Pharmacokinetics of tectorigenin, tectoridi, irigenin, and iridin in mouse blood after intravenous administration by UPLC-MS/MS

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ABSTRACT
Tectorigenin, tectoridin, irigenin, and iridin are the four most predominant compounds present in She Gan. She Gan has been used in traditional Chinese medicine because of its anti-inflammatory, hepatoprotective, anti-tumor, antioxidant, phytoestrogen-like properties. In this paper, a UPLC-MS/MS method was developed to measure the pharmacokinetics of tectorigenin, tectoridin, irigenin, iridin after intravenous administration in mice. A UPLC BEH C18 (50 mm × 2.1 mm, 1.7 μm particle size) chromatographic column was utilized for separation of the four target analytes and internal standard (IS), and the analysis of blood plasma samples; the mobile phase consisted of an acetonitrile-water (w/0.1% formic acid) gradient elution. Electron spray ionization (ESI) positive-ion mode and multiple reaction monitoring (MRM) mode was used for quantitative analysis of the analytes and internal standard. The four compounds were administered intravenously (sublingual) at doses of 5 mg/kg. After blood sampling, samples were processed and then analyzed by UPLC-MS/MS. The linearity of the method was robust over the concentration range of 2–5,000 ng/mL. The intra-day precision of the analysis was within 15%, the inter-day precision was within 12%, and the accuracy was between 92% and 110%. The recoveries were 65–68%, and the matrix effect was 93–109%. The established UPLC-MS/MS detection method was then successfully applied to study the pharmacokinetics of tectorigenin, tectoridin, irigenin, iridin in mice.

KEYWORDS
tectorigenin, tectoridin, irigenin, iridin, UPLC-MS/MS, pharmacokinetics

INTRODUCTION
She Gan (Iris domestica (L.) Goldblatt & Mabb), also known as Wushan, Bianzhu, Jiaojiancao, Shanpushan, Yexuanhua, and Hudiehua, is a perennial herb that predominantly grows in the plains and grassland regions at low elevations in Eastern Asia [1, 2]. As a traditional Chinese medicine, its use was first recorded in the Shennong Materia Medica and is still being widely used in the clinic to date. Among the complicated chemical composition of She Gan, such as tectorigenin and irigenin, are the main active ingredients and exhibit anti-inflammatory, liver protection, anti-tumor, and antioxidant properties [3, 4]. However, the components and diverse metabolites of the crude plant extract are too complex for analysis. Therefore, we selected four active compounds produced in She Gan, tectorigenin, tectoridin, irigenin, and iridin, and investigated their pharmacokinetics in mice after intravenous administration.

There are various methods designed to measure the concentration of tectorigenin, in rat blood plasma and study their pharmacokinetics after administration. Yang et al. developed a
simple, reliable, and sensitive UPLC-MC/MS method to analyze the pharmacokinetic profiles of *Iris tectorum* Maxim extract in mice [5]. Compared the pharmacokinetic characteristics of tectorigenin in rat plasma between iris extract and pure tectoridin, which demonstrated that coexisting components in the iris extract significantly boost the absorption of the isoflavone of interest. Wang et al. measured the 9 tectorigenin metabolites and the concentrations of six primary metabolites in rat plasma [6]. They found that the total blood concentrations of the tectorigenin-bound metabolites were much higher than irigenin; extensive phase II metabolism played a vital role in the pharmacokinetics of tectorigenin in vivo. Li et al. developed a selective and convenient HPLC-MS/MS method to simultaneously measure galuteolin, tectoridin, tectorigenin, iridin, irigenin, arctiin, irisflorentin and arctigenin in rat plasma using acetonitrile to prepare the plasma samples by protein precipitation in order to effectively assess the pharmacokinetic profiles of the main components of SheJinliyan granules [7]. Zhang et al. established a sensitive and reliable ultra-high performance liquid chromatography-electrospray ionization tandem mass spectrometry (UHPLC-ESI-MS/MS) method that was capable of simultaneously determining the three active components in tectorigenin, irigenin, and irisflorentin, which was applied in the investigation of the pharmacokinetics of the compounds in rat plasma and urine after administering She Gan extract to the rats [8].

However, currently, there is no research related to the pharmacokinetics of tectorigenin, irigenin, and iridin in mice after sublingual intravenous injection. Therefore, in this article, a UPLC-MS/MS-based method was developed to measure the concentrations of tectorigenin, tectoridin, irigenin, and iridin in mice and determine the pharmacokinetic parameters of the compounds.

**EXPERIMENTAL**

**Reagents and animals**

Tectorigenin, tectoridin, irigenin, iridin, and midazolam (IS) (Fig. 1) were all purchased from Chengdu Must Pharmaceutical Co., Ltd (Chengdu, China). HPLC-grade methanol and acetonitrile were purchased from Merck Company (Darmstadt, Germany). Ultrapure water (resistance >18 mΩ) was produced by a Millipore Milli-Q purification system (Bedford, MA, USA). Mice (male, 22–25g) were obtained from the Animal Experiment Center of Wenzhou Medical University.

![Chemical structure of tectorigenin (A), tectoridin (B), irigenin (C), iridin (D) and internal standard (E)](image)

Fig. 1. Chemical structure of tectorigenin (A), tectoridin (B), irigenin (C), iridin (D) and internal standard (E)
Instrumentation and chromatographic conditions

The four compounds of interest were analyzed on a XEVO TQ-S micro triple quadrupole tandem mass spectrometer (Waters Corp., USA). The Masslynx 4.1 software (Waters Corp., USA) was utilized for data collection and instrument control. The chromatographic separation of the analytes was conducted on a UPLC BEH C18 (50 mm × 2.1 mm, 1.7 μm particle size). The temperature of the column was maintained at 40°C, and the mobile phase of the chromatography was acetonitrile-water (0.1% formic acid) with a flow rate of 0.4 mL/min. The UPLC method comprised 10% acetonitrile isocratic from 0.0 to 0.2 min, 10–90% acetonitrile from 0.2 to 1.2 min, 90% acetonitrile isocratic from 1.2 to 2.0 min, 90–10% acetonitrile from 2.0 to 2.2 min, followed by 10% acetonitrile from 2.2 to 3.0 min.

For the mass spectrometer, nitrogen was used as the cone gas (50 L/h) and desolvation gas (900 L/h). The capillary voltage was set to 3.0 kV, the ion source temperature was 150°C, and the desolvation temperature was 550°C. ESI positive-ion mode was used for analyte detection under the MRM mode by monitoring the transitions of m/z 301.1 → 286.0 (cone voltage 52 V, collision voltage 26 V) for tectorigenin, m/z 463.2 → 301.1 (cone voltage 10 V, collision voltage 16 V) for tectoridin, m/z 361.1 → 232.1 (cone voltage 52 V, collision voltage 42 V) for irigenin, m/z 523.2 → 361.1 (cone voltage 8 V, collision voltage 14 V) for iridin, and m/z 326.2 → 291.4 (cone voltage 25 V, collision voltage 25 V) for midazolam.

Calibration curve

Calibration curves of each of the four analytes plus the internal standard were generated to measure the concentrations of the analytes in mouse plasma. First, stock solutions (1.0 mg/mL) of tectorigenin, tectoridin, irigenin, iridin, and midazolam (internal standard) were prepared in 1:1 (1.0 mg/mL) of tectorigenin, tectoridin, irigenin, iridin, and midazolam (internal standard) were prepared in a 1:1 mixture of acetonitrile and water. Then, the stock solutions were diluted with acetonitrile to prepare a range of standard working solutions (20, 40, 50, 200, 1,000, 4,500, 5,000, 10,000, 20,000, 45,000, and 50,000 ng/mL). The resulting working solutions were stored at 4°C until use. The standard working solutions of the four compounds were diluted 10-fold in blank mouse plasma to prepare a series of solutions of the compounds (2, 5, 20, 100, 500, 1,000, 2,000, and 5,000 ng/mL) in mouse blood for the standard curve. Three quality control (Q.C.) samples at three different concentrations (4, 450, and 4,500 ng/mL) in mouse plasma were prepared analogous to the calibration solutions.

Sample preparation

Aliquots (20 μL) of the mouse blood and 100 μL of acetonitrile (containing 50 ng/mL midazolam) were added to a 1.5 mL Eppendorf tube, which was vortexed for 1.0 min, and centrifuged at 13,000 rpm/min and 4°C for 10 min. The supernatant (80 μL) was removed, added to the inner liner of the injection bottle, and injected (2 μL injection volume) into the UPLC-MS/MS for quantitative analysis.

Method validation

The UPLC-MS/MS method was validated by determining the selectivity, matrix effect, linearity, precision, accuracy, extraction recovery, and stability of the analytes. The selectivity was evaluated by comparing the chromatographic analysis of the blank mouse plasma samples, blank mouse plasma spiked with the four target compounds and midazolam, and the plasma obtained from the experimental mice. The diluted standard solutions prepared in the blank mouse plasma were injected in equal volume under the same chromatographic conditions. The area under each analyte peak was measured and plotted against the corresponding concentration of the solution, after which a calibration curve was generated. The resulting linear regression equation was used to calculate the concentrations of the experimental samples.

The three Q.C. samples (4, 450, and 4,500 ng/L in blank mouse plasma) were measured using six repetitions to assess the intraday accuracy and precision of the method. The relative error (RE, %) and relative standard deviation (RSD, %) reflected the accuracy and precision, respectively. To determine the matrix effect and the recovery rate, the peak areas of the Q.C. samples were compared to the peak areas of the equal-concentration standard solutions to evaluate the matrix effect and recovery rate. Finally, the stabilities of the working solutions of the four compounds in blank mouse plasma were evaluated in different environments and compared to freshly made solutions in blank mouse plasma. The different environments including storing at room temperature for 2 h, storing at −20°C for 30 d, and three freeze-thaw cycles, after which the respective solutions were injected into the UPLC-MS/MS system to determine the concentrations based on the peak areas of the analytes.

Pharmacokinetics studies

Twenty-four mice were randomly divided into four groups (n = 6). Tectorigenin, tectoridin, irigenin, iridin were administered intravenously (iv) (sublingual) at a dose of 5 mg/kg. At 0.08, 0.5, 1, 1.5, 2, 3, 4, 6, 8, and 10 h, 20 μL of blood was withdrawn from the tail vein, collected in a heparinized test tube, and injected into the UPLC. The pharmacokinetics software (DAS version 2.0) was used to calculate the pharmacokinetic parameters.

RESULTS

Selectivity

Figure 2 displays the UPLC-MS/MS chromatograms of the blank plasma sample and blank plasma spiked with tectorigenin, tectoridin, irigenin, and iridin. The results showed that the endogenous substances in the plasma sample did not have an effect on the analysis of the analytes.

Calibration curve

The calibration curves of the four analytes in blank mouse plasma demonstrated excellent linearity over the range of
concentrations measure (2–5,000 ng/mL). All four of the linear determination coefficients ($R^2$) were greater than 0.999. The regression equations obtained from the calibration curves were: $Y_1 = 0.0003x_1 - 0.0003$ ($R^2 = 0.9996$), $Y_2 = 0.0017x_2 + 0.0029$ ($R^2 = 0.9995$), $Y_3 = 0.0002x_3 + 0.0006$ ($R^2 = 0.9992$), and $Y_4 = 0.0011x_4 + 0.0046$ ($R^2 = 0.9999$), where $x$ represented the concentration of the analytes in the mouse blood, and $y$ represented the ratio of the analyte to the internal standard.

**Precision, accuracy, recovery, and matrix effects**

As shown in Table 1, the intraday and inter-day precisions for all of the analytes were within 15% and 12%, respectively, while the accuracies ranged from 92% to 110%. The recovery rate calculated range from 65% to 68%. In addition, the matrix effect was in the range of 93–109%.

**Stability**

After completing the three freeze-thaw cycles, after being stored in the autosampler for 2 h at room temperature, and after storing at −20°C for 30 d, all samples were found to be very stable under the conditions evaluated, and the method was capable of accurately measuring these different samples, which indicated that the method was robust and met the requirements for the analysis of biological samples, Table 2.

**Pharmacokinetics studies**

Figure 3 shows the concentration-time curves of tectorigenin, tectoridin, irigenin, and iridin in mouse blood after intravenous administration. The main pharmacokinetic parameters of the non-compartmental model are listed in
In this study, the doses of the four drugs injected sublingually into the mice were all 5 mg/kg. The AUC$_{0-t}$ values of tectorigenin, tectoridin, irigenin, and iridin were 219 ± 94, 500 ± 167, 124 ± 59, and 1,219 ± 457 ng/mL·h, which indicated that tectoridin and iridin had a higher absorption than tectorigenin and irigenin. The four compounds also had a relatively fast metabolism, which was demonstrated by their half-lives ($t_{1/2}$) of 0.3-2.4 h.

**Table 1.** Precision, accuracy, recovery and matrix effect of tectorigenin, tectoridin, irigenin, and iridin in mouse blood ($n = 6$)

| Compound | Concentration (ng/mL) | Accuracy (%) | Precision (%) | Matrix effect | Recovery |
|----------|-----------------------|--------------|---------------|---------------|----------|
|          | Intra-day             | Inter-day    | Intra-day     | Inter-day     |          |
| Tectorigenin | 2                      | 95.3         | 97.8          | 5.2           | 8.4      | 100.0 | 73.7 |
|           | 4                      | 100.1        | 107.7         | 14.5          | 6.2      | 99.3  | 69.3 |
|           | 450                   | 101.8        | 109.6         | 5.0           | 5.2      | 93.1  | 70.2 |
|           | 4,500                 | 101.3        | 92.0          | 6.2           | 5.0      | 99.9  | 82.9 |
| Tectoridin  | 2                      | 98.6         | 99.1          | 12.5          | 11.1     | 106.7 | 67.5 |
|           | 4                      | 103.3        | 109.4         | 6.4           | 8.8      | 108.4 | 69.2 |
|           | 450                   | 103.5        | 101.5         | 1.5           | 3.7      | 109.2 | 69.3 |
|           | 4,500                 | 92.3         | 100.1         | 8.2           | 7.7      | 107.2 | 69.4 |
| Irigenin   | 2                      | 95.6         | 106.2         | 7.4           | 6.1      | 105.5 | 64.3 |
|           | 4                      | 96.2         | 107.5         | 13.5          | 7.7      | 103.5 | 72.0 |
|           | 450                   | 96.7         | 99.8          | 7.7           | 6.3      | 94.2  | 65.5 |
|           | 4,500                 | 102.1        | 103.6         | 6.3           | 7.4      | 103.5 | 85.2 |
| Iridin     | 2                      | 107.0        | 106.9         | 9.4           | 6.9      | 97.1  | 67.0 |
|           | 4                      | 103.5        | 101.7         | 5.6           | 10.0     | 100.0 | 65.4 |
|           | 450                   | 101.5        | 99.0          | 6.1           | 9.0      | 107.2 | 71.6 |
|           | 4,500                 | 95.9         | 98.9          | 3.8           | 6.0      | 105.5 | 72.5 |

**Table 2.** Stability of tectorigenin, tectoridin, irigenin, and iridin in mouse blood

| Compound | Concentration (ng/mL) | Ambient (2 h) | Autosampler (4°C, 12 h) | −20°C 30d | Freeze-thaw |
|----------|-----------------------|--------------|-------------------------|------------|-------------|
|          | Accuracy RSD | Accuracy RSD | Accuracy RSD | Accuracy RSD | Accuracy RSD |
| Tectorigenin | 4                     | 100.1        | 4.9                    | 97.5       | 5.2         | 105.2       | 8.7         | 101.2       | 7.2         |
|           | 450               | 101.3        | 5.1                    | 101.3      | 6.1         | 99.9        | 13.0        | 98.0        | 5.1         |
|           | 4,500             | 100.1        | 11.7                   | 101.8      | 8.3         | 104.2       | 9.9         | 97.6        | 3.4         |
| Tectoridin | 4                    | 101.0        | 1.5                    | 101.5      | 8.1         | 100.0       | 8.9         | 106.8       | 5.2         |
|           | 450               | 99.0         | 3.7                    | 104.7      | 6.3         | 99.0        | 11.1        | 103.2       | 8.4         |
|           | 4,500             | 101.4        | 7.1                    | 101.6      | 12.4        | 97.8        | 10.8        | 98.6        | 7.5         |
| Irigenin | 4                    | 101.3        | 7.4                    | 96.2       | 6.1         | 102.1       | 10.7        | 98.6        | 7.9         |
|           | 450               | 97.1         | 6.4                    | 97.1       | 6.5         | 99.7        | 2.6         | 99.4        | 5.0         |
|           | 4,500             | 99.8         | 6.1                    | 101.9      | 3.8         | 100.3       | 5.9         | 103.7       | 7.7         |
| Iridin  | 4                    | 98.0         | 5.0                    | 101.7      | 5.6         | 96.8        | 10.0        | 101.9       | 8.4         |
|          | 450               | 101.4        | 9.0                    | 103.5      | 3.8         | 97.6        | 6.3         | 105.2       | 6.5         |
|          | 4,500             | 102.5        | 9.9                    | 101.5      | 6.8         | 99.0        | 6.0         | 99.1        | 3.9         |

**Fig. 3.** The concentration-time curve of tectorigenin, tectoridin, irigenin, and iridin in the blood of mice

In this study, the doses of the four drugs injected sublingually into the mice were all 5 mg/kg. The AUC$_{0-t}$ values of tectorigenin, tectoridin, irigenin, and iridin were 219 ± 94, 500 ± 167, 124 ± 59, and 1,219 ± 457 ng/mL·h, which indicated that tectoridin and iridin had a higher absorption than tectorigenin and irigenin. The four compounds also had a relatively fast metabolism, which was demonstrated by their half-lives ($t_{1/2}$) of 0.3-2.4 h.

**DISCUSSION**

In this study, a UPLC-MS/MS method was developed for the separation and quantitation of compounds in biological samples; compared to traditional HPLC, UPLC-MS/MS is significantly faster and more sensitive [9–13]. Only 3.5 min
Table 3. The main pharmacokinetic parameters of mice after sublingual intravenous injection of tectorigenin, tectoridin, irigenin, and iridin

| Parameters | Unit     | Tectorigenin | Tectoridin | Irgenin | Iridin |
|------------|----------|--------------|------------|---------|--------|
| AUC(0-t)   | ng/mL·h  | 219 ± 94     | 500 ± 167  | 124 ± 59| 1,219 ± 457 |
| AUC(0-∞)   | ng/mL·h  | 226 ± 91     | 501 ± 167  | 131 ± 63| 1,220 ± 457 |
| MRT (0-t)  | h        | 1.3 ± 0.5    | 0.271 ± 0.133| 1.0 ± 0.5| 0.3 ± 0.2 |
| MRT (0-∞)  | h        | 1.5 ± 0.6    | 0.271 ± 0.132| 1.4 ± 0.4| 0.4 ± 0.2 |
| t1/2z      | h        | 2.4 ± 1.5    | 0.438 ± 0.17| 2.0 ± 0.8| 0.3 ± 0.08|
| CLz        | L/h/kg   | 26.0 ± 12.3  | 11.3 ± 5.1 | 48.6 ± 29.1| 5.3 ± 4.0 |
| Vz         | L/kg     | 93.0 ± 69.6  | 7.4 ± 4.8  | 123.2 ± 54.6| 2.5 ± 1.9 |
| Cmax       | ng/mL    | 349.0 ± 172.4| 1,282.2 ± 667.9| 168.7 ± 148.5| 3,121.8 ± 1,868.6 |

AUC is area under curve, MRT is mean residence time, t1/2 is half time, CLz is clearance, Vz is apparent volume of distribution and Cmax is Maximum Concentration in blood.

was required to complete the analysis of the four compounds in mouse plasma samples. In addition, running the analysis in MRM mode enabled the quick screening of target components in plasma samples with extremely high sensitivity and specificity, especially in complex mixtures, such as blood.

The determination of whether to use positive- or negative-ion mode in electrospray ionization is dependent on the analytes [14–16]. Herein, ESI positive-ion mode was more sensitive than the negative-ion mode with respect to tectorigenin, tectoridin, irigenin, and iridin, so we developed the method using ESI positive-ion mode for detection and analysis.

Theoretically, there should be as much separation between the retention times of the endogenous substances, the analyte, and internal standard as possible during chromatographic separation of the mixtures [17–21]. The column and mobile phase will determine the chromatographic behavior. Strong chromatographic peak intensities and shapes, as well as long retention times, were demonstrated using a BEH C18 column and a mobile phase consisting of methanol–water (w/0.1% formic acid). Gradient elution mode was selected because it proved thorough for removing impurities from the column and therefore protecting the chromatographic system [22–24].

The pretreatment of the blood samples had a decisive influence on the determination accuracy of the drug concentration in plasma [17, 25]. In the early stage of this experiment, methanol, acetonitrile, and methanol-acetonitrile (1:1) were used to directly precipitate the proteins in the blood plasma. We found that acetonitrile precipitation method was the most effective compared to the other solvents tested. When the ratio of blood to acetonitrile was 1:5, the matrix effect was avoided.

Compared the pharmacokinetic characteristics of tectorigenin and irigenin in rat plasma [7], the AUC of tectorigenin and irigenin in rat are higher than mouse, the t1/2 of tectorigenin and irigenin in rat are longer than mouse, the Cmax of tectorigenin and Irgenin in rat are higher than mouse.

In this study, a direct precipitation method that contained acetonitrile was established to process the mouse blood samples. In addition, midazolam was used as the internal standard for quantitative analysis, and UPLC-MS/MS technology was applied to measure the concentrations of tectorigenin, tectoridin, irigenin, and iridin in the blood of mice after administration. The accuracy, precision, selectivity, and linearity of the method were verified, and the pharmacokinetics of the tectorigenin, tectoridin, irigenin, and iridin in mice were reported for the first time.

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