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Determination and pharmacokinetics of orientin in rabbit plasma by liquid chromatography after intravenous administration of orientin and *Trollius chinensis* Bunge extract

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Abstract

A high-performance liquid chromatography (HPLC) method was developed and validated for the determination of orientin in rabbit plasma using ultraviolet (UV) absorbance detection. Orientin is the active constituent of purified herbal extract (TRO PE) from the flower of *Trollius chinensis* Bunge. Protein precipitation was used as the sample preparation technique. A Diamonsil C_{18} column (150 mm × 4.6 mm, 5 μm) was equilibrated with a mobile phase composed of 0.1% acetic acid/methanol/acetonitrile (80/5/15, v/v/v). The calibration curve of orientin in rabbit plasma was linear in the concentration range of 0.530–53.0 μg/mL. This validated method was successfully applied to a pharmacokinetic study in rabbits after the intravenous administrations of orientin and TRO PE at three different doses.

Keywords: Orientin; Liquid chromatography with ultraviolet detection; Rabbit plasma; Pharmacokinetics

1. Introduction

Orientin exists widely in plants of many families [1–6] and has a wide variety of biological activities such as radioprotection, vasorelaxant, antioxidative property, free-radical-inhibiting and antiviral activity [2,5,7–10]. For example, literatures indicate that very low non-toxic dose of orientin provides efficient protection of normal tissues in radiotherapy [7]. In addition, pharmacological studies suggest that orientin protects against foetal irradiation-induced genomic damage and instability, thereby reducing the delayed chromosomal abnormalities and tumorigenesis in adult [9].

The dried flower of *Trollius chinensis* Bunge listed in the medical literature in *Zhong Hua's Herbal Classic*, possesses antimicrobial and antiviral actions and has been used widely for a long time to treat cold, fever, chronic tonsillitis and acute tympanitis [11]. The purified herbal extract (TRO PE) from the flower of *T. chinensis* Bunge is being developed as an anti-inflammatory and anti-febrile agent. Orientin, as one of the main active constituents of TRO PE, is responsible for pharmacological effects of the herbal extracts.

Several analytical methods, including liquid chromatography –mass spectrometric method (LC–MS) [12–15], thin-layer chromatography (TLC) [16], high-speed counter-current chromatography (HSCCC) [17], high-performance thin layer chromatography (HPTLC) [18] and high-performance liquid chromatography (HPLC) [19–23], have been used for identification, determination and isolation of orientin from various herbal medicines and foods. However, to our knowledge, there is no published method for the determination of orientin in biological fluids and little literature information on the pharmacokinetics of orientin in humans or animals. It is plausible that an elucidation of the pharmacokinetics study of orientin would lead to a better understanding of the mechanism of action and facilitate further research and development of orientin, *T. chinensis* Bunge and Chinese herbal compound formulations in which *T. chinensis* Bunge is the major ingredient.

The aim of this study is to develop a validated method for determination of orientin in rabbit plasma and apply this method to the pharmacokinetic study of orientin in rabbit plasma after intravenous administration of orientin and TRO PE at three doses, respectively. Therefore, the present study represents the

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first in vivo study in which the pharmacokinetics of orientin was characterized after orientin and TRO PE dosing.

2. Experimental

2.1. Drugs and reagents

Orientin (purity >98% by HPLC) was isolated from *Trollius ledibouri* Reichb. in our laboratory [12]. The internal standard (I.S.), puerarin (99% purity by HPLC) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The chemical structures of orientin and puerarin are given in Fig. 1. Acetonitrile and methanol used for the mobile phase (HPLC grade) and other reagents used for the sample preparation (analytical grade) were obtained from Yuwang Reagent Company (Shandong, China). Double distilled water was used for all the preparations. The dried flower of *T. chinensis* Bunge was collected from the Good Agricultural Practices (GAP) Bases of Traditional Chinese Medical Materials in Chengde (Chengde, Hebei, China) and a voucher specimen (JLHCD0409) is deposited in Shenyang Pharmaceutical University for future reference. TRO PE was prepared as follows in our laboratory. The dried flower of *T. chinensis* Bunge (100 g) was refluxed with boiling water (1.2 L) for 1 h. The extraction was repeated for three times and the extract was filtered, concentrated under reduced pressure, and then precipitated with aqueous ethanol. The precipitate was discarded and the supernatant was loaded on an AB-8 macroporous resin (Nankai University, Tianjin, China) column (70 cm × 3.0 cm i.d.) eluting with aqueous ethanol. The eluate was lyophilized to obtain the TRO PE. The yield of the extract prepared by the above procedure was 7% (w/w). The content of orientin in the dried flower of *T. chinensis* Bunge was determined as 2.2% and that in the TRO PE was 20% using HPLC method.

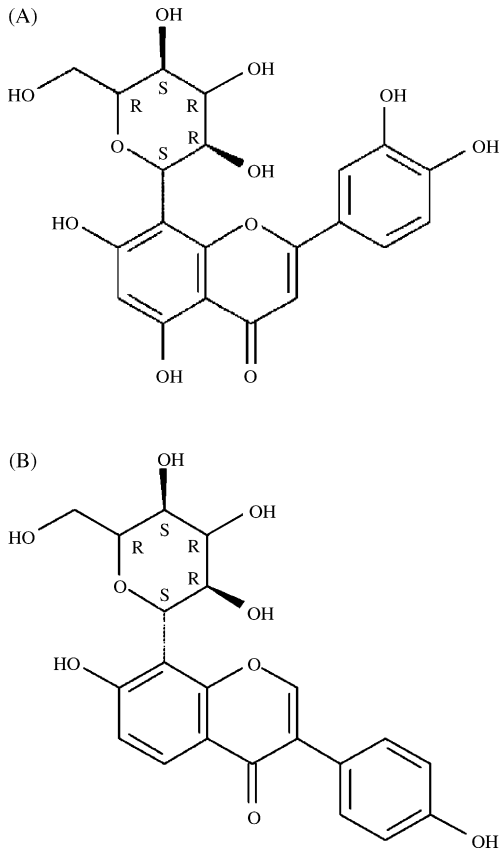

Fig. 1. Chemical structures of orientin (A) and puerarin (I.S.) (B).

2.2. Chromatographic conditions

The HPLC system consisted of a Model LC-10AP vp pump coupled to a Model SPD-10A vp ultraviolet–visible detector (Shimadzu, Kyoto, Japan). Chromatographic separation of orientin and the internal standard was achieved on a Diamonsil C_{18} column (150 mm × 4.6mm, 5 μm) from Dikma Technologies (Beijing, China) protected by a guard column (Phenomenex SecurityGuard™, C_{18} ODS, 4 mm × 3.0 mm, Cheshire, UK). Detection was performed at a wavelength of 340 nm at room temperature. The mobile phase consisted of 0.1% acetic acid/methanol/acetonitrile (80/5/15, v/v/v) at a flow rate of 1.0 mL/min. A 20 μL volume of sample was injected for each separation. Data acquisition and management were achieved with a CBM-102 chromatographic workstation (Shimadzu, Kyoto, Japan).

2.3. Preparation of standard and quality control samples

Stock solution of orientin was prepared in methanol at the concentration of 212 μg/mL and stored at 4 °C and it was further diluted in methanol to make working standards. Solution of internal standard was prepared and diluted to 40 μg/mL with methanol. Calibration curves were prepared by addition of working standards of orientin to blank plasma giving final concentrations of 0.53, 1.06, 2.12, 5.30, 13.3, 26.3 and 53.0 μg/mL. The QC samples were separately prepared in blank plasma at the concentrations of 1.06, 5.30 and 42.4 μg/mL. The spiked plasma samples were stored at −20 °C prior to analysis.

2.4. Preparation of plasma samples

To 100 μL of plasma, 50 μL of internal standard solution (puerarin, 40 μg/mL in methanol) and 250 μL methanol was added and vortexed for 1 min. The mixture was centrifuged at 10,000 × *g* for 10 min and 20 μL of the supernatant was injected into the HPLC.

2.5. Method validation

2.5.1. Selectivity

Selectivity was investigated by comparing chromatograms of blank plasma obtained from rabbits prior to dosing with those of corresponding standard plasma sample spiked with orientin and I.S. (40 μg/mL) and plasma sample from rabbits after intravenous doses of orientin and TRO PE. Blank samples of all matrixes were extracted to ensure the absence of interfering peaks.

2.5.2. Linearity and LLOQ

To evaluate linearity, calibration standards in plasma at seven concentration levels of orientin ranged 0.530–53.0 μg/mL were prepared and assayed on 3 consecutive days. Calibration curves for orientin in plasma were generated by plotting the peak area ratio (y) of orientin to puerarin versus those nominal concentrations (x) in the standard plasma by the $1/x^2$ weighted least-square linear regression. The LLOQ was defined as the lowest concentration of orientin for which an acceptable accuracy (RE) within ±20% and a precision (R.S.D.) below 20% were obtained.

2.5.3. Precision and accuracy

The accuracy and precision were assessed by determine quality control (QC) samples at three concentration levels of orientin (1.06, 5.30 and 42.4 μg/mL) on 3 consecutive days. Precision was expressed as relative standard deviation (R.S.D.) and accuracy as [(mean found concentration − added concentration)/(added concentration)] × 100%. Intra-day precision and accuracy were determined by repeated analysis of QC samples on 1 day ($n = 6$), while inter-day precision and accuracy by repeated analysis on 3 consecutive days ($n = 6$ series per day).

2.5.4. Extraction recovery

Recovery data were determined by comparing the peak area of orientin obtained from plasma samples spiked with analyte before extraction with those from the standards diluted with water at the same concentrations. This procedure was repeated for samples at three concentrations of 1.06, 5.30 and 42.4 μg/mL.

2.5.5. Stability

The stability of orientin and I.S. stock solutions was evaluated after storage at room temperature (15∼25 °C) for 4 h and at 4 °C for 14 days. The stability of orientin and I.S. working solutions was investigated at room temperature for 4 h. QC plasma samples of three concentration levels were subjected to the conditions below. Short-term stability was assessed by analyzing QC plasma samples kept at room temperature for 4 h that exceeded the routine preparation time of samples. Long-term stability was determined by assaying QC plasma samples after storage at −20 °C for 14 days. Freeze–thaw stability was investigated after three freeze (−20 °C)–thaw (room temperature) cycles. Post-preparative stability was assessed by analyzing the extracted QC plasma samples kept at room temperature for 4 h.

2.6. Pharmacokinetic application

Healthy New Zealand white rabbits (2.3 ± 0.5 kg) were provided by the Center of Laboratory Animal of Shenyang Pharmaceutical University. All the animal experiments were conducted with the approval of the Committee of Ethics of Animal Experimentation of Shenyang Pharmaceutical University. These animals were divided into six groups. Each group comprising six animals (three males and three females) were used to conduct the pharmacokinetics studies for each dose level ($n = 6$). All animals were individually housed and maintained with water and standard rabbit feed ad libitum and fasted overnight before the experiments. Just prior to the experiment, rabbits were weighted and restrained in rabbit restrainers. In the PK study day animals were intravenously administered via the marginal vein of the ear with orientin at dose of 2.69, 5.38 and 10.75 mg/kg and TRO PE at dose of 13.44, 26.88 and 53.75 mg/kg (equivalent to 2.69, 5.38 and 10.75 mg/kg orientin), respectively. The TRO PE and orientin were freshly dissolved in 0.9% sodium chloride injection under pH 8.2 and passed through 0.22 μm filter, respectively. Blood samples (0.4 mL) were collected from the marginal vein of the ear into heparinized just prior to dosing and at 4, 8, 12, 16, 30, 45, 60, 90, 120, 150 and 180 min after intravenously administration of orientin and TRO PE. The sampling interval was set according to a preliminary test. Plasma samples were immediately separated by centrifugation at 10,000 × g for 10 min and stored at −20 °C until analysis.

Area under the curve (AUC_{0-t}) was calculated using the linear-trapezoidal rule. One-way analysis of variance (ANOVA) was used to assess statistical significance and a P-value of less than 0.05 was considered as statistically significant.

3. Results and discussion

3.1. Method development

Liquid–liquid extraction and protein precipitation are the commonly used extraction techniques. Liquid–liquid extraction usually offers much cleaner sample that in turn makes the method more robust and scalable. However, during the course of our method development, we found that the use of liquid–liquid extraction technique (solvents tested including ether, ethyl acetate and *n*-butanol) resulted in a relatively low recovery for orientin and the internal standard due to the polarity. The relatively high recovery was obtained with the addition of acidifying reagent, but at the cost of the variable recovery or more interfering peaks which make the precision or specificity of the method unacceptable. Therefore, the method of protein precipitation was considered. The use of methanol as the plasma protein precipitating reagent afforded the acceptable recovery for both orientin and internal standard, clear supernatant and without interfering peaks. So plasma samples were subjected to a simple protein precipitation procedure using the extraction routine described in Section 2.4.

During the method development stage, several glycosides such as icariside, phillyrin, raringin, hespeidin, icariin, syringin and puerarin which have the similar chemical structures as orientin were initially tested as internal standards. Among these, puerarin has been chosen as the most appropriate in the present analysis because it was well separated from orientin and does not exist endogenously in plasma during pharmacokinetic studies. Moreover, the elution time of puerarin, in the present study, was shorter than that of orientin while the elution time of the other tested substances was much longer than that of orientin resulting in considerable elongation of the total analytical time.

The mobile phase comprised of 0.1% acetic acid/methanol/acetonitrile (80/5/15, v/v/v) was used. The appropriate content

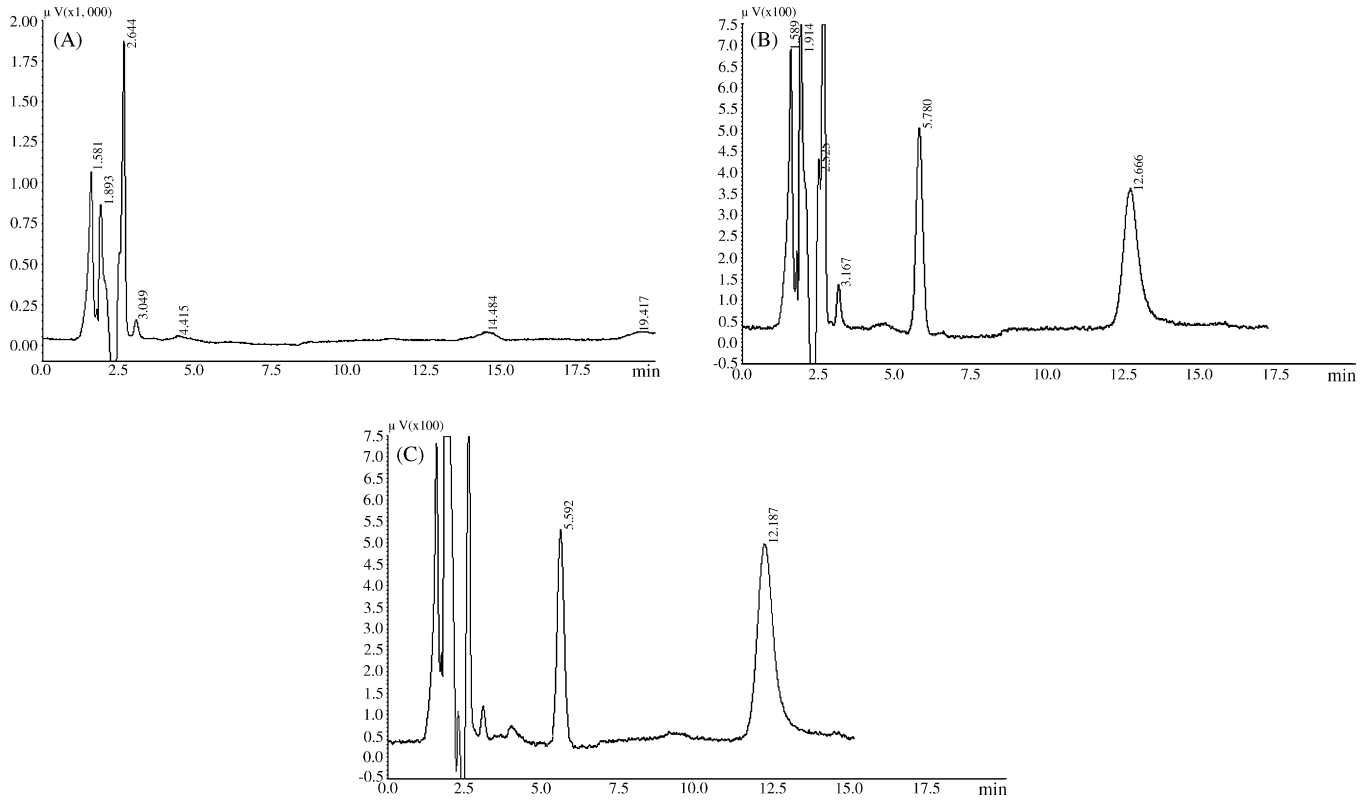

Fig. 2. Represent chromatograms of orientin and puerarin (I.S.) in a blank plasma sample (A); a blank plasma sample spiked with orientin at 5.30 μg/mL and I.S. (40 μg/mL) (B) and a plasma sample from a rabbit 4 min after intravenous administration of TRO PE at 13.44 mg/kg (C). No interferences were observed at the retention times of the analytes. The retention times for orientin and I.S. were 5.8 and 12.7 min, respectively.

of acetic acid was used to achieve satisfactory peak symmetry and peak resolution simultaneously. Eventually, the 0.1% acetic acid and the ratio of the final composition of the mobile phase was chosen to achieve the acceptable peak shape, appropriate retention time and satisfactory separation of orientin from endogenous components in rabbit plasma.

3.2. Method validation

3.2.1. Specificity

The specificity of the method was determined by comparing the chromatograms of blank plasma with the corresponding spiked plasma. Typical chromatograms of blank plasma, a spiked plasma standard and a real plasma sample from a pharmacokinetic study are shown in Fig. 2. Good resolution for every peak and its nearest ones was confirmed by resolution values, *R*s, which were greater than 1.5.

3.2.2. Linearity and LLOQ

Linear calibration curves for orientin were obtained throughout the concentration range studied (0.530–53.0 μg/mL). Representative regression equation for the calibration curve was $y = 4.015 \times 10^{-1}x - 3.511 \times 10^{-2}$ ($r = 0.9956$). The lower limit of quantification (LLOQ) for orientin was 0.530 μg/mL with precision and accuracy presented in Table 1.

3.2.3. Precision and accuracy

The data of intra- and inter-day precision and accuracy for orientin (Table 1) showed that the intra- and inter-day precisions (R.S.D.s) were less than 3.0 and 11.4%, while the corresponding accuracy (RE) was within ±11.4%, indicating acceptable precision and accuracy of the present method.

3.2.4. Extraction recovery and stability

The mean extraction recoveries of orientin from rabbit plasma were 73.3 ± 6.5, 77.2 ± 0.5 and 67.1 ± 1.5% at concentration

Table 1
Precision and accuracy for the determination of orientin in rabbit plasma (intra-day: $n = 6$; inter-day: $n = 6$ series per day, 3 days)

Added C (μg/mL)	Found C (μg/mL)	Intra-day R.S.D. (%)	Inter-day R.S.D. (%)	Accuracy RE (%)
0.530 (LLOQ)	0.540 ± 0.008	0.7	3.5	1.9
1.06 (low)	1.00 ± 0.02	1.7	3.8	−5.2
5.30 (middle)	4.69 ± 0.12	2.5	1.1	−11.4
42.4 (high)	40.8 ± 2.0	3.0	11.4	−3.8

Table 2
Stability of orientin in rabbit plasma at three QC levels ($n = 5$)

Stability	Accuracy (mean ± S.D.)		
	1.06 μg/mL	5.30 μg/mL	42.4 μg/mL
Short-term stability	1.00 ± 0.02	5.02 ± 0.27	43.1 ± 1.00
Long-term stability	1.07 ± 0.04	5.00 ± 0.37	41.0 ± 0.98
Freeze–thaw stability	1.03 ± 0.05	4.76 ± 0.16	43.7 ± 3.94
Post-preparative stability	0.970 ± 0.06	4.70 ± 0.04	47.0 ± 0.57

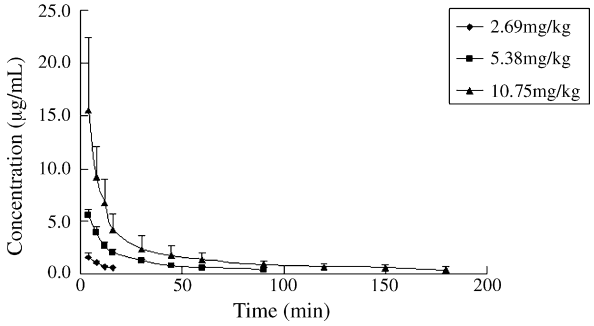

Fig. 3. Mean plasma concentration–time profiles of orientin in rabbits after intravenous administration of 2.69, 5.38 and 10.75 mg/kg of orientin.

levels of 1.06, 5.30 and 42.4 μg/mL, respectively. Table 2 summarizes the results of short-term stability, long-term stability, freeze–thaw stability of orientin in plasma and post-preparative stability. All the results well met the criterion for stability measurements.

3.3. Pharmacokinetic application

The developed method has been successfully used for the pharmacokinetic study of orientin after an intravenous administration of orientin at doses of 2.69, 5.38 and 10.75 mg/kg while TRO PE at doses of 13.44, 26.88 and 53.75 mg/kg to rabbits. Due to the extremely low plasma concentrations after 2.69 mg/kg of orientin, the pharmacokinetic profiles of orientin could not be characterized completely. The concentration–time curves (mean ± S.D.) of orientin after intravenous administration of orientin and TRO PE in rabbits are shown in Figs. 3 and 4, respectively. After administered with 13.44, 26.88 and 53.75 mg/kg of TRO PE, significant

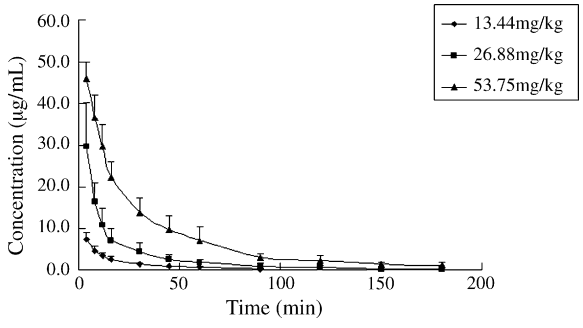

Fig. 4. Mean plasma concentration–time profiles of orientin in rabbits after intravenous administration of 13.44, 26.88 and 53.75 mg/kg of TRO PE (equivalent to containing 2.69, 5.38 and 10.75 mg/kg of orientin).

increase ($P < 0.05$) in dose-normalized AUC_{0-t} (11.4 ± 2.1, 21.5 ± 7.3 and 27.6 ± 4.6 μg min/mL, respectively) was found across the investigated dosage range which was consistent with dose-dependent, non-linear plasma pharmacokinetics. There were significant differences in pharmacokinetics parameters between the two preparations (orientin and TRO PE) at the same orientin dosage levels. The 4.5-fold (577.4 ± 197.4 μg min/mL versus 126.0 ± 13.2 μg min/mL) and 4.0-fold (1485.6 ± 246.5 μg min/mL versus 363.2 ± 126.1 μg min/mL) enhancements of AUC_{0-t} were observed from TRO PE as compared with orientin at the same dose containing 5.38 and 10.75 mg/kg of orientin, respectively. This was probably caused by the effect of the other constituents in TRO PE. Moreover, several orientin-based flavonoid *O*-glycosides (such as 2″-*O*-β-L-galactopyranosylorientin, 2″-*O*-β-arabinopyranosylorientin, 2″-*O*-(3‴, 4‴-dimethoxybenzoyl)orientin and 2″-*O*-(2‴-methylbutyryl)orientin) were found in TRO PE by the published method [12] and they could be transformed into orientin in vivo which consequently caused the increase in AUC.

4. Conclusion

We succeeded in the development of the method for the quantitative determination of orientin in rabbit plasma. The method was validated for linearity, specificity, accuracy, precision, LLOQ and recovery and good results were obtained.

The method has been successfully applied to the analysis of orientin in rabbit plasma after intravenously administration of orientin and TRO PE at three doses. The results of present study indicated that orientin showed non-linear pharmacokinetics in rabbits in the studied dose range. Significant increase in AUC_{0-t} was found from TRO PE dosing compared with orientin dosing at the same dosage levels in the comparative pharmacokinetic study. The mechanism is still ambiguous and further profound researches are required. The pharmacokinetic results are useful for the further study of the clinical applications of the orientin and *T. chinensis* Bunge.

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