To evaluate the bioavailability of ellagic acid loaded supersaturatable self-microemulsifying drug delivery system (S-SMEDDS), its pharmacokinetic properties were studied in rats with an ultra-high performance liquid chromatography-quadrupole time-of-flight tandem mass spectrometry. The plasma samples were treated by solid-phase extraction method, and gallic acid was used as the internal standard when determining the concentration of ellagic acid. Results showed that the established analytical method was sensitive and accurate, which is applicable to the pharmacokinetic study of ellagic acid. The drug was found to be absorbed rapidly in vivo, and the plasma concentration-time curve showed double peaks, indicating that ellagic acid were reabsorbed by entero-hepatic circulation after oral administration. Compared with ellagic acid suspension, the apparent clearance of ellagic acid-loaded S-SMEDDS and SMEDDS reduced significantly, and the $AUC_{0\rightarrow t}$ of them were 4.7 and 5.8-fold increase, respectively. Therefore, the bioavailability of ellagic acid-loaded S-SMEDDS was higher than that of the suspension and SMEDDS.

Keywords: ellagic acid, supersaturatable self-microemulsification, pharmacokinetics, UHPLC-Q-TOF-MS

Supersaturatable self-microemulsifying drug delivery system (S-SMEDDS) is an extension of the self-microemulsifying drug delivery system (SMEDDS), which can inhibit the precipitation and improve the solubility of water-insoluble drugs by adding a precipitation inhibitor into the system (1). In recent years, S-SMEDDS has been used in the oral delivery of many hydrophobic active compounds extracted from herbs or Traditional Chinese Medicine (TCM), increasing the oral bioavailability about 7-fold more than the single drug (2, 3). Ellagic acid (EA), a natural active ingredient, is isolated from the extraction of pomegranates, black raspberries, raspberries, strawberries, walnuts or almonds, et al. (4). However, the water solubility of EA is about 0.01 mg mL$^{-1}$ at pH 1.2 and 0.02 mg mL$^{-1}$ at pH 12.

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pH 6.8. EA shows rapid absorption, distribution and elimination after oral administration at a dose of 85.3 mg kg$^{-1}$ in rats, and it may be metabolized by intestinal micro-organisms (5). More than 50 % of EA is bound to plasma proteins, and ionized EA occurs in the stomach and intestine (6). Yan and Yin (7) have found that EA is poorly absorbed after oral administration (50 mg kg$^{-1}$ in rats), and the pharmacokinetic profile fits the two-compartment model. Its abundant pharmacological activities might be limited by undesirable solubility and bioavailability. S-SMEDDS could be considered as an oral delivery system for ellagic acid to improve its bioavailability and achieve a relatively slower and more sufficient absorption.

Recently, liquid chromatography tandem mass spectrometry (LC-MS/MS) has been widely used for the quantification of pharmaceutical preparations, TCM, biological drugs, et al. (7, 8). The common methods used for the treatment of plasma samples include protein precipitation, liquid-liquid extraction and solid-phase extraction (SPE) method (9). Based on the theory of liquid-solid chromatography, the SPE method can adsorb and elute samples selectively, which helps improve the recovery of biological samples (9, 10). In this study, a specific and reliable ultra-high performance liquid chromatography-quadrupole time-of-flight tandem mass spectrometry (UHPLC-Q-TOF-MS) method is established to study the pharmacokinetics of EA-S-SMEDDS in rats. It is the first time to evaluate the oral bioavailability of EA-S-SMEDDS in vivo.

**EXPERIMENTAL**

**Reagents**

Ellagic acid (HPLC $\geq$ 98 %) was purchased from Shanghai Yuanye Biological Co. Ltd. (Shanghai, China). Ethyl oleate, Tween 80 and polyvinylpyrrolidone (PVP) K30 were obtained from Hunan Er-Kang Pharmaceutical Co. Ltd. (Changsha, China). Methanol, acetonitrile (Merck, Germany) and other used solvents were of chromatographic grade.

**Preparation of EA-S-SMEDDS**

EA-S-SMEDDS was prepared by the previously described method (11). Briefly, the surfactant and co-surfactant (67.5 % Tween 80 and 22.5 % PEG 400) were uniformly mixed with a magnetic stirrer at room temperature. Then, the oil phase (10 % ethyl oleate) was added into the mixture to prepare a blank SMEDDS. After dissolving precipitation inhibitors (0.5 % PVP K30) into the blank SMEDDS, 4 mg g$^{-1}$ ellagic acid was mixed by vigorous vortexing until a transparent solution was reached, and then the EA-S-SMEDDS was obtained. Meanwhile, EA-SMEDDS was prepared by the same method without the use of precipitation inhibitors. Additionally, the morphologies of EA-S-SMEDDS and EA-SMEDDS were investigated by the HT7700 transmission electron microscope (TEM, Hitachi, Japan), and the average particle size of the emulsions was measured using the Mastersizer 2000 particle size analyzer (Malvern Instruments Ltd., UK). The morphology of emulsified EA-S-SEDDS and EA-SMEDDS presented a round shape and non-aggregated, displaying a narrow particle size distribution with the average size of about 45 nm.
**UHPLC-Q-TOF-MS conditions**

To analyze the content of ellagic acid in rat plasma, an Agilent 1290 Infinity liquid chromatography system, equipped with Agilent 6530 Q-TOF mass spectrometric system (Agilent Technologies, USA) was used in this study.

An Infinitylab EC-C$_{18}$ column (2.10 mm × 100 mm, 2.7 μm) (Agilent, USA) with a gradient elution was applied to conduct the separation. The sample injection volume was 20 μL and the flow rate was 0.2 mL min$^{-1}$. The column temperature was set at 30 °C and the UV-detection wavelength was 254 nm. The mobile phase was composed of acetonitrile (A) and 0.1 % formic acid containing 10 mmol L$^{-1}$ ammonium acetate (B). The solvent gradient program of UHPLC analysis is shown in Table I.

The MS detection was performed on a Q-TOF mass spectrometer with ESI source in the negative ionization mode. The optimal MS parameters were as follows: mass spectrum scanning range: m/z 100–1200; nebulizer gas (nitrogen) temperature: 350 °C; nitrogen flow: 11 L min$^{-1}$; nebulizer gas pressure: 50 psi; capillary voltage: 4000 V. The selected ionization pairs (m/z) of ellagic acid and gallic acid were 301.2 ([M-H]$^-$) and 169.1 ([M-H]$^-$), respectively.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>A (%)</th>
<th>B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5</td>
<td>95</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>6</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>9</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>11</td>
<td>5</td>
<td>95</td>
</tr>
<tr>
<td>16</td>
<td>5</td>
<td>95</td>
</tr>
</tbody>
</table>

The mobile phase: A – acetonitrile, B – 0.1 % formic acid containing 10 mmol L$^{-1}$ ammonium acetate

**SPE method for the extraction of plasma samples**

The C$_{18}$ SPE cartridges (1 mL, 30 mg, Beijing Dikema Technology Co., Ltd., China) was activated by 1 mL of methanol and equilibrated by 2 mL of deionized water. The rat plasma (200 μL) was spiked with 100 μL of gallic acid (internal standard, 100 ng mL$^{-1}$) using a vortex for 30 s. The mixed solution was applied to the pre-activated SPE cartridges. Then the cartridges were washed with 2 mL of deionized water to remove the endogenous impurities in plasma. Finally, 1 mL of methanol/0.1 % formic acid (9:1, V/V) was used to wash the SPE cartridges, and 1 mL eluents were collected and stored at –20 °C before the analysis.

**Preparation of standard solution**

The 100 μg mL$^{-1}$ stock solution of ellagic acid was diluted to various concentrations with acetonitrile/0.1 % formic acid (1:9, V/V) to obtain the working standard solutions.
Then, 100 μL of the working standard solution was mixed with 100 ng mL$^{-1}$ internal standard solution (gallic acid) and 200 μL of blank rat plasma, followed by treating with the above-mentioned SPE method to prepare the calibration standard solutions (5, 10, 40, 80, 160, 640 and 800 ng mL$^{-1}$).

**Method validation**

The specificity of the method for determining the concentration of ellagic acid in rat plasma was investigated by preparing and analyzing the blank plasma, blank plasma with ellagic acid working standard solution and drug-containing plasma. The drug-containing plasma of rats was collected at 1 h after oral administration of 40 mg kg$^{-1}$ EA-S-SMEDDS. The linearity of ellagic acid was analyzed with the peak area ratio (ellagic acid/gallic acid) as the ordinate and the concentration of ellagic acid as the abscissa.

A proper volume of low, medium and high concentration of ellagic acid working standard solution (15, 160 and 640 ng mL$^{-1}$) and internal standard solution, were added into 200 μL of blank plasma. And the hybrid solution was treated according to the method of the ‘SPE method for the extraction of plasma samples’ to prepare the standard plasma samples (quality control samples).

To investigate the precision of the determination method, the standard plasma samples were measured 5 times in 24 hours to calculate the intra-day precision, and tested by the same method for 5 consecutive days to determine the inter-day precision. The recovery rate of the samples was performed by comparing the peak area of extracted quality control samples with the peak area of extracted blank plasma spiked with standards at the same amount. The matrix effect was evaluated by the ratio of peak areas of the plasma sample (the extracted blank plasma spiked with analytes) versus the working standard solution at the concentration of 15, 160 and 640 ng mL$^{-1}$, respectively. The blank matrix samples were prepared from 6 different groups of rats, with each group containing 4 individual animals.

The short-term stability of the plasma samples was investigated after preserving the standard plasma samples at 4 °C for 24 h and analyzing the samples before and after treatment by the SPE method. The long-term stability was carried out similarly by preserving the three standard plasma samples at −80 °C for 2 weeks before being treated by the SPE method. To examine the freeze-thaw stability, the standard plasma samples were stored at 4 °C for 24 hours and thawed at room temperature. The freeze-thaw operation was repeated for three times.

**Animals and pharmacokinetic study**

Twelve healthy male Sprague-Dawley rats (200 ± 20 g), obtained from Jinan Pengyue Experimental Animal Breeding Co. Ltd. (Jinan, China), were randomly divided into three groups and adaptively fed for one week prior to the experiments. After being kept under fasting with free access to water overnight, the animals were orally administrated with EA-S-SMEDDS, EA-SMEDDS and EA suspension (4 mg mL$^{-1}$ EA dissolved into 0.5 % CMC-Na solution) at a single dose of 40 mg kg$^{-1}$. About 0.5 mL blood sample was obtained from rat orbital venous plexus at 0.083, 0.167, 0.333, 0.667, 1, 2, 4, 6, 8, 12 and 24 h. Then the blood samples were centrifuged for 15 min at 6262 g. The supernatant plasma was collected and stored at −80 °C until analysis. The animal care and all the handling followed the institutional guidelines of the Animal Care and Use Committee of Shandong University.
Data analysis

GraphPad Prism 7 software (GraphPad Software, USA) was applied for the statistical analysis (Student’s t-test). The pharmacokinetic parameters were analyzed by DAS 2.0 (BioGuider Co., China). Values of $p < 0.05$ were considered to be statistically significant.

RESULTS AND DISCUSSION

Method validation

Fig. 1. displays the chromatograms of ellagic acid and gallic acid. It shows that ellagic acid and the internal standard could be determined in 20 min without any interference from the endogenous substances in rat plasma. The calibration curve for the determination of EA was linear within 5~800 ng mL$^{-1}$ ($R^2 = 0.9989$). And the regression equation was $y = 0.0267x - 0.1851$, whereas $y$ meant the ratio of the peak area of ellagic acid and gallic acid, and $x$ meant the concentration of ellagic acid in rat plasma.

The intra- and inter-day precision for ellagic acid at different concentrations were in the range of 2.54~4.92 % and 3.78~6.04 % (relative standard deviation, RSD), respectively. The extraction recoveries of ellagic acid ranged from 88.11 to 92.34 % ($RSD < 6.22 \%$). And the matrix effect values of ellagic acid at low, medium and high concentrations were 89.21, 90.99 and 90.73 %, respectively ($RSD < 7.10 \%$). These results revealed that the content determination method for ellagic acid in rat plasma was reproducible with a negligible matrix effect.

The results of stability displayed that ellagic acid in plasma samples remained unchanged after 24 h at 4 °C with relative errors ($RE$) lower than 8.28 %. The $RE$ values ranged from $-7.33$ to $-10.89 \%$ for the long term freezer at $-80 \, ^\circ\mathrm{C}$ for two weeks, and from $-6.85$ to $-9.10 \%$ after three freeze-thaw cycles. All these results meet the requirements for analyzing the biological samples.

Pharmacokinetics study

All the plasma samples were collected at predetermined time points and treated before the determination. The concentration-time curve of ellagic acid exhibited an initial upward and double peaks (Fig. 2), which indicated a rapid absorption of the drug after oral administration, and might be reabsorbed through enterohepatic circulation (12). In addition, the drug concentration-time curves of EA-SMEDDS and EA-S-SMEDDS at each time point were higher compared with EA suspension.

The mean pharmacokinetic parameters for the EA suspension, EA-SMEDDS and EA-S-SMEDDS are displayed in Table II. The peak time was within 1 h, and the mean residence time ($MRT$) was 4.653 h, which suggests that ellagic acid could be absorbed and eliminated quickly. Compared with the EA suspension, the $MRT$ of EA-SMEDDS and EA-S-SMEDDS were longer and the clearance was significantly reduced. The increase in $T_{max}$ and $MRT$ might due to the slower release rate of EA from the inner phase of the micro-emulsion, thereby prolonging and delaying the process of penetration and distribution of EA into tissues and into the circulation (13).

The $AUC_{0-\infty}$ of EA-SMEDDS and EA-S-SMEDDS were 5.0 and 6.7 times higher than that of EA suspension. The two emulsion formulations also exhibited about a 3-fold
Fig. 1. The chromatograms of ellagic acid and gallic acid: a) blank rat plasma, b) blank rat plasma combined with ellagic acid reference and internal standard solution, c) plasma sample 1 h after oral administration of 40 mg kg$^{-1}$ EA-S-SMEDDS.
increase for \( C_{\text{max}} \) values compared with EA. The results of the in vivo bioavailability study showed that the peak time of the two emulsions was longer than EA, which might owe to the fact that \( T_{\text{max}} \) is the time required for the plasma concentration to reach the maximum value, when the absolute value of ‘absorbed dose minus distribution, metabolism and excretion’ is maximum. For insoluble drugs, the dissolution process is usually the limiting step of drug absorption, and a higher dissolution rate plays an important role in improving bioavailability (14). In addition, the improved bioavailability of S-SMEDDS could be influenced not only by the quick dissolution but also by the proportion of the dissolution. When S-SMEDDS is emulsified in water, it provides a supersaturation condition and increases the interfacial area for drugs, which helps reduce the precipitation of ellagic acid and further improve the solubility and bioavailability of it (15). And S-SMEDDS could dissolve more completely than the other two groups. Therefore, the oral absorption of ellagic acid in S-SMEDDS was the best among EA suspension, EA-SMEDDS and EA-S-SMEDDS.

Fig. 2. Mean plasma concentration-time curve of ellagic acid in rats after oral administration of EA-S-SMEDDS, EA-SMEDDS and EA suspension at a dose of 40 mg kg\(^{-1}\) (\( n = 4 \)).

Table II. Pharmacokinetic parameters of ellagic acid in rats after oral administration of S-SMEDDS, SMEDDS and the suspension (\( n = 4 \), \( \bar{X} \pm S \))

<table>
<thead>
<tr>
<th>Parameters</th>
<th>EA suspension</th>
<th>EA-SMEDDS</th>
<th>EA-S-SMEDDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>( AUC_{0-t} ) (( \mu g \text{ mL}^{-1} \text{ h} ))</td>
<td>0.436 ± 0.042</td>
<td>2.079 ± 0.064(^{**})</td>
<td>2.552 ± 0.280(^{**})</td>
</tr>
<tr>
<td>( AUC_{0-\infty} ) (( \mu g \text{ mL}^{-1} \text{ h} ))</td>
<td>0.496 ± 0.028</td>
<td>2.489 ± 0.239(^{**})</td>
<td>3.40 ± 0.442(^{**})</td>
</tr>
<tr>
<td>( \text{MRT}_{0-\infty} ) (h)</td>
<td>4.653 ± 0.605</td>
<td>6.03 ± 0.900(^{**})</td>
<td>7.906 ± 1.226(^{**})</td>
</tr>
<tr>
<td>( T_{\text{max}} ) (h)</td>
<td>0.667 ± 0.000</td>
<td>1.000 ± 0.000</td>
<td>1.000 ± 0.000</td>
</tr>
<tr>
<td>( C_{\text{max}} ) (( \mu g \text{ mL}^{-1} ))</td>
<td>0.184 ± 0.027</td>
<td>0.572 ± 0.023(^{**})</td>
<td>0.604 ± 0.057(^{**})</td>
</tr>
<tr>
<td>( \text{CL/F} ) (L h(^{-1}) kg(^{-1}))</td>
<td>80.820 ± 4.583</td>
<td>16.180 ± 1.510(^{**})</td>
<td>12.152 ± 1.772(^{**})</td>
</tr>
</tbody>
</table>

\(^{*} p < 0.05, \quad ^{**} p < 0.01\), statistical significance compared with the EA suspension

\(^{*} p < 0.05\), statistical significance compared with EA-SMEDDS
CONCLUSIONS

A specific and reliable UHPLC-Q-TOF-MS method was established in this study to determine the concentration of ellagic acid in rat plasma with gallic acid as the internal standard. The plasma samples containing ellagic acid held high stability under various conditions. Compared with the EA suspension, EA-S-SMEDDS exhibited a prolonged residence time and higher AUC values in vivo. Therefore, S-SMEDDS could enhance the oral absorption of EA, and the biopharmaceutical advantages of S-SMEDDS indicated promising prospects in the development of EA.

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