Journal of Chromatography B, 868 (2008) 13–19

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

A sensitive liquid chromatography–mass spectrometry method for simultaneous determination of two active chromones from *Saposhnikovia* root in rat plasma and urine

Jinna Dai, Xiaohui Chen, Weiming Cheng, Xiao Liu, Xue Fan, Zhenduo Shen, Kaishun Bi*

School of Pharmacy, Shenyang Pharmaceutical University, 103 Wenhua Road, Shenyang, Liaoning 110016, PR China

ARTICLE INFO

Article history:
Received 3 January 2008
Accepted 30 March 2008
Available online 15 April 2008

Keywords:
Saposhnikovia divaricata (Turcz.) Schischk.
Prim-*O*-glucosylcimifugin
4′-*O*-D-glucosyl-5-*O*-methylvisamminol
LC–MS
Pharmacokinetics

ABSTRACT

A sensitive and efficient liquid chromatography–mass spectrometry method was developed and validated for the simultaneous determination of two active chromones (prim-*O*-glucosylcimifugin and 4′-*O*-D-glucosyl-5-*O*-methylvisamminol) from *Saposhnikovia* root in rat plasma and urine. The plasma or urine samples were prepared by protein precipitation. Chromatographic separation of the two active chromones from matrix interferences was achieved on an Angilent TC-C_{18} column with a mobile phase consisted of methanol, water and 0.1% formic acid. Puerarin was added as the internal standard. The method was validated with the concentration range 1.0–100 ng/mL in rat plasma and 10–1000 ng/mL in urine for prim-*O*-glucosylcimifugin, 1.5–150 ng/mL in plasma and 15–1500 ng/mL in urine for 4′-*O*-D-glucosyl-5-*O*-methylvisamminol. The lower limit of quantitation (LLOQ) of prim-*O*-glucosylcimifugin and 4′-*O*-D-glucosyl-5-*O*-methylvisamminol was 1.0 and 1.5 ng/mL in plasma, 10 and 15 ng/mL in urine, respectively. The intra- and inter-day precision across three validation days over the entire concentration range was lower than 9.0% as terms of relative standard deviation (R.S.D.). Accuracy determined at three quality control concentrations (2.0, 25 and 75 ng/mL for prim-*O*-glucosylcimifugin; 3.0, 37.5 and 112.5 ng/mL for 4′-*O*-D-glucosyl-5-*O*-methylvisamminol) ranged from −1.9 to 3.9% as terms of relative error (R.E.). The LC–ESI–MS method was further applied to assess pharmacokinetics and urine excretion of the two chromones after oral administration of Fangfeng extract to rats. Practical utility of this new LC–MS method was confirmed in pilot pharmacokinetic studies in rats following oral administration.
© 2008 Elsevier B.V. All rights reserved.

1. Introduction

Fangfeng, the root of *Saposhnikovia divaricata* (Turcz.) Schischk., is an important member of traditional Chinese medicines (TCM). In the classic Chinese Materia Medica, Shen Nung Pen Tsao Ching, Fangfeng is listed as a high-grade drug, which means high effectiveness and little toxicity. It is widely applied for headache, febrility, vertigo and arthralgia due to "wind, cold and dampness" in China, Japan and other Asian countries. And it is also used in various prescriptions, including those for the treatment of generalized aching, inflammatory symptoms and cardiovascular diseases [1–4].

Modern pharmacological experiments using the extract of Fangfeng have proved its various activities, such as suppression of adjuvant arthritis, inhibitory effects on the CNS and peptic ulcers and analgesic, anti-convulsant, anticancer, anti-inflammatory and anticoagulant activities, etc. [5–10]. Many chemical studies on this herb have also been done, chromones, coumarins, polyacetylenes and some other compounds were isolated [11–13]. In recent years, it has been identified that the chromones are the main active constituents which contribute most to its pharmacological efficacy, such as analgesic, antifebric and anti-inflammatory activities. Prim-*O*-glucosylcimifugin and 4′-*O*-D-glucosyl-5-*O*-methylvisamminol are the two major active chromones isolated from Fanfeng (Fig. 1), which were demonstrated to possess analgesic, anti-inflammatory effect and suppression of platelet aggregation and they may possess the potential clinical applications such as treating acute and dull aching, infarction, and anti-inflammatory conditions [5,14]. Because of widely utilization of Fangfeng and its preparation in and out of China, there are a number of reports about determination of the two active chromones in the herb and its preparations by using thin layer chromatography (TLC) and HPLC [15–18]. However, those methods were not sensitive enough for pharmacokinetic studies. Up to now, no analytical method has been reported for quantitation

* Corresponding author. Tel.: +86 24 23986259; fax: +86 24 23986259.
E-mail addresses: bikaishun@yahoo.com, kaishunbi@yahoo.com.cn (K. Bi).

1570-0232/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2008.03.031

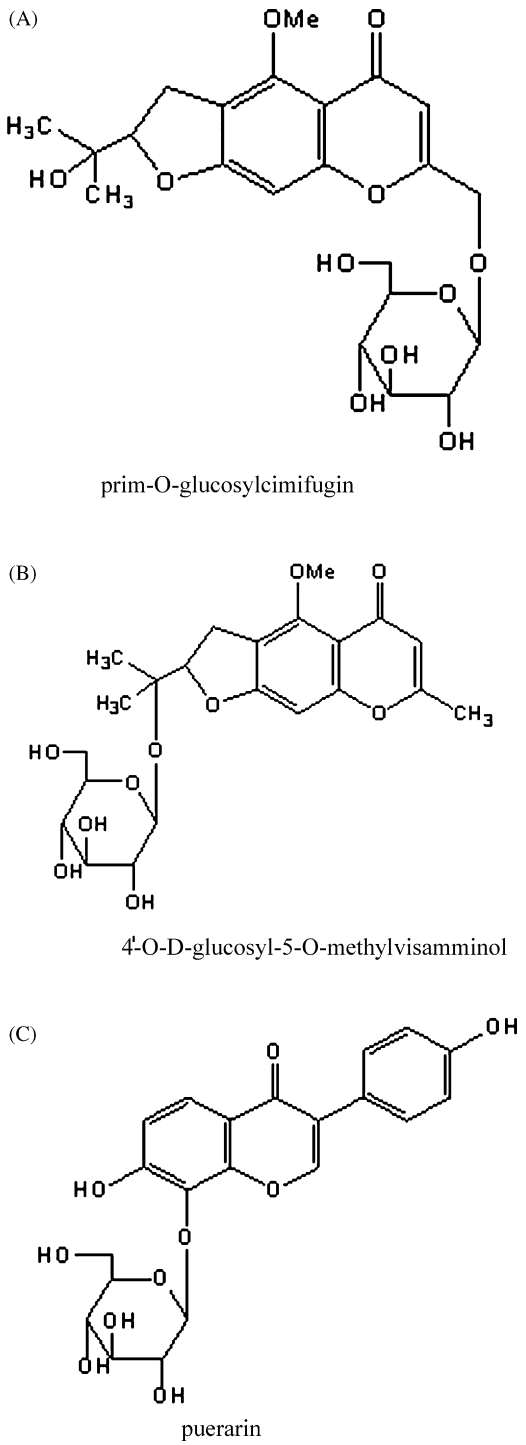

Fig. 1. Chemical structures of prim-*O*-glucosylcimifugin, 4′-*O*-D-glucosyl-5-*O*-methylvisamminol and puerarin (internal standard) for the analysis.
[Please note: (A) structure of prim-*O*-glucosylcimifugin; (B) structure of 4′-*O*-D-glucosyl-5-*O*-methylvisamminol; (C) structure of puerarin (internal standard)].

of these two active constituents, even for any chromone compound in biological samples.

To elucidate the ADME process of Fangfeng in vivo, a selective and sensitive high performance liquid chromatographic technique coupled with mass spectrometry detection (LC–MS) method was developed for the simultaneous determination of prim-*O*-glucosylcimifugin and 4′-*O*-D-glucosyl-5-*O*-methylvisamminol in rat plasma and urine. Using the method described, pharmacokinetic study of these two active chromones in rat was investigated after single oral administration of Fangfeng extract.

2. Experimental

2.1. Chemicals and drugs

Prim-*O*-glucosylcimifugin and 4′-*O*-D-glucosyl-5-*O*-methylvisamminol (Lot No. 111522 and 111523, respectively) were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Shanghai, China). Puerarin (purity > 95%, internal standard, IS) was provided by Beiqi Co. (Dandong, China). Methanol and acetonitrile (HPLC grade) were obtained from TEDIA Company (Tedia Fairfield, OH, USA). All other reagents were analytical purity. Distilled water, prepared from demineralized water, was used throughout the experiment.

2.2. Animals

Twelve male pathogen-free Wistar rats (200–220 g) were provided by the Experimental Animal Center of Shenyang Pharmaceutical University (Shenyang, China), and six for each group were housed to a cage with a standard laboratory diet and water for at least 3 days before the experiments. Animal study was carried out in accordance with the Guidelines for Animal Experimentation of Shenyang Pharmaceutical University (Shenyang, China) and the protocol was approved by the Animal Ethics Committee of the institution.

2.3. Standard solutions

A stock solution containing prim-*O*-glucosylcimifugin and 4′-*O*-D-glucosyl-5-*O*-methylvisamminol was prepared in methanol at the concentration of 0.10 and 0.15 mg/mL, respectively. Then it was serially diluted with methanol to 1.0, 2.0, 10, 25, 50, 100 ng/mL for prim-*O*-glucosylcimifugin, and 1.5, 3.0, 15, 37.5, 75, 150 ng/mL for 4′-*O*-D-glucosyl-5-*O*-methylvisamminol. Internal standard puerarin (Fig. 1) was prepared in methanol at a concentration of 0.04 mg/mL.

Quality control (QC) solutions of prim-*O*-glucosylcimifugin (2.0, 25 and 75 ng/mL) and 4′-*O*-D-glucosyl-5-*O*-methylvisamminol (3.0, 37.5, and 112.5 ng/mL) were independently diluted. All solutions were stored at 4 °C prior to use.

2.4. Apparatus and chromatographic conditions

The HPLC system consisted of a DGU-14 AM degasser, two Shimadzu 10ADvp Pumps, a high pressure mixer, an automatic thermostatic column compartment and a Shimadzu 10ATvp Autoinjector (Shimadzu, Kyoto, Japan). Chromatographic separation of the analytes was performed on an Angilent TC-C_{18} column (150 mm × 4.6 mm i.d., particle size 5 μm, Angilent, USA), equipped with an ODS security guard column (10 mm × 4.6 mm i.d., particle size 5 μm, Zhonghuida Co., China). The mobile phase was a methanol–water mixture containing 0.1% formic acid. The column was eluted under a simple gradient condition with a flow rate of 0.8 mL/min at ambient temperature (Table 1.).

Table 1
Elution program for HPLC analysis

Time (min)	Volume percent of methanol (%)
0	30
3.5	45
7.5	45
9.5	30

A Shimadzu 2010 liquid chromatograph–mass spectrometer (Shimadzu, Kyoto, Japan) equipped with an electrospray ionization (ESI) interface, and a Q-array-Octapole-Quadrupole mass analyzer (QoQ system) was used. Mass spectrometric conditions were optimized to obtain the maximum sensitivity. The analytes were detected with electrospray ionization in positive-ion mode. Mass spectra were obtained at a dwell time of 0.2 s in selected-ion monitoring (SIM) mode and 1 s in full-scan mode. For both modes, the settings for the mass spectrometer were as follows: curve dissolution line (CDL) voltage 10.0 kV, CDL temperature 250 °C, heat block temperature 200 °C, detector voltage 1.50 kV, nebulizing gas flow rate 2.5 L/min. The analytes were ionized to $[M+H]^+$ and monitored at m/z 469, 453 and 417 for prim-*O*-glucosylcimifugin, 4′-*O*-D-glucosyl-5-*O*-methylvisamminol and IS, respectively. Data analysis was performed by LC–MS Solution Version 3.0.

2.5. Sample preparation

2.5.1. Plasma sample

The internal standard puerarin 20 μL, precipitant acetonitrile 300 μL and methanol 100 μL (volume of the corresponding standard solution for calibration curve and QC samples) were added to 100 μL of plasma sample. The mixture was vortexed for 3 min and centrifuged at 1000 × *g* for 5 min. The supernatant (400 μL) was transferred to a 1 mL polypropylene tube, and evaporated to dryness under nitrogen at 35 °C. The residue was reconstituted in 100 μL of methanol, centrifuged at 10,000 × *g* for 3 min and transferred to an autosampler microvial for injection into the HPLC system. The ratio of respective peak area of two substances over the internal standard was used for quantitative analysis.

2.5.2. Urine sample

The sample processing of urine samples was the same as plasma samples as described above except that the urine was diluted to 10 times volume with the corresponding blank matrix prior to sample preparation.

2.6. Method validation

The calibration curves were generated to confirm the linear relationship between the peak area ratio and the concentration of the two substances in the test samples on three separate days. A series of standard solution were added to blank plasma to yield final concentrations of 1.0, 2.0, 10, 25, 50, 100 ng/mL for prim-*O*-glucosylcimifugin, and 1.5, 3.0, 15, 37.5, 75, 150 ng/mL for 4′-*O*-D-glucosyl-5-*O*-methylvisamminol in 0.1 mL plasma. The plasma samples, with known amount of the two compounds, were prepared and analyzed as described above. Calibration was performed by least-squares linear regression of the peak area ratios of the two drugs to the IS versus the nominal standard concentration with a weighted (1/square of concentration) factor. The standard curve was conducted by plotting the peak area ratio against the drug concentration prepared.

The QC samples at low, medium and high concentrations and the LLOQ samples were analyzed to validate the accuracy and precision of the described method. Intra-day and inter-day accuracy and precision were determined over a period of 3 consecutive days with six replications at each concentration per day (n = 18). The precision was evaluated as the relative standard deviation (R.S.D.), while the accuracy was expressed as the relative error (R.E.).

The extraction recovery of internal standard puerarin, prim-*O*-glucosylcimifugin and 4′-*O*-D-glucosyl-5-*O*-methylvisamminol was determined by comparing the response of the analytes extracted from replicating QC samples (n = 18) with the response of the analytes from non-extracted standard solutions at equivalent concentration.

2.7. Stability testing

The stability of prim-*O*-glucosylcimifugin, 4′-*O*-D-glucosyl-5-*O*-methylvisamminol in rat plasma was studied under different storage conditions: modeling three freeze–thaw (−20 °C) (ambient temperature) cycles, 12 h storage at room temperature and frozen (−20 °C) for a week.

2.8. Method application

2.8.1. Drug administration and sampling

All the rats were deprived of food but free access to water for 12 h before and during the experiment. After given 10 mL/kg of Fangfeng extract (containing 25.3 μg/mL of prim-*O*-glucosylcimifugin and 23.6 μg/mL of 4′-*O*-D-glucosyl-5-*O*-methylvisamminol) to each rat orally, six rats were used to collect the blood samples, and the other six were used for the urine samples. The plasma samples (0.3 mL) were obtained at 0, 0.17, 0.33, 0.50, 0.75, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, and 12 h after drug given, then they were placed in heparinized tubes and separated by centrifugation at 1000 × *g* for 5 min, finally stored at −20 °C until analysis. The urine samples were collected during the following time range 0–2, 2–4, 4–6, 6–8, 8–12, 12–24, 24–48, and 48–72 h after oral administration of the same dose. The actual volume of each urine sample was accurately recorded and the samples were stored −20 °C until analysis.

2.8.2. Pharmacokinetics analysis

All the pharmacokinetic parameters were determined by non-compartmental analysis using DAS 2.0 pharmacokinetic program (Chinese Pharmacological Society). 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.

3. Results and discussion

3.1. Sample preparation

Because of little report on chromones, in the early method development stage, we investigated different extraction ways according to the studies of the other TCM [19–21], such as liquid–liquid extrac-

Table 2
Extraction recovery (%) of prim-*O*-glucosylcimifugin and 4′-*O*-D-glucosyl-5-*O*-methylvisamminol on different extracting way

	Liquid–liquid extraction (LLE)				Protein precipitation (PPT)		Aqueous bath
	Aether	EAc	EAc-IPA (95:5, v/v)	*n*-Hexane–DCM–IPA (5:4:1, v/v/v)	Me	AN	100 °C 10 min
Prim-*O*-glucosylcimifugin	15.6	26.7	22.4	19.6	67.6	65.1	11.4
4′-*O*-D-glucosyl-5-*O*-methylvisamminol	21.7	28.6	26.5	18.5	80.3	84.6	22.5

EAc: ethyl acetate; IPA: isopropanol; DCM: dichloromethane; Me: methanol; AN: acetonitrile.

tion (LLE), protein precipitation (PPT), aqueous bath extraction. Aether, ethyl acetate, ethyl acetate–isopropanol (95:5, v/v), and *n*-hexane–dichloromethane–isopropanol (5:4:1, v/v/v) was also tested as the extraction solvent. But the recovery of the analytes was less than 30% and the sensitivity was not enough for analysis (Table 2). That may be due to the polarity reason, and the water-soluble character of two drugs makes it difficult to extract from plasma by LLE. Aqueous bath extraction is a new method for extracting water-soluble ingredients from biological samples after oral administration of TCM. But prim-*O*-glucosylcimifugin was unsteady at high temperature, and the recovery was not satisfactory either. Comparatively, protein precipitation is a simple method and easy to control operating conditions. Finally acetonitrile was chosen as protein precipitant. Adequate sensitivity was obtained for the HPLC–MS analysis.

3.2. LC–ESI–MS optimization

The high specificity and sensitivity of the established LC–ESI–MS method has made it possible to simultaneously determine the two active chromones in rat plasma and urine. In this paper, prim-*O*-glucosylcimifugin, 4′-*O*-D-glucosyl-5-*O*-methylvisamminol and internal standard puerarin were well separated by gradient elution and detected by electrospray positive-mode ionization mass spectrometry. Full-scan positive-ion mass spectra of prim-*O*-glucosylcimifugin, 4′-*O*-D-glucosyl-5-*O*-methylvisamminol and internal standard puerarin showed predominate fragments at *m*/*z* 469, 453 and 417 ($[M+H]^+$ peak), respectively (Fig. 2). Typical chromatograms of rat plasma and urine samples showed well-shaped and base-line separated peaks, without any interference (Fig. 3).

APCI interface was also tested, but the sensitivity was lower and the response was nonlinear during the test range as used with ESI interface. That may be due to the structure of glycoside in the two drugs was not stable enough and easily yielded some other fragments when ionized by APCI. In addition, isocratic elution was utilized originally, but the endogenous substances interfered with the analysis of the two compounds, so we generated a simple gradient elution program for the better separation. Addition of 0.1% formic acid enhanced the sensitivity and the symmetry of the peaks significantly.

Compared the chromatograms of rat urine samples with the plasma samples, some new peaks (Fig. 3C and D) were detected. They increased with time firstly and then decreased until disappeared, indicating that they might be the metabolites of prim-*O*-glucosylcimifugin and 4′-*O*-D-glucosyl-5-*O*-methylvisamminol. The metabolic process of the two active constituents in vivo is on-going investigation.

3.3. Method validation

The standard curve of prim-*O*-glucosylcimifugin was linear with $r \geq 0.997$ during the quantitive range from 1.0 to 100 ng/mL in rat plasma, from 10 to 1000 ng/mL in urine. The standard curve of 4′-*O*-D-glucosyl-5-*O*-methylvisamminol was linear with $r \geq 0.998$ during the quantitive range from 1.5 to 150 ng/mL in rat plasma, from 15 to 1500 ng/mL in urine (Table 3). The LLOQ of prim-*O*-glucosylcimifugin and 4′-*O*-D-glucosyl-5-*O*-methylvisamminol was 1.0 and 1.5 ng/mL in plasma; 10 and 15 ng/mL in urine. Precision and accuracy data confirmed the good reproducibility of the described method (Table 4).

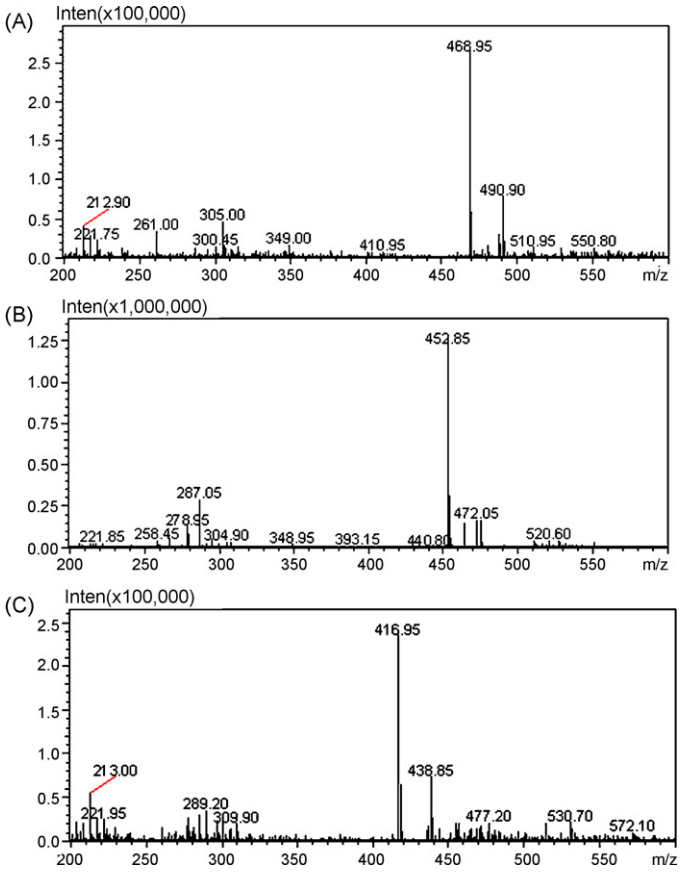

Fig. 2. The product ion mass spectra of prim-*O*-glucosylcimifugin (A), 4′-*O*-D-glucosyl-5-*O*-methylvisamminol (B) and puerarin (C), internal standard for the analysis.
[Please note: (A) mass spectra of prim-*O*-glucosylcimifugin; (B) mass spectra of 4′-*O*-D-glucosyl-5-*O*-methylvisamminol; (C) mass spectra of puerarin (internal standard)].

The recovery of prim-*O*-glucosylcimifugin and 4′-*O*-D-glucosyl-5-*O*-methylvisamminol was 53.1–77.2 and 75.2–97.3%, respectively (Table 4). The mean extraction recovery of internal standard puerarin was 74.3%.

3.4. Stability

The stability study showed that the two active chromones were stable within three freeze–thaw cycles, and showed no significant degradation for 12 h at ambient temperature, even for 7 days at −20 °C (Table 5). Prim-*O*-glucosylcimifugin was reported unstable,

Table 3
Calibration curves for analysis of prim-*O*-glucosylcimifugin and 4′-*O*-D-glucosyl-5-*O*-methylvisamminol in rat plasma ($n = 6$)

	Prim-*O*-glucosylcimifugin			4′-*O*-D-glucosyl-5-*O*-methylvisamminol		
	Slope	Intercept	*r*	Slope	Intercept	*r*
1st day	0.0014	0.0008	0.9981	0.0027	0.0013	0.9984
2nd day	0.0012	0.0017	0.9986	0.0023	−0.0006	0.9983
3rd day	0.0011	0.0018	0.9978	0.0022	0.0015	0.9980

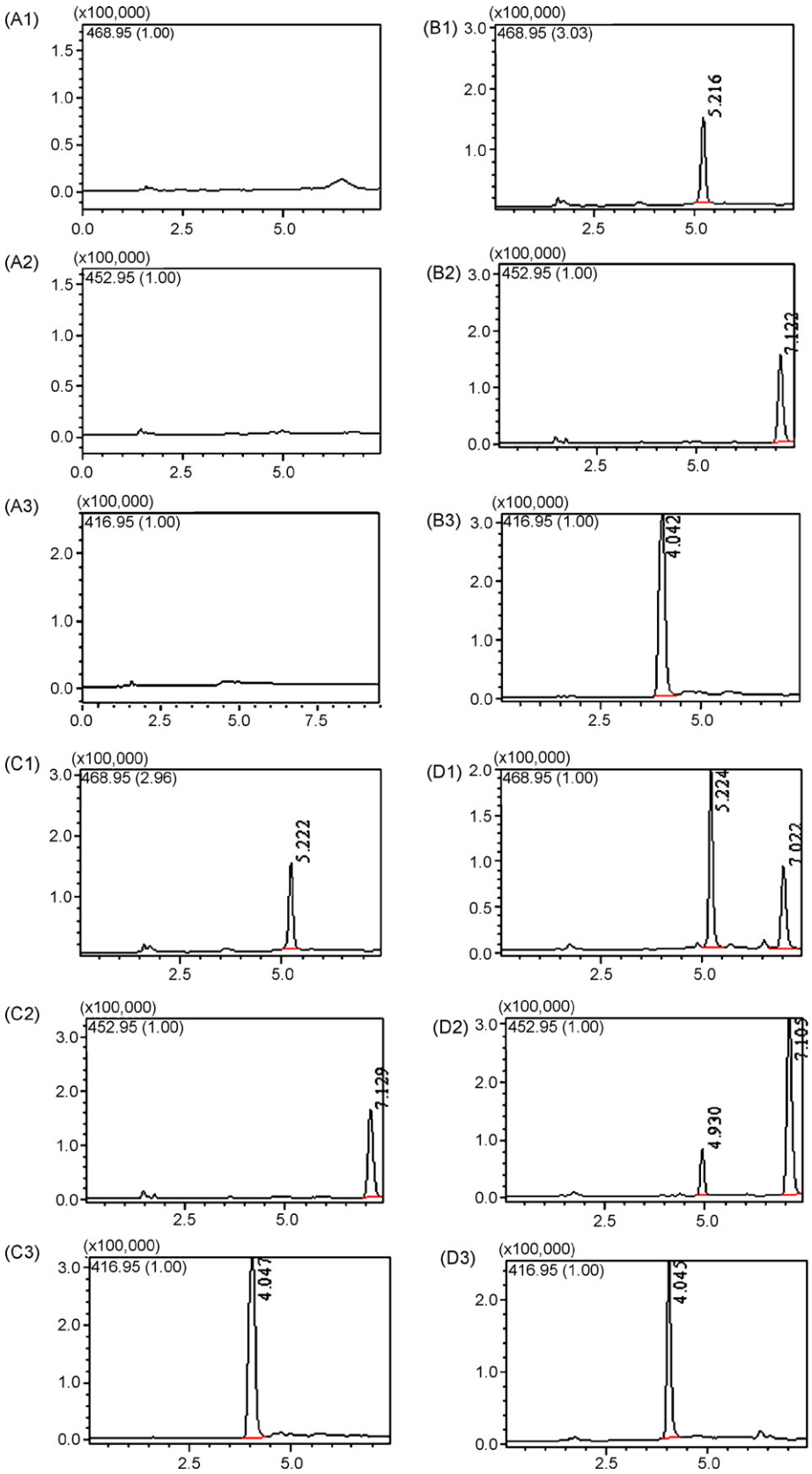

Fig. 3. Chromatograms of blank plasma samples (A), blank plasma added with prim-*O*-glucosylcimifugin, 4′-*O*-D-glucosyl-5-*O*-methylvisamminol and IS puerarin (B), plasma samples from a rat (0.33 h after oral administration of Fangfeng extract) (C) and urine samples from the rat (4 h after oral administration of Fangfeng extract) (D).
1: Prim-*O*-glucosylcimifugin; 2: 4′-*O*-D-glucosyl-5-*O*-methylvisamminol; 3: IS puerarin.
[Please note: from (A-1) to (A-3). Chromatograms of blank plasma samples of prim-*O*-glucosylcimifugin, 4′-*O*-D-glucosyl-5-*O*-methylvisamminol and puerarin; from (B-1) to (B-3) chromatograms of blank plasma added with the three; from (C-1) to (C-3) chromatograms of plasma samples from a rat of the three; from (D-1) to (D-3) chromatograms of urine samples from the rat of the three].

Table 4
Precision and accuracy for analysis of prim-*O*-glucosylcimifugin and 4′-*O*-D-glucosyl-5-*O*-methylvisamminol in rat plasma ($n = 3$ day, six replicates per day)

	Concentration spiked (ng/mL)	Inter-day R.S.D. (%)	Intra-day R.S.D. (%)	Accuracy (%)	Recovery% (mean ± S.D.)
Prim-*O*-glucosylcimifugin	2.0	3.6	7.7	3.9	61.7
	25	4.1	0.5	−1.6	63.4
	75	4.8	2.6	−1.9	61.5
4′-*O*-D-glucosyl-5-*O*-methylvisamminol	3.0	4.8	6.2	−1.0	88.3
	37.5	2.5	9.0	−0.4	87.7
	112.5	2.8	4.0	1.8	89.5

Table 5
Stability for analysis of prim-*O*-glucosylcimifugin and 4′-*O*-D-glucosyl-5-*O*-methylvisamminol in rat plasma ($n = 3$)

	Concentration spiked (ng/mL)	Three freeze–thaw cycles, RE (%)	12 h at ambient temperature, RE (%)	7 days at −20 °C, RE (%)
Prim-*O*-glucosylcimifugin	2.0	2.0	−1.2	−1.8
	25	−2.0	−5.6	−6.0
	75	−4.3	4.3	4.1
4′-*O*-D-glucosyl-5-*O*-methylvisamminol	3.0	5.3	−2.4	8.6
	37.5	−3.0	4.5	−6.0
	112.5	−0.8	−0.2	0.4

Table 6
Pharmacokinetic parameters of prim-*O*-glucosylcimifugin and 4′-*O*-D-glucosyl-5-*O*-methylvisamminol in rats after oral administration of Fangfeng extract

	$T_{1/2}$ (h)	C_{max} (ng/mL)	T_{max} (h)	$AUC_{(0-t)}$ (ng/mL h)	$AUC_{(0-\infty)}$ (ng/mL h)
Prim-*O*-glucosylcimifugin	1.31	39.92	0.54	66.77	75.63
4′-*O*-D-glucosyl-5-*O*-methylvisamminol	1.96	41.53	0.56	65.65	73.05

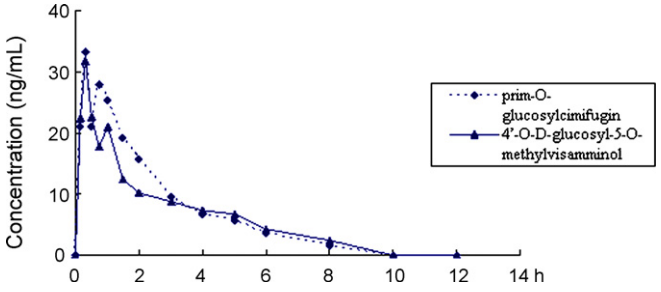

Fig. 4. The concentration–time curve of prim-*O*-glucosylcimifugin and 4′-*O*-D-glucosyl-5-*O*-methylvisamminol in rat plasma after oral administration of Fangfeng extract.

and may change to its aglycone over 45 °C. During our study, the temperature was strictly controlled throughout the experiment, and the results showed that the procedures described above are satisfactory with respect to accuracy, precision and stability.

3.5. Pharmacokinetics study

The plasma drug concentrations of prim-*O*-glucosylcimifugin and 4′-*O*-D-glucosyl-5-*O*-methylvisamminol can be determined by the established LC–ESI–MS method. The concentration versus time curve is presented in Fig. 4. The pharmacokinetic parameters calculated by DAS 2.0 software, and pharmacokinetic parameters are shown in Table 6.

The urine drug concentrations of prim-*O*-glucosylcimifugin and 4′-*O*-D-glucosyl-5-*O*-methylvisamminol can also be determined by the established LC–ESI–MS method. The accumulation of two drugs excreted in the urine after oral administration of Fangfeng extract is presented in Fig. 5. The percent of two drugs excreted in the urine over the dose administered was 49.42 and 56.69% following oral administration.

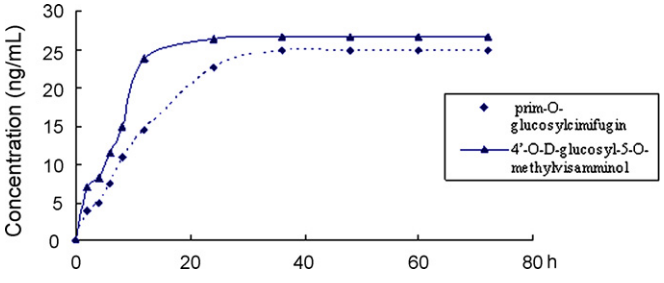

Fig. 5. Accumulation excretion of prim-*O*-glucosylcimifugin and 4′-*O*-D-glucosyl-5-*O*-methylvisamminol into urine after oral administration of Fangfeng extract.

4. Conclusion

A sensitive and accurate analytical LC–MS method was developed for simultaneous determination of the two major active chromones from Fangfeng in rat plasma and urine, and it was successfully applied to a pharmacokinetics study in rats after oral administration of Fangfeng extract. Good linearity and the low LLOQ (1 ng/mL for prim-*O*-glucosylcimifugin, 1.5 ng/mL for 4′-*O*-D-glucosyl-5-*O*-methylvisamminol in plasma) make this method of real practical use for quantification and pharmacokinetics studies in vivo. To our knowledge, it is the first report on simultaneous quantitation of chromone compounds in biological samples. And two metabolites were observed in the rat urines during the experiment, the identification of the metabolites and metabolic process is still under further study in our laboratory.

References

[1] Z.Z. Wu, Sichuanzhongyi 2 (1992) 33.
[2] X.W. Guan, C.M. Gong, J.Z. Dai, S.F. Li, D.C. Gu, Zhongyaoyaoliyulinchuang 6 (1989) 6.
[3] S.X. Chu, Shanghaizhongyiyaozazhi 4 (1991) 31.
[4] R.S. Chen, China J. Chin. Mater. Med. 9 (2000) 574.
[5] E. Okuyama, T. Hasegawa, T. Matsushita, H. Fujimoto, M. Ishibashi, M. Yamazaki, Chem. Pharm. Bull. 2 (2001) 154.
[6] Y. Gao, W.M. Li, X.L. Rong, Q.H. Wu, Y.F. Feng, X.L. Guo, Q. Meng, Chin. Tradit. Herbal Drugs 2 (2005) 254.

[7] L. Zhang, Y. Zhou, X.Q. Ma, J.Z, Niu, Y.R. Lu, L.Y. Zhao, D.Y. Ge, X.Z. Yan, Zhongguoshiyanlinchuanmianyixuezazhi, 5 (1997) 53.
[8] H. Ma, Q. Zhu, X.Y. Sun, Zhongguoyaoliyulinchuang 6 (1998) 21.
[9] L.Q. Guo, M. Taniguchi, Q.Y. Chen, K. Baba, Y. Yamazoe, Jpn. J. Pharmacol. 85 (2001) 399.
[10] H.J. Zhu, H.Y. Zhang, G. Wu, Y.K. Liu, Z.J. Wu, G.Z. Jin, Y.H. Zheng, Zhongcaoyao 12 (1998) 813.
[11] G.Z. Jin, J.D. Li, G.H. Piao, Zhongguozhongyaozazhi 1 (1992) 38.
[12] D.A. Guo, Z.A. Liu, Z.C. Lou, J. Chin. Pharm. Sci. 1 (1992) 81.
[13] Y.Q. Xiao, L.X. Yang, S.L. Cui, X.H. Liu, D. Liu, Zhongguozhongyaozazhi 5 (1995) 294.
[14] B.W. Xue, W. LI, L. Li, Y.Q. Xiao, China J. Chin. Mater. Med. 5 (2000) 297.
[15] L. Li, Y.Q. Xiao, Chin. Pharm. J. 10 (2000) 656.
[16] L. Li, Y.Y. Liu, L.D. Geng, Y.Q. Xiao, China J. Chin. Mate. Med. 3 (2006) 194.
[17] J. Tian, F.M. Li, Chin. Tradit. Patent Med. 9 (2002) 671.
[18] S.X. Liu, J. Wan, Chin. Tradit. Herbal Drugs 12 (2004) 1370.
[19] L. Li, J.L. Zhang, Y.X. Sheng, G. Ye, H.Z. Guo, D.A. Guo, J. Chromatogr. B 808 (2004) 177.
[20] R. Liu, J.H. Sun, K.S. Bi, D.A. Guo, J. Chromatogr. B 829 (2005) 35.
[21] M. Ramadan, B. Watzer, J. Chromatogr. A 1133 (2006) 221.