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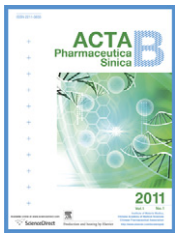

ORIGINAL ARTICLE

HPLC–MS and HPLC–MS/MS analysis of seven active constituents of Xiao-Xu-Ming decoction and application to a pharmacokinetic study after oral administration to rat

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Cimifugin;
5-*O*-methylvisammiol;
Glycyrrhizic acid;
Glycyrrhetinic acid

Abstract Xiao-xu-ming decoction (XXMD) is a traditional Chinese medicine that has been widely used to treat theoplegia and its sequelae. This paper reports the development of three separate assays based on reversed phase high-performance liquid chromatography–mass spectrometry (HPLC–MS) and HPLC–MS/MS for the determination of seven active constituents of XXMD *viz* oroxylin A-7-*O*-glucuronide, wogonoside, liquiritigenin, cimifugin, 5-*O*-methylvisammiol, glycyrrhizic acid and glycyrrhetinic acid in rat plasma. All calibration curves were linear ($r > 0.99$) with lower limits of quantitation (LLOQs) < 12.4 ng/mL. Intra- and inter-day precisions (as relative standard deviation) were all $< 10.7\%$ with recoveries in the range of 88.7–113%. In addition, the seven analytes were shown to be stable in rat plasma samples under relevant storage conditions. The validated methods were successfully applied to a pharmacokinetic study in rat after oral administration of XXMD.

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1. Introduction

Traditional Chinese medicine (TCM) is widely prescribed in China and some Asian countries. The medicines are complex systems in which the various constituents exert a synergistic or antagonistic effect[1]. Studies of the pharmacokinetics (PK) of these multi-component systems usually focus on the active constituents in single-item formulations or simple prescriptions[2]. However, few studies have investigated the PK of complex prescriptions containing multiple herbs. The aim of the current work was to study the PK of the active constituents in the multi-herbal prescription known as Xiao-Xu-Ming Decoction (XXMD).

The use of XXMD was first recorded in the medical text *Bei Ji Qian Jin Yao Fang*, written in the Tang Dynasty by the ancient Chinese therapist, Simiao Sun. The formula includes the following 12 herbs: *Saposhnikovia divaricata* (Turcz.) Schischk., *Scutellaria baicalensis* Georgi, *Paeonia lactiflora* Pall., *Glycyrrhiza uralensis* Fisch., *Zingiber officinale* Rosc., *Stephania tetrandra* S. Moore, *Panax ginseng* C. A. Mey., *Cinnamomum cassia* Presl, *Prunus armeniaca* L. var. *ansu* Maxim., *Ephedra sinica* Staph, *Ligusticum chuanxiong* Hort. and *Aconitum carmichaeli* Debx. in the ratio 3:3:3:3:3:3:6:6:6:9:9:9 on a dry weight basis. The active fraction was screened by Wang et al.[3], and shown to exert a similar pharmacological effect to that of XXMD in treating theoplegia and its sequelae.

In a previous study[4], we reported the active compounds and their metabolites after oral administration of the active fraction of XXMD to rats. Oroxylin A-7-*O*-glucuronide, wogonoside, liquiritigenin, cimifugin, 5-*O*-methylvisammiol, glycyrrhizic acid and glycyrrhetinic acid were all the active components of the active fraction. Oroxylin A showed the neuroprotective or memory enhancing effects through activation of synaptic NMDA receptor and improved Aβ25–35 peptide-induced memory impairment through the GABAergic neurotransmitter system[5,6]. Oroxylin A-7-*O*-glucuronide was the major metabolite of Oroxylin A[7]. Wogonoside has been reported to display anti-allergic and anti-oxidative activities[8,9]. Liquiritigenin showed neuroprotective effect against Aβ25-35-induced neurotoxicity and decreased the secretion of Aβ1-40[10]. Cimifugin and 5-*O*-methylvisammiol, two chromones derived from *Saposhnikovia divaricata* (Turcz.) Schischk., show similar activites such as significant analgesic, antipyretic, anti-inflammatory and anti-platelet aggregation[11]. Glycyrrhizic acid and glycyrrhetinic acid exhibited the activities of anti-allergy, anti-oxidation, anti-inflammatory, anti-viral and anti-cancer[12–16]. Since the activity of the above compounds related to the activity of XXMD, they might be the material basis of XXMD effects. The pharmacokinetic study of the above components would help to elucidate the mechanism of XXMD and provide reference for other research of traditional Chinese medicine.

So far, no analytical method has been reported for systematic pharmacokinetics study of the seven compounds from XXMD by HPLC–MS and HPLC–MS/MS. In this paper, we developed and validated three methods for quantification and pharmacokinetic studies of oroxylin A-7-*O*-glucuronide, wogonoside, liquiritigenin, cimifugin, 5-*O*-methylvisammiol, glycyrrhizic acid and glycyrrhetinic acid in plasma after oral administration of XXMD to rats in order to provide scientific evidence for elucidating the material basis and pharmaceutical mechanism of XXMD and improve the understanding of TCM.

2. Materials and methods

2.1. Chemicals and reagents

The active fraction of XXMD and oroxylin A-7-*O*-glucuronide (purity > 99%) were kind gifts from Prof. Hailin Qin (Institute of Materia Media, Chinese Academy of Medical Science & Peking Union Medical College, Beijing, China). Cimifugin, glycyrrhetinic acid and three internal standards (IS, phenacetin, carbamazepine and icariin) were purchased from National Institute for the Control of Pharmaceutical and Biological Products (purities > 99%, Beijing, China). Wogonoside (purity > 99%) was ordered from the Shanghai Usea Biotech Co., Ltd. (Shanghai, China). Liquiritigenin (purity > 99%) was obtained from the Dalian Fusheng Pharmaceutical Co., Ltd. (Dalian, China). Glycyrrhizic acid (purity > 98%) was purchased from Shanghai Winherb Medical S&T Development Co., Ltd (Shanghai, China). 5-*O*-methylvisammiol was isolated and purified in our laboratory with the purity more than 98% by HPLC-DAD. The chemical structures of 7 compounds from XXMD and IS are shown in Fig. 1.

Acetonitrile of LC/MS reagent grade was obtained from Mallinckrodt Baker, Inc. (Phillipsburg, NJ, USA). Deionized water was purified using a Millipore water purification system (Millipore, Billerica, MA, USA). Analytical grade acetic acid and formic acid were obtained from Merck Inc. (Darmstadt, Germany). Analytical grade methanol and ethyl acetate were purchased from Beijing Chemical Corp. (Beijing, China).

The active fraction of XXMD for oral administration was dissolved in redistilled water with a final concentration of 250 mg/mL, and 0.5% Tween 80 was added as solution adjuvant. The concentrations of liquiritigenin, oroxylin A-7-*O*-glucuronide, wogonoside, cimifugin, 5-*O*-methylvisammioside, glycyrrhizic acid and glycyrrhetinic acid in dosed solution were 0.84, 1.00, 1.50, 1.06, 3.12, 1.60 and 1.13 mg/mL, respectively, determined by HPLC.

2.2. Instrument and analytical conditions

For the determination of cimifugin and 5-*O*-methylvisammiol, the assay was performed on a Thermo Finnigan LTQ system (Thermo Fisher, Co., Ltd., San Jose, CA, USA) consisting of an LC connected to a linear ion trap MS analyzer with an electrospray ionization (ESI) interface available in either positive-ionization or negative-ionization mode. An Xcalibur 1.4 workstation (Thermo Fisher, Co., Ltd., San Jose, CA, USA) was used for LC–MS control and data acquisition. The HPLC separations were achieved using an Inertsil ODS-3 column (100 mm × 2.1 mm, 3 μm). The mobile phase consisted of acetonitrile and 0.1% aqueous formic acid (30:70, *v*/*v*) with a flow rate of 0.2 mL/min for 6 min. The injection volume was set at 10 μL. The temperature of column oven was maintained at 30 °C. The optimum operating parameters of the ESI interface in positive mode were as follows: sheath gas flow rate at 35 arb, aux gas flow rate at 10 arb, sweep gas flow rate at 5 arb, spray voltage at 4.0 kV, capillary temperature at 275 °C, capillary voltage at 4.0 kV and tube lens at 90 V. Quantification was achieved using full scan mode of ion for 5-*O*-methylvisammiol at m/z 290.5–291.5, for cimifugin at m/z 306.5–307.5 and for phenacetin (IS) at m/z 179.5–180.5.

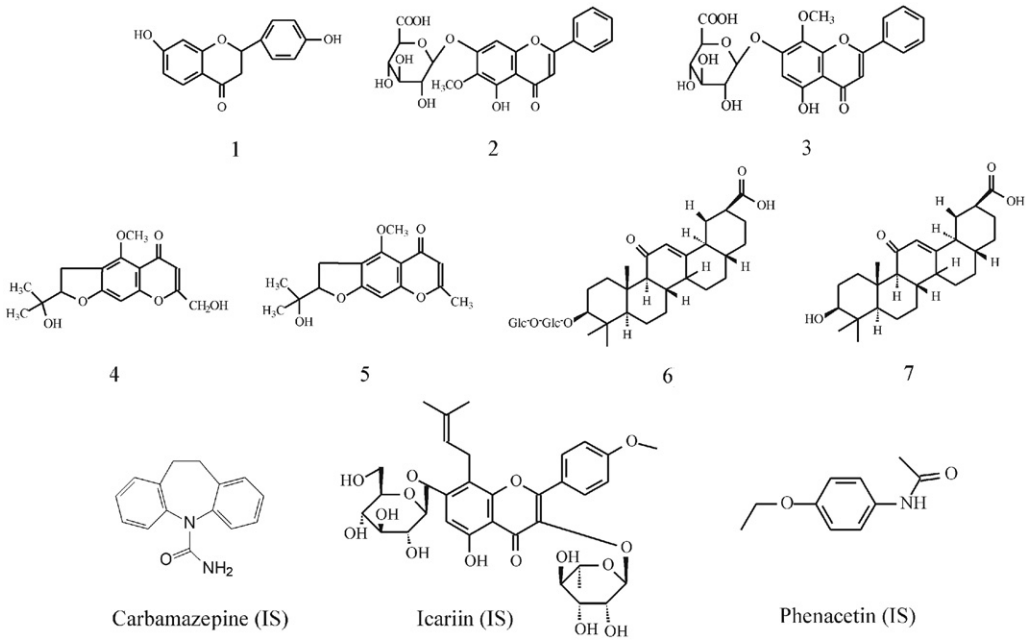

Figure 1 The chemical structures of the seven analytes and three internal standards (IS). **1**, liquiritigenin; **2**, oroxylin A-7-*O*-glucuronide; **3**, wogonoside; **4**, cimifugin; **5**, 5-*O*-methylvisammiol; **6**, glycyrrhizic acid; **7**, glycyrrhetinic acid.

Table 1 Optimized mass parameters for liquiritigenin, oroxylinA-7-*O*-glucuronide, wogonoside, glycyrrhizic acid glycyrrhetinic acid and their internal standards (IS).

Analyte	Precursor ion (m/z)	MS1 Res	Product ion (m/z)	MS2 Res	Dwell (ms)	Fragmentor (V)	CE (V)	Polarity
Liquiritigenin	255	wide	119	Uint	50	120	25	Negative
Oroxylin A-7-*O*-glucuronide	459	wide	268	Uint	50	120	30	Negative
Wogonoside	459	wide	268	Uint	50	120	30	Negative
Icariin(IS)	675	wide	513	Unit	50	180	3	Negative
Glycyrrhizic acid	823.1	Unit	453.3	Unit	200	150	30	Positive
Glycyrrhetinic acid	471	Unit	291.3	Unit	200	135	30	Positive
Carbamazepine (IS)	237.1	Unit	179	Unit	200	135	35	Positive

For the determination of oroxylin A-7-*O*-glucuronide, wogonoside, liquiritigenin, glycyrrhizic acid and glycyrrhetinic acid, the research were performed on an Agilent 6410B triple quadrupole LC–MS system (Agilent Corporation, MA, USA) consisting of an Agilent 1200 RRLC system (Agilent Corporation, MA, USA) connected to a triple quadrupole MS analyzer with an electrospray ionization (ESI) interface usable in either positive-ionization or negative-ionization mode. A MassHunter workstation was used for LC–MS/MS control and data acquisition (Agilent Corporation, MA, USA).

The separation of oroxylin A-7-*O*-glucuronide, wogonoside and liquiritigenin was carried out on a Waters Symmetry C18 column (100 mm × 2.1 mm, 3.5 μm). The mobile phase consisted of acetonitrile and 0.2% aqueous acetic acid (30: 70, *v*/*v*) with a flow rate of 0.3 mL/min for 10 min; the column oven was maintained at 30 °C. The injection volume was set at 5 μL. The optimum operating parameters of the ESI interface were as follows: Source, ESI; Polarity, Negative; Nebulizer, 35 psi; Dry gas, 9 L/min; Dry Temp, 350 °C; Capillary voltage, 4000 V; Delta EMV, 300 V; The LC eluent flow over a period of 0.0–1.0 min was not introduced to the mass spectrometer for data acquisition. Quantification was achieved using multiple-reaction-monitoring (MRM) mode. The operating parameters of MRM were listed in Table 1.

The separation of glycyrrhizic acid and glycyrrhetinic acid was achieved on an Agilent ZORBAX SB-C18 column (100 mm × 2.1 mm, 3.5 μm) with acetonitrile (A)–0.4% aqueous formic acid (B) as mobile phase at a flow rate of 0.2 mL/min, using a liner gradient of 15% B over 0–0.5 min, 15–30% B over 0.5–1 min, 30% B over 1–1.5 min, 30–90% B over 1.5–5 min, 90% B over 5–7.4 min, and the composition returned to the initial condition within 0.1 min and then was maintained for 7.5 min. The injection volume was set at 10 μL. The optimum operating parameters of the ESI interface were as follows: Source, ESI; Polarity, Positive; Nebulizer, 40 psi; Dry gas, 9 L/min; Dry Temp, 350 °C; Capillary voltage, 4000 V; Delta EMV, 300 V. The LC eluent flow over a period of 0.0–3.0 min was not introduced to the mass spectrometer

for data acquisition. Quantification was achieved using multiple-reaction-monitoring (MRM) mode. The operating parameters of MRM were listed in Table 1.

2.3. Sample preparation

To analyze liquiritigenin, oroxylin A-7-*O*-glucuronide and wogonoside, a 500 μL aliquot of plasma was transferred into an Eppendorf tube, and then 50 μL of icariin (IS) working solution was added and vortex-mixed thoroughly. The plasma sample was loaded onto an SPE cartridge, which had been preconditioned with 1 mL methanol followed by 1 mL water. The cartridge was washed with 1 mL methanol–water (5:95, *v*/*v*) followed by elution with 3 mL methanol under reduced pressure(−30 kPa). The methanol eluant was collected, and evaporated to dryness at 35 °C under a gentle nitrogen stream. Then, the residue was reconstituted in 100 μL methanol–water (50:50, *v*/*v*). After centrifugation at 15,493 × *g* for 10 min at 23 °C, 5 μL of the supernatant aliquot was injected into the HPLC–MS/MS system.

To analyze cimifugin and 5-*O*-methylvisammiol, plasma sample (50 μL) was spiked with 950 μL of physiological saline and 50 μL of phenacetin (IS) solution, vortex-mixed for 30 s, followed by addition of 3 mL acetic ether, then vortex-mixed for 60 s and centrifugated at 1,721 × *g* for 10 min at room temperature. 2.5 mL organic phase was transferred to a clean flask and evaporated to dryness at 35 °C under vacuum. The residue was reconstituted in 100 μL of acetonitrile–water (40:60, *v*/*v*) and 10 μL of the aliquot was injected into the LC–MS system.

To analyze glycyrrhizic acid and glycyrrhetinic acid, 20 μL of the carbamazepine (IS) solution and 3 mL of methanol were added to 500 μL of plasma sample, then mixed by vortex for 30 s and centrifuged at 1,721 × *g* for 10 min at room temperature (23 °C). The supernatant was dried under a stream of N_2. The residue was reconstituted in 100 μL of methanol, and then centrifuged at 15,493 × *g* for 10 min at room temperature (23 °C). 10 μL of the supernatant aliquot was injected into the HPLC–MS/MS system.

2.4. Stock solution, calibration curve and quality control (QC) samples

Liquiritigenin, oroxylin A-7-*O*-glucuronide, wogonoside, cimifugin, 5-*O*-methylvisammiol, glycyrrhizic acid and glycyrrhetinic acid were all prepared in methanol at final concentrations of 102.0, 75.0, 100.0, 104.0, 115.0, 5.02 and 12.38 μg/mL, respectively. A series of working solutions of these analytes were obtained by further diluting the stock solutions with methanol. The internal standard solution of icariin, phenacetin and carbamazepine was prepared at final concentrations of 500, 80 and 100 ng/mL in methanol. All solutions were stored at 4 °C prior to analysis.

For the determination of liquiritigenin, oroxylin A-7-*O*-glucuronide and wogonoside, the standard calibration curves and QC samples were prepared by spiking the 500 μL of blank rat plasma with 50 μL of standard working solutions and 50 μL icariin (IS) standard solution. The plasma concentration ranges of liquiritigenin, oroxylin A-7-*O*-glucuronide and wogonoside were 2.55–2.04 × 10^3, 1.88–1.50 × 10^3 and 2.50–2.00 × 10^3 ng/mL, respectively. The QC samples were prepared at concentrations of 5.10, 30.6, 306 and.53 × 10^3 ng/mL for liquiritigenin, 3.75, 22.5, 225 and 1.12 × 10^3 ng/mL for oroxylin A-7-*O*-glucuronide and 5.00, 30.0, 300 and 1.50 × 10^3 ng/mL for wogonoside.

For the determination of cimifugin and 5-*O*-methylvisammiol, to prepare biological calibration curves and QC samples, 50 μL blank rat plasma was added with 950 μL of physiological saline, 10 μL of respective working solution of 5-*O*-methylvisammiol and cimifugin, and 50 μL of the phenacetin (IS) solution. The final concentrations of biological calibration standards of 5-*O*-methylvisammiol and cimifugin were within the ranges of 11.5–230.0 ng/mL and 10.4–936.0 ng/mL, respectively. QC samples were prepared at concentrations of 11.5, 46.0, 115.0 and 230.0 ng/mL for 5-*O*-methylvisammiol and 10.4, 83.2, 312.0 and 624.0 ng/mL for cimifugin.

For the determination of glycyrrhizic acid and glycyrrhetinic acid, the samples for standard calibration curves and QC were prepared by spiking the blank 500 μL rat plasma with 20 μL of standard working solutions and 20 μL of carbamazepine (IS) standard solution. The final biological calibration standards of glycyrrhizic acid and glycyrrhetinic acid were within the range of 5.02–803 ng/mL and 12.4–2229 ng/mL, respectively. The QC samples were prepared at concentrations of 20.1, 80.3 and 402 ng/mL for glycyrrhizic acid and at concentrations of 49.5, 198 and 991 ng/mL for glycyrrhetinic acid in the same manner.

2.5. Method validation

2.5.1. Specificity

Blank rat plasma, blank plasma spiked with 7 analytes and the IS, and plasma sample after administration were analyzed by the established LC–MS or LC–MS/MS methods to observe the interference from endogenous substances with the analytes.

2.5.2. Linearity and lower limits of quantification (LLOQ)

Linearity of the method was evaluated by calibration standards and determined by plotting the peak area ratio of analytes to IS in the rat plasma. A least-squares linear regression method ($1/x^2$ weighting) was used to determine the slope, intercept and correlation coefficient (*r*) of linear regression equation. The lower limit of quantification (LLOQ) was determined by spiked blank plasma samples.

2.5.3. Precision and accuracy

The precision and accuracy of the methods were assessed by analysis of QC samples at three or four concentration levels, along with an independent calibration curve for quantitation. The precision was evaluated by the intra- and inter-day variability with relative standard deviation (RSD). For intra-day precision, the samples were consecutively analyzed six times in a single day; for inter-day precision, this procedure was repeated on six consecutive days. The accuracy was expressed as the percentage of measured concentration in spiked concentration. The criteria for acceptability of the data included accuracy within 85–115%, and precision with RSD less than 15%, except for LLOQ, with accuracy within 80–120%, and RSD less than 20%.

2.5.4. Extraction recovery

The extraction recoveries of 7 analytes were determined by comparing the measured concentration from blank plasma spiked with analytes with nominal concentration.

2.5.5. *Stability*

The stability of analytes was investigated by analysis of QC samples with three or four concentration levels, including (a) intra-day stability: stability of the extracted samples at room temperature for 24 h, (b) inter-day stability: stability of the extracted samples at room temperature for 6 days, (c) freeze–thaw stability: stability of after three freeze–thaw cycles with the frozen temperature of −80 °C and thawing temperature of 23 °C and (d) long term stability: stability of plasma samples at −80 °C for 10 days. All the assays were performed in three replicates.

2.6. *Application of the method and pharmacokinetic study*

Male Wistar rats (190±20 g) were obtained from the Institute of Laboratory Animals Sciences, Chinese Academy of Medical Sciences and Peking Union Medical College (Beijing, China). They were kept in an environmentally controlled breeding room for 3 days before starting the experiments and fed with standard laboratory food and water. Prior to the experiments rats were fasted in a metabolic cage and maintained with physiological saline for 24 h. 75 Rats were randomized according to the body weight, grouped five per three time points, so that the blood sample at three different time points would be collected from the same group. Then the rats were given the active fraction of XXMD at a dose of 2.0 g/kg (the weight of the active fraction of XXMD/ body weight) by oral administration. Rat blood samples were collected after administration at 2, 5, 10, 15, 20, 30, 40, 45 min, 1, 1.25, 1.5, 1.75, 2.1, 1.5, 2, 3, 4, 5, 6, 7, 7.5, 8, 8.5, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 22, 24, 26, 30 and 32 h. Blood samples were immediately heparinized and centrifuged at 1,721 × *g* for 10 min at room temperature (23 °C). The supernatants were placed into 1.5 mL polypropylene tubes and stored at −80 °C until the assay was performed. All protocols and procedures were approved by The Animal Care & Welfare Committee, Institute of Materia Medica, CAMS & PUMC.

3. Results and discussion

3.1. *Method validation*

3.1.1. *Specificity*

The 7 constituents exhibited different physical and chemical properties, so three analytical methods were established and validated for rapid and accurate determination of the 7 analytes in rat plasma. Typical chromatograms of blank rat plasma, blank plasma spiked with the 7 analytes and IS, and plasma sample after administration are shown in Figs. 2–4. Under the assay conditions, there was no significant chromatographic interference around the retention times of the 7 analytes and IS in blank plasma.

3.1.2. *Linearity and LLOQs*

The standard curves of the 7 analytes all exhibited good linearity with the coefficients of correlation (r) better than 0.996. The LLOQs are appropriate for quantitative detection of the 7 analytes in the pharmacokinetic studies. Linear ranges, regression equations, LLOQs, and correlation coefficients obtained from typical calibration curves are shown in Table 2.

3.1.3. *Precision and accuracy*

The measured concentrations of QC samples of the 7 analytes were calculated from the biological calibration curves. The results are shown in Table 3. The RSD of the overall intra- and inter-day precisions were less than 11%, and the accuracy ranged 88.7–113%.

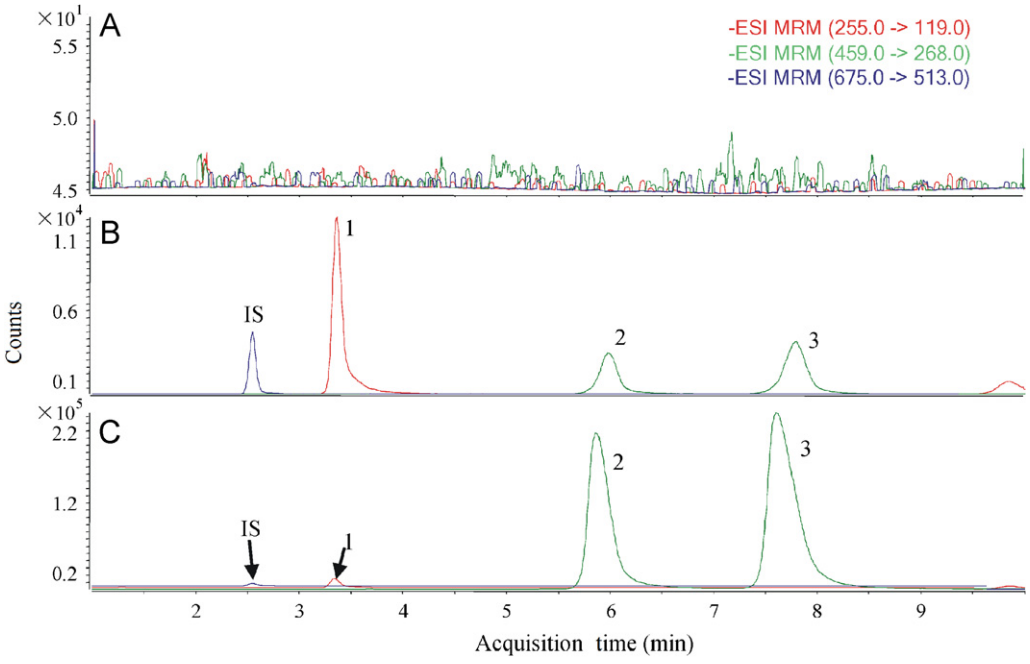

Figure 2 Representative chromatograms of (A) blank plasma, (B) blank plasma spiked with mixed standard and icariin (IS), and (C) a plasma sample taken 2 min after oral administration of the active fraction of XXMD. 1, liquiritigenin; 2, oroxylin A-7-*O*-glucuronide; 3, wogonoside.

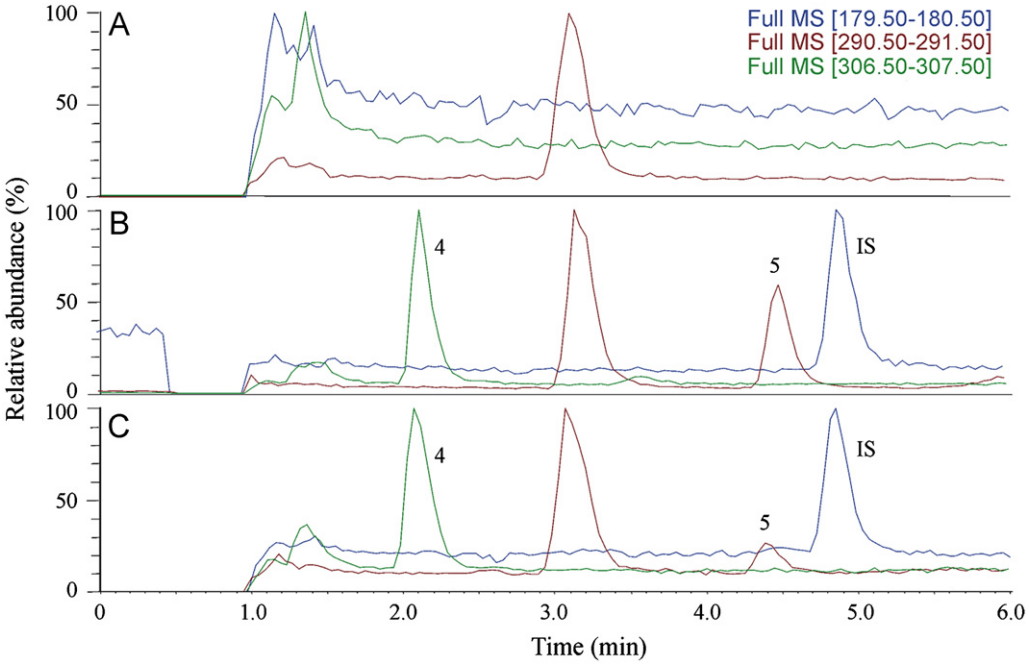

Figure 3 Representative chromatograms of (A) blank plasma, (B) blank plasma spiked with a mixed standard (cimifugin, 5-*O*-methylvisamminol) and phenacetin (IS), and (C) a plasma sample taken 5 min after oral administration of the active fraction of XXMD. 4, cimifugin; 5, 5-*O*-methylvisammiol.

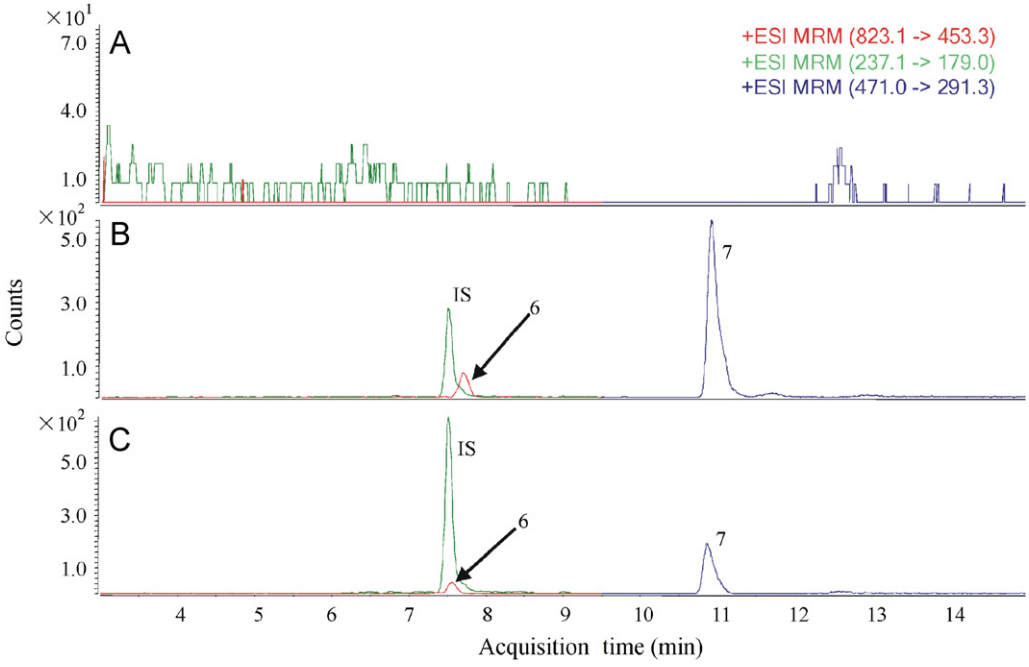

Figure 4 Representative chromatograms of (A) blank plasma, (B) blank plasma spiked with a mixed standard and carbamazepine (IS), and (C) a plasma sample taken 11 h after oral administration of the active fraction of XXMD. 6, glycyrrhizic acid; 7, glycyrrhetinic acid.

3.1.4. Extraction recovery

The extraction recoveries determined for the 7 analytes are shown in Table 4. At three or four concentration levels of the 7 analytes, the extraction recoveries were all in the range of 89.7–113%. These results demonstrated that the values were all in the acceptable ranges.

3.1.5. Stability

The stability was evaluated by analysis of QC samples at three or four levels for intra-day stability, inter-day stability, freeze–thaw stability and long term stability. The RSD of the stability precisions were less than 15%, and the accuracy ranged from 85% to 115%.The results

Table 2 Results of linear regression of calibration curves of the seven analytes in rat plasma.

Analyte	Linear range (ng/mL)	Regression equation	r	LLOQ (ng/mL)
Liquiritigenin	2.55–2040	$Y=0.0116X+0.101$	0.999	2.55
Oroxylin A-7-*O*-glucuronide	1.88–1500	$Y=0.0053X+0.059$	0.998	1.88
Wogonoside	2.50–2000	$Y=0.0061X+0.049$	0.999	2.50
Cimifugin	10.4–936	$Y=0.0299X+0.219$	0.998	5.20
5-*O*-Methylvisammiol	11.5–230	$Y=0.0572X-0.0843$	0.997	5.75
Glycyrrhizic acid	5.02–803	$Y=0.0052X+0.0036$	0.996	5.02
Glycyrrhetinic acid	12.4–2229	$Y=0.0002X-0.0003$	0.999	12.4

Table 3 Precision and accuracy of the seven analytes in rat plasma.

Analyte	Plasma conc. (ng/mL)	Intra-day ($n=6$)			Inter-day ($n=6$)		
		Found[a] (ng/mL)	RSD (%)	Accuracy[b] (%)	Found[a] (ng/mL)	RSD (%)	Accuracy[b] (%)
Liquiritigenin	5.10	5.30±0.12	2.4	104	5.02±0.21	4.2	98.5
	30.6	30.0±0.81	2.7	98.0	30.0±1.02	3.4	97.9
	306	314±3.14	1.0	103	316±8.32	2.6	103
	1530	1.64×10^3±36.1	2.2	107	1.57×10^3±43.6	2.8	102
Oroxylin A-7-*O*-glucuronide	3.75	4.18±0.12	2.9	111	3.97±0.26	6.5	106
	22.5	23.4±0.95	4.0	104	23.5±0.78	3.3	104
	225	226±6.17	2.7	101	220±13.6	6.2	97.8
	1120	1.24×10^3±36.6	3.0	110	1.17×10^3±62.4	5.3	104
Wogonoside	5.00	5.16±0.49	9.4	103	5.12±0.18	3.4	103
	30.0	26.8±0.42	1.6	89.2	28.5±1.43	5.0	95.1
	300	290±5.67	2.0	96.7	298±9.41	3.2	99.4
	1500	1.60×10^3±52.6	3.3	107	1.53×10^3±54.9	3.6	102
5-*O*-Methylvisa-mminol	11.5	12.18±0.43	3.6	106	11.97±1.08	9.0	104
	46.0	41.15±1.37	3.3	89.5	43.69±4.46	10	95.0
	115.0	106.36±5.06	4.7	92.5	102.41±9.96	9.7	89.1
	230.0	239.03±9.13	3.8	104	224.15±10.90	4.9	97.5
Cimifugin	10.4	10.31±0.57	5.5	99.1	11.33±1.06	9.4	109
	83.2	87.34±1.30	1.5	105	85.97±8.65	10	103
	312.0	319.21±9.98	3.1	102	308.13±32.91	11	98.8
	624.0	602.06±13.47	2.2	96.5	609.55±15.88	2.6	97.7
Glycyrrhizic acid	20.1	21.4±0.5	2.4	106	19.1±0.8	4.4	95.3
	80.3	82.6±7.5	9.0	103	79.2±5.7	7.1	98.5
	402	417±9.3	2.2	104	438±25.8	5.9	109
Glycyrrhetinic acid	49.5	52.1±3.5	6.7	105	52.2±4.1	7.8	105
	198	186±5.2	2.8	93.9	183±13.0	7.1	92.4
	991	1150±12.8	1.1	116	909±40.1	4.4	91.8

[a]The values are means±SD of six experiments.
[b]Accuracy (%)=mean of measured concentration/nominal concentration × 100%.

suggested that analytes maintained at these conditions were stable.

3.2. *Pharmacokinetic study*

The established method was successfully applied to analyze all the 7 analytes in rat plasma samples after oral administration of the active fraction of XXMD. The mean concentration ($n=5$) versus time profile was shown in Fig. 5. The non-compartmental pharmacokinetic parameters were obtained according to the mean plasma concentrations of the 7 analytes by Drug and Statistics for Windows (DAS) Ver2.0 program (Table 5).

The compounds existed in the active fraction of XXMD are very complex, and the pharmacokinetics of the 7 analytes are also remarkably non-typical. The double-peak or multi-peak phenomenon was observed from the concentration–time curves of the 7 analytes. And there was an obvious selectivity for the absorption of the 7 compounds. 5-*O*-methylvisammioside is the

Table 4 Extraction recovery of the seven compounds in plasma ($n=6$).

Analyte	Plasma conc. (ng/mL)	Mean ± SD (RSD)	Recovery[a] (%)	RSD (%)
Liquiritigenin	5.10	5.78 ± 0.26	113	4.5
	30.6	30.1 ± 2.19	98.4	7.3
	306	292 ± 6.04	95.5	2.1
	1.53×10^3	$1.44 \times 10^3 \pm 30.6$	94.2	2.1
Oroxylin A-7-*O*-glucuronide	3.75	4.00 ± 0.41	107	10
	22.5	23.1 ± 0.30	103	1.3
	225	218 ± 12.6	96.8	5.8
	1.12×10^3	$1.13 \times 10^3 \pm 58.4$	100	5.2
Wogonoside	5.00	4.91 ± 0.32	98.2	6.4
	30.0	27.8 ± 1.07	92.6	3.9
	300	281 ± 13.4	93.8	4.8
	1.50×10^3	$1.44 \times 10^3 \pm 81.3$	95.8	5.7
5-*O*-methylvisamminol	11.5	11.97 ± 0.28	104	2.4
	46.0	41.25 ± 1.00	89.7	2.4
	115.0	104.81 ± 7.07	91.1	6.7
	230.0	240.22 ± 8.20	104	3.4
Cimifugin	10.4	10.15 ± 0.70	97.6	6.9
	83.2	87.77 ± 2.26	106	2.6
	312.0	320.95 ± 12.79	103	4.0
	624.0	606.20 ± 13.79	97.1	2.3
Glycyrrhizic acid	20.1	19.8 ± 1.6	98.8	8.2
	80.3	90.4 ± 6.9	113	7.6
	402	399 ± 28.9	99.4	7.3
Glycyrrhetinic acid	49.5	51.2 ± 6.3	103	12
	198	177 ± 5.4	89.2	3.0
	991	878 ± 24.2	88.7	2.8

[a]Recovery (%) = mean of measured concentration/nominal concentration × 100%.

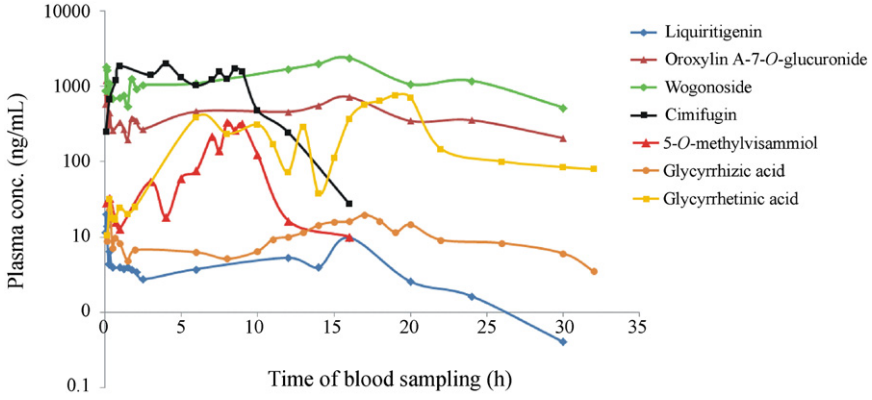

Figure 5 Mean plasma concentration–time profile of the seven active components in rat plasma after oral administration of the active fraction of XXMD.

richest component in the active fraction of XXMD; however, it could not be detected, and 5-*O*-methylvisammiol was detected in dosed rat plasma. It means that 5-*O*-methylvisammiol was the metabolite of 5-*O*-methylvisammioside, so the T_{max} of 5-*O*-methylvisammiol was very long, at 8 h. Wogonoside and glycyrrhizic acid were the secondary richest components in active fraction, and wogonoside showed the highest C_{max} and AUC_{0-t} The concentration–time curves of wogonoside showed double-peak phenomenon (T_{max} of first peak at 0.15 min and the second peak at 16 h), and was similar with that of wogonoside after oral administration of Huangqin decoction to rats[17].

Both glycyrrhizic acid and glycyrrhetinic acid were contained in the active fraction; the content of glycyrrhizic acid (1.6 mg/mL) in XXMD was a little higher than glycyrrhetinic acid (1.13 mg/mL). The profiles of concentration–time of

Table 5 Mean pharmacokinetic parameters of 7 analytes in rat plasma ($n=5$).

Parameter*	Oroxylin A-7-*O*-glucuronide	Wogonoside	Liquiritigenin	5-*O*-Methylvisammiol	Cimifugin	Glycyrrhizic acid	Glycyrrhetinic acid
AUC_{0-t} (min ng/mL)	12525	38749	112.9	1299	18342	298.6	7438
$AUC_{0-\infty}$ (min ng/mL)	14829	44912	114.7	1299	18441	355.5	9415
MRT_{0-t} (h)	14.2	14.5	12.5	7.8	7.1	16.2	15.7
$MRT_{0-\infty}$ (h)	18.4	18.5	12.8	7.9	7.1	18.4	24.4
$T_{1/2}$ (h)	7.7	8.4	3.1	0.7	2.5	7.8	17.4
T_{max} (h)	0.083	16	0.083	8	3	0.33	19
V_z/F (L/kg)	1499.6	542.2	77051	1549.4	392.1	63757	5322.1
CL_z/F (L/H/kg)	134.9	44.53	17434	1539.1	108.5	5625.5	212.4
C_{max} (μg/L)	1200	2362	20.3	338.0	2018	23.24	760.7

*All pharmacokinetic parameters are calculated from plasma concentration–time profiles.

glycyrrhizic acid and glycyrrhetinic acid in rat plasma were similar and showed multiple-peak phenonmen; however, the concentration of glycyrrhetinic acid in rat plasma was significantly higher than that of glycyrrhizic acid and T_{max} of glycyrrhetinic acid (19 h) was the extremely longest among the 7 constituents. Glycyrrhizic acid showed the lowest C_{max} and AUC among the 7 constituents. Takeda et al.[18] reported that glycyrrhetinic acid with higher concentration than glycyrrhizic acid was detected in rat plasma after oral administration of glycyrrhizic acid to rats, and glycyrrhizic acid was transformed to glycyrrhetinic acid in rats. These two compounds have the same parent nucleus, but two glucuronic acid groups contained in glycyrrhizic acid structure rather than glycyrrhetinic acid, so the metabolism easily happened between glycyrrhizic acid and glycyrrhetinic acid. Due to easy absorption of glycyrrhetinic acid in rat plasma and the each other's biotransformation, the concentration of glycyrrhetinic acid in rat plasm was enriched.

The concentrations of liquiritigenin, oroxylin A-7-*O*-glucuronide and cimifugin were at similar level about 1 mg/mL in active fraction, but these three components showed very different pharmacokinetic characterization. Oroxylin A-7-*O*-glucuronide and cimifugin exhibited higher C_{max} and AUC; however, liquiritigenin showed the lowest C_{max} and AUC. Their concentration–time curves also exhibited the double-peak phenomenon.

4. Conclusions

In this study, determination of the 7 analytes by HPLC–MS and HPLC–MS/MS methods in rat plasma have been established and validated for the first time. The established methods are simple, rapid, sensitive and reliable, which have been successfully applied to pharmacokinetic study after oral administration of the active fraction of XXMD to rats. The pharmacokinetic study of the 7 components after oral administration of the active fraction of XXMD to rats showed that the pharmacokinetics of active components such as wogonoside, glycyrrhizic acid and glycyrrhetinic acid kept similar characterization of multiple-peak regardless of whether extract of herbs or pure compound was administrated to rats. The different components with similar concentration levels such as liquiritigenin, oroxylin A-7-*O*-glucuronide and cimifugin in complex prescription exhibited different exposure levels to rats, and might be due to the effects of complex matrix on absorption. This research could provide useful references for the pharmacokinetic study of traditional Chinese medicine.

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References

1. Feng NP, Di B, Liu WY. Drug metabolism and modernization of traditional Chinese medicine. *World Sci Technol Mod Tradit Chin Med* 2003;**5**:5–7.
2. Sun H, Wu ZM, Lv HT, Wang XJ. Chinese medicine and its metabolism: current status and future development. *World Sci Technol Mod Tradit Chin Med* 2006;**8**:1–6.
3. Wang YH, Zhang HX, Li Q, Ding Y, Hu JJ, Du GH. High-throughput screening assay for groups of effective components extracted from Xiaoxuming Recipe. *J Chin Integ Med* 2006;**4**:64–7.
4. Wang Y, Ding C, Du K, Xiao Y, Wu C, Zhang J, et al. Identification of active compounds and their metabolites by high-performance liquid chromatography/electrospray ionization fourier transform ion cyclotron resonance mass spectrometry from Xiao-xu-ming decoction (XXMD). *Rapid Commun Mass Spectrom* 2009;**23**:2724–32.
5. Jeon SJ, Rhee SY, Seo JE, Bak HR, Lee SH, Ryu JH, et al. Oroxylin A increases BDNF production by activation of MAPK-CREB pathway in rat primary cortical neuronal culture. *Neurosci Res* 2011;**69**:214–22.
6. Kim DH, Kim S, Jeon SJ, Son KH, Lee S, Yoon BH, et al. The effects of acute and repeated oroxylin A treatments on Aβ(25–35)-induced memory impairment in mice. *Neuropharmacology* 2008;**55**:639–47.
7. Liu W, Xu X, Feng F, Wu C. Simultaneous quantification of oroxylin A and its metabolite oroxylin A-7-*O*-glucuronide: application to a pharmacokinetic study in rat. *Chromatographia* 2011;**74**:75–81.
8. Jang SI, Kim HJ, Hwang KM, Jekal SJ, Pae HO, Choi BM, et al. Hepatoprotective effect of baicalin, a major flavone from *Scutellaria radix*, on acetaminophen-induced liver injury in mice. *Immunopharmacol Immunotoxicol* 2003;**25**:585–94.

9. Kim DH, Cho KH, Moon SK, Kim YS, Kim DH, Choi JS, et al. Cytoprotective mechanism of baicalin against endothelial cell damage by peroxynitrite. *J Pharm Pharmacol* 2005;**57**:1581–90.
10. Liu RT, Zou LB, Lu QJ. Liquiritigenin inhibits Abeta(25-35)-induced neurotoxicity and scretion of Abeta(1-40) in rat hippocampal neuns. *Acta Pharmacol Sin* 2009;**30**:899–906.
11. Xue BY, Li W, Li L, Xiao YQ. A pharmacodynamic research on chromone glucosides of Fangfeng. *China J Chin Mater Med* 2000;**25**:297–9.
12. Kim SC, Byun SH, Yang CH, Kim CY, Kim JW, Kim SG. Cytoprotective effects of *Glycyrrhizae radix* extract and its active component liquiritigenin against cadmium-induced toxicity (effects on bad translocation and cytochrome c-mediated PARP cleavage). *Toxicology* 2004;**197**:239–51.
13. Jeong HG, You HJ, Park SJ, Moon AR, Chung YC, Kang SK, et al. Hepatoprotective effects of 18β-glycyrrhetinic acid on carbon tetrachloride-induced liver injury: inhibition of cytochrome P450 2E1 expression. *Pharmacol Res* 2002;**46**:221–7.
14. Dai JH, Iwatani Y, Ishida T, Terunuma H, Kasai H, Iwakula Y, et al. Glycyrrhizin enhances interleukin-12 production in peritoneal macrophages. *Immunology* 2001;**103**:235–43.
15. Jung GD, Yang JY, Song ES, Par JW. Stimulation of melanogenesis by glycyrrhizin in B16 melanoma cells. *Exp Mol Med* 2001;**33**:131–5.
16. Raphael TJ, Kuttan G. Effect of naturally occurring triterpenoids glycyrrhizic acid, ursolic acid, oleanolic acid and nomilin on the immune system. *Phytomedicine* 2003;**10**:483–9.
17. Lu T, Song J, Xie L, Wang GJ, Liu XD. Simultaneous determination of baicalin and wogonoside by HPLC in rat plasma administered with Huangqin Decoction and its pharmacokinetic study. *Chin Tradit Herb Drugs* 2005;**36**:870–3.
18. Takeda S, Ishchara K, Wakui Y, Amagaya S, Maruno M, Akao T, et al. Bioavailability study of glycyrrhetic acid after oral administration of glycyrrhizin in rats: relevance to the intestinal bacterial hydrolysis. *J Pharm Pharmacol* 1996;**48**:902–5.