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Original article

Bioanalysis and Pharmacokinetics of Eight Active Components from Huanglian Jiedu Decoction in Rat Plasma by LC-ESI-MS/MS Method

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ABSTRACT

Objective To develop a sensitive and rapid liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) method for the simultaneous determination of wogonin, coptisine, berberine, palmatine, jatrorrhizine, phellodendrine, magnoflorine, and wogonoside in rat plasma and to evaluate the pharmacokinetic characteristics of Huanglian Jiedu Decoction (HJD). **Methods** LC separation was performed on an Acquity HSS T3 column (100 mm × 2.1 mm, 1.8 μm) using gradient elution with the mobile phase consisting of acetonitrile and 0.1% formic acid water. The detection was accomplished by using positive electrospray ionization in multiple-reaction monitoring mode. Plasma samples were pretreated by protein precipitation. **Results** The method showed a good linearity over a wide concentration range ($r^2 > 0.99$). The lower limits of quantification were 0.20 ng/mL for coptisine and phellodendrine, 0.48 ng/mL for berberine, 0.10 ng/mL for jatrorrhizine, 0.32 ng/mL for magnoflorine, 0.30 ng/mL for palmatine, and 4.80 ng/mL for wogonin and wogonoside, respectively. The intra- and inter-day precision of the analytes was less than 12.11%, while the accuracy was between −14.46% and 4.86%. The mean recovery of all the analytes ranged from 93.10% to 110.91%. **Conclusion** This validated method offers the advantages of high sensitivity. It is successfully applied to evaluating the pharmacokinetic properties of HJD.

Key words

alkaloid; bioanalysis; flavonoid; Huanglian Jiedu Decoction; LC-ESI-MS/MS; pharmacokinetics

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1. Introduction

As an important resource for prevention and treatment of diseases, traditional Chinese medicines (TCMs) have been used for more than 2000 years. However, the inherent characteristics such as complexity of chemical composition, uncharacterized active constituents, different disposition process, and complex workings of this ancient medical system hinder its pharmacokinetics (PK). Typically, TCMs consist of several types of components. "Multiple components hit multiple targets and exert synergistic therapeutic efficacies" is the unique mechanism of action (Wang et al, 2008). Therefore, to establish reliable analytical methods to support the PK study of TCMs does add to its scientific knowledge (Wang et al, 2012a).

As a famous multi-herb prescription in China, Huanglian Jiedu Decoction (HJD) is an aqueous extract of four herbal materials, *Coptidis Rhizoma*, *Scutellariae Radix*, *Phellodendri Cortex*, and *Gardeniae Fructus* with the ratio of 3:2:2:3. It has been widely used in the treatment of gastrointestinal disorders, cardiovascular diseases, and Alzheimer's disease in China (Hu et al, 2012). Modern pharmacological research also demonstrated that HJD had various bioactivities such as anti-inflammation, hepatoprotection, gastrointestinal tract protection, and radical scavenging (Hu et al, 2012). Even with so many outstanding achievements, the PK evaluation of HJD was rare for not only the chemical complexity and diversity, but also the trace concentration of the chemical components *in vivo*.

HJD contains multiple bioactive secondary metabolites, mainly including alkaloids from *Coptidis Rhizoma* and *Phellodendri Cortex*, flavonoids from *Scutellariae Radix*, and terpenes from *Gardeniae Fructus*. We reported that the combined active compounds including jasminoidin, jatrorrhizine, palmatine, chlorogenic acid, berberine, baicalin, phellodendrine, magnoflorine, wogonoside, wogonin, coptisine, baiclein, and crocin of HJD (Patent number: ZL 2009 1 0195343.7) were effective on rheumatoid arthritis. In our previous study, LC-DAD coupled with electrospray tandem mass spectrometry and plasma pharmacochemistry based approach have been applied for screening the potential bioactive components of HJD (Hu et al, 2012). Also modern pharmacological research demonstrated that the eight components including wogonin, coptisine, berberine, palmatine, jatrorrhizine, phellodendrine, magnoflorine, and wogonoside possessed antiobesity (Liu et al, 2008), antitumor (Liu et al, 2008; Zhu et al, 2011), anti-inflammatory (Liu et al, 2008; Zhu et al, 2011; Lu et al, 2011), gastrointestinal tract protective (Ohta et al, 1999; Watanabe et al, 2009), and neuroprotective (Liu et al, 2008; Zhu et al, 2012) activities. Thus, eight components from 13 were selected as makers to investigate the PK property of HJD. This would substantially clarify the active mechanism of the whole prescription.

For the PK evaluation of HJD, development of a sensitive and reliable analytical method to determine the bioactive components in biological fluids is the key prerequisite. Earlier publications have described various methods for analysis of the chemical components of HJD in biological samples using HPLC based technologies (HPLC-DAD, HPLC-UV, LC-ESI-MS, and LC-MS/MS) (Zhu et al, 2012; Deng et al, 2006; Lu et al, 2006; Zhu et al, 2013; Tan et al, 2007; Li et al, 2011; Zhou et al, 2012; Chuang et al, 1996; Yu et al, 2007; Zhu et al, 2010; Zhang et al, 2011; Deng et al, 2008; Yuan et al, 2012; Kim et al, 2006; Chung et al, 2012; Tong et al, 2012; Feng et al, 2010; Zan et al, 2011). However, the poor absorption and extensive metabolism from the gastrointestinal tract cause extremely low plasma concentration of alkaloids after oral administration of HJD to rat, Beagle's dogs, and human (Yu et al, 2007; Zuo et al, 2006). To our knowledge, the above methods are only concerned for some of the eight analytes in biological samples and not sensitive for oral PK study of HJD. Although a method for simultaneous determination of 14 major chemical constituents in HJD by HPLC-DAD has been developed (Kwok et al, 2013), and long time (about 30 min) and low sensitivity indicate that the method is not suitable for the quantification of biological samples.

In this paper, a sensitive and rapid LC-ESI-MS/MS method was firstly developed and validated for the simultaneous determination of wogonin, coptisine, berberine, jatrorrhizine, phellodendrine, magnoflorine, palmatine, and wogonoside in rat plasma. The simple, sensitive, and reproducible method was successfully applied to PK study after oral administration of HJD to rats. To the best of our knowledge, this is the first report of PK properties of coptisine, jatrorrhizine, phellodendrine, magnoflorine, and wogonin after oral administration of HJD. This is also the first report of PK properties of phellodendrine and magnoflorine after oral administration.

2. Materials and methods

2.1 Chemicals and reagents

Wogonin, luteolin (internal standard, IS), coptisine, berberine, jatrorrhizine, phellodendrine, magnoflorine, palmatine, tetrahydropalmatine (IS), and wogonoside (purity > 98%) were obtained from the National Institute for Food and Drug Control (Beijing, China). Their chemical structures are shown in Figure 1. Acetonitrile from Honeywell Burdick & Jackson Muskegon (USA) was of LC-MS grade. Methanol from J&K Scientific Ltd. (Beijing, China) was of HPLC grade. Formic acid of HPLC grade was purchased from Sigma-Aldrich (USA). All other reagents were of analytical grade. Purified water prepared by a Milli-Q50 SP Reagent Water System (USA) was used throughout the study.

Coptidis Rhizoma, *Scutellariae Radix*, *Phellodendri Cortex*, and *Gardeniae Fructus* were purchased from Bozhou (Anhui province, China) and identified by Prof. Han-ming Zhang (Second Military Medical University, Shanghai, China). The voucher specimens were stored in Department of Natural Pharmaceutical Chemistry, School of Pharmacy, Second Military Medical University.

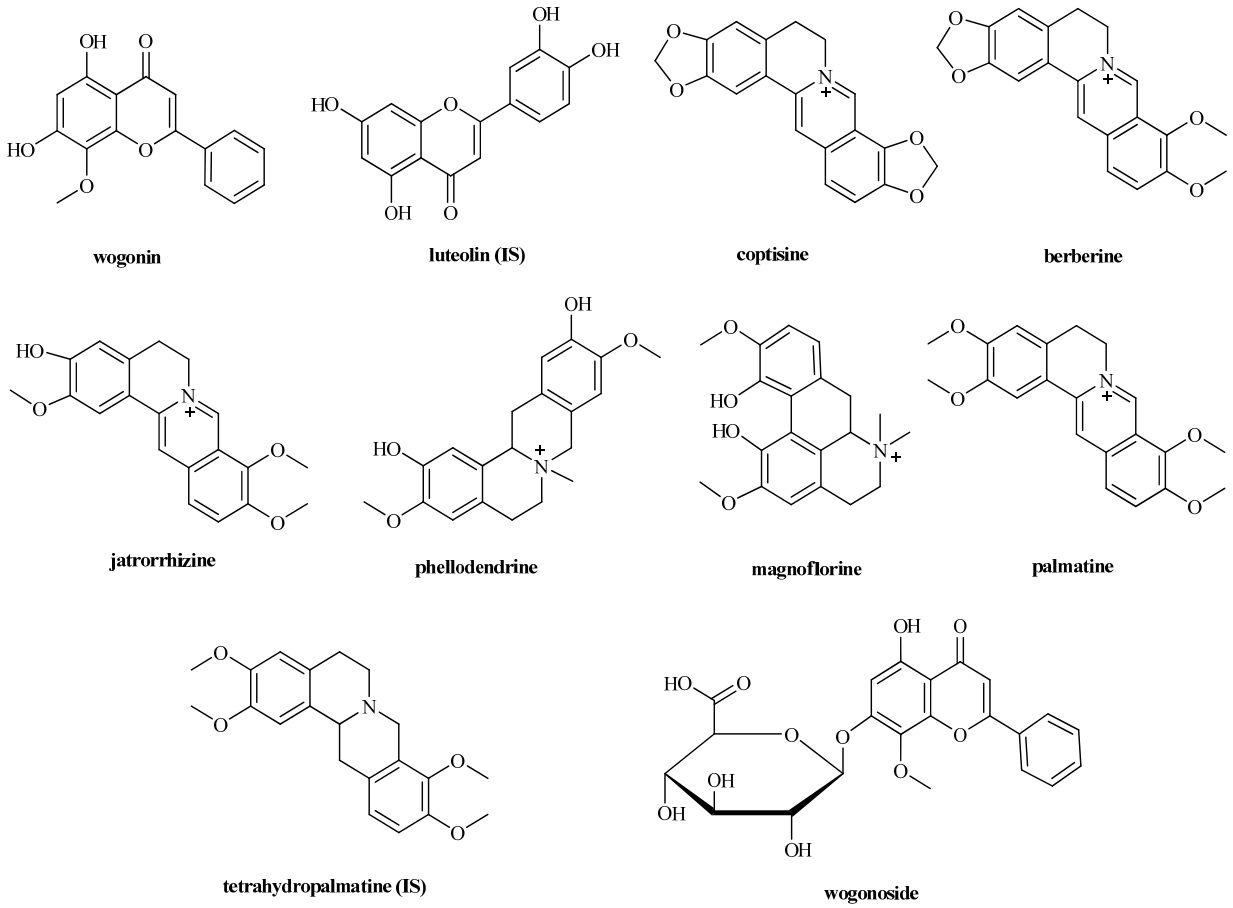

Figure 1 Chemical structures of analytes and IS

2.2 Preparation of HJD

A mixture of *Coptidis Rhizoma* (60 g), *Scutellariae Radix* (40 g), *Phellodendri Cortex* (40 g), and *Gardenia Fructus* (60 g) was decocted twice with boiling water (1:10 and then 1:5) for 1.5 h, and the extracted solution was filtered through six layer gauzes. The combined filtrations were concentrated to give an extract of 42.3 g. The dried powder was stored at about 4 °C before use.

2.3 Content of eight components in HJD

The contents of wogonin, coptisine, berberine, jatrorrhizine, phellodendrine, magnoflorine, palmatine, and wogonoside in HJD for oral administration were determined using the LC-ESI-MS/MS method described in the paper. Accurately the dry powder (1.00 mg) was dissolved with 5 mL 50% methanol and ultrasonicated for 30 min. The sample was centrifuged at 13 800 × *g* for 10 min, and the supernatant was filtered through a 0.22 μm membrane filter. Finally, an aliquot of 2 μL of supernatant was injected into the LC-ESI-MS/MS system. After analyzing three replicate samples, the contents of wogonin, coptisine, berberine, jatrorrhizine, phellodendrine, magnoflorine, palmatine, and wogonoside in HJD were 3.99, 12.69, 65.42, 6.43, 1.86, 4.30, 10.46, and 4.02 mg/g, respectively.

2.4 Preparation of calibration standards and quality control (QC) samples

Stock solutions of the analytes and IS at 200 μg/mL were separately prepared by dissolving appropriate amount reference compounds in methanol and stored at about 4 °C. The stock solutions of the eight analytes were then mixed together and serially diluted with methanol to obtain a series of standard working solutions at appropriate concentration. The two kinds of IS were separately diluted to 1 μg/mL as the working solutions.

Calibration standards and QC samples were prepared by spiking 5 μL of the standard working solutions into 95 μL of blank rat plasma to provide final concentration in the ranges of 4.80−1600 ng/mL for wogonin, 0.20−30 ng/mL for coptisine, 0.48−180 ng/mL for berberine, 0.10−24 ng/mL for jatrorrhizine, 0.20−24 ng/mL for phellodendrine, 0.32−100 ng/mL for magnoflorine, 0.30−40 ng/mL for palmatine, and 4.80−2800 ng/mL for wogonoside. QC samples at three kinds of concentration (12, 120, and 1200 ng/mL for wogonin; 0.2, 2, and 20 ng/mL for coptiside, jatrorrhizine and phellodendrine; 1.2, 12, and 120 ng/mL for berberine; 0.8, 8, and 80 ng/mL for magnoflorine; 0.3, 3, and 30 ng/mL for palmatine; 12, 240, and 2400 ng/mL for wogonoside) were also prepared in the same way. All calibration standards and QC samples were stored at about −20 °C until analysis.

2.5 Apparatus and LC-ESI-MS/MS conditions

The LC-ESI-MS/MS system was composed of an Agilent 1200 Rapid Resolution Liquid Chromatography (USA) equipped with a binary pump, an on-line vacuum degasser, an auto-sampler, a column oven enabling temperature control, and a G6410 triple quadrupole mass spectrometer equipped with an electrospray ion source (ESI) (Agilent, USA). All data processing was performed with Masshunter version B.03.01 Software from Agilent Technologies (USA).

Chromatographic separation was carried out on an Acquity HSS T3 column (100 mm × 2.1 mm, 1.8 μm) with the column temperature at 30 °C. The mobile phase consisted of 0.1% formic acid water (solvent A) and acetonitrile (solvent B). A linear gradient elution was applied for the separation at a flow rate of 0.3 mL/min. The elution program was as follows: 20%−50% B at 0−1.0 min; 50% B at 1.0−2.5 min; 50%−20% B at 2.5−4.0 min; 20% B at 4.0−9.0 min. The injection volume was 2 μL and the total chromatographic running time was 9.0 min per sample. A divert value was used to divert the eluent to waste from 0 to 1.0 min, and to MS from 1.0 to 9.0 min.

Mass spectrometer was operated in positive ionization mode and quantification was obtained using multiple reaction monitoring (MRM) mode. The optimized MRM parameters for each compound including precursor-to-product ion transition, fragment electric voltage and collision energy are described in Table 1. The dwell time of each MRM transition was 60 ms. The other optimal ionization parameters were as follows: drying gas (N_2) flow rate, 10 L/min; drying gas temperature, 350 °C; nebulizing gas (N_2) pressure, 2.76×10^5 Pa; capillary voltage, 3500 V.

Table 1 MRM transitions and parameters for detection of analytes and IS (dwell time of 60 ms)

Analytes	MRM transitions/ (*m*/*z*)	Fragmentor/ V	Collision energy / eV
wogonin	285.0→270.0	140	24
luteolin (IS)	287.1→153.0	160	34
coptisine	320.1→292.0	160	30
berberine	336.0→320.0	140	30
jatrorrhizine	338.1→322.1	150	28
phellodendrine	342.1→192.0	130	22
magnoflorine	342.1→297.0	130	16
palmatine	352.1→336.1	150	28
tetrahydropalmatine (IS)	356.1→192.0	150	24
wogonoside	461.1→285.1	120	14

2.6 Sample preparation

Plasma samples were pretreated employing a simple protein precipitation technique. After thawing at room temperature, 100 μL aliquot of the plasma sample was spiked with 200 μL acetonitrile containing 100 ng/mL of the IS luteolin and 10 ng/mL of tetrahydropalmatine in a 1.5 mL Eppendorf tube. The mixture was vortex mixed for 5 min and centrifuged at 13 800 × *g*, 4 °C for 10 min. Then 200 μL of the supernatant was added to equivalent volume of water. After mixing, the solution was transferred into autosampler vials. Finally, 2 μL of the mixed solution was injected into the LC-ESI-MS/MS system.

2.7 Method validation

A full validation according to the industrial guidelines for bioanalytical method validation from the US Food and Drug Administration (FDA) was carried out for the assay in rat plasma (US Food and Drug Administration, 2001).

The selectivity of the method was evaluated by analyzing six different batches of blank rat plasma. To investigate potential interference of endogenous compounds, they were compared with the corresponding spiked plasma samples at the concentration of lower limit of quantification (LLOQ) and actual plasma samples from the rats after oral administration of HJD at peak regions for each analyte. The signal intensity at LLOQ level was at least five times higher than that of blank plasma sample.

Linearity was assessed by analyzing calibration curves in rat plasma in five replicates. Each calibration curve composed of seven non-zero concentration was constructed by plotting peak-area ratios of each analyte to IS (wogonin, wogonoside to luteolin) versus plasma concentration using an unweighted linear least-squares regression model. LLOQ was defined as the lowest concentration producing a signal-to-noise (S/N) ratio larger than 10 with a relative standard deviation (RSD) below 20% and a deviation from the nominal concentration within ± 20% by five replicate analyses.

The intra-day precision and accuracy were evaluated by determining QC samples at low, medium, and high concentration in five replicates on the same day, while the inter-day precision and accuracy were estimated on consecutive 3 d. The precision was calculated as RSD within the required criteria of ± 15%, and the accuracy was defined as the relative error (RE) within ±15% from the nominal values except for LLOQ, where it should be within ± 20% of accuracy and less than 20% of precision.

The extraction recovery was determined in three replicates at three QC levels by comparing the peak areas obtained from plasma samples spiked before extraction to those of analytes from solutions spiked in post-extracted blank plasma at equivalent concentration. The matrix effect was defined as the ion suppression/enhancement on the ionization of analytes, which was measured via comparison of the peak response from post-extraction blank plasma samples spiked with standard solutions at three levels in three replicates to those of the corresponding concentration neat standard solutions. The same procedure was applied for IS at a single concentration. The matrix effect is not negligible if the ratio is less than 85% or more than 115%.

All stability study was conducted by analyzing five replicates of each low, medium, and high concentration QC samples. The short-term stability of unprocessed QC samples stored for 4 h at room temperature was determined. The QC

samples were subjected to three freeze (at about −20 °C) to thaw (at room temperature) cycles for freeze and thaw stability. The processed samples kept in the auto-sampler at ambient temperature (about 22 °C) for 12 h were reanalyzed to assess post-preparative stability. The long-term stability was examined by analyzing samples stored at about −20 °C for 30 d. For all stability testing of QC samples, the concentration was compared with those of freshly prepared QC samples and the percentage concentration deviations were calculated to evaluate stability.

2.8 Application to PK study

This PK study protocol was in accordance with the Guidelines for the Care and Use of Laboratory Animals approved by the Animal Ethics Committee of the Second Military Medical University. Seven male Sprague-Dawley rats (SPF) weighing (200 ± 20) g were provided by the Laboratory Animal Center of the Chinese Academy of Sciences (Shanghai, China). The rats were maintained in one cage under controlled environmental conditions (temperature of (22 ± 2) °C, and relative humidity of 50% ± 10%) with a 12 h dark-light cycle and available standard laboratory food and water spontaneously for 5 d acclimation. Before the experiment, seven rats were fasted for 12 h and had free access to water. The HJD extract was suspended in 0.5% carboxymethyl cellulose sodium salt (CMC-Na) aqueous solution to yield a concentration of 267 mg/mL. After single oral administration of HJD at a dose of 5.34 g/kg (equivalent to 21.3 mg/kg of wogonin, 67.8 mg/kg of coptisine, 349.3 mg/kg of berberine, 34.3 mg/kg of jatrorrhizine, 9.9 mg/kg of phellodendrine, 23.0 mg/kg of magnoflorine, 55.9 mg/kg of palmatine, and 21.5 mg/kg of wogonoside) by gavage, blood samples (about 250 μL) were collected in heparin pretreated polypropylene tubes via the postorbital venous plexus veins from each rat before dosing (0 min) and at 10, 20, 30 min, and 1, 1.5, 2, 4, 6, 8, 10, 12, 18, 24 h after dosing. All blood samples were immediately centrifuged at 13 800 × *g* for 10 min at 4 °C and stored frozen at about −70 °C until analysis.

PK parameters, including observed peak concentration (C_{max}), time to reach peak concentration (T_{max}) obtained directly, elimination half-life ($T_{1/2}$), mean residence time (MRT), area under the plasma concentration-time curve to the last measureable plasma concentration (AUC_{0-t}), and area under the plasma concentration-time curve to time infinity ($AUC_{0-\infty}$), were calculated by WinNonlin 5.2 Software from Pharsight Co. (USA) based on non-compartmental analysis of plasma concentration versus time data. All data were presented as $\bar{x} \pm s$.

3. Results and discussion

3.1 Method development

To optimize ESI conditions for MRM mode analysis, a 200 ng/mL neat standard solution containing individual analyte or IS along with the mobile phase was directly infused into the mass spectrometer. Both the positive and negative ion modes were conducted. The results of the full-scan spectra showed the response of positive ion mode was much more sensitive and selective for all compounds. Protonated molecular ions $[M + H]^+$ were considered as the most abundant ions. Thus, $[M + H]^+$ ions were selected as the precursor ions. Figure 2 shows that the predominant best product fragments were *m/z* 270.0 for wogonin, *m/z* 153.0 for luteolin (IS), *m/z* 292.0 for coptisine, *m/z* 320.0 for berberine, *m/z* 322.1 for jatrorrhizine, *m/z* 192.0 for phellodendrine, *m/z* 297.0 for magnoflorine, *m/z* 336.1 for palmatine, *m/z* 192.0 for tetrahydropalmatine (IS), and *m/z* 285.1 for wogonoside. The MRM transitions and following detailed optimization of MRM parameters including fragmentor and collision energy are all described in Table 1. Other parameters such as drying gas flow rate, drying gas temperature, nebulizing gas pressure, and capillary voltage were optimized to obtain the highest intensity of protonated molecular of analytes.

To achieve short retention time, symmetric peak shape and satisfactory ionization, some chromatographic conditions including types of reversed-phase chromatographic column, mobile phase composition, choice of additives, column temperature, and flow rate of mobile phase were optimized. Different types of reversed-phase chromatographic column were tried, including Zorbax Eclipse Plus C_{18} column (100 mm × 3.0 mm, 1.8 μm), Zorbax Eclipse XDB-C_{18} column (100 mm × 2.1 mm, 1.8 μm) and Acquity HSS T3 column (100 mm × 2.1 mm, 1.8 μm). As a result, the Acquity HSS T3 column was found to be most suitable, which could give good peak separation and high intensity. Several combinations of acetonitrile, methanol, formic acid, and acetic acid were investigated to optimize the mobile phase. Finally, acetonitrile-water system provided best chromatographic behavior (peak shape and resolution). Moreover, the addition of 0.1% formic acid to the water phase increased remarkably analyte signal response and improved peak shape. Therefore, acetonitrile-water containing 0.1% formic acid system was selected as the mobile phase. For the analysis of multiple components, a linear gradient program was employed with a high water content of 80% at the beginning of the gradient cycle. This dramatically improved the LC separation and removed the interfering substance. When the flow rate was set at 0.3 mL/min with column temperature at 30 °C, better separation and shorter running time were obtained.

Sample preparation is a critical step for developing an accurate and reliable LC-ESI-MS/MS method to eliminate interference from the sample matrix and achieve satisfactory recovery. Several sample preparation methods including protein precipitation (PPT) with acetonitrile or methanol, liquid-liquid extraction (LLE) using different extraction solvents (ethyl acetate, tert-butyl methyl ether, or their combinations), and solid phase extraction (SPE) with Waters Oasis HLB cartridges (Milford, MA, USA) were tried and compared to select an appropriate preparation method. The results showed that LLE with ethyl acetate produced the best recovery for wogonin, wogonoside, and their IS (luteolin), while PPT using acetonitrile provided the best recovery for other six alkaloids and their IS (tetrahydropalmatine).

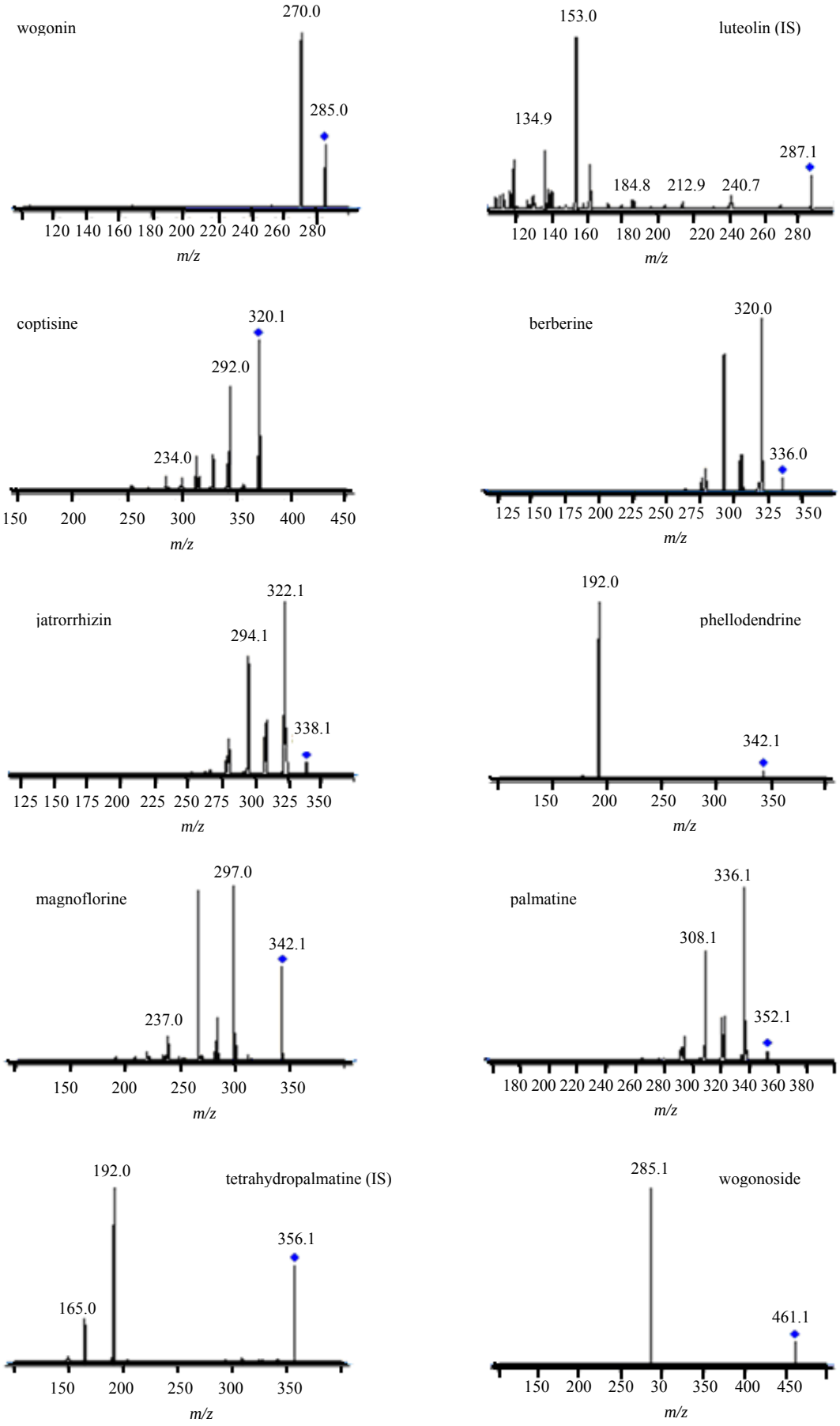

Figure 2 Product ion mass spectra of analytes and IS

However, PPT using acetonitrile could also give wogonin, wogonoside, and luteolin suitable recovery and little interference. In addition, PPT was much simpler and less time-consuming than LLE. Therefore, PPT using acetonitrile was considered as the optimal sample preparation method owing to satisfactory recovery and little matrix effect for all analytes and IS. In terms of peak shape, adding an equivalent volume of water to the supernatant before being injected into the LC-ESI-MS/MS system could yield symmetric peak shape.

IS is needed for accurate quantitative analysis of analytes in biological samples. However, in our study, as the analytes belong to two different groups, it is necessary to select two kinds of IS. Luteolin was finally selected as IS due to its similarity with wogonin and wogonoside in chemical structure, chromatographic retention time, and mass spectrographic behavior. Moreover, luteolin could not be found in the plasma sample of HJD, and is stable during the process of sample preparation. Based on the same reasons, tetrahydropalmatine was selected as another IS for quantification of the six alkaloids.

3.2 Method validation

Under the conditions described above, the representative MRM chromatograms of blank plasma, blank plasma sample spiked with eight analytes and IS, and plasma sample of oral administered rat are presented in Figure 3. The retention time was 5.25 min for wogonin, 5.28 min for luteolin (IS), 4.55 min for coptisine, 4.94 min for berberine, 4.46 min for jatrorrhizine, 1.43 min for phellodendrine, 1.65 min for magnoflorine, 4.85 min

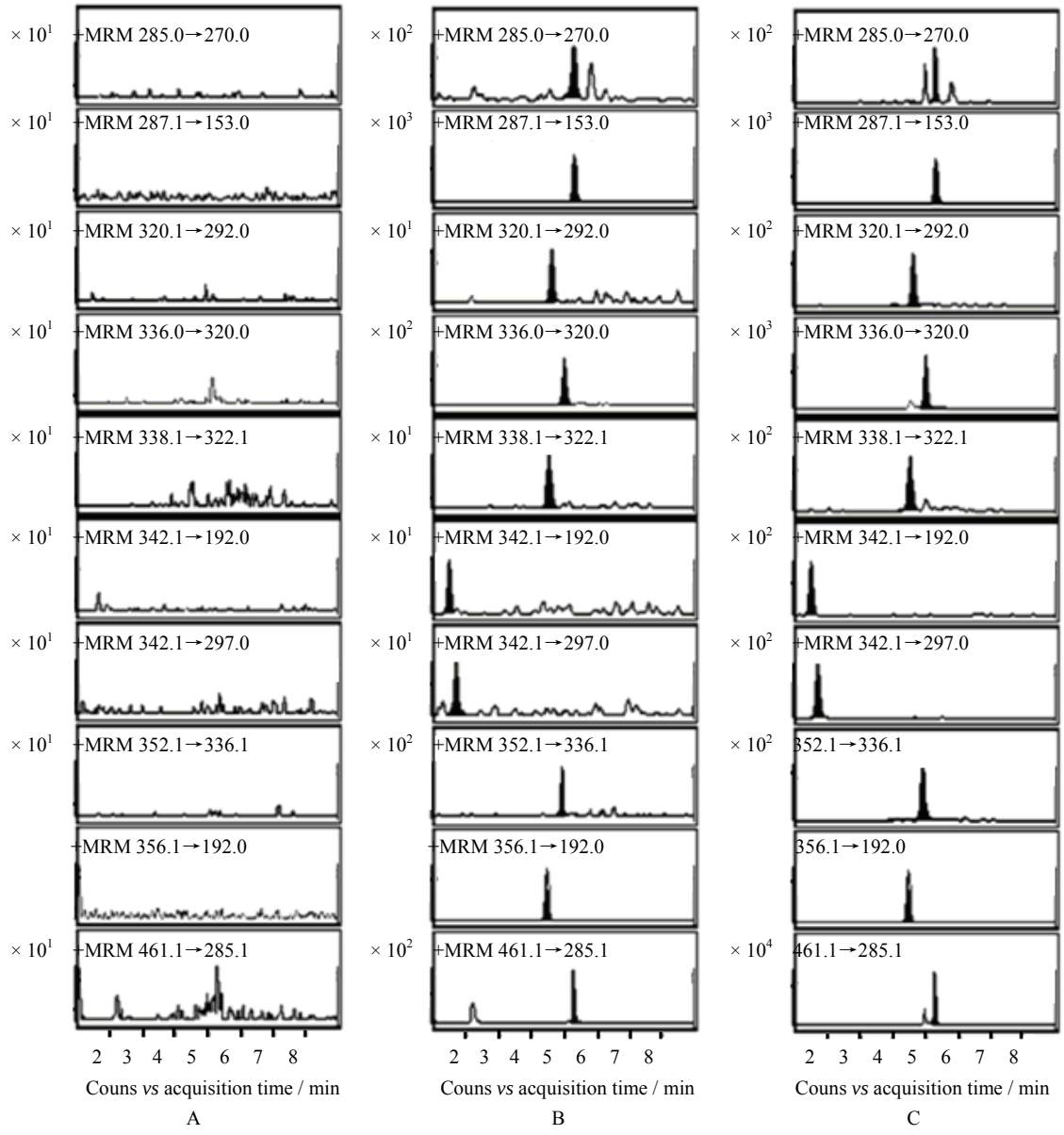

Figure 3 Representative MRM chromatograms of analytes and IS

A: blank plasma sample; B: blank plasma sample spiked with eight analytes at LLOQs and IS; C: plasma sample of oral administered rat at time point of 30 min post dose

for palmatine, 4.40 min for tetrahydropalmatine (IS), and 5.22 min for wogonoside. No significant interference from endogenous substances or other sources were found at the same mass transitions and retention time of the analytes and two kinds of IS, suggesting good selectivity of the developed method.

The linear ranges, regression equations, correlation coefficients and LLOQs of all analytes are listed in Table 2. All the calibration curves exhibited good linearity with correlation coefficients above 0.9959. The LLOQs for wogonin, coptisine, berberine, jatrorrhizine, phellodendrine, magnoflorine, palmatine, and wogonoside were 4.80, 0.20, 0.48, 0.10, 0.20, 0.32, 0.30, and 4.80 ng/mL, respectively, which were sufficient for the PK study of the eight active constituents following oral administration of HJD to rats. Table 3 summarizes the intra- and inter-day precision and accuracy values of the eight analytes at different concentration. The intra- and inter-day precision (RSD) values of the analytes were all no more than 12.01% and 12.11%, respectively. The intra- and inter-day accuracies (RE) ranged from −14.01% to 4.52% and from −14.46% to 4.86%, respectively. All the assay values were within the acceptable criteria, demonstrating that an accurate, reliable, and reproducible method was established for the determination of all the analytes in rat plasma.

As shown in Table 4, the extraction recoveries of eight analytes were from 93.10% to 110.91% with RSD values less than 8.12%, which indicated that the overall extraction recovery of PPT with acetonitrile was efficient, consistent, and reproducible. The matrix effects of analytes ranged from 85.10% to 100.77% at the three QC levels with the RSD values less than 10.76%. No significant matrix effect for all the analytes was observed, which indicated that no co-eluting substance could influence the ionization of the analytes.

The stability of all the analytes under various conditions is presented in Tables 5 and 6. The consistent results indicated that these analytes were all stable in plasma at room temperature for 4 h, after three freeze-thaw cycles at about

Table 2 Regression equations, correlation coefficients, linear ranges and LLOQs of analytes ($\bar{x} \pm s$, $n = 5$)

Analytes	LLOQ / (ng·mL^{-1})	Linear range / (ng·mL^{-1})	Linear regression equation	r^2
wogonin	4.80	4.80−1600	$y = 0.8009x + 0.1355$	0.9982
coptisine	0.20	0.20− 30	$y = 0.2675x + 0.0234$	0.9962
berberine	0.48	0.48− 180	$y = 0.7330x + 0.0401$	0.9979
jatrorrhizine	0.10	0.10− 24	$y = 1.0209x + 0.0014$	0.9959
phellodendrine	0.20	0.20− 24	$y = 3.1792x - 0.0101$	0.9976
magnoflorine	0.32	0.32− 100	$y = 0.6302x + 0.0609$	0.9987
palmatine	0.30	0.30− 40	$y = 1.0547x + 0.0216$	0.9983
wogonoside	4.80	4.80−2800	$y = 4.6970x + 3.1404$	0.9994

Table 3 Precision and accuracy for analytes in rat plasma ($\bar{x} \pm s$, n = 15, five replicates per day for 3 d)

Analytes	Nominal concentration / (ng·mL^{-1})	Intra-day			Inter-day		
		Concentration / (ng·mL^{-1})	RSD / %	RE / %	Concentration / (ng·mL^{-1})	RSD / %	RE / %
wogonin	12	12.28 ± 0.26	2.16	2.34	12.25 ± 0.07	0.60	2.05
	120	120.81 ± 0.60	0.49	0.68	120.06 ± 0.66	0.55	0.05
	1200	1031.89 ± 15.84	1.53	−14.01	1026.51 ± 11.91	1.16	−14.46
coptisine	0.2	0.20 ± 0.02	12.01	1.67	0.19 ± 0.02	12.11	−6.50
	2	1.99 ± 0.08	3.87	−0.45	1.99 ± 0.01	0.51	−0.27
	20	20.33 ± 0.54	2.64	1.64	20.84 ± 0.44	2.12	4.19
berberine	1.2	1.22 ± 0.05	4.02	1.53	1.21 ± 0.01	1.03	0.82
	12	12.15 ± 0.38	3.17	1.21	12.14 ± 0.15	1.24	1.16
	120	120.21 ± 0.96	0.80	0.17	120.21 ± 0.38	0.32	0.17
jatrorrhizine	0.2	0.21 ± 0.01	5.74	3.90	0.20 ± 0.01	3.77	0.10
	2	2.03 ± 0.04	1.81	1.63	2.09 ± 0.05	2.53	4.65
	20	19.99 ± 0.21	1.07	−0.06	20.00 ± 0.03	0.15	0.01
phellodendrine	0.2	0.19 ± 0.01	3.15	−3.35	0.20 ± 0.01	4.72	1.10
	2	2.02 ± 0.02	0.73	1.04	2.02 ± 0.00	0.23	1.15
	20	20.04 ± 0.34	1.68	0.18	19.97 ± 0.06	0.31	−0.14
magnoflorine	0.8	0.82 ± 0.05	6.27	1.89	0.81 ± 0.00	0.61	1.38
	8	8.04 ± 0.15	1.82	0.45	8.01 ± 0.04	0.48	0.13
	80	83.61 ± 0.99	1.18	4.52	83.89 ± 0.24	0.29	4.86
palmatine	0.3	0.31 ± 0.02	4.85	1.93	0.31 ± 0.01	1.63	2.83
	3	3.07 ± 0.06	1.78	2.34	3.05 ± 0.05	1.73	1.83
	30	30.02 ± 0.49	1.63	0.08	29.89 ± 0.17	0.56	−0.34
wogonoside	12	12.19 ± 0.18	1.50	1.59	12.39 ± 0.45	3.66	3.21
	240	240.04 ± 4.76	1.98	0.02	240.39 ± 1.36	0.57	0.16
	2400	2318.17 ± 36.29	1.57	−3.41	2353.77 ± 34.47	1.46	−1.93

Table 4 Extraction recovery and matrix effect for analytes in rat plasma ($\bar{x} \pm s$, $n = 3$)

Analytes	Nominal concentration / ($ng \cdot mL^{-1}$)	Extraction recovery / %	RSD / %	Matrix effect / %	RSD / %
wogonin	12	102.49 ± 2.10	2.15	91.27 ± 5.35	5.86
	120	102.72 ± 1.14	1.11	94.75 ± 2.64	2.78
	1200	101.89 ± 1.61	1.58	94.55 ± 2.14	2.26
coptisine	0.2	101.91 ± 8.27	8.12	91.64 ± 4.76	5.19
	2	110.91 ± 8.42	7.59	85.10 ± 6.06	7.11
	20	103.73 ± 4.67	4.50	86.70 ± 6.19	7.14
berberine	1.2	100.49 ± 1.01	1.01	97.30 ± 2.23	2.30
	12	100.95 ± 2.12	2.10	88.81 ± 5.22	5.88
	120	97.96 ± 1.62	1.65	90.01 ± 3.47	3.86
jatrorrhizine	0.2	96.63 ± 2.71	2.80	88.34 ± 2.98	3.37
	2	103.36 ± 3.17	3.06	96.69 ± 7.34	7.59
	20	103.21 ± 1.01	0.98	99.33 ± 1.30	1.31
phellodendrine	0.2	103.02 ± 3.07	2.98	98.71 ± 8.20	8.31
	2	101.48 ± 0.60	0.59	96.82 ± 0.90	0.93
	20	104.41 ± 3.89	3.72	98.53 ± 1.22	1.24
magnoflorine	0.8	105.60 ± 2.45	2.32	93.91 ± 7.40	7.88
	8	105.68 ± 1.42	1.34	93.51 ± 1.37	1.46
	80	102.33 ± 3.16	3.09	99.22 ± 0.19	0.19
palmatine	0.3	93.10 ± 5.57	5.98	90.09 ± 8.36	9.28
	3	98.41 ± 2.13	2.17	85.58 ± 4.87	5.69
	30	93.46 ± 1.42	1.52	86.42 ± 9.30	10.76
wogonoside	12	101.97 ± 0.56	0.55	100.77 ± 3.86	3.83
	240	100.49 ± 0.53	0.52	97.66 ± 1.50	1.53
	2400	101.03 ± 0.57	0.56	99.11 ± 0.54	0.55

Table 5 Stability of analytes in rat plasma ($\bar{x} \pm s$, $n = 5$)

Analytes	Nominal concentration / ($ng \cdot mL^{-1}$)	Short-term stability (4 h at room temperature)		Freeze-thaw stability (three cycles)	
		Concentration / %	RSD / %	Concentration / %	RSD / %
wogonin	12	94.41 ± 4.57	4.84	97.89 ± 4.07	4.16
	120	93.57 ± 2.70	2.88	96.63 ± 1.54	1.59
	1200	97.48 ± 0.58	0.60	97.12 ± 0.93	0.95
coptisine	0.2	91.85 ± 9.17	9.98	103.74 ± 15.21	14.66
	2	100.00 ± 14.45	14.45	102.76 ± 11.08	10.78
	20	96.26 ± 4.69	4.87	95.50 ± 4.13	4.33
berberine	1.2	90.78 ± 12.42	13.68	103.16 ± 11.45	11.10
	12	96.26 ± 3.67	3.81	96.44 ± 10.84	11.24
	120	102.15 ± 3.44	3.36	96.06 ± 6.14	6.39
jatrorrhizine	0.2	92.02 ± 10.36	11.25	99.15 ± 4.18	4.21
	2	100.07 ± 0.58	0.58	96.31 ± 7.66	7.95
	20	98.02 ± 2.53	2.58	98.77 ± 1.87	1.90
phellodendrine	0.2	100.78 ± 7.23	7.18	102.20 ± 6.78	6.63
	2	97.44 ± 0.72	0.74	96.23 ± 3.27	3.39
	20	100.17 ± 0.87	0.86	96.54 ± 1.48	1.53
magnoflorine	0.8	88.33 ± 5.98	6.78	87.29 ± 7.02	8.04
	8	98.57 ± 1.13	1.15	99.20 ± 6.56	6.61
	80	102.88 ± 0.37	0.36	97.57 ± 2.94	3.02
palmatine	0.3	100.43 ± 2.42	2.41	94.14 ± 6.56	6.97
	3	97.24 ± 0.77	0.79	93.29 ± 0.46	0.49
	30	100.35 ± 0.42	0.42	98.59 ± 1.38	1.40
wogonoside	12	98.09 ± 0.82	0.84	99.54 ± 2.09	2.10
	240	97.32 ± 1.37	1.41	97.63 ± 4.62	4.73
	2400	101.99 ± 1.31	1.29	98.14 ± 2.26	2.30

Table 6 Stability of analytes in rat plasma ($\bar{x} \pm s$, $n = 5$)

Analytes	Nominal concentration / ($ng \cdot mL^{-1}$)	Autosampler stability (12 h at ambient temperature)		Long-term stability (30 d at −20 °C)	
		Concentration / %	RSD / %	Concentration / %	RSD/%
wogonin	12	94.32 ± 3.71	3.94	94.38 ± 4.61	4.88
	120	93.50 ± 3.28	3.51	91.58 ± 1.81	1.98
	1200	98.06 ± 0.87	0.89	96.08 ± 0.73	0.76
coptisine	0.2	96.08 ± 8.90	9.26	94.64 ± 2.45	2.59
	2	93.49 ± 8.32	8.90	92.64 ± 7.04	7.60
	20	96.78 ± 3.66	3.78	93.81 ± 1.80	1.92
berberine	1.2	90.86 ± 10.56	11.62	95.54 ± 7.59	7.94
	12	94.48 ± 10.75	11.38	95.54 ± 3.79	3.97
	120	97.25 ± 3.59	3.70	97.86 ± 4.48	4.58
jatrorrhizine	0.2	91.83 ± 11.83	12.88	95.28 ± 4.65	4.88
	2	94.77 ± 13.17	13.90	96.22 ± 7.31	7.60
	20	98.58 ± 3.20	3.24	97.81 ± 2.25	2.30
phellodendrine	0.2	93.00 ± 10.94	11.76	93.69 ± 4.59	4.90
	2	98.30 ± 2.13	2.16	96.00 ± 2.96	3.09
	20	98.83 ± 1.72	1.74	96.74 ± 1.88	1.94
magnoflorine	0.8	87.53 ± 10.58	12.08	91.42 ± 3.56	3.89
	8	94.34 ± 5.02	5.32	96.94 ± 8.25	8.51
	80	99.13 ± 1.39	1.40	97.12 ± 2.73	2.82
palmatine	0.3	95.01 ± 2.38	2.50	94.61 ± 6.79	7.18
	3	94.40 ± 0.98	1.03	95.76 ± 2.26	2.36
	30	96.74 ± 1.87	1.93	95.88 ± 2.01	2.09
wogonoside	12	93.56 ± 1.67	1.79	94.69 ± 3.07	3.24
	240	93.57 ± 2.37	2.54	91.95 ± 2.48	2.70
	2400	98.22 ± 2.74	2.79	99.20 ± 4.03	4.06

−20 °C for 30 d, and in the autosampler at ambient temperature (about 22 °C) for 12 h after protein precipitation with the values in the range of 87.29%−103.74% (RSD value was less than 15%).

3.3 PK study

The well-validated method was successfully applied to the PK study of wogonin, coptisine, berberine, jatrorrhizine, phellodendrine, magnoflorine, palmatine, and wogonoside following single oral administration of HJD at 5.34 g/kg to seven male rats. The test articles could be monitored in rat plasma for up to 24 h post-dose. The mean plasma concentration-time profiles of the eight active constituents are illustrated in Figure 4 and the corresponding PK parameters are listed in Table 7.

As shown in Figure 4, multiple plasma concentration peaks were observed in both individual and mean plasma concentration curves of the quaternary protoberberine-type alkaloids (coptisine, berberine, jatrorrhizine, phellodendrine, and palmatine). Previous studies reported multiple blood concentration peaks in alkaloid PK and attributed it to the distribution, re-absorption and/or enterohepatic circulation (Deng et al, 2008; Feng et al, 2010; Zan et al, 2011; Cai et al, 2010; Wang et al, 2012b). However, elucidation of the mechanism of the phenomenon in PK study needs further detailed studies. Owing to the similar structures, coptisine, berberine, jatrorrhizine, phellodendrine, and palmatine had parallel PK parameters *in vivo*, being absorbed and eliminated with the similar rate. The T_{max} values were within 20 min for the five quaternary protoberberine-type alkaloids. The results indicated that the absorption of the five alkaloids might be rapid.

However, the C_{max} values were observed as (13.21 ± 5.65) ng/mL for coptisine, (59.84 ± 27.87) ng/mL for berberine, (6.63 ± 3.41) ng/mL for jatrorrhizine, (2.13 ± 1.06) ng/mL for phellodendrine, and (13.02 ± 7.94) ng/mL for palmatine, respectively.

The AUC_{0-t} values were (62.44 ± 41.01) ng/(mL·h) for coptisine, (291.44 ± 153.65) ng/(mL·h) for berberine, (30.63 ± 16.35) ng/(mL·h) for jatrorrhizine, (9.77 ± 7.79) ng/(mL·h) for phellodendrine, and (82.51 ± 38.01) ng/(mL·h) for palmatine. These showed that the five alkaloids shared low plasma concentration. Previous studies concluded that the poor absorption and extensive metabolism might result in low plasma concentration of berberine after oral administration (Yu et al, 2007; Zuo et al, 2006). Because of their similar structures with berberine and lower contents for administration, the plasma concentration of the other four protoberberine-type alkaloids was lower. The values of $T_{1/2}$ ranged from 12.01 to 30.91 h. The results of the reports cited here were vastly different from previous study (Lu et al, 2006), which may be explained by individual differences in rats and/or the administered HJD accounting for different proportions of compounds.

Magnoflorine, an aporphine alkaloid, presented remarkably different mean plasma concentration-time profiles

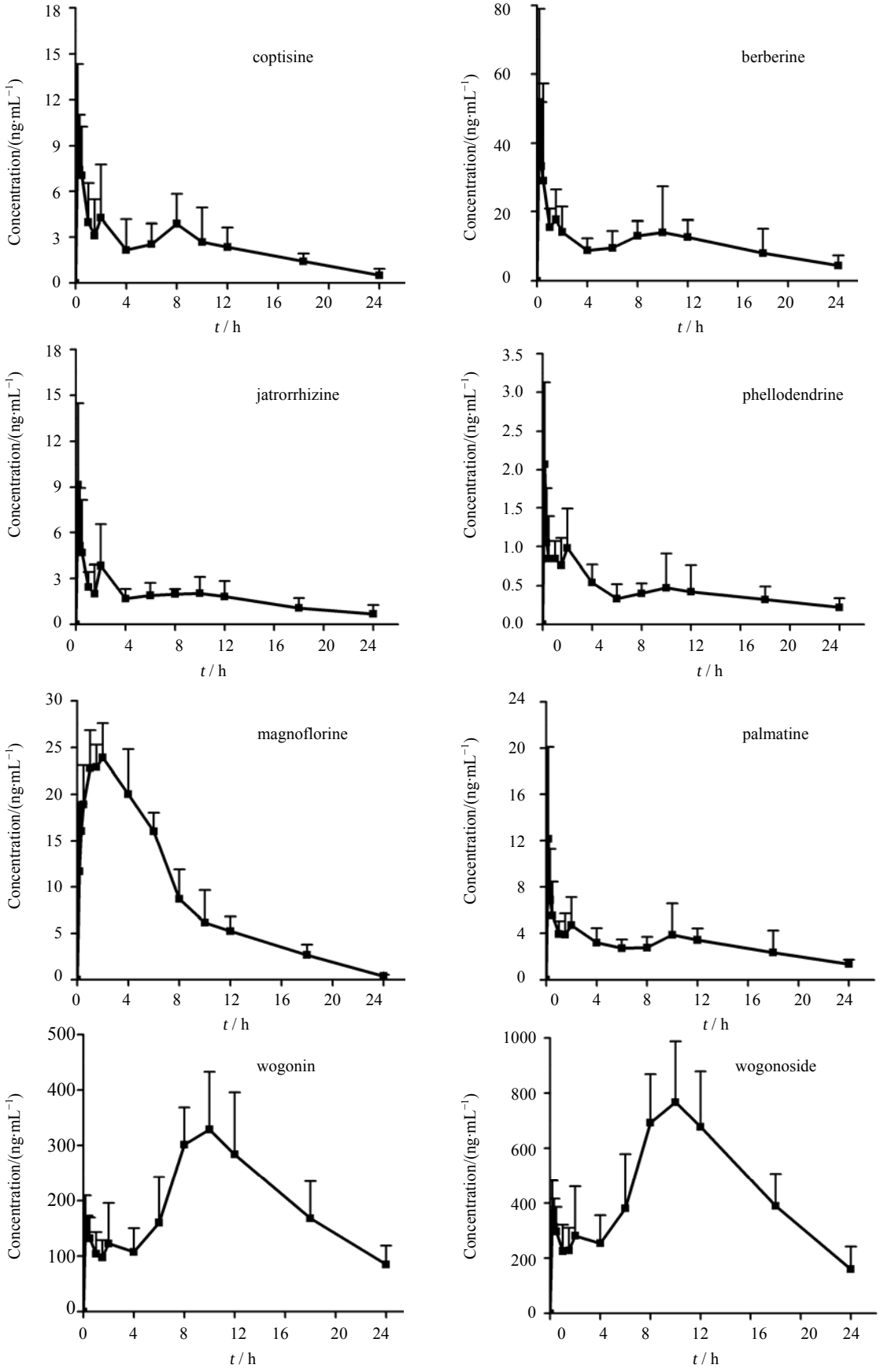

Figure 4 Plasma concentration-time profiles of analyte208s following single oral administration of HJD to rats ($\bar{x} \pm s$, $n = 7$)

Table 7 PK parameters of eight analytes following single oral administration of HJD to rats ($\bar{x} \pm s$, $n = 7$)

Analytes	T_{max} / h	C_{max} / (ng·mL^{-1})	AUC_{0-t} / (ng·mL^{-1}·h^{-1})	$AUC_{0-\infty}$ / (ng·mL^{-1}·h^{-1})	$T_{1/2}$ / h	MRT / h
coptisine	0.31 ± 0.16	13.21 ± 5.65	62.44 ± 41.01	114.49 ± 107.71	12.01 ± 10.83	19.98 ± 15.72
berberine	0.28 ± 0.17	59.84 ± 27.87	291.44 ± 153.65	830.56 ± 823.69	30.91 ± 29.39	45.58 ± 41.79
jatrorrhizine	0.27 ± 0.15	6.63 ± 3.41	30.63 ± 16.35	56.75 ± 31.70	15.35 ± 9.54	23.34 ± 13.99
phellodendrine	0.20 ± 0.07	2.13 ± 1.06	9.77 ± 7.79	25.30 ± 20.04	16.50 ± 10.99	26.55 ± 17.02
palmatine	0.20 ± 0.07	13.02 ± 7.94	82.51 ± 38.01	164.45 ± 64.38	22.68 ± 8.49	34.45 ± 11.73
magnoflorine	1.83 ± 0.68	25.78 ± 3.25	194.81 ± 47.59	202.95 ± 47.70	3.14 ± 1.61	5.62 ± 1.80
wogonin	9.00 ± 1.10	353.17 ± 93.22	4705.49 ± 944.99	5658.33 ± 1039.32	9.20 ± 1.50	16.37 ± 3.34
wogonoside	8.67 ± 1.63	816.67 ± 200.30	10691.19 ± 2089.32	11733.40 ± 2476.81	7.52 ± 1.22	13.81 ± 2.97

and PK parameters. It is the first time that PK study of magnoflorine after oral administration have been reported. Figure 4 shows that magnoflorine performed a typical single-peak concentration-time curve. The analyte exhibited relative slow absorption and rapid elimination through the values of T_{max} and $T_{1/2}$. In addition, both the C_{max} and AUC_{0-t} values were higher than those of initial dose. The results might be presumably due to the different physicochemical properties of compounds or the PK interaction of the prescribed chemical constituents.

As shown in Figure 4, wogonin and wogonoside, the two flavonoids, still performed multiple peaks phenomenon in plasma concentration-time profiles, which might be owing to glucuronidation, enteric circulation, and enterohepatic circulation (Chung et al, 2012; Tong et al, 2012). The values of T_{max} were 9.00 h for wogonin and 8.67 h for wogonoside. The results are apparently different from previous literature (Lu et al, 2007), which may be explained by individual differences in rats and/or administered HJD consisting of components with different proportions. The difference of $T_{1/2}$ indicated that wogonoside was eliminated relatively easier than wogonin, which was in conformity with the previous literature (Tong et al, 2012).

3.3 Method comparison with existing reports

There are several reports on assaying some of eight analytes *in vivo* (Zhu et al, 2012; Deng et al, 2006; Lu et al, 2006; Zhu et al, 2013; Tan et al, 2007; Li et al, 2011; Zhou et al, 2012; Chuang et al, 1996; Yu et al, 2007; Zhu et al, 2010; Zhang et al, 2011; Deng et al, 2008; Yuan et al, 2012; Kim et al, 2006; Chung et al, 2012; Tong et al, 2012; Feng et al, 2010; Zan et al, 2011), but no method for the simultaneous determination of wogonin, coptisine, berberine, jatrorrhizine, phellodendrine, magnoflorine, palmatine, and wogonoside in biological samples exists.

However, simultaneous determination of the eight active compounds in the HJD in biological samples is essential. A method for the simultaneous determination of 14 major chemical constituents in HJD by HPLC-DAD has been developed (Kwok et al, 2013). However, the method was applied *in vitro*. The LLOQs were very high for all the analytes. The chromatographic running time was 30 min. So the method is not suitable for assaying *in vivo*. In earlier published methods, LLOQs of coptisine, jatrorrhizine, phellodendrine, and magnoflorine were all relatively higher, and the four analytes in plasma were not determined. In the paper, the LLOQs were 0.20 ng/mL for coptisine and phellodendrine, 0.10 ng/mL for jatrorrhizine, and 0.32 ng/mL for magnoflorine. To the best of our knowledge, the LLOQs of the four analytes were the lowest, indicating the method was sensitive enough for PK study. The chromatographic running time was within 9 min for all the eight compounds.

4. Conclusion

For the first time, a rapid, sensitive, and convenient LC-ESI-MS/MS method for the simultaneous determination of wogonin, coptisine, berberine, jatrorrhizine, phellodendrine, magnoflorine, palmatine, and wogonoside in rat plasma has been developed and validated. The method offers the advantages of high sensitivity and simple plasma sample preparation. It is successfully applied to simultaneously evaluating the PK properties of the eight bioactive components after oral administration of HJD. The PK parameters obtained from this study and the validated method would be useful in clinical applications of HJD and other related TCM preparations.

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