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Short communication

Simultaneous determination of seven flavonoids in dog plasma by ultra-performance liquid chromatography–tandem mass spectrometry and its application to a bioequivalence study of bioactive components in *Herba Epimedii* and Er-Xian Decoction

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ABSTRACT

In this study, a sensitive and specific ultra-performance liquid chromatography–tandem mass method has been developed and validated for the identification and determination of 7 flavonoids in dog plasma for the first time: epimedin A, epimedin B, epimedin C, icariin, sagittatoside B, 2″-*O*-rhamnosyl icariside II, and baohuoside I. Chromatographic separation was accomplished on an Agilent Zorbax-SB C_{18} column (50 mm × 2.1 mm, 1.8 μm) with a gradient elution system composed of 0.3% acetic acid and 0.3% acetic acid in acetonitrile at a flow rate of 0.4 mL/min. Detection was based on a triple quadrupole mass spectrometer using a multiple reaction monitoring mode with an electrospray ionization source. All of the calibration curves showed good linearity ($r > 0.99$) within the tested concentration ranges. The lower limits of quantification of the seven analytes were all lower than 0.0654 ng/mL. The relative standard deviations for intra- and inter-batches of the seven analytes were less than 13.7% and 14.9%, respectively, at four concentration levels of quality control samples, and the recoveries were between 92.8% and 114.5%, respectively. In addition, the seven flavonoids were found to be stable in dog plasma samples under short- and long-term storage and processing conditions. The validated method was successfully applied to a bioequivalence study in dog plasma after the oral administration of extracts of *Herba Epimedii* and Er-Xian Decoction.

1. Introduction

Herba Epimedii of the family *Berberidaceae*, which include the dried aerial parts of *Epimedium brevicornum* Maxim., *Epimedium sagittatum* Maxim., *Epimedium pubescens* Maxim., *Epimedium wushanense* T.S. Ying and *Epimedium koreanum* Nakai., is a well-known traditional Chinese medicine [1]. This plant has been widely used as a tonic, aphrodisiac and anti-rheumatic in China for over 2000 years. To date, flavonoids are believed to be the active components in *Herba Epimedii*, and more than 60 flavonoids have been isolated from it [2]. According to our previous study [3,4], the main pharmacological components in *Herba Epimedii* were four parent flavonoids: epimedin A, epimedin B, epimedin C and icariin, and their metabolites, including 2″-*O*-rhamnosylicariside II, sagittatoside B, and baohuoside I. Consequently, it would be useful to establish a rapid, sensitive and accurate method for the determination of these seven flavonoids in biological samples to facilitate their continued study. Elucidation of the pharmacokinetic characteristics of *Herba Epimedii* and its prescription derivatives, such as Er-Xian Decoction (EXD), on the basis of multiple active marker components would help to understanding their mechanism of action and predict their efficacy.

Several methods have been described in the literature for determining the flavonoid content in *Herba Epimedii* extracts and in biological samples after the administration of *Herba Epimedii*. These methods include capillary zone electrophoresis (CZE) [5], high-performance liquid chromatography (HPLC) [6,7], ultra-performance liquid chromatography (UPLC) [8] and high performance liquid chromatography–mass spectrometry (LC–MS) [2–4,9–11]. In addition, pharmacokinetic studies of epimedin C [6], icariin [7,9–11] and baohuoside I (sometimes referred to as icariside II) [9–11] have been investigated using HPLC-UV and LC–MS/MS.

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To our knowledge, there has been no report on the simultaneous determination of the seven flavonoids in biological samples using UPLC–MS/MS, nor has there been a pharmacokinetic study of epimedin A or epimedin B.

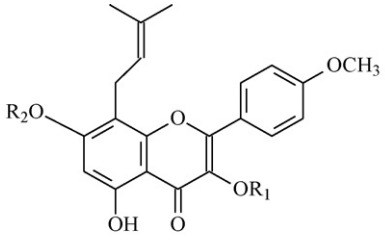
In this study, a simple and rapid UPLC–MS/MS method was established and validated to determine the seven main flavonoids present in dog plasma: epimedin A (1), epimedin B (2), epimedin C (3), icariin (4), sagittatoside B (5), 2″-*O*-rhamnosylicariside II (6), and baohuoside I (7). The validated method was applied to study the pharmacokinetics of the seven main flavonoids after the oral administration of an extract of *Herba Epimedii* and its prescription, EXD, to dogs.

2. Experimental

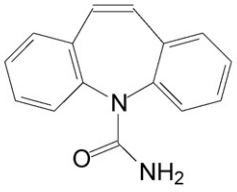
2.1. Materials

The purity of the seven flavonoids and carbamazepine, which was used as the internal standard (IS), was more than 99% as determined by HPLC. The structures of these compounds are shown in Fig. 1. The materials of *Herba Epimedii* (*E. wushanense* T.S. Ying) were collected from Chongqing, one province of China. EXD, a traditional Chinese medicinal formula, is composed of six Chinese herbs, including *Herba Epimedii*, *Rhizoma Curliginis*, *Radix Morindae Officinalis*, *Radix Anemarrhenae*, *Cortex Phellodendri*, and *Radix Angelicae sinensis*, at a ratio of 5:5:3:3:3:3. The other five herbs were purchased from TongRenTang pharmaceutical group (Beijing, China).

Acetonitrile of LC/MS reagent grade was obtained from Mallinckrodt Baker Inc. (Phillipsburg, NJ, USA). Deionized water was purified using a Millipore water purification system (Millipore, Billerica, MA, USA). Analytical grade acetic acid was obtained from Merck Inc. (Darmstadt, Germany). Analytical grade methanol was purchased from Beijing Chemical Corp. (Beijing).



R_1 = -Rha(2-1)Glc	R_2 = -Glc	Epimedin A (**1**)
R_1 = -Rha(2-1)Xyl	R_2 = -Glc	Epimedin B (**2**)
R_1 = -Rha(2-1)Rha	R_2 = -Glc	Epimedin C (**3**)
R_1 = -Rha	R_2 = -Glc	Icariin (**4**)
R_1 = -Rha(2-1)Xyl	R_2 = -H	Sagittatoside B (**5**)
R_1 = -Rha(2-1)Rha	R_2 = -H	2″-*O*-rhamnosylicariside II (**6**)
R_1 = -Rha	R_2 = -H	Baohuoside I (**7**)



Carbamazepine (**IS**)

Fig. 1. The chemical structures of the seven flavonoids (compounds **1**–**7**) and carbamazepine (IS).

2.2. Instrumentation and UPLC–MS/MS analytical conditions

The assay was performed on an Agilent 6410B triple quadrupole LC–MS system (Agilent Corporation, MA, USA) consisting of an Agilent 1200 RRLC system (Agilent Co.) connected to a triple quadrupole MS analyzer with an electrospray ionization (ESI) interface usable in either positive-ionization or negative-ionization mode. A MassHunter workstation was used for LC–MS control and data acquisition (Agilent Co.).Chromatographic separation was achieved using an Agilent Zorbax SB-C_{18} column (50 mm × 2.1 mm, 1.8 μm). The mobile phase consisted of solvent A (0.3% acetic acid) and solvent B (acetonitrile with 0.3% acetic acid) delivered at a flow rate of 0.4 mL/min. The gradient elution started with 23% B and reached 30% B at 1.0 min, 38% B at 3.0 min, 50% B at 6.0 min, 60% B at 6.5 min and then quickly returned to 23% B at 6.6 min, which was maintained for 10 min. The column temperature was maintained at 30 °C, and the sample injection volume was 3 μL.The following were the optimum operating parameters of the ESI interface in positive mode: nebulizer, 45 psi; dry gas, 10 L/min; dry temp, 325 °C; capillary voltage, 4000 V; delta EMV, 350 V; the LC eluent flow during the period from 0.0 to 1.5 min was not introduced to the mass spectrometer for data acquisition. The following precursor-to-product ion transitions were subjected to multiple reaction monitoring: for epimedin A, *m*/*z* 838.8–368.9 (fragmentor, 150 V; collision energy, 42 V); for epimedin B, *m*/*z* 808.8–368.9 (150 V; 42 V); for epimedin C, *m*/*z* 822.8–368.9 (150 V; 42 V); for icariin, *m*/*z* 676.8–368.9 (140 V; 25 V); for sagittatoside B, *m*/*z* 646.8–368.9 (110 V; 10 V); for 2″-*O*-rhamnosylicariside II, *m*/*z* 660.8–368.9 (110 V; 12 V); for baohuoside I, *m*/*z* 514.8–368.9 (100 V; 8 V); for carbamazepine (IS), *m*/*z* 236.8–194.0 (120 V; 18 V).

2.3. Stock solutions, calibration curve and quality control (QC) samples

Stock solutions of the seven flavonoids were prepared in ethanol–water (7:3, v/v) mixture at a concentration of 0.1 mg/mL. Appropriate amounts of the above stock solutions were then mixed and diluted with ethanol–water (7:3, v/v) solution to obtain working solutions with the desired series of concentrations (0.6, 2, 6, 10, 30, 50, 100 and 200 ng/mL). The IS solution was prepared at a final concentration of 20 ng/mL in ethanol–water (7:3, v/v). All solutions were stored at 4 °C prior to analysis.

Next, 50 μL of the above working solutions at varying concentrations, and 50 μL of the IS solution, were added to 500 μL of blank dog plasma to prepare the biological calibration curve and QC samples. The biological calibration curve samples of the seven flavonoids with concentrations of 0.06, 0.2, 0.6, 1, 3, 5, 10 and 20 ng/mL were prepared to construct the corresponding calibration curves. QC samples were prepared at concentrations of 0.2, 1, 3 and 10 ng/mL.

2.4. Sample preparation

To prepare samples, 50 μL of the IS solution and 3 mL of methanol were added to 500 μL of plasma sample; the mixture was vortexed for 30 s and centrifuged at 1721 × *g* for 10 min at room temperature (23 °C). The supernatant was dried under a stream of N_2. The residue was reconstituted in 100 μL of ethanol–water (7:3, v/v) and then centrifuged at 15,493 × *g* for 10 min at room temperature (23 °C); 3 μL aliquots of the supernatant were then injected into the RRLC–MS/MS system.

2.5. Application of the method and pharmacokinetic study

Male beagle dogs weighing 9 ± 1 kg were obtained from Beijing Marshall Biotechnology Co., Ltd. (Beijing). They were kept in an

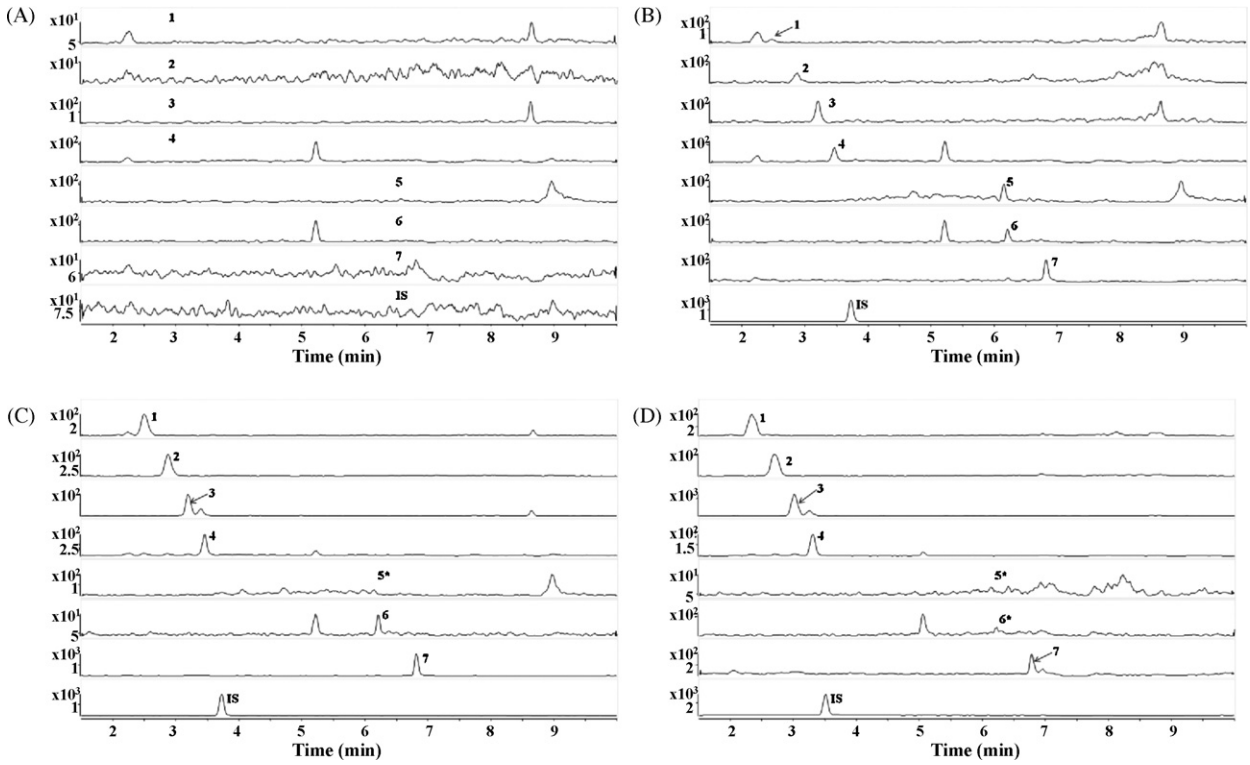


Fig. 2. Representative MRM chromatograms for (A) blank dog plasma; (B) LLOQ, 0.06 ng/mL for analytes with 2 ng/mL IS in dog plasma; (C) plasma sample 30 min after administration of *Herba Epimedii*; (D) plasma sample 30 min after administration of EXD. The numbers assigned to the compounds are the same as in Fig. 1. *The concentration of analytes in plasma was under the LLOQ.

environmentally controlled breeding room for 30 days before starting the experiments and fed with standard laboratory food and water. Prior to the experiments, the dogs were fasted and maintained with physiological saline for 12 h. Dogs were randomized according to body weight, and extract solutions of *Herba Epimedii* and Er-Xian Decoction were orally administered at a dosage of 0.5 g/kg (the weight of raw herb material of *Herba Epimedii*/body weight).

Dog blood samples were collected after administration of the extracts at 1 min, 5 min, 10 min, 20 min, 30 min, 45 min, 1 h, 1.5 h, 2 h, 2.5 h, 3 h, 3.5 h, 4 h, 5 h, 6 h, 8 h, 10 h and 12 h. Blood samples were immediately heparinized and centrifuged at 1721 × *g* for 10 min at room temperature (23 °C). The supernatants were placed into 1.5 mL polypropylene bottles and stored at −80 °C until the assay was performed. Pharmacokinetic parameters, including the area under the concentration–time curve (AUC), maximum plasma concentration (C_{max}) and time to reach the maximum concentration (T_{max}), were estimated by means of a non-compartmental analysis using Drug and Statistics 2.0 (DAS 2.0).

3. Results and discussion

3.1. Method development

To obtain chromatograms with good resolution for the seven flavonoids and IS, the UPLC conditions were selected using the standard mixture and the extract solution of *Herba Epimedii*. Because various kinds of chemical constituents were present in the extract solution, different gradient elution programs and different mobile phases were tested. A mobile phase consisting of 0.3% acetic acid and acetonitrile containing 0.3% acetic acid was found to improve the separation. The gradient elution program was necessary to rapidly separate the seven flavonoids and internal standard.

Optimization of the MS parameters was also a necessary step to obtain good signal response for the protonated ion. The standard solutions of the seven flavonoids and IS at a concentration of 100 ng/mL in acetonitrile were infused directly into the ESI source at a flow rate of 10 μL/min. The values of parameters for MS, such as dry temperature, capillary voltage, and delta EMV, and the parameters of the MRM mode were optimized.

Simple and rapid separation of the seven flavonoids and IS was achieved under the specified UPLC–MS/MS conditions within 10 min. Representative MRM chromatograms are shown in Fig. 2.

3.2. Validation of the developed method

3.2.1. Linearity and carryover

Satisfactory linearities of the seven flavonoids were observed within a concentration range of 0.06–21.8 ng/mL. The linear correlation coefficients were all better than 0.99, and the lower limit of quantification (LLOQ) were all lower than 0.0654 ng/mL for the seven flavonoids. The peaks of the seven flavonoids and IS in blank plasma (injected following the upper limit of quantification standard) were not detected. The results demonstrate that the studied samples were not influenced by carryover.

3.2.2. Matrix effect and recovery

Results comparing the peak responses of the post-extraction, spiked samples with those of the pure standards prepared in ethanol–water (7:3, v/v) indicate that a negligible matrix effect occurred when using this method. For all of the tested concentrations, the ratios of the peak response were within the acceptable range (83.3–113.4%). The relative standard deviations (RSD) for the tested concentrations were less than 14.4% (data not shown). These results indicate that no co-eluting endogenous substances significantly influenced the ionization of the seven flavonoids in this analytical method.

Table 1
Intra-batch and inter-batch precision and accuracy of seven flavonoids in dog plasma (intra-batch: $n = 6$; inter-batch: $n = 6$ duplicates per batch, three batches).

Analytes	Plasma conc. (ng/mL)	Intra-batch ($n = 6$)			Inter-batch ($n = 6$)		
		Observed[a] (ng/mL)	RSD (%)	Accuracy[b] (%)	Observed (ng/mL)	RSD (%)	Accuracy (%)
Epimedin A	0.0612 (LLOQ)	0.0662 ± 0.0038	5.8	108.1	0.0662 ± 0.0038	13.6	105.1
	0.204 (QC)	0.193 ± 0.026	13.5	94.5	0.210 ± 0.031	14.9	103.0
	1.02 (QC)	0.968 ± 0.046	4.8	94.9	0.992 ± 0.018	1.9	97.3
	3.06 (QC)	2.99 ± 0.14	4.6	97.8	3.06 ± 0.13	4.4	99.9
	10.2 (QC)	10.4 ± 0.2	2.3	101.8	10.8 ± 0.6	5.2	106.4
Epimedin B	0.0654 (LLOQ)	0.0625 ± 0.0076	12.1	95.5	0.0675 ± 0.0103	15.8	103.2
	0.218 (QC)	0.194 ± 0.013	6.8	88.9	0.216 ± 0.031	14.6	99.3
	1.09 (QC)	1.02 ± 0.05	4.9	93.1	1.14 ± 0.07	5.8	104.6
	3.27 (QC)	3.08 ± 010	3.1	94.3	3.21 ± 0.20	6.3	98.1
	10.9 (QC)	10.9 ± 0.2	1.7	100.4	10.8 ± 0.5	4.6	98.7
Epimedin C	0.0618 (LLOQ)	0.0684 ± 0.0109	16.0	110.6	0.0627 ± 0.0090	14.3	101.5
	0.206 (QC)	0.212 ± 0.029	13.7	102.8	0.205 ± 0.025	12.2	99.7
	1.03 (QC)	0.996 ± 0.025	2.6	96.7	1.04 ± 0.03	2.9	101.4
	3.09 (QC)	2.95 ± 0.09	3.2	95.4	2.98 ± 0.11	3.7	96.6
	10.3 (QC)	10.4 ± 0.3	2.8	100.7	10.3 ± 0.2	1.8	100.3
Icariin	0.0642 (LLOQ)	0.0685 ± 0.0092	13.4	106.7	0.0685 ± 0.0110	16.1	106.7
	0.214 (QC)	0.213 ± 0.027	12.8	99.7	0.242 ± 0.029	12.0	113.2
	1.07 (QC)	1.13 ± 0.04	3.4	105.2	1.22 ± 0.07	6.1	113.9
	3.21 (QC)	2.98 ± 0.15	5.1	92.8	3.07 ± 0.18	5.8	95.5
	10.7 (QC)	11.1 ± 0.3	2.9	103.5	10.6 ± 0.2	1.7	98.7
Sagittatoside B	0.0648 (LLOQ)	0.0713 ± 0.0077	10.9	110.0	0.0638 ± 0.0106	16.6	98.5
	0.216 (QC)	0.178 ± 0.022	12.5	82.2	0.215 ± 0.031	14.5	99.7
	1.08 (QC)	1.07 ± 0.09	8.3	98.9	1.01 ± 0.13	13.2	93.2
	3.24 (QC)	3.10 ± 0.14	4.7	95.8	3.17 ± 0.31	9.7	98.0
	10.8 (QC)	10.1 ± 0.6	6.1	93.5	10.6 ± 0.8	7.1	98.4
2″-*O*-Rhamnosylicariside II	0.0648 (LLOQ)	0.0578 ± 0.0087	15.2	89.2	0.0634 ± 0.0096	15.1	97.8
	0.216 (QC)	0.227 ± 0.014	6.2	104.9	0.229 ± 0.030	13.1	105.8
	1.08 (QC)	1.04 ± 0.06	5.9	96.5	1.12 ± 0.12	11.1	103.3
	3.24 (QC)	3.12 ± 0.11	3.5	96.3	3.31 ± 0.39	11.8	102.1
	10.8 (QC)	10.6 ± 0.4	3.6	97.9	10.7 ± 0.8	7.7	99.0
Baohuoside I	0.0612 (LLOQ)	0.0651 ± 0.0075	11.6	106.3	0.0592 ± 0.0083	14.1	96.7
	0.204 (QC)	0.170 ± 0.019	11.4	83.5	0.187 ± 0.022	11.6	91.7
	1.02 (QC)	0.870 ± 0.028	3.3	85.3	1.07 ± 0.12	11.2	105.0
	3.06 (QC)	3.04 ± 0.05	1.8	99.3	3.14 ± 0.35	11.0	102.5
	10.2 (QC)	10.5 ± 0.6	5.5	103.0	10.2 ± 0.6	6.4	100.5

[a] The values were means ± SD of six experiments.
[b] Accuracy (%) = 100 × mean of measured concentration/spiked concentration.

Table 2
Pharmacokinetic parameters for major flavonoids in *Herba Epimedii*.

Analytes	PharmacokineticParameter	Measuring unit	Test preparation[a]Mean ± SD[b]	Reference product[c]Mean ± SD	90% confidence interval (%)
Epimedin A	$AUC_{(0-t)}$	μg/L h	1.31 ± 1.01	4.58 ± 1.76	10.1–63.0%
	$AUC_{(0-\infty)}$	μg/L h	1.56 ± 1.05	4.82 ± 1.77	4.7–36.9%
	T_{max}	h	0.25 ± 0.09	0.76 ± 0.62	–
	C_{max}	μg/L	1.05 ± 0.86	2.85 ± 1.28	7.2–53.6%
Epimedin B	$AUC_{(0-t)}$	μg/L h	0.95 ± 0.83	3.80 ± 2.28	7.3–67.1%
	$AUC_{(0-\infty)}$	μg/L h	1.08 ± 0.80	4.92 ± 2.22	8.5–46.1%
	T_{max}	h	0.28 ± 0.13	0.61 ± 0.70	–
	C_{max}	μg/L	0.94 ± 0.89	2.47 ± 1.61	10.5–124.1%
Epimedin C	$AUC_{(0-t)}$	μg/L h	1.57 ± 1.22	3.71 ± 2.32	13.5–140.3%
	$AUC_{(0-\infty)}$	μg/L h	1.92 ± 1.30	4.89 ± 2.84	15.5–104.2%
	T_{max}	h	0.28 ± 0.13	0.28 ± 0.13	–
	C_{max}	μg/L	1.31 ± 1.22	4.57 ± 5.89	9.3–142.4%
Icariin	$AUC_{(0-t)}$	μg/L h	1.26 ± 1.04	4.99 ± 7.71	8.2–170.8%
	$AUC_{(0-\infty)}$	μg/L h	1.40 ± 1.04	6.47 ± 7.71	6.9–104.4%
	T_{max}	h	0.75 ± 1.35	1.58 ± 3.15	–
	C_{max}	μg/L	1.35 ± 1.67	2.81 ± 2.61	11.5–191.2%
Baohuoside I	$AUC_{(0-t)}$	μg/L h	2.55 ± 1.43	3.90 ± 2.05	43.4–100.0%
	$AUC_{(0-\infty)}$	μg/L h	3.01 ± 1.70	4.49 ± 2.21	44.8–93.8%
	T_{max}	h	0.28 ± 0.13	0.86 ± 1.05	–
	C_{max}	μg/L	0.89 ± 0.40	1.31 ± 0.85	41.3–133.7%

[a] EXD.
[b] The values were means ± SD of six male beagle dogs.
[c] *Herba Epimedii*.

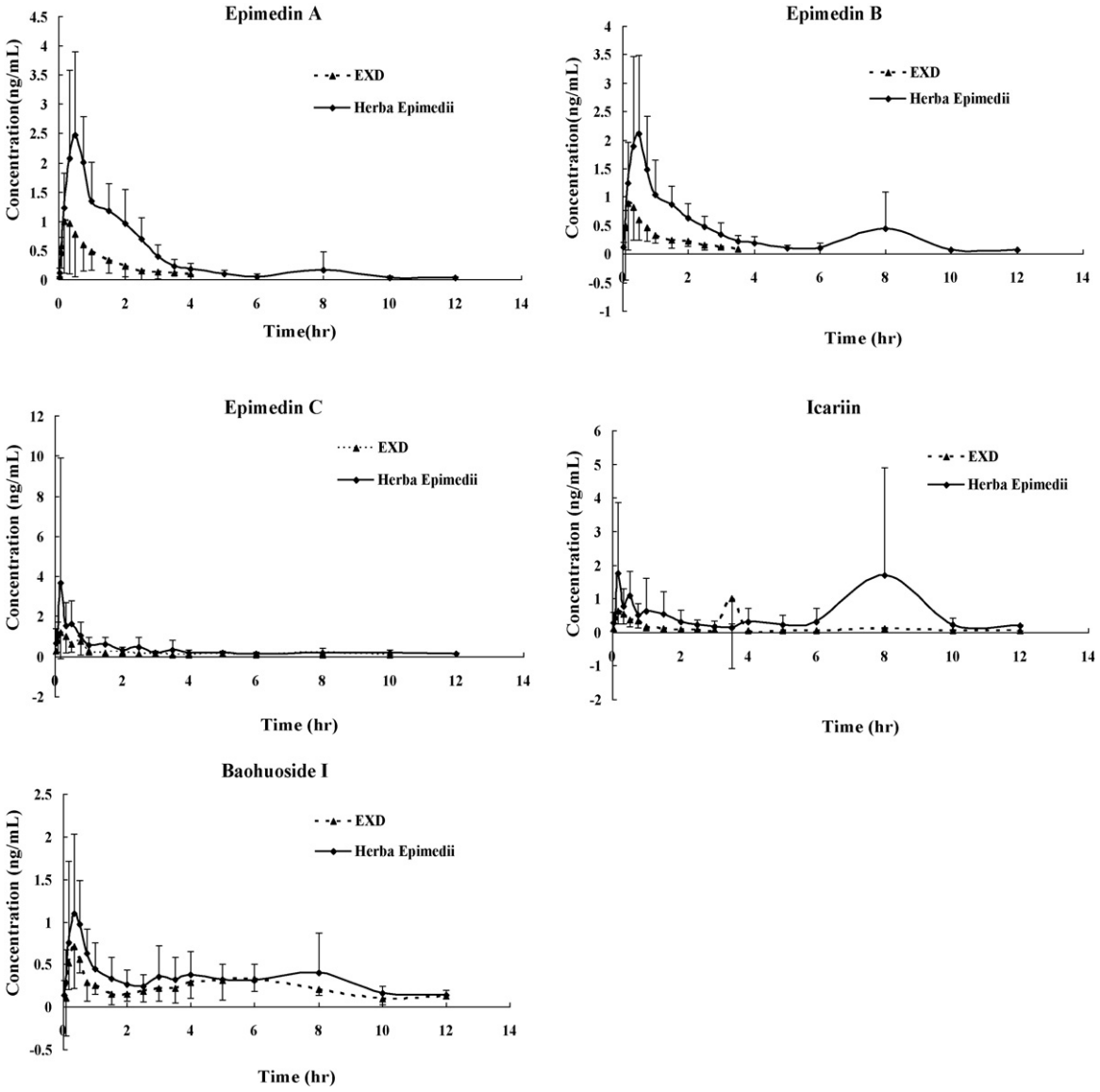


Fig. 3. Mean plasma concentration–time profiles for epimedin A, epimedin B, epimedin C, icariin and baohuoside I in 6 male beagle dogs.

The relative recoveries of the seven flavonoids were investigated by analysis of QC samples at the four concentrations of 0.2, 1, 3 and 10 ng/mL. The measured concentrations of the seven flavonoids were calculated from the biological calibration curves. The recoveries were obtained by dividing the measured concentrations by the spiked concentrations. The average relative recoveries of the seven standards spiked into blank dog plasma ranged from 92.8% to 114.5% (data not shown).

3.2.3. Precision and accuracy

The precision and accuracy of the method were assessed by performing replicate analyses of samples at four concentration levels of QC and LLOQ. The upper limits of the intra-batch and inter-batch samples were less than 15% with the exception of the LLOQ, where it was fixed at 20%, according to the FDA guideline [12]. Table 1 summarizes the accuracy and precision data. The results showed that the accuracy and precision of the method were within acceptable limits.

3.2.4. Stability

The sample stability was evaluated by analysis of QC samples at four levels for short-term stability, long-term stability, freeze–thaw stability and post-preparation stability. The upper limit of the concentrations of analytes in plasma deviated less than 15% with the exception of the low QC sample, which was fixed at $\leq$20%. The stability results (data not shown) showed that the concentrations of the seven flavonoids were between 91.4% and 113.3% of the initial values with the exception of the low QC samples, which ranged from 85.3% to 118.3%, indicating that the analytes maintained at these conditions were stable.

3.3. Pharmacokinetic study

The established method was successfully applied to analysis of the seven flavonoids in dog plasma. Four main flavonoids (epimedin A, epimedin B, epimedin C, icariin) from extracts and their metabolite (baohuoside I) can be determined accurately in dosed-dog plasma with relatively high concentrations. However, the other two metabolites (sagittatoside B and 2″-*O*-rhamnosylicariside II) appeared in dosed-dog plasma with concentrations lower than the LLOQ and could not be determined except at some time points.

The mean plasma concentration–time curves of five flavonoids (epimedin A, epimedin B, epimedium C, icariin, baohuoside I) are

shown in Fig. 3 after oral administration of the extracts of *Herba Epimedii* and EXD to dogs. The pharmacokinetic parameters derived from these profiles are presented in Table 2. Because the 90% confidence intervals for the ratios of EXD to *Herba Epimedii* for AUC_{0-t}, $AUC_{0-\infty}$ and C_{max} were generally less than 80.0%, these data are not consistent with the bioequivalence of *Herba Epimedii* and EXD. In addition, the ratios (EXD/*Herba Epimedii*) of the concentrations of each analyte were within 95.2–97.6% (using the method developed in a previous study [3]). These results indicate that five major, active flavonoids were more highly absorbed from the *Herba Epimedii* extract than from the EXD extract. These data might helpful to explain and predict the different clinical effects of *Herba Epimedii* and EXD. In addition, we can identify multi-peak phenomena in the concentration–time profiles for these flavonoids. These phenomena may be caused by the enterohepatic cycle, or they may arise because the 5 major flavonoids may actually be the metabolites of other flavonoids (e.g., the metabolite of icariin is baohuoside I) [4].

4. Conclusion

In this paper, an UPLC–MS/MS method for the simultaneous determination of seven flavonoids in dog plasma has been established and validated for the first time. The established method is simple, rapid, sensitive and reliable. This method has been successfully applied to a pharmacokinetic study following the oral administration of *Herba Epimedii* and EXD extracts to dogs. This method is suitable for analyzing high throughput biological samples with multiple marker components. Lastly, pharmacokinetic data for epimedium A and epimedium B have been reported for the first time.

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