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Pharmacokinetic Investigation of Main Bioactive Components from Epimedium-Derived Flavonoids in Rabbit Serum by Ultra-Performance Liquid Chromatography Tandem Mass Spectrometry

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Abstract

Background: Total Epimedium-derived flavonoids (EF) were reported to be a group of major bioactive constituents present in genus Epimedium for the treatment of osteonecrosis. The present study aimed to investigate the pharmacokinetics of main bioactive components of EF in rabbit.

Methods: A rapid and sensitive method for the simultaneous determination of icariin, icariside I, icariside II and icaritin in serum was established and validated by ultra-performance liquid chromatography coupled with quadrupole time-of-flight tandem mass spectrometry (UPLC-Q-TOF/MS). Serum samples were pretreated with water-saturated ethyl acetate. Furthermore, sample separation was achieved on an Waters BEH C18 column (50 mm × 2.1 mm, 1.7 μm) by gradient elution with acetonitrile-water (both including 0.1% formic acid) as mobile phase. Finally, pharmacokinetics of EF in rabbit serum was investigated.

Results: All calibration curves displayed excellent linearity (r^2>0.99) within 1.2~24 ng/mL. The assay was specific, precise and accurate, as demonstrated with intra-run precisions not more than 12.6%, inter-run precisions less than 9.9% and accuracies between -4.4% and 9.3%. Meanwhile, the matrix effects, extraction recoveries and stabilities were all satisfactory. Moreover, this validated method was successfully applied to analyse the pharmacokinetic of EF in rabbit serum after oral dosage of 80 mg/kg.

Conclusion: These results would enlarge our knowledge about main bioactive components of EF for the treatment of osteonecrosis in rabbit.

Keywords: Epimedium-derived flavonoids; Osteonecrosis; Main bioactive components; Pharmacokinetic; Rabbit; UPLC-Q-TOF/MS

Introduction

Herba Epimedii, the dried aerial parts of Epimedium L. (Berberidaceae), are a widely used Chinese medicine for impotence and bone loss in East Asian countries for thousands of years [1-3]. Epimedium-derived flavonoids (EF) was reported to be a group of major bioactive constituents presents in Epimedium for promoting osteoblastogenesis, preventing bone loss and steroid-associated osteonecrosis [4-6]. In clinics, a 24-month randomized double-blind placebo-controlled clinical trial had proved that the Epimedium-derived phytoestrogen flavonoids could exert beneficial effects on preventing bone loss in late postmenopausal women, without resulting in a detectable hyperplasia effect on the endometrium [7]. Among these Epimedium-derived flavonoids, icariin and icaritin were considered as the potential agents for treating steroid associated osteonecrosis [8-13].

These biological activities above had stimulated increasing interest in the *in vivo* metabolism of EF. Poor oral bioavailability and first-pass effect of prenylflavonoids had been performed by human intestinal Caco-2 and perfused rat intestinal models [14]. Meanwhile, the amount and bioactivity of intestinal flora and enzymes changed in ovariectomized rats, which affected the intestinal absorption and hydrolysis of epimedium total flavonoids [15]. In previous study, we found that icariin, icarisid I, icarisid II and icaritin were the main absorbed components in rabbit's serum after intragastric administration of EF [16]. In rabbit's intestine, the main metabolic pathways of EF were the sequential deglycosylation metabolism in rat intestine to form secondary glycosides and final product icaritin [17]. Additionally, icaritin was easily conjugated with a glucuronic acid to form phase II metabolites in liver, which indicated that biliary clearance was one of the major routes of excretion [18]. To better understand the metabolic fate, the pharmacokinetics of EF were conducted in this study. In the past decade, ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC/Q-TOF-MS) had been widely introduced to rapidly identify and quantify trace components in complex samples, which exhibited a lot of superiority (superior resolution, high sensitivity and sample throughout) [19,20]. In this study, a rapid and feasible method for icariin, icariside I, icariside II and icaritin was established and validated. Furthermore, the method was successfully applied to investigate the pharmacokinetics of the main components of EF in rabbit serum after oral administration by UPLC/Q-TOF-MS. Taken together, this study would provide a basis

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of the mechanisms of action and further pharmacological studies of EF for the treatment of osteonecrosis.

Materials and Methods

Materials and reagents

Epimedium-derived flavonoid (EF) glycosides (icariin ≧ 83%, epimedoside A ≦ 1.65%, hexandraside F<0.91%, epimedin A<1.18%, epimedin B ≦ 1.52%, epimedin C<4.1% and icariside II<1.84%) were provided by Beijing TongRenTang Health Pharmaceutical Corp., Ltd (Beijing, China). Icariin, icariside I, icariside II and icaritin (shown in Figure 1) were all purchased from Shanghai Winherb Medical Science Corp., Ltd. (Shanghai, China) with purity above 98%. Acetonitrile and Methanol were of HPLC grade and purchased from Dikma Technologies Inc. (Beijing, China). Watsons water was purchased from Beijing Watsons Water Corp., Ltd. (Beijing, China). Other reagents were of analytical grade.

Animals

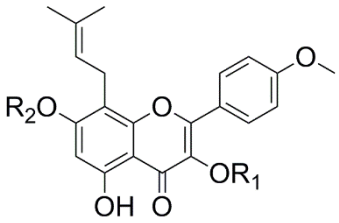
Specific pathogen free (SPF) male New Zealand rabbits (2.75 ± 0.25 kg) were provided by Guangdong Medical Laboratory Animal Center (Guangdong, PR China). The rabbits were kept in a designated animal room at constant temperature (25 ± 2)°C and humidity (55 ± 10)% with 12 h of light/dark per day and free access to water and food. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Jinan University. All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health).

Preparation of standard solutions

Stock solutions of four flavonoids were prepared separately in methanol at a concentration of 36 μg/mL. Calibration samples were prepared by spiking 100 μL of the appropriate working solution to 2 mL of blank rabbit serum. The obtained serum concentrations were 1.2, 3.0, 6.0, 9.0, 12.0, 18.0 and 24.0 ng/mL. The quality control samples (QCs) were independently prepared in the same manner to obtain the serum concentrations of 3.0, 9.0 and 18.0 ng/mL representing low, medium and high concentration levels, respectively. All the solutions were stored at 4°C before use.

Serum sample preparation

Each serum sample (2 mL) was transferred to a 15 mL polypropylene tube containing 100 μL of methanol. Water-saturated ethyl acetate (4 mL) was added, then vortex-mixed vigorously for 1 min and centrifuged at 10000 rpm for 10 min. The supernatant was then transferred and evaporated to dryness under nitrogen stream at room temperature. The residue was re-dissolved in 500 μL of methanol, and and 4 μL aliquots were then injected into the UPLC-Q-TOF/MS system.



icariin: R_1=Rha, R_2=Glc
icariside I: R_1=H, R_2=Glc
icariside II: R_1=Rha, R_2=H
icaritin: R_1=H, R_2=H

Figure 1: Chemical structures of icariin, icariside I, icariside II and icaritin.

UPLC/Q-TOF-MS conditions

UPLC was performed using an Acquity™ UPLC system (Waters Corporation, Manchester, U.K.) with an auto-sampler at 4°C. Chromatographic separation was performed on an Acquity UPLC BEH C_{18} column (1.7 μm, 2.1 × 50 mm; Serial No: 01963110215567, Waters Corporation, USA) coupled with a VanGuard™ pre-column (1.7 μm, 2.1 × 5 mm; Waters Corporation, USA). The mobile phase consisted of solvent A (water including 0.1% formic acid) and solvent B (acetonitrile including 0.1% formic acid) delivered at a flow rate of 0.6 mL/min. The gradient program was as follows: 0~0.3 min, 30% B; 0.5 min, 40% B; 2 min, 52% B; 3 min 60% B; 3.4 min, 100% B; 3.8 min, 100% B; 3.81 min, 30% B; 4 min, 30% B. The column temperature was maintained at 25°C and the injection volume was 4 μL. The UPLC system was coupled to a hybrid quadrupole orthogonal time-of-flight (Q-TOF) tandem mass spectrometer (SYNAPT™ G2 HDMS, Waters, Manchester, U.K.) with electrospray ionization (ESI). The ESI source was operated in the positive ionization mode and optimized conditions for maximum detection of metabolites were as follows: capillary voltage, 3.0 kV; sampling cone, 35 V; extraction cone, 4.0 V; source temperature, 100°C; desolvation temperature, 300°C. The cone and desolvation gas (N_2) flows were set at 50 and 800 (L/h). The full scan mass range was 50~1200 Da. Selective reactions monitoring (SRM) parameter of icariin, icariside I, icariside II and icaritin were as follow. The *m/z* 677.244 → 369.134 transition with a collision energy (CE) of 25 eV for icariin form 0.50 to 0.84 min; *m/z* 531.186 → 369.134 transition with a CE of 25 eV for icariin form 1.10 to 1.44 min; *m/z* 369.134 → 313.071 transition with a CE of 30 eV for icariin form 1.36 to 1.70 min; *m/z* 369.134 → 313.071 transition with a CE of 25 eV for icariin form 3.00 to 3.50 min. The method employed lock spray with leucine enkephalin (*m/z* 556.2771 in positive ion mode and *m/z* 554.2615 in negative ion mode) to ensure mass accuracy.

Method validation

The method was validated for specificity, linearity, matrix effects, extraction recovery, precision, accuracy and stability according to the Guidance for Industry: bioanalytical method validation from the US Food Drug Administration (FDA) [21]. To ensure there were no significant endogenous interferences, specificity was determined by comparing the chromatograms obtained for each blank serum sample (from six different rabbits), rabbit serum with standard solutions at LLOQ concentrations and serum samples collected 0.5 h after oral administration of EF with a dose of 80 mg/kg.

For the linearity, calibration curves were constructed by plotting peak area of analytes (y) *versus* respective serum concentrations (x) using a $1/x^2$ weighting factor and linear least-squares regression analysis, and the slope, intercept and correlation coefficient of each curve were determined. The LLOQ was defined as the lowest concentration in the standard curve that can be measured with acceptable accuracy and precision. The accuracies and inter/intra-day precisions of the assay were evaluated by determining six replicates of QC samples (at low, middle and high concentrations) on three consecutive days.

Extraction recoveries (ER) and matrix effects (ME) were evaluated using a published experimental protocol [22]. The peak areas of three different concentrations of analytes within quality control (QC) samples (3.0, 9.0 and 18.0 ng/mL) were defined as A1. A2 referred to the peak areas pertaining to analytes within extracted control serum samples reconstituted with standard solutions at three concentrations.

A3 corresponded to the responses of analytes obtained by directly injecting the corresponding pure reference standards at three QC levels. Extraction recoveries and matrix effects were calculated as follows: ER%=A1/A2 × 100%, and ME%=A2/A3 × 100%. The percentage of the relative standard deviation (RSD%) was used to report the precision.

The stability of icariin, icariside I, icariside II and icaritin in rabbit serum were analyzing triplicate QC samples (n=3) at concentrations of 3.0, 9.0, 18.0 ng/mL stored for 8 h at ambient temperature, for 48 h at -80°C and also following three cycles of freezing at -80°C and thawing, for 24 h at ambient temperature after water-saturated ethyl acetate extraction. Concentrations of these serum samples following the above storage process were compared to those of freshly prepared serum samples to verify the assay stability.

Pharmacokinetic analysis

The proposed method was applied to investigate the pharmacokinetics of EF in rabbit serum. Three New-Zealand male rabbit (SPF degree) was given to rabbit with EF at a dose of 80 mg/kg. Blood samples were collected in blood collection tubes before and 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0 h time points post-dosing. After collecting, rabbit was added the same amount of saline each time. The serum samples were separated by incubation for 2 h at 37°C and centrifugation at 3000 rpm for 10 min, which were stored at -80°C until analysis.

Results and Discussion

Specificity

The specificity result showed that no significant interferences from endogenous substances were observed at the retention times of icariin, icariside I, icariside II and icaritin. The selective reaction monitoring (SRM) chromatograms of icariin, icariside I, icariside II and icaritin were shown in Figure 2.

Linearity of calibration curves and lower limits of quantification

The calibration curves were linear over the concentration range of 1.2~24.0 ng/mL for icariin, icariside I, icariside II and icaritin. Correlation coefficients generated by linear regression with a $1/x^2$ weighting factor ranged from 0.9914 to 0.9945. The detailed linear correlation paramete s and LODs and LOQs of four analytes in rabbit serum after oral administration of EF were displayed in Table 1. The LLOQ was confirmed to be 1.2 ng/mL, at which the intra- and inter-run accuracies were within ± 20%. Intra- and inter-run precisions at the LLOQ level were not more than 20%.

Precision and accuracy

Accuracy and precision of the assay were summarized in Table 2. Both intra- and inter-run accuracies for all tested concentrations were

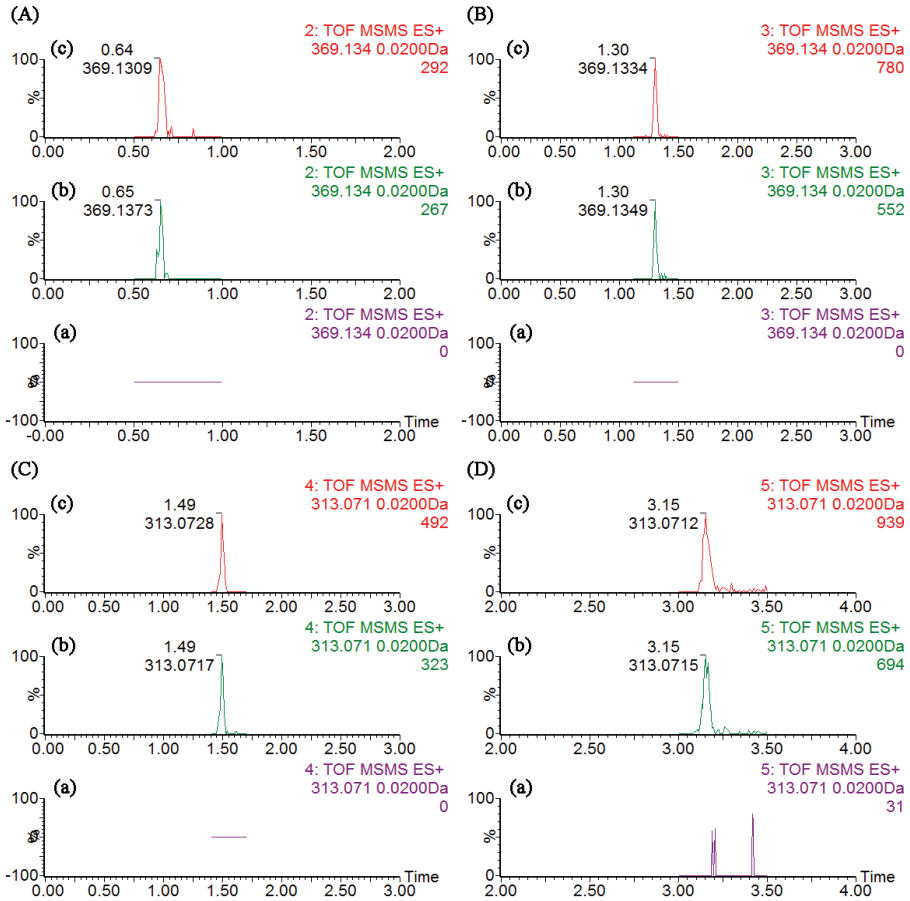


Figure 2: Selective reaction monitoring (SRM) chromatograms of icariin (A), icariside I (B), icariside II (C) and icaritin (D) in blank serum (a), drug serum at 0.5 h after oral administration; (b), blank serum spiked with standards resolution at LLOQs (c).

between -4.4% and 9.3%. Intra-run precisions were not more than 12.6% and inter-run precisions were not more than 9.9%. These results demonstrated that accuracy and precision of the assay were within acceptable limits.

Matrix effects and extraction recovery

The results of the matrix effect and extraction recovery were both summarized in Table 3, which meant that no significant matrix effect for icariin, icariside I, icariside II and icaritin in rabbit serum. The matrix effect of four analytes were from 94.5% to 113.9% with RSD values less than 14.8%. The extraction recovery of icariin, icariside I, icariside II and icaritin were all above 89.1% with RSD values no more than 14.4%. The results demonstrated that the recoveries obtained were consistent and reproducible and did not indicate significant enhancement or suppression of ionization for any of the analytes studied.

Stability

The results of stability experiments were given in Table 4. The data indicated that the serum samples was stable after frozen at -80°C for 48 h, after three freeze-thaw cycles, after water-saturated ethyl acetate

Compound	Slope	Intercept	Concentration range (ng/mL)	r^2	LODs (ng/mL)	LOQs (ng/mL)
icariin	3.1998	0.1275	1.2~24.0	0.9914	0.3	0.6
icariside I	4.3561	-0.9366	1.2~24.0	0.9926	0.3	0.6
icariside II	1.2632	-0.5109	1.2~24.0	0.9945	0.3	0.6
icaritin	7.0131	1.6212	1.2~24.0	0.9939	0.3	0.6

Table 1: Linear correlation parameters and LLOQs of four analytes in rabbit serum after oral administration of Epimedium-derived flavonoids extracts.

Compounds	Conc. (ng/mL)	Intra-run (n=5)			Inter-run (n=15)		
		Measured Conc. (ng/mL)	RE (%)	RSD (%)	Measured Conc. (ng/mL)	RE (%)	RSD (%)
icariin	3.0	3.1 ± 0.32	10.4	3.3	3.04 ± 0.30	9.9	1.3
	9.0	9.1 ± 0.73	8.1	1.1	9.3 ± 0.64	6.9	3.3
	18.0	17.2 ± 1.37	8.0	-4.4	17.7 ± 1.07	6.1	-1.7
icariside I	3.0	3.06 ± 0.36	11.8	2.0	3.08 ± 0.27	8.8	2.7
	9.0	9.08 ± 1.14	12.6	0.9	9.02 ± 0.72	8.0	0.2
	18.0	17.54 ± 0.72	4.2	-2.6	17.43 ± 1.19	6.9	-3.2
icariside II	3.0	2.88 ± 0.33	11.5	-4.0	3.05 ± 0.30	9.9	1.7
	9.0	8.76 ± 0.99	11.4	-2.7	9.2 ± 0.91	9.9	2.2
	18.0	17.54 ± 1.00	5.8	-2.6	17.75 ± 0.82	4.7	-1.4
icaritin	3.0	3.28 ± 0.26	8.0	9.3	3.14 ± 0.26	8.3	4.7
	9.0	9.22 ± 0.63	6.9	2.4	9.19 ± 0.60	6.6	2.1
	18.0	18.58 ± 1.18	6.4	3.2	18.11 ± 0.91	5.1	0.6

Table 2: Inter/intra-day accuracy and precision for icariin, icariside I, icariside II and icaritin in rabbit serum after oral administration of Epimedium-derived flavonoids extracts.

Compound	Concentration (ng/mL)	ME (%)	RSD (%)	ER (%)	RSD (%)
icariin	3.0	113.2 ± 9.7	14.4	98.7 ± 6.1	14.3
	9.0	101.4 ± 6.9	6.8	104.0 ± 6.3	6.1
	18.0	104.7 ± 6.1	5.8	89.1 ± 6.2	7.9
icariside I	3.0	112.1 ± 11.5	14.7	110.7 ± 7.1	10.8
	9.0	113.9 ± 8.4	6.8	99.3 ± 10.3	10.4
	18.0	111.3 ± 7.4	6.1	113.8 ± 6.1	5.3
icariside II	3.0	94.5 ± 15.9	14.8	100.6 ± 10.6	13.5
	9.0	108.0 ± 10.8	10.0	101.9 ± 10.7	11.5
	18.0	103.0 ± 8.9	8.7	90.1 ± 7.6	8.4
icaritin	3.0	106.1 ± 9.5	14.4	116.1 ± 9.6	14.4
	9.0	106.7 ± 9.9	8.3	99.8 ± 5.5	5.5
	18.0	108.9 ± 10.7	7.2	105.6 ± 4.9	9.6

Table 3: Matrix effect (ME) and extraction recovery (ER) of icariin, icariside I, icariside II and icaritin in rabbit serum (n=5).

Compound	Conc. (ng/mL)	At -80°C for 48 h		Three freeze-thaw cycles		Extracted samples at room temperature for 24 h		At room temperature for 8 h	
		RE(%)	RSD(%)	RE(%)	RSD(%)	RE(%)	RSD(%)	RE(%)	RSD(%)
icariin	3.0	-4.3	11.2	10	6.1	10	13.4	1	8.3
	18.0	4.4	9.1	6.7	0.9	3.3	0.6	6.8	1.4
icariside I	3.0	-2.3	8.6	10	3.1	-2.3	7.2	3.3	11.7
	18.0	3.3	5.5	4.6	5.4	2.4	6.4	6.5	3.0
icariside II	3.0	4.3	8.0	11	6.4	0	13.4	2.3	9.5
	18.0	0.6	4.2	-8.5	5.2	-6.3	4.9	5.7	2.5
icaritin	3.0	12.3	3.6	4.3	11.2	14.3	1.8	-10	6.3
	18.0	1.7	6.1	-4.1	2.5	-5.7	7.9	0.2	0.7

Table 4: Stability results of icariin, icariside I, icariside II and icaritin in rabbit serum (n=3).

extraction at ambient temperature for 24 h and under storage for 8 h at ambient temperature.

Pharmacokinetic application

The validated method was successfully applied to investigate the pharmacokinetic of icariside II, the main bioactive component in rabbit serum after oral administration of EF with a single dose of 80 mg/kg. As the main absorbed bioactive component, icariside II could be determined accurately in dosed-rabbit serum. However, the other three xenobiotics (icariin, icariside I and icaritin) appeared in dosed-rabbit serum with concentrations lower than the LLOQ and could not be accurately determined except individual time points. But we still estimate the concentration of icariin, icariside I and icaritin preliminarily by data extrapolation of calibration curves, which could help investigate the dynamic concentration profiles in rabbit serum.

The mean concentration-time profiles of icariin, icariside I, icariside II and icaritin were shown in Figure 3 after oral administration of EF with a single dose of 80 mg/kg. The pharmacokinetic parameters of icariside II were summarized in Table 5. The T_{max} and $t_{1/2}$ values were 4.0 ± 0.0 h and 1.03 ± 0.076 h, respectively. The C_{max} value was 20.83 ± 3.96 ng/mL. In addition, AUC_{0-t} and $AUC_{0-\infty}$ values were 83.92 ± 5.34 and 87.08 ± 5.17 ng•h/mL, respectively. MRT_{0-t} and $MRT_{0-\infty}$ values were 4.28 ± 0.27 and 4.47 ± 0.25 h. These results indicate that the main bioactive metabolite, icariside II was more easily absorbed from the EF, while the pharmacokinetic parameters were not available for icariin, icariside I and icariside II. Previous study have indicated that poor oral bioavailability and first-pass effect of prenylflavonoids had been performed by human intestinal Caco-2 and perfused rat intestinal models [14]. Generally, the *in vivo* metabolism of Herba Epimedii and its prenylflavonoids could easily be metabolized in gastrointestinal tract following deglycosylation reaction. In this study, EF contain many prenylflavonoids, such as icariin (≥ 83%), epimedoside A (≤ 1.65%), hexandraside F (<0.91%), epimedin A (<1.18%), epimedin B (≤ 1.52%), epimedin C (<4.1%) and icariside II (<1.84%). Icariside II have proved be one of the major intermediate decomposition products of these prenylflavonoids [23]. This may explain the higher absorption of C_{max} observed for icariside II in the present study compared to previously published reports [24].

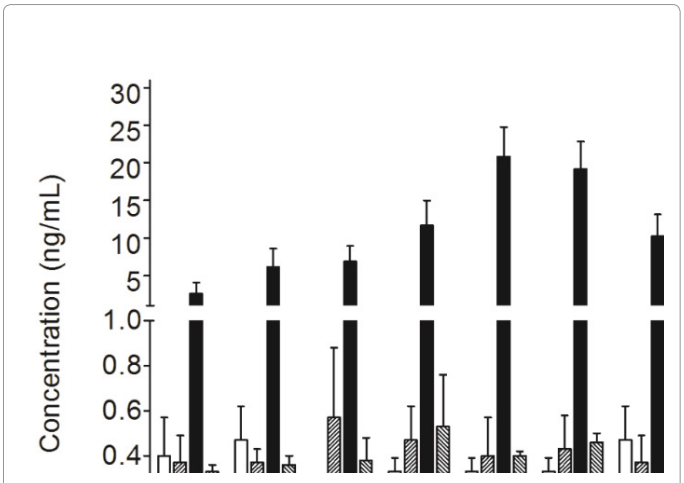


Figure 3: Mean concentration-time profiles of icariin, icariside I, icariside II and icaritin in rabbit serum after oral administration of Epimedium-derived flavonoids extracts.

Parameters	Values
T_{max} (h)	4.0 ± 0.0
C_{max} (ng/mL)	20.83 ± 3.96
$t_{1/2}$ (h)	1.03 ± 0.076
AUC_{0-t} (ng h/mL)	83.92 ± 5.34
$AUC_{0-\infty}$ (ng h/mL)	87.08 ± 5.17
MRT_{0-t} (h)	4.28 ± 0.27
$MRT_{0-\infty}$ (h)	4.47 ± 0.25

Table 5: Pharmacokinetic parameters of icariside II in rabbit serum after oral administration of Epimedium-derived flavonoids extracts.

In conclusion, a rapid and sensitive UPLC-Q-TOF/MS method has been established for simultaneous analysis of icariin, icarisid I, icarisid II and icaritin in rabbit serum. Furthermore, the validated method was successfully applied for the pharmacokinetics of EF in rabbit serum. This study can enlarge our knowledge of *in vivo* metabolic fate of EF after oral administration. Furthermore, the bioactivity evaluation would be contributed to clarifying how the EF reduce the risk of Steroid-induced Osteonecrosis in rabbit.

Acknowledgements

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