Self-microemulsifying drug delivery system for improved oral bioavailability of oleanolic acid: design and evaluation

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Abstract: Oleanolic acid is a poorly water-soluble drug with low oral bioavailability. A self-microemulsifying drug delivery system (SMEDDS) has been developed to enhance the solubility and oral bioavailability of oleanolic acid. The formulation design was optimized by solubility assay, compatibility tests, and pseudoternary phase diagrams. The morphology, droplet size distribution, zeta potential, viscosity, electrical conductivity, and refractive index of a SMEDDS loaded with oleanolic acid were studied in detail. Compared with oleanolic acid solution, the in vitro release of oleanolic acid from SMEDDS showed that the drug could be released in a sustained manner. A highly selective and sensitive high-performance liquid chromatography-mass spectrometry method was developed for determination of oleanolic acid in rat plasma. This method was used for a pharmacokinetic study of an oleanolic acid-loaded SMEDDS compared with the conventional tablet in rats. Promisingly, a 5.07-fold increase in oral bioavailability of oleanolic acid was achieved for the SMEDDS compared with the marketed product in tablet form. Our studies illustrate the potential use of a SMEDDS for delivery of oleanolic acid via the oral route.

Keywords: oleanolic acid, self-microemulsifying drug delivery system, formulation design, in vitro release, bioavailability

Introduction

Oleanolic acid [(3β)-3-hydroxyolean-12-en-28-oic acid], is a triterpenoid compound found widely in food, medicinal herbs, and other plants in the form of free acid or aglycones of triterpenoid saponins. In 1975, the hepatoprotective effects of oleanolic acid were first identified during the study of Swertia mileensis, a traditional herbal medicine used for hepatitis. Of the three compounds isolated from this herb, oleanolic acid was the most effective in protecting against CCl4-induced liver injury in rats. The hepatoprotective effects of oleanolic acid allow its use in the People’s Republic of China as an over-the-counter oral drug for the treatment of human liver disorders such as acute and chronic hepatitis.

A variety of other pharmacologic actions of oleanolic acid have been reported, including antihypertensive, antiatherosclerotic and antioxidant activity, cardiotonic and antidysrhythmic effects, immunomodulatory activity, and inhibitory effect on α-glucosidase, cytochrome P450,12-o-tetradecanoylphorbol-13-acetate-induced gene expression.

However, the application of oleanolic acid has been limited by its poor water solubility and low bioavailability (0.7% in rats) after oral administration. To improve its solubility and bioavailability and increase its clinical efficacy, several approaches...
have been reported, including development of nanosuspensions and solid dispersions containing oleanolic acid.

Recently, with successful clinical use of the cyclosporine formulations Sandimmune® and Neoral® (Novartis Pharmaceuticals Corporation, East Hanover, NJ, USA), the self-microemulsifying drug delivery system (SMEDDS), an isotropic mixture of oil, surfactant, cosurfactant (or solubilizer), and drug, has attracted great interest in the pharmaceutical industry. When diluted in aqueous phases under gentle agitation or by digestive motility in the gastrointestinal tract, a SMEDDS can rapidly form an oil/water (o/w) microemulsion with a droplet size of less than 100 nm and have a large surface area. SMEDDS increases the oral bioavailability of a lipophilic drug by enhancing its solubility, having a small droplet size which provides a large interfacial surface area, and promoting intestinal lymphatic drug transport. In recent decades, SMEDDS have been reported to improve the oral absorption and bioavailability of several drugs, including oridonin, simvastatin, and silymarin. The relative bioavailability of silymarin in a SMEDDS increased dramatically by 1.88-fold and 48.82-fold compared with silymarin in polyethylene glycol 400 solution and suspension, respectively.

In this study, an oleanolic acid-loaded SMEDDS was prepared, and its characteristics and release in vitro were investigated. The oral bioavailability of oleanolic acid in this SMEDDS was evaluated in rats and compared with that of commercial tablets. A sensitive high-performance liquid chromatography (HPLC)-mass spectrometry method was established for determination of oleanolic acid in rat plasma.

Materials and methods

Materials

Oleanolic acid was sourced from Xieli Co, Ltd (Sichuan, People’s Republic of China) with a purity of 99%. Polyoxymethylene glycerol triricinoleate 35 castor oil (Cremophor EL®) was purchased from Sigma Chemical Co (St Louis, MO, USA). Polyoxethylene octylphenyl ether and oleic acid were obtained from Kemiu Chemical Reagent Co, Ltd (Tianjin, People’s Republic of China). Labrasol® (caprylocapryl macrogol-8 glyceride) was provided by Gattefosse (Saint-Priest, France). Octyl and decyl glycerate was purchased from Heshibi Chemical Reagent Co, Ltd (Shanghai, People’s Republic of China). Isopropyl myristate was obtained from Fluka Chemie AG (Buchs, Switzerland). Ethanol, Tween® 80, and ethyl oleate were obtained from Guangcheng Chemical Reagent Co, Ltd (Tianjin, People’s Republic of China). The reference substance of oleanolic acid and benorilate were provided by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, People’s Republic of China). HPLC-grade acetonitrile and methanol were supplied by Merck (Darmstadt, Germany). Deionized water was prepared using a Milli-Q purification system from Millipore (Molsheim, France). All other chemicals were of analytical grade. Commercially available tablets (Lot 0706301) each containing 20 mg of oleanolic acid were supplied by Jiangsu Pengyao Pharmaceutical Co (Jiangsu, People’s Republic of China).

Animals

Male Sprague Dawley rats (weighing 220 ± 10 g, about 8 weeks of age) were supplied by the Laboratory Animal Center of Shandong University. The animals were housed individually under normal conditions with food and water ad libitum. All experimental procedures were approved by the institutional animal ethics committee and were in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Solubility studies

The solubility of oleanolic acid in different oils, surfactants, and cosurfactants was determined. An excess amount of oleanolic acid was added to 2 g of the selected vehicles. The resulting mixture was shaken at 60°C to facilitate solubilization, and then kept at 25°C for 48 hours, followed by centrifugation at 10,000 rpm for 10 minutes. The supernatant was filtered through a membrane filter (0.45 µm) to remove the remaining insoluble oleanolic acid. The saturated solubility of oleanolic acid was then determined using an HPLC analysis system consisting of a 515 series HPLC pump (Waters, Milford, MA, USA) and a model 500 series detector (Shimadzu Scientific Instruments Inc, Columbia, MD, USA). Oleanolic acid was separated at 210 nm using an ODS analytical column (250 mm × 4.6 mm ID, 5.0 µm, Phenomenex Inc, Torrance, CA, USA). Mobile phase was 4% acetic acid and acetonitrile, the volume ratio was 95:5, the flow rate was 1.0 mL per minute, the sample size was 20 µL, and the retention time was about 9.8 minutes.

Compatibility tests

To assess the compatibility of the system, different surfactants and oils were mixed using a magnetic stirrer at three ratios of surfactant to oil (90:10, 80:20, 70:30). Next, 0.1 mL of each formulation was pipetted into 10 mL of purified water at 37°C, and agitated gently. Each formulation was assessed visually according to the rate of emulsification and the final appearance
of the emulsion. The performance of the formulations in vitro was visually assessed following the grading system.

**Selection of cosurfactants**

In order to choose the optimal cosurfactant, pseudoternary phase diagrams of surfactant/cosurfactant, oil, and water were constructed using the titration method. The mixed surfactant was prepared at a fixed weight ratio (2:1) of surfactant to cosurfactant (Km). The weight ratio of oil to mixed surfactant was varied as 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, and 1:9, respectively. Each mixture was titrated with water and observed visually for phase clarity and flowability. The concentration of water at which turbidity-to-transparency and transparency-to-turbidity transitions occurred was derived from the weight measurements. These values were then used to determine the boundaries of the microemulsion domain.

**Formulation optimization**

A ternary phase diagram of surfactant, cosurfactant, and oil was constructed to determine the concentration of components for the existing range of SMEDDS. First, the mixed surfactants were prepared at different K values, which varied as 10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, and 1:9. The mixture containing oil and mixed surfactant was then prepared with weight ratios of oil to mixed surfactant of 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, and 1:9, respectively. Next, 0.1 mL of each mixture was pipetted into 10 mL of purified water at 37°C under gentle stirring. The diluted solution which appeared clear or slightly bluish was the formed microemulsion.

**Preparation of oleanolic acid-loaded SMEDDS**

The SMEDDS consisted of Cremophor EL, ethanol, and ethyl oleate at a weight ratio of 50:35:15. The SMEDDS was prepared by mixing the formulation components together with gentle stirring at 40°C for 20 minutes. Oleanolic acid was then added, and the product was stirred until clear and a transparent solution was obtained. The final concentration of oleanolic acid in the SMEDDS was 1% (w/w). The mixture was stored at room temperature until used.

**Physicochemical characterization of SMEDDS**

**Morphologic characterization**

The oleanolic acid-loaded SMEDDS (1 mL) was diluted with distilled water (100 mL) to form a microemulsion. The morphology of the resulting microemulsion was observed using a transmission electron microscope (JEM-100CXII, JEOL, Tokyo, Japan). One drop of diluted sample was deposited on a film-coated copper grid, stained with a 2% aqueous solution of phosphotungstic acid, and allowed to dry before being observed under the electron microscope.

**Droplet size and zeta potential determination**

The droplet size distribution was determined using a photo-correlation spectroscopy instrument (BI-200SM, Brookhaven Instruments, Holtsville, NY, USA) at 25°C. The effect of different types of dilution medium and volume on the droplet size of the microemulsion was determined.

**Viscosity, electrical conductivity, and refractive index determination**

Viscosity was measured at 25°C using a NDJ-8S digital viscometer (Shanghai Precision and Scientific Instrument, Shanghai, People’s Republic of China) with a No 1 rotor set at 60 rpm. Electrical conductivity was determined using a conductivity meter (DDS-11C, Shanghai San-Xin Instrumentation Inc, Shanghai, People’s Republic of China), and the refractive index was measured with an Abbe refractometer (Shijiazhuang Optical Instrument Factory, Xiamen, People’s Republic of China).

**In vitro release studies**

A dialysis method was used to study the release of oleanolic acid from the SMEDDS in vitro. The oleanolic acid-loaded SMEDDS (diluted with release medium) was put into the dialysis bag (molecular weight cutoff 10,000, Spectrum Medium Industries Inc, Peabody MA, USA), which was firmly sealed and placed in 100 mL of 0.5% sodium dodecyl sulfate solution (the solubility of oleanolic acid in which was 318.42 µg/mL and conformed to sink condition) at 37°C with a rotating speed of 100 rpm. At predetermined intervals, 0.5 mL of release sample was withdrawn and the same volume of fresh release medium was replenished. The release characteristics of oleanolic acid from the SMEDDS was compared with oleanolic acid solution containing the same amount of drug dissolved in 0.5% sodium dodecyl sulfate solution. The samples were analyzed by HPLC. Release percentages were calculated as the ratio of the amount of drug released to the total initial amount of drug in the dialysis bag.

**Determination of oleanolic acid in rat plasma by HPLC-mass spectrometry**

In this study, HPLC-mass spectrometry was used to determine the concentration of oleanolic acid in rat plasma. The HPLC-mass spectrometry system comprised an Agilent...
Physicochemical characterization of SMEDDS
The results of transmission electron microscopic imaging are shown in Figure 3. The microemulsion droplets appeared as a perfect round shape without aggregation.

Table 1 Solubility of oleanolic acid in different vehicles (mg/g)

<table>
<thead>
<tr>
<th>Vehicle</th>
<th>Solubility</th>
<th>Vehicle</th>
<th>Solubility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oleic acid</td>
<td>5.40 ± 0.07</td>
<td>Tween® 80</td>
<td>5.54 ± 0.20</td>
</tr>
<tr>
<td>Ethyl oleate</td>
<td>5.06 ± 0.12</td>
<td>Cremophor EL®</td>
<td>5.65 ± 0.17</td>
</tr>
<tr>
<td>Caprylic/Capric</td>
<td>2.60 ± 0.11</td>
<td>Alcohol</td>
<td>9.84 ± 0.09</td>
</tr>
<tr>
<td>Triglyceride</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isopropyl myristate</td>
<td>2.34 ± 0.15</td>
<td>1,2-Propanediol glycol</td>
<td>1.94 ± 0.14</td>
</tr>
<tr>
<td>Labrasol®</td>
<td>8.08 ± 0.15</td>
<td>Glycerol</td>
<td>0.85 ± 0.08</td>
</tr>
<tr>
<td>Polyoxyethylene octylphenyl ether</td>
<td>4.85 ± 0.09</td>
<td>PEG 400</td>
<td>0.14 ± 0.03</td>
</tr>
</tbody>
</table>

Abbreviation: PEG, polyethylene glycol.

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The size distribution of the oleanolic acid-loaded SMEDDS formulation is shown in Figure 4. Droplet size distribution is a critical factor when evaluating a SMEDDS, and it is known that droplet size has a significant effect on drug absorption in the gastrointestinal tract. The smaller the droplet size, the larger the interfacial surface area provided for drug absorption, and this has a strong relationship with oral bioavailability. The average droplet size in the microemulsion dispersed from the oleanolic acid-loaded SMEDDS was less than 100 nm and showed a Gaussian distribution. The effects of dilution on droplet size in distilled water were measured. When the SMEDDS was diluted 25, 100, 400 times, the droplet size seemed to be unchanged, indicating that the microemulsion structure remained stable when dilution was as large as 400-fold. The effect of different types of dilution medium (dilution 100-fold) on droplet size was also investigated when the SMEDDS formulation was dispersed in distilled water, normal solution, 0.1 mol/L hydrochloride solution, and phosphate-buffered saline (pH 6.8); the resulting droplet sizes were 53.1 ± 4.2 nm, 47.5 ± 4.5 nm, 52.2 ± 5.5 nm, and 52.8 ± 4.9 nm, respectively.

![Diagram](https://via.placeholder.com/150)

**Figure 1** Pseudoternary phase diagrams for different cosurfactants. (A) Alcohol, (B) PEG 400, (C) 1,2-propylene glycol, and (D) glycerol, with the shadow area representing the microemulsion region.

**Abbreviation:** PEG, polyethylene glycol.
There was no significant difference between the four types of dilution medium, indicating that the formulation was not affected by pH and ionic strength.

The physicochemical characteristics of the oleanolic acid-loaded SMEDDS formulation on 100-fold dilution with distilled water were as follows: 1.78 ± 0.05 mm²/sec for viscosity, 92.7 ± 1.6 µsec/cm for electrical conductivity, and 1.396 ± 0.004 for refractive index, respectively.

In vitro release studies

An in vitro release study was carried out to investigate the characteristics of release of oleanolic acid from the SMEDDS formulation. However, a conventional release protocol cannot be used for a SMEDDS, because free drug and microemulsion-associated drug are not able to be separated before analysis. At present, a dialysis method is used for in vitro study of drug release from a SMEDDS. 26, 30 Drug release profiles for the SMEDDS and control formulations are shown in Figure 5.

Our results indicate that release of oleanolic acid from both the SMEDDS formulation and the control solution followed Weibull kinetics, and the equations were...
$\ln[-\ln(1-Q\%)] = 1.0034\ln t - 3.3897 \ (r = 0.9992)$ and $\ln[-\ln(1-Q\%)] = 0.89632\ln t - 1.6963 \ (r = 0.9994)$, respectively. Compared with the control solution, oleanolic acid could be released in a sustained manner from the SMEDDS. It is suggested that oleanolic acid may exist in internal oil phase and the interfacial film of a microemulsion droplet, and that drug release may proceed via the route of internal oil phase to interfacial film to external water phase, which delays the release of oleanolic acid.

**Determination of oleanolic acid in rat plasma by HPLC-mass spectrometry**

Oleanolic acid and the internal standard were separated well from impurities in the plasma extracts, and the mass spectra and typical chromatograms (Figure 6) show a retention time of about 4.65 minutes and 2.13 minutes for oleanolic acid and the internal standard, respectively. In the concentration range of 0.5–500 ng/mL, the peak area ratio (R) of oleanolic acid to the internal standard correlated well with...

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**Figure 6** Mass spectra and chromatograms for plasma. (A) Mass spectra of oleanolic acid (A) and benorilate (B, internal standard); (B) is blank plasma; (C) is plasma added with 0.5 ng/ml oleanolic acid and 50 ng/mL benorilate; (D) is plasma sample after administration of the oleanolic acid-loaded self-microemulsifying drug delivery system. (1 represents oleanolic acid with m/z 455.2 → 455.4, and 2 represents benorilate as internal standard with m/z 312.0 → 370.2).
plasma concentration, ie, R = 14.259C - 2.7683 (r = 0.9993). The absolute recovery, and intraday and interday precision for determination of oleanolic acid in rat plasma at 1.0, 25.0, and 400 ng/mL levels are shown in Table 4. The stability of oleanolic acid in plasma was tested by analysis of the control samples at 1.0, 25.0, and 400 ng/mL levels. It was found that oleanolic acid was stable in plasma at ~40°C for at least 30 days and during three freeze-thawing cycles.

Bioavailability results
The pharmacokinetic parameters for oleanolic acid-loaded SMEDDS and tablets were compared in rats. The mean plasma concentration-time curves for the oleanolic acid-loaded SMEDDS formulation and the control tablets are shown in Figure 7. The major pharmacokinetic parameters for oleanolic acid in the SMEDDS and tablet formulations are shown in Table 5. As indicated, the maximum plasma concentration and area under the plasma concentration-time curve for oleanolic acid in SMEDDS was 209.80 ng/mL and 1740.06 ng h/mL, which is 2.71-fold and 5.07-fold that of the commercial tablet, respectively.

The relative bioavailability of oleanolic acid in SMEDDS was dramatically increased compared with the commercial oleanolic acid tablet. There may be several reasons for the high bioavailability of SMEDDS. First, SMEDDS can markedly increase the solubility of a lipid-soluble drug. For a poorly water-soluble drug, absorption is often inadequate due to insufficient dissolution in the gastrointestinal tract. Therefore, the dissolution process may be an important factor influencing absorption. The SMEDDS transforms into microemulsion in the gastrointestinal tract, which keeps the drug in a dissolved form, which is beneficial for enhanced absorption. Second, the SMEDDS forms a fine o/w microemulsion with a droplet size of less than 100 nm when it emerges in the gastrointestinal tract. The small droplet size provides a large interfacial surface area for the drug to come into contact with the cell membrane, which might increase drug release and absorption. Third, the high surfactant content in a SMEDDS may increase permeability by disturbing the cell membrane.30 Drug absorption mechanisms may include the transcellular and paracellular routes, and hydrophobic drugs with a low molecular weight, such as oleanolic acid, are absorbed transcellularly in most cases. The main barrier to their absorption is considered to be the tight junctions of cells. The surfactant in SMEDDS can reduce the interfacial surface tension and enhance penetration of the drug into epithelial cells.24 The structural characteristics of surfactant allow it to partition between lipid and protein domains.30 Surfactant can interact with the polar head groups in the lipid bilayers, and modify hydrogen bonding and ionic forces. It may also insert between the lipophilic tails of the lipid bilayers, which could disrupt the lipid-packing arrangement. Eventually, the inclusion of a lipid in a SMEDDS formulation may stimulate formation of lipoproteins and chylomicrons, subsequently promoting the lymphatic pathway for drug absorption, which may reduce the opportunity for hepatic first-pass metabolism and thereby enhance oral drug bioavailability, which has been confirmed by Hauss et al.31

Table 3 Design and results of formulation optimization

<table>
<thead>
<tr>
<th>Formula composition %</th>
<th>Drug content (mg/g)</th>
<th>Droplet size (nm)</th>
</tr>
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<tbody>
<tr>
<td>Number</td>
<td>Cremophor EL®</td>
<td>Alcohol</td>
</tr>
<tr>
<td>1</td>
<td>80</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>75</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>70</td>
<td>15</td>
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<td>4</td>
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<td>20</td>
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<td>5</td>
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<td>25</td>
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<td>6</td>
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<td>7</td>
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<td>10</td>
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<td>50</td>
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<tr>
<td>11</td>
<td>30</td>
<td>55</td>
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Table 4 Absolute recovery, precision (n = 5) of the assay method

<table>
<thead>
<tr>
<th>Concentration (ng/mL)</th>
<th>Absolute recovery Mean (%)</th>
<th>RSD (%)</th>
<th>Precision, RSD (%)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Intraday</td>
<td>Interday</td>
<td>Intraday</td>
</tr>
<tr>
<td>1.0</td>
<td>35.28</td>
<td>46.24</td>
<td>2.66</td>
</tr>
<tr>
<td>25.0</td>
<td>34.68</td>
<td>2.20</td>
<td>2.51</td>
</tr>
<tr>
<td>400.0</td>
<td>36.60</td>
<td>3.03</td>
<td>2.08</td>
</tr>
</tbody>
</table>

Abbreviation: RSD, relative standard deviation.
Table 5 Major pharmacokinetic parameters of oleanolic acid after oral administration of oleanolic acid-loaded SMEDDS and a commercial tablet in rats

<table>
<thead>
<tr>
<th></th>
<th>SMEDDS</th>
<th>Tablet</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC&lt;sub&gt;0-&lt;inf&gt;∞&lt;/inf&gt; (ng h/mL)</td>
<td>1740.06 ± 163.25</td>
<td>308.19 ± 55.04</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0-&lt;inf&gt;∞&lt;/inf&gt; (ng h/mL)</td>
<td>1775.26 ± 157.91</td>
<td>350.13 ± 73.67</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; (ng/mL)</td>
<td>209.80 ± 47.19</td>
<td>77.60 ± 16.79</td>
</tr>
<tr>
<td>T&lt;sub&gt;max&lt;/sub&gt; (hours)</td>
<td>2.00 ± 1.00</td>
<td>2.75 ± 0.50</td>
</tr>
<tr>
<td>Relative bioavailability (%)</td>
<td>507.03%</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: AUC, area under the curve; C<sub>max</sub>, peak plasma concentration; T<sub>max</sub>, time to peak plasma concentration; SMEDDS, self-microemulsifying drug delivery system.

Conclusion

A SMEDDS formulation was developed, in which the optimal ratio of ethyl oleate to Cremophor EL to alcohol in the formulation was 50:35:15 (w/w). When diluted with water, the oleanolic acid-loaded SMEDDS could spontaneously form small particles. Neither dilution volume nor type of dilution medium had a significant effect on particle size or self-microemulsifying behavior. In vitro release of oleanolic acid from SMEDDS determined using a dialysis method showed typical sustained-release characteristics. The plasma concentration of oleanolic acid in the rat was determined by HPLC-mass spectrometry. The results of a pharmacokinetic study showed that the relative bioavailability of oleanolic acid was markedly improved, being approximately 507% that of the oleanolic acid tablet. This study provides evidence that a SMEDDS is valuable for oral delivery of oleanolic acid, and could potentially be used to enhance the oral absorption of poorly water-soluble drugs.

Acknowledgment

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Disclosure

The authors report no conflicts of interest in this work.

References