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Journal of Pharmaceutical and Biomedical Analysis 45 (2007) 667–672
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
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Short communication

LC–MS/MS method for the simultaneous determination of icariin and its major metabolites in rat plasma

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Received 10 May 2007; received in revised form 1 July 2007; accepted 5 July 2007
Available online 10 July 2007

Abstract

A rapid and sensitive method using liquid chromatography–tandem mass spectroscopy (LC–MS/MS) was developed and validated for the simultaneous quantitative determination of icariin and its two major metabolites, icariside I and icariside II in rat plasma. The analytes were extracted by liquid–liquid extraction with ethyl acetate after internal standard (daidzein) spiked. The separation was performed by a ZORBAX SB-C_{18} column (3.5 μm, 2.1 mm × 100 mm) and a C_{18} guard column (5 μm, 4.0 mm × 2.0 mm) with an isocratic mobile phase consisting of acetonitrile–water–formic acid (50:50:0.05, v/v/v) at a flow rate of 0.25 mL/min. The Agilent G6410A triple quadrupole LC–MS system was operated under the multiple reaction monitoring (MRM) mode using the electrospray ionization technique in positive mode. The nominal retention times for icariin, icariside I, icariside II and daidzein were 1.21, 1.88, 2.34 and 1.35 min, respectively. The lower limits of quantification (LLOQ) of icariin, icariside I and icariside II of the method were 1.0, 0.5 and 0.5 ng/mL, respectively. The method was linear for icariin and its metabolites with correlation coefficients >0.995 for all analytes. The intra-day and inter-day accuracy and precision of the assay were less than 12.5%. This method has been applied successfully to a pharmacokinetic study involving the intragastric administration of icariin to rats.

Keywords: LC–MS/MS; Icariin; Icariside I; Icariside II; Pharmacokinetics

1. Introduction

Icariin (Fig. 1) is a major constituent of flavonoids isolated from *Epimedium brevicornum* Maxim (Berberidaceae), which is used as a traditional Chinese medicine to nourish the *kidney* and reinforce *yang* for over 2000 years. *E. brevicornum* Maxim is recorded in the Pharmacopoeia of PR China and icariin is regarded as its marker compound [1]. *E. brevicornum* Maxim and its complex formulation are widely used in China for the prevention and treatment of osteoporosis, coronary artery disease and male sexual dysfunction [2,3]. Modern pharmacological study shows that icariin has a lot of pharmacological and biological activities, including prevent osteoporosis, penile erection, anti-cancer and anti-depression [4–8].

Up to now, *E. brevicornum* Maxim formulation and its complex formulation are usually taken orally, while the oral bioavailability of icariin is poor [9]. Our previous study showed that icariin could be metabolized to icariside II by the bacterium in rat intestine [10] and in rat plasma two main metabolites (Fig. 1) were found after intragastric administration of icariin to rats. Due to the high concentration of icariin's metabolites in plasma, when the pharmacokinetics of icariin is studied, it is necessary to consider its metabolites as well. Furthermore, to study the pharmacokinetics of icariin's metabolites will do help for the understanding of icariin's *in vivo* behavior and for the better application of the drug.

Though LC–UV and CZE (capillary zone electrophoresis)–UV methods have been developed for the quantitative determi-

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doi:10.1016/j.jpba.2007.07.007

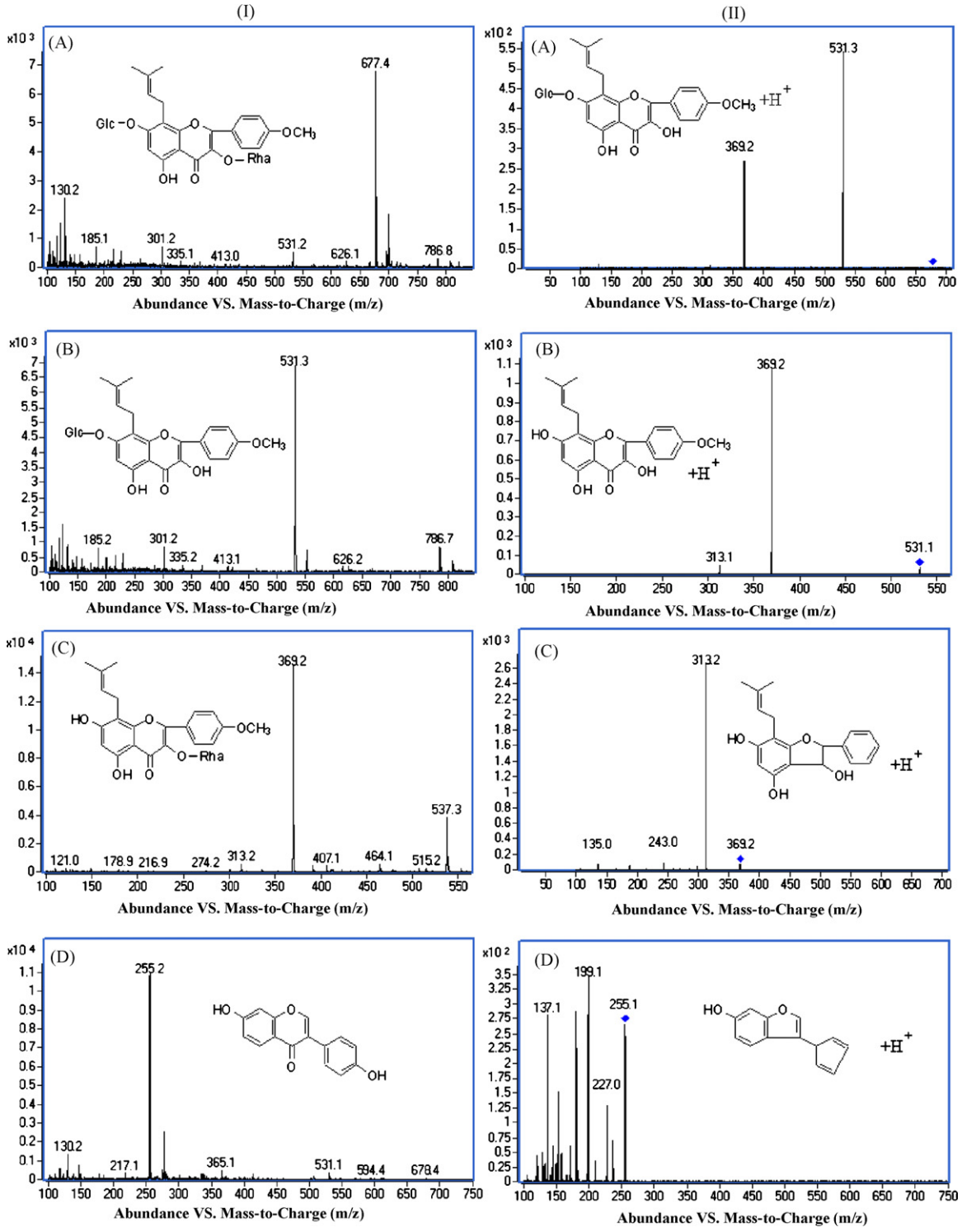

Fig. 1. (I) Full scan mass spectra by MS and chemical structure of icariin (A), icariside I (B), icariside II (C) and daidzein (D). (II) Full scan product ion mass spectra by MS/MS and estimated chemical structure of each compound. Rha and Glc are the abbreviations of rhamnose and glucose, respectively.

nation of icariin in biological samples [9,11], these methods are time consuming and not sensitive enough for the quantitative determination of icariin and its metabolites. To our knowledge, no paper about the simultaneous quantitative determination of icariin and its metabolites or LC–MS method for the determination of icariin in biological samples has been reported. So for the study of the pharmacokinetics of icariin, we developed and validated an LC–MS/MS method for the simultaneous determination of icariin and its two metabolites. This method is sensitive, rapid and specific and can be applied for the pharmacokinetic study on the drug and its metabolites.

2. Experimental

2.1. Materials

Icariin (>99% purity) and daidzein (>99% purity, I.S.) were purchased from National Institute for the Control of Pharmaceuticals and Biological Products (Beijing, China). Icariside I (>98% purity) and icariside II (>98% purity) were extracted from *E. brevicornum* Maxim (Berberidaceae) and refined in our laboratory (identified by NMR and MS). HPLC-grade ethyl acetate was purchased from Tianjin Kermel Chemical Reagents Development Centre (Tianjin, China). HPLC-grade acetonitrile was purchased from Merck Company (Darmstadt, Germany). HPLC-grade formic acid was purchased from Tedia Company Inc. (Fairfield, USA). All other reagents were of analytical grade.

2.2. LC–MS/MS instrumentation and analytical conditions

An Agilent 6410A triple quadrupole LC–MS system (Agilent Corporation, MA, USA) equipped with G1311A quaternary pump, G1322A vacuum degasser, G1329A autosampler and G1316A therm. column compartments was used for all analyses. The system was controlled by MassHunter software (Agilent Corporation, MA, USA).

The separation was performed by a ZORBAX SB-C_{18} column (3.5 μm, 2.1 mm × 100 mm, Agilent Corporation, MA, USA) and a C_{18}-guard column (5 μm, 4.0 mm × 2.0 mm, Phenomenex, CA, USA) with an isocratic mobile phase consisted of acetonitrile–water–formic acid (50:50:0.05, v/v/v) at a flow rate of 0.25 mL/min. The column was maintained at 35 °C and the injection volume was 10 μL.

Ionization was achieved using electrospray in the positive mode with the spray voltage set at 4000 V. Nitrogen was used as nebulizer gas and nebulizer pressure was set at 40 psi with a source temperature of 105 °C. Desolvation gas (nitrogen) was heated to 350 °C and delivered at a flow rate of 10 L/min. For collision-induced dissociation (CID), high purity nitrogen was used as collision gas at a pressure of 0.1 MPa. Quantification was performed using multiple reaction monitoring (MRM) mode at *m/z* 677 → 531 for icariin, *m/z* 531 → 369 for icariside I, *m/z* 369 → 313 for icariside II and *m/z* 255 → 199 for daidzein. The fragmentor energies of MS^1 for icariin, icariside I, icariside II and daidzein were set at 140, 140, 200 and 160 V, respectively. The optimized collision energies of 18, 18, 30 and 30 eV were used for icariin, icariside I, icariside II and daidzein, respectively. The peak widths of precursor and product ions were maintained at 0.7 amu at half-height in the MRM mode.

2.3. Preparation of standard and quality control (QC) samples

The standard stock solutions of icariin, icariside I, icariside II and daidzein were prepared in methanol to final concentrations of 100 μg/mL for each analyte. The stock solution of icariin was further diluted with water to achieve standard working solutions at concentrations of 5.00, 25.0, 50.0, 250.0, 500.0, 2500 and 5000 ng/mL. While the stock solutions of icariside I and icariside II were further diluted with water to achieve standard working solutions at concentrations of 2.50, 5.00, 25.0, 50.0, 250.0, 500.0, 2500 and 5000 ng/mL, quality control samples were also prepared in the same way, using a separately weighed stock solution. Internal standard working solution (1500 ng/mL) was prepared by diluting the stock solution of daidzein with water. All the working solutions were kept at 4 °C.

The standard solutions (20 μL) were used to spike blank plasma samples (100 μL), either for calibration curves of both analytes or for QCs in the pre-study validation and during the pharmacokinetic study.

2.4. Sample preparation

To a 100 μL aliquot of plasma sample, 20 μL of water and 20 μL of the IS working solution were added. Samples were then vortex-mixed for 30 s and extracted with 3.0 mL ethyl acetate by vortex-mixing for 1.0 min. After centrifugation at 2000 × *g* for 10 min, the upper organic layer was transferred to another tube and evaporated to dryness at 45 °C under a gentle stream of nitrogen. The residue was reconstituted in 100 μL mobile phase followed by vortex-mixing for 1.0 min and centrifugation at 2000 × *g* for 10 min. Then, a 10 μL aliquot of supernatant was injected onto the LC–MS/MS system.

2.5. Assay validation

Standard curves ranging from 1.00 to 1000 ng/mL icariin and from 0.50 to 1000 ng/mL icariside I and icariside II were run on three separate days. Calibration curves were constructed from the peak area ratios of each analyte to IS versus plasma concentrations using a $1/x^2$ weighted linear least-squares regression model.

Six replicates of QC samples at three levels icariin, icariside I and icariside II were included in each run to determine the intra-day and inter-day precision of the assay. The accuracy was determined as the percentage difference between the mean detected concentrations and the nominal concentrations. The lower limit of quantification (LLOQ) is defined as the lowest concentration of standard that can be measured with an acceptable accuracy and precision (≤20% for both parameters).

The extraction recoveries of the three analytes at three QC levels were determined by comparing peak areas obtained from plasma samples with those found by direct injection of a standard solution of the same concentration.

The stability of three analytes in plasma was assessed by analyzing triplicate QC samples stored for 6 h at ambient temperatures, three cycles of freezing at −20 °C and thawing, reconstituted extract at room temperature for 24 h and stored for 1 month at −20 °C, respectively. Concentrations following storage were compared with freshly prepared samples of the same concentrations.

2.6. Application of the analytical method

Male Wistar rats, weighing approximately 230–250 g, were provided by Shanghai SLAC Lab Animal Co. Ltd. (Shanghai, China). The animal experimentation was approved by the Second Military Medical University Animal Ethics Committee (Shanghai, China). Rats ($n = 6$) received an intragastric administration of 50 mg/kg icariin (2.5 mL/kg). The drug was dissolved into polyethylene glycol 400. Blood samples (250 μL) were withdrawn from thigh vein using cannulation from each rat at 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 9 and 12 h after administration. The blood samples were placed in heparinized tubes and immediately centrifuged and then the plasma samples were stored at −20 °C until analysis. Plasma collected from six vehicle-administrated rats served as blank.

All pharmacokinetic parameters were determined by non-compartmental analysis. The peak plasma level (C_{max}) and the time to reach the peak plasma concentration (t_{max}) were obtained directly from the concentration–time data. The elimination rate constant (K_e) was calculated from the slope of the logarithm of the plasma concentration versus time using the final four points. The apparent elimination half-life ($t_{1/2}$) was calculated as $0.693/K_e$. The area under the plasma concentration–time curve (AUC) was calculated by the trapezoidal rule. Each value is expressed as mean ± S.D.

3. Results and discussion

3.1. LC–MS/MS optimization

Icariin, icariside I, icariside II and daidzein (IS) were at first characterized by MS^2 scan and MS–MS product ions to ascertain their precursor ions and to select product ions for use in MRM mode, respectively. To get the richest relative abundance of precursor ions and product ions, the parameters for fragmentor energies and collision energies were optimized, and the MRM transition were chosen to be *m/z* 677 → 531 for icariin, *m/z* 531 → 369 for icariside I, *m/z* 369 → 313 for icariside II and *m/z* 255 → 199 for daidzein.

Usually LC–MS/MS method has high specificity because only selected ions produced from analytes of interest are monitored. For icariin, icariside I and icariside II are with similar chemical structures, they cannot be well separated by the MS–MS method if they are not entirely separated by LC method. For example, Icariin can produce the precursor ions of *m/z* 677 and *m/z* 531 which is also a precursor ion of icariside I, so, if icariin and icariside I are not separated by the LC method, the peak area of icariside I will be overrated, while the peak area of icariin is not interfered by icariside I. Similarly, the peak area of icariside II will be overrated if it is not separated with icariside I. So in the optimized LC method icariin, icariside I and icariside II were well separated. The nominal retention times for icariin, icariside I, icariside II and daidzein were1.21, 1.88, 2.34 and 1.35 min, respectively. Comparison of the chromatograms of six independent plasma samples and spiked rat plasma matrices indicated no significant interferences at the retention times of the analytes and IS (Fig. 2).

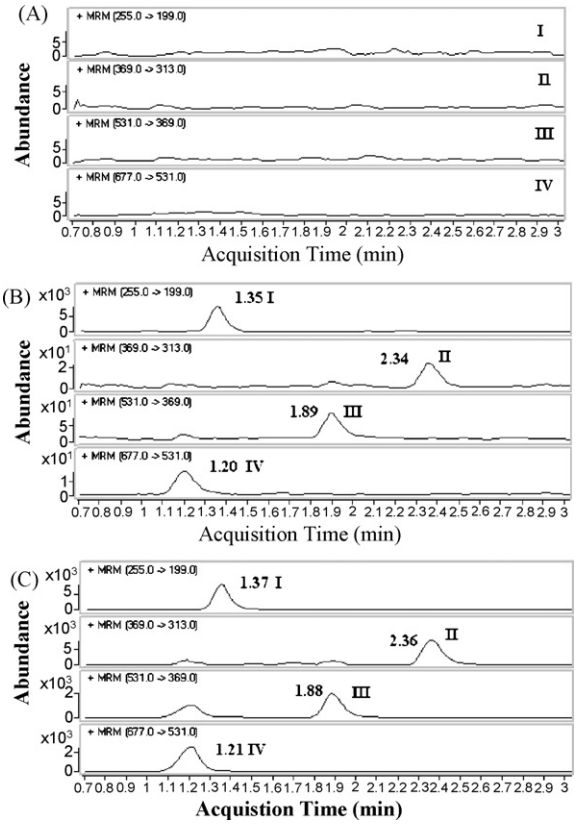

Fig. 2. Representative MRM chromatograms of icariin (IV), icariside I (III), icariside II (II) and IS (I) in rat plasma. (A) A blank plasma sample, (B) a blank plasma sample spiked with icariin, icariside I and icariside II at the lower limit of quantification and IS, and (C) plasma sample from a rat 1 h after intragastric administration of icariin at a dose of 50 mg/kg.

3.2. Method validation

3.2.1. Linearity of calibration curves and lower limit of quantification (LLOQ)

The standard calibration curve for spiked rat plasma containing icariin was linear over the range 1.00–1000 ng/mL with a correlation coefficient (r) > 0.995. And those of icariside I and icariside II were linear over the range 0.50–1000 ng/mL with a correlation coefficient (r) > 0.995. Typical equations for the calibration curve were as follows:

$$\text{Icariin}: \quad Y = 2.98 \times 10^{-3}X + 5.78 \times 10^{-4}, \quad r = 0.9982$$

$$\text{Icariside I}: \quad Y = 2.08 \times 10^{-2}X + 1.03 \times 10^{-3}, \quad r = 0.9965$$

$$\text{Icariside II}: \quad Y = 1.39 \times 10^{-2}X + 9.96 \times 10^{-4}, \quad r = 0.9967$$

where X is the plasma concentration of each analyte (ng/mL) and Y is the peak-area ratios of each analyte to IS.

The lower limit of quantification for determination of icariin, icariside I and icariside II in plasma, defined as the lowest concentration analyzed with accuracy within ±20% and a precision ≤20%, were 1.00, 0.50 and 0.50 ng/mL, respectively. These limits are sufficient for the pharmacokinetic studies of icariin and its major metabolites following an intragastric administration of icariin.

Table 1
Summary of precision and accuracy from QC samples of rat plasma extracts ($n = 3$ days and six replicates per day)

Analyte	Added C (ng/mL)	Found C (ng/mL)	Intra-day R.S.D. (%)	Inter-day R.S.D. (%)	Relative error (%)
Icariin	5.00	5.07	10.6	12.5	1.4
	50.0	49.3	9.4	8.6	−1.4
	500.0	513.9	3.8	7.6	2.8
Icariside I	1.00	0.97	4.5	4.8	−3.2
	50.0	50.8	1.9	3.8	1.6
	500.0	509.4	2.7	4.3	1.9
Icariside II	1.00	1.03	11.4	9.8	3.0
	50.0	50.5	4.2	7.6	1.1
	500.0	498.1	5.6	4.7	−0.4

The limits of detection (LOD) were calculated on the basis of a signal-to-noise ratio of 3:1, resulting in 0.25, 0.15 and 0.10 ng/mL for icariin, icariside I and icariside II.

3.2.2. Assay precision and accuracy

Intra- and inter-day precision was assessed from the results of QCs by using a one-way analysis of variance (ANOVA). The mean values and R.S.D. for QC samples at three concentration levels were calculated over three validation runs. Six replicates of each QC level were determined in each run. The accuracy of the method was determined by calculating the percentage deviation observed in the analysis of QCs and expressed as the relative error (R.E.). Table 1 summarizes the intra- and inter-day precision and accuracy for icariin, icariside I and icariside II from the QC samples. The intra-day precisions were less than 10.6% for each QC level of icariin, less than 4.5% for each QC level of icariside I and less than 11.4% for each QC level of icariside II. The inter-day precision was less than 12.5% for icariin, icariside I and icariside II. The accuracy derived from QC samples was within ±3.2% for each QC level of icariin, icariside I and icariside II.

3.2.3. Extraction recovery

The extraction recoveries of icariin under the liquid–liquid extraction conditions were 91.6 ± 3.4, 93.3 ± 1.9 and 92.8 ± 2.5% at concentrations of 5.00, 50.0 and 500 ng/mL (QC samples), respectively. The extraction recoveries of icariside I and icariside II were 95.7 ± 7.2/96.3 ± 5.8, 94.9 ± 4.1/97.7 ± 2.4 and 95.2 ± 3.9/96.1 ± 4.79% at concentrations of 1.00, 50.0, 500 ng/mL, respectively. The recovery of the internal standard was 90.3 ± 6.3% in rat plasma ($n = 6$).

3.2.4. Analyte stability

The stability of icariin, icariside I and icariside II in rat plasma and mobile phase were investigated. The analyte was found to be stable in rat plasma stored for 1 month at −20 °C and in reconstituted mobile phase at room temperature for 24 h (<5% reduction). After storage at 1–4 °C for 2 month, no obvious reduction was found in the stock and working solutions. The analyte was found to be stable after three freeze-thaw cycles with a reduction of less than 10%. The analytes were also shown to be stable in rat plasma at room temperature for at least 6 h with a reduction of less than 10%.

Table 2
The main pharmacokinetic parameters of icariin, icariside I and icariside II after intragastric administration of icariin to six rats at a dose of 50 mg/kg (mean ± S.D.)

	Icariin	Icariside I	Icariside II
C_{max} (ng/mL)	162.4 ± 42.8	9.6 ± 2.8	137.8 ± 46.1
t_{max} (h)	1.12 ± 0.31	0.96 ± 0.29	0.67 ± 0.20
K_e (1/h)	0.36 ± 0.10	0.36 ± 0.05	0.21 ± 0.04
$t_{1/2}$ (h)	2.08 ± 0.73	1.95 ± 0.25	3.42 ± 0.66
$AUC_{0\to t}$ (ng h^2/mL)	524.6 ± 132.5	30.8 ± 6.2	415.6 ± 171.9
$AUC_{0\to\infty}$ (ng h/mL)	532.7 ± 132.2	31.3 ± 6.2	457.5 ± 195.8

3.3. Application of the analytical method to pharmacokinetic studies

The LC–MS/MS method developed was used to investigate the pharmacokinetics of icariin and its main metabolite icariside I and icariside II after an intragastric administration of 50 mg/kg icariin solution to six rats. Table 2 shows the main pharmacokinetic parameters of icariin, icariside I and icariside II and Fig. 3 shows the mean plasma concentration–time profiles of icariin, icariside I and icariside II after intragastric administration of icariin to six rats at a dose of 50 mg/kg. The method with the LLOQ of 1.00 ng/mL for icariin is more sensitive than the reported ones (LLOQ = 1000 ng/mL) [9,11]. Therefore, it

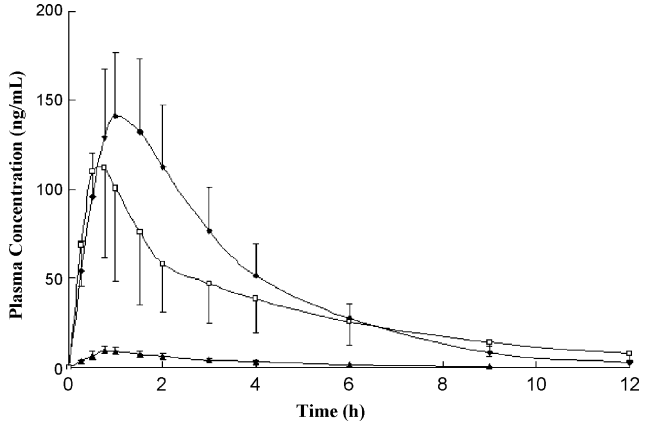

Fig. 3. Mean plasma concentration–time profiles of icariin (◆), icariside I (□) and icariside II (▲) after intragastric administration of icariin to six rats at a dose of 50 mg/kg.

allows the determination of icariin up to 12 h after an intragastric administration.

From the mean plasma concentration–time profiles of icariin, icariside I and icariside II we can see that icariin and icariside II were absorbed rapidly and inadequately. The peak times of metabolites were shorter than that of parent compound. That might be likely due to that icariin could be rapidly metabolized to icariside II by the bacterium in rat intestine [10] and icariside II was absorbed faster than icariin. The value of T_{max} of icariside I was between that of icariin and icariside II, and icariside I could not be produced in rat intestine neither from icariin nor from icariside II [10], so we presume that icariside I was metabolized in epithelial cell of intestine or liver from icariside II.

Due to the high concentration of icariside II in plasma, when the pharmacokinetics of icariin is studied, it is necessary to consider icariside II as well. Though icariin's another metabolite icariside I was not found much in rats' plasma, it may be involved in the process of absorption and metabolism of icariin as well as its metabolites. So determine the plasma concentration of icariside I may be helpful in explaining the oral absorption and metabolic mechanism of icariin as well as its metabolites and may be useful when different species of tested animals are used.

4. Conclusion

A sensitive, accurate and precise procedure based on LC–MS/MS has been developed and validated for the simultaneous determination of icariin and its main metabolites in rat plasma with the LLOQ of 1.00, 0.50, 0.50 ng/mL for icariin, icariside I, icariside II, respectively. The limit of detection of icariin was 0.25 ng/mL, which was 2000–4000 folds lower than those of reported methods [9,11]. Our proposed method is also a rapid one, the icariin and its two metabolites were assayed with a analytical time less than 3 min which was 5–10 folds shorter than those of reported methods [9,11]. This method was used successfully to evaluate the pharmacokinetics of icariin, icariside I and icariside II after an intragastric administration of icariin.

Acknowledgements

The work was supported by program for Changjiang Scholars and Innovative Research Team in University (PCSIRT), "973" program of China (2007CB507400), National 863 Program (2006AA02Z338) and in part by the Scientific Foundation of Shanghai China (05DZ19733, 06DZ19717, 06DZ19005).

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