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Sensitive and rapid method to quantify icaritin and desmethylicaritin in human serum using gas chromatography–mass spectrometry

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Abstract

The prenyl-flavones, icaritin and desmethylicaritin, are bioactive compounds from the traditional Chinese medicinal herb, *Epimedium*, extracts of which can enhance bone health in animal models. In order to examine their bioavailability in humans, we have developed and validated a sensitive method to quantify icaritin and desmethylicaritin in human sera, using gas chromatography–mass spectrometry. The serum samples were extracted with ethyl acetate and then derivatized with BSTFA in pyridine (4:1). With genistein as internal standard, calibration curves with good linearity ($R^2 > 0.99$) within the concentration range of 0.15–10 nM in the selective ion monitoring mode were obtained. The limits of detection and quantization were 11 and 33 pM for icaritin, and 23 and 70 pM for desmethylicaritin, respectively; inter- and intra-assay variabilities were <15%, and accuracies were between 89 and 110%. Icaritin, but not desmethylicaritin, was detected from 1 h, increasing to a peak at 8 h (1.51 ± 1.6 nM) in sera of human volunteers after ingestion of an aqueous decoction of *Epimedium*. This sensitive method can be used to quantify serum levels of icaritin and desmethylicaritin for pharmacokinetic studies.

Keywords: Icaritin; Desmethylicaritin; Prenyl-flavones; Gas chromatography–mass spectrometry (GC–MS); Selective ion monitoring (SIM) mode; Human serum

1. Introduction

Prenyl-flavones are a new class of phytoestrogens originally isolated from hop (*Humulus lupulus* L.) extracts and beer [1]. They are potent stimulators of the estrogen receptor [2] and can affect the activities of a number of key regulatory proteins including tyrosine phosphatase 1B [3], nitric oxide synthase through I-kappa-B-alpha degradation [4], phospholipase C gamma 1 [5], MRP-1 [6] and PDR5p multidrug transporter [7]. Subtle modifications of the prenyl moiety of the flavone can lead to impressive changes in biological activity including distinct changes in agonist and antagonist activity profiles for estrogen receptors [8], increased affinity for biological membranes and to an improved interaction with proteins [9].

Prenyl-flavones are major constituents of the traditional Chinese medicinal herb, *Epimedium* (Berberidaceae), consisting of the dried aerial parts of *E. pubescens* Maxim. and related species [10]. Because of its potent estrogenic activity and inhibitory effects on the proliferation of breast and cancer cells [11], consumption of prenyl-flavones may be of utility for menopause, where the sudden drop in physiological estrogens results in hot flashes, atrophy of the genital tissues, and osteoporosis. There are very few therapeutic options for menopause because of side effects ascribed to current synthetic estrogenic compounds. The estrogenicity of *Epimedium* extracts was mechanistically consistent with the findings from rat models where *Epimedium* flavonoids enhance estrogen-mediated processes such as the osteogenic differentiation of rat primary bone marrow stromal cells [12] and prevention of OVX-induced osteoporosis [13].

The flavonoid glycosides, icariin, epimedin A, B, and C (Fig. 1), are major constituents of *Epimedium* and form about 10% (wt/wt) of extracts [10]. Glycosides and conjugates of flavonoids exhibit weaker biological activities than the free form [14–16]. However the flavonoids, aglycones, icaritin and desmethyicaritin (Fig. 1), occur only in trace amounts in the aerial parts of some *Epimedium* species [17,18]. Icaritin can

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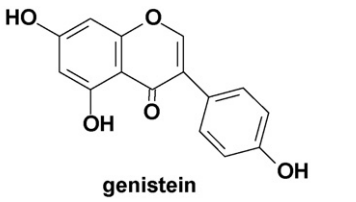
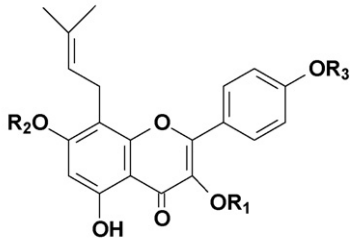


genistein



	R_1	R_2	R_3
icaritin	H	H	CH_3
desmethylicaritin	H	H	H
icariin	Rha	Glu	CH_3
epimedin A	Rha-Glu	Glu	CH_3
epimedin B	Rha-Xyl	Glu	CH_3
epimedin C	Rha-Rha	Glu	CH_3

Fig. 1. Structures of genistein, icaritin, desmethylicaritin and their glycosides from *Epimedium*; Rha: rhamnose; Glu: glucose; Xyl: xylose.

readily be formed by progressive hydrolysis of icariin, epimedin A, B, and C in the intestine [19–22] and oral cavity [23]. The 4′-methyl of icaritin can be removed by colonic bacterial flora to form desmethylicaritin [24]. Unlike its parent glycosides, icaritin and desmethylicaritin, exhibit estrogen-like effects. They up-regulate progesterone receptor mRNA expression and stimulate cell proliferation in MCF-7 cells [24–28] and acting through estrogen-dependent pathways can exert neuroprotective effects against beta amyloid-induced neurotoxicity in an Alzheimer' disease model [29]. It is plausible that effects of *Epimedium* may be mediated through icaritin and desmethylicaritin, and that appearance of the bioactive metabolites in sera may be an important index for the biological efficacy of *Epimedium* extracts, acting through the estrogen receptor or other key regulatory proteins.

To elucidate the possible pharmacological properties of *Epimedium* in humans, it is necessary to devise sensitive methods to measure icaritin and desmethylicaritin levels in serum. Capillary zone electrophoresis has been used to measure icaritin, but was not sufficiently sensitive to quantify its concentration in serum [30]. For the first time, we report a rapid and sensitive GC–MS method to quantify icaritin and desmethylicaritin simultaneously in human serum samples. Since our method requires only small quantities of sera, does not need isotopically-labeled standards, and can be performed on standard equipment; it can be routinely used for pharmacokinetic studies on *Epimedium*. The utility of this assay to detect picomolar quantities of icaritin in human volunteers after oral ingestion of a traditional decoction of *E. pubescens* was demonstrated. Such pharmacodynamic data provide the scientific foundation for large scale clinical studies on botanical extracts for menopause, such as *Epimedium*.

2. Experimental

2.1. Chemicals

Purified icaritin (purity >98%) and desmethylicaritin (purity >98%) were kind gifts from Dr. Willmar Schwabe Pharmaceuticals (Karlsruhe, Germany). Genistein (purity >99%), anhydrous acetonitrile and pyridine were purchased from Sigma (St. Louis, MO, USA). Methanol (HPLC grade), ethyl acetate (analytical grade) and acetonitrile (HPLC grade) were obtained from Merck (Darmstadt, Germany). Bis-(trimethylsilyl) trifluoroacetamide (BSTFA) was purchased from Supelco (Bellefonte, PA, USA). *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) was obtained from Pierce (Rockford, IL, USA).

Stock concentrated standard solutions in acetonitrile were wrapped in foil stored at −20°C under nitrogen gas.

2.2. Human serum samples after Epimedium ingestion

Serial serum samples were from one arm of a prospective randomized-crossover clinical study that will be described in detail elsewhere (manuscript under preparation). Briefly, healthy male volunteers, after phytoestrogen-free diets for 3 days, were administered a standardized water decoction prepared from 50 g of dried leaves of *E. pubescens*. The concentrations of major flavonoid glycoside and the two active aglycones in dried water decoction were determined using methods previously described [10]. Dried water decoction contained icariin: 2.00 mg/g; epimedin (A) 1.40 mg/g; epimedin (B) 1.57 mg/g; epimedin (C) 4.74 mg/g; icaritin: 119 μg/g; desmethylicaritin: 31 μg/g. Following *Epimedium* ingestion, blood sampling was performed at 0.5, 1, 2, 4, 6, 8, 12, 24, and 48 h time points. Sera were extracted and stored at −80 °C for analysis.

2.3. Extraction and preparation of serum samples

All serum samples were thawed at room temperature, spiked with internal standard (Genistein, 10 nM), and equilibrated at 37 °C for at least 1 h before extraction. Analytes were extracted from 0.5 mL serum aliquots by liquid–liquid partition using water-saturated ethyl acetate (4 mL × 2). The ethyl acetate partitions were dried by gently blowing with nitrogen (N_2) gas at room temperature. The residues were re-dissolved in MeOH (200 μL × 2) and transferred into 2 mL GC sample vials. After MeOH was thoroughly removed by N_2 gas, 25 μL of the mixture of BSTFA and pyridine (4:1) was added into sample vials. The derivatization reaction was performed at 60 °C for 1 h. After cooling to room temperature, reaction mixtures were subjected to GC–MS analysis.

2.4. Instrumentation

GC–MS analysis of icaritin and desmethylicaritin was performed on an Agilent 6890N GC coupled to a 5975 inert XL mass selective detector (MSD) (Agilent Technologies, Palo Alto, CA, USA). A HP-5MS capillary column (5% phenyl-

methyl-siloxane) (30 m × 0.25 mm × 0.25 μM film thickness) (J&W, Folsom, CA, USA) was used. Purified helium gas (purity 99.9999%, Soxal, Singapore) was used as the carrier gas at a constant column flow-rate of 3 mL/min. Standards and samples were injected using an Agilent 7683B series autosampler into a 900 μL splitless single-taper liner. The injection volume was 4.0 μL and the solvent delay was kept for 4 min. The pulsed splitless mode was selected for sample injection with a pulse pressure of 60 psi and the purge time delay of 2 min. The initial oven temperature was set at 150 °C, followed by a ramp up to 300 °C at 30 °C/min, and a final hold for 4 min. The front inlet and MSD transfer line temperature were kept at 280 °C and 300 °C, respectively.

The temperature of electron ionization (EI) source and MS quadrupole were kept at 230 °C and 150 °C, respectively. For qualitative analysis, the MSD was operated in full-scan mode from *m/z* 50 to 700 at 2.28 scans/s. For quantitative analysis, the MSD was operated in selected ion monitoring (SIM) mode with a dwell time of 100 ms for the selected ions. A time event for sensitivity was set at 7.65 min with an increase of 400 units on Electron Multiplier Voltage (EMV). Genistein, the internal standard (IS), icaritin and desmethylicaritin standards were used to obtain the quantitative ions for SIM mode.

2.5. Validation

2.5.1. Linearity

Control pooled male human serum (Sigma, St. Louis, MO) was analyzed in full scan and SIM mode to verify the absence of genistein, icaritin and desmethylicaritin. To generate calibration samples, increasing doses of icaritin and desmethylicaritin were added to 0.5 mL of control pooled sera to final concentrations ranging from 0.15 to 10 nM. All standards contained 10 nM of genistein. Calibration standards were tested and found to be stable at −20 °C under nitrogen gas for at least 3 months. A calibration curve with at least six concentration points was constructed every experimental day. The least regression method and the squared correlation coefficient (R^2) were used to estimate linearity.

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

The limit of detection and limit of quantification were defined as: LOD = 3.3σ/S, LOQ = 10σ/S, where σ = the standard deviation of the background response and S = the slope of the calibration curve [31]. The background response was measured by analyzing control pooled human sera and the standard deviation was calculated from 10 independent assays. The average of the slope of four independent calibration curves was used as S for LOD and LOQ calculation.

2.5.3. Accuracy and precision

Low, medium and high quality control (QC) samples, containing 0.15, 0.5 or 1 nM of icaritin and desmethylicaritin, respectively and internal standard in pooled human sera, were placed at each assay for determination of accuracy and precision [32]. The mean values and coefficient of variation (CV) were obtained from six independent assays within one experimental day (intra-day) or independent assays from five consecutive days (inter-day).

3. Results and discussion

3.1. Sample extraction

Liquid–liquid (LLE) [33–37] and solid-phase (SPE) [38–40] extractions are widely used to obtain flavonoids from biological fluids. The extraction efficiency of LLE using ethyl acetate was 80–90% higher than that using C18-Sep-Pak SPE cartridges (<70%). Ethyl acetate was chosen as the extraction solvent because of its low toxicity, good solubility for the genistein, icaritin and desmethylicaritin, and lower cost compared to SPE cartridge.

3.2. Derivatization

Trimethylsilyl (TMS) reagents have been traditionally used to derivatize flavonoids [41]. We investigated the derivatization methods with BSTFA and MSTFA, and found that the tri-TMS products of BSTFA were more stable (>48 h) than those of MSTFA. To have a complete reaction, pyridine was used as a catalyst (BSTFA/pyridine: 4/1). To optimize derivatization time, a time-course study was performed. At 30 min, intermediate TMS products (di-TMS-genistein, di-TMS-icaritin, tri-TMS-desmethylicaritin) were still observed, while at 60 min, only the complete TMS derivatives were detected (Fig. 2).

3.3. GC–MS characterization of derivatized genistein, icaritin and desmethylicaritin

Due to the unavailability of isotope-labeled icaritin, genistein (Fig. 1), a flavonoid not naturally present in *Epimedium*, was chosen as the internal standard. A total ion chromatogram in full scan mode was generated using a mixture of genistein, icaritin and desmethylicaritin calibration standards. The TMS derivatives for genistein, icaritin and desmethylicaritin were completely separated and eluted at 6.278, 7.832 and 8.022 min, respectively (Fig. 3a). From the full scan EI mass spectra, the most abundant and unique ion for each derivative (471, 569 and 627 for genistein, icaritin and desmethylicaritin, respectively) was selected for the subsequent SIM experiments (Fig. 3b–d). The exact mass for genistein, icaritin and desmethylicaritin were determined by SIM experiments (Table 1), and used for calibration curve construction and subsequent clinical sample analyses. Positive and negative chemical ionization modes were also investigated, but neither method provided improved sensitivities for icaritin and desmethylicaritin than EI (data not shown). Pooled human sera used for construction of calibration curve did not contain any detectable genistein, icaritin or desmethylicaritin.

3.4. Validation of GC–MS

Good linearity was obtained for icaritin and desmethylicaritin over the range of 0.15–10 nM in pooled human

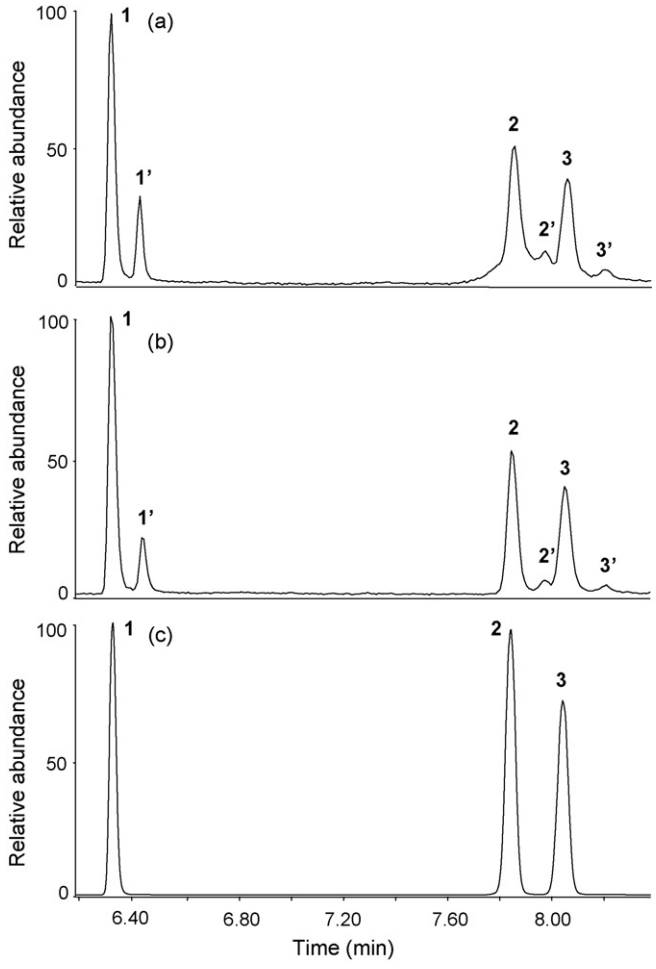


Fig. 2. Chromatograms of TMS derivatives of genistein (gen), icaritin (ict) and desmethylicaritin (dict) in full-scan mode after (a) 5 min, (b) 30 min and (c) 60 min of derivatization. (1) Tri-TMS-gen, (1′) di-TMS-gen, (2) tri-TMS-ict, (2′) di-TMS-ict, (3) tetra-TMS-dict; (3′) tri-TMS-dict.

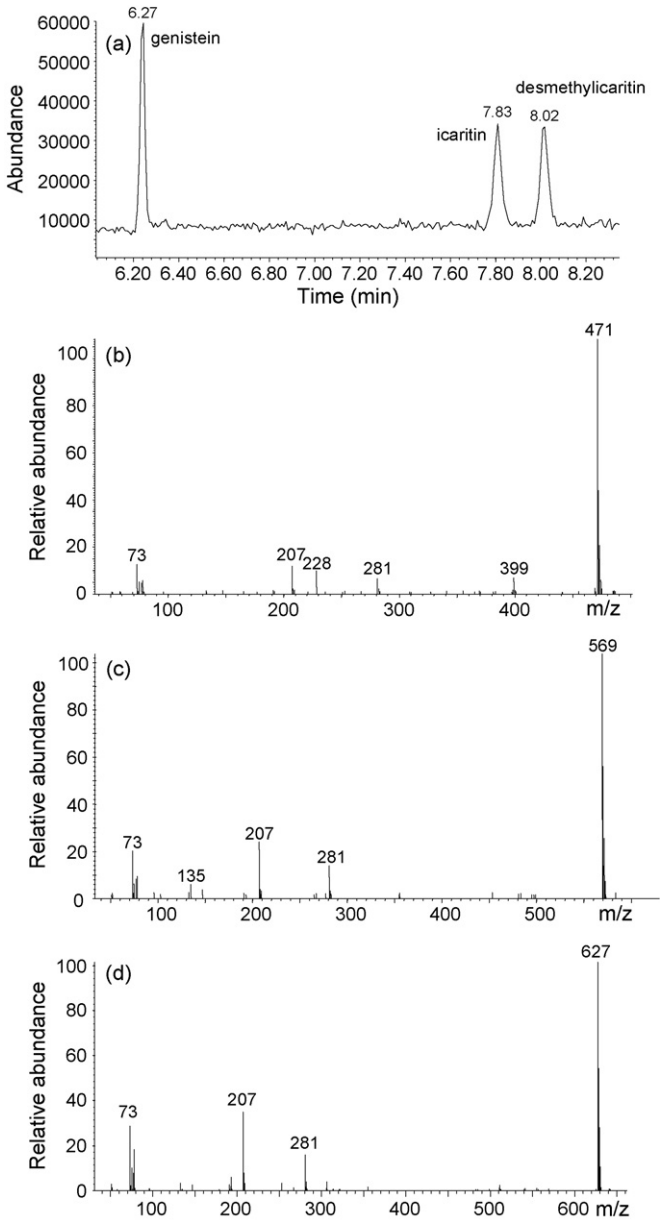


Fig. 3. Chromatograms of derivatized icaritin, desmethylicaritin and genistein standards and corresponding EI MS spectra in full-scan mode: (a) total ion chromatogram of TMS derivatives; MS spectra of (b) tri-TMS-genistein, (c) tri-TMS-icaritin and (d) tetra-TMS-desmethylicaritin.

serum, the calibration equations were $y = (2.8290 \pm 0.1505)x + (0.0158 \pm 0.0057)$ and $y = (1.9082 \pm 0.0836)x + (0.0183 \pm 0.0068)$, with R^2 of >0.994 and >0.998 for icaritin and desmethylicaritin, respectively. The limit of detection (LOD) and quantification (LOQ) for icaritin were estimated to be 11 pM and 33 pM, respectively, with $\sigma = 0.0096$ nM, and $S = 2.8290$. Corresponding values for desmethylicaritin were 23 and 70 pM, respectively, with $\sigma = 0.0134$ nM, and $S = 1.9082$. The chromatogram of blank serum and serum spiked with 50 pM of icaritin and desmethylicaritin standards, which is relatively close to the LOQs, were shown in Fig. 4, demonstrating the sensitivity of the method. Accuracy and precision were determined by analyzing QC samples [32]; intra- and inter-day precisions for determination of icaritin and desmethylicaritin in human sera were <15% and the mean values of accuracy were between 110 and 81% (Table 2).

Table 1
Selected ions (*m*/*z*) of internal standard, icaritin and desmethylicaritin in SIM mode

Compound	Class	MW	MW, tri-TMS product	SIM ions (RA, %)	Retention time (min)
Genistein	Internal standard	270	486	471.2 (100)	6.28
Icaritin	Target	368	584	569.3 (100)	7.83
Desmethylicaritin	Target	354	642	627.4 (100)	8.02

MW: molecular weight; RA: relative abundance.

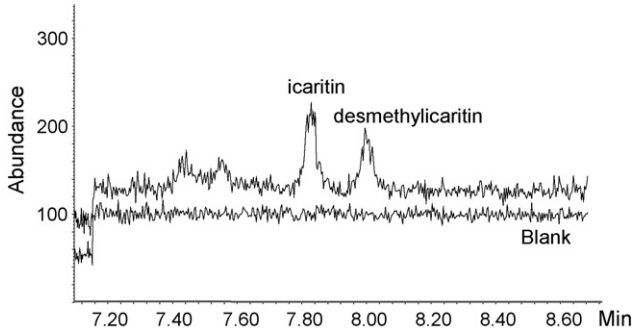


Fig. 4. Representative selective ion monitoring (SIM) chromatograms of blank serum and serum spiked with 50 pM of icaritin (*m/z*: 569.3) and desmethylicaritin (*m/z*: 627.4).

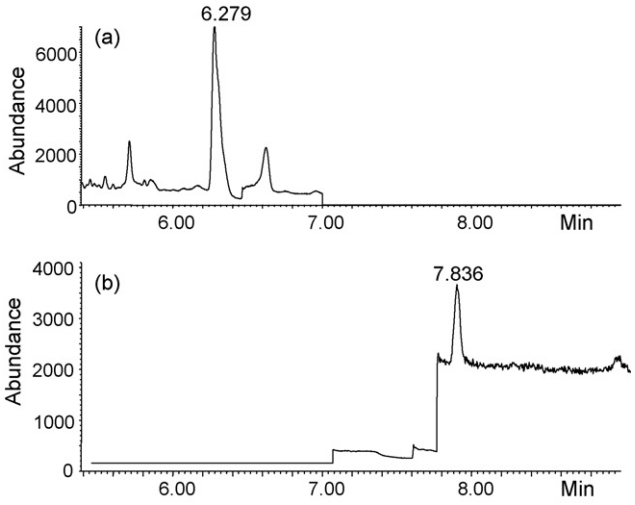


Fig. 5. Typical chromatograms of human serum sample using proposed GC–MS method: (a) SIM chromatogram at *m/z* 471.2 for genistein; (b) SIM chromatogram at *m/z* 569.3 and 627.4 for icaritin and desmethyicaritin, respectively. Note presence of a peak at 7.83 min, but not at 8.02 min, indicating the presence of icaritin and the absence of desmethylicaritin.

3.5. Measurement of icaritin and desmethylicaritin in clinical trial samples

Human sera, obtained from volunteers ($n = 7$) over a 48 h period following ingestion of a traditional *Epimedium* decoction, were analyzed for icaritin and desmethylicaritin content. Each subject consumed 500 mL of decoction containing icariin: 12.6 mg, epimedin (A) 8.86 mg, epimedin (B) 9.9 mg, epimedin (C) 30.0 mg, icaritin: 0.75 mg, and desmethylicaritin: 0.19 mg. Fig. 5 shows a typical GC–EI-MS chromatogram of a human serum sample in SIM mode. Icaritin and desmethylicaritin, but not their glycosides or conjugates, were measured since only the aglycone exerts significant estrogenic activities [24,26]. In SIM mode, icaritin was undetectable at 30 min and was first detected at 1 h (mean value: 0.24 ± 0.21 nM, $n = 7$). This suggests that very little of the aglycone was directly absorbed since directly absorbed flavonoids, such as genistein or diosmin, exhibit high concentrations in blood within 1 h [42] This was also consistent with the low levels of the aglycone in *Epimedium* decoction (119.9 μg/g). Rather icaritin levels were observed to rise rapidly after 4 h, reaching a peak at 8 h (1.51 ± 1.6 nM) and returning

Table 2
Intra- and inter-day accuracy and precision for quantification of icaritin and desmethylicaritin at different concentrations

Spiked conc. (nM)	Accuracy (%)		CV (%)		*n*
	Icaritin	Desmethylicaritin	Icaritin	Desmethylicaritin	
Intra-day					
1	89.95	98.44	9.71	3.95	6
0.5	91.47	97.95	3.36	4.50	6
0.15	92.94	89.55	12.42	9.91	6
Inter-day					
1	102.66	102.00	5.73	7.19	5
0.5	106.64	110.08	13.11	14.06	5
0.15	89.00	81.67	11.01	10.79	5

n: Number of independent assays.

Table 3
Icaritin concentrations in human serum samples

Time point (h)	A	B	C	D	E	F	G	Mean ± SD (nM)
0.5	<0.01	<0.01	<0.01	<0.01	0.09	<0.01	<0.01	<0.01
1	0.39	0.61	<0.03	0.19	0.22	<0.01	<0.03	0.24 ± 0.21
2	0.73	0.40	0.12	0.34	0.10	0.26	0.33	0.33 ± 0.21
4	0.50	0.77	0.26	0.66	0.35	0.58	0.29	0.49 ± 0.19
6	1.17	0.88	0.78	1.07	3.22	1.31	0.34	1.25 ± 0.92
8	1.04	1.09	0.65	1.18	5.11	0.89	0.61	1.51 ± 1.60
12	0.97	0.66	0.71	0.57	2.45	0.49	0.31	0.88 ± 0.72
24	0.29	0.40	0.34	0.43	0.21	0.47	0.34	0.35 ± 0.19
48	0.17	0.12	0.15	0.07	0.41	0.53	0.29	0.25 ± 0.17

A–G: human subject code, SD: standard deviation.

to almost baseline (0.25 ± 0.17 nM) after 48 h (Table 3). Our hypothesis for the late appearance of icaritin in sera of human volunteers is that metabolism of icariin to icaritin occurs mainly in the intestine. This view is supported by animal data showing that icariin is stable in gastric juice, and that hydrolysis of icariin to icaritin occurs in the intestine [21,22]. The aglycone is formed through successive hydrolysis of the di-glycoside icariin and tri-glycosides (epimedin A, B, and C) by intestinal enzymes. Significant differences in peak concentrations between subjects were observed ranging from 5.11 nM in subject E to 0.61 nM in subject G (Table 3). These may reflect differences in enzymatic activity contributed by inter-individual variability in bacteria and enzyme activities [23]. Desmethylicaritin was not detected in human serum samples. One contributory reason may be because the amount of desmethyicaritin (0.19 mg) in the water decoction was only a quarter that of icaritin (0.75 mg). This is the first measurement of icaritin in human serum following ingestion of an *Epimedium* decoction.

4. Conclusion

A method with GC–EI-MS in SIM mode has been shown, for the first time, to be sensitive for quantification of icaritin and desmethylicaritin to low picomolar concentrations in human sera. The successful detection of icaritin at different time points after ingestion of *Epimedium* extracts indicates that this method can be used for pharmacokinetic studies and clinical evaluation of *Epimedium* preparations. Our data provide the first evidence that the *Epimedium* glycosides are pro-drugs and can be metabolized and absorbed as icaritin after oral administration.

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