



A liquid chromatography–tandem mass spectrometric method for the simultaneous quantitation of five components of *Ixeris sonchifolia* (Bge.) Hance in rat plasma and its application to a pharmacokinetic study



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ARTICLE INFO

Article history:

Received 22 November 2012

Accepted 30 April 2013

Available online 20 May 2013

Keywords:

LC–MS/MS

Ixeris sonchifolia (Bge.) Hance

Pharmacokinetics

Rat

ABSTRACT

A rapid and sensitive liquid chromatography–tandem mass spectrometric (LC–MS/MS) method for the simultaneous quantitation of five major active ingredients of *Ixeris sonchifolia* (Bge.) Hance in rat plasma has been developed and validated. After liquid–liquid extraction of 50 μ L plasma with ethyl acetate, analytes and internal standard (I.S.), astilbin, were chromatographed on a Zorbax SB-C18 column (150 mm \times 4.6 mm, 5 μ m) using acetonitrile – 10 mM ammonium acetate (60:40, v/v, pH 5.6) as mobile phase. The five analytes: chicoric acid, luteolin 7-O- β -D-glucuronide, luteolin 7-O- β -D-glucopyranoside, luteolin 7-O- β -D-glucopyranosyl-(1 \rightarrow 2)- β -D-glucopyranoside, apigenin 7-O- β -D-glucuronide and I.S., were detected by negative ion electrospray ionization followed by multiple reaction monitoring of the ions with m/z 473.0 \rightarrow 311.0, 461.0 \rightarrow 285.0, 447.0 \rightarrow 285.0, 609.1 \rightarrow 285.0, 445.1 \rightarrow 269.0 and 449.1 \rightarrow 150.9, respectively. The method was linear for all analytes in the concentration range 10–3000 ng/mL with intra- and inter-day precision (as relative standard deviation) \leq 8.99% and accuracy (as relative error) \leq 4.00%. The limits of detection (LOD) were 5, 1, 5, 5, 2 ng/mL for chicoric acid, luteolin 7-O- β -D-glucuronide, luteolin 7-O- β -D-glucopyranoside, luteolin 7-O- β -D-glucopyranosyl-(1 \rightarrow 2)- β -D-glucopyranoside, apigenin 7-O- β -D-glucuronide, respectively. The method was successfully applied to a pharmacokinetic study of the five analytes in rat after a single intravenous dose of Kudiezi Injection.

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1. Introduction

Ixeris sonchifolia (Bge.) Hance is a herbal medicine commonly used in China for its haemostatic and anti-inflammatory effects [1–4]. On the basis of previous researches, chicoric acid (I) and flavonoid glycosides (luteolin 7-O- β -D-glucuronide

(II), luteolin 7-O- β -D-glucopyranoside (III), luteolin 7-O- β -D-glucopyranosyl-(1 \rightarrow 2)- β -D-glucopyranoside (IV) and apigenin 7-O- β -D-glucuronide (V)) have been reported as the most important active components in *I. sonchifolia* (Bge.) Hance [2,3]. These compounds are present in Kudiezi Injection which is made from the extract of *I. sonchifolia* (Bge.) Hance and has been shown to possess antibacterial [5,6], antioxidant [5–7], anti-inflammatory and antitumor activities [2,5–12].

Currently, there are several reports concerning the determination of some constituents of *I. sonchifolia* (Bge.) Hance in natural plant materials using high performance liquid chromatography (HPLC) with UV [13,14] and MS detection [15]. However, there are few *in vivo* studies on the analysis of flavonoid glycosides in *I. sonchifolia* (Bge.) Hance [16–19]. Furthermore, no research was

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currently available concerned with the simultaneous determination of these five active components in biological sample. The therapeutic action of the traditional Chinese medicine is based on the interaction of multi-component [1,2,19]. In order to better investigate the pharmacokinetic behavior of Kudiezi Injection, it is important to simultaneous analysis of multiple active ingredients in plasma.

This paper reports the first application of liquid chromatography tandem mass spectrometry (LC–MS/MS) to the simultaneous quantitation of the five analytes in rat plasma. The method has been validated and applied to a pharmacokinetic study of the five analytes in rat after an intravenous administration of Kudiezi Injection.

2. Experimental

2.1. Chemicals and reagents

Compounds I–V (purity >95.0%) and Kudiezi Injection were provided by the Jilin Medical Academy (Changchun, China). The concentrations (% w/v, $\mu\text{g}/100\ \mu\text{L}$) of ingredients I–V in Kudiezi Injection were 45.3, 47.7, 26.7, 17.5 and 10.6, respectively. Astilbin (purity >99.5%) for use as internal standard (I.S.), was provided by Shandong Luye Pharmaceutical Co. (Yantai, China). The structures of analytes and I.S. are shown in Fig. 1. Acetonitrile (HPLC-grade) was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Distilled water, prepared from demineralized water, was used throughout the study. All other chemicals were of HPLC grade.

2.2. LC–MS/MS conditions

The LC–MS/MS system consisted of an Agilent 1100 Series HPLC (Agilent Technologies, Palo Alto, CA, USA) coupled to an API 4000 mass spectrometer (Applied Biosystems Sciex, Ontario, Canada) equipped with a TurbolonSpray source for ion production. Data acquisition and integration were controlled by Analyst Software.

Chromatography was performed on a Zorbax SB-C18 column (150 mm \times 4.6 mm, 5 μm) maintained at 40 °C using a mobile phase of acetonitrile – 10 mM ammonium acetate (60:40, v/v, pH 5.6) at a flow rate of 1.0 mL/min. In order to enhance the efficiency of ionization, the eluent was split to allow 0.5 mL/min to enter the mass detector. The MS was operated in the negative ion mode and parameters were optimized by infusing a standard solution of analytes and I.S. using a syringe pump. Optimal parameters were as follows: nebulizer, heater and curtain gas flow rates 45, 45 and 20 units, respectively; dwell time 100 ms; ionspray needle voltage –4500 V; heater gas temperature 500 °C. Declustering potentials (V) and collision energies (eV) were: I 45, 16; II 65, 35; III 95, 40; IV 100, 55; V 60, 35; I.S. 70, 35. Transitions (m/z , amu) for multiple reaction monitoring were: I 473.0 \rightarrow 311.0; II 461.0 \rightarrow 285.0; III 447.0 \rightarrow 285.0; IV 609.1 \rightarrow 285.0; V 445.1 \rightarrow 269.0 and I.S. 449.1 \rightarrow 150.9.

2.3. Preparation of calibration standards and quality control (QC) samples

All solutions were prepared in methanol:water (50:50, v/v). A mixed stock solution of I–V (1 mg/mL in each) was prepared and kept at 4 °C when not in use. Standard solutions were prepared by diluting the stock solution to 0.5, 1.5, 5, 15, 50 and 150 $\mu\text{g}/\text{mL}$. QC solutions at concentrations of 1.5, 15, and 120 $\mu\text{g}/\text{mL}$ were prepared independently in the same way. Calibration standards with concentrations of 10, 30, 100, 300, 1000 and 3000 ng/mL were prepared by mixing 50 μL aliquots of standard solutions with 2450 μL blank plasma. Low, medium and high QC samples with concentrations of 30, 300, and 2400 ng/mL were prepared from QC solutions

in a similar manner. All plasma samples were stored at –80 °C prior to use.

2.4. Sample preparation

Frozen plasma samples were thawed at room temperature and subjected to liquid–liquid extraction (LLE). To a 50 μL aliquot in a glass tube, 50 μL I.S. solution and 100 μL 1 M HCl were added. By the addition of HCl, the plasma is being precipitated slightly. The mixture was vortexed for 30 s and extracted with 3 mL ethyl acetate by shaking for 10 min. After centrifugation at 3500 \times g for 5 min, the upper organic layer was transferred to another tube and dried under nitrogen at 40 °C. The residue was reconstituted in 100 μL mobile phase, and 20 μL was injected into the LC–MS/MS system.

2.5. Method validation

The method was fully validated according to the Food and Drug Administration (FDA) guidance [20] for biological method validation.

Specificity was assessed by analysis of blank plasma samples from six rats. Linearity was evaluated by preparation of calibration curves based on peak area ratios of analytes to I.S. Curves were prepared in duplicate on three separate days and assessed by linear least-squares regression with a weighting index of $1/x^2$. Accuracy (as relative error (R.E.)) and intra- and inter-day precision (as relative standard deviation (R.S.D.)) were based on assay of six replicate QC samples on three different days. The lower limit of quantitation (LLOQ) was defined as the lowest concentration that could be determined with accuracy $\pm 20\%$ and precision $< 15\%$. Matrix effects were evaluated by comparing peak areas of analytes and I.S. in post-extraction spiked samples with those in standard solutions. Recovery was determined by comparing peak areas of QC samples with those of post-extraction blank plasma spiked at corresponding concentrations.

Stability of analytes in rat plasma was evaluated in QC samples placed on storage for 1 month at –80 °C, for 2 h at room temperature (25 °C) and after three freeze/thaw cycles. Stability of analytes in processed samples on storage in autosampler vials at room temperature for 2 h was also evaluated. The effect of dilution was estimated by analysis of six replicates of rat plasma spiked with analytes at 1 mg/mL and diluting with blank rat plasma to concentrations of 30, 300, and 2400 ng/mL.

2.6. Pharmacokinetic study

Six rats (three males, three females, weight 200 ± 15 g) were obtained from the Animal Center of Jilin University. Animal welfare and experimental procedures were carried out in accordance with the guidance for the care and use of laboratory animals of the National Research Council of USA, 1996 and related ethical regulations of Jilin University. After a 24 h fast, each rat was given a single caudal vein intravenous (i.v.) of 1 mL Kudiezi Injection which contained 453 μg I, 477 μg II, 267 μg III, 175 μg IV, 106 μg V. Blood samples (150 μL) were collected into heparinized tubes before the dose and at 3, 8, 15, 30, 45, 60, 90, 120, 240, 360 and 480 min after dosing. Plasma was separated by centrifugation at 2500 \times g for 10 min and stored at –80 °C until analysis. Pharmacokinetic parameters were calculated using the software DAS 3.0.

3. Results and discussion

3.1. Sample preparation

Protein precipitation with methanol, acetonitrile and perchloric acid (HClO_4) was evaluated and found to produce asymmetrical

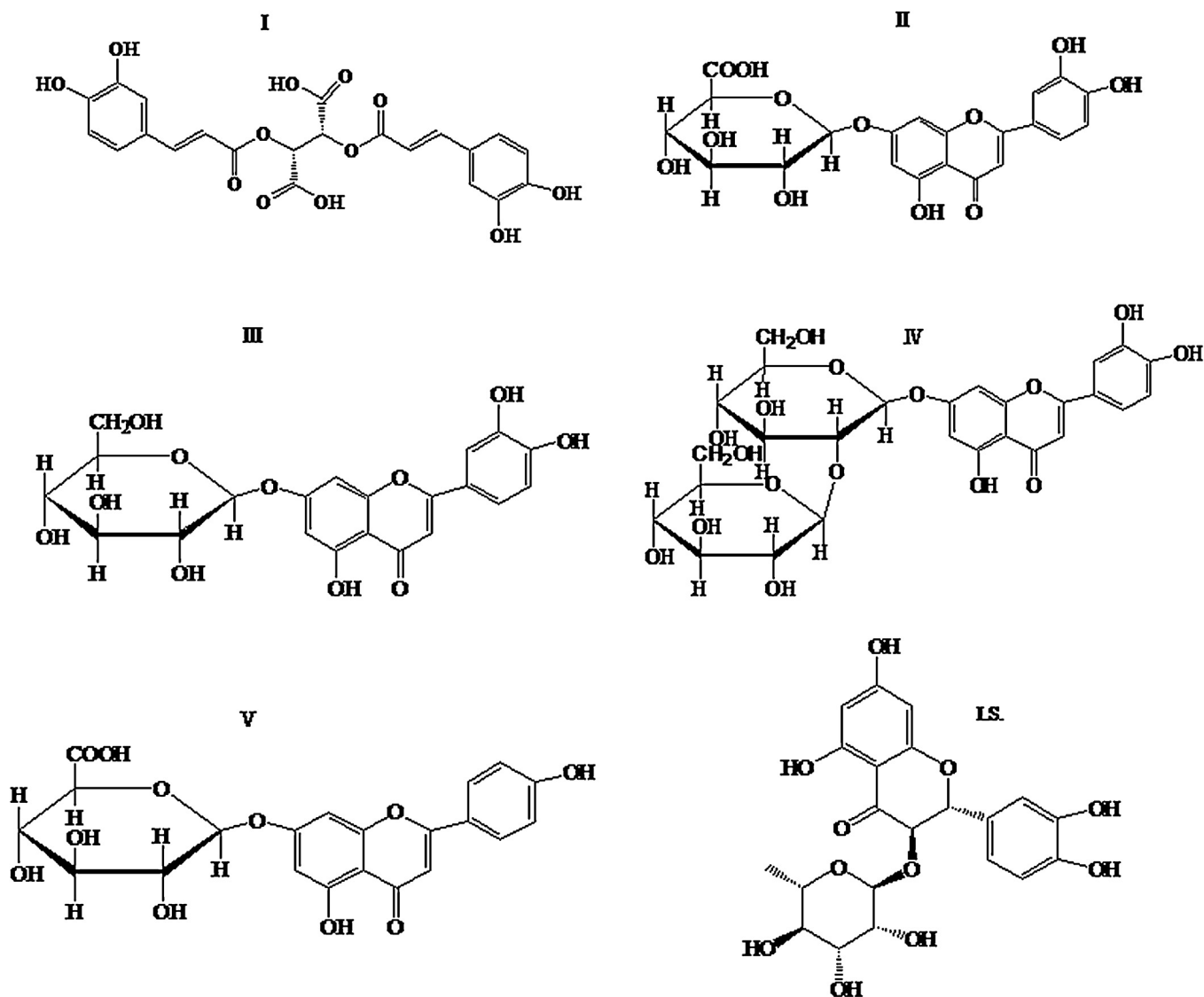


Fig. 1. Chemical structures of the analytes I chicoric acid, II luteolin 7-O-β-D-glucuronide, III luteolin 7-O-β-D-glucopyranoside, IV luteolin 7-O-β-D-glucopyranosyl-(1→2)-β-D-glucopyranoside, V apigenin 7-O-β-D-glucuronide, and I.S., astilbin.

peak shapes with poor sensitivity and significant matrix effects. LLE with different organic solvents (dichloromethane, diethyl ether, hexane and ethyl acetate) was evaluated and found to give relative stable and reproducible extraction of analytes and I.S. from acidified sample using ethyl acetate. It also provided minimal interference, low matrix effects and high sensitivity.

3.2. LC-MS/MS conditions

In this study, analytes and I.S. responded well to negative ionization, giving deprotonated molecule as the major species. MRM utilized transitions to the principal product ions for all analytes and I.S.

3.3. Chromatographic conditions

To optimize chromatographic conditions, a number of commercially available C18 columns (Zorbax SB-C18, Zorbax SB-Aq, Zorbax extend-C18, Venusil MP-C18) and various mobile phases were evaluated. The latter involved testing different ratios of organic solvent (methanol, acetonitrile) to water and inclusion of pH modifiers

(ammonium acetate, formic acid, ammonia) in the water phase. On addition of formic acid, ionization efficiency and sensitivity of all analytes decreased and, on addition of ammonia, retention of I, II and IV was too low. The Zorbax SB-C18 column and elution with acetonitrile – 10 mM ammonium acetate (60:40, v/v, pH 5.6) at 1.0 mL/min gave adequate retention, symmetrical peak shape and satisfactory mass spectrometric responses of analytes and I.S.

3.4. Selection of I.S.

Astilbin was adopted as I.S. due to the similarity of its retention time and extraction efficiency with the analytes, and its efficient ionization in the negative ionization mode.

3.5. Method validation

Compounds I–V and I.S. eluted within 2 min and total run time of 2.5 min allowed high sample throughput. Typical MRM chromatograms of a plasma sample from a rat 3 min after i.v. administration of Kudiezi Injection are shown in Fig. 2. The assay was linear for analytes I–V in the range 10–3000 ng/mL with

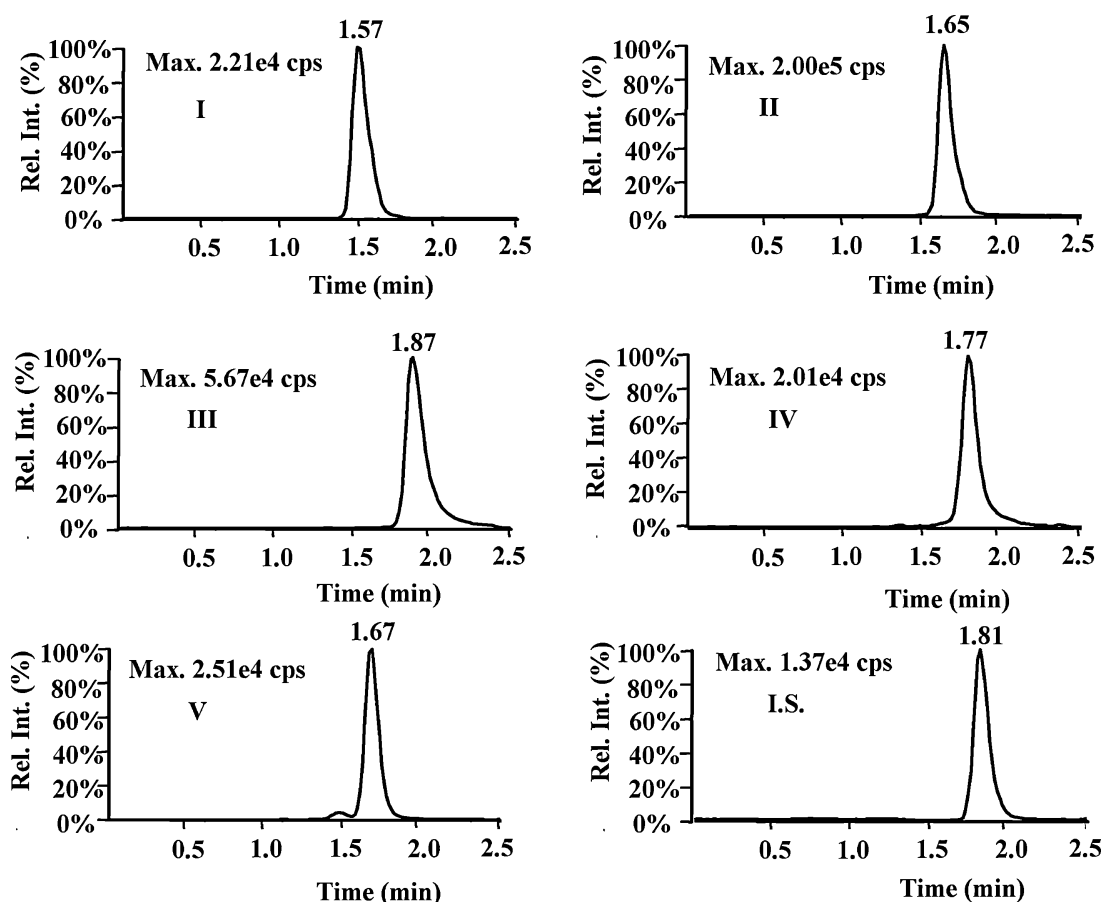


Fig. 2. Representative MRM chromatograms of I chicoric acid, II luteolin 7-*O*- β -D-glucuronide, III luteolin 7-*O*- β -D-glucopyranoside, IV luteolin 7-*O*- β -D-glucopyranosyl-(1 \rightarrow 2)- β -D-glucopyranoside, V apigenin 7-*O*- β -D-glucuronide and astilbin (I.S.) from a rat 3 min after intravenous administration of Kudiezi solution.

Table 1

Absolute recoveries (%) for I chicoric acid, II luteolin 7-*O*- β -D-glucuronide, III luteolin 7-*O*- β -D-glucopyranoside, IV luteolin 7-*O*- β -D-glucopyranosyl-(1 \rightarrow 2)- β -D-glucopyranoside and V apigenin 7-*O*- β -D-glucuronide in rat plasma. (Data are mean \pm S.D. for $n=6$).

	Recovery (%)		
	Low QC	Medium QC	High QC
I	94.4 \pm 1.7	94.8 \pm 1.9	94.6 \pm 2.0
II	83.0 \pm 1.8	84.3 \pm 2.2	81.9 \pm 1.2
III	47.4 \pm 1.7	48.7 \pm 2.5	48.2 \pm 1.3
IV	36.2 \pm 1.9	36.7 \pm 1.9	35.9 \pm 1.3
V	67.4 \pm 2.4	65.1 \pm 2.3	66.9 \pm 1.8

typical regression equations of $y=0.00231x-0.00037$ ($r=0.9996$), $y=0.0016x+0.0948$ ($r=0.9987$), $y=0.00184x-0.00605$ ($r=0.9984$), $y=0.000417x-0.00172$ ($r=0.9993$), $y=0.000773x-0.00111$ ($r=0.9981$), respectively. The LLOQs were 10 ng/mL for all analytes. The limits of detection (LOD) were 5, 1, 5, 5, 2 ng/mL for analytes I–V. The intra- and inter-day accuracy was <8.2 and 9.0%, respectively.

The recoveries for each analyte are shown in Table 1. The results show that although the recoveries are markedly different, they are all reproducible across the concentration range studied. The recovery for the I.S. was $95.3 \pm 0.4\%$.

In terms of matrix effects, actual concentrations (mean \pm S.D.) as percentage of nominal concentration for low, medium and high QC samples respectively were as follows: I, 90.9 ± 3.1 , 94.1 ± 3.2 , 93.5 ± 2.9 ; II, 83.4 ± 1.3 , 82.2 ± 1.3 , 84.6 ± 2.3 ; III, 98.2 ± 3.4 , 97.0 ± 1.1 , 97.4 ± 2.0 ; IV, 90.1 ± 2.7 , 90.2 ± 1.1 , 89.2 ± 1.7 ;

Table 2

Mean pharmacokinetic parameters for I: chicoric acid, II: luteolin 7-*O*- β -D-glucuronide, III: luteolin 7-*O*- β -D-glucopyranoside, IV: luteolin 7-*O*- β -D-glucopyranosyl-(1 \rightarrow 2)- β -D-glucopyranoside and V: apigenin 7-*O*- β -D-glucuronide in rat plasma after an intravenous administration of 1 mL Kudiezi Injection.

Parameters	C_{max} ($\mu\text{g/mL}$)	$AUC_{0-\infty}$ ($\mu\text{g} \times \text{min/mL}$)	CL (mL/min/kg)	$T_{1/2}$ (min)
I	8.4 \pm 1.1	904 \pm 17.5	2.1 \pm 0.5	112 \pm 16
II	14.2 \pm 3.7	233 \pm 68.2	12.1 \pm 1.3	30.1 \pm 4.5
III	3.5 \pm 0.4	44.1 \pm 3.8	26.5 \pm 1.6	21.6 \pm 3.1
IV	3.3 \pm 0.4	45.8 \pm 8.0	16.1 \pm 1.8	25.0 \pm 4.2
V	2.0 \pm 0.3	37.2 \pm 10.5	17.9 \pm 5.2	13.4 \pm 2.2

V, 94.7 ± 2.5 , 95.1 ± 2.2 , 96.5 ± 1.9 . The results were all within $\pm 20\%$ of nominal the theoretical values, indicating that plasma matrix effects were negligible.

In terms of stability, concentrations under the various test conditions were all within $\pm 12.1\%$ of nominal concentrations indicating no significant degradation of the analytes was observed under any of the storage conditions tested.

3.6. Pharmacokinetic study

Plasma concentration–time profiles for the five analytes are illustrated in Fig. 3 with corresponding pharmacokinetic parameters summarized in Table 2. Concentrations of compounds II–V are close to the LLOQ from 200 min after the dose. They are glucose or glucuronic acid conjugates which make them highly polar and soluble in water. Their higher clearances probably reflect efficient renal

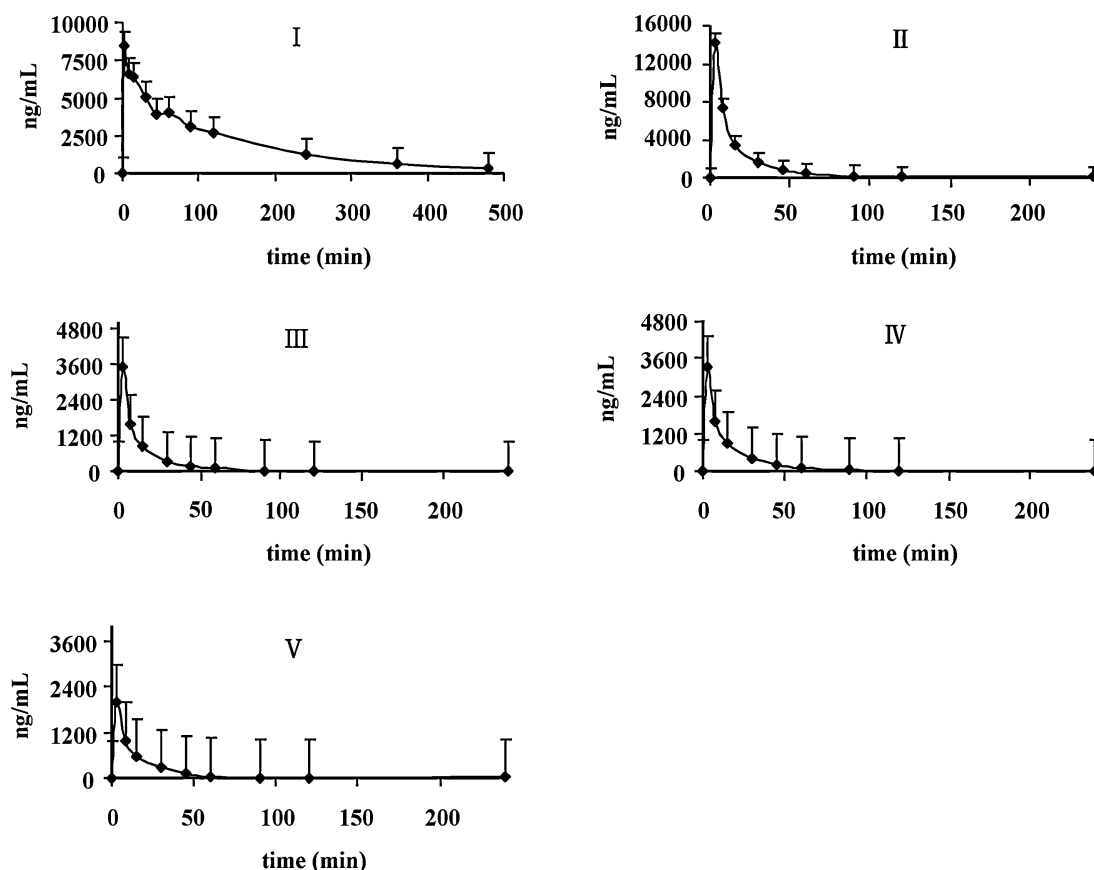


Fig. 3. Mean plasma concentration–time curves for I chicoric acid, II luteolin 7-*O*- β -D-glucuronide, III luteolin 7-*O*- β -D-glucopyranoside, IV luteolin 7-*O*- β -D-glucopyranosyl-(1 \rightarrow 2)- β -D-glucopyranoside and V apigenin 7-*O*- β -D-glucuronide after intravenous administration of 1 mL Kudiezi Injection. Data are mean + S.D. for six rats.

elimination. Conversely, the clearance of compound I is the lowest and presumably reflects a slower liver metabolic biotransformation prior to renal clearance. In general, the relative clearance and $t_{1/2}$ values are consistent. The $t_{1/2}$ values of II–IV are shorter than those reported in literature [19], which studied in beagle dogs. The phenomenon may attribute to species variance. The reason why the clearance of compound III is the fastest is unclear. The results demonstrate that the method is suitable for pharmacokinetic studies of the five analytes after intravenous administration of Kudiezi Injection.

4. Conclusions

A sensitive, high throughput LC–MS/MS assay for the simultaneous determination of five major active compounds of *I. sonchifolia* (Bge.) Hance in rat plasma has been developed and validated. The method involves simple sample preparation and gives high sensitivity using only 50 μ L plasma. It also has a short run time (2.5 min) allowing high throughput analysis. The method has been successfully applied to a pharmacokinetic study in rats after intravenous administration of Kudiezi Injection.

Acknowledgments

This research was supported by the National Natural Science Foundation of China (Grant nos. 30973587 and 81102383), the Science and Technology Major Specialized Projects for “significant new drugs creation” of the 12th five-year plan (2012ZX09303-015) and the National Key Technology R&D Program of the Ministry of Science and Technology (2012BAI30B00).

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