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Simultaneous determination of icariin, icariside II and osthole in rat plasma after oral administration of the extract of *Gushudan* (a Chinese compound formulation) by LC–MS/MS

Man Liu, Huiping Liu, Xiumei Lu, Chao Li, Zhili Xiong, Famei Li *

Department of Analytical Chemistry, Shenyang Pharmaceutical University, Shenyang 110016, PR China

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Abstract

A sensitive, specific and accurate LC–MS/MS method was developed for simultaneous determination of icariin, icariside II and osthole in rat plasma. With carbamazepine as the internal standard, plasma samples were prepared by protein precipitation with acetonitrile. Analysis was carried out on an ACQUITY UPLC^{TM} BEH C_{18} column with a linear gradient and 0.1% aqueous acetic acid and acetonitrile were used as mobile phase. Detection was performed by means of electrospray ionization mass spectrometry in positive ion mode with multiple reaction monitoring. Linear calibration curves of icariin, icariside II and osthole were obtained over the concentration ranges of 2.00–200, 2.00–200 and 2.00–500 ng/ml, respectively. The intra- and inter-day precisions were within 8.0% and 14%, and the accuracy was from −6.0% to 9.0%. The method was successfully applied to pharmacokinetic studies of icariin, icariside II and osthole in rats after oral administration of *Gushudan* extract.
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Keywords: Icariin; Icariside II; Osthole; *Gushudan*; LC–MS/MS; Pharmacokinetics

1. Introduction

Traditional Chinese medicine (TCM) has been used in clinical practice for several thousand years and the healing benefits of more than 7000 kinds of herbs have been documented. TCM is used mostly in combination in China and has played an indispensable role in the prevention and treatment of diseases, especially the complicated and chronic ones. Pharmacokinetic study on active constituents in herbal preparations is a good way for us to explain and predict a variety of events related to the efficacy and toxicity of TCM.

Gushudan is a Chinese composite formulation based on the theory of TCM and clinical practice and formed according to "the basic concept of establishing platforms for operational techniques in systems of elaborately selecting small formulations of traditional Chinese medicine" [1]. It is a kidney-tonifying and bone-strengthening formulation used for the treatment of osteoporosis. The study on ovariectimized rats treated with *Gushudan* formulation showed that it inhibited high bone turnover and stimulated bone formation effectively. And it exhibited an activity of promoting the proliferation of osteoblast cells *in vitro* [2]. The formulation is composed of Herba Epimedii, Fructus Cnidii, Rhizoma Drynariae and Radix Salviae Miltiorrhizae. Icariin is known as an indicative constituent of the *Epimedium* genus. It has been reported that icariin promotes the proliferation [3,4] and stimulates the expression of OPN mRNA and type I collagen of rat osteoblasts [5]. It also has effects of accelerating the osteogenic differentiation of rat bone marrow stromal cells [6] and inhibiting the proliferation of preosteoclast [7]. Icariside II is a metabolite of icariin [8–10]. It also has proliferative activity on osteoblasts [4] and inhibiting effect on the proliferation of preosteoclast [7]. Osthole is one of the active compounds in Fructus Cnidii. Pharmacological studies showed that osthole prevented osteoporosis in ovariectomized rats [11]. It has effects of stimulating the OPG mRNA expression of osteoblasts, decreasing RANKL mRNA level [12] and increasing osteoblast proliferation, ALP activity and collagen synthesis [13].

A high-performance liquid chromatography (HPLC) [14] and a capillary zone electrophoresis (CZE) method [9] have been developed to determine icariin in biological samples. Phar-

* Corresponding author. Tel.: +86 24 2398 6289; fax: +86 24 2398 6289.
E-mail address: fameili@163.com (F. Li).

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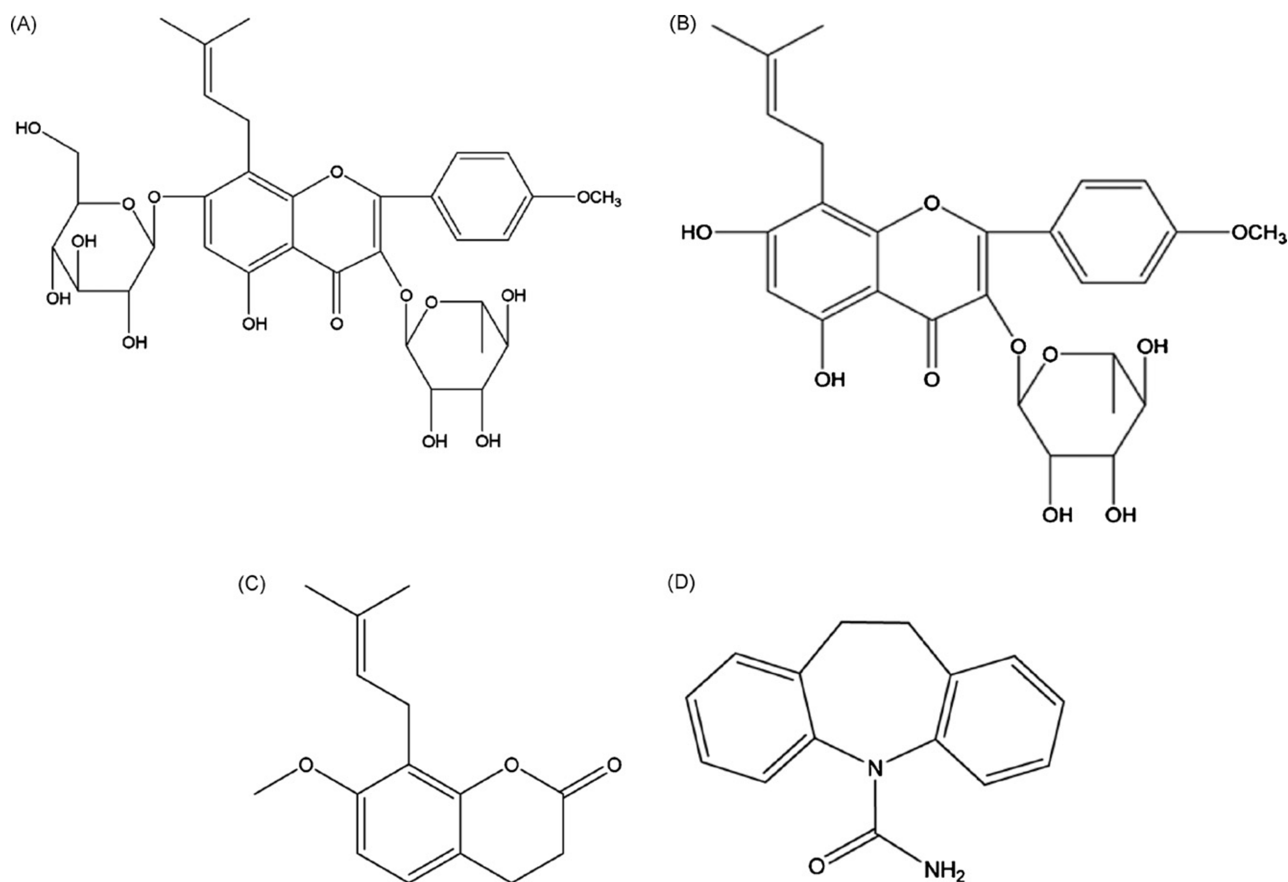

Fig. 1. Chemical structures of icariin (A), icariside II (B), osthole (C) and carbamazepine (IS) (D).

macokinetic study of osthole has been investigated by HPLC with UV detection [15,16] and β-CD inclusion fluorescence [17]. To our knowledge, there is no report on simultaneous determination of icariin, icariside II and osthole in rat plasma using LC–MS/MS and the pharmacokinetic study of icariside II.

In the present study we developed and validated a sensitive, specific and accurate method for the simultaneous determination of icariin, icariside II and osthole in rat plasma. The method was successfully applied to evaluate the pharmacokinetics of icariin, icariside II and osthole after oral administration of *Gushudan* extract to rats.

2. Experimental

2.1. Materials and reagents

Herba Epimedii was purchased from Baoji (Shannxi, China). Rhizoma Drynariae was purchased from Anhui (China). Fructus Cnidii and Radix Salviae Miltiorrhizae were purchased from Shenyang (Liaoning, China). The raw materials were identified by Professor Qishi Sun (School of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University, China) as the herb of *Epimedium brevicornum* Maxim., the fruits of *Cnidium monnieri* (L.) Cuss., the Rhizoma of *Drynaria fortunei* (Kunze) J. Sm. and the Radix of *Salvia miltiorrhiza* Bge. Icariin, osthole and carbamazepine (internal standard) were supplied by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Icariside II was isolated and purified in our laboratory. The purity was 99% determined by HPLC. Their structures are given in Fig. 1. Acetonitrile (HPLC grade) used for protein precipitation was purchased from Yuwang (Chemical Reagent Plant, Shandong, China). HPLC-grade acetonitrile and glacic acetic acid used for LC–MS/MS were purchased from Tedia (USA) and Dima (USA), respectively. Water was purified by redistillation and passed through a 0.22 μm membrane filter before use. Healthy Wistar rats were obtained from the Experimental Animals Center of Shenyang Pharmaceutical University (Shenyang, China).

2.2. Instrumentation and operating conditions

2.2.1. Chromatographic conditions

The chromatography was performed on an ACQUITY UPLC^{TM} system (Waters Corp., Milford, MA, USA) with cooling autosampler and column oven. An ACQUITY UPLC^{TM} BEH C_{18} column (50 mm × 2.1 mm, 1.7 μm; Waters Corp., Milford, MA, USA) was employed. The column temperature was maintained at 40 °C and a gradient elution of A (0.1% aqueous acetic acid) and B (acetonitrile) was used. The linear gradient was as follows: 25–30% B over 0–4 min, 30–90% B over

4–7 min, and then returned to 25% B at 7 min immediately. The flow rate was set at 0.25 ml/min. The autosampler was conditioned at 4 °C and the injection volume was 5 μl.

2.2.2. Mass spectrometric conditions

Triple-quadrupole tandem mass spectrometric detection was carried out on a Micromass® Quattro micro™ API mass spectrometer (Waters Corp., Milford, USA) with an electrospray ionization (ESI) interface. The ESI source was operated in positive ionization mode. Quantification was performed using multiple reaction monitoring (MRM) of the transitions of *m/z* 677 → 531 for icariin, *m/z* 515 → 369 for icariside II, *m/z* 245 → 189 for osthole and *m/z* 237 → 194 for the internal standard (IS), respectively, with a scan time of 0.1 s per transition. The MS parameters were as follows: capillary 3.0 kV, cone 20 kV, source temperature 120 °C and desolvation temperature 300 °C. Nitrogen was used as the desolvation and cone gas with a flow rate of 300 and 50 l/h, respectively. Argon was used as the collision gas at a pressure of approximately 0.385 Pa. The optimized collision energy chosen for the four compounds was 15 eV. All data collected in centroid mode were processed using MassLynx™ NT 4.0 software with a QuanLynx™ program (Waters Corp., Milford, MA, USA).

2.3. Preparation of standard solutions, calibration samples and quality control samples

Stock solutions of icariin, icariside II and osthole were all prepared in methanol at concentration of 500 μg/ml. Standard solutions were prepared by diluting the stock solutions mentioned above with methanol. The 500 μg/ml stock solution of IS was prepared in methanol and then diluted with methanol to prepare the working internal standard solution containing 500 ng/ml of IS. All solutions were stored at 4 °C, protected from light. Calibration samples for icariin and icariside II were prepared by spiking appropriate amount of the standard solutions in blank rat plasma to achieve the final plasma concentrations of 2.00, 5.00, 10.0, 50.0, 100 and 200 ng/ml for each and 2.00, 5.00, 10.0, 50.0, 100 and 500 ng/ml for osthole. The quality control (QC) samples were prepared in the same way as the calibration samples to make low, medium and high concentrations of 5.00, 50.0 and 160 ng/ml for icariin and icariside II and 5.00, 100 and 400 ng/mL for osthole.

2.4. Preparation of Gushudan extract

The dried powder of Herba Epimedii, Fructus Cnidii, Rhizoma Drynariae and Radix Salviae Miltiorrhizae was mixed according to the formulation and extracted with 75% ethanol under thermal reflux for 1.5 h and then filtered. The extraction was repeated twice. The extracted solutions were combined and ethanol was removed under reduced pressure. The residue was dissolved in 0.5% sodium carboxylmethyl cellulose to give an extract with a concentration of 2.5 g/ml (expressed as the weight of raw materials).

2.5. Plasma sample preparation

A 20-μl aliquot of the IS solution (carbamazepine, 500 ng/ml) and 40 μl of methanol were added to 200 μl of plasma samples. After 400 μl of acetonitrile was added the sample was vortex-mixed vigorously for 30 s and centrifuged at 14,000 × *g* for 10 min. The supernatant was separated and evaporated to dryness under a stream of N_2 gas at 50 °C. The residue was reconstituted in 100 μl of acetonitrile–water (30:70, v/v), vortex-mixed and centrifuged at 14,000 × *g* for 5 min. The supernatant was transferred into 700-μl glass vials and 5 μl was injected into the LC–MS/MS system for analysis.

2.6. Method validation

2.6.1. Selectivity

Selectivity was investigated by comparing chromatograms of blank plasma obtained from six rats with those of standard plasma sample spiked with icariin, icariside II, osthole and IS and plasma sample after oral administration of *Gushudan* extract.

The matrix effect on the ionization of analytes was evaluated by comparing peak areas of blank plasma extracts spiked with analytes at three concentration levels (5.00, 50.0 and 160 ng/ml for icariin and icariside II and 5.00, 100 and 400 ng/ml for osthole), dried and reconstituted with 100 μl of acetonitrile–water (30:70, v/v), to those of the standard solutions dried directly and reconstituted with the same solvent. The matrix effect of IS was evaluated using the same procedure.

2.6.2. Linearity of calibration curve and lower limit of quantification

Calibration curves were prepared by assaying standard plasma samples at six concentrations over the range of 2.00–200 ng/ml for icariin and icariside II and 2.00–500 ng/ml for osthole. The linearity of each calibration curve was determined by plotting the peak area ratios of analyte/IS versus plasma concentrations of icariin, icariside II and osthole. Weighted ($1/conc^2$) least-squares linear regression analysis was used to determine the slope, intercept and correlation coefficient.

The lower limit of quantification (LLOQ) is defined as the lowest concentration on the calibration curve at which an acceptable accuracy within ±20% and a precision below 20% were obtained.

2.6.3. Precision and accuracy

The precision and accuracy of the method were assessed by determination of QC samples at low, medium and high concentrations on three different validation days. To determine intra-day precision and accuracy, the assays were carried out on QC samples at different times during the same day. Inter-day precision and accuracy were determined by assaying the QC samples over three consecutive days. The concentration of each sample was determined using a calibration curve prepared on the same day. The precision was expressed as relative stan-

dard deviation (RSD%) and the accuracy as the relative error (RE%).

2.6.4. Recovery

The recoveries of icariin, icariside II and osthole were determined by comparing the peak areas obtained from blank plasma spiked with analytes before extraction to those from samples to which analytes were added after extraction. This procedure was performed at three QC levels. The recovery of IS was determined in a similar way.

2.6.5. Stability

QC samples of three concentration levels were subjected to the conditions below. Short-term stability was assessed by analyzing QC samples kept at room temperature for 2 h that exceeded the routine preparation time of samples. Long-term stability was determined by assaying QC samples after storage at −20 °C for 10 days. Freeze–thaw stability was investigated after three freeze (−20 °C)–thaw (room temperature) cycles. Post-preparation stability was assessed by analyzing the extracted QC samples kept in the autosampler at 4 °C for 8 h. The number of replicates employed for each determination was three.

2.7. Application of the analytical method to a pharmacokinetic study of icariin, icariside II and osthole in rats

Twelve Wistar rats (six males, six females, body weight 250–300 g) were randomly divided into two groups with three males and three females in each and fasted for 12 h prior to administration of the drug extract. *Gushudan* extract was given orally at 27 g (expressed as the weight of raw materials approximately containing 100 mg icariin and 70 mg osthole)/kg body weight. Animals had free access to water during the experiment. Blood (0.5 ml) was collected from the suborbital vein before administration and at 0.08, 0.17, 0.25, 0.33, 0.42, 0.5, 0.67, 0.83, 1.0, 3.0, 6.0, 8.0, 9.0, 10.0, 12.0, 14.0, 16.0 and 24.0 h after dosing. Six rats (three males and three females) were used for the initial 9 time points and the other six rats (three males and three females) were used for the remaining 10 time points to make up for the blood loss. Blood samples were collected into heparinized tubes and immediately centrifuged at 1400 × *g* for 10 min. The plasma was transferred into clean tubes and stored at −20 °C until analysis.

The elimination half-life ($t_{1/2}$) was 0.693/k_e, where k_e, the elimination rate constant, was calculated by least-square regression of the plot of logarithms of concentration against time for the last four measurable points. Maximum drug plasma concentrations (C_{max}) and time to reach the maximum concentrations (T_{max}) were taken directly from the observed data. The area under the curve ($AUC_{0-\infty}$) was calculated by the trapezoidal rule between first (0 h) and last sampling time plus C_n/k_e, where C_n is the last measurable concentration, that is $AUC_{0-t} = \sum(C_i + C_{i-1}) \times (t_i - t_{i-1})/2$, $AUC_{0-\infty} = AUC_{0-t} + C_n/k$.

3. Results and discussion

3.1. Chromatography and mass spectrometry

ESI in both negative and positive modes was attempted in the method development. Different buffers of various proportions were used in positive and negative mode turning to optimize the response signals of analytes. The tested buffers included 0.1% or 0.2% acetic acid and formic acid, 5 mM ammonium acetate. It was found that the positive mode is more sensitive than negative mode under all conditions. Using MS scan mode, protonated molecular ions $[M+H]^+$ of icariin, icariside II, osthole and IS gave sharp signals at *m/z* 677, 515, 245 and 237, respectively. Under the product ion scan mode, the most abundant product ions were *m/z* 531 from *m/z* 677, *m/z* 369 from *m/z* 515, *m/z* 189 from *m/z* 245 and *m/z* 194 from *m/z* 237. The mass spectrometric parameters were optimized to obtain the higher signal for both precursor ions and product ions mentioned above. Fig. 2(A)–(D) shows the product ion mass spectra of icariin, icariside II, osthole and IS obtained in positive ion ESI mode, respectively.

The presence of a small amount of acetic acid in the mobile phase could improve the sensitivity in positive ion mode of LC–MS/MS analysis. When acetic acid was used the responses of icariin, icariside II and osthole were stronger (around 1.8 times) than those with formic acid of the same proportion. The tested concentration range of acetic acid in the aqueous phase was from 0% to 0.2%. When 0.1% acetic acid with acetonitrile was used as the mobile phase the highest detection signals were observed for all analytes and IS, which were about twice of those produced by using 0.2% acetic acid or water only with acetonitrile as the mobile phase. Therefore a binary mobile phase of 0.1% acetic acid and acetonitrile was employed. Gradient elution was employed because of the much different polarity of the analytes and it could also extend the column life. As shown in Fig. 3, a small peak (identified as Epmediin B compared with reference standard) could be detected in channel 2 with a retention time of 2.14 min in rat plasma collected at some time points after oral administration of *Gushudan* extract. Therefore a low percentage of acetonitrile and a moderate gradient were employed in the beginning to avoid the possible interfering of icariin. Afterwards the proportion of acetonitrile increased sharply to achieve a fast elution of icariside II and osthole. The overall chromatographic run time was 9 min, which is relatively shorter than that achieved in HPLC method [9,14]. At higher flow rate (such as 0.35 ml/min) the analysis time could be shortened further, however, in consideration of the column backpressure and the resolution needed for quantification of icariin a flow rate of 0.25 ml/min was employed.

Some compounds with structural similarity to the analytes have been considered as the internal standard, flavonoid glycosides being tested included rutin, hesperidin and linarin. However, linarin could not be detected under the selected LC–MS/MS conditions and the other compounds exhibited faster elution (0.7–1.7 min) than icariin. Therefore other reference compounds available in our laboratory were also tested as IS candidates. Carbamazepine was selected as the internal standard for it exhibited a reasonable retention time between

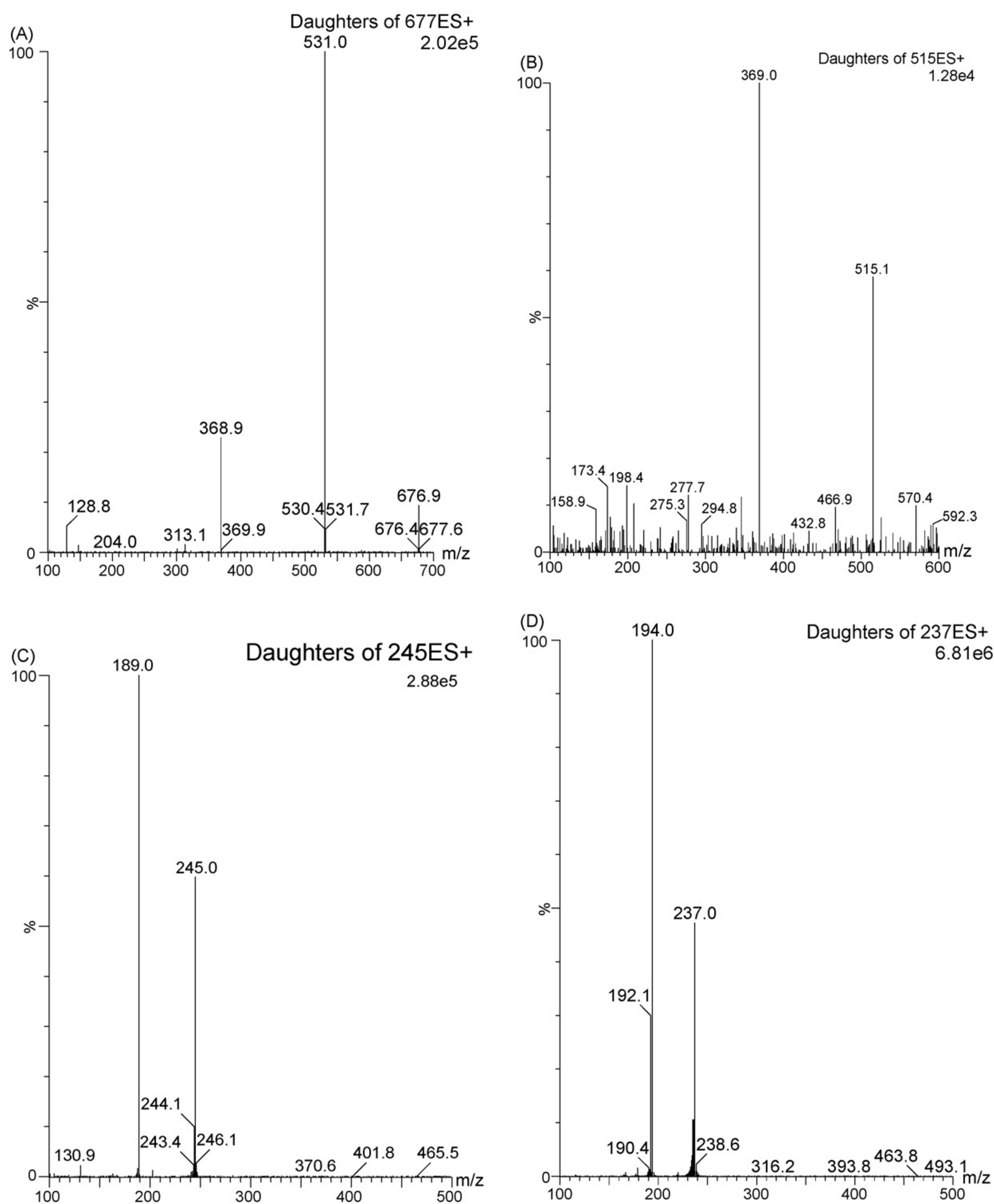

Fig. 2. Product ion mass spectra of $[M+H]^+$ of icariin (A), icariside II (B), osthole (C) and IS (D).

icariin and icarisede II, a best response under the same ESI ionization conditions as those optimal for analytes and a high and stable extraction recovery of 82.8 ± 5.3%, which demonstrated its behaviors similar to the analytes during the sample preparation, chromatographic separation and MS detection. Although its retention time appeared much different from osthole and one more IS nearer osthole would be a good choice, the precision (RSD% below 8%) and accuracy (RE% from −6.0% to 0.9%) of osthole quantification indicted that the variation of the method for osthole was also well assessed with carbamazepine as the IS.

3.2. Method validation

3.2.1. Selectivity

Typical chromatograms are shown in Fig. 3(A)–(C). Four channels were used for recording and the retention times for

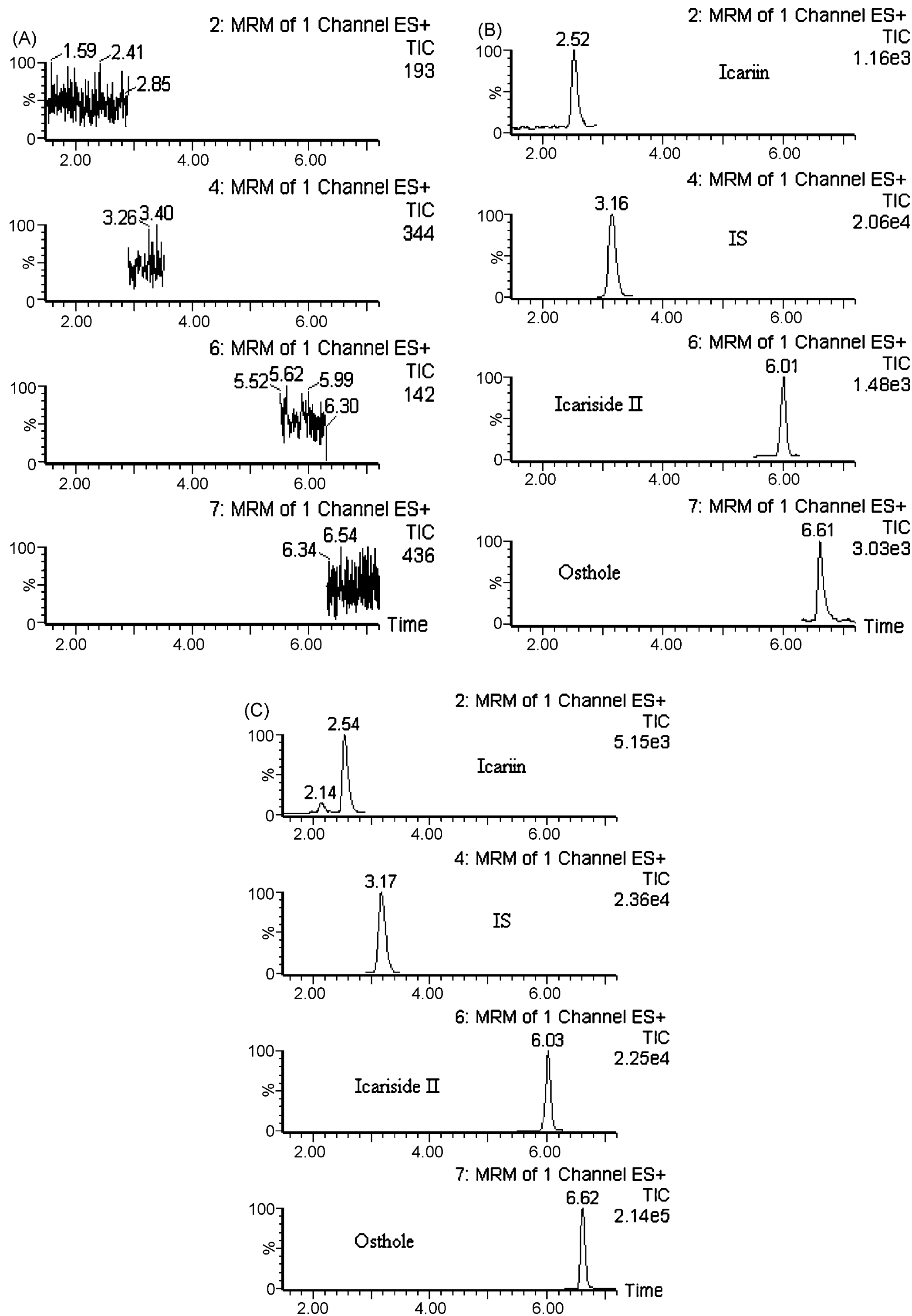

Fig. 3. Representative MRM chromatograms of icariin, IS, icariside II, osthole in rat plasma: (A) blank plasma; (B) blank plasma spiked with icariin, icariside II, osthole (LLOQ) and IS (500 ng/ml); (C) plasma from a rat 0.42 h after oral administration of *Gushudan* extract.

icariin, IS, icariside II and osthole were around 2.54, 3.17, 6.03 and 6.62 min, respectively. As shown in Fig. 3, icariin, icariside II, osthole and IS were detected with excellent resolution as well as peak shapes, and no interfering peaks were observed at the retention times of the analytes. At the same time, due to the high selectivity of MRM mode, there was no interference from another component of the formulation, and/or their metabolites.

As for the evaluation of matrix effect, the peak area ratios of blank plasma extracts spiked with icariin, icariside II, osthole and IS post extraction compared to those of the standard

Table 1
Precision and accuracy for the determination of icariin, icariside II and osthole in rat plasma (intra-day: $n = 6$; inter-day: $n = 6$ series per day, 3 days)

Added concentration (ng/ml)	Found concentration (ng/ml)	Intra-run, RSD (%)	Inter-run, RSD (%)	Accuracy, RE (%)
Icariin				
5.00	5.17 ± 0.44	5.0	14	3.5
50.0	53.2 ± 3.4	6.3	4.3	6.4
160	169 ± 10	4.7	9.8	5.6
Icariside II				
5.00	5.45 ± 0.18	2.2	6.9	9.0
50.0	49.6 ± 3.5	6.7	6.3	−0.8
160	170 ± 11	5.8	7.9	6.2
Osthole				
5.00	5.05 ± 0.41	7.9	6.4	0.9
100	94.0 ± 5.1	5.0	6.2	−6.0
400	393 ± 21	4.5	7.7	−1.8

solutions were within 85–115%, indicating that the developed method was free from matrix effect.

3.2.2. Linearity and LLOQ

Linear responses were obtained in concentration range from 2.00 to 200 ng/ml for both icariin and icariside II and from 2.00 to 500 ng/ml for osthole. Typical equations for the calibration curves were: $y = 19.07x + 0.01017$, $r = 0.9982$ for icariin, $y = 1.404x + 0.01438$, $r = 0.9975$ for icariside II and $y = 68.28x + 0.01969$, $r = 0.9991$ for osthole.

The LLOQ was found to be 2.00 ng/ml for icariin, icariside II and osthole with 5 μL of sample solution injected into the UPLC column, much lower than those reported in the literature where LLOQ of 1 μg/ml for icariin by using HPLC [14], 0.1 μg/ml [15] or 52 ng/ml [16] for osthole with HPLC method and 10 ng/ml with fluorescence method [17] were reported. The precision (RSD%) and accuracy (RE%) of LLOQ in this study were less than 10% and within ±11.8%, respectively.

3.2.3. Precision, accuracy, recovery and stability

The data of intra- and inter-day precision and accuracy for icariin, icariside II and osthole quantification are shown in Table 1. For each QC level, the intra- and inter-day precisions were within 8.0% and 14%, respectively, with relative errors from −6.0 to 9.0%, indicating an acceptable precision and accuracy of the present method for determination of icariin, icariside II and osthole in rat plasma.

The mean recoveries of icariin, icariside II and osthole ranged from 81.9% to 98.4%, from 83.7% to 94.4% and from 85.1% to 87.9%, respectively. The mean recoveries with SD of icariin, icariside II and osthole at three QC levels are shown in Table 2. The recovery of the IS was 82.8 ± 5.3%.

Table 3 summarizes the results of short-term stability, long-term stability, freeze–thaw stability of icariin, icariside II and osthole in plasma and post-preparative stability. All the results indicated that the analytes were stable under these conditions.

Table 2
The mean recoveries of icariin, icariside II and osthole in rat plasma ($n = 5$)

	Concentration (ng/ml)	Recovery (%, mean ± SD)
Icariin	5.00	81.9 ± 5.2
	50.0	84.5 ± 3.8
	160	98.4 ± 5.8
Icariside II	5.00	83.7 ± 2.6
	50.0	92.2 ± 3.0
	160	94.4 ± 4.2
Osthole	5.00	87.9 ± 4.7
	100	85.1 ± 2.5
	400	86.3 ± 1.5

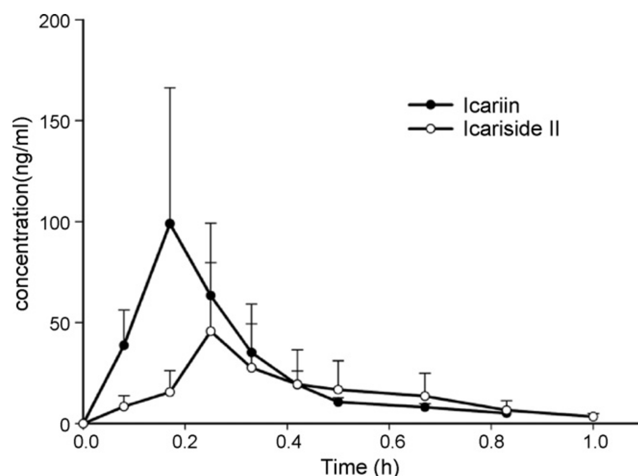

Fig. 4. Mean (±SD) plasma concentration–time curves of icariin and icariside II in rats after oral administration of *Gushudan* extract.

3.3. Application in pharmacokinetic studies of icariin, icariside II and osthole in rats

This validated method was applied to monitor the plasma concentrations of icariin, icariside II and osthole in rats after a single oral administration of *Gushudan* extract at a dose of 27 g/kg body weight. The mean plasma concentration–time curves are illustrated in Figs. 4 and 5. The pharmacokinetic parameters

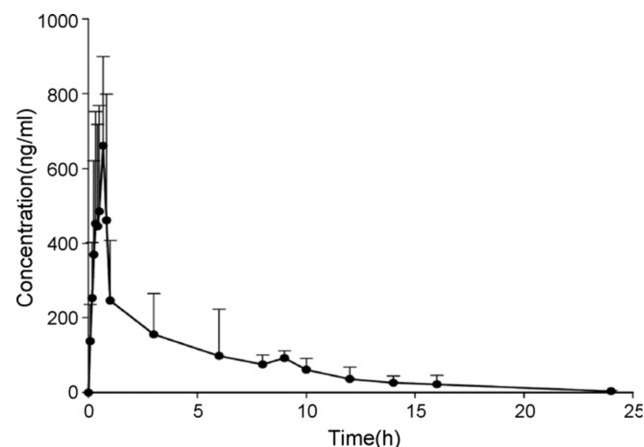

Fig. 5. Mean (±SD) plasma concentration–time curve of osthole in rats after oral administration of *Gushudan* extract.

Table 3
Stability of icariin, icariside II and osthole in rat plasma ($n = 3$)

Concentration (ng/ml)	Accuracy (%, mean ± SD)***			
	Short-term stability	Long-term stability	Freeze–thaw stability	Post-preparative stability
Icariin				
5.00	104 ± 7	95.2 ± 3.9	104 ± 9	93.5 ± 3.8
50.0	104 ± 7	106 ± 6	108 ± 5	103 ± 4
160	104 ± 5	105 ± 2	106 ± 3	92.2 ± 2.9
Icariside II				
5.00	99.8 ± 9.0	105 ± 9	101 ± 11	91.9 ± 5.4
50.0	102 ± 3	97.1 ± 5.5	98.8 ± 5.6	103 ± 8
160	103 ± 7	105 ± 8	94.2 ± 2.8	92.0 ± 4.5
Osthole				
5.00	96.9 ± 5.3	100 ± 5	101 ± 8	92.7 ± 2.6
100	94.1 ± 7.7	94.7 ± 2.5	99.4 ± 9.5	94.1 ± 3.4
400	96.5 ± 3.4	97.8 ± 3.7	100 ± 5	102 ± 8

Table 4
Pharmacokinetic parameters (mean ± SD) of icariin, icariside II and osthole in rats after oral administration of *Gushudan* extract ($n = 6$)

Parameters	Icariin	Icariside II	Osthole
C_{max} (ng/ml)	102 ± 64	46.3 ± 33.2	672 ± 247
T_{max} (h)	0.16 ± 0.04	0.24 ± 0.03	0.70 ± 0.07
$t_{1/2}$ (h)	0.28 ± 0.09	0.28 ± 0.10	4.83 ± 1.78
k_e (h^{-1})	2.64 ± 0.86	2.81 ± 1.22	0.16 ± 0.06
$AUC_{0-\infty}$ (ng h/ml)	23.2 ± 5.8	16.3 ± 11.9	1.81×10^3 ± 873

are presented in Table 4. After oral administration, icariin and icariside II appeared to be absorbed and eliminated fast, while osthole was absorbed fast but eliminated slowly, which is in agreement with the results in published pharmacokinetic studies [15,17].

4. Conclusions

A sensitive, specific and accurate LC–MS/MS method was developed for the analysis of icariin, icariside II and osthole in rat plasma with a chromatographic run time of 9 min. The method has advantages of high sensitivity with an LLOQ of 2.00 ng/ml, satisfactory selectivity and simple sample preparation. The analytical procedure was then successfully applied to the pharmacokinetic study of icariin, icariside II and osthole in rats after oral administration of *Gushudan* extract, which will play an important role in investigating the action mechanism of *Gushudan* and supply a suitable reference in clinical application of the formulation .

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