Analysis of Bioactive Components, Simultaneous Determination and Pharmacokinetic Study of Six Components After Intragastric Administration of Qiling Wenshen Xiaonang Granule.

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Research

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Abstract

**Background:** Qiling Wenshen Xiaonang Granule (QLWSG), a compound preparation of traditional Chinese medicine, which was recently approved by the National Medical Products Administration for clinical trials of polycystic ovary syndrome (PCOS). It is necessary to do further researches about QLWSG for better understanding.

**Methods:** In this study, qualitative and quantitative analysis of QLWSG in rat plasma were conducted by ultra-fast liquid chromatography coupled with triple quadrupole mass spectrometry (UFLC-QqQ-MS). The multi-reaction monitoring mode/electrospray ionization source of the triple quadrupole mass spectrometer was used for detection. The plasma samples were prepared by precipitating protein with organic solvent.

**Results:** 21 prototypes in rat plasma were identified after intragastric administration of QLWSG, and most of the identified components are flavonoids and phenolic acids. Furthermore, a sensitive and specific UFLC-QqQ-MS method was also built, which was used for simultaneous determination and pharmacokinetic study of six active components, astragaloside IV, calycosin, calycosin-7-O-β-D-glucoside, icariin, epimedin C and rosmarinic acid in rat plasma after intragastric administration of QLWSG with three doses (8, 16, 24 g/kg). All the calibration curves showed good linearity ($r > 0.9942$) in the range of concentration measured. The validated method is stable and reliable, meanwhile, successfully applied for pharmacokinetic evaluation of tested compounds. The results showed that there was no significant difference in half-lives ($t_{1/2}$) and clearance (CL/F) of six analytes at the three doses were observed.

**Conclusions:** It can be inferred that the pharmacokinetic behavior of QLWSG is positively correlated to dose at the range of 8–24 g/kg. Simultaneous determination of the six compounds in vivo for the first time by UFLC-QqQ-MS. The validated method and the results of this study would benefit therapeutic material basis and clinical application for QLWSG. And the validated method would also be applied to the analysis of other Chinese medicine compound prescriptions.

1. **Background**

   Traditional Chinese medicine (TCM), which has been used by practitioners for thousands of years, plays an important role in clinical treatment and has attracted increasing attention due to its almost no side effects and beneficial therapeutic effect on western medicines [1]. Polycystic ovarian syndrome (PCOS) is a common endocrine disorder with three cardinal features: hyperandrogenism, anovulation, and polycystic ovary (PCO) morphology [2], which affects 5–10% of women of reproductive age and leading to the increased risk of anovulatory infertility and recurrent pregnancy loss [3, 4]. However, there are currently no TCMs on the market for PCOS treatment. Qiling Wenshen Xiaosang, formerly known as Bushen Huatan Formula (BHF), was a decoction according to Professor Lihui Hou's (First Affiliated Hospital, Heilongjiang University of Chinese Medicine) clinical experience in treating PCOS for many years. Tasly Pharmaceutical Co. Ltd revised the decoction to granules during their cooperation with First
Affiliated Hospital of Heilongjiang University of Chinese Medicine. Qiling Wenshen Xiaonang Granule (QLWSG), a compound preparation of traditional Chinese medicine, which was recently approved by the National Medical Products Administration for clinical trials of polycystic ovary syndrome (PCOS). The preclinical trial of QLWSG is being developed, and a good clinical effect is achieved. Moreover, some publications have shown that QLWSG was proved effective to PCOS by improving the inflammatory reaction and oxidative stress [5]. It has also been reported that QLWSG can improve insulin resistance and regulate ovulation function in PCOS patients. [6]. Combined with clinical biochemical data, QLWSG has unique advantages in improving the clinical indicators of PCOS patients. So, this granule is expected to complete the new drug registration and fill a gap in the market for TCM treatment of PCOS.

It is widely accepted that TCMs are mostly used in combination, and the composite formula will produce therapeutic effect [7]. QLWSG formula is composed by Radix Astragali, Epimedii folium, Salvia miltiorrhiza, Atractylodes rhizomes and Poria cocos. Among them, Radix Astragali was used as the sovereign drug, which played a major therapeutic role against PCOS and was reported to improve insulin secretion and lower blood glucose [8]. Epimedii folium, as the minister drug, the extract and active components of which was commonly used for regulating hormone level, enhancing sexual function and treating hyperglycemia [9–13]. It is well known that formulation is a complex mixture containing different chemical constituents which are responsible for therapeutic effects. Previous studies have identified 36 chemical constituents about QLWSG, including 19 flavonoids, 11 phenolic acids, 4 triterpenoid saponins, etc. [14], and it is demonstrated that the major active components of QLWSG are flavonoids such as calycosin-7-O-β-D-glucoside, calycosin, icariin and epimedin C [15]. In particularly, Astragaloside IV and icariin have been recognized as a unique chemical marker for the quality control of Radix Astragali and Epimedii folium in the Chinese Pharmacopoeia, respectively [16]. TCMs combination is used to cure diseases by their holistic effects [17]. Phenolic acids from Salvia miltiorrhiza in QLWSG are also play an important role. Rosmarinic acid is one of phenolic acids which are the main type of hydrophilic components from Salvia miltiorrhiza, and phenolic acids was reported has plenty of biological activities, was used for treatment of coronary arteriosclerosis, angina pectoris and hyperlipaemia [18, 19]. Therefore, it is necessary to do further research on the above components.

Pharmacokinetic study on active constituents in herbal preparations, which is a good way to explain and predict correlations between efficacy and toxicity of TCMs. A clear understanding of pharmacokinetics will guide prescriber to choose doses and dose intervals which made the target tissues be exposed to appropriate drug concentrations for a sufficient length of time, and will also help for the better application of the drug. [20, 21]. As far as we know, the investigations of this formulation were mainly focused on clinical and pharmacological aspects, but no previous study was carried on pharmacokinetics of QLWSG components. Therefore, pharmacokinetics properties of bioactive ingredients in QLWSG remain unclear.

In the present study, 21 prototypes were identified from the plasma of QLWSG, and a sensitive, specific and accurate method was also developed and validated for simultaneous determination of astragaloside IV, calycosin-7-O-β-D-glucoside, calycosin, icariin, epimedin C and rosmarinic acid in rat plasma using chloramphenicol and diazepam as internal standards (IS). The method was successfully applied to
evaluate the pharmacokinetics behaviors of astragaloside IV, calycosin-7-O-β-D-glucoside, calycosin, icariin, epimedin C and rosmarinic acid after oral administration of QLWSG to rats, which would provide a solid foundation for further investigation of this TCM formula.

2. Experimental Materials And Methods

2.1. Materials and reagents

QLWSG was supplied by Tasly Pharmaceutical Co. Ltd (Tianjin, China). Astragaloside IV, calycosin-7-O-β-D-glucoside, icariin, rosmarinic acid, chloramphenicol (as IS) and diazepam (as IS) were obtained from the National Institutes for Food and Drug Control (Beijing, China). Calycosin and epimedin C were acquired from Tianjin Yifang science and technology Co. Ltd. According to HPLC/UV analysis, the purity of the standards was > 97.1%. Acetonitrile was acquired from Merck (Merck Serono, Germany, LCMS-grade). Formic acid was acquired from Sigma (LC-grade, Saint Louis, Mo, USA). Deionized water was achieved from a Milli-Q system (Millipore, Billerica, USA). The other reagents used were of analytical grade.

2.2. Instruments and analytical conditions

A SHIMAZU UFLC system outfitted with an LC-30AD binary pump, a DGU-20 degasser, an SIL-30AC autosampler and a CTO-30AC column oven (Shimadzu Corp, Tokyo, Japan) was used on chromatographic analysis. Chromatographic separation was implemented on a WATERS ACQUITY UPLC BEH C18 (2.1 × 100 mm, 1.7 µm) column with a guard cartridge at temperature of 30 °C. The mobile phase consisted of 0.05% formic acid-water (A) and 0.05% formic acid acetonitrile (B) using a gradient elution of 5% (v/v) B at 0–1 min; 5–65% B at 1.01-10 min; 65–95% B at 10–11 min; 95% B at 11–12 min; 95 – 5% B at 12-12.5 min; 5% B at 12.5–13 min. The flow rate and the injection volume were set at 0.4 mL/min and 2µL, respectively.

A Q-Trap® 5500 triple quadrupole mass spectrometry allocated with electrospray ionization (ESI) was applied on both in the positive and negative ionization mode (AB Sciex, CA, USA). MS conditions were installed as follows: Ion-spray voltage was set to -4.5 kV in negative ionization mode; Ions-pray voltage was set to 4.8 kV in positive ionization mode; ion source temperature was set to 550 °C; the nebulizer gas and heater gas (both are nitrogen ) was set to 45 psi; and curtain gas, was set to 20 psi.

All the operations, the receiving and analysis of data on the instrument were controlled by Analyst software (AB Sciex, USA).

2.3. Preparation of various samples

With methanol as the solvent, the required amount of reference standard was added to prepare the mixed reserve standard solution containing astragaloside IV (0.5 mg/mL), calycosin-7-O-β-D-glucoside (0.5 mg/mL), calycosin (0.2 mg/mL), icariin (0.2 mg/mL), epimedin C (0.2 mg /mL), and rosmarinic acid (0.5 mg/mL). The IS solution (1.0 mg/mL) was also prepared with methanol as solvent. A series of
working solutions were prepared by diluting reserve solutions with methanol, in which the concentrations of astragaloside IV, calycosin-7-0-β-D-glucoside and rosmarinic acid ranged from 1 to 5000 ng/mL, while the concentrations of calycosin, icariin and epimedin C ranged from 1 to 2000 ng/mL. The IS working solution (500 ng/mL) was also prepared by diluting the reserve solution with methanol. All the above solutions were kept at 4 °C before used.

The samples for standard calibration curves were prepared by gradually adding appropriate amount of the standards solutions to 150 µL blank plasma to final concentrations of 1-5000 ng/mL for astragaloside IV, calycosin-7-0-β-D-glucoside and rosmarinic acid; and 1-2000 ng/mL for calycosin, icariin and epimedin C. As above, the quality control (QC) samples were prepared with blank plasma at concentrations of 2, 250, 4000 ng/mL for astragaloside IV, calycosin-7-0-β-D-glucoside and rosmarinic acid; 2, 100, 1000 ng/mL for icariin, calycosin and epimedin C. The above spiked samples were treated according to the following parts.

2.4. Processing of plasma samples

After thawing the plasma samples at room-temperature, the method of precipitating protein with organic solvent was used for the plasma sample preparation. After 100 µL IS solution (500 g/mL) was added into 150 µL plasma sample, the mixture was swirled and mixed for 1 min to achieve uniform mixing state. Then 500 µL 0.2% pre-cooled formic acid acetonitrile was added. After 1 min of vortex mixing, the mixture was centrifuged at 14,100 rpm (15°C) for 10 min. The supernatant after centrifugation was removed to an eppendorf tube, 500 µL 0.2% pre-cooled formic acid acetonitrile was added for the second time about protein precipitation. After vortex and centrifugation as above, the supernatant was transferred to a 5 mL tube and evaporated to dryness at 30 °C under a gentle stream of nitrogen. The residue in the tube was redissolved in 80 µL mobile phase, then the supernatant was obtained by vortex mixing and centrifugation under the above conditions. And 2 µL of the supernatant was injected into the UFLC- QqQ-MS system for further analysis.

2.5. Validation of the method

2.5.1. Specificity and selectivity

The specificity of method, which was tested by analyzing the chromatograms of six separate blank rat plasma samples, plasma samples mixed with the analytes and IS, and plasma samples after a dosage by gavage. Blank rat plasma samples were used to analyze endogenous interference.

2.5.2. Linearity and lower limits of quantification (LLOQ)

Each calibration curve was built by scheming the peak area ratio of the analytes to IS (Y-axis) relative to the nominal concentration(X-axis) of astragaloside IV, calycosin-7-O-β-D-glucoside, calycosin, icariin, epimedin C and rosmarinic acid. Then the above curves were evaluated by weighted least-squares linear regression analysis with a weighed factor (1/x2). The lower limit of quantification (LLOQ) was
defined as the lowest concentration, which with an acceptable precision within 20% and accuracy of 80–120%.

2.5.3. Precision and accuracy

The precision and accuracy (intra-day and inter-day) of the method were calculated by analyzing the three QC concentrations (LQC, MQC and HQC) for plasma in six repetitions within a day and three days. Tolerable precision (relative standard deviation, RSD) and accuracy (RE) values were set within ±15% of the nominal concentration, except LLOQ (within ±20%).

2.5.4. Extraction recovery and matrix effect

The extraction recovery was determined by calculating the ratio of the amounts of QC samples (LQC, MQC and HQC) finally obtained against those originally spiked in the blank plasma. The effect of matrix was assessed by comparing the peak area acquired from samples where the processed matrix was mixed with standard solutions to those acquired from the unmixed reference standards solutions at the equally concentration.

2.5.5. Stability of samples

The stabilities of low, medium and high QC samples were analyzed under different storage and process states. The short-term stability of QC samples was analyzed by placing them at room-temperature for 24 h. The freeze/thaw stability was evaluated by analyzing QC samples after undergoing three cycles of freeze (-20 °C)-thaw (25 °C). Long-term stability in rat plasma stored at -80 °C was studied for a period of one month employing QC samples.

2.6. Identification of components in plasma of QLWSG

Two female Wistar rats (190 ± 10 g, age from 9 to 10 weeks) were acquired from Beijing Vital River Laboratory Animal Technology Co. Ltd. Rats were housed in a 12 h light/12 h dark cycle at an ambient temperature (25 °C) and 60% RH. All animal care and experiments met the requirements of the NIH guidelines for the Care and Use of Laboratory Animals, and were also supported by the Animal Ethics Committee of Tasly academy (Tianjin, China). The rats were adapted to the lab for 1 week and allowed to drink water freely with fasted overnight before experiments. The gavage dose of QLWSG was twice the clinical equivalent dose (16 g/kg). QLWSG was dissolved in water. Plasma samples (400 µL) were obtained from orbital venous plexus of rats at 0.417, 1, 2, 4 h after gavage, and stored in 1.5 mL heparinized polythene tubes. The target plasma sample was supernatant obtained by centrifugation (4900 rpm, 10 min), which was then stored at -80 °C until advanced analysis. After centrifugation at 4900 rpm for 10 min, plasma samples were separated and stored at -80 until further analysis.

2.7. The study of Pharmacokinetic

Eighteen female Wistar rats (190 ± 10 g, age from 9 to 10 weeks) were acquired from Beijing Vital River Laboratory Animal Technology Co. Ltd. Rats were housed in a 12 h light/12 h dark cycle at an ambient temperature (25 °C) and 60% RH. All animal care and experiments met the requirements of the NIH
guidelines for the Care and Use of Laboratory Animals, and were also supported by the Animal Ethics Committee of Tasly academy (Tianjin, China). The rats were adapted to the lab for 1 week and allowed to drink water freely with fasted overnight before experiments. 18 rats were randomly assigned to 3 groups (6 rats /group). Intragastric administration of QLWSG at low dose (clinical equivalent dose, 8 g/kg), medium dose (twice the clinical equivalent dose, 16 g/kg), and high dose (three times the clinical equivalent dose, 24 g/kg), respectively. QLWSG was dissolved in water. Blood samples (400 µL) were obtained from orbital venous plexus of rats at 0.167, 0.417, 0.667, 1, 2, 4, 6, 8, 12, 24 h after gavage, and stored in 1.5 mL heparinized polythene tube. The target plasma sample was supernatant obtained by centrifugation (4900 rpm, 10 min), which was then stored at -80 °C until advanced analysis.

3. Results And Discussion

3.1. Identification of components in plasma of QLWSG

Using the MRM (multi reaction monitoring) mode of UFLC-QqQ-MS, the reference standards were analyzed to determine the information of the precursor ion and the product ion, and the parameters were optimized. The plasma samples collected at different time points after mixing was treated in 2.4 terms, and the sample was analyzed. From the reference material information, also based on information of products ions, 21 prototypes were identified from the plasma of QLWSG, including 5 components from Radix Astragali, 8 components in Epimedii folium, 1 component in Atractylodes rhizomes, and 9 components in Salvia miltiorrhiza. Most of the above components are flavonoids and phenolic acids. The results are shown in Table. 1.
| No. | Identification                | Source            | Formula     | Molecular mass | Precursor ion (m/z)          | Product ion (m/z)          |
|-----|------------------------------|-------------------|-------------|----------------|------------------------------|----------------------------|
| 1   | Formononetin                 | Radix Astragli    | C_{16}H_{12}O_{4} | 268.3         | 269.1[M+H]^+                  | 237.1[M+H-CH_3]^+          |
| 2   | Calycosin                    | Radix Astragli    | C_{16}H_{12}O_{5} | 284.3         | 285.0[M+H]^+                  | 137.1[M+H-C_9H_6O_2]^+      |
| 3   | Ononin                       | Radix Astragli    | C_{22}H_{22}O_{9} | 430.4         | 431.2[M+H]^+                  | 269.1[M+H-Glc]^+            |
| 4   | Calycosin-7-O-β-D-glucoside  | Radix Astragli    | C_{22}H_{22}O_{10} | 446.4         | 447.1[M+H]^+                  | 285.2[M+H-Glc]^+            |
| 5   | astragaloside IV             | Radix Astragli    | C_{41}H_{68}O_{14} | 784.9         | 785.5[M+H]^+                  | 437.4[M+H-Glc-Xyl-2H_2O]^+  |
| 6   | Icariin                      | Epimedi folium    | C_{33}H_{40}O_{15} | 676.2         | 721.2[M+H-COO]^+              | 513.0[M-H-2H_2O-Glc-Rha]-^  |
| 7   | Epimedin A                   | Epimedi folium    | C_{39}H_{50}O_{20} | 838.3         | 839.4[M+H]^+                  | 369.0[M+H-2Glc-Rha]^+       |
| 8   | Epimedin B                   | Epimedi folium    | C_{38}H_{48}O_{19} | 808.3         | 853.5[M+H-COO]^+              | 645.3[M-H-Rha]-^            |
| 9   | Epimedin C                   | Epimedi folium    | C_{39}H_{50}O_{19} | 822.3         | 823.4[M+H]^+                  | 369.2[M+H-Glc-2Rha]^+       |
| 10  | Baohuoside I                 | Epimedi folium    | C_{27}H_{30}O_{10} | 514.2         | 515.1[M+H]^+                  | 369.1[M-H-Rha]^+            |
| 11  | Sagittatoside A              | Epimedi folium    | C_{33}H_{40}O_{15} | 676.2         | 677.2[M-H]-^                  | 340.5[M+H-H_2O-Glc-Rha]+    |
| 12  | Sagittatoside B              | Epimedi folium    | C_{32}H_{38}O_{14} | 646.2         | 647.3[M+H]^+                  | 313.2[M+H-Rha-Xyl]+^        |
| 13  | Chlorogenic acid             | Epimedi folium    | C_{16}H_{18}O_{9}  | 354.1         | 353.1[M-H]-^                  | 191.0[M-H-C_9H_6O_3]+^      |
| 14  | Atractyloside A              | Atractylodes         | C_{21}H_{36}O_{10} | 448.2         | 493.3[M+H]^                  | 447.3[M-H]-^                |
| No. | Compound                        | Family              | Formula       | Molecular Mass | Fragmentation                        |
|-----|--------------------------------|---------------------|---------------|---------------|-------------------------------------|
| 15  | Tanshinone IIA                  | Salvia miltiorrhiza | C_{19}H_{18}O_{3} | 294.1         | 295.1[M+H]+                          |
|     |                                |                     |               |               | 206.3[M+H−2CO-CH_{3}-H_{2}O]+        |
| 16  | Protocatechuic aldehyde         | Salvia miltiorrhiza | C_{7}H_{6}O_{3}  | 138.1         | 136.8[M−H]^−                          |
|     |                                |                     |               |               | 109.0[M−H-CO]^-                      |
| 17  | Lithospermic acid               | Salvia miltiorrhiza | C_{27}H_{22}O_{12} | 538.1         | 537.1[M−H]^−                          |
|     |                                |                     |               |               | 295.2[M−H-CO_{2}-C_{9}H_{10}O_{5}]^- |
| 18  | Danshensu                      | Salvia miltiorrhiza | C_{9}H_{10}O_{5} | 198.2         | 197.1[M−H]^−                          |
|     |                                |                     |               |               | 135.1[M−H-C_{9}H_{10}O_{5}]^-        |
| 19  | Rosmarinic acid                 | Salvia miltiorrhiza | C_{18}H_{16}O_{8} | 360.3         | 359.1[M−H]^−                          |
|     |                                |                     |               |               | 161.0[M−H-C_{9}H_{10}O_{5}]^-        |
| 20  | Salvianolic acid A              | Salvia miltiorrhiza | C_{26}H_{22}O_{10} | 494.1         | 493.2[M−H]^−                          |
|     |                                |                     |               |               | 295.1[M−H-C_{9}H_{10}O_{5}]^-        |
| 21  | Salvianolic acid B              | Salvia miltiorrhiza | C_{36}H_{30}O_{16} | 718.6         | 717.2[M−H]^−                          |
|     |                                |                     |               |               | 519.2[M−H-C_{9}H_{10}O_{5}]^-        |

Rha: Rhamnose; Glc: Glucose; Xyl: Xylose

### 3.1.1 Identification of flavonoids

Flavones and their glycosides were mostly detected in positive ion mode. In the positive ionization mode of the compound, the precursor ion showed a peak of m/z 447.1 [M + H]^+ and the secondary mass spectrum showed that the precursor ion at m/z 447.1 loss the glucose group (162 Da) to generated the main fragment ion [M + H-Glc]^+ at m/z 285.2. Therefore, it was determined that the molecular mass of the compound was 446. Also compared with the reference standards, it was determined that the compound was calycosin-7-O-β-D-glucoside as a prototype from *Radix Astragali*.

### 3.1.2 Identification of phenolic acids

Phenolic acids were mostly detected in negative ion mode. In the negative ionization mode of the compound, the precursor ion showed a peak of m/z 717.2 [M−H]^−. And the main fragment ion [M-H-C_{9}H_{10}O_{5}]^- at m/z 519.2 was generated after one molecule of Danshensu (198 Da) was broken with the precursor ion at m/z 717.2. Based on the reference standards, it was speculated that the compound was salvianolic acid B as a prototype from *Salvia miltiorrhiza*. Furthermore, combining the information of the reference standards with the similar fragmentation pattern mentioned above, rosmarinic acid was also determined as a prototype from *Salvia miltiorrhiza*. 
3.2. Pharmacokinetic study

On the basis of the identification results above, the pharmacokinetic study of six main components (astragaloside IV, calycosin-7-O-β-D-glucoside, calycosin, icariin, epimedin C and rosmarinic acid) were carried out. Their structures and characteristic ion pairs were shown in Fig. 1.

3.2.1 Optimization of UFLC-QqQ-MS method

The responses of calycosin-7-O-β-D-glucoside, calycosin observed in negative ionization mode were lower than that in positive ionization mode. But the responses of astragaloside IV, icariin, epimedin C and rosmarinic acid observed in negative ionization mode were higher than that in positive ionization mode. So, we also use two ionization modes at the same time.

MS parameters of collision energy (CE), declustering potential (DP), entrance potential (EP) and cell exit potential (CXP) were adjusted by injecting the standard solution respectively, so that the response of the precursor and product ions of each component reached the maximum (Table 2). Furthermore, the precursor and product ions of astragaloside IV, calycosin, calycosin-7-O-β-D-glucoside, icariin, epimedin C, rosmarinic acid and IS were shown in Fig. 2.

Table 2: Optimized MRM parameters, collision energy (CE), declustering potential (DP), entrance potential (EP) and cell exit potential (CXP) of astragaloside IV, calycosin, calycosin-7-O-β-D-glucoside, icariin, epimedin C, rosmarinic acid and chloramphenicol, diazepam (IS).

| Analytes                      | Precursor ion (m/z) | Product ion (m/z) | DP(V) | EP(V) | CE(V) | CXP(V) |
|-------------------------------|---------------------|-------------------|-------|-------|-------|--------|
| astragaloside IV              | 783.5               | 829.5             | -13   | -6    | -39   | -14    |
| rosmarinic acid               | 359.1               | 161               | -73   | -8    | -25   | -14    |
| icariin                       | 721.2               | 513               | -41   | -12   | -23   | -18    |
| epimedin C                    | 867.4               | 659.3             | -53   | -12   | -33   | -12    |
| calycosin                     | 285                 | 137.1             | 225   | 7     | 43    | 9      |
| calycosin-7-O-β-D-glucoside   | 447.1               | 285.2             | 45    | 7     | 21    | 26     |
| Chloramphenicol (IS)          | 321                 | 152               | -87   | -12   | -20   | -17    |
| Diazepam (IS)                 | 285.1               | 193.2             | 171   | 14    | 43    | 17     |

3.2.2 Specificity and selectivity
Representative chromatograms received from a blank sample, a spiked plasma sample with analytes (at LLOQ) and IS, and a plasma sample after intragastric administration of low-dose QLWSG were showed in Fig. 3. The retention times for astragaloside IV, calycosin-7-O-β-D-glucoside, calycosin, icariin, epimedin C and rosmarinic acid, chloramphenicol (IS) and diazepam (IS) were 8.38, 5.79, 6.18, 7.29, 7.16, 6.56, 7.02 and 9.44 min, respectively. The interference of endogenous peaks was not found in blank plasma sample, it showed that the UFLC-QqQ-MS with high selectivity.

3.2.3 Linearity and lower limits of quantification

Linear responses were obtained in concentration range from 1 to 5000 ng/mL for astragaloside IV, calycosin-7-O-β-D-glucoside, and rosmarinic acid, from 1.00 to 2000 ng/ml for calycosin, icariin and epimedin C. The calibration curves of astragaloside IV, calycosin-7-O-β-D-glucoside, calycosin, rosmarinic acid, icariin and epimedin C were $y = 0.000271x + 0.000108 (r = 0.9991)$, $y = 0.00309x + 0.00328 (r = 0.9983)$, $y = 0.0007x + 0.00104 (r = 0.9942)$, $y = 0.00377x + 0.00194 (r = 0.9997)$, $y = 0.00442x + 0.00388 (r = 0.9995)$, $y = 0.00165x + 0.000473 (r = 0.9991)$, respectively.

The LLOQ of astragaloside IV, calycosin-7-O-β-D-glucoside, calycosin, rosmarinic acid, icariin and epimedin C were 1 ng/mL, which were sufficient for pharmacokinetic studies in rats following the administration of three oral doses of QLWS extract. The RSD values of all analytes for precision were less than 10.61%, and the accuracy of all analytes ranged from 86.91–113.01%, which in accordance with the requirements of the Guideline on bioanalytical method validation (EMEA).

3.2.4 Precision and accuracy

The results of intra- and inter-day precision and accuracy for astragaloside IV, calycosin, calycosin-7-O-β-D-glucoside, rosmarinic acid, icariin and epimedin C are shown in Table 3. The intra- and inter-day precisions (RSD) were all less than 14.74%, and the accuracy ranged from 86.50–114.60%. The results indicated that the precision and accuracy of this method were acceptable.
### Table 3

| Analytes                | Precursor ion(m/z) | Product ion (m/z) | DP(V) | EP(V) | CE(V) | CXP(V) |
|-------------------------|--------------------|-------------------|-------|-------|-------|--------|
| astragaloside IV        | 783.5              | 829.5             | -13   | -6    | -39   | -14    |
| rosmarinic acid         | 359.1              | 161               | -73   | -8    | -25   | -14    |
| icariin                 | 721.2              | 513               | -41   | -12   | -23   | -18    |
| epimedin C              | 867.4              | 659.3             | -53   | -12   | -33   | -12    |
| calycosin               | 285                | 137.1             | 225   | 7     | 43    | 9      |
| calycosin-7-O-β-D-glucoside | 447.1          | 285.2             | 45    | 7     | 21    | 26     |
| Chloramphenicol (IS)    | 321                | 152               | -87   | -12   | -20   | -17    |
| Diazepam (IS)           | 285.1              | 193.2             | 171   | 14    | 43    | 17     |

Precision and accuracy of intra-day (n = 6) and inter-day (n = 6)

### 3.2.5 Extraction recovery and matrix effect

The extraction recoveries and matrix effects of astragaloside IV, calycosin-7-O-β-D-glucoside, calycosin, rosmarinic acid, icariin, epimedin C and IS from rat plasma were shown in Table 4.
Table 4

| Analytes                  | Spiked (ng/ml) | Intra-day (n = 6) | Inter-day (n = 6) |
|--------------------------|----------------|-------------------|-------------------|
|                          | Conc. Measured (ng/ml) | Precision (%) | Accuracy (%)     | Conc. Measured (ng/ml) | Precision (%) | Accuracy (%)     |
|                          | Precisio n (%) |                    |                   | Pre cisio n (%) |                    |                   |
|                          | Accurac y (%) |                    |                   | Accurac y (%) |                    |                   |
| astragalside IV          | 2              | 2.08 ± 0.20        | 9.37              | 104.23        | 2.05 ± 0.23        | 11.04            | 102.50           |
|                          | 250            | 277.00 ± 22.52     | 8.13              | 110.67        | 283.67 ± 16.77     | 5.91             | 113.47           |
|                          | 4000           | 3903.33 ± 568.54   | 14.57             | 97.58         | 4093.33 ± 603.43   | 14.74            | 103.50           |
| calycasin               | 2              | 2.09 ± 0.13        | 6.23              | 104.50        | 1.83 ± 0.24        | 10.61            | 92.50            |
|                          | 100            | 90.65 ± 6.54       | 10.15             | 91.65         | 88.21 ± 7.34       | 4.35             | 88.21            |
|                          | 1000           | 1111.8 ± 40.18     | 8.98              | 111.18        | 938 ± 50.21        | 7.66             | 93.80            |
| calycosin-7-O-β-D-glucoside | 2    | 2.27 ± 0.28        | 12.14             | 113.67        | 2.19 ± 0.28        | 12.95            | 109.38           |
|                          | 250            | 249.00 ± 14.80     | 5.94              | 99.60         | 250.50 ± 12.45     | 4.97             | 100.20           |
|                          | 4000           | 3586.67 ± 347.90   | 9.70              | 89.65         | 3600.00 ± 347.90   | 9.66             | 90.01            |
| icariin                  | 2              | 2.22 ± 0.07        | 3.19              | 110.50        | 2.23 ± 0.23        | 10.16            | 110.50           |
|                          | 100            | 109.50 ± 7.19      | 6.56              | 109.50        | 109.50 ± 7.19      | 6.56             | 109.50           |
|                          | 1000           | 887.33 ± 57.77     | 6.51              | 88.73         | 959.00 ± 53.11     | 5.54             | 95.90            |
| epimedin C               | 2              | 2.03 ± 0.24        | 11.86             | 101.60        | 2.16 ± 0.08        | 3.86             | 107.83           |
|                          | 100            | 88.28 ± 2.40       | 2.71              | 88.28         | 107.03 ± 10.75     | 10.05            | 107.03           |

Extraction recoveries and matrix effects of analytes
| Concentration | Peak Area (± SD) | Retention Time | Calibration Slope (± SD) | Calibration Intercept (± SD) | Extraction Recovery (± SD) |
|---------------|-----------------|----------------|--------------------------|-------------------------------|---------------------------|
| 1000          | 872.67 ± 20.55  | 2.35           | 87.27 ± 54.34            | 5.86 ± 92.80                  |
| Rosmarinic Acid | 2.14 ± 0.22     | 10.31          | 106.83 ± 1.98 ± 0.05     | 2.55 ± 106.83                 |
| 250           | 256.50 ± 12.02  | 4.69           | 102.60 ± 241.33 ± 33.50  | 13.88 ± 95.47                 |
| 4000          | 4347.50 ± 197.99| 4.55           | 108.68 ± 3940.00 ± 461.45| 11.71 ± 104.73                |

Extraction recoveries and matrix effects of analytes

The average recoveries of the samples were over 65.85% and the mean extraction recovery of IS was 76.27%. The above data indicated that the extraction recoveries of calycosin-7-O-β-D-glucoside, calcosin, astragaloside IV, rosmarinic acid, icariin, epimedin C and IS from the plasma were independent of concentration within the range of concentration assessed and were acceptable.

As displayed in Table 4, matrix effects of the all analytes ranged from 86.97 to 112.6%, and extraction recoveries of all analytes ranged from 65.85–103.44%. The results showed that the interference of matrix in rat plasma was negligible.

### 3.2.6 Stability

As presented in Table 5, the results of short-term stability, freeze/thaw stability and long-term stability were all within acceptable ranges. The results showed that the six compounds were steady under every tested situation.
Table 5

| Analytes                           | Extraction recovery (n = 6) | Matrix effect (n = 6) |
|------------------------------------|-----------------------------|-----------------------|
|                                   | Mean ± SD (%)               | Mean ± SD (%)         |
|                                   | Low QC                      | Medium QC             | High QC                     | Low QC      | Medium QC | High QC |
| astragalo side IV                  | 90.75 ± 7.33                | 70.46 ± 4.19          | 85.18 ± 3.41                | 89.29 ± 7.04 | 100.00 ± 4.78 | 98.77 ± 3.55 |
| calycosin                          | 93.78 ± 2.03                | 82.87 ± 3.45          | 90.11 ± 7.45                | 102.2 ± 2.97  | 95.60 ± 10.34  | 95.92 ± 0.25  |
| calycosin-7-O-β-D-glucoside        | 103.44 ± 13.12              | 87.48 ± 8.45          | 98.71 ± 2.44                | 86.83 ± 7.70  | 98.17 ± 1.82  | 97.27 ± 0.59  |
| icariin                            | 82.54 ± 8.36                | 72.14 ± 3.05          | 65.85 ± 0.69                | 96.40 ± 10.60 | 94.49 ± 4.33  | 91.67 ± 4.78  |
| epimedin C                         | 88.83 ± 3.25                | 101.36 ± 1.92         | 97.50 ± 2.49                | 108.62 ± 2.16 | 94.52 ± 6.27  | 100.74 ± 4.39 |
| rosmarinic acid                    | 71.37 ± 11.72               | 87.82 ± 4.66          | 67.88 ± 3.61                | 87.05 ± 14.18 | 93.94 ± 91.22 | 86.97 ± 7.95  |
| IS                                 | 94.24 ± 3.86                | 82.72 ± 3.66          | 107.35 ± 4.12               | 108.03 ± 4.76 | 101.82 ± 0.45 | 98.63 ± 0.53  |
| IS                                 | 87.30 ± 2.60                | 76.28 ± 1.13          | 82.92 ± 0.32                | 98.05 ± 0.02  | 104.06 ± 3.73 | 89.89 ± 0.25  |

Stability of analytes

3.2.7 Pharmacokinetic study of QLWSG

The validated method was successfully applied to pharmacokinetic study of astragaloside IV, calycosin-7-O-β-D-glucoside, calycosin, rosmarinic acid, icariin, epimedin C in rat plasma after intragastric administration of QLWSG with doses of 8 g/kg, 16 g/kg, 24 g/kg. The pharmacokinetic parameters were computed and analyzed by the DAS Software (version 3.2.6, National Medical Products Administration) using non-compartmental model. The mean plasma concentration–time curves (n = 6) of the six analytes were shown in Fig. 4. The main pharmacokinetic parameters in rats were presented in Table 6, including the mean retention time (MRT), the area under the time concentration curve (AUC), half-life time ($t_{1/2}$), clearance ($CL/F = Dose / AUC_{0-\infty}$), the maximum concentration ($C_{max}$), and the time to reach maximum concentration ($T_{max}$). The significant differences of each parameter among three dose groups were measured by SPSS 19.0. And data with P value less than 0.05 were judged to have statistical significance. Calycosin-7-O-β-D-glucoside and calycosin plasma concentrations were both lower than the limit of quantitation at 24 h for three doses, so only the data no more than 12 hours were adopted for analysis. As results indicated in Table 6, no significant differences of $t_{1/2}$ and CL/F among three doses
(Table 6) after intragastric administration of QLWSG. At low, medium and high doses, the $t_{1/2}$ of calycosin-7-$O$-$\beta$-$D$-glucoside was $1.10 \pm 0.07$ h, $1.76 \pm 0.32$ h, $1.09 \pm 0.63$ h, respectively, suggesting that it was speedily eliminated from rat plasma. And it may also imply that calycosin-7-$O$-$\beta$-glucoside distributed to various tissues or promoted metabolic reactions in a short time that resulting its decline in plasma. The Tmax of astragaloside IV was shorter than 0.417 h. And at low, medium and high doses, astragaloside IV eliminated slower in plasma than the other analytes with the $t_{1/2}$ of $6.586 \pm 1.64$ h, $8.33 \pm 2.89$ h, $6.97 \pm 0.79$, respectively. It demonstrated that astragaloside IV absorbed rapidly in rats, and the elimination process is mainly. The results are similar to those reported in oral administration of astragaloside IV in rats [22].
| Analytes                  | Spiked (ng/ml) | Short-term stability | Freeze/thaw stability | Long-term stability |
|--------------------------|----------------|----------------------|-----------------------|---------------------|
|                          | (ng/ml)        | Measured Conc. (ng/ml) | RSD(%)               | Measured Conc. (ng/ml) | RSD(%) |
| astra galoside IV        | 2              | 2.08 ± 0.23          | 10.90                 | 1.96 ± 0.09         | 4.70   |
|                          | 250            | 269.00 ± 8.72        | 3.24                  | 265.67 ± 14.47      | 5.45   |
|                          | 4000           | 3990.00 ± 519.71     | 13.03                 | 4253.33 ± 328.84    | 7.73   |
| caly cosin               | 2              | 2.21 ± 0.33          | 6.77                  | 2.09 ± 0.28         | 4.30   |
|                          | 100            | 93.2 ± 3.67          | 5.02                  | 91.27 ± 4.88        | 6.02   |
|                          | 1000           | 978.67 ± 39.21       | 4.33                  | 970.31 ± 41.23      | 5.17   |
| caly cosin-7-O-β-D-glucoside | 2              | 2.19 ± 0.28          | 12.95                 | 2.26 ± 0.29         | 12.94  |
|                          | 250            | 242.50 ± 17.75       | 7.32                  | 254.25 ± 16.01      | 6.30   |
|                          | 4000           | 3715.50 ± 347.90     | 9.36                  | 3827.50 ± 190.00    | 4.96   |

Pharmacokinetic parameters (mean ± SD) of astragaloside IV, calycosin, calycosin-7-O-β-D-glucoside, icariin, epimedin C, and rosmarinic acid, after intragastric administration of QLWSG (n = 6)
Pharmacokinetic parameters (mean ± SD) of astragaloside IV, calycosin, calycosin-7-0-β-D-glucoside, icariin, epimedin C, and rosmarinic acid, after intragastric administration of QLWSG (n = 6)

| Analytes | Dose (µg) | AUC(0-t) (µg/L*h) | AUC(0-∞) (µg/L*h) | MRT(0-t) (h) | MRT(0-∞) (h) | t1/2 (h) | CL/F (L/h/g) | Cmax (µg/L) | Tmax (h) |
|----------|-----------|-------------------|-------------------|--------------|--------------|----------|-------------|------------|---------|
| icarin   | 2         | 2.19 ± 0.16       | 7.09              | 2.07 ± 0.07  | 3.42         | 1.94 ± 0.10 | 5.09        |
|          | 100       | 108.25 ± 4.79     | 4.42              | 99.75 ± 6.24 | 6.25         | 102.75 ± 6.70 | 6.52        |
|          | 1000      | 967.00 ± 46.36    | 4.79              | 982.33 ± 35.70 | 3.63     | 1053.00 ± 66.01 | 6.27        |
| epimeden C | 2         | 2.07 ± 0.07       | 3.48              | 1.97 ± 0.11  | 5.48         | 2.19 ± 0.06 | 2.74        |
|          | 100       | 106.53 ± 10.36    | 9.72              | 109.50 ± 7.42 | 6.77     | 103.48 ± 10.00 | 9.66        |
|          | 1000      | 935.00 ± 58.81    | 6.29              | 1001.33 ± 92.34 | 9.22     | 994.33 ± 81.25 | 8.17        |
| rosmarinic acid | 2         | 1.96 ± 0.03       | 1.56              | 1.98 ± 0.05  | 2.55         | 2.05 ± 0.07 | 3.52        |
|          | 250       | 232.33 ± 31.13    | 13.40             | 241.33 ± 33.50 | 13.88    | 236.00 ± 28.79 | 12.20       |
|          | 4000      | 3907.50 ± 347.75  | 8.90              | 4077.50 ± 111.80 | 2.74     | 3986.25 ± 461.45 | 11.50       |
| astragaloside IV | low       | 75.0 ± 13.80      | 83.2 ± 18.40      | 8.26 ± 1.94  | 0.76* # #    | 0.7 ± 0.23 | 0.16        |
|          | 6*        | 0 ± 0            | 0 ± 0            | 0 ± 0        | 0 ± 0        | 0 ± 0     | 0 ± 0        |
|                  | mean | 95% CI | mean | 95% CI | mean | 95% CI | mean | 95% CI | mean | 95% CI | mean | 95% CI | mean | 95% CI |
|------------------|------|--------|------|--------|------|--------|------|--------|------|--------|------|--------|------|--------|
|                   |      |        |      |        |      |        |      |        |      |        |      |        |      |        |
| **Pharmacokinetic parameters (mean ± SD)** |      |        |      |        |      |        |      |        |      |        |      |        |      |        |
| astragaloside IV  | 137.3 ± 16.4 | 114.6 ± 19.3 | 8.56 ± 1.98 | 8.33 ± 2.89 | 39.0 ± 33.1 | 42.8 ± 8.0 | 9.0 ± 1.2 |
| calycosin-7-O-β-D-glucoside | 116.5 ± 30.1 | 242.1 ± 17.0 | 1.31 ± 0.79 | 51.8 ± 1.9 | 59.8 ± 7.0 | 14.7 ± 3.67 |
| icariin           | 1164.5 ± 753.4 | 1114.8 ± 565.9 | 62.9 ± 2.55 | 16.4 ± 6.54 | 857.0 ± 50.0 | 16.4 ± 4.04 | 30.1 ± 4.0 |
| epimedin C        | 1231.5 ± 273.3 | 1266.4 ± 257.9 | 4.37 ± 4.04 | 20.3 ± 1.0 | 609.0 ± 75.0 | 1.0 ± 0.01 | 34.7 ± 0.35 |
| rosmarinic acid   | 1222.7 ± 84.2 | 222.7 ± 15.5 | 8.0 ± 3.56 | 452.7 ± 14.7 | 59.8 ± 14.7 | 3.67 ± 0.17 | 6 ± 7 |

Pharmacokinetic parameters (mean ± SD) of astragaloside IV, calycosin, calycosin-7-O-β-D-glucoside, icariin, epimedin C, and rosmarinic acid, after intragastric administration of QLWSG (n = 6)
|          | low       | medium   | high      |
|----------|-----------|----------|-----------|
| epimedin C | 6.20 ± 1.62 | 317.45 ± 147.77 | 363.41 ± 93.09 |
|          | ± 1.63*   | 09 ± 23  | 33 ± 92.8  |
|          | #         | 45 ± 135 | 41 ± 92.8  |
|          |           | 77       | 90         |
|          | 1.78*     | 10.9     | 0.66       |
|          | ±         | ±        | ±          |
|          | 2.43 ± 1.82* | 6.85 ± 3.77 | 5.65 ± 0.93 |
|          | ±         | ±        | ±          |
|          | 4.38 ± 2.86 | 5.39 ± 4.08 | 5.87 ± 0.66 |
|          | ±         | ±        | ±          |
|          | 1414 ± 436.36 | 54.1 ± 16.8 | 114.85 ± 85.6 |
|          | 0.87 ± 36 | #         | 6 ± 18.96  |
|          | 0         | 0        | 0          |
|          | 2.86      | #        | 2.20       |
|          |           | 106*     | 3.20       |
|          |           | ±        | ±          |
|          | 11.4 ± 67 | 313.25 ± 209.94 | 184.25 ± 41.5 |
|          | 0.16      | 0.16     | 0.54       |
|          | 0 ± 0     | 0        | 0.25       |
| rosmarinic acid | 72.7 ± 5.78 | 266.33 ± 201.27 | 563.50 ± 288.34 |
|          | ± 5.82*   | 08 ± 55  | 96 ± 295.09 |
|          |           | 33 ± 201 | 34 ± 295  |
|          |           | 9        | 0         |
|          | 1.81 ± 0.96 | 5.33 ± 3.41 | 5.86 ± 2.35 |
|          | ±         | ±        | ±          |
|          | 1.82 ± 0.97 | 5.41 ± 3.34 | 6.25 ± 2.52 |
|          | ±         | ±        | ±          |
|          | 2.27 ± 0.66 | 2.26 ± 1.35 | 3.84 ± 0.96 |
|          | ±         | ±        | ±          |
|          | 114 ± 85.6 | 85.6 ± 45.1 | 53.6 ± 26.7 |
|          | ±         | 0 ±       | 0 ±        |
|          | 66.6 ± 3 | 45.1 ± 37 | 209 ± 55  |
|          | 0.16      | 0.16     | 1.18       |
|          | 7 ± 0     | 7 ± 0    | 8 ± 9      |
|          |           | 1        | 9         |

*: p < 0.05, compared with high dose.

#: p < 0.05, compared with medium dose.

Pharmacokinetic parameters (mean ± SD) of astragaloside IV, calycosin, calycosin-7-O-β-D-glucoside, icariin, epimedin C, and rosmarinic acid, after intragastric administration of QLWSG (n = 6)

As indicated in Table 6, the AUC$_{0-\infty}$ of icariin were 1.39 ± 0.35, 1164.22 ± 537.27, 1266.46 ± 257.95, respectively, and its exposure level in plasma is the highest among the six analytes. The above results may indicate that icariin have the longest effect time in vivo compared with other five compounds in QLWSG. In Fig. 4, Rosmarinic acid showed a double-peak phenomenon in the high dose group, it may be due to excessive dose and enterohepatic circulation [23], etc. By normalizing the value of AUC$_{0-\infty}$ with the corresponding dose, the results showed that the AUC$_{0-\infty}$ values of the six analytes were proportional to the doses as shown in Fig. 5. And the results of regression analysis showed that the correlation between the AUC$_{0-\infty}$ value of Rosmarinic acid and its dose was the highest (correlation coefficient was 0.9981). Since there was no significant difference in t1/2, we could infer that the pharmacokinetic behavior of QLWSG is positively correlated with dose in the range of 8–24 g/kg based on the above results.

Compared with published pharmacokinetic studies of individual compounds, the pharmacokinetic parameters of six compounds have some differences [24–26]. It could be the effect of herb–herb
interactions in QLWSG. In addition, this is the first time of the six compounds of QLWSG have been simultaneously determined and analyzed in vivo by an UFLC-QqQ-MS method.

4. Conclusions

In this study, a total of 21 prototypes of plasma of QLWSG were identified by the MRM mode of UFLC-QqQ-MS. These results provide information on the identification of bioactive compounds of QLWSG. And combining with the previous studies of the chemical constituents, the results of identification in vivo would benefit therapeutic material basis for QLWSG.

In addition, based on identification of prototypes and described a simple, sensitive and validated UFLC-QqQ-MS method for simultaneous determination of astragaloside IV, calycosin, calycosin-7-O-β-D-glucoside, rosmarinic acid, icariin, epimedin C in rat plasma after intragastric administration of QLWSG, as well as investigated on their pharmacokinetic studies. The assay provided adequate recovery with good precision and accuracy. The established UFLC-QqQ-MS method could also be used for pharmacokinetic study of metabolites and provide a basis for metabolomics of QLWSG. Moreover, the validated UFLC-QqQ-MS method and the all results of this study have important reference value for the exploration of efficacy, clinical research and application of QLWSG.

Overall, simultaneous determination of these six bioactive components in rat plasma for pharmacokinetic investigations is then required, combined with the subsequent metabolomics research results of the QLWSG, not only to provide information on the identification of more bioactive compounds and to promote the clinical applications of QLWSG, but also to receive a better comprehension of the pharmacological action mechanism and to deduce the mechanism of the action of TCMs, explaining various events correlated with the efficacy of TCMs.

Abbreviations

QLWSG
Qiling Wenshen Xiaonang Granule
PCOS
Polycystic ovary syndrome
UFLC-QqQ-MS
Ultra-fast liquid chromatography coupled with triple quadrupole mass spectrometry
TCM
Traditional Chinese Medicine
BHF
Bushen Huatan Formula
IS
Internal standards
ESI

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Electrospray ionization
LLOQ
Lower limit of quantification
RSD
Relative standard deviation
MRM
Multi reaction monitoring
CE
Collision energy
DP
Declustering potential
EP
Entrance potential
CXP
Cell exit potential
EMEA
Guideline on bioanalytical method validation
MRT
Mean retention time
AUC
The area under the time concentration curve
t1/2
Half-life time,
Cmax
The maximum concentration
Tmax
The time to reach maximum concentration

Declarations

Ethics approval and consent to participate

All animal care and experiments met the requirements of the NIH guidelines for the Care and Use of Laboratory Animals, and were also supported by the Animal Ethics Committee of Tasly academy (Tianjin, China).

Consent for publication

Not applicable.
**Availability of data and materials**

All data generated or analysed during this study are included in this submitted manuscript [includes table and figure files].

**Competing interests**

The authors declare that they have no competing interests.

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**Authors' contributions**

Jiawen Shi and Yu Wang finished the experiment and analysed the data regarding the pharmacokinetic study. Jiawen Shi was a major contributor in writing the manuscript. Yiqian Zhang performed the part of the animal's experiment. Yuanxue Liu and Zhipeng Huo gave some advices for writing. And all authors read and approved the final manuscript.

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Figures
Figure 1

The structures and characteristic ion pairs of astragaloside IV, calycosin-7-O-β-D-glucoside, calycosin, icariin, epimedin C and rosmarinic acid.
Figure 2

Precursor and product ions of six analytes and IS.
Figure 3

Representative MRM chromatograms of astragaloside IV, calycosin, calycosin-7-O-β-D-glucoside, icariin, epimedin C and rosmarinic acid and IS. (A) Blank plasma sample; (B) blank plasma sample spiked with LLQC of 1.00 ng/mL astragaloside IV, calycosin-7-O-β-D-glucoside, calycosin, icariin, epimedin C, rosmarinic acid and IS; (C) rat plasma sample obtained at 25 min after intragastric administration of QLWSG (8g/kg).
Figure 4

Mean plasma concentration-time curves of six analytes in rat plasma after intragastric administration of QLWSG at different doses (each point represents mean ± S.D., n = 6).
**Figure 5**

The results of normalizing the AUC(0-∞) values of six analytes with the different doses.