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Oleanolic acid nanosuspensions: preparation, in-vitro characterization and enhanced hepatoprotective effect

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Abstract

Oleanolic acid is a naturally derived triterpene used clinically in the treatment of hepatitis in China, but its poor solubility often leads to poor bioavailability. In the present study, oleanolic acid nanosuspensions were prepared by the nanoprecipitation method and then systematically characterized. The average particle size of the obtained nanosuspensions was 284.9 nm, with a polydispersity index of 0.216. Transmission electron microscopy and atomic force microscopy showed that the drug existed as spherical or near-spherical nanoparticles in the nanosuspensions. Differential scanning calorimetry and X-ray diffraction studies indicated that oleanolic acid was present in an amorphous state in the lyophilized nanosuspensions. At 25°C, the saturation solubility of oleanolic acid was increased by about 6 times after nanoation (25.72 μg mL^{-1} vs 4.37 μg mL^{-1}). In the in-vitro drug release experiments, the lyophilized nanosuspensions showed a faster drug dissolution rate than that of the coarse drug powder (approx. 90% vs 15% during the first 20 min), and nearly 95% of the oleanolic acid was released by 120 min. As evidenced by the lower serum alanine aminotransferase activity and liver malondiadehyde content, pre-treatment with oleanolic acid nanosuspensions significantly enhanced the hepatoprotective effect of oleanolic acid against carbon tetrachloride-induced liver injury.

Introduction

Oleanolic acid, a naturally derived triterpene, is found in many traditional Chinese medicines. Its many pharmacological activities include hepatoprotective, anti-tumour, antibacterial, anti-inflammatory and antiulcer effects. In China, oleanolic acid is commonly used in the treatment of hepatitis (Wang & Jiang 1992; Liu 1995). Because of its poor solubility in water, the oral administration of oleanolic acid often results in erratic pharmacological activity. Various formulations for the oral delivery of oleanolic acid have been used to improve its bioavailability. However, commonly used approaches such as solid dispersions and cyclodextrin inclusions do not give satisfactory results (Yan et al 1995; Xiang et al 2002; Guo et al 2003; Ruanshi & Zhao 2003).

Enhancing the absorption of poorly water-soluble drugs is a real challenge for pharmaceutical research, with the aim of improving drug therapeutic effectiveness as well as creating new market opportunities. Nanonization is a promising formulation approach for poorly water-soluble drugs and could have numerous advantages, such as increased saturation, solubility and drug dissolution velocity, improved bioavailability and dose proportionality, reduced fed/fasted variability, and inter-subject variability, compared with the coarse or micronized drug powder (Liversidge & Cundy 1995; Liversidge et al 2003; Müller et al 2001).

There are various approaches used to generate nanometer-sized drug particles: nanosuspensions, nanocrystals or dispersion of nanoparticles (Liversidge et al 1991; Fessi et al 1992; Müller et al 1998; Rogers et al 2001; Patravale et al 2004). Nanoprecipitation can be carried out in laboratories without expensive apparatus, offering reproducible submicronial particle size with a narrow distribution. Most of the other techniques require special equipment, such as a high-pressure homogenizer, pearl mill, high-shear media mill, or supercritical fluid extraction device, which may restrict their wider application.

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In this study, oleanolic acid nanosuspensions were prepared using the nanoprecipitation method and systematically characterized by means of photon correlation spectroscopy (PCS), Zeta potential, differential scanning calorimetry (DSC), transmission electron microscopy (TEM), atomic force microscopy (AFM) and X-ray diffraction (XRD). In addition, drug saturation solubility and drug dissolution tests were performed to give a more detailed account of the nanoparticulate drug delivery system. The hepatoprotective effect of the oleanolic acid nanosuspensions was also investigated.

Materials and Methods

Materials

Oleanolic acid (>95%, HPLC) was a kind gift from Professor Dingxian Han (Department of Chemistry, Huazhong University of Science and Technology). Tween 80 was purchased from Wuhan Jiangbei Chemical Factory (Wuhan, China). Acetonitrile was HPLC grade. Assay kits for serum alanine aminotransferase (ALT) and liver malondiadehyde (MDA) content were purchased from Nianjing Jiancheng Bioengineering Institute (Nanjing, China). All other reagents were of analytical grade and unless otherwise noted were used without further purification.

Nanoprecipitation method for oleanolic acid nanosuspensions

Oleanolic acid (100 mg) was dissolved in 50 mL of ethanol (95%). The organic phase was added at 6–8 mL min^{-1} under moderate stirring into an aqueous phase prepared by dissolving 100 mg of Tween 80 in 40 mL bidistilled water at room temperature. The organic solvent was then removed by rotary evaporation under reduced pressure at a temperature below 40°C. The volume of the resulting dispersion was adjusted to 20 mL. Aggregates were excluded by filtration through 0.8-μm hydrophilic syringe filters. Freeze-drying was performed on a Freezone Freeze Dry System (Labconco Corp., Kansas City, MO) for 72 h, with the temperature between −40°C and 50°C, pressure 50–80 × 10^{-3} mBar. No excipients were added.

Particle size analysis and Zeta potential

The particle size, polydispersity index, and Zeta potential measurements were performed on a Nano-zs90 (Malvern Instruments) thermostated at 25°C. The sample was diluted 20 times with bidistilled water before the measurements. All values were measured at an analysis angle of 90° in a 10-mm diameter cell. Each value reported is the average of three measurements.

TEM imaging

A drop of the diluted nanosuspensions containing 0.01% (w/v) phosphotungstic acid was deposited on a Cu grid coated with carbon film, and air dried at room temperature. The TEM imaging of the particle size morphology was carried out on a JEM-100CX II (JEOL Ltd., Tokyo, Japan).

AFM imaging

Samples for AFM imaging were prepared by depositing 10 μL of the diluted oleanolic acid (80 times) nanosuspensions onto a freshly cleaved mica plate, followed by drying with hot air at 60°C. Imaging was performed on a SPA400 atomic force microscope (Seiko Instruments Inc., Matsudo, Chiba, Japan) using a linearized 100-μm scanner in tapping mode and a 139-kHz pyramidal cantilever with silicon probes having force constants of 17 N m^{-1}. The scanning speed was set at 2 Hz.

DSC

DSC of the lyophilized oleanolic acid nanosuspensions and the coarse oleanolic acid crystalline powder was carried out on a DSC-7 (Perkin-Elmer, Boston, MA). An empty aluminium pan was used as a reference. Scans were recorded at a heating and cooling rate of 10 K min^{-1}.

XRD

The X-ray analysis of the lyophilized oleanolic acid nanosuspensions and the coarse oleanolic acid crystalline powder were conducted on a D/MAX III B powder diffractometer (Rigaku Corp., Tokyo, Japan). A Cu Ka radiation source was used. The scanning rate (2θ min^{-1}) was set at 10°C min^{-1}.

HPLC determination of oleanolic acid

HPLC was performed on an Agilent 1100 series (Palo Alto, CA) equipped with an automatic sampling system and a variable wavelength UV detector, with the detection wavelength set at 215 nm. Chromatographic separation was carried out with a Hypersil column (ODS C18, 5 μm, 25 cm × 4.6 mm; Dalian Elite Analytical Instruments, Dalian, China) eluted at a rate of 0.8 mL min^{-1} with a solvent of acetonitrile/1% aqueous H_3PO_4 (9:1, v/v) at 25°C. Tween 80 did not have any effect on the separation of oleanolic acid.

Standard solutions of oleanolic acid were prepared by diluting the appropriate volume of stock solution of oleanolic acid in methanol (1 mg mL^{-1}) with methanol to give a final concentration of 1, 2, 4, 8, 16, 32 and 64 μg mL^{-1}. Then, 20 μL of the standard solutions was injected and run for the calibration curve. The regression equation of the plotted calibration curve was $y = 55.7961x - 102.87$, with a determination coefficient of 0.9956. The precision and reproducibility of the HPLC method were satisfactory, with intraday and interday relative standard deviations of <3%. The relative standard deviation of recovery was 2.1%.

Saturation solubility test

Excessive lyophilized nanosuspensions (0.5 g) and coarse oleanolic acid powder (0.5 g) were added to 10 mL of bidistilled water at 25°C. To determine the solubilization effect of Tween 80 on the solubility of oleanolic acid, 0.5 g coarse powder was added to 10 mL of 2% Tween 80 dissolved

bidistilled water. Each system was kept at 25°C under stirring for 4 consecutive days to ensure saturation. After the equilibrium solubility was attained, the saturated solutions were immediately and rapidly filtered through 0.22-μm membranes. Then, 2 mL of the filtered samples was dried at 60°C under N_2 flow. After drying, the residue was dissolved in 2 mL methanol for HPLC determination.

Drug dissolution test

Dissolution studies were carried out in a ZRS-8G drug dissolution apparatus (Tianjin University Radio Factory, Tianjin, China) using the Chinese Pharmacopoeia 2000 paddle method. Coarse drug powder and lyophilized nanosuspensions equivalent to 20 mg oleanolic acid were transferred into 900 mL of distilled water stirred at 100 rev min^{-1} at 37°C. At predetermined time intervals, 3-mL samples were withdrawn, filtered through a 0.22-μm Millipore filter and immediately replaced with an equal volume of fresh dissolution medium. Then, 2 mL of the filtered samples was treated as described above for the saturation solubility test.

Hepatoprotection study

Animal tests were performed according to Good Clinical Practice. Adult male Kunming mice (25–30 g) were obtained from the Experimental Animal Center of Tongji Medical College (Wuhan, China). They were allowed free access to food and water, and were maintained on a 12-h light/dark cycle at a temperature of approximately 22°C and 50% relative humidity. The mice were randomly divided into groups of 10 animals each. The normal control group was treated orally with saline. In the treated groups, mice were pre-treated orally with coarse oleanolic acid suspension or oleanolic acid nanosuspensions at the same daily doses of 100 mg kg^{-1} for 2 consecutive days. At 24 h after the final dose, mice in the carbon tetrachloride (CCl_4) groups were injected subcutaneously with CCl_4 at a dose of 0.01 mL kg^{-1}. At 24 h after the CCl_4 challenge, blood was removed by cardiac puncture to determine the serum ALT activity; hepatic tissue homogenate was prepared by homogenizing 0.6 g of hepatic tissue in 6 mL ice-cold homogenizing buffer to measure lipid peroxidation by the formation of the thiobarbituric acid reactive material, MDA.

Statistical analysis

Differences between treatment groups were compared using the Mann–Whitney *U*-test (SPSS version 11 software, SPSS Inc., Chicago). The Mann–Whitney *U*-test was selected because the relatively small group sizes did not allow for parametric assumptions of normality. Statistical significance was set at a level of $P < 0.05$.

Results and Discussion

Particle size and morphology

Nanoparticles were spherical or near spherical in shape. No free drug crystals were detectable. The nanoparticles characterized by PCS had an average particle size of 284.9 nm, with a polydispersity index of 0.216; the Zeta potential was -27.6 ± 7.2 mV. The nanoparticle size observed by TEM (Figure 1) and AFM (Figure 2) correlated well with that given by PCS. At 90 days later, the particle size was 298.0 nm, with a polydispersity index of 0.224.

The nanoprecipitation method can achieve reproducible submicron-sized particles with a narrow distribution, and avoids or minimizes the use of potentially toxic components (such as chlorinated solvents and surfactants). It involves a spontaneous gradient-driven diffusion of amphiphilic organic solvent into the continuous aqueous phase. This process leads to rapid dispersion of the drug in the form of nanodroplets. Because of its good solubility in ethanol and poor aqueous solubility, oleanolic acid appears to be a good candidate for the nanoprecipitation method. Nanosuspensions of aphidicolin, atovaquone and buparvaquone prepared by high-pressure homogenization have been reported (Kayser 2000; Müller et al 2001). This is the first report on the preparation and characterization of a nanoparticulate drug delivery system of a naturally

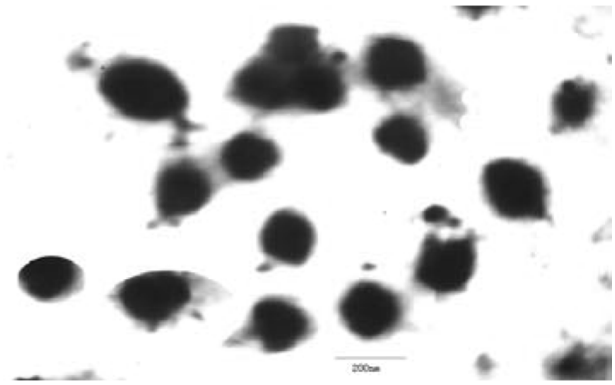

Figure 1 Transmission electron microscopy image of oleanolic acid nanosuspensions.

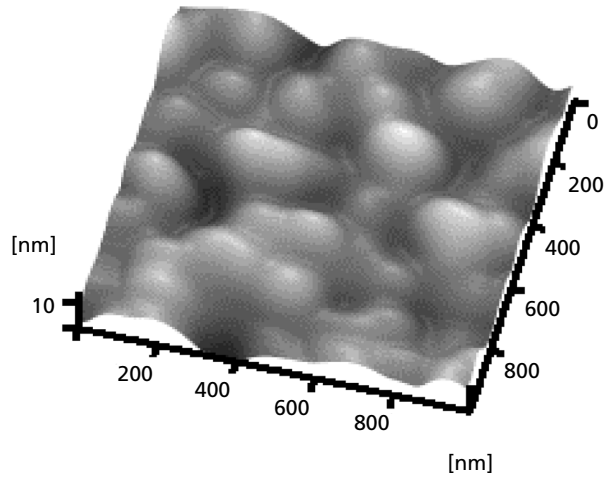

Figure 2 Three-dimensional atomic force microscopy image of oleanolic acid nanosuspensions.

derived active pharmaceutical ingredient (API) using the nanoprecipitation method.

Tween 80 was used as a stabilizer in the preparation of the nanosuspensions. We identified the surfactant concentration, organic phase injection rate, volume ratio of organic phase/aqueous phase and water content in the organic phase as size determinants (data not given). In the present study, using the optimized formulation variables, we produced oleanolic acid nanosuspensions with small particle size and good dispersion (polydispersity index = 0.216). The aggregations in the AFM images may be due to the sample preparation method before the investigation (Dubes et al 2003; Montasser et al 2003). The Zeta potential of -27.6 ± 7.2 mV is sufficient to keep the system stable, together with the steric stabilization offered by the absorbed polymer molecule on the drug particles (Müller et al 2001). After redispersion of the lyophilized nanosuspensions, the particle size and polydispersity index changes by PCS were negligible (data not given).

XRD and DSC experiments

Because of its liquid existence at ambient conditions, DSC and XRD studies were not performed with Tween 80. Figure 3 shows the XRD patterns of each sample. The powder diffraction patterns of coarse oleanolic acid showed characteristic high-energy diffraction peaks at 2θ values between 6° and 22°, which indicates the crystalline structure of the coarse oleanolic acid. In the lyophilized nanosuspensions, the sharp peaks of pure oleanolic acid were not observed, indicating the disappearance of crystalline characteristics. This suggests that oleanolic acid may be present in an amorphous state in the lyophilized oleanolic acid nanosuspensions.

The DSC investigation (Figure 4) gave similar results to the XRD study. The coarse drug powder exhibited a sharp melt process, with an onset temperature of 314.29°C and a peak of 317.78°C, and a melt enthalpy of 101.85 J g^{-1}. No melt process was observed for the lyophilized oleanolic acid nanosuspensions, indicating that there was no crystalline oleanolic acid in the lyophilized nanosuspensions; polymorphism transition may have taken place after the nanoprecipitation process.

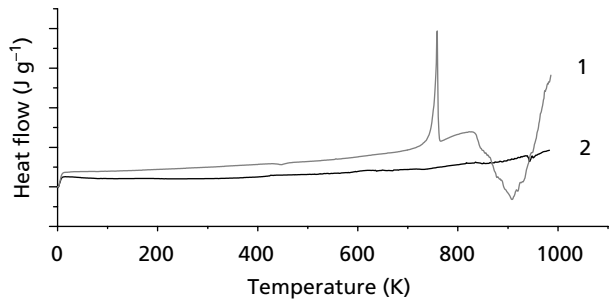

Figure 4 Differential scanning calorimetry curves of coarse oleanolic acid crystalline powder (1) and lyophilized oleanolic acid nanosuspensions (2).

Drug saturation solubility and drug dissolution investigation

At 25°C, the aqueous solubility of coarse oleanolic acid was 4.37 μg mL^{-1}, which is in good agreement with the literature (Xia et al 1988). The aqueous solubility of the lyophilized oleanolic acid nanosuspensions was 25.72 μg mL^{-1}; the aqueous solubility of coarse oleanolic acid in 2% Tween 80 was 5.87 μg mL^{-1}.

The cumulative dissolved drug for coarse oleanolic and lyophilized nanosuspensions is shown in Figure 5. About 90% of the lyophilized oleanolic acid nanosuspensions was released during the first 20 min, and nearly 95% of the drug was dissolved at 120 min. For the coarse oleanolic acid powder, the values were 15% and 20%, respectively.

A general approach used for many years is the micronization of poorly soluble drugs by mills (Rasenack et al 2003; Reverchon et al 2003). This can increase the dissolution velocity due to the increase in surface area but does not change the saturation solubility. Nanonization of poorly soluble drugs can circumvent this problem.

According to the Ostwald-Freundlich equation (Equation 1), the saturation solubility increases with decreasing particle size in the range below 1 μm:

$$\log C_s/C_\infty = 2v\sigma/2.303RT\rho r \quad (1)$$

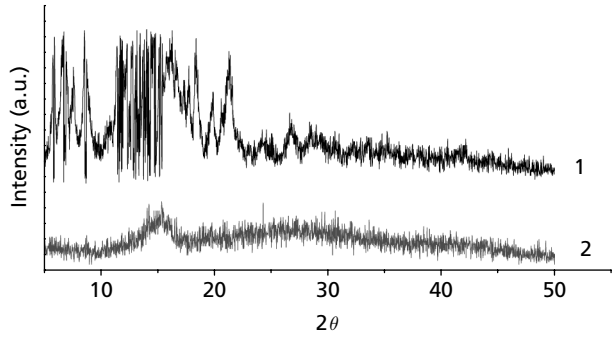

Figure 3 X-ray diffraction patterns of coarse oleanolic acid crystalline powder (1) and lyophilized oleanolic acid nanosuspensions (2).

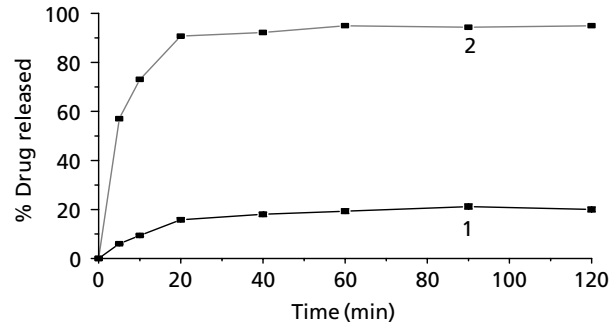

Figure 5 Cumulative oleanolic acid dissolved from coarse powder (1) and lyophilized nanosuspensions (2) in bidistilled water at 37°C.

where C_s is the solubility, C_∞ is the solubility of the solid consisting of large particles, σ is the interfacial tension, ν is the molar volume of the particle material, R is the gas constant, T is absolute temperature, ρ is the density of the solid, and r is the radius (Müller & Peters 1998).

Differences in interfacial tensions explain the different saturation solubility of polymorphic forms. From the equation above, we can also get another explanation for the increased solubility. When disrupting the more or less ideal drug microcrystals to nanoparticles, lyophobic surfaces from the inner of the crystal will be exposed to the aqueous dispersion medium; then the high-energy surfaces created lead to increased interfacial tension, which contribute to the increased solubility.

The dissolution rate of an API into an aqueous solution depends on the diffusion coefficient (D), the surface area (A), the local equilibrium concentration of the API in the diffusion layer surrounding the particle (C_s), and the diffusion layer thickness (h) (Chen et al 2004):

$$dm/dt = (DA/h)(C_s - C) \quad (2)$$

where C is the concentration of the API in bulk solution. So, the increase in C_s described above and the decrease in particle size (large surface area, A) lead to an increased dissolution velocity.

Furthermore, according to the Prandtl equation (Equation 3), a decrease in particle size results in a thinner hydrodynamic layer around particles and then an increase of the area specific dissolution rate (Mosharra & Nyström 1995):

$$h = k\,(\sqrt{L}/\sqrt{V}) \quad (3)$$

where k denotes a constant, L is the length of the surface in the direction of flow, V represents the relative velocity of the flowing liquid against a flat surface, and h is the hydrodynamic boundary layer thickness.

Hepatoprotection of coarse oleanolic acid powder and nanosuspensions

CCl_4 is widely used as a model chemical in the study of acute liver injury in mammals (Raucy et al 1993). Numerous researchers have validated the hepatoprotective effect of oleanolic acid (Hunan Medical Institute 1975; Hye 1999). Here, we selected two simple and most representative parameters to determine the hepatoprotective effect of oleanolic acid nanosuspensions. Pre-treatment with coarse oleanolic acid ($100\,mg\,kg^{-1}$, p.o., 2 days) resulted in no change in serum ALT activity and liver MDA content compared with the normal control (Table 1). Also, there was no difference between untreated mice and mice treated with 2% Tween 80 (data not shown). CCl_4 treatment caused hepatocellular damage in mice, as indicated by the rise in serum ALT and liver MDA content. Pre-treatment with $100\,mg\,kg^{-1}$ oleanolic acid before CCl_4 administration led to a 61% and 43% decrease in CCl_4-induced rise in serum ALT and liver MDA content, respectively. After pre-treatment with oleanolic acid nanosuspensions, the values were 80% and 66%, respectively. Thus, the hepatoprotective effect of oleanolic acid nanosuspensions was greater than that of the coarse oleanolic acid powder (Table 1).

Table 1 Hepatoprotective effect of pre-treatment with oleanolic acid or oleanolic acid nanosuspensions on carbon tetrachloride (CCl_4)-induced hepatotoxicity

Group	Serum alanine aminotransferase ($U\,L^{-1}$)	Malondiadehyde ($nmol\,g^{-1}$)
Normal control	45.4 ± 2.7	6.9 ± 0.6
CCl_4	253.3 ± 11.3^{a}	21.3 ± 3.6^{a}
Oleanolic acid + CCl_4	$126.6 \pm 8.5^{a,b}$	$15.1 \pm 0.8^{a,b}$
Oleanolic acid nanosuspensions + CCl_4	$88.1 \pm 14.6^{a,b,c}$	$11.8 \pm 1.1^{a,b,c}$

[a]Significantly different compared with normal control. [b]Significantly different compared with CCl_4 group. [c]Significantly different compared with oleanolic acid + CCl_4 group.

The hepatoprotective effect of oleanolic acid on CCl_4 hepatotoxicity was dose dependent (Hye 1999; Yim et al 2001). The increased saturation solubility, increased drug dissolution velocity, and the probable bioadhesive interaction of the particles with the membrane of the alimentary tract prevented oleanolic acid from being directly eliminated (Ponchel & Irache 1998; Müller et al 2001; Liversidge et al 2003) (i.e. increased absorption of oleanolic acid was achieved for nanosuspensions). A potential benefit of the increased absorption is a more beneficial pharmacodynamic effect of nanosized drug particles.

Conclusion

Nanoprecipitation can be used to prepare stable oleanolic acid nanosuspensions with a narrow particle distribution. The amorphous state as well as the small particle size in the nanometer range bring about the increased drug solubility and dissolution velocity of the lyophilized oleanolic acid nanosuspensions. The prepared oleanolic acid nanosuspensions had enhanced hepatoprotective effects compared with the coarse oleanolic acid powder. The greater absorption of nanosized oleanolic acid drug particles may account for the enhanced hepatoprotective effect of oleanolic acid nanosuspensions. Based on this technique, new oleanolic acid dosage forms with enhanced pharmacokinetic and pharmacodynamic behaviour could be developed.

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