A UPLC−MS/MS approach for simultaneous determination of eight flavonoids in rat plasma, and its application to pharmacokinetic studies of Fu-Zhu-Jiang-Tang tablet in rats

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Abstract
The purpose of this study is to establish and validate a UPLC−MS/MS approach to determine eight flavonoids in biological samples and apply the method to pharmacokinetic study of Fu-Zhu-Jiang-Tang tablet. A Waters BEH C18 UPLC column was employed with methanol/0.1% formic acid–water as mobile phases. The mass analysis was carried out in a triple quadrupole mass spectrometer using multiple reaction monitoring with negative scan mode. A one-step protein precipitation by methanol was used to extract the analytes from blood. Eight major flavonoids were selected as markers. Our results showed that calibration curves for 3′-hydroxyquercitrin, mirificin, puerarin, 3′-methoxyquercitrin, daidzin, rutin, astragalin and daidzein displayed good linear regression (r² > 0.9986). The intra- and inter-day precisions (RSD) of the eight flavonoids at high, medium and low levels were <8.03% and the bias of the accuracies ranged from −5.20 to 6.75%. The extraction recoveries of the eight flavonoids were from 91.4 to 100.5% and the matrix effects ranged from 89.8 to 103.8%. The validated approach was successfully applied to a pharmacokinetic study in Sprague–Dawley rats after oral administration of FZJT tablet. Double peaks were emerged in curves of mean plasma concentration for 3′-methoxyquercitrin, which was reported for the first time.

KEYWORDS
Fu-Zhu-Jiang-Tang tablet, pharmacokinetics, UPLC−MS/MS

1 | INTRODUCTION

Fu-Zhu-Jiang-Tang (FZJT) tablet, which consists of six well-known traditional Chinese medicines – Pueraria lobata (PL), Morus alba (MA), Panax notoginseng (PN), Astragalus membranaceus (AM), Momordica charantia (MC) and Cortex lycii (CL) – has been proven to show beneficial effects on patients with type II diabetes. A number of articles have been published on the traditional use of the six individual herbs for the treatment of diabetes (Habicht, Ludwig, Yang, & Krawinkel, 2014; Li, Wang, Xue, Gu, & Lin, 2011; Ulbricht et al., 2015; Uzayisenga, Ayeka, & Wang, 2014; Zhang, Pugliese, Pugliese, & Passantino, 2015). For instance, the nonanthocyanin phenolics of MA known to date are rutin, quercetin and 2-cafeoylquinic acid isomers, which exhibit anti-diabetic effects through inhibition of α-glucosidase (Zhang, Han, He, & Duan, 2008). Isoflavones, such as puerarin and daidzin, are the major constituents of PL, and exert hypoglycemic and hypolipemic roles by elevating insulin expression and maintaining metabolic homeostasis (Wu et al., 2013a). Ginsenosides, which belong to PN, can improve lipid profiles, inhibit peroxidation and increase the activity of antioxidant enzymes (Xia, Sun, Zhao, & Hypolipidemic, 2011). Astragalosides, which are the major constituents of AM, show protective effects on kidney of diabetic rats (Motomura et al., 2009). The extract of MC displays insulin secretagog and insulominimetic activity, which may be ascribed to steroidal compounds and polypeptides (Raman & Lau, 1996). Our previous study characterized and determined the major constituents of FZJT tablet using UPLC−Q−TOF/MS and HPLC−UV (Tao et al., 2016). Several analytical approaches have been established for pharmacokinetic determination of the above-mentioned anti-diabetic constituents in vivo. For example, He et al. developed a sensitive
LC–MS/MS method to simultaneously determine rutin and astragalin in rat plasma after oral administration of total flavonoids from mulberry leaves (He et al., 2013). Xu, Li, and Zhang, (2015) quantified puerarin and daidzin in rat plasma using LC–MS/MS and applied them to a pharmacokinetic study of Gegenqinlian decoction. Furthermore, Yan et al. (2014) established an LC–MS/MS method to simultaneously quantify puerarin, daidzin and daidzein in female rat plasma following oral administration of Ge-Gen decoction. These studies just focus on one or two herbal. There is no report on the method for the pharmacokinetic study of the six-herb preparation – FZJT tablet. The pharmacokinetic behavior of FZJT tablet after oral administration remains unclear.

Pharmacokinetics is a branch of pharmacology dedicated to determining the fate of substances administered externally to a living organism, which is of great importance in drug development and can explain a variety of events related to the efficacy of herbal medicines (Katz, Murray, Bhathena, & Sahelijo, 2008). The composition of herbal preparations is complex and different constituents may have interactions. The pharmacokinetics of multi-herbal preparations may provide valuable information for their clinical usage. In this study, our aim was to develop, optimize and validate a simple, sensitive and specific UPLC–MS/MS method for the simultaneous determination of the bioactive compounds in rat plasma after oral administration of the FZJT total fraction.

Our previous study showed that steroidal compounds and polyphenols were not detected using UPLC–Q–MS/MS. Therefore, only compounds which can be detected were used for pharmacokinetic analysis. Meanwhile, these eight flavonoids showed anti-diabetic effects. For instance, rutin and astragalin exhibited anti-diabetic effects through inhibition of α-glucosidase (Tao, Zhang, Cheng, & Wang, 2013). Puerarin, daidzin and daidzein have been found to significantly decrease the baseline fasting plasma glucose and total cholesterol and increase insulin levels in several in vivo models (Liu et al., 2015). 3′-Hydroxypuerarin, 3′-methoxypuerarin, mirificin and daidzein exhibited anti-diabetic effects by reducing oxidative stress to which the pancreatic cells were exposed (Bebrevska et al., 2010). In addition, standards for the eight flavonoids were available. Thus, the eight flavonoids were used as the determining index.

2 | EXPERIMENTAL

2.1 | Chemicals and reagents

3′-Hydroxypuerarin, puerarin, 3′-methoxypuerarin, mirificin, daidzin and daidzein were obtained from Chengdu Purechem standard Co. Astragalin and 2,3,5,4′-tetrahydroxy-stilbene-2-O-β-D-glucoside (IS) were obtained from Sichuan Weikeqi Biotechnology Co. Rutin was purchased from Chengdu Must Bio-technology Co. The purity of each standard substance was >98%. FZJT tablet (lot no. 150320) and FZJT total fraction were obtained from By-Health Co. Methanol and acetonitrile were obtained from Merck (Darmstadt, Germany). Ultra-pure water used in the experiment was generated by a Milli-Q water purification system (Millipore Corporation, Billerica, MA, USA). All other chemicals were of analytical grade.

Sprague-Dawley rats (280 ± 20 g) were obtained from Slaccas Experiment Animal Company (Shanghai, China). The rats were maintained in an air-conditioned room under the constant room temperature (23 ± 2°C), relative humidity level (50 ± 15%) and lighting (12 h light/dark cycle) with free access to food and water for 7 days before the experiment. Before the herb extract administration, the rats were fasted overnight but had access to water ad libitum. The experimental protocols were reviewed and approved by the Animal Ethics Committee of Nanjing University of Chinese Medicine.

2.2 | UPLC–MS/MS conditions

Liquid chromatography was carried out on a Shimadzu ultra performance liquid chromatography (UPLC) unit with an Acquity BEH C18 column (2.1 × 50 mm, 1.7 μm particle size) and inline 0.2 μm stainless steel frit filter. The elution solvents were optimized using methanol-water or acetonitrile-water (with or without 0.1% formic acid) as the mobile phase system. Finally, a gradient program was employed with the mobile phase of solvent A (0.1% formic acid in water) and solvent B (methanol) as follows: 5–30% B (0–2 min), 30% B (2.0–2.5 min), 30–100% B (2.5–6.0 min), 100% B (6.0–7.0 min). A subsequent re-equilibration time (1 min) should be performed before next injection. The flow rate was 0.30 mL/min and the injection volume was 5 μL. The column temperature was maintained at 35°C.

An AB scieix QTRAP 5500 triple quadrupole mass spectrometer was employed for mass spectrometric detection. The detection was operated in the multiple reaction monitoring (MRM) mode under unit mass resolution in the mass analyzers. The mass parameters for each compound were optimized, including cone voltage, collision energy and so on. The MRM analysis was conducted by monitoring the precursor ion to product ion transitions of m/z 431.0 → 311.0 for 3′-hydroxypuerarin, 415.0 → 267.0 for puerarin, 547.0 → 295.0 for mirificin, 445.1 → 325.0 for 3′-methoxypuerarin, 415.2 → 251.9 for daidzin, 609.2 → 299.9 for rutin, 447.1 → 283.9 for astragalin, 253.1 → 224.0 for daidzein, 405.2 → 243.0 for 2,3,5,4′-tetrahydroxy-stilbene-2-O-β-D-glucoside (IS), respectively. Analyst (version 1.5.2, AB Scieix) and PeakView software (version 1.2, AB Scieix) was used for data acquisition and instrument control.

2.3 | Stock solutions

Reference standards of 3′-hydroxypuerarin, puerarin, mirificin, daidzin, rutin, astragalin, 3′-methoxypuerarin, daidzein and IS were separately weighed and dissolved in methanol to prepare stock solutions. A series of standard solutions were obtained by further dilution of the stock solution with methanol. Calibration samples were prepared by adding the series standard solutions (10 μL) to blank rat plasma (190 μL) to obtain concentrations of 1.2–156.5 ng/mL for 3′-hydroxypuerarin, 2.0–1251 ng/mL for mirificin, 1.1–188.4 ng/mL for puerarin, 1.0–1250 ng/mL for 3′-methoxypuerarin, 0.8–250 ng/mL for daidzin, 1.8–250.8 ng/mL for rutin, 1.5–125.2 ng/mL for astragalin and 1.3–500.4 ng/mL for daidzein.

Quality control (QC) samples were prepared independently at concentrations of 4.5, 50 and 120 ng/mL for 3′-hydroxypuerarin, 10.5, 90 and 1050 ng/mL for mirificin, 3.5, 40 and 165 ng/mL for puerarin, 3.2, 45 and 150 ng/mL for 3′-methoxypuerarin, 3, 35 and 195 ng/mL for daidzin, 8.5, 75 and 195 ng/mL for rutin, 5.2, 65 and 100 ng/mL for astragalin.
105 ng/mL for astragalin and 5.5, 55 and 350 ng/mL for daidzein using the same method.

2.4 Sample preparation

Protein precipitation and liquid–liquid extraction methods were attempted and compared during sample preparation. For protein precipitation, the effects of methanol and acetonitrile on extraction rate were investigated. For liquid–liquid extraction, ethylacetate, chloroform and n-hexane were used to extract the compounds from plasma samples.

As a result, 100 μL of collected plasma sample was transferred to a 1.5 mL centrifuge tube. An aliquot of 300 μL of the IS working solution (30 ng/mL in methanol) was added to the plasma sample. The tubes were vortexed for 3 min and then centrifuged at 13,000 g for 5 min. The supernatant was injected into the UPLC-MS/MS system for analysis.

2.5 Method validation

2.5.1 Selectivity and specificity

The selectivity of the method was evaluated by comparing the chromatograms of blank plasma with the corresponding plasma samples spiked with the eight flavonoids, as well as real samples collected from treated rats.

Specificity toward the endogenous plasma matrix constituents was assessed by comparing the chromatograms of blank rat plasma from six sources, blank plasma spiked with the analytes at the concentrations of the lower limit of quantification (LLOQ) and the plasma samples from the rats after oral administration of FZJT total fraction.

2.5.2 Linearity and sensitivity

Calibration curves were constructed by plotting the peak-area ratio of the eight flavonoids in both positive and negative ionization modes against the concentrations of the flavonoids. The regression equations were achieved using a weighted least-squares linear regression (the weighting factor, 1/x²). The lower limit of quantification (LLOQ) was determined based on at least 10 times of signal-to-noise ratio at which the precision (expressed by relative standard deviation, RSD) and accuracy (calculated by relative error, RE) were lower than ±20%.

2.5.3 Precision and accuracy

Three validation batches, each containing six replicates of QC samples at low, medium and high concentration levels (4.5, 50 and 120 ng/mL for 3′-hydroxyauratin, 10.5, 90 and 1050 ng/mL for mirificin, 3.5, 40 and 165 ng/mL for puerarin, 3.2, 45 and 150 ng/mL for 3′-methoxyauratin, 3, 35 and 195 ng/mL for daidzin, 8.5, 75 and 195 ng/mL for rutin, 5.2, 65 and 105 ng/mL for astragalin and 5.5, 55 and 350 ng/mL for daidzein) were assayed to assess the precision and accuracy of the method on three different days. The precision is expressed by relative standard deviation (RSD) between the replicate measurements. Accuracy is defined as relative error (RE), which is calculated using the formula RE = [(measured value – theoretical value)/theoretical value] × 100.

2.5.4 Matrix effect and extraction recovery

The extraction recoveries of the analytes at the three QC levels were evaluated by comparing the peak area ratios of the analytes with the IS in the post-extraction spiked samples with those acquired from the pre-extraction spiked samples. The matrix effects were measured by comparing the peak areas of the analytes in the post-extraction spiked samples with those of the standard solutions. The extraction recovery and matrix effect of the analytes at the three QC levels were repeated for six replicates.

2.5.5 Stability

Six QC samples of each concentration at low, medium and high levels were prepared in the blank plasma and stored at −20°C for the freeze–thaw experiments. Three freeze–thaw cycles were carried out within 36 h to evaluate the freeze–thaw stability. Short-term stability was conducted by storing the QC samples at room temperature for 4 h. Long-term stability was assessed on the same QC samples which had been stored at −20°C for 30 days. Autosampler stability was performed by setting pretreated QC samples in autosampler (the temperature was 4°C) for 24 h before analysis.

2.6 Pharmacokinetic study of FZJT tablet

Six Sprague–Dawley rats weighing 280 ± 20 g were fasted for 12 h before the experiment, with the exception of free access to water. The experiment was approved by the Animal Ethics Committee of Nanjing University of Chinese Medicine. According to the manufacturer, 1 g FZJT tablet consisted of 0.5 g FZJT total fraction and 0.5 g excipients. TIC chromatograms in negative and positive mode of FZJT total fraction are shown in Figure 1 (in the Supporting Information). The routine dose for human is 6 g FZJT tablet/day. Thus, six rats received an intragastric administration of 2.7 g/kg FZJT total fraction (equivalent to 8.30 mg/kg of 3′-hydroxyauratin, 2.69 mg/kg of mirificin, 66.90 mg/kg of puerarin, 53.35 mg/kg of 3′-methoxyauratin, 9.40 mg/kg of daidzin, 7.35 mg/kg of rutin, 5.48 mg/kg of astragalin and 0.65 mg/kg of daidzein). The FZJT total fraction was suspended in 0.5% carboxymethyl cellulose sodium (w/v). Blood samples (about 500 μL) were collected in heparinized tubes via the postorbital venous plexus veins from each rat before the dose and at 0.08, 0.176, 0.333, 0.67, 1.0, 2.0, 4.0, 6.0, 8.0, 12.0 and 24.0 h after administration, and were immediately centrifuged. Plasma was transferred into clean tubes and stored at −80°C until analysis. The pharmacokinetic parameters were calculated by the noncompartmental analysis of plasma concentration vs time data using the DAS 2.0 software package.

3 RESULTS AND DISCUSSION

3.1 Optimization of mass spectrometry conditions

The chemical structures of the eight flavonoids and IS are displayed in Figure 1. The mass responses of the eight flavonoids in both positive and negative modes were investigated, with better response obtained in the negative ionization mode (see Figure). This phenomenon may be explained by the loss of a proton to yield [M − H]⁻ being easier than...
adding an proton to produce \([M + H]^+\) for the eight flavonoids under soft ionization mode. Throughout the development of the method, the selectivity, reproducibility and robustness of the MS method were monitored and adjusted to establish methods that could be validated and applied to a pharmacokinetic study. The following MRM modes were found to be specific and intense for the analysis of 

- 3′-hydroxypuerarin \((m/z 431.0–311.0)\), puerarin \((m/z 415.0–267.0)\), mirificin \((m/z 547.0–295.0)\), 3′-methoxypuerarin \((m/z 431.0–311.0)\), daidzin \((m/z 445.1–325.0)\), rutin \((m/z 609.2–299.9)\), astragalin \((m/z 447.1–283.9)\), daidzein \((m/z 253.1–224.0)\) and IS \((m/z 405.2–243.0)\). The details are presented in Table 1.

### 3.2 Optimization of liquid chromatography conditions

First, gradient elution was carried out using methanol–water or acetonitrile–water as the mobile phase system and the results are shown in Figure 1. For compounds 1, 3, 4 and 5, the methanol–water mobile phase system yielded better signal intensity than that of acetonitrile–water. For compounds 6–8, the acetonitrile–water mobile phase system yielded better signal intensity than that of methanol–water. Moreover, the signal intensities of the compounds were investigated by adding 0.1% formic acid to the water phase. The addition of 0.1% formic acid significantly increased the signal intensity of the eight compounds, especially for the methanol–water system. Meanwhile, the addition of 0.1% formic acid was beneficial to the peak shape of the compounds. Thus, the methanol–water (0.1% formic acid) system was selected as the elution solvent.

### 3.3 Optimization of extraction procedure

Protein precipitation and liquid–liquid extraction methods were attempted and compared during sample preparation. As shown in Figure 1, the result of the liquid–liquid extraction method was unsatisfactory, which may be due to the compounds having a high chemical polarity and not being able to be extracted by ethyl acetate, chloroform and n-hexane. Thus, protein precipitation was chosen to prepare plasma on account of its high recovery. The extraction rates of various organic solvents (methanol, acetonitrile) were compared. Finally, methanol was chosen as a reliable solvent for protein precipitation owing to its excellent precipitation, preferable recovery and small matrix effect.

### TABLE 1 Multiple reaction monitoring transitions and parameters for the detection of the analytes and IS

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Precursor ion ((m/z))</th>
<th>Product ion ((m/z))</th>
<th>Cone voltage (V)</th>
<th>Collision energy (eV)</th>
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<tr>
<td>3′-Hydroxypuerarin</td>
<td>431.0</td>
<td>311.0</td>
<td>-150</td>
<td>-32</td>
</tr>
<tr>
<td>Puerarin</td>
<td>415.0</td>
<td>267.0</td>
<td>-175</td>
<td>-42</td>
</tr>
<tr>
<td>Mirificin</td>
<td>547.0</td>
<td>295.0</td>
<td>-195</td>
<td>-38</td>
</tr>
<tr>
<td>3′-Methoxypuerarin</td>
<td>445.1</td>
<td>325.0</td>
<td>-175</td>
<td>-32</td>
</tr>
<tr>
<td>Daidzin</td>
<td>415.2</td>
<td>251.9</td>
<td>-200</td>
<td>-36</td>
</tr>
<tr>
<td>Rutin</td>
<td>609.2</td>
<td>299.9</td>
<td>-185</td>
<td>-48</td>
</tr>
<tr>
<td>Astragalin</td>
<td>447.1</td>
<td>283.9</td>
<td>-120</td>
<td>-38</td>
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<tr>
<td>Daidzein</td>
<td>253.1</td>
<td>224.0</td>
<td>-25</td>
<td>-34</td>
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<tr>
<td>IS</td>
<td>405.2</td>
<td>243.0</td>
<td>-90</td>
<td>-26</td>
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</table>

FIGURE 1 Chemical structures of eight compounds and 2,3,5,4′-tetrahydroxy-stilbene-2-O-β-D-glucoside (IS)
3.4 | Validation of the method

3.4.1 | Selectivity and specificity

At the retention times of 3'-hydroxypuerarin (2.18 min), mirificin (2.38 min), puerarin (2.41 min), 3'-methoxypuerarin(2.46 min), daidzin (2.67 min), rutin (2.86 min), astragalin (3.19 min), daidzein (3.75 min) and IS (2.98 min), no obvious endogenous interferences were observed from blank plasma. Representative plasma chromatographs, such as blank plasma, plasma at the LLOQ level and plasma samples obtained from intragastric administration of FZJT total fraction in rats are shown in Figure 2.

3.4.2 | Linearity and LLOQs

The calibration curves were constructed by plotting the ratio of peak areas of the eight flavonoids to that of IS vs the nominal concentration of calibration standards and fitted to linear regression (y = ax + b). The concentrations of analytes in test samples were calculated by the regression parameters from the calibration curves. The lowest limit of quantification (LLOQ) was defined as the lowest concentration of analyte in spiked plasma resulting in a signal-to-noise ratio of 10:1. The results in Table (Supporting Information) show that all of the analyte in spiked plasma resulting in a signal quantification (LLOQ) was defined as the lowest concentration of regression parameters from the calibration curves. The lowest limit of concentrations of analytes in test samples were calculated by the LLOQ level and plasma samples obtained from intragastric administration of FZJT total fraction in rats are shown in Figure 2.

3.4.3 | Precision and accuracy

The intra-day and inter-day precisions and accuracy of the eight flavonoids were investigated by analyzing three levels of QC samples. All the data are shown in Table S2. All the results of the tested samples were within the acceptance criterion of ±15%.

3.4.4 | Extraction recovery and matrix effect

The mean extraction recoveries of eight analytes were determined using six replicates of QC samples of each level. The results are shown in Table S3. The mean extraction recoveries of the eight flavonoids were from 91.4 to 100.5% and the matrix effects ranged from 89.8 to 103.8%. As seen from Table S3, the values of matrix effect >100% were only detected at the lower QC concentration of 3'-hydroxypuerarin, puerarin and rutin. This phenomenon may be explained by the systematic error of the UPLC-MS/MS instrument or the sampling error. The results indicated that the plasma matrix peaks did not affect the quantification of the eight flavonoids in the experimental conditions.

3.4.5 | Stability

The stability of the flavonoids during the sample processing procedures and storage was evaluated by analysis of three levels of QC samples. As shown in Table S4, the results indicated that the eight flavonoids in rat plasma were all stable for 4 h at room temperature, for 30 days' storage at −80°C, after three freeze–thaw cycles and at 4°C for 24 h, with accuracy in the range of −2.31–7.08, −7.81–9.36, −2.94–10.55 and −6.22–9.16%, respectively. The results suggest that the eight flavonoids are stable under these conditions.

3.5 | Pharmacokinetic analysis

This developed approach was applied to determine the plasma concentrations of the eight flavonoids after intragastric administration of FZJT tablet. The mean plasma concentration–time profiles of 3'-hydroxypuerarin, mirificin, puerarin, 3'-methoxypuerarin, daidzin, rutin, astragalin and daidzein are illustrated in Figure 3. In addition, the derived pharmacokinetic parameters are summarized in Table 2.

The assay was sensitive for the simultaneous determination of 3'-hydroxy puerarin, mirificin, puerarin, 3'-methoxy puerarin, daidzin, rutin, astragalin and daidzein in rat plasma after oral administration of FZJT total fraction.
The results in Table 2 indicate that flavonoids had homogeneous absorption rates, and the rank order of elimination half-life ($t_{1/2}$) was daidzein > 3′-methoxypuerarin > mirificin > rutin > puerarin > 3′-hydroxyypuerarin > daidzin > astragalin. The mean plasma concentration–time curve profiles of puerarin, 3′-methoxypuerarin, 3′-hydroxyypuerarin and mirificin exhibited a consistent trend in vivo owing to their parallel chemical structures. As shown in Figure 3 (A–D), puerarin, 3′-hydroxyypuerarin, mirificin and 3′-methoxypuerarin were quickly absorbed into the body and could be detected 5 min after oral administration. The four compounds achieved the maximum plasma concentration between 0.28 and 0.67 h. Meanwhile, the plasma concentrations of the four compounds were eliminated rapidly, within 4 h of intragastric administration. However, the pharmacokinetic parameters of 3′-methoxypuerarin were not entirely in accordance with the data reported in previous literature (Zhao, Zhao, Liu, Han, & Simultaneous, 2012). Double peaks emerged in curves of mean plasma concentration for 3′-methoxypuerarin (see Figure 3D), which is reported for the first time. A previous report showed that 3′-methoxypuerarin can be transformed into 3′-methoxy-6″-O-acetylpuerarin by Caco-2 cells (Wu, Xu, & Yang, 2013b). The double PK peak phenomenon of 3-methoxypuerarin might not be attributed to bacteria-mediated biotransformation, but can be explained by distribution, reabsorption and enterohepatic circulation.

As shown in Figure 3(E and F), daidzin and daidzein achieved the maximum plasma concentration at 0.56 and 0.67 h, respectively. The values of $t_{1/2}$ of daidzin and daidzein were 0.36 and 6.44 h, respectively. Meanwhile, the area under concentration–time curve (AUC) of daidzin was much higher than that of daidzein. Double peaks emerged in curves of mean plasma concentration for daidzein. This phenomenon was also been reported in the literature (Yan et al., 2014). The double PK peak phenomenon of daidzein might be attributed to the biotransformation of most daidzin into daidzein. It was reported that daidzin, isoflavone O-glycoside, was easily hydrolyzed by glucosidases in small intestine (Miao et al., 2013). Meanwhile, puerarin and mirificin were reported to be transformed into the same aglycone, daidzein, by Caco-2 cells (Wu, Wu, Wang, & Yang, 2015).
Astragalin and rutin are flavonoid glycosides. As shown in Figure 3 (G and H), the times to reach the maximum plasma concentration ($t_{\text{max}}$) of astragalin and rutin were 0.39 ± 0.14 and 0.44 ± 0.18 h, respectively. Meanwhile, their $t_{\frac{1}{2}}$ ranged from 0.31 to 0.76 h. It was demonstrated that two flavonoid glycosides were rapidly absorbed and eliminated in rat plasma after oral administration of FZJT total fraction.

Compared with several previous studies, the established method had three advantages (see Table 2). First, LLOQs of 3′-hydroxypuerarin, puerarin, mirificin, 3′-methoxypuerarin, daidzin and daidzein in this study were lower than those of previous studies. Second, a one-step protein precipitation method instead of liquid–liquid extraction method was employed in this study. The recoveries of 3′-hydroxypuerarin, puerarin, mirificin, 3′-methoxypuerarin, rutin and astragalin were much higher than those of previous studies. Lastly, the validated method can be applied to simultaneously determine the concentrations of eight flavonoids in plasma. The information described in the present study should be informative for future application of FZJT tablets in clinical therapy.

## 4 CONCLUSIONS

A multi-component pharmacokinetic study was conducted for the simultaneous determination of 3′-hydroxypuerarin, puerarin, mirificin, puerarin, 3′-methoxypuerarin, daidzin, rutin, astragalin and daidzein in rat plasma in negative ionization mode after oral administration of FZJT tablet by UPLC-MS/MS. The excellent selectivity, sensitivity, precision, accuracy and extraction recovery proved that the method is suitable for pharmacokinetic study. The analysis was fast and easy owing to the relative short chromatographic running time and straightforward sample pretreatment. The rapid absorption of bioactive constituents gave a reasonable explanation for the rapid effects of FZJT tablet in the treatment of type II diabetes. The co-existing compounds in FZJT tablet could alter the pharmacokinetic behaviors of these eight constituents compared with other prescriptions of traditional Chinese medicine. The results will be helpful for the further investigation of the mechanism of action of FZJT tablet and may provide a useful tool for its clinical application.

## ACKNOWLEDGMENTS

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## REFERENCES


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1. Replace (Ins) Tool – for replacing text.
   Strikethrough (Del) Tool – for deleting text.
   Add note to text Tool – for highlighting a section to be changed to bold or italic.
   Add sticky note Tool – for making notes at specific points in the text.

How to use it
- Highlight a word or sentence.
- Click on the Replace (Ins) icon in the Annotations section.
- Type the replacement text into the blue box that appears.
- Highlight a word or sentence.
- Click on the Strikethrough (Del) icon in the Annotations section.
- Click at the point in the proof where the comment should be inserted.
- Type the comment into the yellow box that appears.

How to use it
- Highlight a word or sentence.
- Click on the Add note to text icon in the Annotations section.
- Type instruction on what should be changed regarding the text into the yellow box that appears.
- Click on the Add sticky note icon in the Annotations section.
- Click at the point in the proof where the comment should be inserted.
- Type the comment into the yellow box that appears.
USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

5. **Attach File Tool** – for inserting large amounts of text or replacement figures.

- Inserts an icon linking to the attached file in the appropriate pace in the text.

  **How to use it**
  - Click on the **Attach File** icon in the Annotations section.
  - Click on the proof to where you’d like the attached file to be linked.
  - Select the file to be attached from your computer or network.
  - Select the colour and type of icon that will appear in the proof. Click OK.

6. **Add stamp Tool** – for approving a proof if no corrections are required.

- Inserts a selected stamp onto an appropriate place in the proof.

  **How to use it**
  - Click on the **Add stamp** icon in the Annotations section.
  - Select the stamp you want to use. (The **Approved** stamp is usually available directly in the menu that appears).
  - Click on the proof where you’d like the stamp to appear. (Where a proof is to be approved as it is, this would normally be on the first page).

7. **Drawing Markups** Tools – for drawing shapes, lines and freeform annotations on proofs and commenting on these marks.

- Allows shapes, lines and freeform annotations to be drawn on proofs and for comment to be made on these marks.

  **How to use it**
  - Click on one of the shapes in the **Drawing Markups** section.
  - Click on the proof at the relevant point and draw the selected shape with the cursor.
  - To add a comment to the drawn shape, move the cursor over the shape until an arrowhead appears.
  - Double click on the shape and type any text in the red box that appears.

For further information on how to annotate proofs, click on the Help menu to reveal a list of further options: