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Simple and sensitive liquid chromatography–tandem mass spectrometry assay for simultaneous measurement of five *Epimedium* prenylflavonoids in rat sera

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ABSTRACT

A rapid and sensitive method to separate and quantify icariin, icariside I, icariside II, icaritin and desmethylicaritin in rat sera was developed using liquid chromatography–tandem mass spectrometry. Serum samples were extracted with ethyl acetate without further derivatization. Using coumestrol as an internal standard, calibration curves with good linearity ($r^2 > 0.99$) within the concentration range of 0.78–12.5 nM for icariin, icaritin and desmethylicaritin, and 0.78–100 nM for icariside I and II, were obtained in the multiple reaction monitoring mode. For all analytes, the limits of detection and quantification were <1 nM and 1–2 nM, respectively. Inter- and intra-assay variabilities were <15% and accuracies were between 94% and 114%, respectively. This method was successfully applied to quantify levels of icariin, icariside I, icariside II, icaritin and desmethylicaritin in rat sera after oral administration of an *Epimedium* preparation.

1. Introduction

Plants of the genus *Epimedium* (Berberidaceae) are traditionally used in East Asian countries to improve bone health and for other indications such as sexual dysfunction [1,2]. The principal bioactive constituent of *Epimedium* is the flavonoid glycoside, icariin [3–8]. Icaritin is the aglycone of icariin. Removal of rhamnose or glucose residues from icariin results in icariside I or icariside II, respectively. Icaritin can be demethylated to desmethylicaritin [3]. Metabolism of icariin can result in the presence of its deglycosylated and demethylated derivatives *in-vivo* [4]. Icariside II can inhibit hypoxia-inducible factor-1α in human osteosarcoma cells [5], and melanogenesis in melanocytes [6]. Icaritin and desmethylicaritin have estrogenic properties and can stimulate MCF-7 breast cancer cell proliferation [3,7], affect osteoblastic/osteoclastic activity [8] on bone cells and exert protective effects on rat neuronal cells [9].

The effects of icariin and its derivatives on key signaling processes have stimulated increasing interest in the pharmaceutical investigation [10] of these compounds for hormone replacement therapy and erectile dysfunction, as well as, therapeutic agents for bone [11], cardiovascular, neurological and endocrinological diseases. HPLC-UV methods have been successfully applied for detection and quantification of *Epimedium* flavonoids in herbal extracts [12,13], however, the sensitivities of the HPLC-UV methods used in biological samples were unsatisfactory [14–16]. Several LC–MS/MS methods have been reported for detection and quantification of icariin and its metabolites *in-vivo* [17–20]. Icariin, together with its major metabolites, icariside I and icariside II, were detected and quantified in the plasma of rats after administration of icariin [18]. Besides LC–MS/MS, capillary zone electrophoresis (CZE) technique coupled with ESI-MS and GC/MS were also applied to study the metabolism of icariin *in-vivo*, and icaritin and desmethylicaritn were identified as the metabolites of icariin [4,21]. However these methods tracked icariin alone, measured only one or two of its metabolites, used large quantity of sample, or depended on time- and sample-consuming derivatization steps. A simple, sensitive and reliable method to simultaneously quantify icariin and its derivatives in small quantities of serum is required to fully understand the pharmacokinetic disposition of bioactive compounds following oral administration of *Epimedium* prenylflavonoids.

In this study, we developed and validated a LC–MS/MS method that can be used to quantify icariin and its four derivatives simultaneously via a single injection without complicated sample preparation steps, such as derivatization. This method is rapid, simple, and sensitive enough to detect less than 1 nM and accurately quantify 1–2 nM of all the five analytes with good precision and accuracy. The validated method was successfully applied to

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Table 1
Main working parameters for mass spectrometry.

Parameter	Period I	Period II
Curtain gas (CUR) (psi)	15	15
Ion spray voltage (IS) (V)	4500	4500
Source gas temperature (TEM) (°C)	550	550
Ion source gas (Gas 1) (psi)	40	30
Heater gas (Gas 2) (psi)	65	55
Collision associated dissociation (CAD) (psi)	3	5
Declustering potential (DP) (V)	70	50
Entrance potential (EP) (V)	10	10
Collision energy (CE) (V)	50	50
Mode of analysis	(−)	(−)

Parameter	ICA	IS	ICAR I	ICAR II	DICT	ICT
Collision cell exit potential (CXP) (V)	4	2	4	4	2	2
MRM ion pair *m*/*z*	513 → 351	267 → 211	529 → 367	513 → 351	353 → 136	367 → 175

ICA: icariin; IS: internal standard; ICAR I and ICAR II: icariside I and II; DICT: desmethylicaritin; ICT: icaritin.

quantify the five target prenylflavonoids in rat sera after oral administration of a flavonoid-enriched *Epimedium* preparation.

2. Experimental

2.1. Chemicals

Icariside I, icariside II, icaritin and desmethylicaritin (purity >98%) were provided by Dr. Willmar Schwabe Pharmaceuticals (Karlsruhe, Germany). Icariin (purity ∼98%) was purchased from 3B Scientific Corporation (Libertyville, IL, USA). Coumestrol (purity >99%), formic acid (purity ∼98%) and ammonium formate (purity ∼98%) were purchased from Sigma (St. Louis, MO, USA). Solvents such as methanol (HPLC grade), ethyl acetate (analytical grade), acetonitrile (HPLC grade) and acetone (analytical grade), were obtained from Merck (Darmstadt, Germany).

Stock solutions of compounds were made using methanol, and stored in foiled-wrapped containers under nitrogen gas at −20 °C.

2.2. Animal study protocol

Female Sprague-Dawley rats, ovariectomized at 6 weeks of age, were treated with 300 mg of *Epimedium* standardized preparation per kilogram of body weight at 8 weeks of age by gavage. The *Epimedium* preparation (PSC1929/L01-60/B/Wo 06-002-28) was derived from *Epimedium brevicornu* dry leaves that were extracted with 60% ethanol. The preparation contained 143.3 mg/g of icariin, 0.157 mg/g of icariside I, 18.68 mg/g of icariside II, 0.009 mg/g of icaritin, and 0.122 mg/g of desmethylicaritin, respectively (determined by method described below). Following *Epimedium* administration, blood sampling was performed at 0, 0.5, 1, 2, 4, 8, 24, 48, and 72 h time points (four rats/each time point). Sera were separated and stored at −80 °C until analysis. Animal experiments were performed in the laboratories of Dr. Willmar Schwabe Pharmaceuticals (Karlsruhe, Germany) in accordance with German Federal law for animal welfare. Written approvals for the study were granted by veterinary authority of Baden-Wurttemberg, Germany; and the Institutional Animal Care and Use Committee, National University of Singapore.

2.3. Sample preparation

All serum samples were thawed at room temperature, spiked with internal standard (IS) (coumestrol, 100 nM) and equilibrated at 37 °C for 1 h before extraction. Analytes were extracted together with IS from 0.5 mL serum aliquots thrice via liquid–liquid partition (1 mL × 3) using water-saturated ethyl acetate (EtOAC) for 1 min inside a 2 mL polypropylene centrifuge tube. After each round of solvent–solvent partition, the mixture was centrifuged for 1 min at 10,000 × *g* using a centrifuge from Eppendorf (Microcentrifuge 5424, Eppendorf, NY, USA) to clearly separate the organic layer from the aqueous layer. The organic layers obtained from the three rounds of solvent–solvent partition were combined and dried with a gentle stream of nitrogen gas. The residue was solubilized using 100 μL of methanol by vortexing for 1 min. This was then transferred into a 200 μL borosilicate glass insert placed inside a sample vial for LC–MS/MS analysis.

2.4. Instrumentation

LC–MS/MS analysis of icariin, icariside I, icariside II, icaritin and desmethylicaritin was performed on an Agilent 1200 LC system (Palo Alto, CA, USA) coupled to an API3200 Triple-Quadrupole Mass spectrometer (Applied Biosystems/MDS SCIEX, Foster City, CA). A Turbo V source with TurboIonSpray (TIS or ESI) and atmospheric pressure chemical ionization (APCI) probes were used for the analysis. Data acquisition was performed with Analyst 1.4.2 software (AB MDS Sciex).

The chromatographic separation was performed on 150 mm × 2.0 mm (i.d.) Phenomenex Synergi 4μMax-RP column (Phenomenex, CA, USA) with a column oven temperature of 35 °C. The mobile phase consisted of two eluents, namely, solvent A (ultrapure water containing 0.5 mM ammonium formate, pH 3) and solvent B (acetonitrile with 0.1% formic acid), delivered at a flow rate of 0.4 mL/min. Before every run, the column was first equilibrated with 45% of solvent B for 4 min before sample injection. The gradient was programmed to increase from 45% to 90% of solvent B in 5 min and maintained at 90% of B for 3 min to complete the separation. Standards or samples were introduced into the LC using an Agilent 1200 G1367B autosampler and injection volume was 10.0 μL.

The ion source was operated in the negative mode. The experiment was divided into two periods to detect two groups of analytes: prenylflavonoid glycosides (0.2–4.5 min) and aglycones (4.51–8 min). Ion-spray voltage and temperature were set constantly at –4500 V and 550 °C for both periods. The details of the MS parameters were summarized in Table 1.

2.5. Assay validation

2.5.1. Linearity

Commercial pooled rat serum (Sigma, St. Louis, MO) was analyzed using full scan and multiple reaction monitoring (MRM) mode to confirm the absence of interference with the IS and the

five analytes. To generate calibration samples, increasing doses of icariin, icariside I, icariside II, icaritin and desmethylicaritin were added to 0.5 mL of pooled rat sera to obtain final concentrations ranging from 0.78 to 100 nM. All standards contained 100 nM of coumestrol as the internal standard. Six calibration curves with seven concentration points were constructed. The final curves for each of the five compounds were generated with the average values and were used for quantification of the analytes. The least squares regression method was used to estimate linearity.

2.5.2. Limits of detection (LOD) and quantification (LOQ)

Using the statistical method based on the calibration curve constructed from standard compounds, the limit of detection and limit of quantification were defined as – LOD = $3.3\sigma/S$, LOQ = $10\sigma/S$, where σ is the standard deviation of the background response and S is the slope of the calibration curve [22]. The background response was measured by analyzing pooled rat sera and the standard deviation was calculated from 10 independent assays. The average of the slope of six independent calibration curves was used as S for LOD and LOQ calculations.

The empirical method for estimation of the LOD and LOQ involved analyzing decreasing concentrations of analytes. The LOD was defined as having a signal-to-noise ratio of 3:1, and a signal-to-noise ratio of 10:1 was defined for LOQ [22]. Three independent assays were performed to obtain the signal-to-noise ratios.

2.5.3. Accuracy and precision

Quality control (QC) samples consisting of a high, medium and low concentration of the five analytes, containing 1, 5 and 10 nM of icariin, icaritin and desmethylicaritin; 5, 30 and 60 nM of icariside I and icariside II, respectively and IS in pooled rat sera, were placed at the beginning and end of each assay for determination of accuracy and precision [23]. The mean values and coefficient of variation (CV or RSD) were obtained from five independent assays within one experimental day (intra-day) or independent assays from 6 consecutive days (inter-day).

2.5.4. Matrix effect

0.5 mL of blank rat serum was extracted three times with 1 mL of EtOAC, and the EtOAC layers were combined and dried with nitrogen gas. The residue was reconstituted with 0.5 mL of MeOH to serve as serum extract. Mixture of five analyte standards and IS were spiked into 0.1 mL of MeOH (neat solution) and 0.5 mL of serum extract, respectively. Neat solution samples were directly injected and analyzed with LC–MS/MS. Samples spiked in serum extract were dried with N_2 gas and reconstituted with 0.1 mL of MeOH and analyzed with LC–MS/MS. Matrix effect was assessed by comparing the peak areas of the analytes and IS between neat solution and serum extract [24]. Each assay was performed with five replicates.

2.5.5. Stability

Analyte standards were spiked into blank serum with low and high concentrations and stored at −80 °C for 1 month. The samples were thawed, processed and analyzed with the developed method. The concentrations calculated from the calibration curve were compared with actual values to determine the long-term stability [25]. For post-preparative stability study, sera spiked with low and high levels of standards were processed and kept at 4 °C in an autosampler for 24 h before analysis [25]. Concentrations of the samples were compared with actual values. All the assays were performed in five replicates.

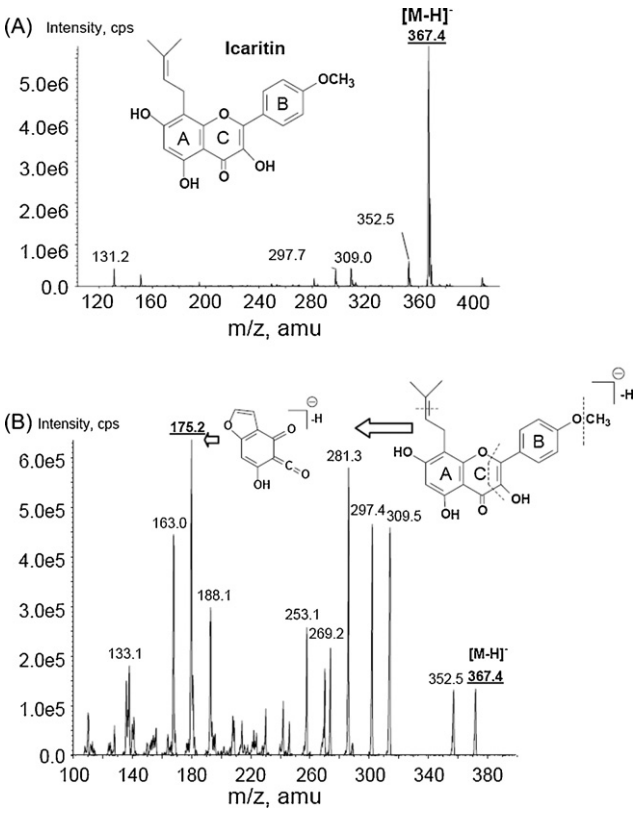

Fig. 1. Full scan mass spectrum of icaritin (A); product ion spectrum of m/z 367 $[M-H]^-$ (B); the possible fragmentation pathway of the precursor ion $[M–H]^-$ and the structure of the main product ion was proposed. Ion pair 367/175 chosen for MRM analysis was shown in bold and underlined.

3. Results and discussion

3.1. LC–MS/MS analysis

In order to obtain good specificities and sensitivities for all the five prenylflavonoids, different ionization methods, including positive and negative modes in ESI and APCI, were optimized and compared. In APCI mode, both positive and negative modes did not induce satisfactory responses for the glycosides (data not shown). However in ESI mode, both the positive and negative modes gave relatively good responses for all the analytes. The ESI negative mode was chosen because it had better signal-to-noise responses at low concentrations of the analytes (data not shown). This is in good agreement with previous observations when measuring flavonoids with LC–MS/MS [26].

The five prenylflavonoid standards were used to select the ion pairs and optimize the MS conditions. In MS full scan mode, the most abundant deprotoned $[M–H]^-$ peaks of icaritin, desmethylicaritin, icariside I and icarised II were observed; while icariin had a base ion peak of losing a glucose, $[M–H–glc]^-$ (Figs. 1(A), 2(A), 3(A) and 4). These most abundant ions were selected and further fragmented and scanned, yielding product ion spectra (Figs. 1(B), 2(B), 3(B) and 4). Based on known fragmentation patterns of related compounds [27–29], the fragmentation pathways of the parent ions and the structures of the product ions were deduced (Figs. 1(B), 2(B), 3(B) and 4). The product ion m/z 175 of m/z 367 in the mass spectrum of icaritin could be formed by cleavage of ring B from m/z 309 (Fig. 1(B)) [27]. The most abundant product ion at m/z 136 in the mass spectrum of desmethylicaritin could be due to the cleavage of B ring and the loss of CO from m/z 298 (Fig. 2(B)). Icariin is a glucoside of icariside II, and the base ion peak of icariin was $[M–H–glc]^-$,

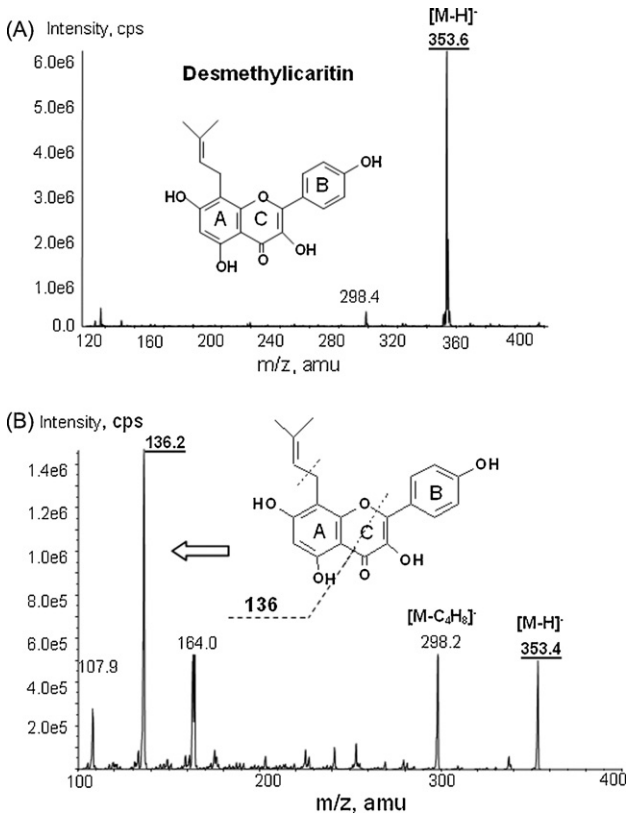

Fig. 2. Full scan mass spectrum of desmethylicaritin (A); product ion spectrum of *m/z* 353 $[M–H]^-$ (B); the possible fragmentation pathway of the precursor ion $[M–H]^-$ and predicted structures of the main product ions were indicated. Ion pair 353/136 chosen for MRM analysis was shown in bold and underlined.

so the same ion pair (513/351) was selected (Fig. 3). The product ion *m/z* 351 in the mass spectrum of icariside II could be due to the loss of one rhamnose and methyl group from *m/z* 513 (Fig. 3). For icariside I, the characteristic product ion *m/z* 367 was due to loss of glucose (Fig. 4(B)).

Coumestrol, a coumarin with similar chemical and physical properties but not naturally present in *Epimedium* and normal rat diet was used as the IS. The LC mobile composition, flow rate and gradient program were carefully optimized to achieve the base-line separation of the five analytes within 8 min. Meanwhile, the compounds and ion source dependent parameters in MS were optimized to give sufficient sensitivities to detect less than 1 nM and accurately quantify 2 nM of the five analytes in rat sera (Fig. 5(A)–(C)). Commercial pooled rat sera used for construction of calibration curve did not contain any detectable analytes or the IS.

3.2. Linearity

Good linearity was obtained for icariin, icaritin and desmethylicaritin over the range of 0.78–12.5 nM with r^2 of 0.996, 0.998, and 0.996, and for icariside I and II, over the range of 0.78–100 nM (Table 2) with r^2 of 0.999 and 0.999, respectively. The limits of detection (LOD) and quantification (LOQ) for the five analytes were estimated with two methods, namely, the statistical method based on calibration curves constructed from standards and the empirical method by analyzing increasingly lower concentrations of the analytes (Table 2). The LOQs generated with calibration curves were relatively similar to those obtained from the second method of signal-to-noise ratios and they are ranged from 0.25 and 0.55 nM for icariin, and 2 and 1.88 nM for icariside II, which were sensitive enough to quantify nanomolar levels of the five compounds in

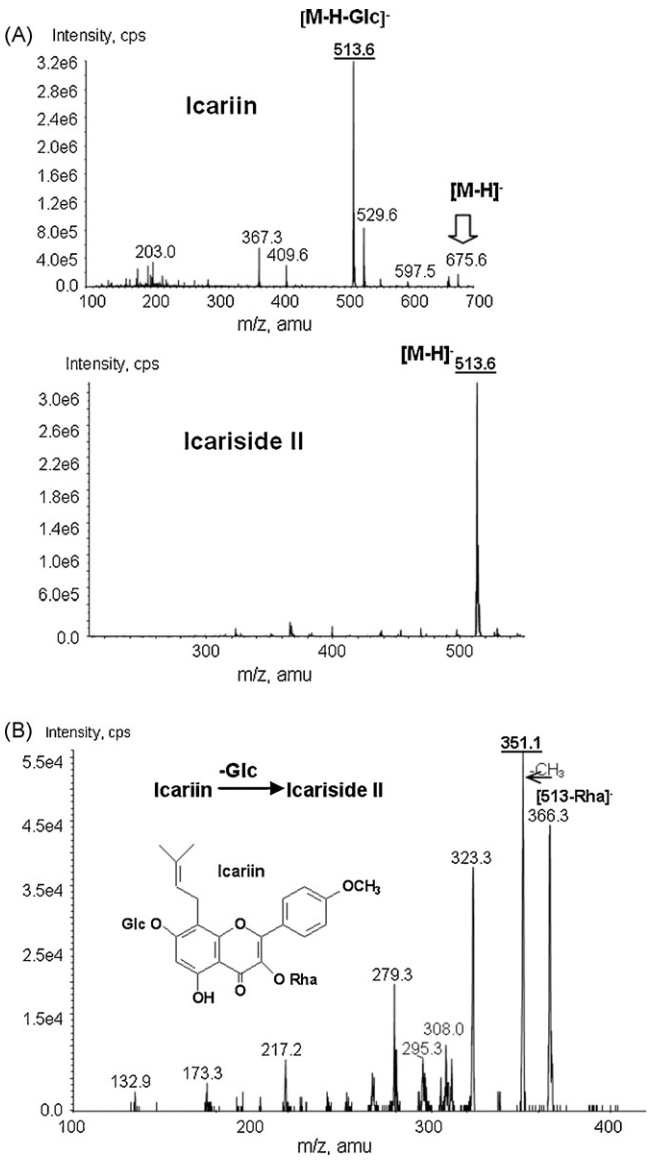

Fig. 3. Full scan mass spectra of icariin and icariside II (A); product ion spectrum of *m/z* 513 (B); the possible fragmentation pathways of the precursor ion and the structures of the main product ions were proposed. Ion pair 513/351 chosen for MRM analysis was shown in bold and underlined.

rat sera (Table 2, Fig. 5(B) and (C)). Accuracy and precision were determined by analyzing high, medium and low concentrations of QC samples throughout the calibrator concentration ranges; intra- and inter-day precisions for determination of all the five prenylflavonoids in rat sera were <15% and the mean values of accuracy ranged between 94.2% and 114.7% (Table 3).

3.3. Matrix effects and stability

Standards of the five analytes were spiked into MeOH (neat solution) and serum extract with low, medium and high concentrations (5, 10 and 100 nM), respectively. The peak areas were compared with samples in neat solution as 100%. The matrix effects (ME = serum extract/neat solution × 100%) for icariside I, icariside II, icariin, icaritin and desmethylicaritin were summarized in Table 4. Ion suppression was observed to range from 80.6% to 97.2% for the five analytes. It was most evident with icaritin but this did not affect assay performance as our method could detect nanomolar concentrations of this compound in serum samples. Similar ranges of matrix effects have also been reported in assays

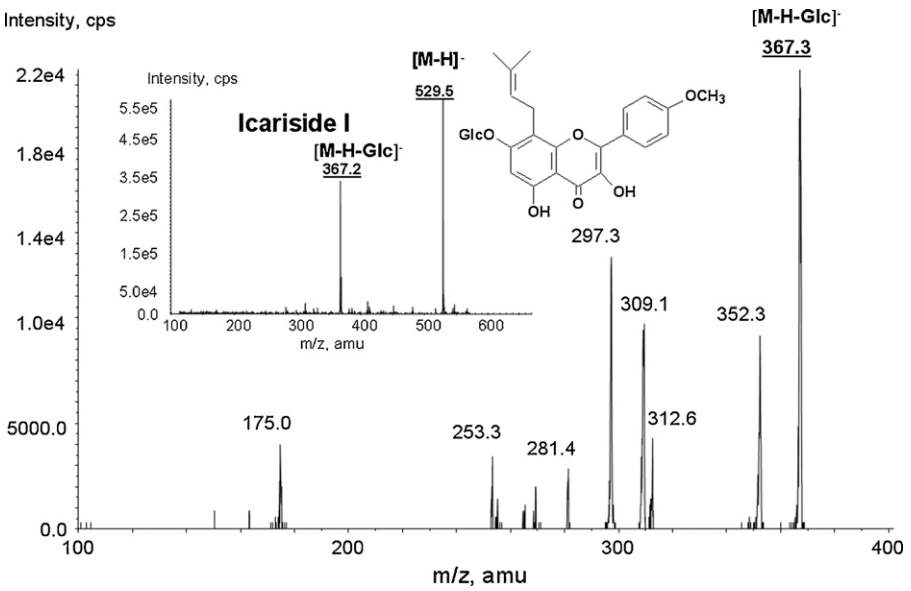

Fig. 4. Full scan mass spectrum of icariside I and product ion spectrum of *m/z* 529 $[M–H]^-$. The possible fragmentation pathway of the precursor ion and the structure of the main product ion were proposed. Ion pair 529/367 chosen for MRM analysis was shown in bold and underlined.

Table 2
Linearity of the calibration curves and sensitivity of the method for the five prenylflavonoids.

IS or analyte	Retention time (min)	Regression equation and test range (nM)	r^2	LOD (nM)		LOQ (nM)	
Coumestrol	2.52	Internal standard	–	S/N = 3	$3.3\sigma/S$	S/N = 10	$10\sigma/S$
ICA	1.37	$Y = 0.0179X + 0.0052$ (0.78–12.5)	0.996	0.125	0.18	0.25	0.55
ICT	6.72	$Y = 0.0421X - 0.004$ (0.78-12.5)	0.998	0.125	0.27	0.5	0.82
DICT	5.12	$Y = 0.0425X - 0.0061$ (0.78–12.5)	0.996	0.25	0.30	1	0.90
ICAR I	3.04	$Y = 0.0103X + 0.0057$ (0.78–100)	0.999	0.5	0.63	2	1.88
ICAR II	4.00	$Y = 0.0254X + 0.0038$ (0.78–100)	0.999	0.125	0.42	0.25	1.25

IS: internal standard; MRM: multiple reaction monitoring; LOD and LOQ: limit of detection and limit of quantification; S/N: signal-to-noise ratio; σ: the standard deviation of the response of blank, $n = 10$; S: the slope of the calibration; ICA: icariin; ICT: icaritin; DICT: desmethylicaritin; ICAR I and II: icariside I and II.

Table 3
Intra- and Inter-day accuracy and precision of the method for the five prenylflavonoids in rat sera.

Intra-day accuracy and precision ($n = 5$)												
Added (nM)	Found (nM)			Accuracy			SD			RSD (%)		
	ICA	ICT	DICT	ICA	ICT	DICT	ICA	ICT	DICT	ICA	ICT	DICT
1	1.00	0.99	0.94	99.6	99.0	94.2	0.11	0.07	0.07	10.9	6.81	7.15
5	4.92	4.99	4.71	98.3	99.9	94.3	0.16	0.31	0.46	3.29	6.35	9.86
10	10.9	9.58	9.86	109.1	95.8	98.6	0.87	0.42	0.7	8.01	4.43	7.13
	ICAR I	ICAR II		ICAR I	ICAR II		ICAR I	ICAR II		ICAR I	ICAR II	
5	5.09	5.73		101.9	114.7		0.18	0.68		3.55	11.8	
30	29.4	33.0		98.0	110.0		1.84	1.57		6.27	4.76	
60	62.2	67.0		103.7	111.7		3.17	3.70		5.10	5.52	
Inter-day accuracy and precision ($n = 6$)												
Added (nM)	Found (nM)			Accuracy			SD			RSD (%)		
	ICA	ICT	DICT	ICA	ICT	DICT	ICA	ICT	DICT	ICA	ICT	DICT
1	1.52	1.58	1.54	97.2	101.2	98.6	0.04	0.05	0.05	2.50	3.20	3.45
5	4.95	4.87	4.81	99.0	97.5	96.1	0.65	0.55	0.32	13.1	11.3	6.65
10	12.8	12.4	12.5	102.4	99.4	100.1	1.13	1.04	0.73	8.85	8.37	5.82
	ICAR I	ICAR II		ICAR I	ICAR II		ICAR I	ICAR II		ICAR I	ICAR II	
5	5.01	5.11		100.0	102.1		0.24	0.73		4.76	14.35	
30	29.6	29.2		98.7	97.3		3.75	3.42		12.7	11.7	
60	61.3	60.1		102.0	100.0		3.42	5.95		5.59	9.91	

n: number of independent assays; ICA: icariin; ICT: icaritin; DICT: desmethylicaritin; ICAR I and ICAR II: icariside I and II.

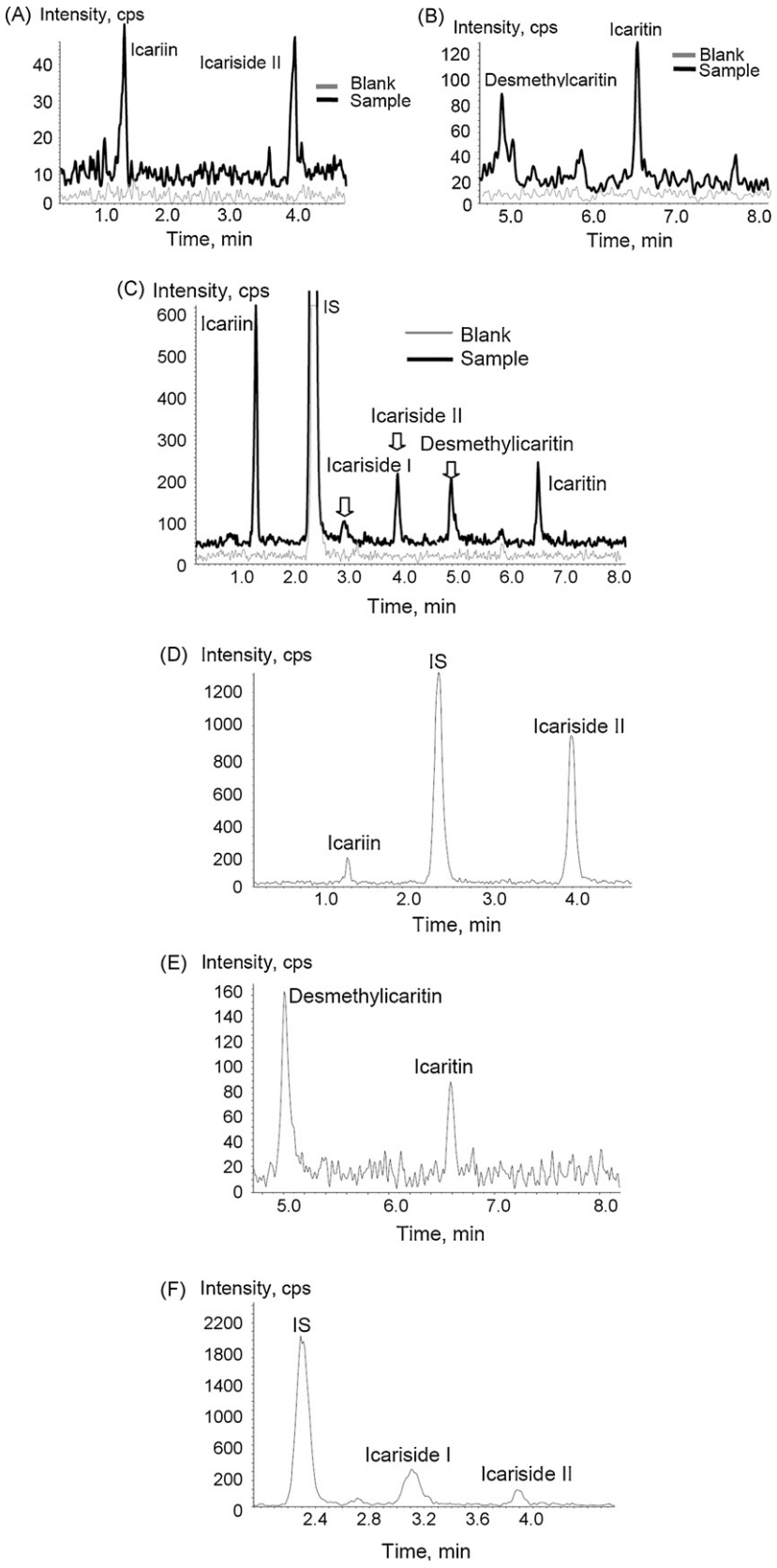

Fig. 5. Typical chromatograms of 0.5 nM of icariin and icariside II spiked in rat serum (A); 1 nM of icaritin and desmethylicaritin spiked in rat serum (B); 2 nM of the five prenylflavonoids with IS (coumestrol, 100 nM) spiked in rat serum (C). Rat serum without (blank) or with spiking of analyte standards (sample) were extracted and analyzed under same conditions. Typical chromatograms of rat serum samples obtained using this LC–MS/MS method: 0.5 h (D); 8 h after *Epimedium* extract administration (E) and (F).

Table 4
Matrix effects on the five prenylflavonoids and internal standard (IS).

Analyte and IS ($n=5$)	QC sample (nM)	ME (%)	RSD (%)
Icariin	5	89.8	8.73
	10	85.5	1.35
	100	87.0	4.53
Icariside I	5	91.2	8.89
	10	86.3	3.19
	100	86.1	4.10
Icariside II	5	89.8	7.85
	10	85.5	1.79
	100	87.0	3.83
Icaritin	5	80.6	2.99
	10	82.2	1.34
	100	82.4	4.10
Desmethylicaritin	5	91.5	1.07
	10	97.2	2.81
	100	97.0	0.85
Coumestrol (IS)	100	97.2	5.76

RSD (%) = (standard deviation/mean) × 100%; ME (%): matrix effect = (analyte peak area of serum extract/analyte peak area of neat solution) × 100%.

Table 5
Stability of the five prenylflavonoids ($n=5$).

Analyte	nM	Long term		Post-preparative	
		Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)
Icariin	5	96.8	6.19	99.7	1.06
	10	94.5	7.19	101	0.71
Icariside I	10	99.1	7.65	98.3	3.39
	100	99.9	10.7	99.1	3.97
Icariside II	10	99.1	9.83	105	9.77
	100	99.7	9.64	97.0	1.29
Icaritin	5	99.8	2.42	100	1.23
	10	98.8	3.02	99.1	1.73
Desmethylicaritin	5	101	10.1	97.5	6.96
	10	99.2	3.34	99.7	1.52

using serum samples [30–33]. The relative recovery of this validated method for the five analytes ranged from 73% to 77%, which accounts for both extraction losses and matrix effects. Relative recovery was determined using RE = (spiked serum/neat solution × 100%).

Table 5 summarized the results of the long-term and post-preparative stability of the five analytes. Long-term stability was investigated by spiking standards into blank sera with low and high concentrations of the five analytes and stored at −80 °C for 1 month. All the analytes were found stable under the storage and analytical conditions. Long-term stability of the analytes ranged from 94.5% to 101%. Similarly, the post-preparative stability ranged from 97.0% to 105%.

3.4. Application of the validated method for biological samples

To test the utility of this assay for biological samples, we measured the concentrations of these five prenylflavonoids in rat sera following oral administration of an *Epimedium* preparation. Serum samples were obtained at defined time points over 72 h following oral administration. Fig. 5(D)–(F) shows typical LC–MS/MS chromatograms of the targeted compounds at 0.5 and 8 h after *Epimedium* extract ingestion. The serum concentrations of the individual analytes are summarized in Table 6. Icariin showed an absorption peak at 0.5 h with a concentration of <2 nM. Comparing with its concentration in *Epimedium* extract, which was 143.3 mg/g and the most abundant compound among the five, the unconjugated form of icariin in rat serum was very low, suggesting that it could be poorly absorbed, or directly absorbed, but quickly metabolized into other forms. Icariside II could be detected from 0.5 h, and showed a peak at 1 h with a concentration of ∼15 nM after *Epimedium* extract ingestion (Table 6), indicating its possible metabolic pathway originally from icariin or other glycosides with more than one sugar moieties. Icariside I, icaritin and desmethylicaritin were undetectable at 0.5 h (Table 6), suggesting that very small amounts of them were directly absorbed. The results were not unexpected as the concentrations of three prenylflavonoids in the *Epimedium* extract were only 0.157, 0.009 and 0.122 mg/g, respectively. The levels of icariside I, icaritin and desmethylicaritin, were observed to rise rapidly after 4 h, and peaked at 8 h with maximum concentration of 18, 1.6 and 1.8 nM, respectively (Table 6). The results of icaritin and desmethylicaritin were consistent with data from our previous study [21], indicating that they could be metabolized from icariin or other glycosides in the extract. It is important to note that this assay measures unconjugated forms of the analytes. Total flavonoid content and possible bioactivities could be higher if conjugated forms after enzyme hydrolysis of these analytes are assayed. These assays form the basis for further dose–response studies to develop formulations of *Epimedium* that can deliver therapeutic doses of bioactive compounds *in-vivo*.

4. Conclusion

A method with LC–MS/MS in ESI negative mode has been established and validated for simultaneous quantification of icariin, icariside I, icariside II, icaritin and desmethylicaritin to low nanomolar concentrations in rat sera. The method is simple, rapid and sensitive. The successful detection of the five prenylflavonoids at different time points after ingestion of *Epimedium* extracts indicates that this method can be used for metabolic and pharmacokinetic studies of the five compounds and is applicable for clinical evaluation of *Epimedium* preparations.

Table 6
Concentrations (nM) of five analytes in rat sera after oral administration of an *Epimedium* preparation.

Time point (h)	Icariin	Icariside I	Icariside II	Icaritin	Desmethylicaritin
0	<0.125	<0.5	<0.125	<0.125	<0.25
0.5	1.75 ± 0.87	<0.5	3.15 ± 1.6	<0.27	<0.30
1	0.81 ± 0.53	<0.5	14.56 ± 9.17	<0.27	0.31 ± 0.32
2	0.32 ± 0.17	0.95 ± 0.66	6.86 ± 4.12	<0.27	<0.30
4	<0.18	9.11 ± 4.34	3.03 ± 1.25	1.47 ± 1.33	0.77 ± 0.71
8	0.51 ± 0.19	17.94 ± 4.8	1.47 ± 0.81	1.66 ± 1.04	1.75 ± 1.09
24	0.33 ± 0.30	2.52 ± 2.15	0.54 ± 0.63	0.42 ± 0.31	0.64 ± 0.25
48	<0.18	<0.63	<0.125	<0.27	0.31 ± 0.22
72	<0.18	1.60 ± 1.83	<0.125	0.29 ± 0.14	<0.30

The concentration was mean ± SD ($n=4$).

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