LC-MS/MS analysis and pharmacokinetic study on five bioactive constituents of Tanreqing injection in rats

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[ABSTRACT] Tanreqing injection (TRQ), a well-known traditional Chinese medicine formula, is commonly used to treat respiratory diseases. In the present study, a rapid, selective, and sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed and validated to simultaneously determinate the plasma contents of 5 major constituents of TRQ, including chlorogenic acid (CHA), caffeic acid (CFA), baicalin (BA), ursodeoxycholic acid (UDCA) and chenodeoxycholic acid (CDCA) in rats after intravenous administration of TRQ. Chromatographic separation was performed on an Agilent Zorbax SB-C18 column (3.5 μm, 100 mm × 2.1 mm), with acetonitrile and 0.1% aqueous formic acid as mobile phase at a flow rate of 0.3 mL·min⁻¹. The calibration curves were linear over the ranges of 27.0–13333.0 ng·mL⁻¹ for CFA, 30.0–14933.0 ng·mL⁻¹ for CHA, 50.0–50333.0 ng·mL⁻¹ for BA, 550.0–55000.0 ng·mL⁻¹ for UDCA, and 480.0–48000.0 ng·mL⁻¹ for CDCA, respectively. Intra- and inter-day precisions (relative standard deviations, RSDs) were from 3.11% to 14.08%. The extraction recoveries were greater than 71% and accuracy (relative recovery) was from 89% to 137% for all analytes, except endogenous bile acids. This validated method was successfully applied to the first pharmacokinetic study of CFA, CHA, BA, UDCA and CDCA in rat plasma after intravenous administration of TRQ.

[KEY WORDS] Tanreqing Injection; Flavones; Phenolic acids; Bile acids; Pharmacokinetics

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Introduction

Traditional Chinese medicine (TCM), which originated in ancient China, has been widely used for prevention and treatment of various diseases in China and other countries[1]. Tanreqing injection (TRQ) is a well-known TCM formula and won a big total sale in 2010. Produced from five herb raw materials, including Scutellariae Radix, Fel selenarcti, Cornu naemorhedi, Lonicerae japonicae Flos, and Forsythiae fructus, TRQ has markedly curative effects on the infections of upper respiratory tract and some serious influenza in China [2-4]. Our previous chemical profile analyses have found that TRQ contains flavones from Scutellariae Radix and Forsythiae fructus, cholic acids from Fel selenarcti, amino acids from Cornu naemorhedi, phenolic acids from Lonicerae japonicae Flos as major constituents. Beside, its fingerprint and qualitative analyses are performed by using a high performance liquid chromatography coupled with photodiode array detection and evaporative light scattering detection (HPLC-DAD-ELSD) method, which is useful for its quality control [5]. Establishing the pharmacokinetic basis for TCM efficacy favors the clinical application [6], but it is challenging to simultaneously determine major chemical markers representing all kinds of compounds in TRQ, in biological fluids within a single run.

Recently, several studies have developed HPLC, LC-MS/MS and/or UPLC-MS/MS methods for major chemical markers in different biological samples, such as baicalin, baicalein, wogonin, wogonoside, oroxylin A, chrysin, forsythiaside A, chlorogenic acid, neochlorogenic acid,
cryptochlorogenic acid, 3, 4-dicaffeoylquinic acid, and 3, 5-dicaffeoylquinic acid (all belong to flavones and phenolic acids), following oral administration of Scutellariae Radix, Forsythiae fructus, Lonicerae japonicae Flos, and their other TCM formulae [7-13]. Pharmacokinetics study about TRQ has been found only in a few reports, which have focused on three compounds including baicalin, ursodeoxycholic acid and chenodeoxycholic acid [14-15], ignoring other bioactive compounds in TRQ.

FDA has strongly encouraged monitoring the active ingredients, representative markers and/or major chemical components of TCM and TCM formulas [16]. An in vitro analytical method for TRQ has been established in our previous work, using chlorogenic acid (CHA), caffeic acid (CFA), baicalin (BA), ursodeoxycholic acid (UDCA) and chenodeoxycholic acid (CDCA) as main bioactive compounds [5] (Fig. 1). The present study was designed to develop a simple, rapid, and reliable LC-MS/MS method for in vivo assay of aforementioned five bioactive compounds in rat plasma which was successfully applied in its pharmacokinetic study after intravenous administration of TRQ.

Fig. 1 Chemical structures of five analytes and two internal standards

**Materials and Methods**

**Chemicals and materials**

Methanol and acetonitrile (MS-grade) were purchased from Merck (Darmstadt, Germany). Deionized water was prepared by a Milli-Q50 Reagent Water System (Bedford, MA, USA). Other reagents were of analytical grade.

Reference standards of CHA (batch number 110753-200413), CFA (110885-200102), UDCA (110755-9003), CDCA (110806-200704), puerarin (PA, internal standard-1, 110648-200954), and rutin (RT, internal standard-2, 110903-200825) were purchased from the Chinese Institute for the Control of Pharmaceutical and Biological Products (Beijing, China); BA (26695) was purchased from Aladdin Reagents (Shanghai, China) (all purities > 98%). TRQ injections (batch number 110616) were obtained from Shanghai Kaibao Pharmaceutical Co., Ltd. (Shanghai, China). Concentrations of CHA, CFA, BA, UDCA, and CDCA used in the present study were 43.9, 192.6, 8 121.0, 7 942.0 μg·mL⁻¹, respectively, using the previous HPLC-DAD-ELSD method [5].

**Instrumentation and chromatographic conditions**

LC-MS system consisted of an Agilent 1200 series HPLC and an Agilent 6410 triple-quadrupole mass spectrometer equipped with an electrospray ionization source, and Agilent 6410 Quantitative Analysis version B.01.02 analyst data processing software was applied for data acquisition and processing (Santa Clara, CA, USA). An Agilent Zorbax SB-C18 column (3.5 μm, 2.1 mm × 100 mm) was kept at 35 ºC. Gradient elution program using acetonitrile with 0.1% formic acid (A) and 0.1% aqueous formic acid solution (B) as mobile phases was as follows: 0–1 min, 10%–55% A; and 1–10 min, 55% A. The flow rate was set at 0.3 mL·min⁻¹ and the injection volume was 10 μL.

MS detection was conducted in multiple reaction monitoring (MRM) in the negative mode with the optimized parameters: capillary voltage, 4.0 kV; nebulizer pressure, 40 psi; gas temperature, 350 °C; drying gas flow, 10 L·min⁻¹; and high-purity nitrogen for collision-induced dissociation, 0.1 MPa. The MRM conditions were m/z 353.1→191.1, 179.0→135.1, 445.1→269.1, 391.3→391.3, 391.3→391.3, 609.1→300.1, and 415.1→267.0 for CHA, CFA, BA, UDCA, CDCA, RT, and PA, respectively (Fig. 1). The values of fragmentation/collision energy were set at 100 V/20 eV, 70 V/13 eV, 120 V/12 eV, 120 V/18 eV and 175 V/20 eV for CHA, CFA, BA, RT, and PA, respectively.

**Preparation of standard and quality control samples**

Stock solutions of CHA, CFA, BA, UDCA, and CDCA were prepared separately in methanol at 1.0 mg·mL⁻¹.
Working standards were prepared by independent dilution of above stock solutions for each compound with 50% methanol aqueous solution in the same volumetric flasks at seven or eight different levels. Calibration samples were prepared by spiking 100 µL of working standards to 900 µL of blank plasma samples to obtain final concentrations of 30.0, 60.0, 149.0, 597.0, 1 493.0, 2 987.0, 5 973.0, and 1 4933.0 ng·mL\(^{-1}\) for CHA, 27.0, 54.0, 133.0, 533.0, 1 333.0, 2 667.0, 5 333.0, and 13 333.0 ng·mL\(^{-1}\) for CFA, 50.0, 201.0, 503.0, 1 006.0, 2 013.0, 10 066.0, 20 133.0, and 50 333.0 ng·mL\(^{-1}\) for BA, 550.0, 1 100.0, 2 200.0, 5 500.0, 11 000.0, 22 000.0, and 55 000.0 ng·mL\(^{-1}\) for UDCA, and 1933.0, 4 833.0, 9 667.0, and 19 333.0 and 48 333.0, and 55 000.0 ng·mL\(^{-1}\) for CDCA, respectively. Quality control (QC) samples were prepared at low, medium and high concentrations. Be specifically, 149.0, 1 493.0, and 5 973.0 ng·mL\(^{-1}\) for CHA, 133.0, 1 333.0, and 5 333.0 ng·mL\(^{-1}\) for CFA, 201.0, 2 013.0, and 20 133.0 ng·mL\(^{-1}\) for BA, 1 100.0, 5 500.0, and 22 000.0 ng·mL\(^{-1}\) for UDCA, and 967.0, 1 933.0, 4 833.0, and 19 333.0 for CDCA. The stock solutions were stored at 4 °C until use.

**Sample preparation**

Rat plasma (100 µL) was transferred to a centrifuge tube (1.5 mL). 300 µL of acidified methanol-acetonitrile (3 : 1, 1/V, 0.1% formic acid), which containing 1 400 ng·mL\(^{-1}\) RT and 1 000 ng·mL\(^{-1}\) PA, was added for protein precipitate. After 2-min vortexing and 10-min centrifugation at 10 000 r·min\(^{-1}\); the supernatant was re-centrifuged at 10 000 r·min\(^{-1}\) for 10 min, and 100 µL of the supernatant was transferred to a new centrifuge tube (1.5 mL), adding 100 µL of water. After 0.5-min vortexing, the supernatant (150 µL) was collected and 10 µL was directly injected onto the LC-MS system for analysis.

**Method validation**

Selectivity was assessed by comparing the chromatograms of blank rat plasma with the corresponding spiked samples. Calibration curves were constructed by plotting the peak area ratios of the CHA, CFA, BA, UDCA and CDCA to RT or PA versus their respective concentrations in plasma. LLOQ of the assay was the lowest concentration of the calibration curve with accuracy and precision within 20%. Precision and accuracy were assessed by QC samples on the same day (intra-day) and on the three consecutive days (inter-day), and expressed as relative standard deviation (RSD %). Extraction recovery was analyzed by the determination of three QC levels, and calculated by comparing the peak area obtained from the pre-spiked with those post-spiked QC samples. Matrix effect was measured as the ratio of the peak area of samples spiked post-extraction with those of standard solutions and the area obtained from standard solutions at equivalent concentrations. Stability was evaluated by measuring three concentrations of QC samples (n = 5) under different conditions: long-term stability for 30 days of storage at −20 °C, short-term stability for 8-h storage at room temperature, three freeze (−20 °C)- thaw (room temperature) cycles, and post-preparation stability at 4 °C for 24 h in the auto-sampler. Carry-over was measured by injecting a blank rat plasma sample following the three injections of QC samples at high level of concentration [17].

**Pharmacokinetic study**

Adult male SD rats, weighing 200–220 g, were obtained from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). The rats were housed in well-ventilated cages at (20 ± 1) °C and (50% ± 10%) air humidity in a 12h/12h light/dark cycle. The study protocols were approved by the Animal Ethics Committee of Second Military Medical University, Shanghai, China. Eight rats were fasted with free access to water for 12 h. Six rats received 2.5 mL·kg\(^{-1}\) of TRQ by intravenous infusion via tail vein, and two rats were intravenously administrated with normal saline. The orbital blood samples (about 0.3 mL) were collected into heparinized tubes before infusion (0 h) and at 5, 10, 15, 20, 25, 30, and 45 min, 1, 1.5, 2, 3, 4, 6, 8, 12, and 24 h after dosing The plasma was immediately separated by centrifugation at 3 000 r·min\(^{-1}\) for 5 min and stored at 20 °C until analysis.

Pharmacokinetic parameters, including C\(_{\text{max}}\), T\(_{\text{max}}\), t\(_{1/2}\), AUC, and MRT, were calculated using Drug and Statistics DAS 3.2.6 (DAS DAS 3.2.6, Mathematical Pharmacology Professional Committee of China, Shanghai, China), using non-compartmental model.

**Results and Discussion**

**Method development**

BA had good responses in both modes, and the intensity obtained in positive mode was higher than that in negative mode. But for the other analytes, the intensities observed in negative mode were better than that in positive mode. Thus, all analytes were analyzed in negative mode. Since formic acid in the mobile phase contributed to overcome the peak-tailing effect for phenolic compounds such, as CHA and CFA [18] and low concentration of formic acid had less impact on ion suppression in negative mode, the best peak shape and ionization were achieved using acetonitrile with 0.1% formic acid. CDCA and UDCA which showed no prominent product ionization were achieved using acetonitrile with 0.1% formic acid. CDCA and UDCA which showed no prominent product ion were assessed using a MS/MS transition without fragmentation, and only the collision energy for each compound was optimized [19]. Owing to highly different chemical properties of five analytes, two ISs were chosen for analyses: RT for CFA and CDA, and PA for BA, CDCA, and UDCA, respectively.

Protein precipitation (PPT), liquid-liquid extraction (LLE) and solid phase extraction (SPE) were tested in the present study. It was found that LLE was time-consuming and SPE was costly and tedious. Therefore, simple and quick PPT was investigated using different kind of precipitants, and a mixture of methanol and acetonitrile (3 : 1, V/V) with 0.1% formic acid gave effective absolute extraction efficiencies for all the compounds and IS.

A certain residual effect was found for BA, which was present at a high concentration in plasma samples. To
minimize this effect, the post-precipitated sample was diluted with equivoluminal water, and the LC-MS system was washed by acetonitrile for 30 min, after every 30 analyses. As endogenous substances, bile acids could be also detected pre-administration. As a result, when concentrations of CDCA and UDCA in TRQ-dosed rats were under two times less than those in the control rats, it was recognized that both bile acids were eliminated. Besides, a simplified method was used to reduce effect caused by the endogenous bile acids in the assay. Actual concentrations of bile acids were defined as the plasma concentration from TRQ-dosed rats minus that from control rats at the equivalent times.

Method validation

Fig. 2 shows the typical chromatograms of blank plasma sample, LLOQ sample and plasma sample at 15 min post-administration of TRQ. For all the samples analyzed, no significant interferences from endogenous substances were observed at the retention times of CHA (3.298 min), CFA (4.189 min), and BA (5.366 min), CDCA (8.411 min), and UDCA (6.607 min), respectively.

The calibration curves were linear over the concentration ranges of 27.0–13 333.0 ng·mL$^{-1}$ for CFA, 30.0–14 933.0 ng·mL$^{-1}$ for CHA, 50.0–50 333.0 ng·mL$^{-1}$ for BA, 550.0–55 000.0 ng·mL$^{-1}$ for UDCA, and 480.0–48 000.0 ng·mL$^{-1}$ for CDCA, respectively, with good linearity being observed for all analytes with coefficients in range of 0.996 3–0.999 9. Representative regression equations were $y = 0.000 1x - 0.002 0$ for CFA, $y = 0.000 1x - 0.003 9$ for CHA, $y = 0.000 1x + 0.001 6$ for BA, $y = 0.000 9x + 0.134 5$ for UDCA, and $y = 0.000 9x - 0.052 4$ for CDCA, respectively.

Intra- and inter-day precisions are presented in Table 1, with all RSDs being within 15% at each QC level. The stability results showed that the relative errors (RE) for all the analytes were below 10% in rat plasma for 30-day-storage at $-20$ °C, below 8% for 8-h-storage at room temperature, below 11% for three freeze-thaw cycle process, and below 9% for 24-h-storage in the autosampler at 4 °C, respectively. These results suggested that the present assay had acceptable
were endogenous substances, had non-neglectable impact on the matrix effect, especially when the analytes were in the low concentration, the values could up to 231.38%. Overall, the results of matrix effects were all in the acceptable ranges.

**Table 1 Matrix effects, recoveries, intra- and inter-day precisions of analytes**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Spiked (ng·mL&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Extration recovery (%)</th>
<th>RE (%)</th>
<th>Matrix effect (%)</th>
<th>Intra-day (a = 5) (%)</th>
<th>Inter-day (a = 5) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHA</td>
<td>149</td>
<td>85.94 ± 16.63</td>
<td>-4.25</td>
<td>112.44 ± 15.46</td>
<td>3.35</td>
<td>-12.05</td>
</tr>
<tr>
<td></td>
<td>1 493</td>
<td>91.01 ± 4.91</td>
<td>-12.58</td>
<td>97.80 ± 5.26</td>
<td>9.20</td>
<td>6.56</td>
</tr>
<tr>
<td></td>
<td>5 973</td>
<td>87.42 ± 1.08</td>
<td>-11.24</td>
<td>97.82 ± 2.12</td>
<td>4.12</td>
<td>5.05</td>
</tr>
<tr>
<td>CFA</td>
<td>133</td>
<td>93.62 ± 3.52</td>
<td>-11.05</td>
<td>99.36 ± 6.87</td>
<td>3.51</td>
<td>-13.91</td>
</tr>
<tr>
<td></td>
<td>1 333</td>
<td>102.53 ± 5.59</td>
<td>-14.20</td>
<td>92.05 ± 11.04</td>
<td>3.17</td>
<td>5.00</td>
</tr>
<tr>
<td></td>
<td>5 333</td>
<td>97.58 ± 0.86</td>
<td>-13.02</td>
<td>89.94 ± 3.52</td>
<td>3.82</td>
<td>6.23</td>
</tr>
<tr>
<td>BA</td>
<td>201</td>
<td>71.49 ± 2.04</td>
<td>-11.82</td>
<td>130.43 ± 7.00</td>
<td>3.19</td>
<td>-12.92</td>
</tr>
<tr>
<td></td>
<td>2 013</td>
<td>73.65 ± 7.81</td>
<td>0.81</td>
<td>128.28 ± 7.14</td>
<td>3.96</td>
<td>-1.45</td>
</tr>
<tr>
<td></td>
<td>20 133</td>
<td>77.45 ± 5.13</td>
<td>8.24</td>
<td>121.11 ± 7.84</td>
<td>3.54</td>
<td>9.84</td>
</tr>
<tr>
<td>UDCA</td>
<td>1 100</td>
<td>85.58 ± 11.92</td>
<td>-5.43</td>
<td>136.30 ± 18.15</td>
<td>4.32</td>
<td>-6.47</td>
</tr>
<tr>
<td></td>
<td>5 500</td>
<td>97.39 ± 3.76</td>
<td>5.37</td>
<td>130.80 ± 6.12</td>
<td>4.36</td>
<td>4.37</td>
</tr>
<tr>
<td>CDCA</td>
<td>967</td>
<td>95.49 ± 7.93</td>
<td>0.36</td>
<td>224.68 ± 6.70</td>
<td>3.98</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>4 833</td>
<td>95.11 ± 4.15</td>
<td>0.90</td>
<td>164.28 ± 7.77</td>
<td>4.81</td>
<td>-0.26</td>
</tr>
<tr>
<td></td>
<td>19 333</td>
<td>95.37 ± 4.36</td>
<td>0.71</td>
<td>138.06 ± 6.09</td>
<td>5.18</td>
<td>-0.02</td>
</tr>
</tbody>
</table>

* RE is expressed as (measured concentration/spiked concentration -1) × 100%

Carry-over effect was negligible when peak areas of five analytes were less than 20% of LOQ for one blank rat plasma sample, which was injected after analysis of high-level QC sample. Although post-precipitated sample was diluted with sample, which was injected after analysis of high-level QC sample, this validated method was successfully applied to a pharmacokinetic study of five bioactive compounds in rat plasma after intravenous administration of TRQ. Mean plasma concentration-time curves are shown in Fig. 3. The plasma pharmacokinetic profiles and parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CFA</th>
<th>CHA</th>
<th>BA</th>
<th>UDCA</th>
<th>CDCA</th>
</tr>
</thead>
<tbody>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; (ng·mL&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>364.21 ± 36.22</td>
<td>261.61 ± 35.98</td>
<td>48 781.0 ± 5 194.5</td>
<td>47 051.4 ± 4 771.8</td>
<td>18 561.5 ± 3 864.9</td>
</tr>
<tr>
<td>t&lt;sub&gt;1/2&lt;/sub&gt; (h)</td>
<td>0.13 ± 0.03</td>
<td>0.30 ± 0.07</td>
<td>2.35 ± 1.08</td>
<td>0.23 ± 0.11</td>
<td>0.18 ± 0.15</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0→&lt;inf&gt;inf&lt;/inf&gt;&lt;/sub&gt; (ng·h·mL&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>57.50 ± 6.13</td>
<td>69.03 ± 11.59</td>
<td>23 364.5 ± 3 653.5</td>
<td>10 135.7 ± 3 929.0</td>
<td>3 059.7 ± 744.1</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0→&lt;inf&gt;inf&lt;/inf&gt;&lt;/sub&gt; (ng·h·mL&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>67.61 ± 6.99</td>
<td>78.01 ± 11.89</td>
<td>23 805.3 ± 3 843.8</td>
<td>10 560.8 ± 4 019.1</td>
<td>3 527.4 ± 1 583.6</td>
</tr>
<tr>
<td>AUMC&lt;sub&gt;0→&lt;inf&gt;inf&lt;/inf&gt;&lt;/sub&gt; (ng·h·mL&lt;sup&gt;-2&lt;/sup&gt;)</td>
<td>4.79 ± 0.48</td>
<td>15.80 ± 5.17</td>
<td>45 000.5 ± 11 469.0</td>
<td>2 265.9 ± 2 732.4</td>
<td>325.5 ± 328.8</td>
</tr>
<tr>
<td>MRT&lt;sub&gt;0→&lt;inf&gt;inf&lt;/inf&gt;&lt;/sub&gt; (h)</td>
<td>0.08 ± 0.00</td>
<td>0.23 ± 0.04</td>
<td>1.91 ± 0.25</td>
<td>0.18 ± 0.13</td>
<td>0.01 ± 0.08</td>
</tr>
</tbody>
</table>
quickly, in parallel to previous publications \[24-25\]. More, UDCA had larger pharmacokinetic parameters than that of CDCA, which was in agreement with the previous reports \[14-15\]. Maybe there were four reasons for this. First, UDCA dose (mean concentration 7 794.0 \(\mu\)g·mL\(^{-1}\) in TRQ) was higher than CDCA dose (1 564.0 \(\mu\)g·mL\(^{-1}\)) after TRQ administration. Second, UDCA could reduce the intestinal absorption of endogenous bile acids \[26\]. Third, hydrophilic UDCA stimulates, but hydrophobic CDCA lowers, the secretion of the hepatic biles \[27-29\]. And lastly, more hydrophilic UDCA stimulates the receptor-dependent LDL uptake in the liver, but more hydrophobic CDCA decreases the LDL receptor activity \[30-31\]. On the other hand, our results indicated that concentrations of both bile acids from TRQ-dosed rats had no significant difference, compared with those from controls at 1 h after administration. It is known that CDCA concentrations in mouse serum are low during most time of the day with highest level at 02:00, and UDCA concentration was higher in the dark phase and lower in the late-light phase \[32-33\]. From our results of the present study that both bile acids were present in low plasma levels in early-light phase with a quick elimination, it was assumed to be influence of the circadian rhythm. As the conjugation/de-conjugation of BAs also exhibit a diurnal rhythm in rats \[21\], the fast elimination might be attributed to the self-regulation in vivo of the organism \[14-15\]. This would make sense that \(t_{1/2}\) (h) values varied in our experiment and Hu’s study.

**Conclusion**

It was the first time that, using a simple, sensitive and validated LC-MS/MS method, CFA, CHA, BA, UDCA, and CDCA were simultaneously determined in a pharmacokinetic study of TRQ after iv administration. The pharmacokinetic evaluation of the target components may contribute to a better understanding of mechanisms for the pharmacological actions and provide important information on clinical efficacy of TRQ.

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