasol and Solutol HS 15 to make HCPT dissolved in the melted lipid phase. We found that Tween 80 was optimal to solubilize HCPT. Oleic acid was added into the formulation of HCPT-NLC to control the pH for maintaining the active lactone form of 10-HCPT, and deoxycholic acid sodium salt was a key factor to improve the stability of NLC by increasing zeta potential. In the course of screening

TABLE 1
The Composition of Two Formulations

Components	NLC-F 68	NLC-Brij 35
10-HCPT (mg)	5	5
Compritol (% w/w)	1	1
Miglyol 812 (% w/w)	1	1
Oleic acid (% w/w)	2	2
Tween 80 (% w/w)	2.5	2.5
Deoxycholic acid sodium salt (% w/w)	0.5	0.5
Poloxamer 188 (% w/w)	1	—
Brij 35 (% w/w)	—	1

the surfactants, we found that HCPT was easily separated from the NLC when lecithin was added into the formulation.

Particle Size, Zeta Potential, and Structural Analyses

The mean particle sizes and zeta potentials of different NLCs are presented in Table 2. The mean diameter of NLC-F 68 was 108 nm, and that of NLC-Brij 35 was 126 nm. Zeta potential is essential to the storage stability of colloidal dispersion, being −28.5 mV and −32.1 mV for NLC-F 68 and NLC-Brij 35, respectively. The electron microscopy micrographs of HCPT-NLC are shown in Figure 2. The shape of two types of NLC was spherical. The particle size approximately ranged from 50 to 200 nm.

The physical dispersion state of incorporated drug in the nanoparticles is an important factor that affects the release profile. Figure 3 shows the results of DSC analysis of two HCPT-NLCs. It is observed from the DSC diagrams that the exothermic peak of 10-HCPT at about 275°C no longer exists in the curve of the drug-loaded nanoparticles. Taking into consideration the drug-crystal-free particle surface, it is apparent that 10-HCPT is amorphously dispersed within the nanoparticles, which is preferable to a controlled release system (Allen, Maysinger, & Eisenberg, 1999; Gref et al., 1994). Similar observations were reported for other drugs entrapped into lipid matrices (Yang & Zhu, 2002). The DSC analysis of camptothecin solid lipid nanoparticles prepared by high-pressure homogenization showed that camptothecin was in its amorphous state.

TABLE 2
Mean Particle Sizes and Zeta Potentials of the NLCs

Formulations	Mean Diameter[a] (nm)	Zeta Potential[b] (mV)
NLC-F 68	108 ± 26.88	−28.5
NLC-Brij 35	126 ± 38.46	−32.1

[a]Mean diameter ± *SD* (*n* = 8).
[b]The mean value of 10 measurements.

Incorporation Efficiency of 10-HCPT

Incorporation efficiency is an important factor for evaluating the nanoparticles delivery systems. It was revealed that NLC could achieve high drug incorporation for lipophilic drugs. The incorporation efficiency may be altered by several factors such as the physicochemical properties of drug and the chemical structure of NLC. In the present study, we investigated the influence of amount of Miglyol 812 and Tween 80 on the incorporation efficiency (Table 3). We found that the incorporation efficiency nearly did not change with increasing amount of Miglyol 812 and was slightly increased when the percentage of Tween 80 increased. This result suggested that drug might be partly entrapped into the core of NLC, and some were concentrated in the layer of interface of surfactant. Thereby, when increasing the amount of Miglyol 812, the percentage of Tween 80 needs to be increased. When the content of Miglyol 812 and Tween 80 are 1% and 2.5% respectively, the incorporation efficiency is 85.7% and 87.4% for NLC-F 68 and NLC-Brij 35, respectively.

The Percentage of Lactone Form at Different pH Values

The percentage of the active lactone form of 10-HCPT raw drug and HCPT-NLC at different pH values is shown in Figure 4 We found that 10-HCPT was easily transferred to the carboxylate form at the physiologic fluid (pH 7.0–7.4), but more than 80% lactone form in two types of HCPT-NLCs was found at pH 8.0. Therefore we concluded that this novel delivery system could effectively protect the active lactone form for improved activity and reduced toxicity of 10-HCPT, which is essential to parenteral delivery system of camptothecin analogs.

Lyophilization

Freeze-drying may generate many stresses that could destabilize colloidal suspension of nanoparticles, especially, the stress of freezing and dehydration. Usually, special excipients must be added to the suspension of nanoparticles before freezing to protect these fragile systems. These excipients are usually added in order to protect the product from freezing stress (cryoprotectant) or drying stress (lyoprotectant) and also to increase its stability upon storage (Abdelwahed, Degobert, Stainmesse, & Fessi, 2006). The lyoprotective effect was attributed to the ability of the sugar additive to form a glassy amorphous matrix around the particles, preventing the particles from sticking together during the removal of water (Konan, Gurny, & Allémann, 2002). Numerous studies have shown the cryoprotective effect of excipients, such as sugars, to prevent particle aggregation during the freeze-drying process. In order to choose the appropriate cryoprotectants to effectively prevent particle aggregation, an optimization procedure of sugars at variable concentrations was performed. NLC-F 68 was selected to screen the effective cryoprotectant.

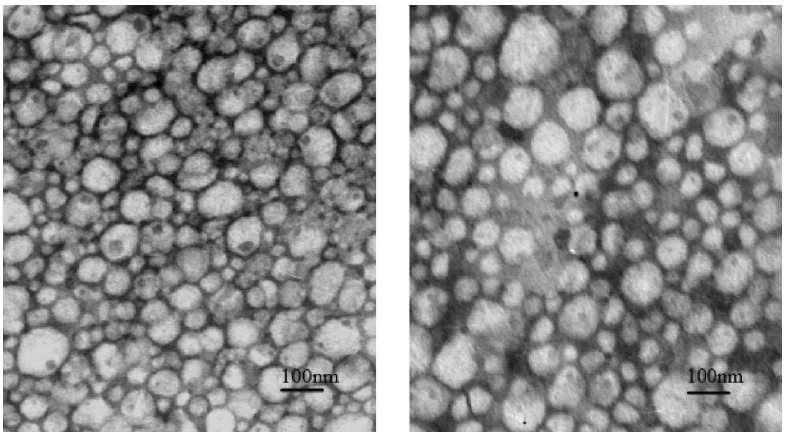

FIGURE 2. Transmission electron microscopy micrographs of nanoparticles aqueous suspensions: NLC-F 68 (left), NLC-Brij 35 (right).

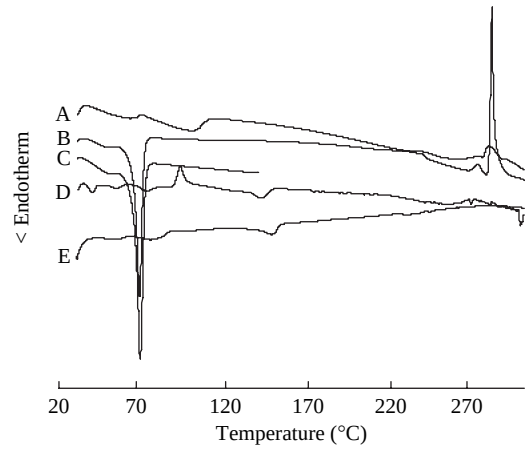

FIGURE 3. Differential scanning calorimetry curves: (A) 10-HCPT; (B) the physical mixture of 10-HCPT and compritol 888; (C) compritol 888; (D) NLC-F 68 loaded with 10-HCPT; (E) NLC-Brij 35 loaded with 10-HCPT.

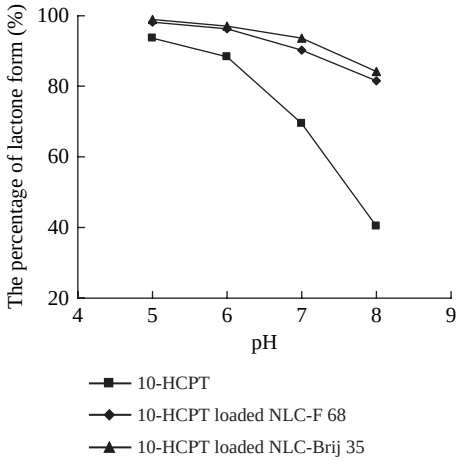

FIGURE 4. The percentage of the lactone form of 10-HCPT raw drug and HCPT-NLC at different pH values.

TABLE 3
Incorporation Efficiency of NLC-F 68 and NLC-Brij 35 with Different Miglyol 812 and Tween 80 Contents

Percentage (%, w/w)	Incorporation Efficiency (%)	
	NLC-F 68	NLC-Brij 35
Miglyol 812		
1	85.7 ± 1.56	87.4 ± 0.98
2	85.4 ± 1.87	86.9 ± 1.65
3	83.1 ± 2.04	84.6 ± 1.39
Tween 80	80.2 ± 1.78	82.4 ± 2.11
1.5		
2.5	85.7 ± 1.56	87.4 ± 0.98
4	86.4 ± 1.49	88.3 ± 1.70

Data represent Mean diameter ± *SD* (n = 3).

Different concentrations of cryoprotectants were tested as shown in Figure 5. We concluded that trehalose was the best cryoprotectant to decrease the particle size after redispersion. The appearance of freeze-dried products was an important aspect. Our results revealed that a slight shrinkage was observed with trehalose, and the freeze-dried product with mannitol provided a satisfied, intact, and fluffy cake. To obtain the desired characteristics of a freeze-dried pharmaceutical form with good appearance and small particle size, we combined trehalose and mannitol for screening (Figure 6). At last, the optimal formulation with 2.5% of trehalose and 2.5% of mannitol presented the smallest particle size (252 nm) after redispersion. Redispersion speed and turbidity change also are important to the freeze-dried products. The final formulation combined with trehalose and mannitol could disperse in distilled water within 1 min without nearly any change of turbidity compared with the initial HCPT-NLC suspension. The results indicated that the combination of cryoprotectants

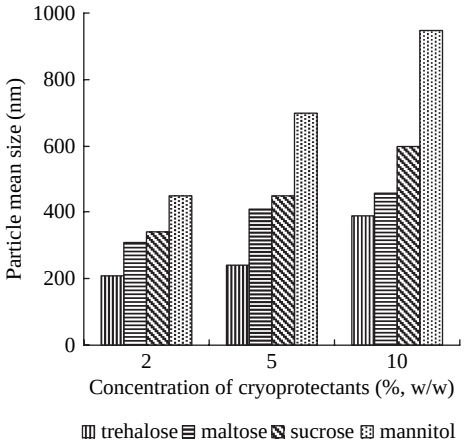

FIGURE 5. Effect of the concentration of different lyoprotectants on nanoparticle size after redispersion.

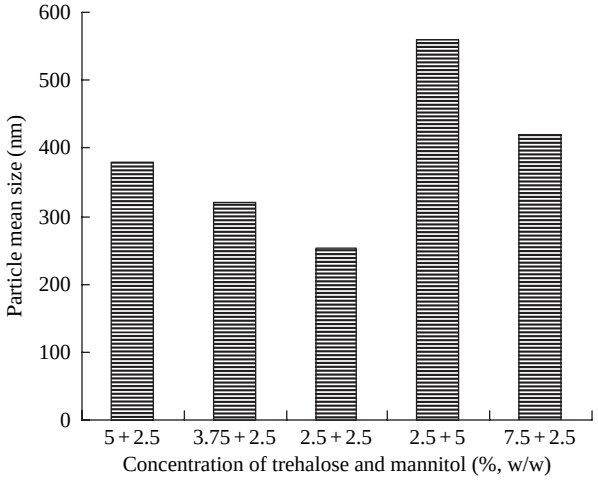

FIGURE 6. Effect of different concentration ratio of trehalose and mannitol on particle size after redispersion.

was effective in preventing particle growth in the freeze-drying process. Changes in particle size by lyophilization could be minimized by optimizing the parameters of the lyophilization process, like freezing velocity and thermal treatment. Thereby, by optimizing critical process parameters and cryoprotectants, NLC suspensions could be freeze-dried, preserving their small particle size.

In Vitro Drug Release

In vitro release curves of two types of drug-loaded nanoparticles are shown in Figure 7. For all release curves, a biphasic drug release pattern was observed, that is, drug rapidly releases at the initial stage and is followed by sustained release at a constant rate. We found that there was turning point at about 8 h. Before 8 h, it was in a fast release stage and nearly 75% of the

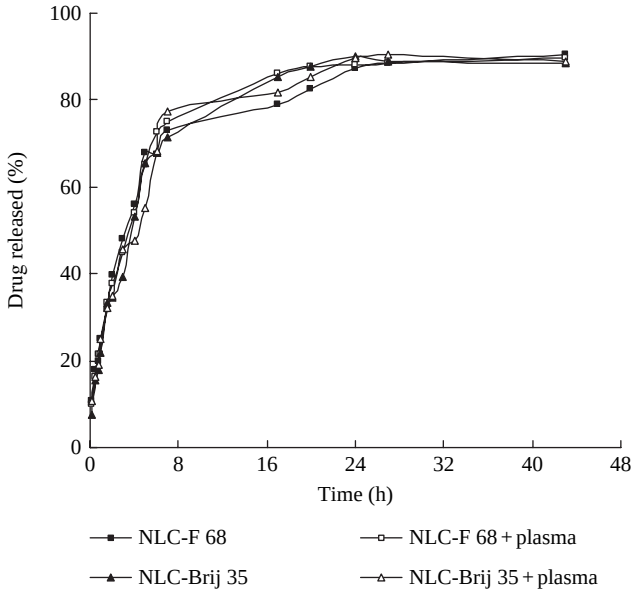

FIGURE 7. In vitro release of 10-HCPT from two types of NLCs with human plasma and without human plasma in PBS (pH 5.0).

drug was released. After 8 h, it was in a sustained release stage and the accumulative release percentage was close to 90%. This release pattern further indicated that the drug, probably concentrated in the layer of interface of surfactant, released easily from NLC and released slowly from the lipid core, subsequently. Aiming at this release behavior, we thought that liquid lipid was not homogenously distributed in nanoparticles matrix. During the cool process from the melted lipid droplet to the formation of the nanostructured lipid carriers, because of the different melting points between solid lipid and liquid lipid, the solid lipid could crystallize first to form a liquid little or free lipid core; finally, most of the liquid lipid and the surfactants located at the outer shell of the nanoparticles, which led to drug-enriched interface layer, related with drug burst release at the initial stage. There also were some studies to improve drug loading using the interface layer (Malzert-Freon et al., 2006; Schubert & Müller-Goymann, 2005). We examined the influence of human plasma on drug release behavior, and found that the existence of human plasma did not obviously affect the drug release from NLC. It was reported that emulsion incorporating Brij 35 slowed down the drug release (Hung et al., 2007), and our results revealed that NLC-Brij 35 displayed the similar release profile to that of NLC-F 68.

CONCLUSION

An emulsification-ultrasonication method was employed to prepare the HCPT-NLC with improved drug incorporation efficiency and release properties. This NLC could effectively protect the lactone ring of 10-HCPT. The drug release behavior from the NLC exhibited a biphasic pattern with burst release at

the initial stage and followed by sustained release. Two types of NLCs have no apparent difference in drug release, and we need to examine the two types of NLCs by pharmacokinetics and toxicity experiments further. These studies indicate that 10-HCPT loaded in NLC lies mostly in the active lactone form and this novel delivery system has a promising potential as an alternative parenteral formulation for 10-HCPT.

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