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Pharmacokinetics of nitrogen-containing metabolites *R*-gentiandiol and *S*-gentiandiol in rat plasma after oral administration of swertiamarin

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ABSTRACT

Objective: A chiral resolution method for enantiomers of two chiral nitrogen-containing metabolites *R*-gentiandiol and *S*-gentiandiol of swertiamarin in plasma was developed, and the pharmacokinetics of the metabolites were studied. **Methods:** The metabolites of swertiamarin *in vivo* were detected by LC-MS/MS using Astec Cyclobond Ⅱ Cyclodextrin column (4.6 mm×100 mm, 5 μm), gradient elution with acetonitrile-water as mobile phase, and monitored by multiple reaction monitoring (MRM) method in positive mode. The ion pairs for quantitative analysis are *R*-gentiandiol (*m/z* 210.04 192.06), *S*-gentiandiol (*m/z* 210.04 192.06) and gentianone (*m/z* 192.02→162.08). **Results:** The linear correlation coefficients of the method developed were greater than 0.999, the precision was less than 7.00%, the recovery was 99.57%-102.65 %, and the matrix effect was 90.94%-91.34%. The peak t_{max} of *R*-gentiandiol and *S*-gentiandiol in rat plasma after oral administration of swertiamarin were (1.63±0.23) h and (1.58±0.21) h, $t_{1/2}$ was (6.23±0.52) h and (5.46±0.38) h, C_{max} was (86.79±20.81) ng/mL and (60.72±18.95) ng/mL, and the *AUC0−24* were (1 094.58±86.37)) (ng • h)/mL and (724.67±58.38) (ng h)/mL, respectively. **Conclusion:** The method was highly sensitive with good accuracy and precision, and it was successfully applied for chiral resolution and pharmacokinetics study of *R*-gentiandiol and *S*-gentiandiol in plasma. The method developed and experimental results will provide scientific basis for the study of pharmacodynamics and pharmacodynamic material basis of swertiamarin, and lay a foundation for clinical application and resource development of TCM monomer.

1. Introduction

Iridoids compounds are abundant in medicinal plants[1] and have a variety of pharmacological effects[2], so they have always been a hot spot in the study of pharmacodynamic substances of traditional Chinese medicine. Swertiamarin is the most representative iridoid[3], widely distributed in Swertia, and is the main component of *Swertia* medicinal plants such as *Swertia pseudochinensis* Hara[4]. Swertiamarin has a variety of pharmacological effects, including anti-inflammatory, analgesic, hepatoprotective, anti-diabetes, anti-oxidation, neuroprotection and gastric protection[5−10], especially in the treatment of hepatobiliary diseases. Studies have reported that the action entity of swertiamarin may be its metabolite[13]. Our previous study found that swertiamarin can be metabolized into enantiomers of two chiral nitrogen-containing metabolites *R*-gentiandiol (RG) and *S*-gentiandiol (SG) *in vivo*[14]. In this study, the chiral separation of RG and SG was carried out, and their pharmacokinetics was studied, which provided data support for the metabolism of swertiamarin *in vivo*, in order to provide reference for further determining the pharmacodynamic material basis of swertiamarin.

2. Materials and methods

2.1 Instruments and reagents

1290UPLC (Agilent, USA); 6400Triple quadrupole mass spectrometer (Agilent, USA); Astec Cyclobond Ⅱ cyclodextrin

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chiral column (4.6 mm×100 mm, 5 μm);Thermo Sorvall ST 16R Cryogenic ultracentrifuge (Thermo Fisher, USA); Ultrasonic cleaner (Shanghai Kedao Instrument Factory); Electronic analytical balance (Mettler. Toledo Instruments); desktop centrifuge (ShangHai Anting Scientific Instrument Factory); Micropipette (Eppendorf); swertiamarin (Beijing Bailingwei Company, 98%); chromatographic grade methanol (Fisher, USA); chromatographic grade acetonitrile (Merck, USA); anhydrous ethanol (Sigma, USA); ammonia (Sigma, USA); tert-butanol (Fisher, USA); ethyl acetate (Sigma, USA); sodium sulfite (Sigma, USA); anhydrous magnesium sulfate (Fisher, USA); AD-mix-β and AD-mix-α (Sigma, USA); the internal standard gentianone was synthesized in the early stage of the laboratory.

2.2 Experimental animals

Male Wistar rats, weighing 180-220 g, license number: SCXK (black) 2018-003, provided by the Safety Evaluation Center of Heilongjiang University of Traditional Chinese Medicine. This experiment was performed with the permission of the Animal Experimentation Ethics Committee (No. 2021121002). The feeding conditions were room temperature (20 ± 2) ℃, humidity (45 ± 5) %. The animals were reared in the form of 12 h light and 12 h dark, feeding in the drug analysis laboratory of Heilongjiang University of Traditional Chinese Medicine with free access to food and water for two weeks of adaptive feeding. The rats were fasted for 12 hbefore intragastric administration.

2.3 Animal grouping administration and blood collection

Wistar rats were randomly divided into blank group and administration group. standard swertiamarin (20 mg) was dissolved in distilled water and configured into oral administration containing 1 mg/mL. There were six rats in the administration group, each of which was treated with swertiamarin by oral administration of 1.0 mL/100 g, and the blank group was treated with distilled water by oral administration of 1.0 mL/100 g. At 0.25, 0.5, 1.5, 3, 5, 8, 12, 24, 36 and 48 h after administration, 0.5 mL of blood was collected from the eyepit and placed in EP tubes infiltrated with 1 mg/mL heparin sodium, centrifuged at 3500 r/min for 15 min, and the supernatant was retained for measurement of various parameters and data processing. The software used was WinNolin.

2.4 Preparation of standard

2.4.1 Preparation of gentianine

RG and SG were prepared by our previous synthesis method[14].

2.4.2 Preparation of reference solution

Two 10 mL volumetric flasks were added with 10 mg of precisely weighed RG and SG, respectively, and diluted with methanol to make RG and SG stock solutions for later use. The SG stock solution was precisely weighed and prepared into standard solutions with concentrations of 100 ng/mL, 200 ng/mL, 400 ng/mL, 800 ng/mL and 1 600 ng/mL. Continue to dilute with rat blank plasma to plasma standard working solution with concentrations of 10 ng/mL, 20 ng/mL, 40 ng/mL, 80 ng/mL, and 160 ng/mL. The concentrations of RG standard solution were 250 ng/mL, 500 ng/mL,000 ng/mL, 2 000 ng/mL, 4 000 ng/mL. Continue to dilute with rat blank plasma to plasma standard working solution with concentrations of 25 ng/mL, 50 ng/mL, 100 ng/mL, 200 ng/mL, and 400 ng/mL.

2.5 Preparation of internal standard solution

The 10 mL volumetric flask was added with 10 mg of standard gentianone, which was diluted and diluted with methanol to a concentration of 1 mg/mL. 0.2 mL of the stock solution was accurately taken and diluted 100 times in the 10 mL volumetric flask to prepare 200 ng/mL gentianone internal standard solution.

2.6 Preparation of quality control solution

The RG and SG stock solution were precisely weighed and diluted into sample solutions of 1 ng/mL, 10 ng/mL, and 100 ng/mL using blank plasma.

2.7 Chromatographic and mass spectrometric conditions

2.7.1 Chromatographic conditions

Chromatographic column: Astec Cyclobond II cyclodextrin chiral column (4.6 mm × 100 mm, 5 μm); mobile phase A: acetonitrile, mobile phase B: water, gradient elution (Table 1); The column temperature: 30 ℃. Flow rate: 1.0 mL/min; Temperature of sample chamber: 4 ℃; Injection volume: 5 μL.

Tab1
The gradient program of the mobile phase

Time (min)	Flow rate (mL/min)	Mobile phase A (%)	Mobile phase B (%)
Initial	1.0	10	90
1	1.0	15	85
3	1.0	20	80
4	1.0	25	75
7	1.0	35	65
10	1.0	100	0
12	1.0	10	90

2.7.2 Mass spectrum conditions

Electrospray ion source (ESI), multiple reaction monitoring (MRM) scanning mode, and positive ion (ESI+) mode were used to collect data. Capillary: 3.5 KV; Spray pressure: 45 psi; Auxiliary gas pressure: 10 psi; Fragmentor: 100 V; Desolvation temperature: 300 ℃; Dessolvent flow rate: 10 L min-1. The detection ion pairs were RG (*m/z* 210.04→192.06), SG (*m/z* 210.04→192.06), and gentianone (*m/z* 192.02→162.08). Collision voltage: 50 eV for RG,

50 eV for SG, and 40 eV for gentianone.

2.8 Preparation of biological samples

A total of 100 μL of plasma standard working solution, 100 μL of blank plasma and 100 μL of quality control sample were taken, and 5 μL of internal standard solution was added to each sample except blank, and vortexed for 5 min. Then 300 μL of methanol (0.1 mol/L) was added to continue vortexing for 1 min, and centrifuged at 12 000 r/min for 15 min. The supernatant was filtered through a 0.22 μm microfiltration membrane and placed in a sample bottle for LC-MS/MS analysis.

3. Results

3.1 Condition optimization of liquid chromatography-mass spectrometry

The chromatographic conditions were further optimized based on the retention time, separation degree, trailing factor and other factors. Acetonitrile and water were selected as mobile phases and gradient elution method was adopted. In the investigation of the data collection mode, the response values of RG, SG and gentianone in the positive and negative ion scanning modes were compared. It was found that the response values in the positive ion mode were higher, and the collection mode was finally determined as the positive ion mode. After optimization, the optimal RG collision voltage is 50 eV, SG is 50 eV and gentianone is 40 eV. The $[M+H]^+$ values of RG, SG and gentianone were 210.04, 210.04 and 192.02, respectively. The ion pairs were determined to be 210.04→192.06 (RG), 210.04→192.06 (SG) and 192.02→162.08 (gentianone) by full scanning of post-collision secondary mass spectrometry (Figure 1 and 2).

3.2 Specificity

Among the many extraction and purification solvents of plasma samples, methanol was found to have the highest extraction efficiency, and plasma samples had the least matrix interference effect. Therefore, methanol was selected as the extraction solvent. The sample was processed under the condition of "2.8", and after LC-MS/MS detection, the chromatogram was shown in Figure 3.

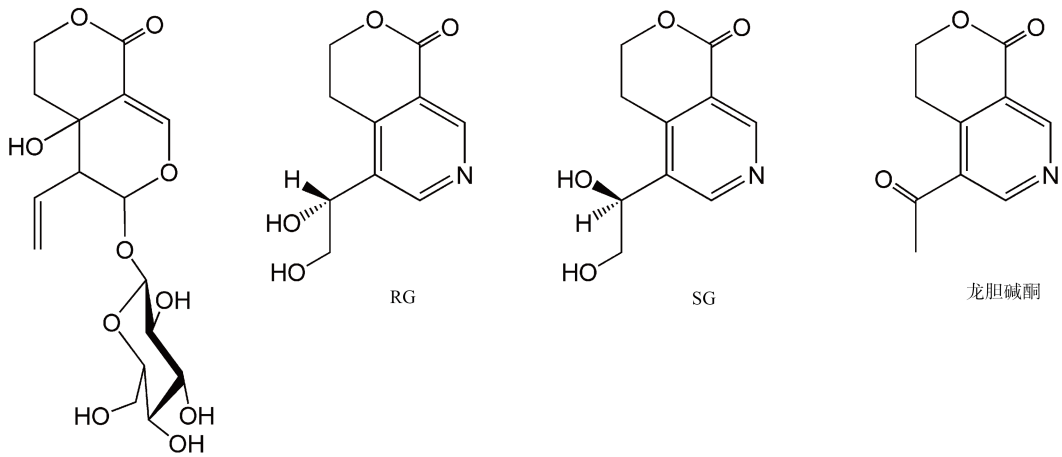
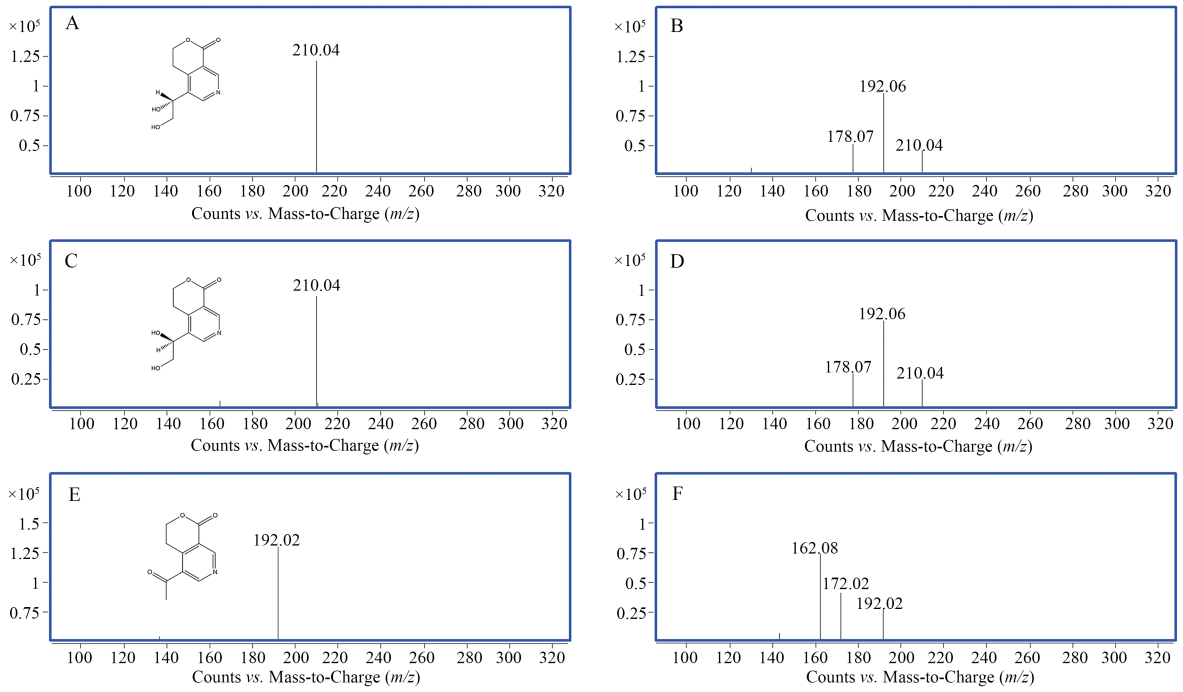


Fig 1 Structures of swertiamarin, RG, SG and gentianone (IS)

The results showed that RG and SG were successfully separated under the chromatographic condition, and the degree of separation was good. The retention times of the three were as follows: RG was 3.1 min, SG was 3.3 min and gentianone was 6.5 min.

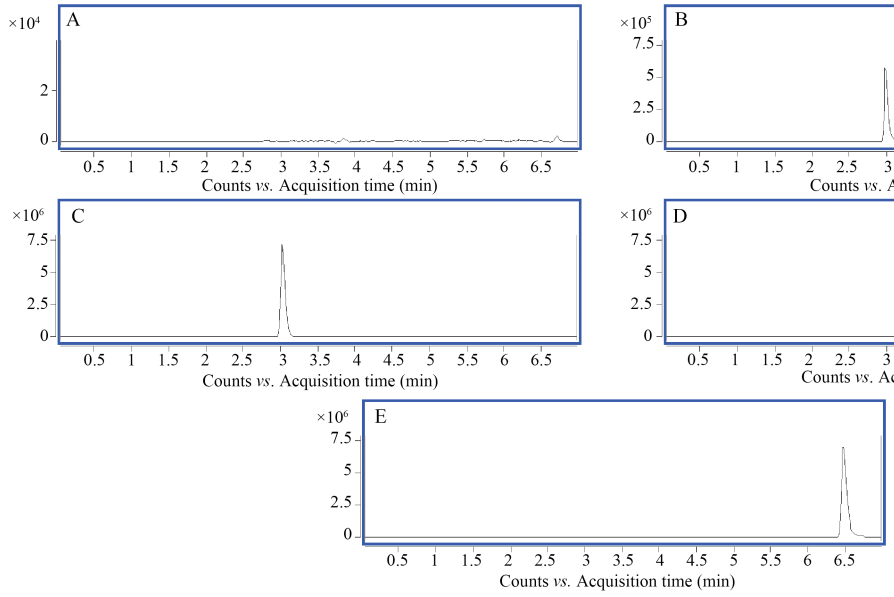
3.3 Linear equation, linear range and limit of quantitation

According to the preparation method of biological samples under the condition of "2.8", several tubes of 100 μL blank plasma were taken, and 5 μL of different amounts of RG, SG and gentianone were precisely added. The corresponding control solution was obtained (RG were 25 ng/mL, 50 ng/mL^{-1}, 100 ng/mL^{-1}, 200 ng/mL^{-1}, 400 ng/mL, SG were 10 ng/mL, 20 ng/mL, 40 ng/mL, 80 ng/mL, 160 ng/mL). 6 parallel samples were set for each concentration, and 5 μL of the above reference solutions were sampled. The ordinate is the ratio of material to internal standard peak area, and the abscissa is the concentration. The regression equations of RG and SG are obtained by using the weighted least square method. The linear relationship of RG and SG was good, and the linear range of RG was 25-400 ng/mL, and SG was 10-160 ng/mL.



A: RG; C: SG; E: gentianone; B: RG; D: SG; F: gentianone.

Fig 2 Mass spectra



A: Blank plasma (*m/z* 210.04→192.06); B: Plasma after oral administration of swertiamarin (*m/z* 210.04→192.06); C: Standard RG (*m/z* 210.04 192.06);

D: Standard SG (*m/z* 210.04→192.06); E: Gentianone (m/z 192.02→162.08).

Fig 3 MRM chromatogram

3.4 Accuracy and precision

The quality control solution prepared under the "2.6" was taken and the quality control samples with concentrations of 1 ng/mL, 10 ng/mL and 100 ng/mL were determined to calculate the accuracy and precision of RG and SG, as shown in Table 2.

Tab 2

Precision and accuracy (n=6)

Compound	concentration (ng/mL)	Measured average (ng/mL)	Accuracy (%)	Precision (%)
RG	1	1.01	101.33	3.67
	10	10.07	100.77	1.35
	100	101.64	101.65	4.46
SG	1	1.01	101.00	6.66
	10	9.95	99.57	1.53
	100	102.65	102.65	3.65

3.5 Extraction recovery and matrix effect

Plasma quality control solutions of rats with concentrations of 1 ng/mL, 10 ng/mL and 100 ng/mL were used for determination, and the peak areas were A1. Adding pure standard solution with concentrations of 1 ng/mL, 10 ng/mL and 100 ng/mL, the peak areas were A2. The concentration of 1 ng/mL, 10 ng/mL and 100 ng/mL pure standards were added into blank plasma, and the peak areas were A3. The data were analyzed and the recovery rate and matrix effect were calculated. The ratio of A1 to A2 is the recovery rate, and the ratio of A3 to A2 is the matrix effect, as shown in Table 3. It was proved that the method had good recovery and small matrix effect.

Tab 3
Recovery rates and matrix effects of RG and SG in rat plasma (n=6)

Compound	1 ng/mL	10 ng/mL	100 ng/mL	Matrix effect
RG	89.63±2.37	86.47±3.94	84.92±4.53	91.34±3.77
SG	89.14±2.41	86.39±3.62	85.21±4.39	90.94±3.18

3.6 Stability

The quality control samples of 1 ng/mL, 10 ng mL, and 100/ ng mL were placed at room temperature at 25 ℃ for 4 h, frozen and placed for 20 d (-20 ℃), and their stability was measured after repeated freezing and thawing (6 times). See Table 4 for details. The results showed that RG and SG were stable in plasma under all conditions.

3.7 Pharmacokinetic studies of RG and SG

The plasma concentrations of RG and SG in rats were determined by the established liquid chromatography-mass spectrometry method. The drug-time curves of RG and SG were drawn with the administration time as the abscissa and the blood concentration as the ordinate. The pharmacokinetic parameters were calculated by WinNolin software, and the results were shown in Figure 4.

The results showed that the peak time of RG was (1.63 ± 0.23) h, the half-life was (6.23 ± 0.52) h, the maximum plasma concentration was (86.79 ± 20.81) ng/mL, and *AUC0–24* was (1 094.58 ± 86.37) (ng • h)/mL. The peak time of SG was (1.58 ± 0.21) h, the half-life was (5.46 ± 0.38) h, the maximum plasma concentration was (60.72 ± 18.95) ng • mL, and *AUC0–24* was (724.67 ± 58.38) (ng • h)/mL.

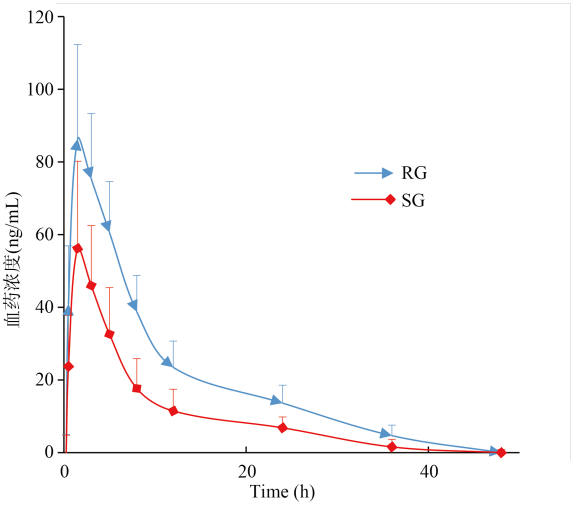


Fig 4 Blood concentration-time curves of RG and SG after oral administration of swertiamarin in rats

Tab 4
Stability of RG and SG in rat plasma (n=6)

Compound	Concentration (ng/mL)	Short-term stability (%)	Long-term stability (%)	Freeze-thaw stability (%)
RG	1	90.31±2.34	91.33±2.52	90.76±3.18
	10	91.14±3.69	91.81±3.27	92.84±2.63
	100	89.92±4.16	90.45±1.22	91.67±4.22
SG	1	90.54±1.82	91.57±2.71	90.48±2.85
	10	91.69±3.27	90.89±1.73	92.34±2.26
	100	89.71±2.73	90.94±2.16	91.53±3.74

4. Discussion

Iridoid compounds contain hemiacetal structure, so their structure changes greatly during metabolism *in vitro* and *in vivo*. In our previous research work, we found that many iridoids can be converted to nitrogen-containing metabolites under the action of intestinal bacteria, such as Geniposide can be converted to Gardenine[15], Geniposide can be converted to Genipinine[15], *etc*. At the same time, we also found that the iridoid compound swertiamarin can be converted into coumarin and alkaloid compounds in rats[14,16], and the alkaloid metabolite gentiandiol is a chiral compound. As one of the characteristics of alkaloids, the optical rotation of alkaloids is related to their biological activity, which has attracted much attention in recent years. Studies have found that the biological activity of the L-isomer of alkaloid compounds is significantly better than that of the D-isomer, which has high biological activity[17]. In order to ensure the safety of drug use, in 1992, FDA of the United States clearly stipulated that the pharmacodynamics and pharmacokinetics research results of chiral new drugs must be provided for both levorotatory and dextrorotatory. In 2006, the drug regulatory department of China also issued corresponding new policies[18]. In order to investigate the correlation between the spatial configuration of gentiandiol and its pharmacokinetics and bioactivity, we attempted to split gentiandiol isomers.

After trying chiral columns such as protein[19] and cellulose[20], RG and SG were successfully separated by Astec Cyclobond Ⅱ cyclodextrin chiral column for the first time, and the pharmacokinetics of RG and SG were studied by LC-MS/MS (MRM scanning). The established method has strong specificity, high sensitivity and good accuracy. The concentration of RG and SG in blood reached the peak rapidly after the administration of swertiamarin, and RG and SG were rapidly excreted. The maximum plasma concentration and AUC of RG were significantly higher than those of SG. Among them, the optical rotation of RG is left-handed and that of SG is right-handed. For many alkaloids, the levorotatory is more bioactive than the dextrorotatory, and the levorotatory RG was the dominant enantiomer in blood in this experiment. The biological activities of RG and SG have not been reported so far. We will further explore the relationship between the biological activities of RG and SG and blood drug concentration. This study provides data support for the study of the acting substances of swertiamarin in vivo, and also provides method reference for the chiral separation of enantiomers with similar structures of RG and SG, the transformation of other iridoids into nitrogen-containing metabolites in vivo, and the study of pharmacokinetics.

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