Pharmacokinetic study and metabolite identification of the bidesmosidic triterpenoid saponin BTS-1 in rat plasma

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Abstract Assays based on high-performance liquid chromatography (HPLC) and liquid chromatography tandem mass spectrometry (LC–MS²) have been developed and validated for the determination and metabolite identification of the bidesmosidic triterpenoid saponin, BTS-1 (3-O-β-D-galactopyranosyl-(1→2)-[β-D-xylpyranosyl-(1→3)]-β-D-glucuronopyranosyl gypsogenin 28-O-α-L-arabinopyranosyl-(1→3)-β-D-xylpyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→2)-β-D-fucopyranoside), in rat plasma. The assay was successfully applied to a pharmacokinetic study in rats given a single oral dose of BTS-1 (400 mg/kg). The results indicated that the compound was rapidly absorbed ($T_{\text{max}} = 1.28 \pm 0.29$ h, $C_{\text{max}} = 37.4 \pm 5.6$ mg/mL) and slowly eliminated ($t_{1/2} = 13.2 \pm 6.6$ h). In addition, secondary glycosides and aglycones of BTS-1 were detected and identified. Since these metabolites are known to be active α-glucosidase inhibitors, they probably play an important role in mediating the pharmacological effects of the saponin.
1. Introduction

Triterpenoid saponins, a large category of secondary plant metabolites, are believed to be the main constituents of many traditional Chinese medicines (TCM) and are considered responsible for numerous pharmacological effects. One such compound known as BTS-1 with the chemical name 3-O-β-D-galactopyranosyl-(1→2)-[β-D-xylopyranosyl-(1→3)]β-D-glucuronopyranosylglycosgenin 28-O-α-L-arabinopyranosyl-(1→3)-β-D-xylopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→2)-β-D-fucopyranoside (Fig. 1) isolated from the roots of Gypsophila oldhamiana (family Caryophyllaceae), a plant widely distributed in the Northern regions of China. The roots have been used as an alternative to the roots of Stellria dichotoma var. Lanceolata Bge (Yin-Chai-Hu), the most common TCM for the treatment of fever, consumptive disease and diabetes.

The triterpenoid saponins of the roots of G. oldhamiana, which contain BTS-1 as a major compound, are known to be oleanane-type, some of which are 3-O-monoglucosides, 28-O-monogluco-sides and 3,28-O-bidesmosides. These saponins have been reported to exhibit anticancer and antidiabetic effects. In our previous research, the 28-O-monogluco-sidic saponins and their aglycones were shown to be the main constituents responsible for α-glucosidase inhibitory activity, while the 3,28-O-bidesmosidic saponins were found to be without this type of activity. Furthermore, we found that the content of the monoglucosidic saponins was much lower than that of the 3,28-O-bidesmosidic saponins.

In order to understand the pharmacological effects of the roots of G. oldhamiana, it is important to determine whether orally administered 3,28-O-bidesmosidic saponins are absorbed from the intestine and metabolized to their monoglucosidic saponins or aglycones. To this end, we carried out a pharmacokinetic study of BTS-1 in rat using a fully validated liquid chromatography mass spectrometric assay and in addition identified its metabolites.

2. Experimental

2.1. Chemicals and reagents

The reference standards were isolated from G. oldhamiana in our laboratory and their structures were fully characterized using chemical and spectroscopic methods (UV, IR, NMR and MS). The purity of the standards was > 98.0% as determined by HPLC. The internal standard (IS), glycyrrhizic acid (purity > 98.0%, Fig. 1) was obtained from the TCM Institute of Chinese Materia Medica (Nanjing, China). Water was purified using a Milli-Q50 SP water purification system (Millipore, MA, USA). HPLC-grade acetonitrile was purchased from TEDIA Company (Tedia Fairfield, OH, USA). The other reagents were of analytical purity and used as received.

2.2. Animals

The animal pharmacokinetic study was approved by the Animal Ethics Committee of China Pharmaceutical University, Nanjing, China. Male Sprague–Dawley rats (weight 180–220 g) were bought from the Shanghai SIPPR/BK Experimental Animal Co., Ltd. The rats were maintained in an air-conditioned animal house at 22 ± 2 °C and a relative humidity of 50 ± 10%. Water and food (laboratory rodent chow, Nanjing, China) were allowed ad libitum. The animals were acclimatized to the facilities for a week and then fasted with free access to water for 12 h prior to an experiment.

2.3. Sample collection

Rats were administrated an oral dose of 400 mg/kg BTS-1 suspended in an aqueous solution of 0.5% carboxymethylcellulose sodium. Blood samples (200 μL) were collected from the oculi chorioideae vein into heparinized Eppendorf tubes before dosing and at 0.084, 0.25, 0.5, 1, 2, 4, 6, 8, 10, 12, 24, 32, 40 and 48 h following the dose. Plasma samples were obtained by centrifuging at 4000 rpm for 10 min and frozen at −20 °C pending analysis.

2.4. Sample preparation

Plasma samples (50 μL) were spiked with 10 μL IS and extracted for 6 min with 1 mL n-butanol. After centrifugation (4000 rpm, 5 min), the supernatant was transferred to another vial and evaporated to dryness under a slow stream of nitrogen at room temperature. The residue was then reconstituted in 100 μL mobile phase (acetonitrile: 0.05% aqueous formic acid 30:70 v/v) and 20 μL injected into the HPLC system. Plasma samples prepared from blood samples taken 1 h after oral administration of BST-1 were used for to investigate metabolites.

2.5. Instrumentation and assay conditions

Chromatography was performed on an LC-2010 instrument (Shimadzu, Japan) equipped with a vacuum degasser, a quaternary pump, an autosampler and a UV detector set at 210 nm. Chromatographic separation was carried out on a C18 column (250 mm × 4.6 mm i.d., 5 μm; Welch Materials Inc., USA) maintained at 30 °C and protected by a C18 guard column (7.5 mm × 4.6 mm i.d.). The mobile phase consisted of (A) acetonitrile and (B) 0.05% (v/v) aqueous formic acid delivered at 1 mL/min according to the following linear gradient: 0–8 min 30–35% A; 8–16 min 35–36% A; 16–46 min 36–53% A; 46–56 min 30% A for re-equilibration. The injection volume was 20 μL.

Figure 1 Chemical structures of BTS-1 and glycyrrhizic acid (IS).
LC–MS\(^n\) \((n=2, 3)\) analysis was performed on an Agilent 1100 HPLC instrument coupled to an LC/MSD trap SL mass spectrometer (Agilent Technologies) via an ESI interface operated in the negative ion mode. Chromatography was carried out as above except the gradient was extended: 0–8 min 30–35% A; 8–16 min 35–36% A; 16–46 min 36–53% A; 46–62 min 53–75% A; 62–85 min 75–95%; 85–95 min 30% A for re-equilibration. The MS conditions were as follows: Nebulizer/drying gas N\(_2\) 9.0 L/min; temperature 350 °C, nebulizer pressure 40 psi; flow rate 0.3 mL/min; scan range 200–1800 amu. Data acquisition was performed using the Chemstation software (Agilent Technologies).

### 2.6. Preparation of calibration standards and quality control samples

A stock solution of BTS-1 was prepared in the initial mobile phase (acetonitrile:0.05% aqueous formic acid, 30:70 v/v) and diluted to appropriate concentrations to give standard solutions. Calibration standards containing BTS-1 (0.264, 11.4, 23.8, 62.0, 103.7 and 198.3 μg/mL) and IS (20.37 μg/mL) were prepared by spiking drug-free plasma (200 μL) with standard solutions (100 μL) and IS (10 μL). Quality control (QC) samples were prepared independently in a similar way at high, medium and low concentrations. All solutions were stored at 4 °C and found to be stable for 4 days.

### 2.7. Assay validation

This HPLC method was validated for selectivity, linearity, accuracy and precision (intra- and inter-day), sensitivity, absolute recovery and stability. Selectivity was initially investigated by comparing chromatograms of pooled blank rat plasma with the same matrix spiked with BTS-1 and IS. Pre-dose plasma samples were also collected from each rat in the pharmacokinetic study \((n=6)\), and the selectivity further evaluated by comparing chromatograms of pre-dose and post-dose plasma samples.

Linearity was assessed by linear regression of calibration curves based on peak areas using GraphPad Prism Version 5.01 (La Jolla, CA 92037, USA) with a 1/x\(^2\) weighting factor. Plasma BTS-1 concentrations in the pharmacokinetic study were all within this calibration range.

Sensitivity was examined by determination of the limit of detection (LOD) and lower limit of quantitation (LLOQ) defined as the concentrations with signal-to-noise ratios of 3 and 10, respectively. Intra- and inter-day accuracy and precision were determined by calculation of the means and relative standard deviations (RSDs) for QC samples (2.64, 23.8 and 103.7 μg/mL) analysed on four different days.

 Absolute recovery was calculated by comparing peak areas of BTS-1 in QC samples with standard solutions of the same concentration. Stability of BTS-1 in rat plasma was evaluated in QC samples stored for 4 days at 4 °C, 2 weeks at −20 °C and after three freeze–thaw cycles (−20 °C to 24 °C) cycles.

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![Figure 2](image-url)  
**Figure 2** HPLC chromatograms of BTS-1: (A) blank rat plasma; (B) blank rat plasma spiked with BTS-1 and glycyrrhizic acid; (C) a plasma sample collected 30 min after oral administration of 400 mg/kg BTS-1.
2.8. Pharmacokinetic study

The analytical method was applied to a pharmacokinetic study in rats administered a single oral dose of 400 mg/kg BTS-1. Blood samples were collected before and at 0.083, 0.25, 0.5, 1, 2, 4, 6, 8, 10, 12, 24, 32, 40 and 48 h after oral administration, transferred to heparinized Eppendorf tubes and centrifuged at 4000 rpm for 10 min. Plasma was then collected and frozen at −20°C until analysis. Pharmacokinetic parameters were determined from concentration–time data by noncompartmental methods using DAS Software (version 1.0, Medical College of Wannan, PR China). \( C_{\text{max}} \) and \( T_{\text{max}} \) values were obtained directly from the data. All results are expressed as mean±standard deviation (SD).

3. Results and discussion

3.1. Sample preparation and chromatography

Two methods of sample preparation were tested viz liquid–liquid extraction (LLE) and protein precipitation. Simple protein precipitation with either methanol, acetonitrile or other reagents did not provide satisfactory recovery. LLE using either ethyl acetate, ethyl acetate–methanol, \( n \)-butanol, ethyl acetate–isopropanol or ethyl acetate–acetone was tested over various extraction times (5, 10, 20 and 30 min) and \( n \)-butanol was found to give the best recovery and reproducibility.

With regard to the chromatography, the inclusion of 0.05% formic acid in the mobile phase was found to improve peak shape by inhibiting the ionization of BTS-1. Other parameters were investigated (column, column temperature, gradient, flow rate and detection wavelength), and it was found that symmetric peaks and better resolution was achieved using an Ultimate XB-C18 column at 30°C with a flow rate of 1 mL/min.

3.2. Assay validation

Chromatographs of blank rat plasma, blank rat plasma spiked with analyte and IS and a sample collected 30 min after oral administration of BTS-1 are shown in Fig. 2. The retention times of BTS-1 and IS were 16.8 and 22.5 min, respectively, with baseline separation and no evidence of interfering peaks. The LOD and LLOQ were found to be 0.032 and 0.089 \( \mu \)g/mL, respectively. This method was validated according to the acceptance criteria of bioanalytical method validation. The assay was linear \( (r^2>0.99) \) with a typical calibration equation of \( y=0.0058x+0.7133 \) where \( x \) was the concentration of BTS-1, \( y \) was the ratio of peak area of BTS-1 to that of IS. Precision and accuracy data based on analysis of QC samples are presented in Table 1. Intra- and inter-day accuracies were in the range 89.7–95.7% and 91.4–100.5% with RSD<10% in both cases indicating the method has acceptable reproducibility. Recovery (Table 2) was in the range 70.0–92.4% with RSD<10%. Stability results indicate that there was no significant degradation of the analyte for 4 days at 4°C and for up to 2 weeks at −20°C (Table 2).

3.3. Pharmacokinetic study

The mean plasma concentration–time profiles of BTS-1 (\( n=5 \)) after a single oral dose are shown in Fig. 3 with corresponding pharmacokinetic parameters presented in Table 3. The mean \( C_{\text{max}} \) Table 1

<table>
<thead>
<tr>
<th>Spiked (( \mu )g/mL)</th>
<th>Intra-day (( n=6 ))</th>
<th>Inter-day (( n=4 ))</th>
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<tbody>
<tr>
<td></td>
<td>Mean* (( \mu )g/mL)</td>
<td>Accuracy (%)</td>
</tr>
<tr>
<td>103.7</td>
<td>103.2±0.1</td>
<td>95.7</td>
</tr>
<tr>
<td>23.8</td>
<td>23.3±0.1</td>
<td>92.5</td>
</tr>
<tr>
<td>2.64</td>
<td>2.39±0.13</td>
<td>89.7</td>
</tr>
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*Data are expressed as mean±SD.

Table 2

<table>
<thead>
<tr>
<th>Spiked (( \mu )g/mL)</th>
<th>Recovery (%)</th>
<th>RSD (%)</th>
<th>Storage at 4°C</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Stability (%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Freeze-thaw cycles</td>
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<tr>
<td>103.7</td>
<td>91.8±2.8</td>
<td>3.65</td>
<td>98.7±2.4</td>
</tr>
<tr>
<td>23.8</td>
<td>92.4±3.3</td>
<td>2.48</td>
<td>93.8±3.5</td>
</tr>
<tr>
<td>2.64</td>
<td>70.0±5.8</td>
<td>3.83</td>
<td>96.2±4.8</td>
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</table>

Figure 3 Mean plasma concentration–time profiles of BTS-1 in rats after a single 400 mg/kg oral dose of BTS-1. Data are mean±SD (\( n=5 \)).
and $T_{\text{max}}$ values, 37.4 ± 5.6 μg/mL and 1.28 ± 0.29 h respectively, were obtained directly from the results. Plasma concentration declined with a $t_{1/2}$ of 13.2 ± 6.6 h. These results indicate that BTS-1 is rapidly absorbed and eliminated slowly in rats.

3.4. Identification of metabolites

Tandem mass spectrometric fragmentation behavior of saponins has been extensively investigated$^{11–13}$, allowing the characterization of unknown compounds in the absence of reference standards. In the present study, ionization in the negative ion mode was found to give greater signal intensity of the triterpenoid saponins than in the positive ion mode.

To identify the metabolites of BTS-1 in rat plasma, LC–MS$^n$ ($n=2, 3$) was utilized to analyze blood samples collected 1 h after oral administration, a time corresponding to the $T_{\text{max}}$ of BTS-1 (Fig. 3). Total ion current (TIC) chromatograms are shown in Fig. 4. The main triterpenoid saponins were well detected and exhibited quasi-molecular [M–H]$^-$ ions. Six metabolites were identified by comparing retention times and MS/MS data (Table 4) with those of authentic compounds or literature data$^{3,4}$. On the basis of these results, the metabolic pathways shown in Fig. 5 are proposed. The findings suggest that some of an orally administered dose of BTS-1 is quickly metabolized to its secondary glycosides and aglycone$^{14}$. These findings imply that although 3,28- O-bidesmosidic saponins have been shown to be inactive as α-glucosidase inhibitors$^3$, they can deglycosylate to monoglucosidic saponins or their alycones in vivo and thereby exert important biological effects. Overall, this study shows that information on the metabolism of TCM is necessary to understand their pharmacologic activities.

4. Conclusions

HPLC and LC–MS$^n$ methods have been successfully developed for the determination of the bidesmosidic triterpenoid saponin BTS-1 and identification of its metabolites in rat plasma. It was found that BTS-1 is rapidly absorbed and eliminated slowly after

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**Table 3** Mean pharmacokinetic parameters for BTS-1 in rats ($n=5$) after a single 400 mg/kg oral dose.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value (mean ± SD)</th>
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<tbody>
<tr>
<td>$T_{\text{max}}$ (h)</td>
<td>1.28 ± 0.29</td>
</tr>
<tr>
<td>$C_{\text{max}}$ (μg/mL)</td>
<td>37.4 ± 5.6</td>
</tr>
<tr>
<td>$V_{d}$ (L)</td>
<td>43.0 ± 3.4</td>
</tr>
<tr>
<td>$Cl_{\text{tot}}$ (L/h)</td>
<td>13.19 ± 6.58</td>
</tr>
<tr>
<td>$AUC_{(0→∞)}$ (μg/mL·h)</td>
<td>121.8 ± 16.8</td>
</tr>
<tr>
<td>$AUC_{(0→∞)}$ (μg/mL·h)</td>
<td>138.3 ± 13.2</td>
</tr>
<tr>
<td>$MRT_{(0→∞)}$ (h)</td>
<td>12.8 ± 1.6</td>
</tr>
</tbody>
</table>

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**Table 4** Retention times and LC–MS$^n$ data for metabolites in rat plasma after a single 400 mg/kg oral dose of BTS-1.

<table>
<thead>
<tr>
<th>No.</th>
<th>$t_R$ (min)</th>
<th>[M–H]</th>
<th>LC–MS$^n$ m/z (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18.4</td>
<td>1495.6</td>
<td>MS$^2[1495]$: 939(58), 1025 (4), 807 (3)</td>
</tr>
<tr>
<td>2 or 2'</td>
<td>19.2</td>
<td>1363.9</td>
<td>MS$^2[1495→939]: 807(51), 563 (42), 469 (34)</td>
</tr>
<tr>
<td>3</td>
<td>26.3</td>
<td>939.6</td>
<td>MS$^2[1364]: 939 (18)</td>
</tr>
<tr>
<td>4</td>
<td>27.6</td>
<td>807.4</td>
<td>MS$^2[1364→939]: 807(46), 713 (31), 583 (58)</td>
</tr>
<tr>
<td>5</td>
<td>28.2</td>
<td>1025.0</td>
<td>MS$^2[939]: 807(43), 479 (75)</td>
</tr>
<tr>
<td>6</td>
<td>48.5</td>
<td>470.1</td>
<td>MS$^2[907]: 645(43)</td>
</tr>
</tbody>
</table>

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Figure 4  Total ion current (TIC) chromatograms of BTS-1 metabolites in a rat plasma sample taken 1 h after a single 400 mg/kg oral dose of BTS-1. Numbers 2–5 denote compounds (see Fig. 5).
oral administration to rats, the elimination involving metabolism to secondary glycosides and aglycones with α-glucosidase inhibitory activity.

Acknowledgments

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References