Trace analysis of icariin in human serum with dansyl chloride derivatization after oral administration of *Epimedium* decoction by liquid chromatography tandem mass spectrometry

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Abstract

Epimedium herbs are a type of complex traditional Chinese medicine (TCM) with high estrogenic bioactivity. The *Epimedium* herbal decoction mixture contains many compounds including icariin that can exert potent effects on numerous physiological processes related to human health. An ultrasensitive liquid chromatography tandem mass spectrometric (LC–MS/MS) method has been developed to determine trace levels of icariin in human serum with dansyl chloride derivatization after oral administration of the *Epimedium* herbal decoctions. The dansyl-icariin showed an intense protonated molecular ion at m/z 910. The collision-induced dissociation of this ion formed a distinctive product at m/z 764, corresponding to a characteristic removal of a rhamnose sugar moiety of icariin. The selected reaction monitoring, based on the m/z 910 → 764 transition, was highly specific and ultrasenstive for icariin in human serum samples. The lower limit of quantitation was 10 pg/mL icariin spiked into blank serum. The ranges of coefficients of variation for interday assays and intraday assays were 0–15.0% and 1.1–17.5%, respectively, for a wide linear range from 10 pg/mL to 4 ng/mL. This method was successfully applied to measure trace levels of icariin in a human serum after oral administration of *Epimedium* decoction within 48 h for the first time.

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Keywords: Trace analysis; Icariin; Liquid chromatography; Tandem mass spectrometry; *Epimedium* decoction; Human clinical trial

1. Introduction

The traditional Chinese medicinal herb, *Epimedium* L. (*Berberidaceae*), is a popular botanical supplement used to improve menopausal symptoms, bone health and erectile function, amongst other indications [1,2]. The key bioactive compound in *Epimedium* is icariin, a prenylated-flavonoid glycoside (Fig. 1). Icariin exerts potent effects on numerous physiological processes including skeletal health, central nervous systems and erectile functions. In terms of bone health,

icariin has estrogenic effects [3], enhances the osteogenic differentiation of rat primary bone marrow stromal cells [4] and stimulates proliferation and differentiation of human osteoblasts [5]. Flavonoids derived from *Epimedium* can prevent osteoporosis in ovariectomized rats [6] and can prevent bone loss in postmenopausal women in a randomized, double blind placebo-controlled trial [7]. In the central nervous system, icariin exerts neuroprotective effects against *beta*-amyloid-induced neurotoxicity in primary cultured rat neuronal cells [8], affects neurite outgrowth activity [9], has antidepressant-like effects, and can affect hypothalamic–pituitary–adrenal axis action and cytokine levels in stressed Sprague–Dawley rats [10]. Icariin affects penile erectile function through its phosphodiesterase-5 activity in cavernous smooth muscle cells. These properties coupled with its reported cytotoxic and anti-proliferative effects on human prostate carcinoma [11], hepatoma, leukaemia cells [12] and

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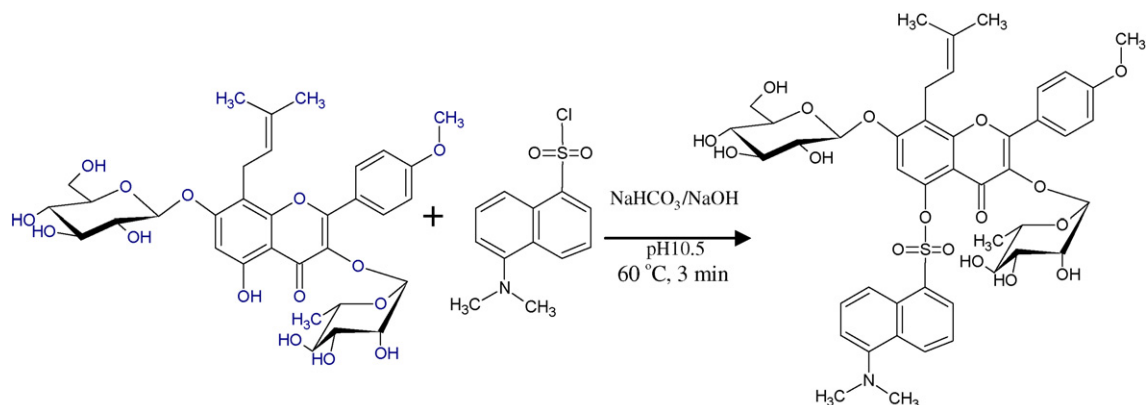*E-mail address:* obggy@nus.edu.sg (Y. Gong).

Fig. 1. Scheme of the chemical reaction of dansyl derivatization of icariin.

breast cancer cells [2], make icariin a compound of considerable pharmacological interest.

Icariin and its related glycosides epimedin A, B, and C are major constituents of *Epimedium*, forming about 10% (w/w) of its extracts [13]. The biotransformation of epimedin A, B and C to icariin is possible via enzymatic or metabolic removal of glucose from the rhamnose-glucose moiety. Thus, the sensitive measurement of icariin levels in serum is of critical importance to evaluate its pharmacological properties. The determination of the icariin is commonly performed by using thin-layer chromatography [14], high-performance liquid chromatography (HPLC) [15], capillary electrophoresis [16,17], and recently LC–MS/MS [18]. However these reported methods are not sensitive enough for the measurement of trace levels of the icariin expected in serum following administration of *Epimedium* water decoction. Thus, an ultrasensitive method to measure trace levels of icariin in complex matrices like sera is needed.

To enhance electrospray ionization efficiency and improve detection sensitivity in LC–MS/MS analysis, a robust method of derivatization of ethinylestradiol [19] in monkey plasma and estradiol [20] in human plasma with dansyl chloride has been reported. Ultrasensitive measurements of ethinylestradiol and estradiol were easily achieved by monitoring transition of m/z $530 \rightarrow m/z$ 171 for dansyl-ethinylestradiol and m/z $506 \rightarrow m/z$ 171 for dansyl-estradiol, respectively. In this paper, by modifying the reported LC–MS/MS method for analysis of estradiol in human plasma [20], we report the first ultrasensitive LC–MS/MS method to quantify trace levels of icariin in the human serum after oral administration of *Epimedium* water decoction via dansyl chloride derivatization and selected reaction monitoring of m/z $910 \rightarrow m/z$ 764. The dansyl derivatization of icariin introduced a basic secondary nitrogen into the molecule that was readily ionized in an acidic LC mobile phase. This greatly enhanced the ionization efficiency under positive turbo ion spray ionization. This ultrasensitive LC–MS/MS method was successfully applied to determine icariin at pg/mL levels in a human clinical trial wherein the subject was administered an *Epimedium* water decoction. This ultrasensitive method is generally applicable for trace analysis of phenolic compounds with sugar chains that are very common in natural products.

2. Experimental

2.1. Instrumentation

HPLC was carried out on an Agilent Technologies (Waldbronn, Germany) Model 1100 liquid chromatograph system with a binary pump. Chromatographic data were recorded and processed by using the Analyst software Version 1.4.1 (ABI-Sciex, Foster City, CA). A Phenomenex (Torrance, CA) Synergi 4 μm Max-RP 80A (150 mm × 2 mm, 4 μm particle size) column was used for separation. The HPLC effluent was analyzed by an API 4000 triple quadrupole mass spectrometer (ABI-Sciex, Toronto, Ontario, Canada) equipped with a turbo ion spray source operated under positive selected reaction monitoring (SRM) mode.

2.2. Chemicals

Icariin was purchased from 3B Medical System, Inc. (Libertyville, IL). Formic acid and HPLC-grade solvents acetonitrile, methanol and acetone were purchased from Merck (Darmstadt, Germany). Deuterated-estradiol (i.e. 17β-estradiol-2,4,16,16,17-d_5) purchased from CDN isotopes (Pointe-Claire, Quebec, Canada), was used as an internal standard. Dansyl chloride (>99.0% HPLC purity), bovine serum albumin, sodium azide and commercial human serum were obtained from Sigma–Aldrich (St. Louis, MO). Ultrapure water was prepared using a Milli-Q (Millipore, Bedford, MA) water purification system. Dried leaves of *E. pubescens*, one of the five major species of *Epimedium* in current use (China State Pharmacopoeia), were obtained from Sichuan Province, China. The herb was authenticated by Dr. Guo Baolin (Institute for Medicinal Plant Development, Chinese Academy of Medical Sciences, Beijing, PR China), and compared with standard samples by genetic fluorescent amplified fragment length polymorphisms (AFLP) analysis [13]. Voucher specimens (SBG-EP02-060110) have been deposited in the Singapore Herbarium.

2.3. Administration of Epimedium herbal decoction

The *Epimedium* decoction was prepared in a traditional way [21]. Briefly, 400 g of the dried leaves of *E. pubescens*

(50 g/subject) were grounded into powder and soaked in 8 L of cold water for 30 min (herb(g):water ratio (mL) = 1:20). After soaking, the herb/water mixture was heated to boiling point and the boiling condition was maintained for 30 min. The herb residues were removed by filtration with sterile cotton gauzes and the decoction was further concentrated to 4 L and stored at 4 °C over night. Next morning, 500 mL of chilled water extract (termed *Epimedium* decoction) was administered to the subject. No additives were added to the decoction. The chemical fingerprints of the major flavonoid glycosides and minor bioactive flavone aglycones were measured as reported [13]. The water decoction was consumed by the subject in the Lilly-NUS Centre for Clinical Pharmacology Pte Ltd. (a partnership of Lilly Research Laboratories and The National University of Singapore). Blood was collected from the human subject at 0, 0.5, 1, 2, 4, 6, 8, 12, 24 and 48 h after administration. The collected serum samples were stored at −20 °C until they were tested. Before sample preparation, the frozen serum was thawed to room temperature.

2.4. Method development and sample preparation

Basing on a reported method for measurement of estrone and estradiol in human plasma with dansyl chloride derivatization [20], an ultrasenstive method for trace analysis of icariin in human serum has been developed with following modifications and improvements. The reported extraction solvent methylene chloride [20] was replaced by ethyl acetate which can provide better extraction yield for icariin and is less toxic to operator and environment. The mobile phase gradient conditions were also changed and optimized to achieve better retention for dansyl-icariin in the LC column. Time segments were applied in MS analysis of dansyl-icariin. The flow system was switched to waste for eluting salts and impurities in the first time segment to improve detection sensitivity. All the ion source and mass spectrometer parameters were changed and optimized to favor the signal of the new daughter ion of m/z 764, corresponding to a characteristic removal of a rhamnose sugar moiety of icariin. Since the deuterium-substituted icariin is not commercially available, deturium-substituted estradiol (d_5-E2) was chosen as internal standard.

The working solutions of icariin were prepared by diluting 1 mg/L stocking solutions in matrix [20] of phosphate-buffered saline containing 1 g/L bovine serum albumin (BSA) and 1 g/L sodium azide. Standards containing icariin in concentration of 0.01, 0.04, 0.1, 0.4, 1, 4 ng/mL were prepared in the same matrix. 0.5 mL of human serum samples, quality controls, and the standards were aliquoted into 13 mL PPE centrifuge tubes. To each of these tubes, 25 μL of 10 μg/L stock solution of d_5-E2 in the matrix was added as an internal standard. Then, 6 mL of ethyl acetate was added into the tube and vigorously vortexed for 2 min. The mixture was then centrifuged for 3 min at 3000 rpm. The upper organic layer was carefully removed and transferred to a clean 13 mm × 70 mm glass screw-cap tube. The solvent was evaporated under nitrogen gas in a 45 °C water bath. The dried residue was redissolved in 50 μL of sodium bicarbonate buffer (100 mmol/L, pH 10.5). Thereafter, 50 μL of 1 g/L dansyl

chloride in acetone was added in the mixture and vortexed for 2 min. The glass tube containing the mixture was then placed in an oven at 60 °C for 3 min. The reaction mixture was allowed to cool to room temperature and transferred to autosampler vial with glass insert. 5 μL of this extract mixture was then injected into the LC–MS/MS system.

2.5. High performance liquid chromatography

HPLC was carried out with the Phenomenex column with a mobile phase that consisted of two eluents, solvent A (950 mL/L acetonitrile–50 mL/L H_2O containing 1 mL/L formic acid) and solvent B (50 mL/L acetonitrile–950 mL/L H_2O containing 1 mL/L formic acid), delivered at a flow rate of 0.25 mL/min. The initial condition of 35% solvent A was slowly increased to 90% solvent A for the first 6 min. This condition was maintained for the next 4 min, before the system was returned to the initial proportion of 35% solvent A over the following 0.5 min, and maintained for the final 5.5 min. The total run time was 16 min. The retention time of dansyl-icariin and dansyl-d_5-E2 were found to be 4.7 and 12.2 min, respectively. In order to reduce the interference of salts and polar impurities and to improve detection sensitivity, three time segments were applied in the analysis. In the first time segment, from 0 to 4 min, the inlet of the MS was automatically switched to waste (bypassing the MS) of each run to elute the salts and polar impurities. In the second time segment, from 4 to 8 min, the inlet was switched to the MS and the dansyl-icariin was eluted and detected. In the third time segment, from 8 to 16 min, the inlet was switched to the MS and the dansyl-d_5-E2 was eluted and detected. Thereafter, the inlet was switched to waste for the next run.

2.6. Mass spectrometry

The icariin and internal standard d_5-E2 were detected using the API 4000 triple–quadrupole MS equipped with a turbo ion spray source operated in the positive selected reaction monitoring mode. All of the source and instrument parameters were optimized by flow injection analysis of derivatization reaction mixture containing dansyl-d_5-E2 or dansyl-icariin. Both Q1 and Q3 were operated under unit mass resolution. Nitrogen was used as the curtain (setting 10), drying gas 1 (setting 50), drying gas 2 (setting 50), and collision gas (setting medium), which was delivered from nitrogen Dewar with the gas regulator maintained at 552 kPa. The electrospray voltage was set to 5000 V for dansyl-icariin in the first and second time segments and 5500 V for dansyl-d_5-E2 in the third time segment. The turbo ion spray interface was maintained at 350 °C for dansyl-icariin in the first and second time segments and 400 °C for dansyl-d_5-E2 in the third time segment. The specific SRM transitions for dansyl-icariin and dansyl-d_5-E2 (internal standard) were monitored (200 ms dwell time/transition) at $m/z\ 910 \rightarrow 764$ and $m/z\ 511 \rightarrow 171$, respectively. The declustering potential, collision energy, and collision exit potentials were optimum at 80, 25, 5 for dansyl-icariin and 50, 50, 10 for dansyl-d_5-E2 respectively. The icariin concentration was quantified using a six-point calibration curve of peak

area ratio for icariin to internal standard d_5-E2 against the concentration.

2.7. Method validation

Triplicate sets of calibration standard curves were obtained to determine the linearity of the assay. Quality control (QC) samples were prepared in a charcoal-treated commercial human male serum and were analyzed for intraday and interday precision and recovery. The LOD, based on a signal noise ratio of 5, was determined using the charcoal-treated blank commercial male serum samples spiked with icariin. For interday precision and accuracy, 3 sets of 6 standard solutions of icariin having concentrations of 0.01, 0.04, 0.1, 0.4, 1, and 4 ng/mL were prepared in the BSA matrix and were determined on each of 3 consecutive days. For intraday precision and accuracy, 5 sets of 6 standard solutions of icariin at concentrations of 0.01, 0.04, 0.1, 0.4, 1, and 4 ng/mL were determined within the same day. To determine the interday precision and recovery of QC samples, 3 sets of 3 QC solutions of icariin having concentrations of 0.4, 2 and 4 ng/mL were prepared in the commercial human serum and determined on each of 3 consecutive days. To determine the intraday precision and recovery of QC samples, 5 sets of 3 QC solutions of icariin having concentrations of 0.4, 2 and 4 ng/mL were prepared in a commercial human serum and analyzed on the same day.

3. Results and discussion

3.1. Development of the ultrasensitive method to measure trace levels of icariin

The major bioactive compound of *Epimedium* extract, icariin, is a weak acidic phenolic molecule (Fig. 1). This property suggests that chromatographic separation of icariin would be enhanced by using acidic additives in the mobile phase. However this could decrease the ionization efficiency under negative electrospray ionization. One solution is to separate in acidic mobile phase conditions, but detect the compound in basic con-

ditions in the MS via online postcolumn pH adjustment by using a T-joint. However this method would reduce separation efficiency. We found that the dansyl derivatization of icariin (Fig. 1) introduced a basic secondary nitrogen into the molecule via reaction of dansyl chloride with the phenolic hydroxyl group of icariin. This greatly enhanced the separation and the ionization efficiency under the positive turbo ion spray ionization. The dansyl-icariin showed an intense protonated molecular ion at *m/z* 910. The collision-induced dissociation of this ion formed a distinctive product at *m/z* 764, corresponding to a characteristic removal of a rhamnose sugar moiety of icariin (Fig. 2). The selected reaction monitoring, based on the *m/z* 910 → 764 transition, was highly specific for icariin in human serum samples. No background signal was observed from blank pooled commercial human serum and the serum samples collected before administration of *Epimedium* decoction in our human trial study.

Estrogens, including estradiol (E2), are naturally present in human sera. To avoid confusion with endogenous estradiol, synthetic d_5-E2 was used as the internal standard. The MS/MS fragmentation of dansyl-icariin was different from that reported for dansyl-d_5-E2 [20]. Dansylated d_5-E2 showed an intense protonated molecular ion [M+H]$^+$ at *m/z* 511 and a strong product ion at *m/z* 171, corresponding to the protonated 5-(dimethylamino)-napthalene moiety due to the cleavage of the C–S bond of the derivatized estradiol dansyl-d_5-E2 [20]. However, the MS/MS spectrum of dansyl-icariin showed the highest peak at *m/z* 764 and a very weak peak at 171 (at noise level). Therefore, we elected to measure icariin content by monitoring specific SRM transitions for dansyl-icariin and dansyl-d_5-E2 at *m/z* 910 → 764 and *m/z* 511 → 171, respectively. Icariin concentrations were quantified using a six-point calibration curve of the peak area ratio of icariin to internal standard, against concentration. A typical mass chromatogram of determination of dansyl-icariin and dansyl-d_5-E2 is shown in Fig. 3.

3.2. Validation of the ultrasensitive LC–MS/MS method

Using a signal/noise ratio of 5, the LOD for icariin was determined as 2 pg/mL. The lower limit of quantization was
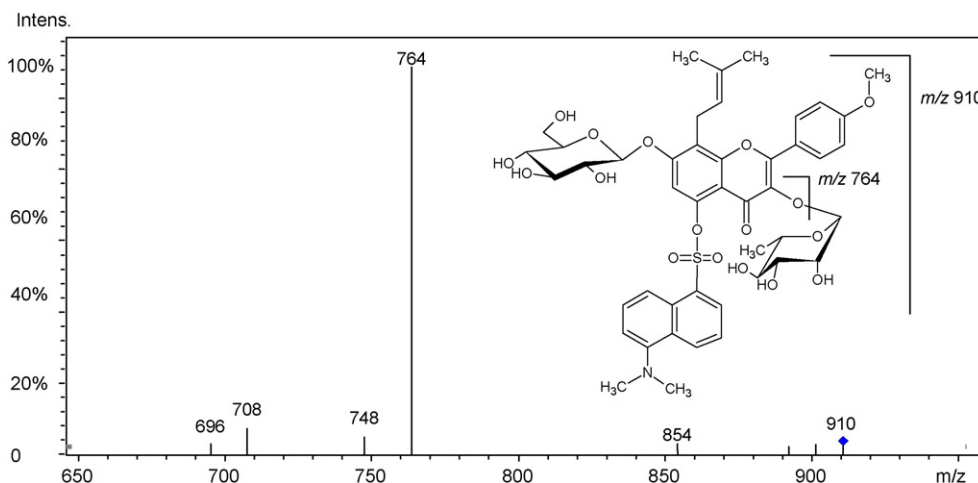

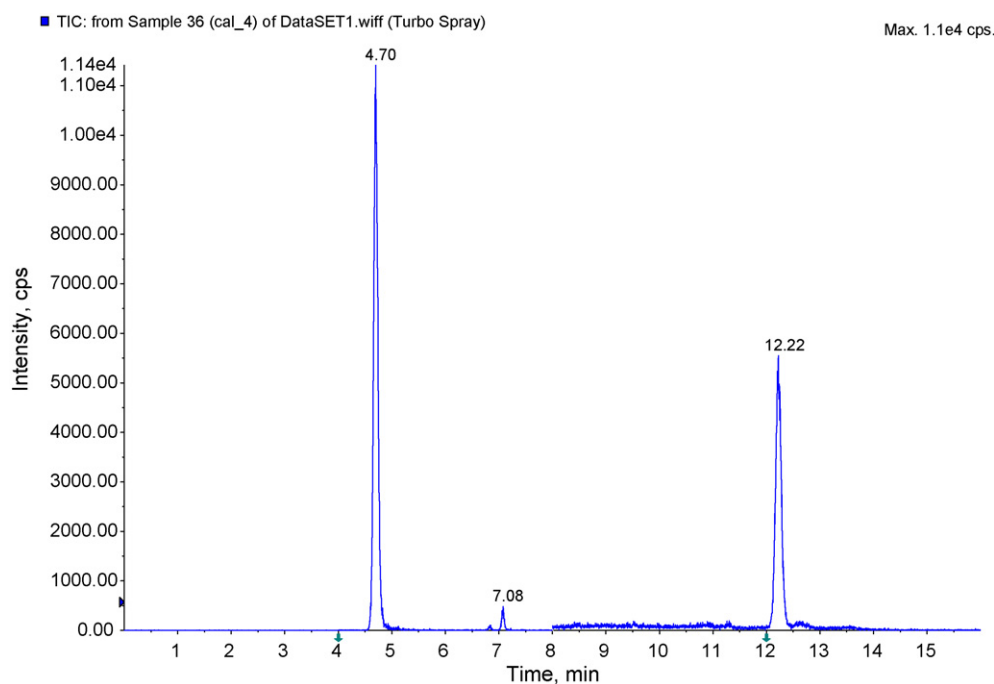Fig. 2. MS/MS fragmentation of dansyl-icariin.

Fig. 3. A typical mass chromatogram of determination of dansyl-icariin and dansyl-d_5-E2. The concentration of icariin in the standard solution is 4 ng/mL. The mass chromatograms for both serum sample and standard solution are similar. The first peak at retention time of 4.7 min is dansyl-icariin. The second peak at retention time of 12.2 min is dansyl-d_5-E2.

Table 1
Summary of the calibration curve validation for interday analysis

Nominal concentration (ng/mL)[a]	Interday estimate concentration[b]			Interday validation summary			
	Day 1	Day 2	Day 3	Mean	S.D.	Mean %CV	Mean %accuracy
0.01	0.0107	0.0086	0.0104	0.0099	0.0011	11.4	98.9
0.04	0.0390	0.0420	0.0473	0.0428	0.0042	9.8	106.9
0.2	0.2008	0.2411	0.2007	0.2142	0.0233	10.9	107.1
0.4	0.4000	0.3999	0.4002	0.4000	0.0002	0.0	100.0
1	0.8300	1.0634	0.8708	0.9214	0.1247	13.5	92.1
4	3.9461	4.2898	5.2466	4.4942	0.6739	15.0	112.4

[a] Three sets of triplicate samples were determined to estimate interday precision and accuracy and linearity of calibration curves.
[b] Values are the mean of the three tests. The correlation coefficient for day 1, 2 and 3 of the validation for each triplicate curve was 0.9992, 0.9998 and 0.9999, respectively.

10 pg/mL for icariins spiked into the commercial human serum samples. This LC–MS/MS method exhibited a good linear range from 10 pg/mL to 4 ng/mL. A typical equation of the calibration curve was $Y = 0.2325X + 0.0016$ ($R^2 = 0.9999$). Interday and intraday precision (%CV) of standard solutions ranged from 0 to 17.5% (shown in Tables 1 and 2). Interday and intraday pre-

cision (%CV) of QC samples prepared in commercial human serum ranged from 0 to 6.3% (shown in Tables 3 and 4). Interday and intraday recoveries for QC samples were 98.2–111.2%. In terms of accuracy and precision, all validation values fell within ±20% for low concentration and ±15% for mid to high concentration. These results indicated that extraction, derivati-

Table 2
Summary of the calibration curve validation for intraday analysis

Nominal concentration (ng/mL)	Intraday estimate concentration					Intraday validation summary			
	1st run	2nd run	3rd run	4th run	5th run	Mean	S.D.	%CV	%Accuracy
0.01	0.0120	0.0118	0.0100	0.0078	0.0092	0.0102	0.0018	17.5	101.6
0.04	0.0382	0.0377	0.0410	0.0418	0.0418	0.0401	0.0020	5.0	100.3
0.2	0.1961	0.2125	0.2284	0.2184	0.1998	0.2110	0.0133	6.3	105.5
0.4	0.3916	0.4087	0.3843	0.4158	0.3999	0.4001	0.0127	3.2	100.0
1	1.1923	1.2736	0.9696	0.9481	1.1074	1.0982	0.1403	12.8	109.8
4	4.7676	4.6987	3.8592	3.7708	4.3211	4.2835	0.4613	10.8	107.1

Table 3
Summary of the quality control validation for interday analysis

Nominal concentration (ng/mL)[a]	Interday estimate concentration[b]			Interday validation summary			
	Day 1	Day 2	Day 3	Mean	S.D.	Mean %CV	Mean %recovery
0.4	0.3999	0.4000	0.4000	0.4000	0.0001	0.0	100.0
2	1.9939	1.9761	1.9218	1.9639	0.0376	1.9	98.2
4	4.4332	4.1490	4.7676	4.4499	0.3096	0.1	111.2

[a] Three sets of triplicate of charcoal-treated commercial male serum samples were spiked with 0.4, 2, and 4 ng/mL icariin on different days and analyzed to estimate the interday precision and recovery of the quality control samples as described in Section 2.
[b] Values are the mean of the three tests.

Table 4
Summary of the quality control validation for intraday analysis

Nominal concentration (ng/mL)[a]	Intraday estimate concentration					Intraday validation summary			
	1st run	2nd run	3rd run	4th run	5th run	Mean	S.D.	%CV	%Recovery
0.4	0.4789	0.3599	0.3708	0.4032	0.3546	0.3935	0.0247	6.3	98.4
2	1.9517	1.9939	2.0046	2.0028	1.9963	1.9899	0.0218	1.1	99.5
4	4.4728	4.0856	4.2454	4.0939	4.0939	4.1983	0.0875	2.1	105.0

[a] Five sets of triplicate of charcoal-treated commercial human male serum samples were spiked with 0.4, 2, and 4 ng/mL icariin on the same day and analyzed to estimate the intraday precision and recovery of the quality control samples as described in Section 2.

zation and quantification were reproducible for the measurement of icariin in human serum. No significant differences in precision and accuracy were found for samples prepared in the matrix and commercial human serum (data not shown).

3.3. Application to measure trace levels of icariin in human serum samples after oral administration of the Epimedium decoction

The validated LC–MS/MS method was applied to determine icariin levels in serum obtained from a human volunteer administered a standardized *Epimedium* water decoction. This decoction contained 12.6 mg of icariin and 61.0 mg of total flavonoids including epimedin A, B, C and icariin. Blood was collected from the human subject at 0, 0.5, 1, 2, 4, 6, 8, 12, 24 and 48 h after administration. Clear peaks of icariin can be detected in
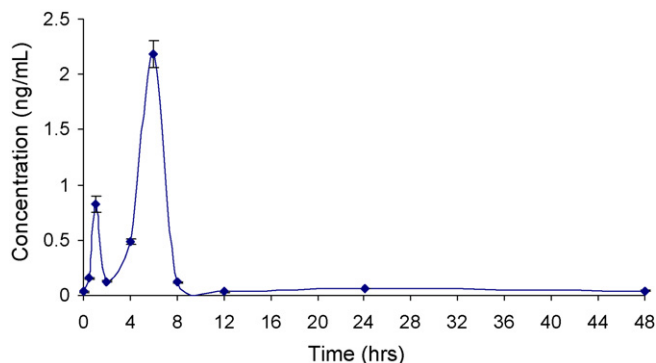

Fig. 4. The concentration profiling of the icariin in human male serum after oral administration of *Epimedium* decoction within 48 h. The oral dosage of the icariin equivalence was 1.8×10^5 ng/kg for the human subject. The serum samples were analyzed as described in Section 2. Every serum sample for each time point was analyzed three times by LC–MS/MS. The error bars are standard deviation of these three measurements.

the serum samples (Fig. 4), indicating the feasibility of our analytical method for detection of this compound in actual patient samples. Studies need to be performed on a larger number of subjects to determine the actual bioavailability and pharmacokinetics of icariin. It is clearly shown in Fig. 4 that the LC–MS/MS method was sensitive and robust to measure the trace level of icariin in human serum samples after oral administration of the herbal decoctions. This technique may be useful for the determination of trace levels of icariin in complex samples with limited volume in animal studies and human clinical trials. Our data indicate that dansyl derivatization may be applicable to glycosides and glycosylated natural products with phenolic rings such as ginsengnosides, genistin and diadzin, because the lability of their sugar moieties would give rise to a strong daughter ion.

4. Conclusions

For the first time, an ultrasensitive LC–MS/MS method has been developed to determine trace levels of icariin in a human serum after oral administration of *Epimedium* decoction within 48 h. Dansyl derivatization of icariin greatly enhanced the separation and ionization efficiency for positive electrospray ionization in LC–MS/MS analysis. This method is useful for determination of trace levels of icariin in complex samples with limited volume in animal study and human clinical trials. This ultrasensitive LC–MS/MS method is generally applicable for phenolic compounds with sugar chains that are very common in natural products.

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