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Preparation and cytotoxic activity of hydroxycamptothecin nanosuspensions

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ABSTRACT

Hydroxycamptothecin is a promising anticancer agent that possesses the ability to inhibit the growth of a wide range of human tumors. Owing to its poor solubility and instability, the pharmaceutical development and clinical utilization of hydroxycamptothecin have been limited. In the present study, a novel precipitation-combined high-pressure homogenization (PCH) technique was used to prepare hydroxycamptothecin nanosuspensions. Based on the homogenization pressure and number of cycles, the process with 10 cycles at 18,000 psi of homogenization pressure was found to be the most efficient method to achieve consistent particle size reduction. It was used to prepare nanosuspensions for characterization and evaluation of the formulation performance. Lyophilization of hydroxycamptothecin nanosuspensions, the shape and crystal form of the drug, and antiproliferative activity were also studied. The mean particle size (z-ave) of the reconstituted freeze-dried powder was small and uniform. The freeze-dried powder might be a good choice for intravenously administrating poorly soluble hydroxycamptothecin, which proved to have higher cytotoxicity against the cancer cells than hydroxycamptothecin injections ($p < 0.001$). Overall, these studies have demonstrated that the PCH technique can be used successfully to prepare hydroxycamptothecin nanosuspensions.

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1. Introduction

Hydroxycamptothecin (HCPT), a camptothecin analog, is a promising anticancer agent that possesses the ability to inhibit the growth of a wide range of human tumors. HCPT acts on topoisomerase I, inhibiting DNA replication and RNA transcription by stabilizing the cleavable complexes formed between topoisomerase I and DNA (Hsiang et al., 1985; Rothenberg, 1997). Therefore, HCPT and other camptothecin analogues have recently undergone extensive clinical evaluation worldwide (Han et al., 2005; Markman, 2005; Yonemori et al., 2005). The preservation of the α-hydroxy-δ-lactone ring of HCPT is crucial for its antitumor activity (Hertzberg et al., 1989). However, the delivery of the lactone form is quite challenging, since the lactone exists in a pH-dependent equilibrium with an open carboxylate, and the lactone form with the closed E-ring has poor solubility. At present, HCPT injections are formulated as a sodium salt of the carboxylate which possess only 10% of the cytotoxic activity of lactone form (Hertzberg et al., 1989), which has exhibited several side-effects during in clinical trials (Wani et al., 1980; Zhang et al., 2001). The pharmaceutical development and clinical utilization of HCPT have been limited because of the effects of its low aqueous solubility and activity *in vivo* on drug hydrolysis and blood–protein interactions (Burke and Mi, 1993). Therefore, the development of a safer and more potent intravenous formulation is necessary.

Recently, specifically designed dosage forms and techniques for camptothecins have been evaluated to overcome their hydrophobic and unstable characteristics. The promising approaches were liposomal formulations (Sadzuka et al., 2005; Saetern et al., 2004; Zhao et al., 2006) and submicron emulsions (Zhao et al., 2007), which can solubilize drugs in the lipid membrane or oily phase, and protect the lactone form of camptothecins. The low loading content and encapsulation efficiency, however, were limited to develop these delivery systems. Although the drug loading content and encapsulation efficiency of nanoparticles (Williams et al., 2003; Yang et al., 2007; Zhang et al., 2007) and microspheres (Mallery et al., 2001) were high, camptothecins were still unstable in these formulations. In addition, the water- and lipid-soluble prodrugs (Croce et al., 2004), cyclodextrines inclusion compounds (Saetern et al., 2004), and polymer-bound derivatives (Minko et al., 2002; Paranjpe et al., 2005) were investigated. However, the solubility and stability of camptothecins in these formulations are also in question.

Nanosuspensions are submicron colloidal dispersions of pure drug nanocrystals (<1000 nm) in an outer liquid phase (Moschwitzer et al., 2004) and have special application to water insoluble drug for various administration routes (e.g., oral, parenteral) (Keck and Müller, 2006). There are several techniques for

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producing drug nanocrystals. Typically, the drug nanocrystals are generated in an aqueous dispersion medium (e.g., by high-energy processes), which should contain certain surfactants or polymers to ensure the stability of drug colloidal system. Aside from the high-energy processes, nanosuspensions can also be prepared directly by crystallization or precipitation. However, efficient removal of the solvent is important in such a process since the presence of solvent can lead to Ostwald ripening (Rabinow, 2004). The micro-precipitation and high-energy approaches can also be combined to exploit the advantages of both processes (Constantinides et al., 2008; Keck and Müller, 2006). Such a combination process can be more efficient than homogenization or precipitation (crystallization) alone. The particle size achieved with the combination process was lower than that seen for direct homogenization. The first combination technology called NANOEDGE™ was developed by Baxter Healthcare (Kipp et al., 2003). Difficulties related with the solvent removal and potential solvent residues are drawbacks of the NANOEDGE™ technology. Recently, a microprecipitation–high-pressure homogenization method was developed to prepared HCPT nanosuspensions (Pu et al., 2009). By employing the method, HCPT nanosuspensions with mean diameters below 200 nm and with very low polydispersity could be prepared. However, the method was more complex and time-consuming. In addition, an organic solvent was also used in the preparation process.

Generally, the precipitation technique needs an organic solvent to dissolve a water insoluble drug, which raises environmental and human safety concerns over residual solvent (Kocbek et al., 2006). However, HCPT can be dissolved in an alkaline solution and crystals are precipitated when the opening of the lactone ring of HCPT transforms it back to the lactone form in acidic pH conditions.

In the present work, we prepared HCPT nanosuspensions by the precipitation-combined high-pressure homogenization (PCH) method, investigated the characteristics of the HCPT nanosuspensions, and compared the cytotoxic effects with that of HCPT injections.

2. Materials and methods

2.1. Materials

Hydroxycamptothecin (purity > 98.6%) was purchased from China Aroma Chemical Co. Ltd., China. Hydroxycamptothecin injections (hydroxycamptothecin sodium salt, SANCTITY®) were from Harbin Sanctity Pharmaceutical Co., Ltd., China. Poloxamer 188 and Solutol® HS 15 were obtained from BASF, Germany. Lipoid S75 was a product of Lipoid GmbH. All other reagents were of the highest grade commercially available.

2.2. Preparation of HCPT nanosuspensions

2.2.1. Precipitation method

HCPT powder (40 mg) was dissolved in 18 ml of 0.02 M sodium hydroxide solution with various stabilizers [0.4% Lipoid S75 and (or) 0.5% Poloxamer 188, 0.25–0.75% Solutol® HS 15, 0.4% Lipoid S75 and 0.5% Solutol® HS 15, w/v]. The pH of the solution was adjusted to 5.2 with a 1.0% solution of acetic acid in order to precipitate HCPT. Meanwhile the suspensions were stirred with Ultra Turrax (T18 basic, IKA, Guangzhou, China) at 10,000 rpm.

2.2.2. High-pressure homogenization method

HCPT powder (40 mg) was dispersed into a 20 ml aqueous solution containing various stabilizers under magnetic stirring. The obtained pre-mixture was homogenized using a Microfluidizer processor M-110L (MFIC, USA). At first, the suspensions were homogenized with 5 cycles at 9000 psi, and then 10 cycles with 18,000 psi. All operations were carried out using a heat exchanger to maintain sample temperature at 25–30 °C.

2.2.3. Precipitation-combined high-pressure homogenization method

A two-steps procedure was followed to prepare the drug nanosuspensions. Firstly, HCPT suspensions were obtained by using the precipitation method. Following that, HCPT suspensions were homogenized and the different potentially influence of the homogenization pressure and the cycle number on the reduction of HCPT microprecipitation was investigated.

2.3. Lyophilization of HCPT nanosuspensions

HCPT nanosuspensions stabilized with Lipoid S75 and Poloxamer 188 were selected to test the possibility of using lyophilization to enhance the stability of HCPT nanocrystals. The freshly prepared nanosuspensions were lyophilized with a cryoprotective agent (mannitol) at different concentrations. Briefly, HCPT nanosuspensions were rapidly cooled to −80 °C for 2 h and then transferred to a freeze-dryer (Heto Drywinner, Heto Holtan, Allerød, Denmark). Drying was performed at a pressure of 0.120 mbar and at the temperature of −24 °C for 24 h to yield a dry sample.

2.4. Particle size analysis

The mean particle size (z-ave) and the polydispersity index (PI) were determined by photon correlation spectroscopy (PCS) with a Malvern Zetasizer (Nano-ZS90, Malvern Instruments, UK). The z-ave is an intensity mean size and the PI is a measure for the width of the particle size distribution. All the measurements were performed with a laser wavelength of 633 nm at 25 °C, and at a scattering angle of 90°. Each sample was measured in triplicate with 11 runs in each measurement and 10 s duration in each run. The measuring range of PCS is approximately 3 nm to 3 μm. Prior to the measurement, the samples were diluted with distilled water to a suitable scattering intensity and redispersed by handshaking before the measurement. In order to investigate how dilution with water affects the size of the particle, we determined the z-ave of samples diluted or redispersed with distilled water and the drug-saturated water at 0, 10, 20 and 30 min.

2.5. Zeta potential analysis

The zeta potential (ZP) is a measure of the electric charge at the surface of the particles indicating the physical stability of colloidal systems. In this study, the same Malvern Zetasizer (Nano-ZS90, Malvern Instruments, UK) was used to measure the ZP values by determining the particle electrophoretic mobility at 25 °C. The measurements were performed in ultrapurified water adjusted to a standardized conductivity of 50 μS/cm of a sodium chloride solution (0.9%, w/v) to avoid changes in ZP values due to day-to-day variations occurring in the conductivity of the water. The pH was in the range of 5.5 ± 0.2. ZP values were calculated using the Helmholtz–Smoluchowsky equation. The sample treatment was the same as that of the "*Particle size analysis*".

2.6. Morphology research

Morphological evaluations of HCPT nanosuspensions prepared with the precipitation method, the high-pressure homogenization method, and the PCH method were conducted through transmission electron microscopy (TEM, JEM2000-FX, JEOL Ltd., Tokyo, Japan). A drop of HCPT nanosuspension was applied to a 300-mesh copper grid, then air dried and examined by TEM.

Table 1
The mean particle size (nm) of nanosuspensions distilled with water and the drug-saturated water after 0, 10, 20 and 30 min.

Time (min)	Nanosuspensions		Freeze-drying powder	
	Distilled water	Drug-saturated water	Distilled water	Drug-saturated water
0	288.9 ± 5.6	283.5 ± 6.8	330.5 ± 18.1	336.3 ± 16.5
10	273.7 ± 7.5	290.3 ± 5.4	324.3 ± 16.4	338.1 ± 19.3
20	243.9 ± 4.5	282.9 ± 4.5	304.5 ± 20.2	330.9 ± 15.8
30	223.9 ± 7.1	292.3 ± 6.5	298.6 ± 19.2	326.3 ± 18.8

Table 2
The influence of different preparation methods on the mean particle size (z-ave), the polydispersity index (PI), and the zeta potential (ZP) values of HCPT nanosuspensions (mean ± S.D., $n = 3$).

Preparation method	z-ave (nm)	PI	ZP (mV)
Precipitation	1176.0 ± 65.5	0.603 ± 0.090	−34.31 ± 5.08
High-pressure homogenization	848.7 ± 28.9	0.46 ± 0.023	−22.59 ± 0.064
Precipitation-combined high-pressure homogenization	286.9 ± 6.1	0.16 ± 0.021	−32.21 ± 0.92

2.7. Differential scanning calorimetry (DSC)

Thermal properties of the powder samples were investigated with a DSC 204 Phoenix® (NETSCH, Germany). Samples of about 5 mg were placed in standard aluminum pans and sealed with a lid. Heating scans by heat runs for each sample were set from 0 °C to 300 °C at 10 °C/min with a nitrogen purge of 20 ml/min.

2.8. Cell culture

The human gastric cancer cell line SGC7901 and the human hepatocellular carcinoma cell line SMMC-7721 were obtained from TCC Cell Bank (Shanghai, China) and grown in RPMI1640 supplemented with 10% (v/v) fetal bovine serum. Cells were incubated in a humidified atmosphere at 37 °C, gassed with 5% CO_2 in air, and subcultured every 2 days with 0.25% trypsin.

2.9. Cytotoxic effects of HCPT nanosuspensions

The *in vitro* cytotoxic effect of HCPT nanosuspensions was evaluated by a proliferation assay utilizing the tetrazolium dye, MTT (Mosmann, 1983; Zhao et al., 2007). In brief, cells were seeded into a 96-well plate, at a density of 1×10^5 cells/well, and incubated with medium containing various concentrations (12.5, 25, 50, 100, 200, 400, 800 and 1600 ng/ml) of HCPT injections and HCPT nanocrystals for 72 h. The medium containing HCPT injections or HCPT nanocrystals was replaced with fresh medium and the cells were incubated for 4 h with 5 mg/ml MTT. The media was then replaced with 150 μl DMSO. The optical densities at 492 nm were determined using a microplate reader (SUNRISE TECAN, Austria). Cell survival was expressed as a percent of control.

3. Results and discussion

3.1. Effect of dissolution on the particle size

In order to show how dilution with water affects the size of the particles, we determined the z-ave of samples diluted or redispersed with distilled water and the drug-saturated water at 0, 10, 20 and 30 min (Table 1). The results show that the z-ave is not significantly changed at 10 min after the samples were diluted or redispersed either with distilled water or the drug-saturated water. In our investigation, the z-ave is determined at 10 min after the samples were diluted or redispersed with distilled water. Teeranachaideekul et al. (2008) also reported that the z-ave of nanosuspensions (nanocrystals) is immediately determined after the samples diluted or redispersed with distilled water.

3.2. Preparation method

In this study, 3 preparation methods of HCPT nanosuspensions stabilized with 0.4% Lipoid S75 and 0.5% Poloxamer 188 were compared. Table 2 shows the effects of the different preparation methods on the z-ave, PI, and ZP of HCPT nanosuspensions. The z-ave and PI of HCPT nanosuspensions prepared with the precipitation and the high-pressure homogenization methods were bigger than that of the HCPT nanosuspensions prepared with the PCH method. This result is consistent with that of the particle size distribution of HCPT nanosuspensions (Fig. 1). The large PI indicates that the distribution of particle size is very broad and heterogeneous. Generally, higher PI values result in a more pronounced Ostwald ripening effect (Teeranachaideekul et al., 2008). Ostwald ripening is the result of the difference in solubility between small

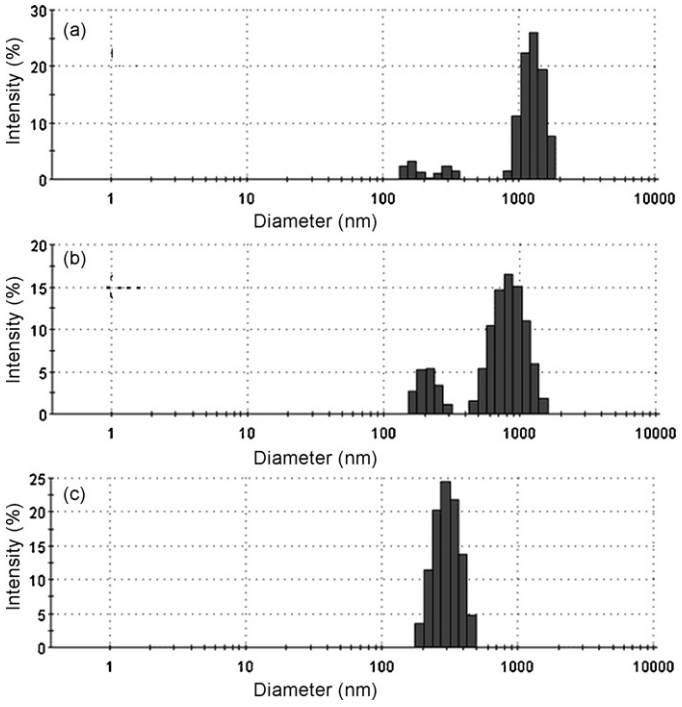

Fig. 1. The particle size distribution of HCPT nanosuspensions obtained from the precipitation method (a), the high-pressure homogenization method (b) and the precipitation-combined high-pressure homogenization method (c). Measurements were performed by photon correlation spectroscopy with a laser wavelength of 633 nm at 25 °C, and at a scattering angle of 90°. Each sample was measured in triplicate with 11 runs.

Table 3
The mean particle size (z-ave), the polydispersity index (PI) and the zeta potential (ZP) values of HCPT nanosuspensions during 3 months storage (mean ± S.D., $n = 3$).

Storage time (month)	z-ave (nm)	PI	ZP (mV)
0	292.35 ± 7.36	0.17 ± 0.043	−33.45 ± 1.12
1	288.72 ± 10.25	0.18 ± 0.061	−33.59 ± 1.22
2	298.46 ± 9.50	0.23 ± 0.092	−34.31 ± 5.08
3	335.16 ± 11.5	0.37 ± 0.085	−32.87 ± 4.39

and large particles due to the higher degree of curvature of the smaller particles leading to a higher solubility compared with the larger particles (Müller and Keck, 2004). As a result, smaller particles dissolve and deposit at the surface of larger particles, which show lower solubility. Subsequently, the small particles disappear while overall particle growth increases during storage. We found that the z-ave of HCPT nanosuspensions prepared with the precipitation or the high-pressure homogenization methods significantly increased and reached values higher than 2 μm after 3 days of storage at room temperature (25 °C). However, the z-ave of HCPT nanosuspensions prepared with the PCH method did not obviously increase. The ZP of HCPT nanosuspensions prepared with the PCH method was higher than that prepared with the high-pressure homogenization method. The high ZP indicates good physical stability of the nanocrystal. The ZP values higher than |30 mV|indicate electrostatic long-term stability of aqueous dispersions (Müller, 1996). In this study, the ZP of HCPT nanosuspensions prepared with the PCH method was −32.21 mV. After 2 months of storage, the z-ave, PI, and the ZP of HCPT nanosuspensions prepared with the PCH method did not significantly change (Table 3). However, a significant increase in the z-ave and PI was observed after 3 months of storage.

TEM micrographs clearly showed the differences between the HCPT nanosuspensions prepared with the three different methods (Fig. 2). The microparticles prepared with the precipitation method and the high-pressure homogenization method, were found to be large (Fig. 2a and b). The microparticles prepared with the precipitation method often exhibited the tendency of continuous crystal growth to the size of a micrometer while crystals and microparticles prepared with the high-pressure homogenization method showed no significant alteration even if the suspensions were homogenized for 20 cycles with a homogenization pressure of 18,000 psi. To avoid further crystal growth, the combined precipitation with the high-pressure homogenization method was used. The size of nanocrystals prepared with the combined method became relatively small and uniform (Fig. 2c). The size of nanocrystals observed by TEM was in good agreement with that by PCS. The combination technology of precipitation and homogenization can achieve reproducible submicron-sized particles with a narrow distribution, and avoid or minimize the use of potentially toxic components (Pu et al., 2009). The small and uniform size, and minimizing the use of potentially toxic components were crucial for the safety of intravenous administration of nanosuspensions. The combination technology of precipitation and homogenization (NANOEDGE^{TM}) was developed by Kipp et al. to avoid further crystal growth and uncertainty of crystalline/amorphous state (Kipp et al., 2003). However, the distinct disadvantages of the NANOEDGE^{TM} technology are the need to remove solvent in a costly process after the homogenization and the organic solvents to raise environmental and human safety concerns over residual solvents. On the other hand, our method is useful only for drugs with an aqueous solution, thereby being safe to environment and human as well as more economical.

The results described above demonstrate that the PCH method is a viable alternative for preparing HCPT nanosuspensions.

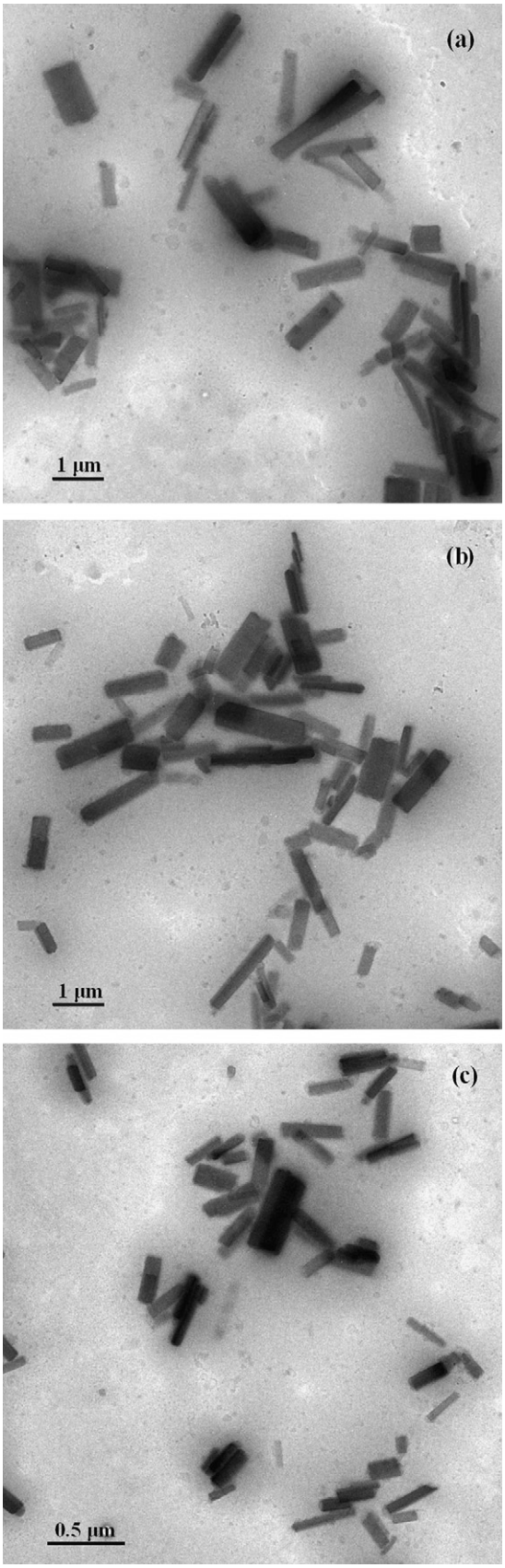

Fig. 2. Transmission electron microscopy (TEM) micrographs of HCPT nanosuspensions obtained from the precipitation method (a), the high-pressure homogenization method (b), and the precipitation-combined high-pressure homogenization method (c). HCPT nanosuspension was applied to a 300-mesh copper grid, then air dried and examined by TEM.

Table 4
The influence of different stabilizers on the mean particle size (z-ave), the polydispersity index (PI), and the zeta potential (ZP) values of HCPT nanosuspensions (mean ± S.D., $n = 3$).

Stabilizer	Concentration (%)	z-ave (nm)	PI	ZP (mV)
Lipoid S75	0.4	365.4 ± 3.3	0.188 ± 0.020	−31.10 ± 0.73
Poloxamer 188	0.5	345.4 ± 16.5	0.157 ± 0.027	−26.98 ± 0.97
Solutol® HS 15	0.25–0.75	Floccules	–	–
Lipoid S75 + Poloxamer 188 (1:1.25)	0.9	283.9 ± 4.5	0.150 ± 0.020	−32.39 ± 1.22
Lipoid S75 + Solutol® HS 15 (1:1.25)	0.9	316.5 ± 13.7	0.203 ± 0.014	−33.15 ± 0.75

3.3. Effect of stabilizers

The choice of a stabilizer is specific to each drug candidate and each formulation procedure. The stabilizer (or mixture of stabilizers) should exhibit sufficient affinity for the particle surface in order to stabilize the nanosuspensions (Kocbek et al., 2006). In this study, three types of stabilizers and mixtures of stabilizers were investigated with the PCH method (Table 4). The type of compound employed for stabilization has a pronounced effect on the particle size and the polydispersity index. Lipoid S75 (0.4%, w/v) and Poloxamer 188 (0.5%, w/v), obtained almost the same z-ave and PI. However, floccules were found in nanosuspensions stabilized with Solutol® HS 15 (concentration from 0.25% to 0.75%). The z-ave and PI of nanosuspensions stabilized with a combination of Lipoid S75 and Poloxamer 188 were smaller than that of a combination of Lipoid S75 and Solutol® HS 15, but the ZP showed no significant alteration. Our results show that the stabilization of nanosuspensions with a combination of stabilizers was better than one alone. Poloxamer 188 is polymeric molecules, which can by adsorption on the particle surface act as a steric barrier, preventing close contact of the particles and later particles. Lipoid S75 can enhance the effect of electrostatic repulsion by increasing the ZP of particle surface. Therefore, the combination of stabilizers is also preferred for long-term stabilization (Kocbek et al., 2006; Müller and Keck, 2004; Rabinow, 2004). Consequently, nanosuspensions stabilized with a combination of Lipoid S75 and Poloxamer 188 were chosen for further studies.

3.4. Homogenization pressure and cycles

High-pressure homogenization is one of the most important techniques to produce drug nanocrystals (Keck and Müller, 2006). The influence of homogenization pressure on the reduction of HCPT microprecipitation was shown in Fig. 3. The z-ave and PI of nanosuspensions prepared with a low homogenization pressure of 3000 psi with 10 cycles were about 501 nm and 0.35, respectively. As the pressure increased from 3000 to 18,000 psi with the same cycle numbers, the z-ave became much smaller (~280 nm). The homogenization pressure simultaneously narrowed the width of the size distribution, i.e. reduced the PI of the bulk population. Therefore, to obtain a higher monodispersity (smaller PI, ~0.16) the homogenization pressure was increased from 3000 to 18,000 psi. Fig. 4 shows the decrease in the z-ave as a function of the cycle number with the homogenization pressure of 18,000 psi for the three batches produced. The z-ave clearly decreased up to cycle number 8 and remained unchanged when the cycle number was raised to 16. There was a batch-to-batch variation in the particle size up to 8 homogenization cycles. However, after 8 cycles the particle size became more uniform. The required cycle number is influenced by the hardness of drug and the size of the starting material (Müller et al., 2001). In this study, HCPT particles prepared with the precipitation were smaller and softer than the raw HCPT. Therefore, the z-ave of nanocrystals prepared with the combined method became relatively small and uniform as compared to the z-ave of nanocrystals prepared with the single step high-pressure homogenization method in the same homogenization pressure and cycle number.

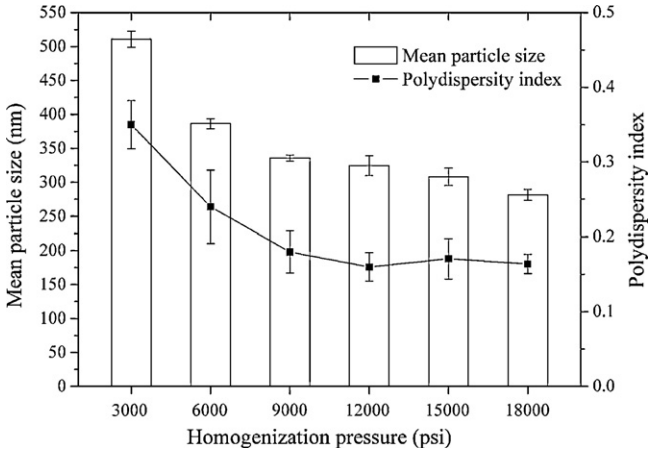

Fig. 3. The influence of the homogenization pressure with 10 cycle numbers on the reduction of the HCPT crystals stabilized with 0.4% Lipoid S75 and 0.5% Poloxamer 188. The mean particle size and the polydispersity index are expressed as a function of the pressure using Microfluidizer processor. Data are expressed as mean ± S.D., $n = 3$.

3.5. Lyophilization of HCPT nanosuspensions

Lyophilization of a nanosuspension can be performed to overcome either physical or chemical incompatibility, permitting recovery of the original particle size after the reconstitution (Peters et al., 2000). Hydrophobic interaction may cause HCPT nanosuspension particles to aggregate during the freeze-dry process. In order to prevent this aggregation, the addition of cryoprotective agents (mannitol) at various concentrations was tested. Table 5 shows the z-ave, PI, and ZP values of HCPT nanosuspensions without a cryoprotective agent and with 2%, 5% and 10% mannitol. After

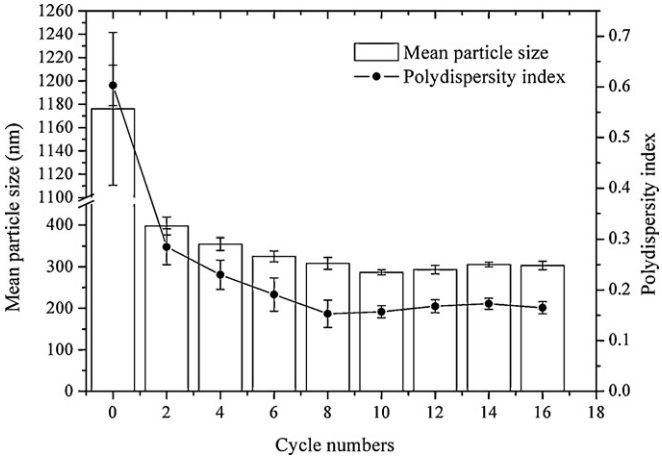

Fig. 4. The influence of cycle numbers at 18,000 psi of the homogenization pressure on the reduction of the HCPT crystals stabilized with 0.4% Lipoid S75 and 0.5% Poloxamer 188. The mean particle size and the polydispersity index are expressed as a function of cycle numbers using Microfluidizer processor. Data are expressed as mean ± S.D., $n = 3$.

Table 5
The mean particle size (z-ave), polydispersity index (PI), and zeta potential (ZP) values of HCPT nanosuspensions stabilized with 0.4% Lipoid S75 and 0.5% Poloxamer 188, immediately after reconstituting the lyophilized nanosuspensions (mean ± S.D., $n = 3$).

HCPT nanosuspension formulations	z-ave (nm)	PI	ZP (mV)
0% mannitol	622.7 ± 3.7	0.279 ± 0.023	−38.70 ± 2.47
2% mannitol	502.2 ± 18.8	0.363 ± 0.061	−38.19 ± 0.67
5% mannitol	394.0 ± 11.8	0.282 ± 0.041	−41.33 ± 2.26
10% mannitol	334.3 ± 21.0	0.258 ± 0.033	−41.24 ± 1.62

the freeze-dry process, the z-ave of the reconstituted powder with mannitol was smaller than that of the powder without a cryoprotective agent, and the appearance of the powder with mannitol was incompact and porous. In the case of the formulation with 10% mannitol, the z-ave and PI were smaller than those of the powder with 2% and 5% mannitol. Although the z-ave and PI of the reconstituted powder with 10% mannitol were slightly increased in comparison to those of the dispersion before lyophilization (particle size from ~293.2 to ~334.3 nm, PI from ~0.15 to ~0.258), they were not significantly increased after 2 months of storage at 25 °C (particle size from ~334.3 to ~341.1 nm, PI from ~0.258 to ~0.262). In this study, sucrose was also investigated as cryoprotective agent. However, agglomeration could be visually observed and the powder was slightly viscous and sticky after the nanosuspensions were dried. The capillary pressure theory is currently the main theory used to explain agglomeration during the drying process (Van Eerdenbrugh et al., 2008; Wang et al., 2005). According to this theory, agglomeration occurs as a result of the capillary forces encountered during the drying process. In this study, adding 10% mannitol to the nanosuspensions is better for preventing particle agglomeration during the drying process and during storage time than adding 0%, 2%, or 5% mannitol. Therefore, nanosuspensions with 10% mannitol yielded optimal results.

For clinical use, the reconstitution was carried out in different media including distilled water, 0.9% NaCl solution, and 5% glucose solution. The z-ave, PI, and ZP were determined after reconstitution with the three media (Fig. 5). Obviously, the z-ave after reconstitution with distilled water or a 5% glucose solution was smaller than the z-ave after reconstitution with a 0.9% NaCl solution. In the electrolyte (0.9% NaCl) solution, the change of the electric double layer and the ZP resulted in the aggregation of particles and increased particle size (Hwang and Liu, 2002). The PI was not significantly affected by the three media. However, the ZP value of the sample reconstituted with 0.9% NaCl solution was drastically small (−11.42 mV) in comparison to that of the sample reconstituted with distilled water (−41.24 mV) or 5% glucose solution (−34.02 mV). The ZP is the electrostatic potential that exists at the shear plane of a particle, which is related to both surface charge and the local environment of the particle (Hwang and Liu, 2002; Zhang et al., 2008). Sodium ions with positive charge are absorbed on surface of particles with negative charge when the sample is reconstituted with a 0.9% NaCl solution. The result will cause the ZP value of particle to decrease. In the experiment, the freeze-dried products were all reconstituted with distilled water.

3.6. Differential scanning calorimetry analysis

To characterize the freeze-dried nanosuspensions, DSC studies of HCPT, Poloxamer 188, mannitol, Lipoid S75, and lyophilized nanosuspensions were performed. The DSC curve of pure HCPT exhibits a single endothermic peak at 274.2 °C, due to its melting (Fig. 6A). Poloxamer 188, mannitol, and Lipoid S75 also have visible melting points at 49.9 °C, 169.8 °C, and 51.4 °C, respectively (Fig. 6B–D). After the nanosuspensions were freeze-dried, the melting endothermic peaks of the excipients disappeared (Fig. 6E), whereas that of HCPT was almost constant. These results demonstrate that the excipients might form an amorphism on

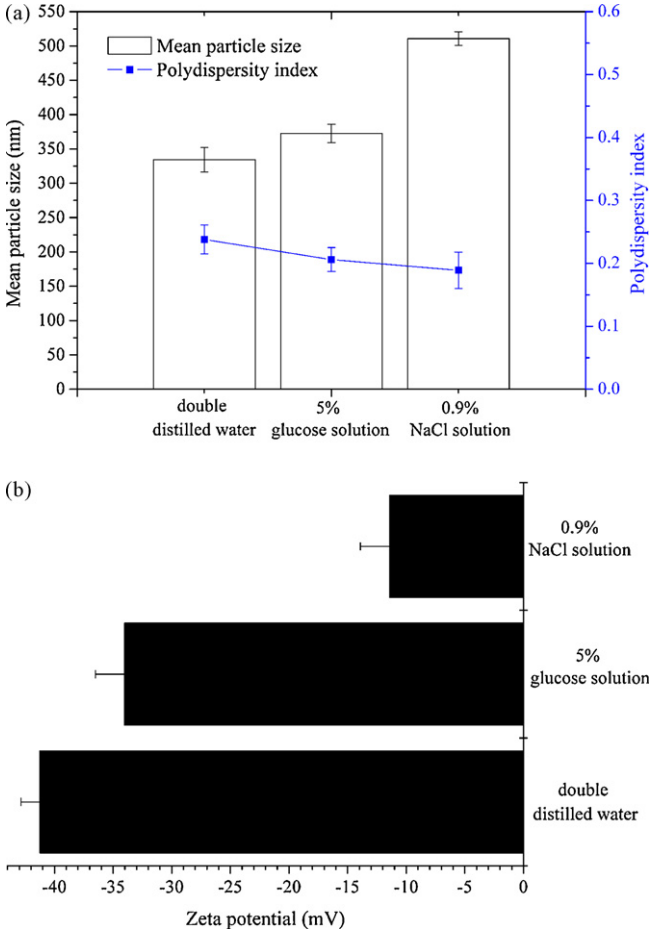

Fig. 5. The mean particle size, polydispersity index (a), and zeta potential value (b) of HCPT nanosuspensions stabilized with 0.4% Lipoid S75 and 0.5% Poloxamer 188 after the reconstitution by different media including distilled water, 0.9% NaCl solution, and 5% glucose solution. Data are expressed as mean ± S.D., $n = 3$.

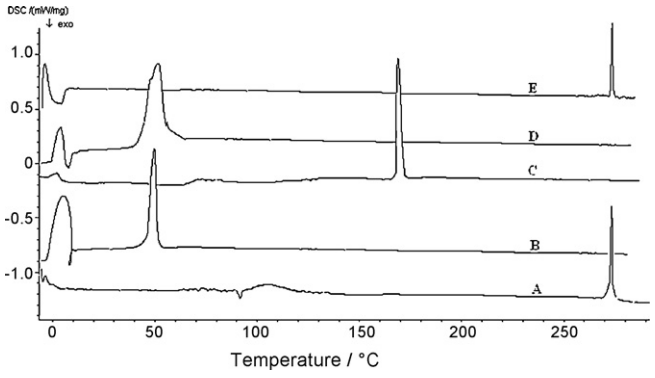

Fig. 6. The differential scanning calorimetry curves of HCPT (A), Poloxamer 188 (B), mannitol (C), Lipoid S75 (D), and lyophilized nanosuspension (E) stabilized with 0.4% Lipoid S75 and 0.5% Poloxamer 188. Heating scans by heat runs for each sample were set from 0 °C to 300 °C at 10 °C/min with a nitrogen purge of 20 ml/min.

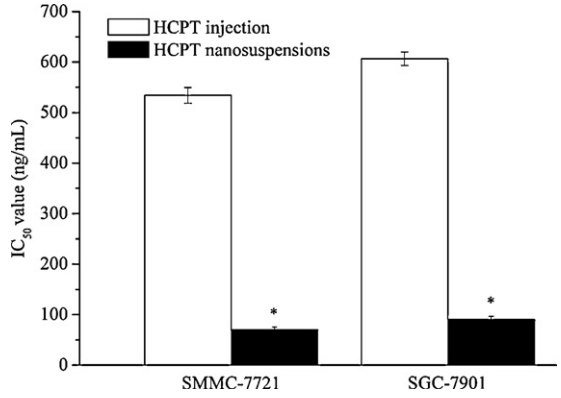

Fig. 7. The IC_{50} values (ng/ml) of HCPT nanosuspensions and HCPT injections in SMMC-7721 and SGC-7901 cells. The cells were incubated with medium containing various concentrations (12.5, 25, 50, 100, 200, 400, 800 and 1600 ng/ml) of HCPT nanocrystals or HCPT injections for 72 h at 37 °C. Data are expressed as mean ± S.D., $n = 4$. $^*p < 0.001$ *vs*. HCPT injections.

the surface of HCTP nanocrystal, but the form of HCPT was not transformed after the precipitation and high-pressure homogenization. Pu et al. (2009) reported that the crystalline form of HCPT was transformed to amorphous state after the precipitation and high-pressure homogenization. The different forms of HCPT in the lyophilized powders for nanosuspension may be relative to the preparation methods of HCPT nanosuspensions.

3.7. Antiproliferative activity of HCPT nanosuspensions

The MTT cytotoxicity assay measured for HCPT indicated that both the injections and the nanosuspensions were cytotoxic against SMMC-7721 and SGC-7901 cells. The IC_{50} values of HCPT nanosuspensions were lower than those of HCPT injections by about 7.6 times against SMMC-7721 cells and 6.7 times against SGC-7901 cells (Fig. 7). HCPT injections formulated in sodium salt of the carboxylate possessed only 10% of the cytotoxic activity of lactone form (Hertzberg et al., 1989). However, HCPT exists as an active lactone form in nanosuspensions and cell uptake of this form may be enhanced by using HCPT nanocrystals. As a result, the cytotoxicity of HCPT nanosuspensions against the cancer cells was greater than the injections. Pu et al. (2009) also reported that the nanosuspension delivery system could effectively protect the active lactone form and may consequently improve the activity of HCPT.

4. Conclusions

The results obtained in this study demonstrated that the PCH method could be used to formulate HCPT suspensions with crystals in the nanometer range. The particle size of HCPT nanosuspensions was depended on the type and concentration of stabilizers, homogenization pressure, and number of homogenization cycles. Furthermore, lyophilization of HCPT nanosuspensions, the shape and crystal form of the drug, and antiproliferative activity were investigated.

The z-ave of the reconstituted freeze-drying powder with a cryoprotective agent was small and uniform. Therefore, the freeze-dried powder might be a good choice for intravenously administrating poorly soluble HCPT, which is proved to have higher *in vitro* cytotoxicity against the cancer cells than that of HCPT injections.

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