A sensitive and specific liquid chromatography–tandem mass spectrometric (LC–MS-MS) method was developed for the determination and pharmacokinetics of amygdalin in rats. Rat plasma pretreated by solid-phase extraction was analyzed by LC–MS-MS with negative electrospray ionization in the multiple reaction monitoring mode. Amygdalin and geniposide [the internal standard (IS)] were separated on a C18 column eluted with a mobile phase of methanol and water (85:15; v/v) at a flow rate of 0.25 mL/min in a run time of 3.0 min. The precursor to product ion transitions were monitored at \( m/z \) 457.2 \( \rightarrow \) 279.1 for amygdalin and \( m/z \) 387.1 \( \rightarrow \) 224.9 for the IS. The calibration curve of amygdalin showed good linearity over a concentration range of 10–2,000 ng/mL. The limit of quantification was 10 ng/mL. Intra-day and inter-day precisions and accuracy (percent relative standard deviation) were both within 10%. The method was fully validated for its selectivity, sensitivity, matrix effect, recovery and stability. This accurate and specific assay produced a useful LC–MS-MS method, which was successfully applied to pharmacokinetic studies after the oral administration of amygdalin to rats.

Introduction

Amygdalin (D-mandelonitrile-β-gentiobioside) is a cyanogenic diglucoside that is abundant in seeds of the prunasin family, plants such as almonds, apricots, cherries, and peaches and other rosaceous plants (1). In traditional Asian medicine, it has been used to treat various illness, such as asthma, bronchitis, emphysema, constipation, leprosy and leucoderma (2–4). Since the 1980s, amygdalin has been advocated as a new anticancer drug in northern Europe, Mexico and Asia (5–7), although there has been controversy about the anticancer effects of amygdalin for its cyanide toxicity and unclear anticancer mechanisms. More scientific researchers are taking an interest in amygdalin as an anticancer drug, and an increasing number of reports have been published that demonstrate the anticancer effects of amygdalin for its cyanide toxicity and unclear anticancer mechanisms. More scientific researchers are taking an interest in amygdalin as an anticancer drug, and an increasing number of reports have been published that demonstrate the anticancer effects of amygdalin (8–11).

Many reports have been conducted regarding the quantitative analysis of amygdalin in plasma and other biological fluids, typically involving gas chromatography (GC), high-performance liquid chromatography (HPLC) and HPLC–mass spectrometry (MS) (12–16). However, these methods possess many disadvantages such as low sensitivity, long retention time and the requirement of large volumes of organic solvent. Compared with these methods, liquid chromatography–tandem mass spectrometry (LC–MS-MS) is a well-established method in bioanalysis as a result of its good specificity, sensitivity and short analytical time (17–18). There have been reports on LC–MS-MS methods, but Ge et al. (19) used the selected ion monitoring (SIM) mode rather than the multiple reaction monitoring (MRM) mode to identify metabolites in rat urine after the intravenous injection of 100 mg/kg doses of amygdalin. During SIM, the MS analysis time is only focused on analytes of specific masses. MRM is one of the most sensitive approaches for the quantification of known analytes. An essential feature of MRM is that both the parent ion and one or more transitional product masses are known. Wen et al. (20) developed a liquid chromatography–quadrupole time-of-flight mass spectrometry (RRLC–Q-TOF-MS) method to identify the absorbed components and metabolites in rat urine after the oral administration of Buyang Huanwu decoction (BYHWDA: a traditional Chinese prescription consisting of seven medicinal crude drugs, including Radix Astragali, Radix Angelicae Sinensis, Radix Paeoniae Rubra, Rhizoma Ligustici Chuanxiong, Flos Carthami, Semen Persicae and Lumbricus in the ratio of 120:6:4:5:3:3:3:3). Although this method was extremely sensitive, amygdalin was not detected in urine. The reasons might be that the ratio of Semen Persicae in the prescription was too low, the concentration of amygdalin was below the limit of detection of the assay or amygdalin was transformed into prunasin in urine.

Concerning the pharmacokinetics of amygdalin, Ames et al. (12) reported a GC–MS method for the determination of plasma and urine concentrations of amygdalin, whole blood concentrations of cyanide and thiocyanate concentrations in serum and urine in cancer patients following the intravenous (4.5 g/m²) and oral (500 mg tablet) administration of amygdalin. Following the intravenous administration of amygdalin, plasma concentrations were very high (1.401 μg/mL). Cyanide was barely detectable in whole blood and thiocyanate concentrations did not increase. Following the oral administration of amygdalin, plasma concentrations and urinary recovery of the parent drug were much lower, with peak values less than 525 ng/mL. Cyanide concentrations increased to values as high as 2.1 μg/mL in whole blood. Rauws et al. (13) reported an HPLC–MS method for the determination of amygdalin and prunasin in plasma ultra-filtrate and urine. After intravenous administration, the unchanged amygdalin was the predominantly excreted glycoside, whereas after oral administration, almost all excreted glycoside was prunasin. Recently, Fang et al. (15–16) reported that amygdalin was rapidly absorbed in prototype to blood after injection; the prototype of amygdalin was not detected after oral administration, but two metabolites were detected, which were isomers of prunasin, as confirmed by MS. Thus far, the pharmacokinetic data are still limited regarding amygdalin after oral administration.
The purpose of this study is to establish and validate an LC–MS-MS method in MRM mode for the determination of amygdalin and successfully apply this method to the pharmacokinetic study of amygdalin in rats after oral administration. This method exhibited excellent results with respect to sensitivity and a wide linear concentration range.

Experimental

Chemicals and materials
Amygdalin (>98% pure) and geniposide (>98% pure; internal standard (IS)) were purchased from Chendu King-Tiger Pharm-Chem Tech Co. (Chendu, China). HPLC-grade methanol was purchased from Merck (Darmstadt, Germany). Deionized water was generated with the Milli-Q Plus system (Millipore, Bedford, MA). All the other reagents were of analytical grade. Drug-free rat plasma was collected from male Sprague-Dawley rats and stored at –20°C before use.

LC–MS-MS instruments and conditions
An Agilent 1200 series liquid chromatographic system, interfaced to an API4000 triple quadruple mass spectrometer and coupled with electrospray ionization (ESI), was used in the study. The chromatographic separation was achieved on a Gemini C18 analytical column (50×2.0 mm, 5 μm; Phenomenex, Torrance, CA) at a temperature of 40°C. The mobile phase consisted of methanol and water (85:15, v/v) with a flow rate of 0.25 mL/min in a run time of 3.0 min. The injection volume was 5 μL. Mass spectrometric detection was conducted with negative mode ESI using MRM. The mass transitions were m/z 457.2→279.1 for amygdalin and m/z 387.1→224.9 for the IS (Figure 1). The optimal MS parameters were as follows: nebulizer (GS1), heater (GS2), curtain gas and collision gas were set at 25, 25, 30 and 6 psi, respectively; ionspray needle voltage was –4,500V; probe temperature was 400°C; N2 was used as the collision gas. The optimal parameters were as follows: declustering potential (DP), entrance potential (EP), collision energy (CE) and collision exit potential (CXP) were –43.9, –9.4, –14.3 and –12.9, and –51.8, –9.9, –11.4 and –14.6 for amygdalin and the IS, respectively.

Animals and pharmacokinetic analysis
Six male Sprague-Dawley rats (280–320 g) were provided by the Medical Laboratory Animal Center (Guangdong, China). Animals studies were conducted in accordance with the Guidelines for Animal Experimentation of Guangzhou University of Chinese Medicine.

The rats were fasted overnight and allowed free access to water. After a single dose by oral administration of 20 mg/kg amygdalin to the rats, blood samples (0.3 mL) were collected into heparinized tubes before administration (0 min) and at different time points after dosing (0.083, 0.167, 0.25, 0.5, 1, 1.5, 2, 4, 6, 8, 12 and 24 h), immediately centrifuged at 3,000 g for 10 min, and the plasma samples were stored frozen at –20°C before analysis.

The dosed plasma samples were thawed and brought to room temperature. A 100 μL aliquot of a plasma sample spiked with 10 μL of a solution of the IS (2 μg/mL) and 800 μL of water were added to 1.5 mL Eppendorf centrifuge tubes. After vortexing for 1 min, the plasma samples were applied to the solid-phase extraction (SPE) cartridges. The plasma samples were treated under the same process as described in the following, and the concentration of amygdalin was determined from the calibration curve on the same day.

The plasma concentrations of the analytes at different time points were expressed as mean ± standard deviation (SD), and concentration versus time curve was plotted. The maximum plasma concentration (Cmax) and the corresponding time (Tmax) after oral administration were obtained directly from the experimental data. The elimination rate constant (Kel) was calculated by the slope of the regression line of best fit. The plasma half-life (t1/2) was calculated by using 0.693/Kel. The area under the plasma concentration versus time curve from zero to infinity (AUC0→∞) was calculated by using the trapezoidal rule with extrapolation to infinity with Kel (21).

Preparation of calibration and quality control samples for LC–MS-MS analysis
Stock solutions of amygdalin and the IS were prepared in methanol–water (50:50, v/v) at concentration of 1 mg/mL. Working solutions with concentrations of 2 μg/mL for the IS and between 0.10 and 20 μg/mL for amygdalin were prepared by diluting the stock solution with methanol–water (50:50, v/v). The
working solutions were miscible liquids of amygdalin and the IS. The concentrations of amygdalin in working solution were between 0.10 and 20 μg/mL. The concentration of the IS was 2 μg/mL in all working solutions. All solutions were protected from light and stored at 4°C.

Calibration standards of amygdalin were prepared by adding appropriate amounts of the mixed working solutions with concentrations of 2 μg/mL for the IS and between 0.10 and 20 μg/mL for amygdalin to drug-free rat plasma (100 μL) to obtain the concentrations of 10, 25, 50, 100, 250, 500, 1,000 and 2,000 ng/mL for amygdalin and 200 ng/mL for the IS. The treatment of calibration standard samples were also extracted by SPE and under the same process as described in the following. Quality control (QC) samples were prepared in the same way as the calibration samples, representing low, medium and high concentrations of amygdalin in plasma at 30, 300 and 1,500 ng/mL. The plasma samples were stored at −20°C and thawed at room temperature before use.

Sample preparation
The SPE cartridges (ProElut C18 200 mg/3 mL 50/ pkg; Dikma Technologies, Lake Forest, CA) were activated by 3 mL of methanol followed by 3 mL of water to prepare the sorbent for use. A 100 μL aliquot of plasma spiked with 10 μL of working solutions of amygdalin with suitable concentrations and 800 μL of water were added to 1.5 mL Eppendorf centrifuge tubes. After vortexing for 1 min, the samples were applied to the SPE cartridges and subsequently washed with 3 mL of water. A 1.5 mL clean tube was positioned below the SPE cartridge and the compounds were eluted with 1 mL of methanol. The eluate was dried with nitrogen at 37°C. The residue was redisolved with 100 μL of the mobile phase and centrifuged for 10 min at 12,000 g, and 5 μL was injected into the LC–MS-MS system for analysis.

Method validation
The method was validated according to the US Food and Drug Administration (FDA) bioanalytical method validation guidance on specificity, sensitivity, precision, recovery and stability (22). Six blank plasma samples obtained from six different drug-free rats were analyzed to evaluate specificity of the assay. Each blank plasma sample was tested to demonstrate that there was no interference from endogenous components.

Linearity, sensitivity and matrix effect
The calibration curve was constructed by plotting the peak area ratios (y) of amygdalin to the IS versus the concentrations (x) of amygdalin in plasma. Linearity was determined by using a linear least-squares regression (weighting factor: 1/x²). The assay was evaluated with an eight-point calibration plot in the concentration range from 10 to 2,000 ng/mL.

The limit of determination (LOD) was defined as the plasma concentration that produced a signal-to-noise ratio (S/N) of 3. The limit of quantitation (LOQ) was defined as the lowest plasma concentration on the calibration curve that produced an S/N of 10 and was assessed by acceptable precision and accuracy (within 20%).

The matrix effect was evaluated by comparing the peak areas of the analytes added to pre-extracted samples of blank plasma with corresponding peak areas obtained from injections of standard solutions at appropriate concentrations.

Precision, accuracy and extraction recovery
The accuracy and precision of the method were validated by the analysis of QC plasma samples at three concentration levels (30, 300 and 1,500 ng/mL) with six replicates in three analytical runs. The accuracy was expressed as the percentage of difference between the mean detected concentrations and the nominal concentrations. The precision was expressed as relative standard deviation (RSD).

The extraction recovery of amygdalin and the IS were determined by comparing the mean peak areas of QC samples at three concentration levels (30, 300 and 1,500 ng/mL) with those of post-extracted blank plasma spiked with the corresponding concentrations.

Stability
The stability of amygdalin in rat plasma was assessed by using QC samples at three concentration levels (30, 300 and 1,500 ng/mL), which were exposed to different temperatures and durations of time. The short-term stability was determined after the exposure of QC samples to 25°C for 2 h and ready-to-inject samples (after extraction in the mobile phase) to the autosampler rack (25°C) for 24 h. The long-term stability was assessed after the storage of QC samples at −80°C for 30 days. The freeze/thaw stability was evaluated after three complete freeze/thaw cycles (−20°C to room temperature).

Results
LC–MS-MS method
Typical chromatograms of rat blank plasma, blank plasma spiked with amygdalin and the IS, and dosed rat plasma samples are presented in Figure 2. The chromatographic run time for the extracted plasma sample was 3 min. No interference from endogenous components was observed at the retention times of amygdalin (1.68 min) and the IS (1.11 min).

Selection of IS
An ideal IS should be a structurally similar to the analyte, but with better recovery under the current conditions. It was difficult to find a compound that could ideally mirror the analyte to serve as a suitable IS. Several compounds were investigated, such as lithospermose and salidroside, but they possessed some disadvantages such as low sensitivity and poor peak shapes under the current conditions. Geniposide was chosen for quantification as the IS because of its similarity to amygdalin in mass spectrographic behavior, retention behavior and extraction recovery.

Method validation
Linearity, sensitivity and matrix effect
The calibration curves were constructed by plotting the peak area ratio (y) of amygdalin to the IS versus the plasma concentration (x) of amygdalin. Good linear calibration curves were obtained over the concentration range from 10 to 2,000 ng/mL. The mean linear regression equation for the calibration curves of
amygdalin was \( y = 0.003x + 0.0214 \) (correlation coefficient \((r^2) = 0.9984, n = 6\) replicates). The LOQ of amygdalin was 10 ng/mL, which was sensitive enough for pharmacokinetic studies of amygdalin in oral administration to rats. The LOD of amygdalin was 1.25 ng/mL.

The matrix effect was estimated by the analysis of QC plasma samples at three concentration levels (30, 300 and 1,500 ng/mL, \( n = 6 \)); the average matrix effects were found to be 94.9 ± 4.1%, 105.5 ± 3.9% and 99.5 ± 4.4% for 30, 300 and 1,500 ng/mL, respectively. The matrix effect of the IS was 92.4 ± 5.3% at the

Figure 2. Chromatograms for amygdalin (left) and the IS (right): blank plasma sample (A); blank plasma sample spiked with 10 ng/mL of amygdalin (at the LOQ) and 40 ng/mL of the IS (B); blank plasma sample spiked with 250 ng/mL amygdalin and 200 ng/mL of the IS (C); rat plasma sample 6 h after oral dose of amygdalin (20 mg) (D).
Table I
Precision and Accuracy of the Method for the Analysis of Amygdalin (n = 6)

<table>
<thead>
<tr>
<th>Spiked concentration (ng/mL)</th>
<th>Intra-day</th>
<th>Inter-day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Measured concentration (ng/mL)</td>
<td>Precision (%)</td>
</tr>
<tr>
<td>30</td>
<td>30.8 ± 0.80</td>
<td>2.6</td>
</tr>
<tr>
<td>300</td>
<td>309.6 ± 7.74</td>
<td>6.4</td>
</tr>
<tr>
<td>1,500</td>
<td>1,452.2 ± 62.44</td>
<td>4.3</td>
</tr>
</tbody>
</table>

Table II
Stability Test of Amygdalin in Rat Plasma (n = 6)

<table>
<thead>
<tr>
<th>Storage conditions</th>
<th>Concentration (ng/mL)</th>
<th>Precision (%)</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spiked</td>
<td>Measured</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Short-term stability (2 h at 25°C)</td>
<td>30 29.3 ± 0.84</td>
<td>2.9</td>
<td>97.7</td>
</tr>
<tr>
<td></td>
<td>300 308.8 ± 10.40</td>
<td>3.4</td>
<td>102.9</td>
</tr>
<tr>
<td></td>
<td>1,500 1,439.3 ± 59.70</td>
<td>4.1</td>
<td>96.0</td>
</tr>
<tr>
<td>Short-term stability (24 h at 25°C)</td>
<td>30 30.7 ± 0.89</td>
<td>2.6</td>
<td>102.3</td>
</tr>
<tr>
<td></td>
<td>300 318.0 ± 19.58</td>
<td>6.2</td>
<td>106.0</td>
</tr>
<tr>
<td></td>
<td>1,500 1,541.3 ± 35.64</td>
<td>2.3</td>
<td>102.8</td>
</tr>
<tr>
<td>Long-term stability (30 days at –80°C)</td>
<td>30 32.1 ± 1.16</td>
<td>3.6</td>
<td>107.0</td>
</tr>
<tr>
<td></td>
<td>300 283.1 ± 25.41</td>
<td>8.9</td>
<td>106.9</td>
</tr>
<tr>
<td></td>
<td>1,500 1,405.7 ± 76.58</td>
<td>5.4</td>
<td>93.7</td>
</tr>
<tr>
<td>Three freeze/thaw cycles (–20°C to room temperature)</td>
<td>30 31.4 ± 1.22</td>
<td>3.8</td>
<td>104.7</td>
</tr>
<tr>
<td></td>
<td>300 320.6 ± 25.41</td>
<td>5.4</td>
<td>110.5</td>
</tr>
<tr>
<td></td>
<td>1,500 1,443.4 ± 64.40</td>
<td>4.5</td>
<td>96.2</td>
</tr>
</tbody>
</table>

Figure 3. Plasma concentration–time curve of amygdalin in rat plasma after oral administration of 20 mg/kg of amygdalin to rats (n = 6).

Table III
Major Pharmacokinetic Parameters of Amygdalin in Rats (n = 6) after Oral Administration (20 mg/kg)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Unit</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cmax</td>
<td>ng/mL</td>
<td>1,702.52 ± 108.06</td>
</tr>
<tr>
<td>Tmax</td>
<td>h</td>
<td>1.50 ± 0.08</td>
</tr>
<tr>
<td>t1/2</td>
<td>h</td>
<td>8.45 ± 0.14</td>
</tr>
<tr>
<td>Ke</td>
<td>1/h</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>AUC0–24</td>
<td>ng h/mL</td>
<td>15,854.88 ± 641.47</td>
</tr>
<tr>
<td>AUC0–∞</td>
<td>ng h/mL</td>
<td>18,355.29 ± 648.35</td>
</tr>
</tbody>
</table>

Precision, accuracy and extraction recovery
The results of intra-day and inter-day precision and accuracy are summarized in Table I. The intra-day and inter-day precision values (RSD) were both within 10%, whereas the assay accuracy values ranged from 95.2 to 109.5%. The data indicated that the method shows good precision and accuracy.

The extraction recovery of amygdalin was calculated by analyzing five replicates at three concentration levels (30,300 and 1,500 ng/mL). The assay values of the extraction recovery values were 67.2 ± 6.1%, 74.4 ± 8.3% and 79.3 ± 7.6% for the low, middle and high concentrations, respectively. The recovery of IS was conducted in the same way and found to be 68.5 ± 4.6%.

Stability
The stability results are summarized in Table II. The amygdalin in rat plasma was found to be stable at 25°C for 2 h, at 25°C for 24 h and at −80°C for 30 days, at three freeze and thaw cycles. The stability of the QC samples (after extraction in the mobile phase) was acceptable after 2 and 24 h at 25°C and after three freeze and thaw cycles. Amygdalin in plasma stored at −80°C was stable for 30 days. The results showed that amygdalin remained stable in all steps of the determination.

Pharmacokinetic application
The presented method was successfully applied to the determination of amygdalin in the plasma of six rats after the administration of a single oral dose of 20 mg/kg of amygdalin. The concentration versus time profile is shown in Figure 3. The pharmacokinetic parameters of amygdalin were calculated by using noncompartmental model analysis and are listed in Table III. According to this table, the Cmax was 1,702.52 ± 108.06 ng/mL and the Tmax was 1.50 ± 0.08 h. The t1/2 was 8.45 ± 0.14 h. The AUC from zero to the last quantifiable time point (AUC0–t) and the AUC0–∞ were 15,854.88 ± 641.47 ng × h/mL and 18,355.29 ± 648.35 ng × h/mL, respectively.

Discussion
Amygdalin possesses satisfactory effects on relieving cough and asthma, lubricating bowels and relieving constipation. It has been used to treat various illness, such as asthma, bronchitis, emphyma, constipation, leprosy and leucoderma. However, few data were available regarding the pharmacokinetic characterization of amygdalin after oral administration. This study developed and validated a sensitive and specific LC–MS-MS method coupled with SPE for the quantification of amygdalin in rat plasma, and the selectivity, precision, accuracy, matrix effect, recovery and concentration of 200 ng/mL. There was no significant matrix effect for the analytes.
stability were found to be acceptable. Compared with previous methods, the major advantages of the present method include the requirement of a small volume of sample (100 μL); sample preparation; short run time (3.0 min) for each sample analysis, which makes it suitable for the routine assay of many biological samples; sensitive detection (LOD was 1.25 ng/mL with S/N > 3; LOQ was 10 ng/mL with S/N > 10); and freedom from the interference of endogenous substances. This method was shown to be adequate and reliable for the application to pharmacokinetic study of amygdalin in rats after oral administration at a dose of 20 mg/kg. Pharmacokinetic results showed that amygdalin can be detected immediately in plasma of rat within 5 min; a shorter $T_{\text{max}}$ (less than 2 h) indicated that amygdalin may be absorbed rapidly after oral administration. Meanwhile, the elimination of amygdalin may be slow in rats because of the longer $t_{1/2}$ values were approximately 8.45 h. The information described previously may be helpful for the further pharmacological studies and for the clinical use of amygdalin. In addition, further research on the elimination and metabolism of this drug is necessary to fully understand its in vivo distribution.

Conclusions
A sensitive and specific LC–MS-MS method has been developed for the quantification of amygdalin in rat plasma using SPE as a sample cleanup procedure. The method is specific, sensitive and accurate over a concentration range of 10–2,000 ng/mL. Furthermore, this method demonstrated a relatively short analysis time with good precision, selectivity, recovery and sample stability. The developed method has been successfully applied to pharmacokinetic studies of amygdalin following oral administration to rats.

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