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RESEARCH PAPER

Preparation and Characterization of Camptothecin Solid Lipid Nanoparticles

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ABSTRACT

Camptothecin (CA), an antitumor drug, was incorporated into solid lipid nanoparticles (SLNs) prepared by high-pressure homogenization. A Taguchi orthogonal experimental design was used to study the influence of four different variables, with each variable having three value levels on nanoparticle size. Analysis of variance (ANOVA) has been used to evaluate the preparation of CA-SLNs and perform product optimization. The optimized CA-SLNs suspension was lyophilized using mannitol and glucose as cryoprotectants. The physicochemical characteristics of CA-SLNs were evaluated using transmission electron microscopy (TEM), electrophoresis, and differential scanning calorimetry (DSC). The release of camptothecin from CA-SLNs in various media was evaluated using a high-performance liquid chromatography (HPLC) method. The results showed that the concentration of emulsifier and the homogenization pressure had a significant influence on the particle size. The optimized CA-SLNs had an average diameter of about 200 nm, exhibited monodispersity with D_w/D_n *of 1.06, and carried a negative charge. The optimal cryoprotectants consisted of 10% mannitol and 5% glucose in nanoparticle suspension. Lyophilized product was reconstituted in distilled water within 0.5 min without change of nanoparticle size. Camptothecin might exist in an amorphous state in SLNs. In vitro results showed that drug release was achieved for up to one week, and the released camptothecin quickly changed to open carboxylate form in the biological pH phosphate buffer. The results indicate that SLNs might be good potential sustained-release delivery vehicles for camptothecin or other lipophilic drugs.*

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Key Words: *Camptothecin; Drug release; Lyophilization; Solid lipid nanoparticles; Taguchi orthogonal experimental design; Zeta potential*

INTRODUCTION

Camptothecin (CA), a plant alkaloid isolated from *Camptotheca acuminata* (1), is the prototype of a novel class of antitumor agents with a unique mechanism of action: stabilization of the binding of topoisomerase I to DNA, leading to DNA fragmentation in the G_2-phase of the cell cycle and to cell death. Inhibition of this enzyme provides a unique therapeutic approach to treatment of slow-growing tumor types (2,3). The lactone functionality appears to play an important role in the biological activity of camptothecin (2). The carboxylate form of camptothecin was found to be significantly less potent than the lactone in in vivo tumor models, and highly toxic in earlier clinical trials (2). Camptothecin lactone opens rapidly and completely to the carboxylate form and almost negligible lactone at equilibrium in human plasma (4). The lactone exists in a pH-dependent equilibrium with an open carboxylate form (Fig. 1). The pharmacokinetics of lactone camptothecin are different from the carboxylate form and are altered by different preparations or dissolving solvents. The absorption of camptothecin from the gastrointestinal tract is very fast. Plasma concentration–time curves of camptothecin and its analogs can be characterized with a two- or three-compartment model (2,5–9).

Because of the poor water solubility of the lactone form and its instability at biological pH, and the low biological activity and severe toxicity of the carboxylate form, the delivery of the active form is quite challenging. To find approaches to address these problems, the drug was prepared as

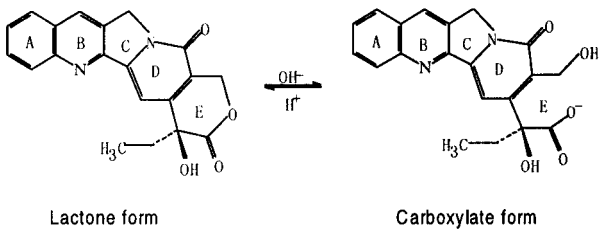

Figure 1. The structure of camptothecin and equilibrium reaction between the lactone form and the ring-opened carboxylate form.

nanocrystalline suspensions (10) incorporated into poly(lactide-co-glycolide) (11) and solid lipid nanoparticles (SLNs) (12,13) or formulated as a suspension in Tween 80–saline or lipid media (14,15).

Recently, increasing attention has focused on SLNs (16–19) because as colloidal drug carriers they combine the advantages of polymeric nanoparticles, fat emulsions, and liposomes, but simultaneously avoid some of their disadvantages. Lipoid matrices composed of physiological and biodegradable compounds have lower toxicity compared to synthetic biodegradable polymers (20,21). Solid lipid nanoparticles possess solid matrices for the controlled release of drugs, avoiding the burst release as obtained with fat emulsions. The pharmacokinetics of a drug can be changed greatly when the drug is incorporated into nanoparticles, owing to the controlled release of the drug from the nanoparticles and the alteration in body distribution of the drug through incorporation into nanoparticles (12,13,22,23).

To design a new formulation in the field of pharmaceutical dosage forms, it is very important to identify the parameters in the preparation since these variables might affect the properties of the final dosage forms. The use of experimental designs is the most common method of simultaneously analyzing the influence of different factors on the properties of the drug delivery system being studied. Taguchi (24) has envisaged a new method of conducting the design of experiments using a special set of arrays called orthogonal arrays. These standard arrays stipulate a way to conduct the minimal number of experiments to give full information on all factors that affect the performance parameters. In this paper, the formulation and homogenization variables were evaluated using a Taguchi orthogonal experimental design. The optimized nanoparticle suspension was lyophilized using glucose, mannitol, and lactose as cryoprotectants. The CA-SLNs were characterized with transmission electron microscopy (TEM), electrophoresis, and differential scanning calorimetry (DSC). The in vitro release of camptothecin from CA-SLNs in different media was evaluated using a high-performance liquid chromatography (HPLC) method.

MATERIALS AND METHODS

Chemicals

Stearic acid was analytical grade. Pure soybean lecithin of medical grade was purchased from Shanghai No. 1 Oils and Fats Factory (Shanghai, China). Poloxamer 188 of medical grade was a gift from the Surfactant Institute of Jinling Petrochemical Co. (Nanjing, China). Camptothecin was purchased from Zhejiang Huangyan Pharmaceutical Plant (Zhejiang, China). All other chemicals used were either analytical or spectroscopic grade. Double-distilled water was filtered through a 0.45 μm (cellulose acetate) membrane before use.

Preparation of CA-SLNs

Nanoparticles were prepared by high-pressure homogenization as described previously (16,17). Briefly, after camptothecin was dissolved in pH 8.0 absolute ethanol adjusted with ammonium hydroxide, stearic acid and soybean lecithin were added. The mixture was heated to above melting point and ultrasonicated to produce a clear melted lipid phase. Poloxamer 188 was dissolved in the aqueous phase containing glycerol as an isotonic agent. A predispersion of the lipid in this aqueous phase, which was previously heated to approximately the same temperature as the melted lipid, was prepared by magnetic stirring and ultrasonication. This premix was passed through a preheated high-pressure homogenizer for five cycles at different pressures. The hot dispersions were filtered through a 0.45 μm membrane and then cooled quickly to 4°C to form a CA-SLNs suspension. The manufacturing process was carried out under nitrogen atmosphere.

Experimental Design

Based on the preliminary study of the effect of parameters on the size of CA-SLNs, the experiments were performed by high-pressure homogenization (15M-8BA, APV, England) (16,17) using a Taguchi orthogonal experimental design (24). The variables, such as emulsifiers, concentration of camptothecin, concentration of emulsifiers, and homogenized pressure, were varied at three levels (Table 1). The Taguchi array which led to the optimized combination through only nine experiments is listed in Table 2. Each experiment was carried out three times.

Table 1

Independent Variables and Their Correspondence Between Real and Orthogonal Values in Taguchi Orthogonal Experimental Design

	Levels		
Variables	1	2	3
Concentration of camptothecin (%) (*A*)	0.02	0.1	0.5
Concentration of emulsifier (%) (*B*)	0.5	2.0	5.0
Emulsifier (*C*)	Tween 20	q[a]	w[b]
Homogenization pressure (psi) (*D*)	2,000	6,000	10,000

[a]q: Lecithin/poloxamer 188 (3:1).
[b]w: Lecithin/poloxamer 188 (2:1).

Lyophilization of CA-SLNs

The CA-SLNs were lyophilized using an FD2 freeze dryer (Germany). Various amounts of cryoprotectants were added to CA-SLNs suspensions before freezing. Twenty milliliters of each sample was rapidly precooled at −25°C, and lyophilized for 24 hr at a temperature of −50°C and a vacuum of 3 mmHg. The resultant lyophilized products were reconstituted in distilled water by manual shaking. The suspension turbidity of reconstituted lyophilized CA-SLNs was measured at 590 nm using a spectrophotometer.

Characterization of CA-SLNs

The diameters of CA-SLNs were determined by TEM (H-7000, Japan). Samples were prepared by placing a drop of CA-SLNs suspension onto a copper grid and air drying, followed by negative staining with a drop of 2 M aqueous solution of sodium phosphotungstate for contrast enhancement. The air-dried samples were then examined directly under the transmission electron microscope. The mean diameter of CA-SLNs was determined by counting 500 particles.

Various amounts of electrolytes were added to CA-SLNs suspensions. The surface charge of nanoparticles was determined by electrophoretic mobility

Table 2

Variables and Results of Taguchi Orthogonal Experimental Design [$L_9(3^4)$]

Trial	Independent Variables				Diameter (nm)			Sum of Diameter
	A	*B*	*C*	*D*	1	2	3	
1	1	1	1	1	326	367	348	1041
2	1	2	2	2	201	223	222	645
3	1	3	3	3	178	191	186	555
4	2	1	2	3	204	215	240	659
5	2	2	3	1	353	322	341	1016
6	2	3	1	2	175	162	170	507
7	3	1	3	2	210	189	190	589
8	3	2	1	3	194	206	201	601
9	3	3	2	1	325	309	310	944

using a U-type tube at 20°C. The electric field strength applied in this experiment was 10 V/cm. From the knowledge of the direction and rate of particle migration, the sign and zeta potential of CA-SLNs in suspension were determined. The zeta potential value was calculated from the Helmholtz–Smoluchowski equation as follows (25):

$$\zeta = \frac{\nu}{E}\frac{4\pi\eta}{\varepsilon}$$

where ζ is the zeta potential, ν is the relative velocity between particles and the surrounding medium, E is the electric field strength, η is the viscosity of the medium, and ε is the dielectric constant of the medium.

Differential scanning calorimetry analyses were carried out on CA-SLNs. In this way, the samples were weighed directly in perforated aluminum pans and scanned between 30 and 300°C at a heating rate of 10°C/min under nitrogen using a DSC-2C (Perkin Elmer, USA). To eliminate the water present in the samples, they were washed three times in distilled water, ultracentrifuged, and dried to constant weight in a desiccator.

Evaluation of the Camptothecin Encapsulation Efficiency

The drug loading and entrapment efficiency of CA-SLNs were determined by ultrafiltration with XHH hollow fiber ultrafiltration membrane (MW 10,000, Reili Separation Instrument Factory, Shanghai, China). The concentrations of camptothecin in CA-SLNs suspension and the ultrafiltrate diluted with methanol were assayed with HPLC (6,12,13,26), performed using an SP 8800 pump and data-get integrator (Spectra Physics Analytical, USA). The analytical column was RP-C18 10 μm, $220 \times 4.6\,mm^2$ (ELITE-TEST, USA). Camptothecin was monitored using a Waters 470 scanning fluorescence detector using an excitation wavelength of 360 nm and an emission wavelength of 430 nm with 18-nm bandwidth. The mobile phase was composed of 55% (v/v) methanol (spectroscopic grade)–double-distilled water (pH 5.5) with a flow rate of 1.5 mL/min. The retention time was 5.8 min. The detection limit of camptothecin was 0.5 ng/mL. Intra- and interday variabilities were $< 2\%$.

In Vitro Release of CA-SLNs

In vitro release of camptothecin from nanoparticles was evaluated using a dialysis bag diffusion technique. Dialysis bags with a molecular weight cut-off of 12,000 (Sigma) were filled with 500 μL of CA-SLNs suspension, and then immersed in 500 mL 0.1 M pH 7.4 phosphate buffer saline, pH 5.5 or pH 3.5 distilled water (adjusted by hydrochloric acid). In vitro release of camptothecin was performed at 37°C using RC drug dissolution tester (Tianjin Medical Instrumental Factory, Tianjin, China) with paddle rotation at 50 rpm. Samples were taken from the outer solution, then added with the same volume of fresh dissolution medium every time. The samples were determined with HPLC before and after acidification with glacial acetic acid.

RESULTS AND DISCUSSION

Preparation of Nanoparticles

Taguchi orthogonal experimental designs [$L_9(3^4)$] offer the possibility of investigating four independent variables at three levels after performing only nine experiments (24). The selection of factors and levels in the design would be based on the results of a preliminary investigation. Depending on the therapeutic application, obtaining a stable suspension with small monodisperse particle size requires information on the effects that formulation and production variables have on the CA-SLNs properties.

The mean diameters of CA-SLNs determined by TEM through counting 500 particles are shown in Table 2, and analysis of variance (ANOVA) of the orthogonal experimental design is presented in Table 3. The concentration of emulsifiers and homogenized pressure had a statistically significant ($P < .01$) influence on the particle size of CA-SLNs. With an increasing concentration of emulsifiers or homogenized pressure, the mean diameter of CA-SLNs decreased significantly. Although the concentration of camptothecin and the various kinds of emulsifiers showed only a slight influence on the size of resultant CA-SLNs, they had a significant influence on the stability of CA-SLNs suspension.

Because of the poor solubility of camptothecin in lipid phase, when its concentration was higher than 0.1%, the resultant suspension would exhibit poor storage stability, resulting in the formation of drug crystals which could be seen under TEM. Emulsifier concentration was a very important parameter in the preparation of CA-SLNs. The higher the amount of emulsifier being added, the smaller the size of nanoparticles. Large amounts of emulsifier, however, should be avoided in the parenteral formulation. The types of emulsifiers had no significant effect on particle size but had significant influence on the stability of CA-SLNs suspension. Considering safety and the stability of the resulting nanoparticle suspension, a combination of lecithin and poloxamer 188 in the ratio 3:1 was chosen as the optimum formulation. The high homogenization pressure and large number of cycles may result in a very small particle size, and the resulting suspension was nearly transparent. But it quickly flocculated and precipitated on storage, which may be because of the considerable increase in surface area and/or the decrease in absolute value of zeta potential of nanoparticles (16,17).

Based on the results of ANOVA, the stability of the resulting suspension, and the safety of the emulsifiers, the optimum setting was A_2 (concentration of camptothecin, 0.1%), B_2 (concentration of emulsifier, 2.0%), C_2 (lecithin/poloxamer 188 = 3:1), and D_2 (homogenization pressure, 6000 psi). The optimum formulation (w/w%) consisted of camptothecin 0.1 g, stearic acid 2.0 g, soybean lecithin 1.5 g, poloxamer 188 0.5 g, glycerol 2.25 g, and double-distilled water to 100 g. The optimum homogenized parameters were five cycles at high pressure of 6000 psi. The optimal experimental setting was performed three times and the resulting CA-SLNs suspensions showed the best response to nanoparticle size and stability of suspension.

Table 3

Analysis of Variance

Source	Sum of Squares	DF[a]	Mean Square	*F*
A	647.2	2	323.6	1.91
B	5,358.4	2	2,679.2	13.83[b]
C	669.0	2	334.5	1.98
D	110,804.5	2	55,402.2	327.24[b]
Error	3,047.1	18	169.3	

[a]DF: degrees of freedom.
[b]At least 99% confidence.

Lyophilization of CA-SLNs

The effects of cryoprotectants on the physical properties and turbidity change of reconstituted CA-SLNs from freeze-dried suspensions are shown in Table 4. According to the physical properties, redispersion speed, and turbidity change, the optimized combination of cryoprotectants was 10% mannitol and 5% glucose. The lyophilized product had excellent physical properties and could disperse in distilled water within 0.5 min without change of turbidity compared with the initial CA-SLNs suspension. The results indicated that the cryoprotectants were effective in preventing particle growth in the freeze-drying process. Changes in particle size during lyophilization could be minimized by optimizing the parameters of the lyophilization process. Zimmermann et al. (27) investigated

Table 4

Effects of the Nature of the Cryoprotectants on the Morphological Characteristics of Reconstituted CA-SLNs from Freeze-Dried Suspension

No.	Cryoprotectant (%)			Physical Properties[a]	Speed of Redispersion[b]	R^c
	Mannitol	Glucose	Lactose			
1	10			++	+	1.63
2	20			+++	+	1.31
3		10		−−	−	−
4		20		−−	−	−
5			10	−	+	2.40
6			20	−	+	1.62
7	10	5		+++	++	1.01
8	10		5	++	+	1.08
9		10	5	−	+	1.62

[a]The appraisal of the different parameters was graded as excellent (+++), very good (++), good (+), bad (−), and very bad (−−).
[b]The speed of redispersion was graded as instantaneous (++), slow (+), and very slow (−).
[c]The turbidity ratio of reconstituted CA-SLNs suspension to initial suspension with the same concentration of CA-SLNs.

the influence of different parameters of lyophilization, like the protective effect of cryoprotectants, freezing velocity, and thermal treatment on changes of particle size. The results demonstrated that, by optimizing critical process parameters, i.v.-injectable SLN dispersions can be freeze-dried, preserving their small particle size. Solid lipid nanoparticles of a quality acceptable for i.v. administration were freeze-dried and the results showed that the sugar trehalose was most effective in preventing particle growth during freezing and thawing, and also in the freeze-drying process (28).

Characterization of Nanoparticles

For fresh CA-SLNs prepared according to the optimized formulation and preparation conditions, CA-SLNs were dense, rigid spheres as shown in Fig. 2. The number average diameter (D_n) was 196.8±21.3 nm. The CA-SLNs showed monodispersity, with D_w/D_n of 1.06. The size of nanoparticles changed greatly with different formulations and preparation conditions. Needle crystals of camptothecin could be seen under TEM as the drug concentration was higher than 0.1% in resulting suspensions. The nanoparticle size of optimal CA-SLNs did not show any significant change under TEM within one month at room temperature.

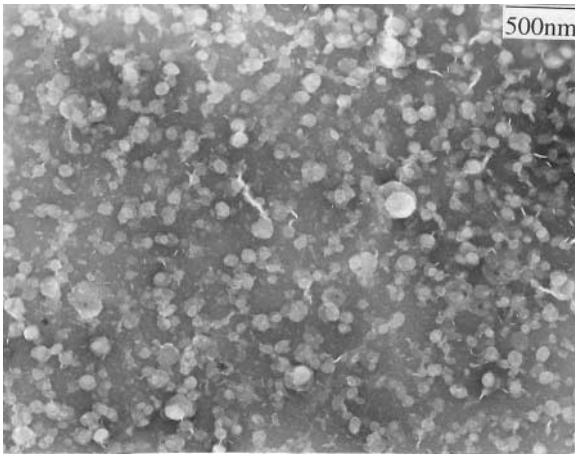

Figure 2. Transmission electron microscopy image of CA-SLNs (×20,000).

Figure 3 shows the effect of the kinds and concentrations of electrolytes on the surface charge of CA-SLNs. The CA-SLNs carried negative charge with a zeta potential of −45.2 mV. The electrolytes of NaCl and Na_2COOCH_3 decreased the zeta potential of CA-SLNs when they were added to the optimum suspension. However, the zeta potential of CA-SLNs increased to about −70 mV as $Na_3C_6H_5O_7$ and Na_3PO_4 were added to the resulting suspension.

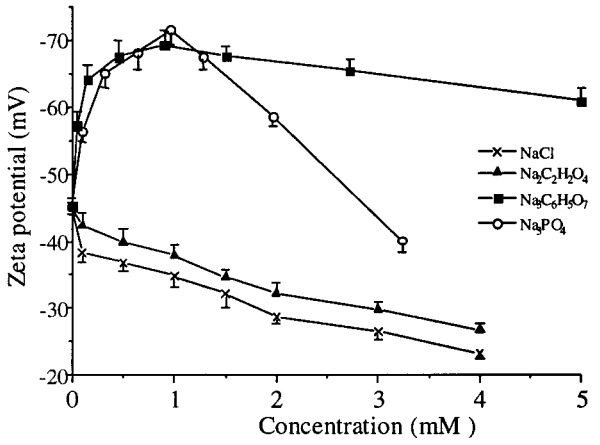

Figure 3. The effect of electrolytes on the zeta potential of CA-SLNs.

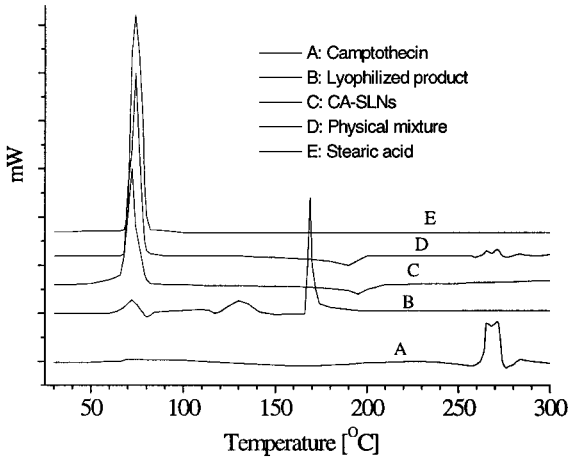

Figure 4. Differential scanning calorimetry thermograms of camptothecin (A), lyophilized sample (B), CA-SLNs (C), physical mixture (D), and stearic acid (E).

These tri-anionic electrolytes, however, could increase the zeta potential of CA-SLNs when the concentrations were higher than 1.0 mM. The tri-anions, co-ions, might be adsorbed or diffused to a stagnant layer and make more contribution to the zeta potential than the monovalent counterion, Na^+, because of the high valence of the tri-anion (25). The phenomenon of zeta potential exhibiting a maximum at an optimum concentration of electrolyte has been studied extensively (29), but a definitive formula is not yet agreed upon. According to DLVO theory, controlling the electrolyte concentration at the optimum level might result in a stable colloidal suspension (25).

The diagram of camptothecin (Fig. 4A) shows endothermic peaks at 258–275°C, typical of camptothecin melting crystals. There are no camptothecin endothermic peaks in the DSC heating run for CA-SLNs (Fig. 4C) and lyophilized product (Fig. 4B), but the endothermic peaks can be seen for a physical mixture (Fig. 4D). This might be due to an amorphous state of camptothecin dispersed in the CA-SLNs and freeze-drying of the product. X-ray powder diffraction and DSC analysis showed that two model drugs, phenothiazine and nifedipine, changed to amorphous when incorporated into lipid nanoparticles (30); DSC analysis showed that hydrocortisone and progesterone were dispersed in SLNs in an amorphous state (31). The crystal–liquid transition of stearic acid in CA-SLNs can be seen in the DSC curve (Fig. 4C), but the melting point and enthalpy decreased compared with pure stearic acid (Fig. 4E). The DSC heating curves of CA-SLNs indicated that solidification of colloidally-dispersed stearic acid under investigation resulted in crystalline material. Polymorph B or the amorphous state of stearic acid might exist in CA-SLNs since the melting point and enthalpy of stearic acid in the nanoparticles decreased. Similar results were reported by Westesen et al. (19) and Cavalli et al. (30). The exothermic peak at about 190°C is the decomposition of lecithin, since it may decompose at above 160°C.

The pH value of the resulting CA-SLNs suspension was 5.5±0.5. The drug loading of CA-SLNs was 4.8±0.3% ($n = 3$) and the entrapment efficiency was 99.6±0.3% ($n = 3$). The high entrapment efficiency might be because of the lipophilic property of camptothecin. Due to the instability of camptothecin in a high pH environment, the pH value can greatly influence the entrapment efficiency.

In Vitro Release of Camptothecin from CA-SLNs

The release of total camptothecin from CA-SLNs was rather slow (Fig. 5A). The release of camptothecin from CA-SLNs in pH 7.4 phosphate buffer saline was a little faster than that in pH 3.5 and pH 5.5 media. In vitro release of camptothecin was up to more than 154 hr in each medium. These results indicated that SLNs have successfully

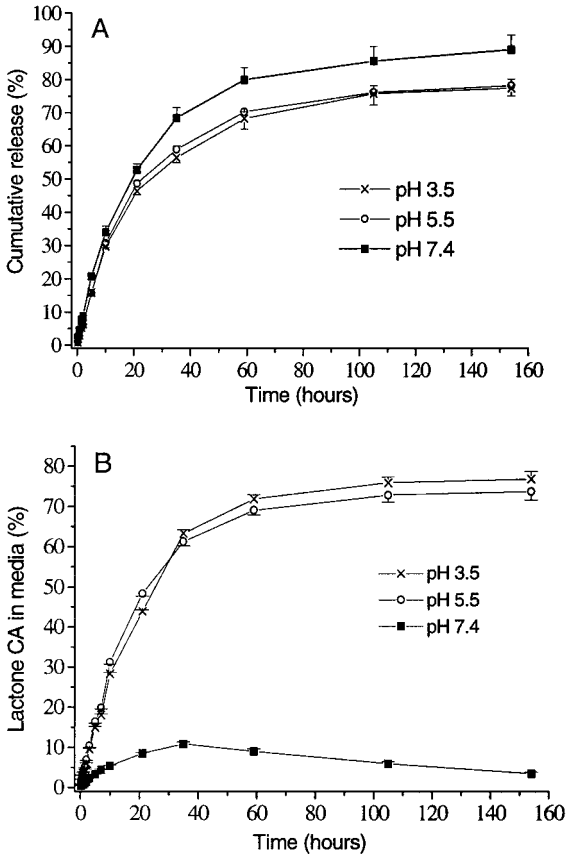

Figure 5. In vitro release of camptothecin from CA-SLNs (A) and the percentage of lactone camptothecin (B) in different pH media at 37°C.

controlled camptothecin release from CA-SLNs. The release of drug from SLNs depends on the nature of the drug and lipid used in SLN. A burst drug release was observed with tetracaine and etomidate SLNs, which was attributed to the large surface area of the nanoparticles and drug enrichment in the outer shell of the particles. In contrast, prednisolone-loaded SLN showed a distinctly prolonged release over a monitored period of 5 weeks. Depending on the chemical nature of the lipid matrix, 83.8% and 37.1% drug were released from cholesterol and compritol, respectively (32). Glyceryl behenate SLN was loaded with vitamin A and the release profiles were studied using Franz diffusion cells to assess the release kinetic over a period of 24 hr. A good correlation between polymorphic transitions and increased drug release was also observed in the study (33). The amount of released lactone camptothecin existing in dissolution medium, however, was significantly lower in pH 7.4 phosphate buffer saline than in pH 3.5 and pH 5.5 media (Fig. 5B). A large amount of released camptothecin was hydrolyzed to the carboxylate form in pH 7.4 phosphate buffer saline. The E-ring of camptothecin is quite labile in neutral and basic environments. There is a pH-dependent equilibrium between the lactone form and the carboxylate form. The carboxylate form is soluble in pH 7.4 dissolution medium. The kinetic investigation of camptothecin lactone ring opening showed that the lactone form hydrolyzed to the carboxylate form in pH 7.4 phosphate buffer saline at 37°C with a half-life value of 23.8 min (4). Not only could SLNs control the release of camptothecin from CA-SLNs, but they might also protect the incorporated lactone camptothecin from hydrolysis, as described by Shenderova et al. (11). In vivo, the lipid matrix will be degraded by enzymes, thus accelerating to some extent the drug release. The contribution of matrix degradation to drug release depends on the type of enzymes and the concentration of enzymes present. The release rate of camptothecin from SLNs would be faster in gut compared to an intramuscular injection site, because lipases exist in the intestinal tract.

CONCLUSION

The formulation and preparation of CA-SLNs were optimized using a Taguchi orthogonal experimental design. The concentration of emulsifiers and the homogenized pressure had a statistically significant influence ($P < 0.01$) on the particle size of CA-SLNs. The optimum homogenized parameters were five cycles at high pressure of 6000 psi, and the optimal formulation (w/w%) consisted of camptothecin 0.1 g, stearic acid 2.0 g, soybean lecithin 1.5 g, poloxamer 188 0.5 g, glycerol 2.25 g, and double-distilled water to 100 g. The resultant CA-SLNs showed monodispersity with an average diameter (D_n) of 196.8±21.3 nm. The CA-SLNs carried a negative charge and the zeta potential increased from −45.2 mV to about −70 mV as $Na_3C_6H_5O_7$ and Na_3PO_4 were added to the CA-SLNs suspension. In vitro release of camptothecin from CA-SLNs in pH 7.4 phosphate buffer saline was a little faster than that in pH 3.5 and pH 5.5 media. The amount of released lactone camptothecin existing in dissolution medium was significantly lower in pH 7.4 phosphate buffer saline than in pH 3.5 and

pH 5.5 media because of the instability of camptothecin in high pH medium.

The results showed that CA-SLNs greatly increased the stability of camptothecin toward hydrolysis and retention of the active lactone. Solid lipid nanoparticles may be considered as a promising carrier for controlled release and targeted delivery of camptothecin and other antitumor drugs.

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