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Research Article
Metabolic Regularity of Bioactive Compounds in *Epimedium* in Rats Based on LC-MS Analysis Technology

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Abstract

Background and Objective: *Epimedium* is an important traditional medicinal and edible plant in China. It is not only used as medicine but is often developed into health care products or functional food for long-term use. However, adverse reaction associated with its long-term use includes thirst or nosebleed. This study aimed to reveal the metabolic regularity of bioactive compounds in *Epimedium*, so as to explore the causes of its adverse reactions. **Materials and Methods:** In the current UPLC-QTOF-MS technique was adopted for qualitatively identifying the prototype components of *Epimedium* in the blood after multiple oral administrations in rats. Thereafter, a method for the simultaneous determination of eight components containing p-hydroxycinnamic acid, salidroside, brevicornin, lauric acid, liquiritigenin, sagittatosdie B, icariside I and icariside II in rat plasma was established by UPLC-QQQ-MS technology. Thereafter, this method was successfully used in the pharmacokinetic study of the eight analytes in rats after multiple oral administrations of *Epimedium*. **Results:** Totally 22 prototype components were determined in rat plasma, of which 16 were flavonoids and a small number of acids, phenols and triterpenes were also detected. The pharmacokinetic parameters of eight components were obtained, as a result, compared with a single administration, multiple administration induced the slow metabolic rate, prolonged Tmax, increased bioavailability and prolonged transformation time of chemical ingredient monomers. **Conclusion:** The relative increases of these ingredients in the whole body may be the reason for adverse reactions after long-term administration of *Epimedium*.

Key words: *Epimedium*, bioactive compounds, metabolic regularity, pharmacokinetics, UPLC-QTOF-MS, UPLC-QQQ-MS

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Epimedium is the dry leaf of *Epimedium brevicornu* Maxim., *Epimedium pubescens* Maxim., *Epimedium koreanum* Nakai. or *Epimedium sagittatum* (Sieb. et Zucc.) Maxim. It is a perennial herb mainly distributed in Asia, the Middle East, the temperate and subtropical regions of Europe and about 85% of the taxa are endemic to China. As an important traditional medicinal and edible plant in China, *Epimedium* has been used in herbal preparations or functional foods for more than 2000 years[1]. *Epimedium* displays a wide range of clinical indications, especially for the treatment of sexual dysfunction and osteoporosis[2,3]. As revealed by modern pharmacological research, *Epimedium* also has the activities of regulating the immune system, improving cardiovascular function, anti-cancer, anti-aging, anti-depression, anti-asthma and anti-senile dementia[4]. However, long-term clinical use of *Epimedium* may cause toxic reactions and symptoms such as thirst and nosebleed[5]. To find out the substances that cause adverse reactions after long-term administration of *Epimedium*, this study adopted the methods of blood component analysis and pharmacokinetic study of main monomer components to explore the change rules of substance metabolism in *Epimedium* caused by the duration of administration, so as to shed more lights on the causes of toxicity and side effects and the reasonable use of *Epimedium* in clinical treatment.

The chemical composition of *Epimedium* is complex, mainly including flavonoids, lignans, organic acids, alkaloids, saccharides and other components[6]. Previous studies have suggested that *Epimedium* possesses a large number of flavonoids, mainly isopentenyl flavonoids, which are generally considered to be the main bioactive ingredients of *Epimedium*, such as icariin, epimedin A, epimedin B and epimedin C. According to the *in vivo* pharmacokinetics studies of *Epimedium*, most of the index components are single or multiple flavonoids like icariin and *Epimedium* A, B, C[7,8], while, other flavonoids (such as ikarisoside A, sagittatoside A and sagittatoside B), phenolic acids and alkaloids are also reported in small amounts. Besides, most of them are given by single administration[9], which cannot scientifically explain the causes of adverse reactions induced by long-term administration.

According to the clinical and people's actual medication habits, *Epimedium* is not only used as medicine but often developed into health care products or functional food for long-term use[1]. Therefore, based on the multiple administration of *Epimedium* in rats, our research team utilized UPLC-QTOF-MS technology to qualitatively identify the prototype components of *Epimedium* in the blood. Thereafter, UPLC-QQQ-MS technology was employed to obtain the blood concentration-time curve and the main pharmacokinetic parameters of the last administration after the blood concentration of rats reached a steady state. Later, differences in chemical composition, C_{max}, T_{max}, $T_{1/2}$, AUC_{0-t}, MRT and other main parameters between multiple administration and single administration were analyzed and compared. Furthermore, the metabolic rules of various bioactive components were discussed and substances that might cause toxic and side effects after long-term use were identified, so as to provide a basis for better utilization and food safety of *Epimedium*.

MATERIALS AND METHODS

Study area: The current work was performed at the analysis and test center, Liaoning University of Traditional Chinese Medicine, China from April, 2021 to January, 2022.

Reagents and materials: *Epimedium* was purchased from Shenzhen Heshun Materia Medica Co. Ltd., (Shenzhen, China). Methotrexate for internal standard (IS), salidroside and oleanolic acid (purities>98%) were provided by the China Institute of Food and Drug Verification and Research (Beijing, China). P-Hydroxycinnamic acid, liquiritigenin, 2, 15-hexadecanedione, icariside II, ikarisoside A, icariin, epimedoside A and lauric acid (purities>98%) were bought from Sichuan Weikeqi Biological Technology, Co. Ltd., (Chengdu, China). Brevicornin, sagittatosdie B, afzelin and icariside I (purities>98%) were provided by Chengdu Biyang Biotechnology Co. Ltd., (Chengdu, China). The MS-grade acetonitrile and methanol were acquired from Merck (Darmstadt, Germany). The MS-grade formic acid was purchased from Thermo Fisher Scientific (China) Co. Ltd., (Shanghai, China).

Animals: Twelve male Sprague-Dawley (SD) rats (200±20 g) were obtained from Liaoning Changsheng Biotechnology Co. Ltd., [number: SCXK (Liao) 2020-0001]. After 1 week of acclimatization, rats were used for experiments. In addition, all the experiments were performed based on the approved animal protocols and guidelines established by the Medicine Ethics Review Committee for Animal Experiments of Liaoning University of Traditional Chinese Medicine under the approval number: 2020YS013(KT)-013-01.

Preparation of *Epimedium* extract solution for intragastric administration: In total, 27 g *Epimedium* powder was reflux

extracted with eight volume of water for 2 hrs, followed by filtering and concentration of the filtrate to 30 mL to obtain the solution for gavage.

Multiple dose plasma sample collection: Twelve SD rats were randomly classified into blank group and an *Epimedium* administration group, with 6 rats in each group. Rats in the administration group were given *Epimedium* extract solution (0.9/200 g) once a day for 6 consecutive days, while those in the blank group were given the same amount of water. After fasting for 12 hrs on the night of day 6, blood was collected from the hepatic portal vein at 0,15 and 30 min, 1 hr, 1.5, 2, 3, 4, 6, 8, 10, 12 and 24 hrs after intragastric administration on day 7 and plasma was collected. After centrifugation at 5000 rpm for 12 min at 4°C, the supernatant was gathered and kept at -80°C for later use. The plasma obtained from the blank group was mixed thoroughly and stored at -80°C.

Treatment of plasma samples for UPLC-QTOF-MS analysis: Briefly, 60 µL of the mixed drug-containing plasma was gathered at 30 min, 1 hr, 2 and 4 hrs, after intragastric administration, respectively. Later, 180 µL precooled methanol was added, followed by ultrasonic treatment for 2 min, vortex for 1 min, centrifugation at 4°C 12000 rpm for 10 min to precipitate protein. Thereafter, the supernatant was taken and vacuum centrifuged at 1500 rpm for drying. After adding 30 µL methanol, the sample was subject to ultrasonic treatment for 2 min, vortex for 1 min and centrifugation at 4°C for 10 min at 12000 rpm. Then, for the purpose of performing the analysis, the supernatant was further collected.

UPLC-QTOF-MS analysis conditions: Plasma samples were characterized on an Agilent-1290 UPLC/6550 QTOF-MS system (Agilent Technologies, Inc., USA). The Agilent ZORBAX Eclipse Plus C18 column (2.1×100 mm, 1.8 µm) was adopted for chromatographic analysis using the system of 0.1% formic acid aqueous solution (A)-acetonitrile (B) in the positive mode, whereas water (A)-acetonitrile (B) in the negative mode. The gradient elution conditions were 0-30 min, 3-100% B, the flow rate of 0.3 mL min^{-1}, the column temperature of 30°C and the injection volume of 2 µL. Under the same conditions as our previous research, the MS experiment was conducted on a dual spray ion source (Dual AJS ESI)[10].

UPLC-QQQ-MS analysis conditions: Pharmacokinetic analysis was conducted on an Agilent-1290 UPLC/6495 QQQ-MS system (Agilent Technologies, Inc., USA). The column, flow rate, column temperature and injection volume remained the same as those of UPLC-QTOF-MS analysis but the system of 0.1% formic acid aqueous solution (A)-acetonitrile (B) with the gradient elution procedure of 0-20 min, 5-100% B was used.

QQQ-MS analysis conditions: Electrospray ionization source (ESI) was used, the positive and negative ion switching mode was used for detection, the drying gas flow rate was 11 L min^{-1}, drying gas temperature was 150°C, the capillary voltage was 3500 V, nebulizer pressure was 25 psig, Sheath Gas Temp was 350°C and Sheath Gas Flow was 12 L min^{-1}. Data acquisition was completed in the multi-reaction monitoring (MRM) mode. With the use of MassHunter Workstation software (V.7.0 Quantitative Analysis, Agilent, USA), all data were processed. The parameter settings of each quantitative component were shown in Table 1.

Calibration standards and quality control sample preparation: Methotrexate (MTX) was collected and dissolved with methanol to produce an internal standard solution at the concentration of 328 ng mL^{-1}.

Through diluting the stock solution with methanol, standard working solutions were prepared. Moreover, calibration standards and QC samples were prepared by mixing blank plasma with the above-described working solutions at proper concentrations.

The concentrations of calibration standard solutions were 0.0975, 0.1950, 0.3900, 0.7800, 1.5600, 3.1200 and 6.2400 µg mL^{-1}, respectively, for p-hydroxycinnamic acid, 0.017, 0.034, 0.068, 0.136, 0.272, 0.544 and 1.088 µg mL^{-1} separately for salidroside, 0.153, 0.306, 0.612, 1.224, 2.448, 4.896 and 9.792 µg mL^{-1}, respectively, for brevicornin, 1.4, 2.8, 5.6, 11.2, 22.4, 44.8 and 89.6 ng mL^{-1}, respectively, for liquiritigenin, 11.5, 23.0, 46.0, 92.0, 184.0, 368.0 and 736.0 ng mL^{-1}, respectively, for sagittatosdie B, 2.475, 4.950, 9.900, 19.800, 39.600, 79.200 and 158.400 ng mL^{-1}, respectively, for icariside I, 1.325, 2.650, 5.300, 10.600, 21.200, 42.400 and 84.800 ng mL^{-1}, respectively, for icariside II, whereas, 0.46, 0.92, 1.84, 3.68, 7.36, 14.72 and 29.44 µg mL^{-1}, respectively, for lauric acid.

For QC samples, the concentrations were 0.235 µg mL^{-1} (low QC), 1.175 µg mL^{-1} (medium QC) and 5.875 µg mL^{-1} (high QC) for p-hydroxycinnamic acid, 0.037 µg mL^{-1} (low QC), 0.187 µg mL^{-1} (medium QC), 0.937 µg mL^{-1} (high QC) for salidroside, 0.362 µg mL^{-1} (low QC), 1.811 µg mL^{-1} (medium QC) and 9.054 µg mL^{-1} (high QC) for brevicornin, 3.2 ng mL^{-1} (low QC), 16.1 ng mL^{-1} (medium QC) and 80.4 ng mL^{-1} (high QC) for liquiritigenin, 27.8 ng mL^{-1} (low QC), 139.1 ng mL^{-1} (medium QC) and 695.5 ng mL^{-1} (high QC) for

Table 1: Precursor ion and product ion information

Compound	Retention times (RT, min)	Precursor ion (m/z)	Product ion (m/z)	Collision energy (CE, V)	Fragmentor voltage (FV)
p-Hydroxycinnamic acid	1.24	165.0 $[M+H]^+$	124.1	10	380
Salidroside	2.68	345.1 $[M-H]^-$	89.2	15	380
			45.3	25	380
Brevicornin	5.23	401.1 $[M+H]^+$	401.1	30	380
Liquiritigenin	7.22	255.0 $[M-H]^-$	135.0	15	380
			119.1	25	380
Sagittatosdie B	9.72	645.2 $[M-H]^-$	366.5	35	380
			351.3	45	380
Icariside I	9.83	531.1 $[M+H]^+$	369.2	30	380
			312.9	35	380
Icariside II	10.55	513.1 $[M-H]^-$	366.4	25	380
			351.4	30	380
Lauric acid	15.81	199.1 $[M-H]^-$	100.7	30	380
			167.0	15	380

sagittatosdie B, 4.624 ng mL^{-1} (low QC), 23.120 ng mL^{-1} (medium QC) and 115.600 ng mL^{-1} (high QC) for icariside I, 3.105 ng mL^{-1} (low QC), 15.526 ng mL^{-1} (medium QC) and 77.629 ng mL^{-1} (high QC) for icariside II, while 1.05 μg mL^{-1} (low QC), 5.26 μg mL^{-1} (medium QC) and 26.32 μg mL^{-1} (high QC) for lauric acid.

Treatment of plasma samples for UPLC-QQQ-MS analysis: In brief, 50 μL plasma was collected, then 25 μL IS and 125 μL precooled methanol were added successively, followed by vortex for 3 min and centrifugation at 4°C 12000 rpm for 10 min to precipitate the protein. Later, the supernatant was collected and vacuum centrifuged at 1500 rpm for drying. Subsequently, 25 μL methanol was added, followed by ultrasonic treatment for 2 min, centrifugation at 4°C for 10 min at 12000 rpm and then the supernatant was collected for analysis.

Method qualification: The method was confirmed following the FDA guidelines for bioanalytical method validation[11,12]. To be specific, specificity was evaluated through comparing the MRM chromatograms of blank plasma with administered plasma and spiked plasma with analytes. The plasma concentration of enantiomer was taken as the abscissa, while the peak area ratio of enantiomer to IS was used as the ordinate to construct the calibration curves by weighted (1/x2) least-squares linear regression. The limit of quantitation (LOQ) was determined as the concentration with a signal-to-noise ratio of 10 and the limit of determination (LOD) was 3. The intra-batch and inter-batch precision and accuracy were carried out by referring to the methods in the literature[13]. Moreover, by investigating QC samples at low, middle and high concentrations, the extraction recovery and matrix effect were evaluated. Preliminary stability tests were performed in rat plasma samples based on different situations, including short-term, long-term and freeze-thaw stability at three different levels of QC. In addition, the short-term stability was identified at room temperature for 24 hrs and at 4°C for 24 hrs, whereas the long-term stability was detected by exploring QC samples kept frozen at -80°C for 30 days. In terms of freeze-thaw stability test, the samples were refrigerated at -80°C for 24 hrs and placed at 25°C for 30 min, later refrigerated at -80°C for 12 hrs and placed at 25°C for 30 min and refrigerated at -80°C for 12 hrs and placed at 25°C for another 30 min.

Pharmacokinetic application: The experiments were carried out according to the confirmed plasma treatment method and mass spectrometry conditions. Thereafter, the peak area and internal standard information of the object to be measured were imported into Agilent QQQ Quantitative software to calculate its concentration at each time point. In addition, the measured blood drug concentration and time information of each component were subsequently imported into DAS 3.2.8 Pharmacokinetic software to calculate the pharmacokinetic parameters.

Statistical analysis: The results were analyzed with SPSS 19.0 software and data was expressed as Mean±SD.

RESULTS

Identification of the prototype components absorbed into plasma: Serum pharmaco-chemical research method, combined with UPLC-QTOF-MS technology, was adopted for studying rat plasma samples after intragastric administration of *Epimedium* extract[14]. The absorption spectra of blood entering components under physiological conditions were established (Fig. 1). Components were identified by comparison with the reference, database and literature search. Finally, a total of 22 prototype components were identified (Table 2), of which 16 were flavonoids and a small amount of acids, phenols and triterpenes were also detected.

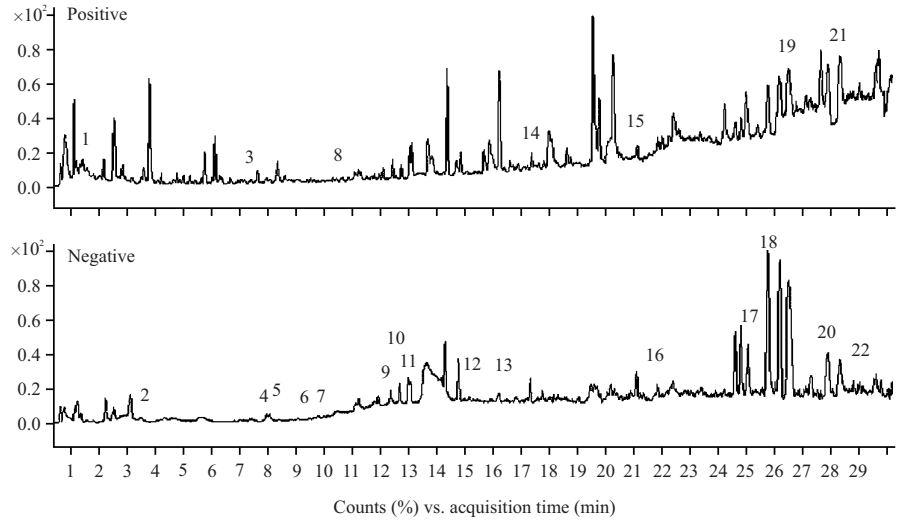


Fig. 1: Total ion current (TIC) diagram of prototype components absorbed in positive and negative mode

Table 2: Prototype components absorbed into plasma

RT (min)	Molecular formula	Ion mode	Theoretical value (m/z)	Measured value (m/z)	Error (ppm)	Fragment ions (m/z)	Compounds	Literature
1.496	$C_9H_8O_3$	$[M+H]^+$	165.0546	165.0543	-1.82	124	p-Hydroxycinnamic acid	*
3.947	$C_{14}H_{20}O_7$	$[M+COOH]^-$	345.1191	345.1181	-2.90	89	Salidroside	*
7.398	$C_{22}H_{24}O_7$	$[M+H]^+$	401.1595	401.1558	-9.22	401	Brevicornin	*
8.017	$C_{21}H_{20}O_{10}$	$[M-H]^-$	431.1984	431.1970	-3.25	285, 255	Afzelin	*
8.531	$C_{27}H_{30}O_{10}$	$[M-H]^-$	513.1766	513.1759	-1.36	366, 351, 323	Icariside II	*
9.787	$C_{38}H_{48}O_{19}$	$[M+COOH]^-$	853.2772	853.2766	-0.70	645, 366	Epimedin B	14
9.788	$C_{32}H_{38}O_{14}$	$[M-H]^-$	645.2189	645.2174	-2.32	366, 351, 323	Sagittatosdie B	*
10.180	$C_{33}H_{40}O_{15}$	$[M+Na]^+$	699.2259	699.2226	-4.72	531, 369	Icariin	*
12.853	$C_{32}H_{38}O_{16}$	$[M-H]^-$	677.2087	677.2131	6.50	515, 353	Ikarisoside B	14
12.970	$C_{32}H_{38}O_{16}$	$[M-H]^-$	677.2087	677.2106	2.81	529	Epimedin E	14
13.064	$C_{15}H_{12}O_4$	$[M-H]^-$	255.0663	255.0652	-4.31	351,135	Liquiritigenin	*
15.367	$C_{21}H_{20}O_6$	$[M-H]^-$	367.1187	367.1186	-0.27	311	Icaritin	14
15.923	$C_{32}H_{38}O_{15}$	$[M-H]^-$	661.2138	661.2175	5.60	499, 353	Epimedoside A	*
17.376	$C_{28}H_{34}O_{12}$	$[M+H]^+$	563.2123	563.2067	-9.94	369	Caohuoside D	14
21.134	$C_{26}H_{28}O_{10}$	$[M+H]^+$	501.1755	501.1786	6.19	353	Ikarisoside A	*
21.903	$C_{12}H_{24}O_2$	$[M-H]^-$	199.1704	199.1705	0.50	199	Lauric acid	*
25.401	$C_{30}H_{48}O_3$	$[M-H]^-$	455.3531	455.3522	-1.98	455	Oleanolic Acid	*
25.781	$C_{16}H_{30}O_2$	$[M-H]^-$	253.2173	253.2181	3.16	253	2,15-hexadecanedione	*
26.548	$C_{27}H_{32}O_{11}$	$[M+H]^+$	533.2017	533.1967	-9.38	369	Icaritin-3-O-rhamnoside	14
28.182	$C_{27}H_{32}O_{12}$	$[M+COOH]^-$	593.1876	593.1822	-9.10	367	Maohuoside A	14
28.353	$C_{27}H_{30}O_{11}$	$[M+H]^+$	531.1861	531.1866	0.94	369	Icariside I	*
28.767	$C_{18}H_{34}O_2$	$[M-H]^-$	281.2486	281.2482	-1.42	281	Oleic acid	14

*Compared with the reference substance

Among these blood entering components, eight components, including p-hydroxycinnamic, salidroside, brevicornin, icariside II, sagittatosdie B, liquiritigenin, lauric acid and icariside I acid, were found in plasma samples at four different time points (30 min, 1, 2, 4 hrs). They met the requirements of plasma quantification and could be used for pharmacokinetic study. As for afzelin, icaritin, epimedoside A and ikarisoside A, their contents were extremely low and could not meet the quantitative requirements by changing the sample processing method. Meanwhile, epimedin B, icariin, caohuoside D, icaritin-3-O-rhamnotide and mahuoside A were only detected in blood samples at 30 min but not found at other time points, making it impossible to carry out pharmacokinetic studies on them. Other components were only confirmed by secondary comparison. As there is no reference substance in the laboratory at present, no further study is conducted.

Table 3: Regression equations, linearity ranges, LODs and LOQs of 4 analytes

Compound	Regression equation	r	Linearity range (μg mL^{-1})	LOQ (μg mL^{-1})	LOD (μg mL^{-1})
p-Hydroxycinnamic acid	Y = 0.3835X+0.0380	0.9965	0.0975-6.2400	0.0975	0.0334
Salidroside	Y = 1.0204X-0.0173	0.9983	0.017-1.088	0.017	0.007
Brevicornin	Y = 0.6925X-0.0015	0.9986	0.153-9.792	0.153	0.062
Lauric acid	Y = 0.0527X+0.0084	0.9963	0.46-29.44	0.46	0.12

Table 4: Regression equations, linearity ranges, LODs and LOQs of 4 analytes

Compound	Regression equation	r	Linearity range (ng mL^{-1})	LOQ (ng mL^{-1})	LOD (ng mL^{-1})
Liquiritigenin	Y = 0.0336X-0.0686	0.9971	1.4-89.6	1.4	0.5
Sagittatosdie B	Y = 0.0059X-0.0470	0.9983	11.5-736.0	11.5	4.6
Icariside I	Y = 0.0195X-0.0466	0.9978	2.475-158.400	2.475	1.046
Icariside II	Y = 0.0211X-0.0004	0.9941	1.325-84.800	1.325	0.722

Table 5: Intra- and inter-day accuracy and precision of 4 analytes in rat plasma (n = 6)

		Intra-day			Inter-day		
Compound	Concentration (μg mL^{-1})	Mean concentration (μg mL^{-1})	Accuracy (%)	Precision (%)	Mean concentration (μg mL^{-1})	Accuracy (%)	Precision (%)
p-Hydroxycinnamic acid	0.235	0.247±0.010	5.035	4.237	0.220±0.007	-6.596	3.019
	1.175	1.204±0.032	2.468	2.675	1.187±0.015	0.979	1.267
	5.875	5.692±0.137	-3.118	2.403	5.810±0.254	-1.106	4.368
Salidroside	0.037	0.038±0.002	2.252	5.130	0.041±0.004	9.459	8.660
	0.187	0.177±0.006	-5.526	3.175	0.188±0.011	0.357	5.696
	0.937	0.923±0.012	-1.530	1.246	0.943±0.013	0.640	1.379
Brevicornin	0.362	0.353±0.011	-2.486	3.041	0.360±0.010	-0.506	2.795
	1.811	1.867±0.073	3.074	3.936	1.830±0.044	1.049	2.391
	9.054	8.936±0.140	-1.305	1.566	8.895±0.220	-1.754	2.470
Lauric acid	1.05	1.12±0.09	6.98	7.96	1.12±0.06	6.19	5.52
	5.26	5.21±0.19	-0.86	3.60	5.39±0.24	2.41	4.48
	26.32	25.71±1.71	-2.32	6.64	25.56±1.13	-2.89	4.40

Table 6: Intra- and inter-day accuracy and precision of 4 analytes in rat plasma (n = 6)

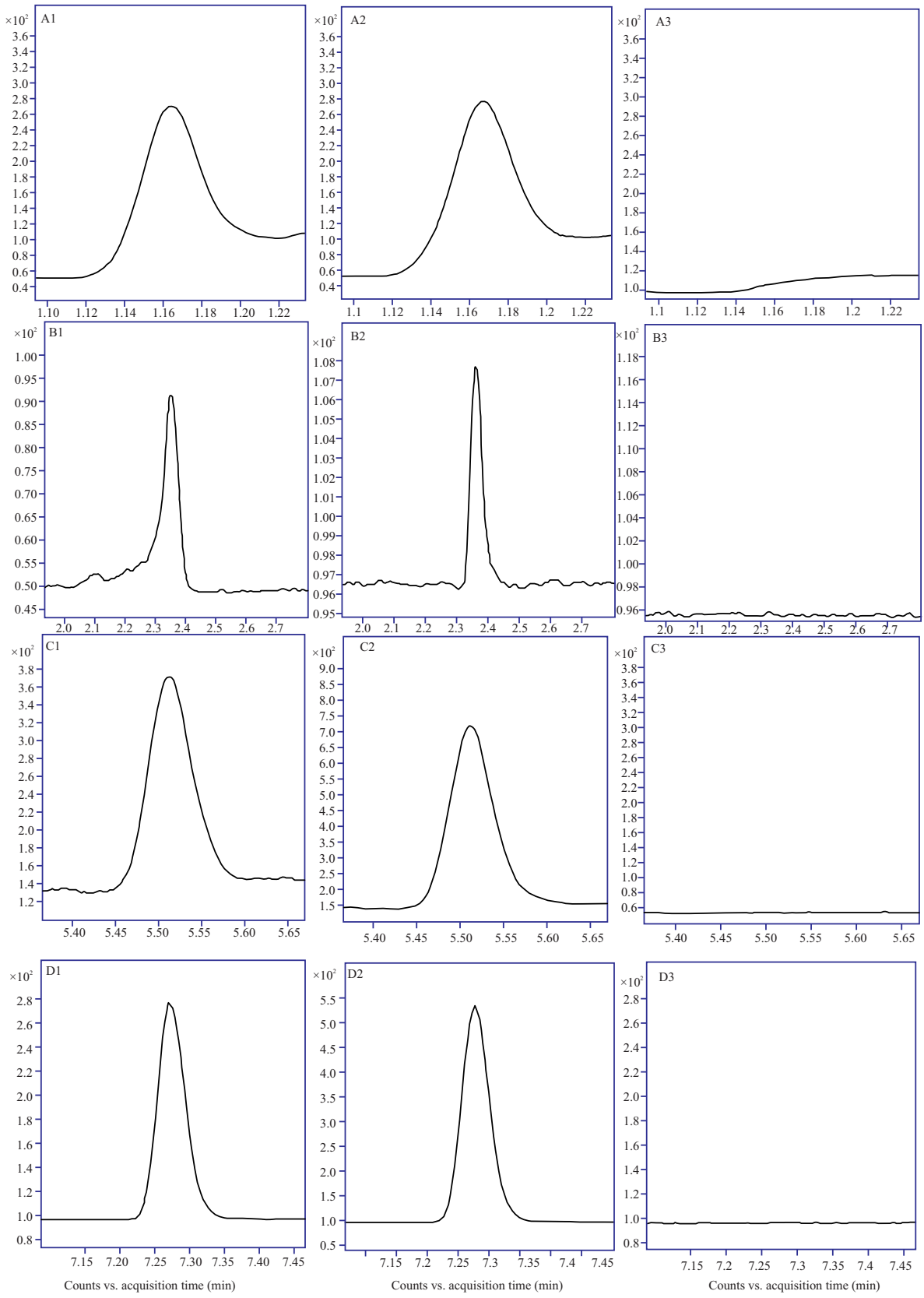
		Intra-day			Inter-day		
Compound	Concentration (ng mL^{-1})	Mean concentration (ng mL^{-1})	Accuracy (%)	Precision (%)	Mean concentration (ng mL^{-1})	Accuracy (%)	Precision (%)
Liquiritigenin	3.2	2.9±0.2	-8.9	7.3	3.0±0.2	-6.8	7.5
	16.1	15.3±1.1	-5.2	7.2	16.2±0.9	0.3	5.9
	80.4	79.5±2.8	-1.1	3.6	79.0±3.1	-1.7	3.9
Sagittatosdie B	27.8	26.4±1.4	-5.1	5.4	26.5±1.5	-4.9	5.7
	139.1	137.0±4.9	-1.5	3.6	138.5±6.1	-0.5	4.4
	695.5	697.0±14.3	0.2	2.1	688.2±12.2	-1.0	1.8
Icariside I	4.624	4.482±0.122	-3.064	2.725	4.469±0.170	-3.352	3.798
	23.120	23.413±1.751	1.269	7.477	23.023±1.178	-0.418	5.118
	115.600	144.936±4.001	-0.575	3.481	113.934±4.527	-1.441	3.973
Icariside II	3.105	3.076±0.192	-0.934	6.247	3.188±0.203	2.673	6.354
	15.526	15.869±1.478	2.210	9.316	15.628±0.702	0.659	4.495
	77.629	76.872±4.342	-0.975	5.649	74.796±3.457	-3.650	4.622

Method validation: Based on the analysis results of blood entering prototype components, eight components commercially available were selected for further study on pharmacokinetics methodology, including p-hydroxycinnamic acid, salidroside, brevicornin, icariside II, sagittatosdie B, liquiritigenin, lauric acid and icariside I.

Through comparing the MRM chromatograms obtained from plasma at 1 hr after intragastric administration of *Epimedium* extract solution, blank plasma and spiked plasma with eight analytes, it was found that the endogenous substances of blank plasma samples were not interfered in the analytes (Fig. 2). The retention time of p-hydroxycinnamic acid, salidroside, brevicornin, icariside II, sagittatosdie B, liquiritigenin, lauric acid and icariside I was 1.24, 2.68, 5.23, 7.22, 9.72, 9.83, 10.55 and 15.81 min, respectively.

Information of the regression equations, linearity ranges, LOQs and LODs was displayed in Table 3 and 4. The linearity with correlation coefficients for all compounds was (r) >0.9941.

The precision and accuracy data of the method were shown in Table 5 and 6. As observed, the intra-and inter-day precision ranged from 1.25 to 9.32% and from 1.27 to 8.66%,



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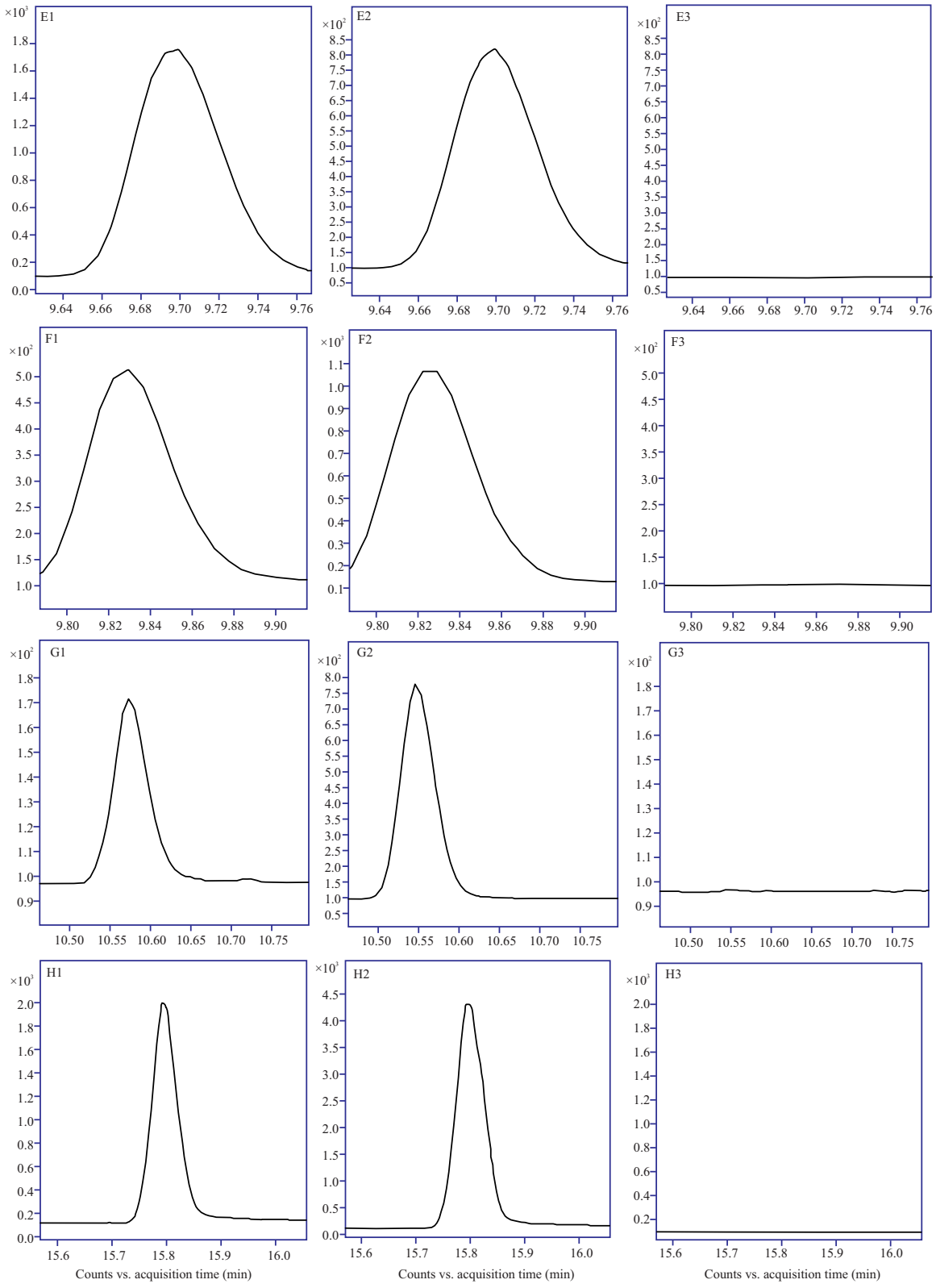

Fig. 2: MRM chromatograms obtained from plasma 1h after intragastric administration of *Epimedium* extract solution (1), blank plasma (3) and spiked plasma with 8 analytes (2)

A: p-Hydroxycinnamic acid, B: Salidroside, C: Brevicornin, D: Lauric acid, E: Liquiritigenin, F: Sagittatosdie B, G: Icariside I and H: Icariside II

Table 7: Extraction recoveries and matrix effects of 4 analytes in rat plasma (n = 6)

		Extraction recovery		Matrix effect	
Compound	Concentration (μg mL^{-1})	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
p-Hydroxycinnamic acid	0.235	82.40	3.18	88.30	3.48
	1.175	86.41	2.76	91.76	2.52
	5.875	87.04	4.87	90.23	2.99
Salidroside	0.037	89.26	2.31	83.92	2.02
	0.187	92.25	1.70	94.47	1.93
	0.937	83.86	1.75	96.25	2.69
Brevicornin	0.362	89.68	2.09	91.99	1.95
	1.811	84.47	1.57	90.52	1.68
	9.054	92.89	0.94	86.41	2.12
Lauric acid	1.05	93.58	1.72	93.12	3.53
	5.26	95.98	1.77	94.90	2.28
	26.32	94.14	2.20	91.27	2.17

Table 8: Extraction recoveries and matrix effects of 4 analytes in rat plasma (n = 6)

		Extraction recovery		Matrix effect	
Compound	Concentration (ng mL^{-1})	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Liquiritigenin	3.2	87.32	1.87	90.73	2.15
	16.1	91.32	1.95	95.85	1.34
	80.4	89.73	2.07	95.30	1.35
Sagittatosdie B	27.8	92.57	1.52	87.58	2.79
	139.1	87.78	2.91	92.71	1.88
	695.5	90.59	1.47	87.45	2.50
Icariside I	4.624	89.59	1.92	89.84	2.11
	23.120	93.00	1.78	86.10	1.31
	115.600	90.28	2.17	89.74	1.76
Icariside II	3.105	93.21	1.89	85.20	2.52
	15.526	89.15	1.59	88.83	2.37
	77.629	90.31	2.20	88.87	2.24

Table 9: Short-term stability of 4 analytes in rat plasma (n = 6)

		24 hrs at room temperature		24 hrs at 4°C	
Compound	Concentration (μg mL^{-1})	Mean concentration (μg mL^{-1})	Accuracy (%)	Mean concentration (μg mL^{-1})	Accuracy (%)
p-Hydroxycinnamic acid	0.235	0.226±0.011	-3.759	0.227±0.014	-3.262
	1.175	1.181±0.011	0.525	1.210±0.066	2.965
	5.875	5.735±0.152	-2.391	5.695±0.215	-3.072
Salidroside	0.037	0.039±0.005	4.054	0.039±0.005	6.306
	0.187	0.193±0.012	3.387	0.183±0.022	-2.406
	0.937	0.929±0.050	-0.818	0.927±0.042	-1.032
Brevicornin	0.362	0.373±0.037	3.039	0.372±0.035	2.808
	1.811	1.822±0.091	0.607	1.856±0.132	2.466
	9.054	8.953±0.262	-1.119	9.051±0.268	-0.037
Lauric acid	1.05	1.04±0.13	-1.11	1.12±0.22	6.35
	5.26	5.42±0.20	3.07	5.42±0.41	3.07
	26.32	25.82±2.10	-1.89	26.44±2.28	0.44

respectively and the accuracy was from -8.90 to 9.46%, demonstrating the feasibility of the method.

The extraction recoveries and matrix effects for the eight components were presented in Table 7 and 8. In addition, the extraction recoveries of three concentrations all ranged from 82.40 to 95.98% and the relative standard deviation (RSD) was 4.87%, demonstrating that the values were all in the acceptable ranges. Their matrix effects were all between 83.92 and 96.25%, with RSD 3.53%, indicating no significant matrix effect observed in all of the substances.

Moreover, the results of short-term, freeze-thaw and long-term stability tests were presented in Table 9-12. According to current results, the eight analytes in plasma were all stable at room temperature for 24 hrs (relative error

Table 10: Short-term stability of 4 analytes in rat plasma (n = 6)

		24 hrs at room temperature		24 hrs at 4°C	
Compound	Concentration (ng mL^{-1})	Mean concentration (ng mL^{-1})	Accuracy (%)	Mean concentration (ng mL^{-1})	Accuracy (%)
Liquiritigenin	3.2	3.1±0.3	-3.7	3.1±0.4	-4.2
	16.1	16.5±0.5	2.3	15.7±0.7	-2.6
	80.4	80.3±3.9	-0.2	79.2±4.1	-1.5
Sagittatosdie B	27.8	26.2±2.5	-5.7	29.0±2.6	4.1
	139.1	145.2±4.5	4.4	134.7±2.2	-3.2
	695.5	688.4±9.2	-1.0	675.0±10.7	-2.9
Icariside I	4.624	4.421±0.238	-4.397	4.758±0.236	2.905
	23.120	21.702±2.142	-6.135	23.768±2.835	2.801
	115.600	117.045±4.590	1.250	114.256±7.224	-1.163
Icariside II	3.105	2.912±0.173	-6.216	3.394±0.267	9.308
	15.526	14.721±1.181	-5.185	14.402±0.654	-7.238
	77.629	74.194±3.076	-4.425	77.658±6.333	0.037

Table 11: Freeze-thaw and long-term stability of 4 analytes in rat plasma (n = 6)

		Three free-thaw cycles		30 days	
Compound	Concentration (μg mL^{-1})	Mean concentration (μg mL^{-1})	Accuracy (%)	Mean concentration (μg mL^{-1})	Accuracy (%)
p-Hydroxycinnamic acid	0.235	0.230±0.018	-2.270	0.237±0.014	0.780
	1.175	1.147±0.124	-2.411	1.220±0.100	3.844
	5.875	6.227±0.469	5.989	5.955±0.123	1.365
Salidroside	0.037	0.037±0.005	0.450	0.039±0.005	5.856
	0.187	0.174±0.011	-6.774	0.169±0.011	-9.447
	0.937	0.929±0.040	-0.854	0.902±0.021	-3.700
Brevicornin	0.362	0.353±0.031	-2.624	0.352±0.017	-2.670
	1.811	1.734±0.109	-4.243	1.776±0.098	-1.914
	9.054	8.964±0.564	-0.998	9.308±0.364	2.800
Lauric acid	1.05	1.11±0.15	5.24	1.10±0.17	4.44
	5.26	5.27±0.20	0.25	5.10±0.25	-3.14
	26.32	24.55±1.27	-6.73	26.13±1.77	-0.74

Table 12: Freeze-thaw and long-term stability of 4 analytes in rat plasma (n = 6)

		Three free-thaw cycles		30 days	
Compound	Concentration (ng mL^{-1})	Mean concentration (ng mL^{-1})	Accuracy (%)	Mean concentration (ng mL^{-1})	Accuracy (%)
Liquiritigenin	3.2	2.9±0.2	-8.9	3.0±0.3	-5.2
	16.1	16.2±1.5	0.6	15.5±0.5	-3.5
	80.4	81.1±5.0	0.9	82.9±1.8	3.1
Sagittatosdie B	27.8	26.9±1.1	-3.4	25.8±1.1	-7.1
	139.1	142.4±5.2	2.4	146.7±4.9	5.5
	695.5	669.3±12.7	-3.8	719.7±8.0	3.5
Icariside I	4.624	4.425±0.197	-4.314	4.481±0.274	-3.093
	23.120	24.305±3.075	5.126	23.475±2.092	1.535
	115.600	125.629±7.630	8.676	120.344±7.904	4.104
Icariside II	3.105	3.098±0.445	-0.242	3.272±0.357	5.368
	15.526	15.913±1.468	2.494	14.632±1.421	-5.756
	77.629	75.728±3.668	-2.448	74.351±2.703	-4.223

(RE): -6.216 to 4.400%), for 24 hrs at 4 (RE: -7.238 to 9.308%), after three freeze-thaw cycles (RE: -8.854 to 8.676%) and storage at -20 for 30 days (RE: -9.447 to 5.856%).

Pharmacokinetic application: The main pharmacokinetic parameters of the eight analytes were shown in Table 13 and 14. The mean concentrations of analytes in plasma samples versus time plot was displayed in Fig. 3. Obviously, the largest component of C_{max} was lauric acid (3.074 μg mL^{-1}), meanwhile, p-hydroxycinnamic acid, salidroside, liquiritigenin, Icariside I and icariside II reached the maximum plasma concentrations between 0.500 and 1.917 hrs, while,

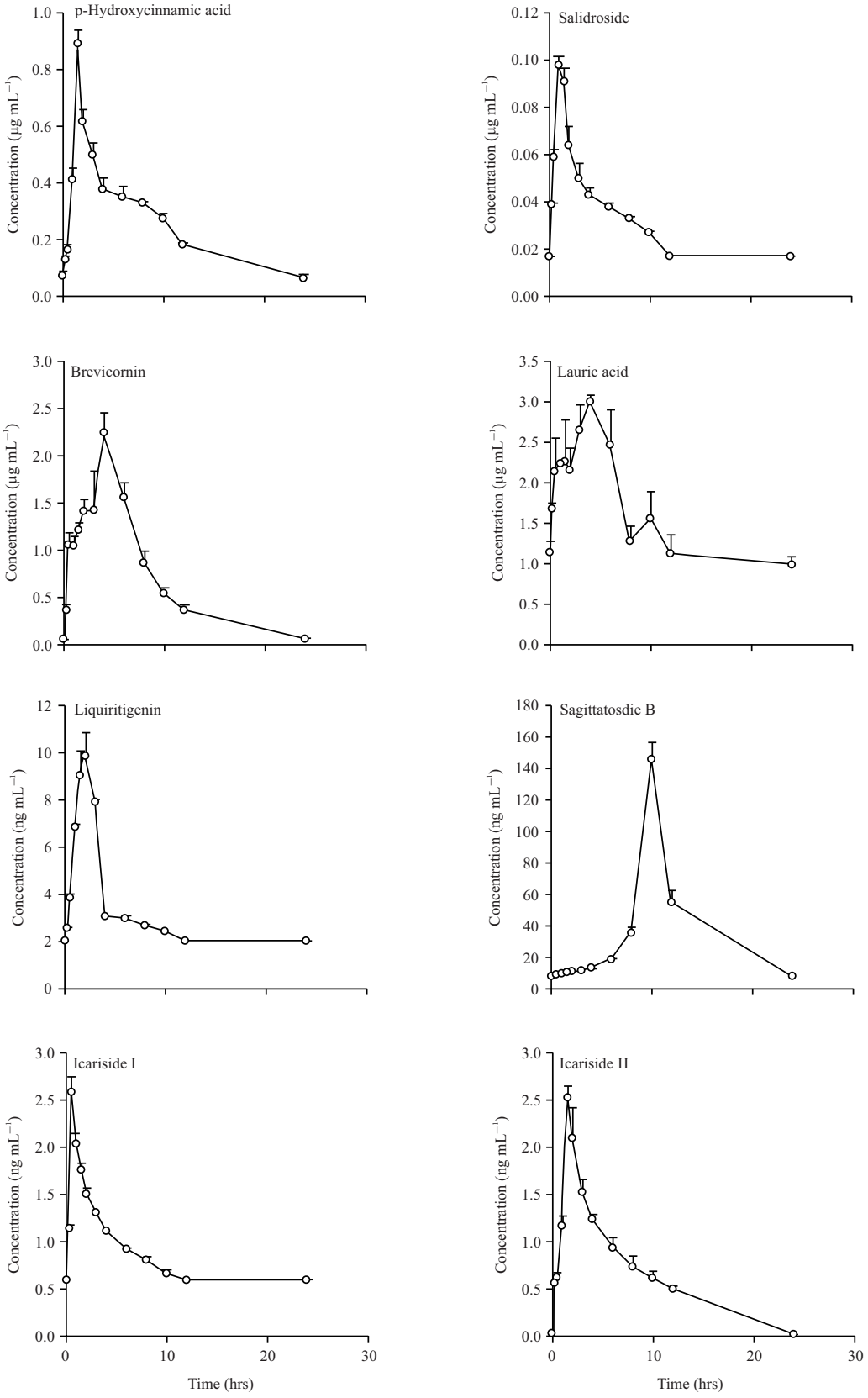

Fig. 3: Drug concentration-time curve of the 8 components in rats after multiple administration of *Epimedium*

Table 13: Pharmacokinetic parameters of 4 analytes in rat plasma after multiple oral administration of *Epimedium* (n = 6)

Compound	C_{max} (μg mL^{-1})	T_{max} (hrs)	AUC_{0-t} (μg hrs mL^{-1})	$T_{1/2}$ (hrs)	MRT (hrs)
p-Hydroxycinnamic acid	0.894±0.048	1.500	5.862±0.087	6.861±0.736	7.723±0.202
Salidroside	0.099±0.003	1.167	0.707±0.022	15.545±5.690	8.530±0.138
Brevicornin	2.248±0.204	3.833	16.309±1.307	3.945±1.006	6.371±0.098
Lauric acid	3.074±0.118	4.167	36.825±3.522	22.620±7.806	9.505±0.361

Table 14: Pharmacokinetic parameters of 4 analytes in rat plasma after multiple oral administration of *Epimedium* (n = 6)

Compound	C_{max} (ng mL^{-1})	T_{max} (hrs)	AUC_{0-t} (ng hrs mL^{-1})	$T_{1/2}$ (hrs)	MRT (hrs)
Liquiritigenin	10.402±0.524	1.917	73.043±0.649	10.126±0.941	9.053±0.062
Sagittatosdie B	145.950±10.941	10.000	888.474±61.426	3.631±0.104	10.820±0.063
Icariside I	10.405±0.646	0.500	79.714±0.764	9.321±1.357	9.505±0.074
Icariside II	2.571±0.156	1.583	15.343±1.149	6.774±2.602	6.352±0.102

brevicornin, lauric acid and sagittatosdie B reached the maximum plasma concentrations between 3.833 and 10.000 hrs. Besides, the $T_{1/2}$ of all compounds ranged from 3.631 to 22.620 hrs. The MRT calculation results suggested that the average residence time of the eight compounds in rat plasma was 6.371 to 10.820 hrs. Among them, the MRT time of brevicornin was the shortest, while that of sagittatosdie B was the longest.

DISCUSSION

Icariin, epimedin A, epimedin B, epimedin C and icariside II are the five main isopentenyl flavonoid monomers contained in *Epimedium*. In most researches on the pharmacokinetics of *Epimedium* given by a single oral administration, these components have received high attention, which are associated with the features of rapid absorption, high peak concentration and moderate elimination. In particular, epimedin A, epimedin B and epimedin C have complex metabolic processes and low bioavailability *in vivo*. It is found that icariin is deglycosylated and metabolized into icariside II in rats. Epimedin A can be metabolized to sagittatoside A and then further converted to icariside II. By contrast, Epimedin B can be metabolized into sagittatoside B and later transformed into icariside II. Epimedin C can be metabolized into 2'-O-rhamnosyl icariside II and then further converted into icariside II. Icariside II can further hydrolyze one molecule of rhamnose to form icaritin[8].

In this study, after multiple oral administration of *Epimedium*, a small amount of prototype components such as icariin and epimedin B were detected in the plasma samples within 30 min, which were then quickly absorbed into the blood for transformation and the pharmacokinetic behaviors of icariside II and sagittatoside B were clearly observed. In addition, glycosides such as afzelin, epimedoside A, ikarisoside A, caohuoside D, icaritin-3-O-rhamnoside and maohuoside A were also detected due to the accumulation of multiple administration. However, the relative exposure in the body was too low to be quantified.

Compared with single administration, the absorption and elimination of icariside II and sagittatoside B in rat plasma after multiple administration slowed down. In addition, apart from the common flavonoids, this work was the first to acquire the pharmacokinetic process of brevicornin and liquiritigenin and the pharmacokinetic parameters of p-hydroxycinnamic acid, salidroside, lauric acid and other components.

These blood entering components are closely related to various biological activities of *Epimedium* in treating sexual dysfunction, cardiovascular and cerebrovascular diseases, osteoporosis and cancer. For example, icariside II can be used to treat erectile dysfunction[15,16], cardiovascular and cerebrovascular diseases[17] and diabetic nephropathy[18]. The P-hydroxycinnamic acid has bone protective effect, promotes bone formation and inhibits bone absorption *in vivo*[19]. Salidroside and liquiritigenin can be adopted to treat cardiovascular diseases, lung cancer, liver cancer, breast cancer, colorectal cancer as well as other cancers[20-23]. Sagittatoside B has the immune anti-tumor activity[24]. Lauric acid can reduce insulin resistance[25].

After long-term use of *Epimedium*, its main pharmacoactive monomers can present a slow metabolic rate, prolonged Tmax, increased bioavailability and longer chemical component transformation time. The relative increases of these components in the whole body may be the root cause for adverse reactions. To avoid the adverse reactions induced by long-term administration, according to the analysis of blood composition rules and the pharmacokinetic parameters of monomers, it is suggested to reduce the dosage and increase the interval time of administration, so as to reduce toxicity and increase efficiency.

CONCLUSION

In the present study, UPLC-QTOF-MS technology was adopted for analyzing the blood entering components of *Epimedium* after multiple oral administration in rats and 22 prototype components were detected. Furthermore, UPLC-QQQ-MS technique was employed with the purpose of establishing a method for the simultaneous quantitative determination of eight active components in rat plasma, namely p-hydroxycinnamic acid, salidroside, brevicornin, lauric acid, liquiritigenin, sagittatosdie B, icariside I and icariside II. The pharmacokinetic parameters of these eight components were obtained. Furthermore, it was found that compared with single administration, multiple administration induced the slow metabolic rate, prolonged T_{max}, increased bioavailability and prolonged transformation time of the main active chemical ingredient monomers. The relative increases of these ingredients in the whole body may be the reason for adverse reactions after long-term administration of *Epimedium*.

SIGNIFICANCE STATEMENT

Epimedium is an important traditional medicinal and edible plant in China and it has been used in herbal preparations or functional foods for over 2000 years. Recently, people found that long-term clinical use of *Epimedium* might cause toxic reactions and symptoms including thirst or nosebleed. Previous literature reports on the pharmacokinetics of *Epimedium* mainly concentrated on single administration. To find out the substances that cause adverse reactions after long-term administration of *Epimedium*, this study adopted UPLC-QTOF-MS and UPLC-QQQ-MS technologies revealed the pharmacokinetics and metabolic regularity of *Epimedium* in rats after multiple administration for the first time, laying a good foundation for the clinical rational application of *Epimedium*.

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