Formulation of dried lignans nanosuspension with high redispersibility to enhance stability, dissolution, and oral bioavailability

SHEN Gang¹,², CHENG Ling¹,², WANG Li-Qiang³, ZHANG Li-Hong¹, SHEN Bao-De¹, LIAO Wei-Bo¹, LI Juan-Juan¹,², ZHENG Juan¹,², XU Rong¹*, YUAN Hai-Long¹*

¹ Department of Pharmacy, Air Force General Hospital of PLA, Beijing 100142, China; ² Pharmacy College, Chengdu University of Traditional Chinese Medicine, Chengdu 611137, China; ³ School of Biomedical Sciences, Huaqiao University, Quanzhou 362021, China

Available online 20 Oct., 2016

[ABSTRACT] Herpetospermum caudigerum lignans (HTL), one of the potential drugs with anti-hepatitis B virus and hepatoprotective effects, has limited clinical applications because of poor aqueous solubility and low bioavailability. Both herpertione (HPE) and herpetin (HPN) are the most abundant ingredients in HTL and exhibit weak acidity. The purpose of the present study was to produce dried preparations of HTL (composed of HPE and HPN) nanosuspensions (HTL-NS) with high redispersibility using lyophilization technology. The HTL-NS was prepared by utilizing precipitation-combined homogenization technology based on acid-base neutralization reactions, and critical formulation and process parameters affecting the characteristics of HTL-NS were optimized. The resultant products were characterized by particle size analysis, SEM, XRD, stability, solubility, dissolution and in vivo bioavailability. HTL-NS showed near-spherical-shaped morphology and the size was 243 nm with a narrow PDI value of 0.187. The dried preparations with a relatively large particle size of 286 nm and a PDI of 0.215 were achieved by using 4% (W/V) mannitol as cryoprotectants, and had a better stability at 4 or 25 ºC for 2 months, compared to HTL-NS. In the in vitro test, the dried preparations showed markedly increased solubility and dissolution velocity. Besides, in the in vivo evaluation, it exhibited significant increases in AUC₀–t, Cmax, MRT and a decrease in Tmax, compared to the raw drug. In conclusion, our results provide a basis for the development of a drug delivery system for poorly water-soluble ingredients with pH-dependent solubility.

[KEY WORDS] Herpetospermum caudigerum lignans; Nanosuspensions; Acid-base neutralization; Saturation solubility; Dissolution; Oral bioavailability


Introduction

Currently, more than 40% of active pharmaceutical ingredients (API) discovered through different drug discovery program suffer from low oral bioavailability due to poor aqueous solubility [1-2]. Although various formulation strategies in pharmaceutical industry have been developed to address this issue, such as amorphous solid dispersion [3], solubilization [4], salt formation [5], co-solvents [6], inclusion complexation with cyclodextrins [7], liposome [8], and microemulsion [9], they have some drawbacks, including low drug loading efficiency, poor physical stability, high cost, complex manufacturing process, and usage of excipients which may lead to adverse side effects. Hence, there are still increasing requirements for innovative yet universal approaches to improving the bioavailability of poor water-soluble drugs.

Based on Noyes-Whitney equation and Ostwald-Freundlich equation, both dissolution and solubility are size dependent and can be markedly enhanced by reducing particle size [10-11]. Therefore, reducing the particle size by using nanosuspension is a promising strategy in formulating a...

poorly water-soluble drug, and a superior in vivo performance via oral delivery in the form of nanoparticles can be reached through achieving quick onset of action, reducing fed/fasted state variability, and enhancing bioavailability [12]. At present, the methods of preparation for drug nanosuspensions can be basically classified into two categories: mechanical comminution of larger particle (top-down) such as high-pressure homogenization [13] and media milling [14], and precipitating drug particles from a supersaturated solution to form nanoparticles (bottom-up) [15]. The top-down approaches are more frequently used because of its simplicity of process and ease of large-scale production, compared with the bottom-up methods, but have some disadvantages, such as large energy consumption, pharmaceutical contamination, and wide distribution of particle size [11]. The bottom-up methods can make up those shortcomings, but addition of toxic organic solvent still restricts their wider application and further commercialization [16]. In fact, many poorly water-soluble drugs are weakly acidic or basic (e.g., azithromycin, aripiprazolei and ketoprofen), and their solubility exhibit in a pH-dependent equilibrium [17]. Thus, utilizing acid-base neutralization reaction to accomplish a bottom-up process for producing nanosuspensions is possible, and has considerable potential in the pharmaceutical industry because of avoidance of toxic solvents and needs for special manufacturing equipment.

There is an increasing interest in delivery traditional medicines using nanotechnology. Herpetospermum caudigerum, as a folk medicine, has been widely used in Tibet for the treatment of liver diseases, cholic diseases, and dyspepsia [18]. In previous chemical studies of its mature seed, many lignans have been isolated and identified [19-21]. Amongst Herpetospermum caudigerum lignans (HTL) identified thus far, herpetrione (HPE) and herpetin (HPN) (Fig. 1) are the most abundant ingredients with significant anti-hepatitis B virus activity [22]. In the previous pharmacodynamic researches, HPE and HPN have been well proven to protect liver and lower aminotransferase levels against the hepatic dysfunction induced by various chemical hepatotoxins [23-25]. However, pharmacokinetic studies in rats show that the drugs exhibit a low oral bioavailability because of their poor solubility and dissolution [25-26]. So far, there has been no effective formulation reported to simultaneously improve their solubility and bioavailability of HTL. Our previous study has shown that HPE and HPN are both weakly acidic compounds and can be easily dissolved in strongly alkaline solution. Accordingly, it is feasible to utilize bottom-up technology based on acid-base neutralization reaction to prepare coarse HTL nanosuspension (HTL-NS).

The purpose of the present study was to produce dried preparations of HTL-NS with high redispersibility by lyophilization technology. The HTL-NS was prepared by utilizing precipitation-combined homogenization technology based on acid-base neutralization reactions, and the critical factors involved in the preparation process and formulation composition, such as the type and concentration of stabilizers, drug concentration, and homogenization pressure and cycles, were optimized through single-factor investigations. The properties of resultant products were characterized by analyses of particle size and zeta potential, scanning electron microscopy, and X-ray diffraction. The in vitro solubility and dissolution behavior and in vivo bioavailability of dried HTL-NS were also evaluated. Additionally, we developed a suitable method for simultaneous quantification of HPE and HPN (Fig. 1).

Materials and Methods

Materials

Herpetospermum caudigerum lignans (HTL), a mixture of two lignans herpetrione (HPE, the purity was up to 65%) and herpetin (HPN, the purity was up to 30%), were prepared in the laboratory of Dr. YUAN (Air Force General Hospital of PLA, Beijing, China). Sodium dodecyl sulfate (SDS), polyvinylpyrrolidone K30 (PVP K30), Poloxamer 188 (F68), and Tween 80 were obtained from BASF Co., Ltd. (Shanghai, China). Hydroxypropyl methylcellulose (HPMC) and polyvinyl alcohol (PVA) were purchased from Shin-Etsu
Chemical Company, Ltd. (Tokyo, Japan). Lecithin was bought from Huatingmeiheen Co., Ltd. (Beijing, China). Mannitol (analytical grade) was provided by Tianjin Guangcheng Chemical Agent Co., Ltd. (Tianjin, China). HPLC-grade methanol and acetonitrile were purchased from Fisher Scientific (Massachusetts, USA). All other reagents were of analytical grade.

**Preparation of HTL-NS**

The HTL-NS was prepared by precipitation-combined homogenization technique based on acid-base neutralization reaction. Briefly, HTL was dissolved in a 10 mL of NaOH aqueous solution (0.20 mol·L\(^{-1}\)) and then slowly added dropwise into 90 mL of HCl aqueous solution (0.025 mol·L\(^{-1}\)) containing a certain amount of stabilizers (HPMC, Lecithin, PVA, Tween80, PVP K30, F68 and DS) under moderate stirring. A flaskwise suspension spontaneously formed with gradually adding alkaline droplets containing HTL. The freshly prepared suspension was further homogenized at appropriate pressure and cycles using a piston-gap high pressure homogenizer (EmulsiFlex-C3, Avestin Inc., Ottawa, Canada) to produce uniform nanosuspensions with small particle size.

**Lyophilization of HTL-NS**

In order to strengthen physical stability of the freshly prepared nanosuspensions, it was necessary to further transform it into solid powders by lyophilization. Mannitol was selected as a cryoprotectant with different concentrations ranging from 0 to 5% (W/V). HTL-NS was pre-frozen at −80 °C in an ultra-low temperature freezer (DW-86L388, Qingdao Haier Co., Ltd., China) for 24 h, transferred to the lyophilizer (FD5-series, Gold SIM, Beijing, China), and kept at −50 °C in vacuum for 72 h to yield dry powder.

**Preparation of physical mixture**

The physical mixture possessed the same proportions and compositions with optimized nanosuspensions formulation. HTL, SDS, and PVP K30 were mixed in 100 mL of deionized water, triturated in a mortar until a homogenous mixture was obtained, and then transformed into dry powder under optimized lyophilization process and additives (the same with that of optimal HTL-NS).

**Particle size and zeta potential analyses**

The mean particle sizes (MPS) and polydispersity indexes (PDI) were determined by photon correlation spectroscopy (3000SH, Malvern Instruments Ltd., UK). All samples were diluted or reconstituted with deionized water to avoid the multi-scattering phenomenon before measurement, and measured at a scattering angle of 90° at room temperature in triplicate. The zeta potential values (ZP) of the samples were measured by electrophoretic mobility method after diluted with deionized water, using the same instrument.

**Scanning electron microscopy (SEM)**

The surface morphologies of the samples were visualized by scanning electron microscopy (SEM) (S-4800, Hitachi Technologies Corporation, Japan). Prior to analysis, all the mounted samples were coated with gold by a sputter coater and dried under vacuum. The SEM was performed at an excitation voltage of 15 kV.

**X-ray diffraction (XRD) analysis**

The XRD patterns of raw drug, dried preparations, physical mixture, and excipients were analyzed using an X-ray diffractometer (D/MAX-2500PC, Rigaku, Tokyo, Japan), with Cu-K\(_{α}\) radiation generated at 30 mA current and 40 kV voltage. The scans were set at 2°·min\(^{-1}\) from 3° ≤ 2θ ≤ 60° angles.

**Saturation solubility analysis**

Excessive HTL, dried preparations (the optimal formulation) and physical mixture were separately dispersed in 5 mL of HCl solution (pH 1.2) and phosphate buffer saline (pH 7.4) and incubated at 25 °C for 48 h under mild magnetic stirring. The samples were then centrifuged at 8 000 r·min\(^{-1}\) for 10 min to remove the undissolved drug, and the obtained supernatant (1 mL) was assayed by HPLC. The experiment was repeated three times.

**Dissolution analysis**

The dissolution analyses of HTL, dried preparations (the optimal formulation), and physical mixture were conducted according to the Ch.P.2010 Edition Appendix XC Method II (the paddle method) using a dissolution apparatus (RC-8, Tianjin Guoming Medicine and Equipment Co., Inc., Tianjin, China). Accurately weighed samples containing the equivalent of 30 mg of HTL were directly added to 900 mL of dissolution media, which were the simulated gastric fluid without enzymes (HCl solution, pH 1.2) and simulated intestinal fluid without enzymes (phosphate buffer saline, pH 7.4). The temperature of media was maintained at 37 (± 0.5) °C, and the stirring speed was set to 100 r·min\(^{-1}\). The samples (2 mL each) were collected at scheduled times (0, 5, 10, 15, 20, 30, 45, and 60 min) and the media were compensated by adding equal volume of the freshly prepared blank, temperature-equilibrated media immediately. Quantitative analyses of HPE and HPN in the samples were accomplished by HPLC after filtered through 0.45-μm microporous membrane filter. Each dissolution analysis was repeated three times.

**Stability analysis**

The optimized nanosuspensions and dried preparations were separately stored at 4 °C (refrigerator) and room temperature (approximately 25 °C, 60% relative humidity) in capped vials. Their stability was assessed by particle size, polydispersity index, zeta potential, and drug contents after 2 months of storage. Each sample was analyzed in triplicate.

**Cytotoxicity assay**

The cytotoxic effect of dried nanosuspension was tested in HepG2.2.15 cells provided by the Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences (Beijing, China) using the MTT assay. The cells were seeded into 96-well plates at a density 2 × 10\(^{4}\) cells per well and cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% FBS in a humidified atmosphere containing 5% CO\(_2\) at 37 °C. After 24 h of incubation, the cells were treated with different concentrations (62.5, 125,
250, 500, 800, and 1 000 µg·mL\(^{-1}\)) of raw drug or dried nanosuspension in serum-free medium for 72 h. The cells in the control group were treated with medium alone. 20 µL of MTT solution (5 mg·mL\(^{-1}\)) was added to each well and incubation continued at 37 °C for 4 h, followed by addition of 150 µL of DMSO to solubilize the formazan. The absorbance (\(A\)) at 490 nm was measured by using an automatic plate reader (BIO-RAD, 168-1000XC, California, USA). The growth inhibition rate was calculated as follows: inhibitory rate (%) = \((A_{\text{control}} - A_{\text{experimental}}) / A_{\text{control}}\) × 100%.

**In vivo pharmacokinetic studies in rats**

The animal experiments of pharmacokinetic evaluation were conducted in full compliance with the NIH Guidelines for the Care and Use of Laboratory Animals, and followed protocol approved by 302 Military Hospital of China Institutional Animal Care and Use Committee with approval number DWFL-2014-D042. Male Wistar rats (weighing 200 g ± 20 g) were obtained from Academy of Military Medical (Beijing, China). These rats were randomly divided into two groups (\(n = 8\)) and housed under standard laboratory conditions (22 °C ± 2 °C, 60% ± 5% relative humidity) for at least 3 days before experiment. The rats were fasted for 12 h with free access to water before the experiment. Two formulations of HTL were orally administrated to the two groups at a dose equivalent to 375 mg·kg\(^{-1}\) body weight of HTL. The nanosuspensions of optimized dried preparations were reconstituted by adding appropriate distilled water and the coarse HTL suspensions (HTL-CS) were prepared by adding the same proportion of excipients with the optimal nanosuspensions formulation to CMC-Na aqueous solution (1%, \(W/V\)) and dispersed uniformly in an ultrasonic bath for 15 min. Blood samples (0.5 mL) of each rat were collected from retro-orbital puncture into heparinized centrifugation tubes at 0 (predose), 0.083, 0.25, 0.5, 0.75, 1, 2, 3, 4, 6, 8, 10, and 12 h after administration and were then centrifuged at 8 000 r·min\(^{-1}\) for 10 min to separate the plasma that was stored at −20 °C until analysis. The plasma samples were treated by deproteinization method. Briefly, the plasma samples (200 µL) were extracted with absolute methanol (400 µL) on a vortex mixer for 2 min. After centrifugation at 3 000 r·min\(^{-1}\) for 10 min, the obtained supernatant was filtered through a 0.45-µm membrane filter and directly injected onto HPLC column for drug analysis.

### HPLC analysis

The contents of HPE and HPN in dissolution media and plasma samples were determined by the same HPLC system that was a prominence LC system (Shimadzu Corporation, Kyoto, Japan) equipped with a quaternary pump, a degassing unit, a diode array detector, and a CBM-20A Chemstation Data system. Chromatographic separation was performed on an Alltima C\(_{18}\) column (4.6 mm × 250 mm, 5 µm, Alltech) protected by an Alltima C\(_{18}\) guard column (4 mm × 20 mm, 5 µm, Alltech). The mobile phase was consisted of acetonitrile and 2% (\(V/V\)) glacial acetic acid aqueous solution (24 : 76, \(V/V\)); the flow rate was set at 1.0 mL·min\(^{-1}\); the column temperate was set at room temperature (25 °C). The injection volume was 10 µL and the detection wavelength was set at 280 nm. The linearity ranges were 0.8–213.0 and 1.2–204.0 µg·mL\(^{-1}\) for HPE and HPN analyses, respectively. The regression equation of the calibration curves were \(Y_{\text{HPE}} = 14493X_{\text{HPE}} - 24290\) and \(Y_{\text{HPN}} = 5024.7X_{\text{HPN}} + 15231\) (where \(Y\) is the peak area and \(X\) is the concentration in µg mL\(^{-1}\), \(R_{\text{HPE}} = 0.9995\), \(R_{\text{HPN}} = 0.9993\), \(n = 7\), respectively).

### Data analysis

The main pharmacokinetic parameters, including peak plasma concentration (\(C_{\text{peak}}\)), the time to reach maximum concentration (\(T_{\text{max}}\)), the area under the plasma concentration-time curve (\(AUC\)), and the mean residence time (\(MRT\)), were analyzed by DAS® 2.0 software (Boying Corporation, Guangzhou, China). The results were expressed as means ± standard deviations (SDs). Analysis of variance (ANOVA) was performed to demonstrate statistical differences between different experimental groups using the SPSS 17.0 software (Chicago, IL, USA). A P value less than 0.05 was considered statistically significant, while less than 0.01 was highly significant.

## Results and Discussion

### Screening of stabilizers

In initial screening trials, seven stabilizers, including HPMC, Lecithin, PVA, Tween80, PVP K30, F68, and SDS, were used to prepare the nanosuspensions at fixed concentration of 2 mg·mL\(^{-1}\) in acidic solution. The MPS of all the products were in nanometer range, and the use of HPMC yielded nanosuspensions with smallest MPS (457 nm; Table 1). However, these samples precipitated and

### Table 1 The Particle size, PDI and macroscopic appearance after 2.5 h of HTL-NS stabilization with different stabilizers

<table>
<thead>
<tr>
<th>Stabilizer (s)</th>
<th>Particle size (nm)</th>
<th>PDI</th>
<th>Macroscopic appearance after 2.5 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPMC</td>
<td>457.5 ± 33.7</td>
<td>0.392 ± 0.072</td>
<td>Fluffy precipitate</td>
</tr>
<tr>
<td>F68</td>
<td>647.3 ± 43.5</td>
<td>0.375 ± 0.086</td>
<td>Aggregate and precipitate</td>
</tr>
<tr>
<td>PVA</td>
<td>679.3 ± 36.1</td>
<td>0.287 ± 0.051</td>
<td>Aggregate</td>
</tr>
<tr>
<td>PVP K30</td>
<td>500.4 ± 48.6</td>
<td>0.270 ± 0.063</td>
<td>Colloidal dispersion</td>
</tr>
<tr>
<td>SDS</td>
<td>625.7 ± 35.7</td>
<td>0.302 ± 0.058</td>
<td>Aggregate and precipitate</td>
</tr>
<tr>
<td>Tween 80</td>
<td>711.9 ± 27.8</td>
<td>0.414 ± 0.091</td>
<td>Precipitate</td>
</tr>
<tr>
<td>Lecithin</td>
<td>896.2 ± 47.3</td>
<td>0.562 ± 0.121</td>
<td>Precipitate</td>
</tr>
</tbody>
</table>
aggregated to different degrees within 2.5 h except for PVP K30. Polymeric stabilizers (HPMC, F68, PVA, and PVP K30), with exception of PVP K30, could not effectively surround the nanoparticles surface due to low affinity for drug, thus, resulting in particles precipitated and aggregated. Surfactant stabilizers, out of providing electrostatic or steric hindrance, had solubilization properties. The solubility enhancement of drug would appear the time-dependent Oswald ripening phenomenon and enlarge particle size [27]. In contrast to other stabilizers, PVP K30 could solely produce stable colloidal dispersion with relatively small MPS (500 nm) and smallest PDI (0.270) (Table 1). This strong drug-polymer interaction originated from hydrogen bond formation between the abundant hydroxyl groups of HTL and the nitrogen atom of PVP K30.

It has been reported that combination with PVP K30 and SDS could form a more condensed polymer-surfactant binary protective layer relied on electrostatic attraction [28]. We evaluated whether a greater extent of particle size reduction could be achieved by PVP-SDS binary system with different ratios under fixed PVP K30 concentration (2 mg·mL$^{-1}$). As shown in Fig. 2, when the volume ratio of PVP K30 to SDS changed from 1 : 0.1 to 1 : 0.2, the change in MPS was not obvious, compared with that of nanosuspensions containing PVP K30 alone; it was most likely that the surface adsorption of two types of stabilizers occurred independently at low SDS concentration and failed to form PVP-SDS binary system [29]. However, with further decreasing the ratios from 1 : 0.2 to 1 : 0.6, the MPS and PDI abruptly reduced, indicating that electrostatic interaction started to take place and gradually strengthened with increase of SDS concentration [30]. Continuing to increase the concentration of SDS, the MPS and PDI slightly changed. As a result, PVP K30 and SDS were combined as stabilizers at ratio of 1 : 0.6 in subsequent experiments.

Optimization of HTL-NS preparation

The effects of concentration of HTL in alkaline solution and concentration of stabilizers in acidic solution on the MPS and PDI values of HTL-NS are shown in Figs. 3A and 3B, when fixed homogenization pressure and cycles were set at 1 000 bar.
and 15 cycles, respectively. When the concentration of HTL changed within 5 to 30 mg·mL$^{-1}$, the MPS firstly decreased from 325 to 264 nm, and then gradually increased at concentrations higher than 15 mg·mL$^{-1}$. The phenomenon was mainly induced by the HTL supersaturation. In order to produce tiny and uniform particles, high supersaturation was needed to accelerate nucleation rate greater than the rate of crystal growth, according to classical crystallization theory [31]. However, when the drug concentration was excessive, a large number of nuclei apace formed on the interface of solid phase and liquid phase and slowed the diffusion from solvent to antisolvent, leading to particles adhesion, reunion, and larger particles generation [32]. Besides, the viscosity of drug solution would increase with enhancement of drug concentration and further prevent particle diffusion [16].

The effects of stabilizer concentration on MPS and PDI were similar to that of drug (Fig. 3B). The MPS and PDI values were firstly decreased and then increased with the increase of stabilizer concentration. Actually, the stabilizers affected MPS in a paradoxical manner. If the stabilizer concentration was too low, it would fail to fully cover on the particle surface, leading to growth of particle size. Within a certain range, the higher the concentration of the stabilizer, the more stabilizer molecules adsorbed onto the particle surface. However, the adsorption would reach equilibrium due to the limited surface area [33]. Therefore, 4 mg·mL$^{-1}$ was selected as optimum concentration of stabilizer in subsequent experiments.

After fixing the concentrations of HTL and stabilizers at 15 and 4 mg·mL$^{-1}$, respectively, the effects of different homogenization pressure and cycles on MPS were investigated and the results are displayed in Figs. 3C and 3D. The MPS was initially reduced with the increasing homogenization pressure and cycles to 1 000 bar and 15 cycles, but the continuous increase might damage the stabilizer layer, so that the MPS gradually enlarged. In fact, low energy homogenization was suitable for breaking up large aggregation, resulting in more transfer units than that of high energy homogenization; the more transfer units existed in the homogenizing system, the greater erosion of external surface of the aggregated particle would be [34]. But further interaction between individual particles of smaller scale would not occur during this process with low energy homogenization. Besides, low energy input could be insufficient to promote stabilizers fully cover fast forming particles, and as a result, the growth and aggregation could occur. Not only could high power applied in the procedure form high speed micro-jets and high pressure shock waves to successfully produce particles with smaller scale, but also intensify external mass transfer and absorption rate of stabilizer for preventing the particle aggregation. These results indicated that particles with smaller size and narrower distribution would be obtained through the combination of different pressures and cycles. Therefore, the selected process parameters were firstly 5 cycles at 600 bar as pre-homogenizing step and then 15 cycles at 1 000 bar, for the preparation of optimal HTL-NS.

**Lyophilization of HTL-NS**

During the lyophilization process, crystallization of ice exerts mechanical stress on the nanoparticles in the cryo-concentrated solution, which can further destabilize the nanosuspension due to aggregation [35]. Therefore, addition of the appropriate water-soluble cryoprotectants can form hydrophilic exipient bridges interconnecting the nanoparticles, which would strongly affect nanaggregate binding force, permitting recovery of the original particle size after reconstitution [36]. The protective effects of mannitol at different concentrations on the redispersed MPS and PDI values are shown in Fig. 4. It was evident that the MPS of formulation without mannitol increased to 562 nm, more than 2 times larger than that of the original nanosuspension (243 nm) before drying. That meant an irreversible aggregation occurred during the lyophilization process, so that the dried powder of the nanoparticles was unable to redisperse into the original particle size. However, the presence of mannitol apparently reduced redispersed MPS and PDI values. The formulation with 5% (W/V) mannitol yielded the smallest particle size (270 nm), but 4% was finally chosen because there was no significant statistical difference in MPS between the two concentrations ($P > 0.05$).

**Fig. 4** The effects of different mannitol concentrations on the particle size and PDI values of reconstituted nanosuspension in distilled water (*$P < 0.05$, **$P < 0.01$)

**Characterization of dried HTL nanosuspensions**

**Morphology and particle size**

From the SEM image, the raw drug was composed of irregular fragment structure with various sizes, ranging from 1 to 100 μm (Fig. 5A), but MPS of the freshly optimized nanosuspension sharply reduced to 243 nm with PDI value of 0.187, indicating the potential improvement of bioavailability of the drug. After freeze-drying, great adhesion and aggregation of individual near-spherical-shaped particles occurred in dried powders without mannitol (Figs. 5B and 5C), eventually causing larger particle size (478 nm). Because of presence of mannitol, a large proportion of particles were split into a single
by the rod-like mannitol (Figs. 5D and 5E), and redispersed particle size only appeared slight change, compared with that of original nanosuspension. Strangely, no matter with mannitol or not, the zeta potential values before and after freeze-drying showed no significant changes and were all less than -20 mV, since zeta potential was governed by both stabilizers and drug that were hardly affected during the freeze-drying process[37]. These results implied that the freeze-drying process using mannitol as cryoprotectant was an effective and feasible means to solidify HTL nanosuspensions.

XRD characterization

X-ray diffraction analysis was performed to determine the crystalline structure of the original drug and nanoparticles (Fig. 6). For raw drug (Fig. 6h), broad, diffuse haloes appeared with an absence of the characteristic crystalline peaks, indicating that HTL was in a highly amorphous state. However, the crystallinity diffraction peaks were clearly observed in dried preparations (Figs. 6f and 6g) that might be due to the existence of SDS or mannitol, since the peaks position were superimposable to that of SDS or mannitol (Figs. 6b and 6c). Accordingly, it could be concluded that HTL exhibited the desired amorphous form in dried preparations with mannitol. The number and intensity of diffraction peaks of the freeze-dried preparations were less than that of the physical mixture (Fig. 6e). This phenomenon might be induced by particle size reduction and cladding reaction of excipients[38]. In addition, it was not difficult to see that no significantly characteristic peaks of sodium chloride (NaCl) deriving from the acid-base neutralization reaction were observed from the diffractogram, which might be because of the integrated influence of HTL and excipients with less content.

Saturation solubility

The enhanced saturation solubility would be capable of achieving equal or better therapeutic effects with much smaller dosage. Both HPE and HPN from the same formulations exhibited the similar saturation solubility tendency in two media (Table 2). It is sufficient to draw the conclusion that reducing particle size could effectively facilitate the drug to dissolve with improved solubility, which was in accordance with the Ostwald-Freundlich equation[11]. However, considerable enhancement of physical mixture was attributed to solubilization and wetting action of the surfactants (SDS). HTL is weakly acidic in nature, so its solubility increases with an increase in pH value. This was illustrated by the fact that the solubility of all the samples at pH 1.2 was lower than that at pH 7.4.

Table 2  Saturation solubility of raw drug, physical mixture and dried preparations in different medium (µg·mL⁻¹) (mean ± SD, n = 3)

<table>
<thead>
<tr>
<th>Formulation</th>
<th>pH 1.2</th>
<th></th>
<th>pH 7.4</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HPE</td>
<td>HPN</td>
<td>HPE</td>
<td>HPN</td>
</tr>
<tr>
<td>Raw drug</td>
<td>22.6 ± 1.57</td>
<td>25.5 ± 2.23</td>
<td>56.3 ± 2.65</td>
<td>51.8 ± 4.43</td>
</tr>
<tr>
<td>Physical mixture</td>
<td>32.5 ± 2.12</td>
<td>51.2 ± 3.03</td>
<td>94.6 ± 4.4</td>
<td>74.7 ± 2.75</td>
</tr>
<tr>
<td>Dried preparations</td>
<td>84.3 ± 3.14</td>
<td>103.6 ± 1.95</td>
<td>253.5 ± 3.33</td>
<td>221.2 ± 2.06</td>
</tr>
</tbody>
</table>
**Dissolution properties**

The saturation solubility of HPE and HPN reached 22.6 and 25.5 µg·mL⁻¹ in HCl solution (pH 1.2), respectively, and 253.5 and 221.2 µg·mL⁻¹ in PBS (pH 7.4), respectively, So 900 mL of medium was adequate to achieve the sink condition for samples equivalent to 30 mg HTL. As displayed in Fig. 7, HPE and HPN in different formulations displayed the similar dissolution profiles. The HPE and HPN from dried preparations in two dissolved environments achieved the highest cumulative dissolution rate (about 60% in pH 1.2 and 90% in pH 7.4) at the shortest time (15–20 min). This phenomenon might be caused by enlargement in the surface area, improvement in saturation solubility, and decrease in the diffusion layer thickness according to the Noyes-Whitney equation [39]. Besides, the dissolution of physical mixture was appreciably higher than that of raw drug in all the test solutions because of presence of SDS.

![Dissolution profiles of Raw drug, Physical mixture, and Dried preparations in different media: (A) HPE dissolution profiles in pH 1.2 HCl solution, (B) HPN dissolution profiles in pH 1.2 HCl solution, (C) HPE dissolution profiles in pH 7.4 phosphate buffer, and (D) HPN dissolution profiles in pH 7.4 phosphate buffer (n = 3)](image)

The order of dissolution rate for all the tested formulations was kept the same in two solutions, but the dissolution degree in PBS was higher than that in HCl solution (Fig. 7). It was explained that obtained saturation solubility of each formulation in PBS was much higher than that in HCl solution (Table 2). It is easy to see that the dissolution rate of dried preparations reached higher degree in both two dissolution media, which, from an in vivo point of view, implied that the dried preparations would be less affected by pH environment variation in the gastrointestinal tract, well dispersed in gastric fluid, and then individually transported to the upper intestine to reach complete dissolution, resulting in an improved bioavailability.

**Stability**

From the appearance of optimized nanosuspension, a loose, thin layer of flavescant sediment appeared at bottom of the glass vials after 2 months of storage at 25 and 4 ºC, and was difficultly dissolved through slight manual shaking alone, inducing a significant increase of particle size and PDI (Fig. 8), when compared to those at an initial time. The phenomenon could be explained by Ostwald ripening, in which small particles in the suspensions would dissolve and molecules were re-deposited to larger particles [40]. From another point of view, nanoparticles were in the amorphous state and existed a recrystallization risk. In a word, the nanosuspension was unsuitable for long-term storage. About dried preparations, no evident changes were observed in particle size and PDI during the stability study (Fig. 8). In contrast to initial time, the zeta potential and contents of HPE and HPN in two formulations were almost unchanged under both storage conditions (data not shown). In order to possess long-term stability, it was necessary to transform nanosuspensions formulation into freeze-dried preparations, avoiding Ostwald ripening and crystalline transformation.
Cytotoxicity

Cytotoxicity of dried nanosuspension was evaluated in HepG2.2.15 cells, compared with that of raw drug. As shown in Fig. 9, the cytotoxic effects of the dried nanosuspension and raw drug increased in parallel with drug concentration. Dried nanosuspension exhibited significantly higher inhibition rates than raw drug, which might be induced by improved cell uptake of drugs following nanolization and presence of SDS which is toxic to cells [41].

In vivo pharmacokinetic properties in rats

To investigate whether the optimized dried nanosuspension could enhance the oral bioavailability of HTL, an in vivo test experiment was executed in rats by gavage administration at a dose of 375 mg·kg\(^{-1}\). Compared with the coarse suspensions, the plasma concentration profiles of HPE and HPN from the nanosuspensions were distinctly improved (Fig. 10), and the main pharmacokinetic parameters of the nanosuspensions showed markedly different from that of the coarse suspensions (Table 3). The mean values of the maximum concentrations of HPE and HPN from the oral nanosuspensions were 12.557 (± 0.412) and

\[ C_{\text{max}} \]

Fig. 8  Mean particle size (MPS) and PDI values of optimized nanosuspension and dried preparations after 7 weeks of storage at 25 °C (A) and 4 °C (B) (n = 3)

Fig. 9  Cytotoxicity of dried nanosuspension and raw drug in HepG2.2.15 cells. Results are expressed as means ± SDs (n = 6). *P < 0.05, **P < 0.01 vs control group; #P < 0.05, ##P < 0.01 vs the same dose of raw drug
Fig. 10  Mean plasma concentration-time profiles of HTL in reconstituted nanosuspension (HTL-NS) and coarse suspension (HTL-CS) after oral administration of a single dose of 375 mg·kg\(^{-1}\) HTL in rats (n = 8)

7.657 (± 0.346) µg·mL\(^{-1}\), respectively, which were 2.35-fold (5.345 ± 0.255 µg·mL\(^{-1}\)) and 1.86-fold (4.122 ± 0.190 µg·mL\(^{-1}\)) higher than that of the coarse suspensions. Meanwhile, the \textit{AUC}\(_{0-t}\) values increase of HPE and HPN in nanosuspensions were 2.14-fold (42.502 ± 1.049 vs 19.857 ± 0.616 µg·h·mL\(^{-1}\)) and 1.90-fold (27.225 ± 1.258 vs 14.319 ± 1.356 µg·h·mL\(^{-1}\)), respectively, compared to the coarse suspensions. The \textit{T}\(_{\text{max}}\) value was almost 1 h after oral administration of the coarse suspensions, but shortened to 0.5 and 0.75 h for HPE and HPN in nanosuspensions, respectively. These results proved that the bioavailability of the reconstituted nanosuspensions was better than that of the coarse suspensions.

For orally administrated BSC II drugs, dissolution is deemed as a critical rate decision step for absorption. In contrast to raw drug, above 50-fold reduction of particle size of the dried preparations could induce a significant increase of dissolution (Fig. 10). This meant that nanoparticles could be easily dispersed to form a comparatively high concentration gradient in the gastrointestinal tract within a short time for facilitated drug absorption. Moreover, obvious increases in adhesive property and the direct contact area between nanoparticles and intestinal epithelium of villi would promote drug molecules continuously to release from nanoparticles, leading to larger drug concentrations for adsorption \[42\], which was demonstrated by MRT extension of dried preparations in comparison with that of coarse suspension (Table 3). Most importantly, nanoparticles could directly cross the gastrointestinal membrane via endocytosis, gain access into the mesenteric lymphatic systems, and enter the blood circulation directly \[43\].

### Table 3  Main values of pharmacokinetic parameters of HTL after oral administration of reconstituted nanosuspensions and coarse suspensions in rats (n = 8)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Reconstituted nanosuspension</th>
<th>Coarse suspension</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HPE</td>
<td>HPN</td>
</tr>
<tr>
<td>\textit{T}(_{\text{max}}) (h)</td>
<td>0.50 ± 0.15(^a)</td>
<td>0.75 ± 0.11(^a)</td>
</tr>
<tr>
<td>\textit{C}(_{\text{max}}) (µg·mL(^{-1}))</td>
<td>12.557 ± 0.412(^a)</td>
<td>7.657 ± 0.346(^a)</td>
</tr>
<tr>
<td>\textit{AUC}(_{0-t}) (µg·h·mL(^{-1}))</td>
<td>42.502 ± 1.049(^a)</td>
<td>27.225 ± 1.258(^a)</td>
</tr>
<tr>
<td>\textit{MRT}(_{0-t}) (h)</td>
<td>3.127 ± 0.066(^a)</td>
<td>2.981 ± 0.140(^a)</td>
</tr>
</tbody>
</table>

\(^a\) \textit{P} < 0.05; \(^b\) \textit{P} < 0.01 vs the corresponding parameters of coarse suspension

### Conclusion

In the present study, we utilized an acid-base neutralization reaction in aqueous solution to prepare coarse HTL-NS. This strategy was a simple process and avoided the use of toxic organic solvents and specialized equipment. The rapid decrease in the solubility of HTL induced by HCl solution resulted in the nanoscale aggregation of particles in the presence of stabilizers (PVP K30 and SDS) with stabilizing and growth inhibition effects. Then, the coarse HTL-NS was changed into uniform nanoparticles with smaller particle size and PDI through high-pressure homogenization. In order to improve its stability, it was necessary to transform HTL-NS into dried preparations with high redispersibility using lyophilization and mannitol as matrix former. The optimal conditions were as follows: PVP K30 to SDS volume ratio of 1 : 0.6, 4 mg·mL\(^{-1}\) of total stabilizers concentration in acidic solution, 15 mg·mL\(^{-1}\) of drug concentration in alkaline solution, homogenization process of 600 bar for the first 5 circles and 1 000 bar for the next 15 circles, and mannitol concentration of 4\% (\textit{W}/\textit{V}). With these optimized process and formulation parameters, dried preparations possessed smaller particle size of 286 nm and had no significant change during 2 months of storage at 4 or 25 °C. The \textit{in vitro} test showed that the dried preparations exhibited significantly increased solubility and dissolution compared to the raw drug, which were the main factors contributing to enhanced oral bioavailability. These positive results demonstrated that this dried drug nanosuspension formulation prepared by precipitation-combined homogenization technology based on acid-base neutralization reactions might be a promising universal method for preparing nanoparticles for the enhancement of oral bioavailability of poorly water-soluble ingredients with pH-dependent solubility in pharmaceutical and chemical industries.
Abbreviations

<table>
<thead>
<tr>
<th>Full name</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Herpetospermum caudigerum lignans</td>
<td>HLT</td>
</tr>
<tr>
<td>Herpetrine</td>
<td>HPE</td>
</tr>
<tr>
<td>Herpetin</td>
<td>HPN</td>
</tr>
<tr>
<td>Nanosuspension</td>
<td>NS</td>
</tr>
<tr>
<td>Coarse suspension</td>
<td>CS</td>
</tr>
<tr>
<td>Scanning electron microscopy</td>
<td>SEM</td>
</tr>
<tr>
<td>X-ray diffraction</td>
<td>XRD</td>
</tr>
<tr>
<td>Mean particle size</td>
<td>MPS</td>
</tr>
<tr>
<td>Polydispersity indexes</td>
<td>PDI</td>
</tr>
<tr>
<td>Hydroxypropyl methylcellulose</td>
<td>HPMC</td>
</tr>
<tr>
<td>Polyvinyl alcohol</td>
<td>PVA</td>
</tr>
<tr>
<td>Poloxamer 188</td>
<td>F68</td>
</tr>
<tr>
<td>Polivinylpyrrolidone K30</td>
<td>PVP K30</td>
</tr>
<tr>
<td>Sodium dodecyl sulfate</td>
<td>SDS</td>
</tr>
</tbody>
</table>

References


[29] Öztekin N, Erüm FB. Determination of critical aggregation concentration in the poly-(vinylpyrrolidone)–sodium dodecyl sulfate system by capillary electrophoresis [J]. J Surf Deter,


Cite this article as: SHEN Gang, CHENG Ling, WANG Li-Qiang, ZHANG Li-Hong, SHEN Bao-De, LIAO Wei-Bo, LI Juan-Juan, ZHENG Juan, XU Rong, YUAN Hai-Long. Formulation of dried lignans nanosuspension with high redispersibility to enhance stability, dissolution, and oral bioavailability [J]. Chin J Nat Med, 2016, 14(10): 757-768.