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Simultaneous determination of six hydrophilic components in rat plasma after oral administration of Jitai tablet by liquid chromatography–electrospray ionization–tandem mass spectrometry: Application to a pharmacokinetic study

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ABSTRACT

A liquid chromatography–electrospray ionization-tandem mass spectrometry (LC–ESI-MS/MS) method was developed and validated for the simultaneous determination of amygdalin (ADL), danshensu (DSS), ferulic acid (FA), hydroxysafflor yellow A (HSYA), salvianolic acid A (SAA) and salvianolic acid B (SAB) in rat plasma. Plasma samples were pretreated by protein precipitation with acetonitrile. LC separation was performed on a Zorbax Eclipse Plus C18 column (3.0 mm × 100 mm I.D, 1.8 μm) with gradient elution using a mobile phase consisting of acetonitrile-0.1% formic acid in water at a flow rate of 0.3 mL/min. ESI-MS spectra was acquired in negative ion multiple reaction monitoring mode. The mass transition ion-pair was followed as *m/z* 456.0 → 323.1, *m/z* 197.3 → 178.8, *m/z* 193.0 → 133.9, *m/z* 611.1 → 325.2, *m/z* 493.0 → 295.0, and *m/z* 717.0 → 519.0 for ADL, DSS, FA, HSYA, SAA and SAB, respectively. All analytes showed good linearity over a wide concentration range ($r > 0.99$). The lower limit of quantification was 7 ng/mL, 2 ng/mL, 4 ng/mL, 1 ng/mL, 2 ng/mL, and 4 ng/mL for ADL, DSS, FA, HSYA, SAA and SAB, respectively. The mean recovery of the analytes ranged from 86.29% to 93.16%. The intra- and inter-day precisions were in the range of 1.50–9.98% and the accuracies were between 91.17% and 99.46%. The validated method was successfully applied to a pharmacokinetic study of the six hydrophilic components in rat plasma after oral administration of Jitai tablet.

1. Introduction

Pharmacokinetic (PK) study dose add to the body of scientific knowledge concerning traditional Chinese medicines (TCMs), and is important considering the fact that a large percentage of world population uses this type of treatment. In general, there is a lack of PK data and little or no PK data on TCMs owing to the challenges including complex compositions, uncharacterized active constituents, differential disposition process, low concentrations in vivo, etc. Thus, it is imperative to establish sensitive and reliable analytical methods to quantify the multiple components of TCMs in vivo [1].

Jitai tablet (JTT), an important TCM prescription in treating opiate addiction since Qing dynasty, has been proved to be very safe and effective in the inhibition of protracted withdrawal symptoms with less harmful side effects [2,3], and good for the rehabilitation of abnormal body functions induced by chronic drug use, including improving immune function, increasing working memory and preventing neurological disorders [4,5]. The prescription consists of fifteen medicinal materials, including *Rhizoma Corydalis*, *Radix Salviae Miltiorrhiae*, *Radix Angelicae sinensis*, *Ligusticum Chuanxiong*, *Semen Persicae*, *Flos Carthami*, *Radix Aconite*, *Radix Ginseng*, *Cortex Cinnamomi*, *Rhizoma Zingiberis*, *Semen Myristicae*, *Flos Daturae*, *Radix Aucklandiae*, *Lignum Aquilariae Resinatrm* and *Margarita*. On the basis of Chinese medical philosophy and practice, they usually serve as principal, deputy, adjuvant and guide roles respectively, in the prevention and treatment of opiate addiction with a rigorous intrinsic mass ratio. Particularly, the principal components, *Radix Salviae Miltiorrhiae*

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and *Radix Corydalis*, exhibit the main therapeutic efficacy, while others raise the efficacy or reduce the toxicity of the principal components [6]. Thus, to profile the PK properties of the active ingredients in the principal components and other adjunctive components would substantially aid clarifying the active mechanism of the whole prescription. To *Radix Corydalis*, a specific and reliable method using liquid chromatography–electrospray ionization–tandem mass spectrometry (LC–ESI-MS/MS) in positive ionization mode has been developed for the PK evaluation of the tertiary alkaloids in rat plasma after oral administration of JTT [7]. Whereas no method could be found for simultaneous evaluation of the PK properties of *Radix Salviae Miltiorrhiae* and other important adjunctive components including *Radix Angelicae sinensis*, *Lignum Chuanxiong*, *Semen Persicae* and *Flos Carthami*, which are aqueous extracts, and the major ingredients could be detected only in negative ionization mode using LC–ESI-MS/MS.

TCMs consist of complex mixtures of phytochemical constituents, selection of marker compounds for PK study is usually affected by subjective assessments, empirical evidences or the commercial availabilities of reference standards. The approach of plasma pharmacochemistry is increasingly applied in the discovery of bioactive components in TCMs with its global research strategy. According to the theory of plasma pharmacochemistry, only the orally absorbed constituents have the chance to become potential bioactive components [8]. Danshensu (DSS), salvianolic acid A (SAA) and salvianolic acid B (SAB) in *Radix Salviae Miltiorrhiae*, amygdalin (ADL) in *Semen Persicae*, ferulic acid (FA) in *Radix Angelicae sinensis* and *Lignum Chuanxiong*, and hydroxysafflor yellow A (HSYA) in *Flos Carthami* could be detected in the rat plasma after oral administration of JTT in our previous study [9]. Previous chemical investigations and pharmacological studies also discovered that the water-soluble compounds, including DSS, SAA and SAB are the representative agents of *Radix Salviae Miltiorrhiae*. Additionally, ADL, FA and HSYA are also the important constituents owing to their antiemetics [10], antiinflammatory [11–13], antinociceptive [14,15], anxiolytic [16], and sedative tranquilizing effects [17]. Therefore, it is of great value to develop an efficient and reliable bioanalytical protocol for simultaneous determination of these active ingredients in biological matrix with hopefully maximum response and resolution, and to study the PK properties of them to advance the therapeutic applications of JTT.

Various analytical methods based on liquid chromatography technology have been developed for the determination of the six compounds in biological fluids, respectively [18–24]. However, these methods have shortens: high limit of quantification, long analysis time, or could not fulfill oral PK study due to the insufficient sensitivity and specificity. It is well known that LC–MS is an effective approach for the PK study of compounds in complex matrix samples. Many studies also developed LC–MS methods for PK evaluation of these six compounds [25–30]. However, these methods usually focused on only one or two main marker components separately, no global methodology could be given for a comprehensive PK study of JTT. The introduction of sub-2 μm analytical column allows greatly speeding up of the analytical process by a factor of nine folds while maintaining similar efficiencies [31]. Therefore, the innovative technology fulfills key requirements in terms of sensitivity, selectivity, and peak–assignment certainty for the rapid determination of analytes at low concentrations in vivo. It is well known that the chemical components in JTT are prescribed combination to obtain synergistic effects or diminish potential adverse reactions. Therefore, an integral investigation into the PK evaluation of the multi components could not only link data from pharmacological assays to clinical effects, but also help designing rational dosage regimens, minimizing harmful side effects and investigating drug–drug interactions. In this paper, an LC–ESI-MS/MS method with triple–quadrupole tandem mass spectrometer in multiple–reaction monitoring (MRM) mode, and a 1.8 μm particles analytical column to ensure its high throughput, was developed for the simultaneous determination of ADL, DSS, FA, HSYA, SAA and SAB in rat plasma to evaluate their PK properties after oral administration of JJT.

2. Experimental

2.1. Materials and reagents

Acetonitrile and methanol of HPLC grade were obtained from Merck (Darmstadt, Germany). Formic acid was purchased from Sigma–Aldrich (St. Louis, MO, USA). All aqueous solutions were prepared with ultra pure water produced from a Milli–Q50 SP Reagent Water System (Bedford, MA, USA). Other reagents were of analytical grade or higher. Reference standards of ADL, DSS, FA, HSYA, SAA, SAB and gallic acid (internal standard, IS) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), their chemical structures are shown in Fig. 1. JTT samples (batch NO.: 050602) were kindly offered by National Engineering Research Center for TCM (Shanghai, China).

2.2. Determination of six hydrophilic components in JTT

The contents of the six compounds in JTT were quantitatively determined to calculate the administration dosage to rats. JTTs were powdered and sieved through a NO. 40 mesh sieve to get a homogeneous size. 80 mg was accurately weighed into a 10 mL volumetric flask. Methanol–water (50:50, V/V) was added and ultrasonic extracted for 30 min, the lost volume was compensated by adding the same solvent when cooled at room temperature, and then centrifuged at 13, 500 × *g* for 10 min, the supernatant was filtered through a syringe filter (0.22 μm). An aliquot of five (5) μL of supernatant was subjected to LC–ESI-MS/MS analysis. The LC–MS/MS conditions of this part were the same conditions applied for determination of the six compounds in rat plasma addressed in part 2.4 and part 2.5. The contents of ADL, DSS, FA, HSYA, SAA and SAB were determined as 4534.0, 626.5, 85.9, 87.7, 998.8 and 2532.1 μg/g, respectively.

2.3. Preparation of calibration standards and quality control samples

The stock solutions of ADL, DSS, FA, HSYA, SAA and SAB were prepared by dissolving requisite amount in methanol at a high concentration (200 μg/mL). Every stock solution was mixed in volumetric flasks with appropriate volumes, and then diluted to volume to make working standard solutions with methanol. Calibration work solutions were prepared by adding the diluted working standard solutions into blank rat plasma (5/95, v/v), final concentration series ranged from 7 ng/mL to 3500 ng/mL, 2 ng/mL to 1000 ng/mL, 4 ng/mL to 2000 ng/mL, 1 ng/mL to 500 ng/mL, 2 ng/mL to 1000 ng/mL, and 4 ng/mL to 2000 ng/mL for ADL, DSS, FA, HSYA, SAA and SAB were obtained, respectively. The IS working solution was prepared with a final concentration of 1 μg/mL.

Quality control (QC) samples including 14, 140 and 1400 ng/mL for ADL, 4, 40 and 400 ng/mL for DSS, 8, 80 and 800 ng/mL for FA, 2, 20 and 200 ng/mL for HSYA, 4, 40 and 400 ng/mL for SAA, and 8, 80 and 800 ng/mL for SAB were also prepared in the same manner. Calibration work solutions and QC samples were stored at 4 °C until LC–ESI-MS/MS analysis.

2.4. LC–ESI-MS/MS instrument and conditions

LC analysis was carried out on an Agilent 1200 Rapid Resolution Liquid Chromatography (Palo Alto, CA, USA) equipped with

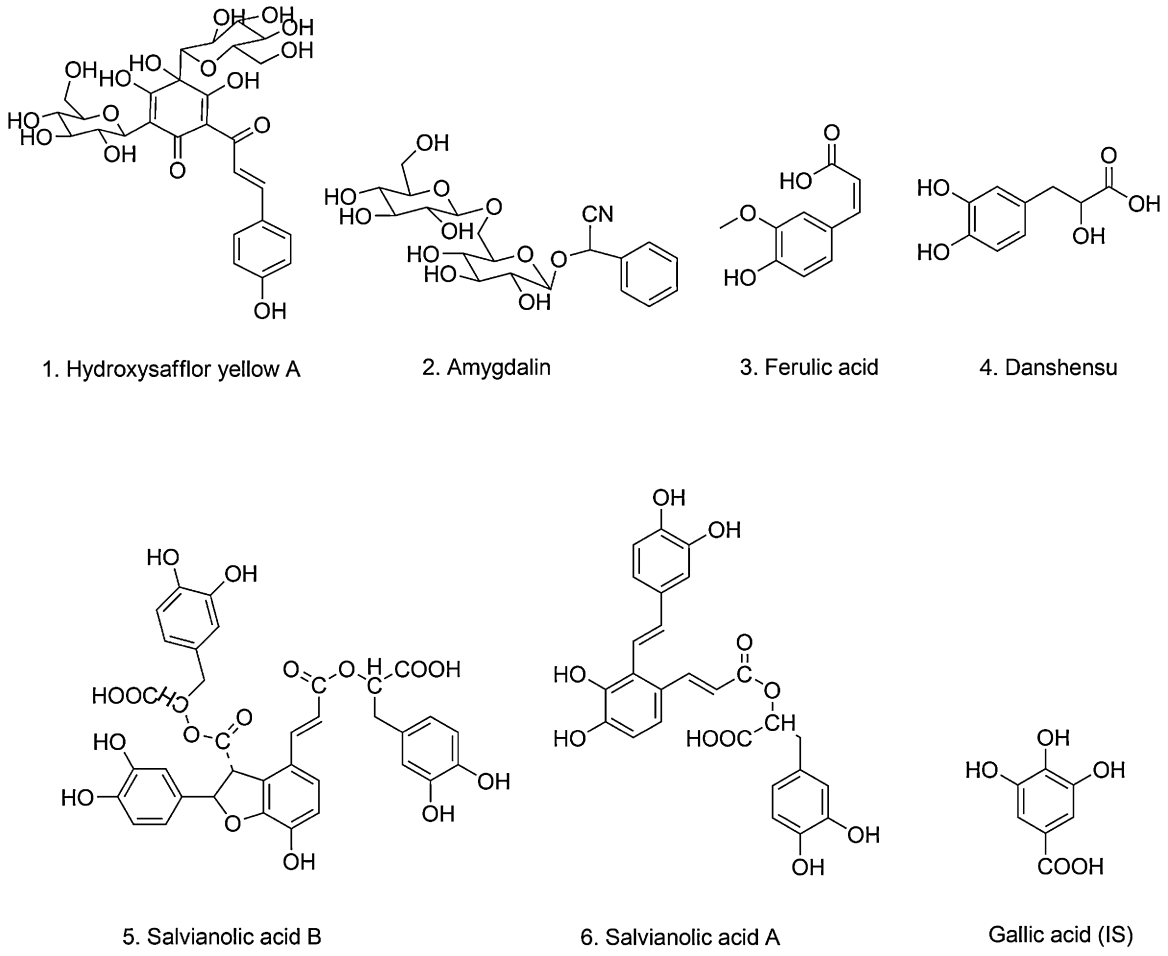

Fig. 1. Chemical structures of the analytes and IS.

a binary pump, an on–line vacuum degasser, an auto–sampler and a thermostated column compartment. Chromatographic separations were performed on an Agilent Zorbax Eclipse Plus C18 column (3.0 mm × 100 mm I.D, 1.8 μm, Agilent). Analytical column was maintained at 35 °C and eluted with a mobile phase consisting of acetonitrile (A) and water containing 0.1% formic acid (B) using the following gradient program: 20% A → 45% A at 0–1.0 min; 45% A at 1.0–2.5 min; 45% A → 20% A at 2.5–4.0 min; 20% A at 4.0–7.0 min at a flow rate of 0.3 mL/min. The total run time was 7 min and the equilibrated time was 2 min. 5 μL aliquot was injected onto the column. The effluent from the LC column was directed from the waste to the mass spectrometer source after the first 1.0 min of each run.

2.5. Mass spectrometric conditions

An Agilent G6410 Triple Quadrupole mass spectrometer equipped with an electrospray ion source (ESI) (Agilent, MA, USA) was applied to quantitative analysis of the analytes. ESI–MS spectra was acquired in negative ion multiple reaction monitoring (MRM) mode. The conditions of MS analysis were designed as follows: capillary voltage, 3500 V; nebulizer pressure, 40 psi; drying gas (N_2), 10 L/min with a temperature of 350 °C; collision gas (N_2); dwell time, 100 ms; mass analyzers Q1 and Q3 operated at unit mass resolution were used for each MRM transition. Compound–dependent parameters are listed in Table 1. Data collection, peak integration, and calculations were performed using Masshunter Workstation Software from Agilent Technologies (version B.03.01) (Palo Alto, CA, USA).

2.6. Sample preparations

One hundred (100) μL aliquot of the plasma sample was treated with two hundred (200) μL acetonitrile containing the IS (40 ng/mL, W/V), formic acid (0.5%, V/V) and vitamin C (200 ng/mL, W/V). The mixture was vortex mixed for 2 min and centrifuged at 13, 500 × *g* for 10 min. 5 μL of supernatant was injected to the LC–ESI-MS/MS system.

2.7. Method validation

The method was validated for linearity, lower limit of quantification (LLOQ), accuracy and precision, extraction recovery, matrix effect and stability following the industrial guidelines for bioanalytical method validation from the U.S. Food and Drug Administration (FDA) [33].

2.7.1. Specificity and selectivity

The specificity and selectivity of the method toward endogenous plasma matrix components was assessed by analyzing chromatograms of blank rat plasma from six sources, blank plasma spiked with the analytes at LLOQ, and plasma samples obtained from PK studies. The interfering peak area should be less than 20% of the peak area for the analytes at LLOQ in rat plasma.

2.7.2. Linearity and LLOQ

The linearity of the method was generated by analysis of five calibration curves containing eight non-zero concentrations. Each calibration curve was analyzed individually by fitting the area ratio

Table 1
MS/MS transitions and parameters for the detection of the analytes and IS.

Analyte	Retention time (min)	Q1 mass (*m/z*)	Q3 mass (*m/z*)	Dwell time (ms)	Fragmentor (v)	Collision energy (v)
Gallic acid (IS)	1.831	169.0	125.0	100	80	10
HSYA	1.975	611.1	325.2	100	190	30
FA	1.963	193.0	133.9	100	90	4
ADL	2.177	456.0	323.1	100	160	6
DSS	5.072	197.3	178.8	100	80	10
SAB	5.666	717.0	519.0	100	150	13
SAA	5.924	493.0	295.0	100	130	10

response for analyte/IS as a function of standard concentration, using least square weighted ($1/x^2$) linear regression and excluding the point of origin. The LLOQ was defined as the lowest concentration yielding a precision with coefficient of variation (CV) less than 20% and accuracy within 20% of the nominal value (i.e. accuracy between 80 and 120%) for each run analysis.

2.7.3. Accuracy and precision

The intra–day accuracy and precision were assessed by analyzing five replicate QC samples at three concentration levels on the same day. The inter–day accuracy and precision were assessed by analyzing five precision and accuracy batches on three consecutive validation days. Accuracy was expressed as percentages of nominal values. Precision was calculated as relative standard deviation (RSD) using one–way analysis of variance with day as the grouping variable. Values below ±15% and at LLOQ below ±20% were accepted.

2.7.4. Extraction recovery and matrix effect

The extraction recovery and matrix effect were assessed according to the procedure described by Matuszewski et al. [34]. Both parameters were evaluated by comparing the mean area response of three sets of solutions at three concentration levels. The extraction recovery was calculated by comparing the mean area response of pre–extraction spiked plasma samples to that of post–extraction spiked plasma samples at each QC level. The matrix effect was assessed by comparing the mean area response of post–extraction spiked plasma samples with mean area of neat standard solutions. It was considered negligible if values below ±15% were observed. The value of matrix effect less than 85% represented ionization suppression, while more than 115% represented ionization enhancement.

2.7.5. Stability

The stability of the analytes in rat plasma was tested by comparison with baseline (=QC samples extracted and analyzed immediately) and by calculation the deviation from nominal concentrations. The QC samples were subjected to three cycles of freezing at −20 °C and thawing for freeze and thaw stability, stored for 4 h maintained at room temperature for short–term temperature stability, and stored for 4 weeks at −20 °C for long–term stability. The autosampler stability was assessed by reanalyzing the pretreated samples after being left in the auto–sampler at room temperature for 12 h.

2.8. PK study in rats

Male Sprague–Dawley (SD) rats (body weight: 220–250 g) were supplied by the Laboratory Animal Center of the Chinese Academy of Sciences (Shanghai, China). The animals were maintained in controlled conditions (temperature 20–25 °C, relative humidity 55–60% and 12 h dark–light cycle) with free access to standard laboratory food and water for 5 days acclimation, and then fasted the night before (12 h prior to the experiment), and the time of recovery of feeding was 4 h post dose. The rats were randomly divided into group 1, 2, 3 and 4, 10 rats in each group to diminish the individual variation. In the pre test, all the blood collects were performed in the same animal. Since 14 collects were performed and the volume of blood was about 0.25 mL in each one, the total withdrawn blood volume was about 3.5 mL per animal, a value considerably high. Considering that hypovolemia and dehydration alter the pharmacokinetic parameters. In addition, multiple collects from the orbital vein caused injuries in the animal eye. Venous blood of each rat in group 1, 2 and 3 was collected for four consecutive time points, while the last two consecutive time points collected in group 4. All rats were intragastric administered JTT suspended in 0.5% carboxymethyl cellulose sodium aqueous solution at 15 g/kg. Venous blood (250 μL) was obtained from the orbit vein and collected in heparin pretreated polypropylene centrifuge tubes at 0 min, 5 min, 15 min, 30 min, 1 h, 1.5 h, 2 h, 3 h, 4 h, 6 h, 8 h, 10 h, 12 h and 24 h post–dose. All blood samples were immediately centrifuged and obtained plasma stored frozen in a freezer set to maintain −20 °C until bioanalysis. The plasma collected from six vehicle–administered rats served as the blank. Animal experiments were carried out according to the Guidelines for the Care and Use of Laboratory Animals, and were approved by the Animal Ethics Committee of the Second Military Medical University.

2.9. Statistical analysis

PK parameters were determined by non compartmental methods using WinNonlin 5.2 from Pharsight Corporation (Sunnyvale, CA, USA), including highest observed plasma concentration (C_{max}), time when highest plasma concentration observed (T_{max}), terminal or elimination half-life ($T_{1/2}$), mean residence time (MRT), area under the plasma concentration–time curve from Time 0 to the last measured concentration (AUC_{0-t}), and area under the plasma concentration–time curve extrapolated to infinity (AUC_{0-INF}).

3. Results and discussion

3.1. Method development

To develop a sensitive and reliable LC–ESI-MS/MS method, it was important to optimize the chromatographic and mass spectrometric conditions, as well as to obtain an efficient and simple extraction procedure for all the analytes and IS. Gallic acid was chosen as IS since structure similarities to compensate for variable extraction yields and to reduce possible effects of ion suppression/enhancement in the MS source.

3.1.1. Optimization of sample pre-treatment

The procedures of sample pre–treatment were focused on minimizing matrix effect and increasing the extraction recovery. Liquid–liquid extraction (LLE) with ethyl acetate and adjusted pH values (pH 1–3) accounted for most of the published methods [35–37]. However, due to the polarity of HSYA, only protein precipitation (PPT) was suitable for the extraction procedures [38,39]. In the present study, several sample pre–treatment methods were also evaluated, including PPT, LLE with ethyl acetate and the mixture of ethyl acetate and acetone, solid phase extraction (SPE) with

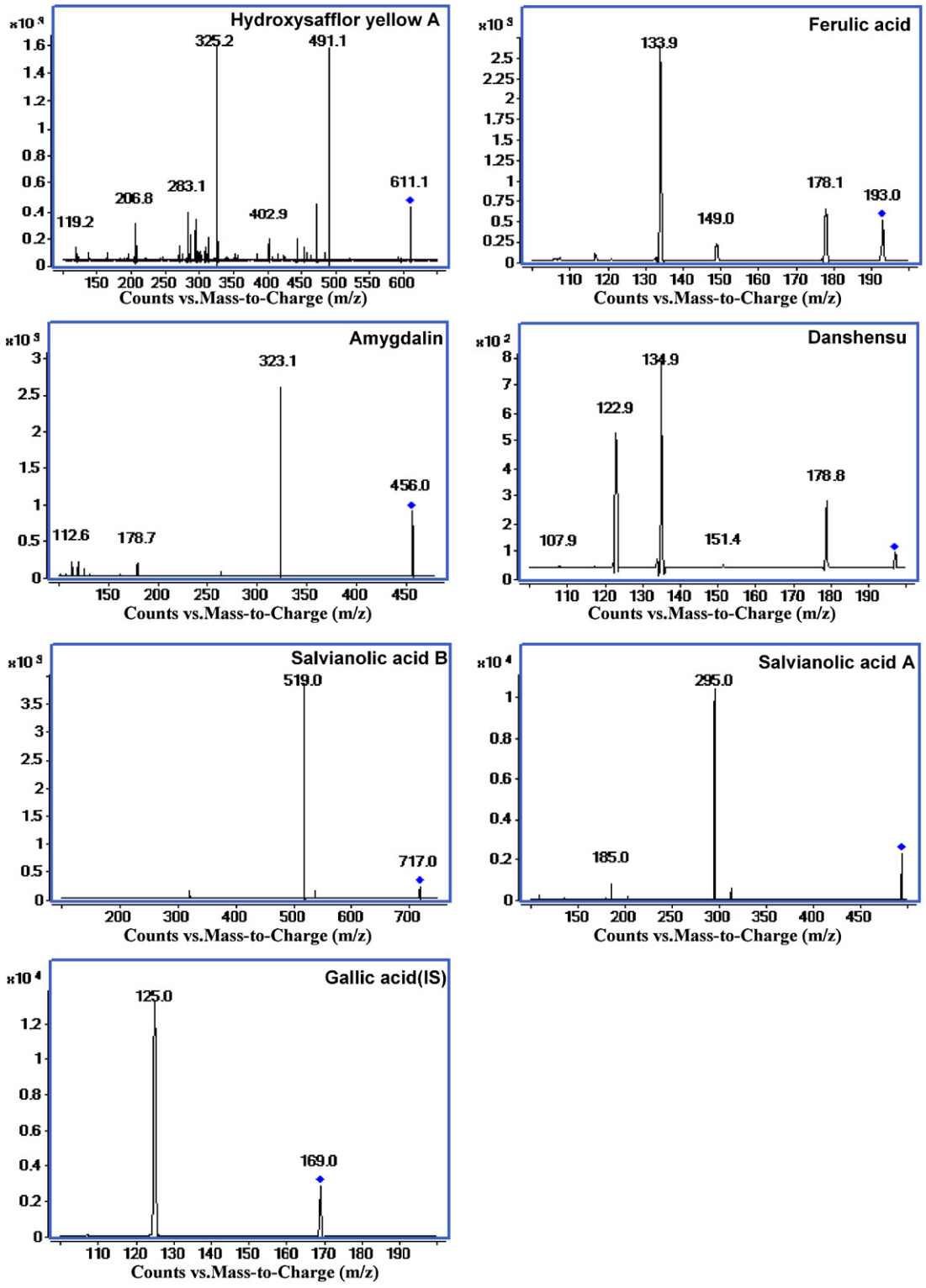

Fig. 2. Product ion mass spectra of the analytes and IS.

Waters Oasis HLB cartridges (Milford, MA, USA). However, SPE and LLE showed limited extraction efficiency due to the polarity of the analytes and extracting solvents, or the complex manual pre–treatment steps. Overall, taking matrix effect and extraction recovery into consideration together, PPT with acetonitrile, formic acid and vitamin C was selected as a promising and feasible approach since it could not only reduce the ionization of phenol, phenolic hydroxyl and carboxyl groups, but also prevent the possible degradation of phenolic acids.

3.1.2. *Optimization of liquid chromatographic conditions*

Liquid chromatographic conditions including stationary phase, the composition and pH value of mobile phase, column temperature and flow rate that could greatly influence the separation

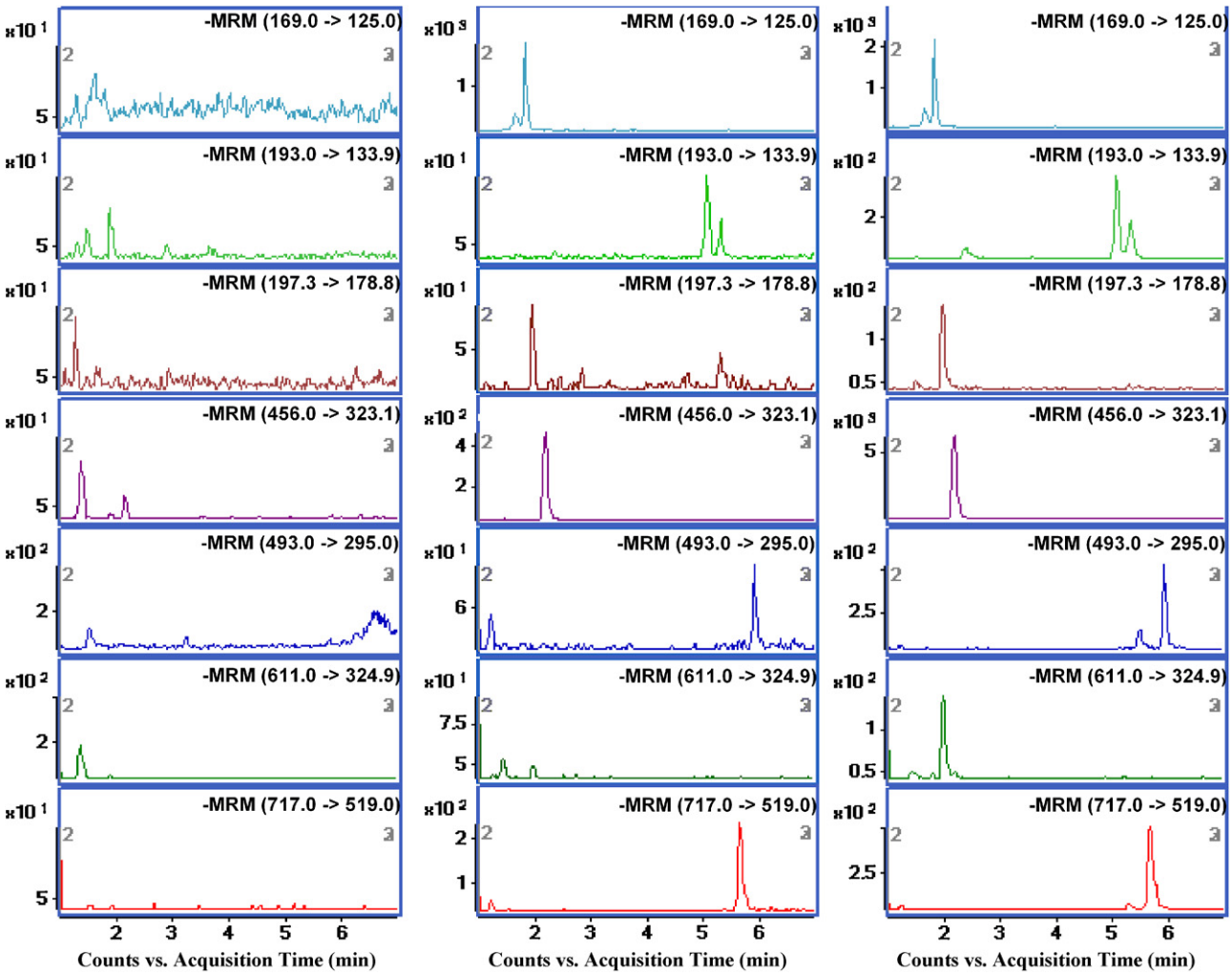

Fig. 3. Representative MRM chromatograms of the analytes and IS: (A) blank plasma sample, (B) blank plasma sample spiked with the analytes at LLOQ and IS, and (C) plasma sample of oral administrated rat at time point of 6 h post dose.

were investigated. Zorbax Eclipse Plus C18 column was chosen in the present study for its high efficiency and improving the peak symmetry in high column temperature. Different mobile phases (methanol–water, acetonitrile–water, methanol–acid aqueous solution and acetonitrile–acid aqueous solution) were examined and compared by performing several trials to obtain efficient chromatography, appropriate ionization and a short run time for the analytes. Addition of acetonitrile could remarkably improve the peak shape for DSS, SAA, SAB and FA. Correspondingly, owing to the carboxyl group and phenolic hydroxyls existed in the analytes, better separation and less interference from other components in the plasma were achieved by addition of 0.1% (V/V) formic acid to decrease the pH value of the mobile phase, acetonitrile–acid aqueous solution was therefore selected as the mobile phase. It was also found that the best separation was obtained when gradient elution was performed and column temperature was kept at 35 °C using a flow rate of 0.3 mL/min.

3.1.3. Optimization of mass spectrometric conditions

The following orders were employed to set up the MRM method for all the analytes and IS. Initially, the precursor and product ions of the analytes and IS were ascertained by infusing 200 ng/mL standard solutions in scan and product ions mode, respectively. The full–scan mass spectra showed that the ionization of all the analytes and IS gave more relative intense signals in negative ion mode than in positive ion mode. The MS/MS product ion spectra of the analytes and IS are shown in Fig. 2. On the basis of that, to get the richest relative abundance of precursor and product ions, the parameters for fragmentor energy and collision energy were further optimized. Table 1 shows the optimized MS/MS transitions and energy parameters of all the analyses. At the end, maximum abundance of the molecular ions of the compounds, acquisition parameters (source temperature and ions spray voltage) were investigated and the highest ion intensity for molecular ion of the analytes were achieved when the source temperature, the nebulizing gas (N_2) pressure, the drying gas flow were set at 350 °C, 40 psi and 10 L/min, respectively.

3.2. Method validation

3.2.1. Specificity and selectivity

In MRM mode, a specific voltage at the optimum value was set to provide the best sensitivity and specificity for each analyte, whereas ion discrimination was not possible by monitoring isotopic ions and fragment ions in addition to the major precursor ions. In the present study, the chromatograms of the blank plasma, spiked plasma sample with all analytes and the IS, and the plasma samples of oral administrated rat were represented in Fig. 3. The mass transition ion–pair was followed as *m/z* 456.0 → 323.1 for ADL, *m/z* 197.3 → 178.8 for DSS, *m/z* 193.0 → 133.9 for FA,

Table 2
Regression data and LLOQ of the analytes.

Analyte	LLOQ (ng/mL)	Linear range (ng/mL)	Slope	Intercept	R^2
HSYA	1	1–500	4.2282	51.8320	0.9894
FA	4	4–2000	2.3583	−52.3500	0.9973
ADL	7	7–3500	19.0382	−642.9695	0.9999
DSS	2	2–1000	13.0019	127.3089	0.9997
SAB	4	4–2000	28.0299	272.4712	0.9999
SAA	2	2–1000	16.6216	−25.7484	0.9998

Table 3
Precision and accuracy for the analytes in rat plasma (n = 15, 5 replicates per day for 3 days).

Analyte	Nominal concentration (ng/mL)	Intra-day				Inter-day			
		Concentration found (ng/mL)		Accuracy (%)	RSD (%)	Concentration found (ng/mL)		Accuracy (%)	RSD (%)
		Mean	SD			Mean	SD		
HSYA	2	1.89	0.09	94.72	4.55	1.88	0.08	93.83	4.23
	20	19.04	0.81	95.20	4.24	19.12	0.92	95.62	4.83
	200	190.23	7.89	95.12	4.15	190.60	6.55	95.30	3.44
FA	8	7.64	0.61	95.52	7.95	7.59	0.74	94.84	9.78
	80	75.52	4.01	94.40	5.31	75.18	3.58	93.98	4.76
	800	767.10	16.61	95.89	2.17	760.52	11.44	95.07	1.50
ADL	14	13.46	0.44	96.11	3.28	13.21	0.59	94.35	4.45
	140	136.95	2.98	97.82	2.18	137.81	2.99	98.43	2.17
	1400	1374.72	21.45	98.19	1.56	1375.61	22.98	98.26	1.67
DSS	4	3.65	0.30	91.17	8.29	3.68	0.14	91.99	3.81
	40	37.50	2.99	93.76	7.99	37.40	2.71	93.50	7.25
	400	397.83	9.32	99.46	2.34	380.48	12.75	95.12	3.35
SAB	8	7.42	0.52	92.74	6.95	7.33	0.61	91.60	8.36
	80	74.53	1.77	93.16	2.38	76.05	3.46	95.06	4.56
	800	759.95	60.84	94.99	8.01	763.48	48.77	95.43	6.39
SAA	4	3.81	0.18	95.29	4.69	3.83	0.38	95.80	9.98
	40	38.35	1.34	95.87	3.48	37.98	2.59	94.95	6.82
	400	384.65	13.49	96.16	3.51	383.84	18.09	95.96	4.71

m/z 611.1 → 325.2 for HSYA, m/z 493.0 → 295.0 for SAA, and m/z 717.0 → 519.0 for SAB, while the retention time was 2.2 min, 5.1 min, 1.9 min, 1.9 min, 5.9 min and 5.7 min for each number, respectively. No interference from endogenous materials or other source was found at the same retention time as the analytes, which indicated that the elaborated procedure was specified and selective.

3.2.2. Linearity and LLOQ

The method was linear over the concentration range of 7–3500 ng/mL for ADL, 2–1000 ng/mL for DSS, 4–2000 ng/mL for FA, 1–500 ng/mL for HSYA, 2–1000 ng/mL for SAA, and 4–2000 ng/mL for SAB. The mean values of linear regression equation of the analytes were listed in Table 2. In which, y was the peak–area ratio, x was the relative concentrations and r was the correlation coefficient. The LLOQ samples of six different rat plasma independent of the calibration curves were analyzed. A signal-to-noise (S/N) > 10 at the LLOQ was observed for all the analytes. It was observed that the LLOQ was 7 ng/mL for ADL, 2 ng/mL for DSS, 4 ng/mL for FA, 1 ng/mL for HSYA, 2 ng/mL for SAA, and 4 ng/mL for SAB, allowing sufficient for PK study of the analytes following oral administration of JTT to rats.

3.2.3. Precision and accuracy

The intra–day and inter–day precisions and accuracies of the QC samples were presented in Table 3. The RSD of QC samples were in the range of 1.50–9.98% and the accuracies were between 91.17% and 99.46%. The results, which were within the acceptable criteria for accuracy and precision, allowed the accurate assay of the analytes in rat plasma.

3.2.4. Extraction recovery and matrix effect

The extraction recoveries of the QC samples were presented in Table 4. The recovery ranged from 86.29% to 93.16%, which indicated that the overall extraction recovery of PPT was efficient, consistent and reproducible. Evaluation of matrix effect on the quantitative analysis in biological fluids plays an important role in bioanalytical method validation. Because of the presence of salts and endogenous materials, ion suppression or enhancement may be greater than that of SPE and LLE when PPT method was utilized in the pretreatment of plasma samples. Table 4 shows the results of matrix effect for all analytes. They were no less than 91.08%, which indicated that no co–eluting unseen compounds significantly influenced the ionization of analytes and IS.

3.2.5. Stability

No significant difference of concentrations for the analytes in rat plasma was observed during the 4–h period of assay, the bias between the nominal concentrations and the concentrations of all analytes stored at −20 °C for 4 weeks was less than 6.11% (shown in Table 5).

The concentrations of all analytes spiked to rat plasma were not significantly affected by the freezing and thawing test. After completion of three freezing and thawing cycles, the recoveries of all analytes were between 91.23% and 97.01%.

3.3. Sample analysis and PK studies

In the present study, the plasma concentrations of ADL, DSS, FA, HSYA, SAA and SAB were successfully determined by the established method. The test articles could be monitored in rat plasma for up to 12 h post–dose (except for SAB) when following single oral

Table 4
Matrix effect and extraction recovery for the analytes in rat plasma ($n = 3$).

Analyte	Nominal concentration (ng/mL)	Matrix effect			Extraction recovery		
		Mean	SD	RSD (%)	Mean	SD	RSD (%)
HSYA	2	91.08	1.66	1.82	88.15	2.17	2.46
	20	93.73	1.28	1.37	92.45	1.52	1.64
	200	93.68	2.09	2.23	93.16	3.73	4.00
FA	8	92.01	3.53	3.84	86.29	1.46	1.69
	80	93.29	1.01	1.08	87.72	4.17	4.75
	800	93.47	1.61	1.72	87.53	2.84	3.25
ADL	14	91.53	1.60	1.75	89.54	2.20	2.46
	140	92.24	1.04	1.13	87.69	1.44	1.64
	1400	92.65	2.04	2.20	92.58	3.70	4.00
DSS	4	95.51	4.08	4.27	88.58	1.50	1.69
	40	94.18	2.40	2.55	91.77	2.40	2.61
	400	93.08	2.03	2.18	92.38	3.25	3.52
SAB	8	92.38	2.00	2.17	88.54	2.25	2.54
	80	92.79	3.04	3.28	90.43	2.20	2.43
	800	92.45	2.01	2.18	89.78	3.61	4.02
SAA	4	91.57	1.43	1.56	89.24	1.79	2.01
	40	93.24	2.02	2.17	91.33	4.34	4.75
	400	93.12	1.56	1.67	90.08	2.93	3.25

Table 5
Stability of the analytes in rat plasma ($n = 5$).

Analyte	Nominal concentration (ng/mL)	Short-term stability (4 h at room temperature)			Long-term stability (4 weeks at −20 °C)			Autosampler stability (12 h at room temperature)			Freeze–thaw stability (3 cycles)		
		Concentration found (ng/mL)		RSD (%)	Concentration found (ng/mL)		RSD (%)	Concentration found (ng/mL)		RSD (%)	Concentration found (ng/mL)		RSD (%)
		Mean	SD		Mean	SD		Mean	SD		Mean	SD	
HSYA	2	96.80	1.13	1.17	95.42	4.29	4.50	96.47	3.15	3.27	91.23	2.12	2.33
	20	95.74	1.73	1.80	93.89	2.43	2.59	95.65	2.72	2.84	93.45	2.54	2.71
	200	99.60	2.09	2.10	94.65	3.08	3.26	96.53	2.67	2.76	94.65	2.15	2.27
FA	8	95.40	1.88	1.97	95.14	2.09	2.19	95.75	2.55	2.66	94.85	3.27	3.45
	80	95.42	1.87	1.96	94.13	2.15	2.28	94.57	1.67	1.77	95.13	1.80	1.89
	800	98.84	1.41	1.42	95.69	3.26	3.41	95.60	2.34	2.45	94.18	2.27	2.41
ADL	14	98.17	2.14	2.18	95.37	1.72	1.80	94.36	2.67	2.83	94.22	3.52	3.74
	140	99.18	2.72	2.75	95.62	2.57	2.69	96.49	1.53	1.59	93.22	2.33	2.50
	1400	98.33	1.66	1.69	96.34	3.21	3.33	97.53	2.25	2.31	92.53	3.12	3.38
DSS	4	97.69	2.38	2.44	95.22	2.45	2.58	96.18	3.72	3.87	94.13	2.74	2.91
	40	93.87	2.39	2.54	95.77	2.54	2.65	96.23	3.12	3.24	96.34	2.42	2.51
	400	96.06	1.42	1.48	96.12	3.30	3.43	98.21	3.05	3.11	97.01	2.26	2.32
SAB	8	95.40	2.00	2.10	95.85	2.27	2.37	95.54	3.02	3.17	96.22	2.28	2.37
	80	95.73	1.24	1.30	96.01	2.39	2.49	96.23	2.05	2.13	95.32	2.34	2.45
	800	98.43	1.81	1.84	95.44	3.35	3.51	97.15	2.46	2.53	92.16	2.23	2.42
SAA	4	95.08	2.36	2.48	96.26	2.64	2.74	95.22	2.13	2.24	92.31	2.54	2.75
	40	99.20	2.16	2.17	96.73	2.80	2.89	96.14	1.86	1.94	93.11	2.02	2.17
	400	98.33	2.53	2.57	96.66	3.02	3.13	98.73	2.20	2.23	95.73	2.08	2.17

administration of JTT at 15 g/kg (ADL 68.01 mg/kg, DSS 9.40 mg/kg, FA 1.29 mg/kg, HSYA 1.32 mg/kg, SAA 14.98 mg/kg and SAB 37.98 mg/kg) to forty rats. The mean plasma concentration–time profiles of all analytes were shown in Fig. 4, and the PK parameters of them were listed in Table 6.

Although some quantitative methods for ADL in biological fluids have been published, the limitation of insufficient selectivity and sensitivity was existed [18]. Therefore, aiming at a more selective method, LC–ESI-MS/MS method was chosen in the present study with its high selectivity and sensitivity because selected ions were monitored even with trace concentration. Following single oral administration of JTT, ADL exhibited maximum plasma concentration ($C_{max} = 1473.40 \pm 575.21$ ng/mL) observed at 0.28 ± 0.13 h post dose (T_{max}), and the corresponding AUC_{0-t}

Table 6
PK parameters of the analytes following single oral administration of JTT to rats ($n = 10$).

Parameters	HSYA		FA		ADL		DSS		SAB		SAA	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Dose (mg/kg)	1.32	0.00	1.29	0.00	68.01	0.00	9.40	0.00	14.98	0.00	37.90	0.00
C_{max} (ng/mL)	88.94	45.11	519.04	255.38	1473.40	575.21	219.14	62.50	100.21	52.48	136.57	75.26
T_{max} (h)	0.31	0.12	0.08	0.00	0.28	0.13	0.28	0.13	0.39	0.09	0.45	0.19
AUC_{0-t} (ng/mL h)	123.34	31.26	400.71	78.34	2274.26	292.59	981.95	106.71	359.44	146.37	103.39	90.58
AUC_{0-INF} (ng/mL h)	137.87	33.64	463.08	127.47	2865.62	375.17	1622.71	283.71	433.02	181.84	128.44	118.34
$T_{1/2}$ (h)	2.27	1.64	2.91	1.98	8.17	3.87	8.51	1.85	12.88	3.24	2.79	2.53
MRT (h)	3.62	3.08	3.99	2.22	7.45	4.16	12.40	2.59	18.55	6.58	3.69	3.28

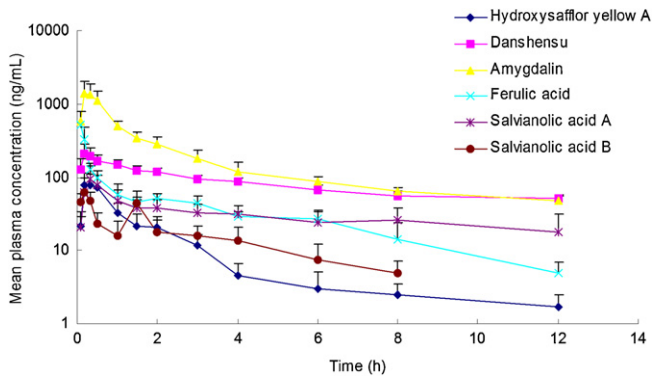

Fig. 4. Plasma concentration-time profiles of the analytes following single oral administration of JTT to rats ($n = 10$).

was 2274.26 ± 292.59 ng/mL h. To our knowledge, the intestinal absorption of ADL was expected to be slight judging from the chemical structure (cyanogenic glycoside). This prediction proved to be correct based on the obtained results, which was also in conformity with the literature reference that intestinal absorption of intact ADL was rather limited [18].

It was reported that the absorption of HSYA was rapid with peak concentrations occurring at 10 min after oral administration [40]. As calculated from the mean plasma concentrations of HSYA following oral administration of JTT, the absorption speed of HSYA was rapid with highest plasma concentration observed (T_{max}) at 0.31 ± 0.12 h (T_{max} less than 1 h). The corresponding C_{max} and AUC_{0-t} were 88.94 ± 45.11 ng/mL and 123.34 ± 31.12 ng/mL h, respectively, which implied consistence with those given in the literature references [40].

Phenolic acids could be classified into monomer and polymer including dimers, trimers and tetramers, which were calculated by the number of phenyls in the structure accordingly [41]. Although the PK profiles of many phenolic acids have been investigated by previous reports, the simultaneous determination and PK study of these components together was rare. In the present study, the PK properties of monomers such as DSS and FA, polymers such as SAA and SAB were investigated by the established method. Significant differences existed in the absorption and elimination of the PK parameters among DSS, FA, SAA and SAB, which might be presumably due to the different physicochemical properties of each compound or the pharmacokinetic interaction of the prescribed chemical constituents.

It was calculated that the maximum plasma concentration (C_{max}) of DSS amounted to 219.14 ± 62.50 ng/mL, the time to maximum plasma concentration (T_{max}) was 0.28 ± 0.13 h, and the elimination half-life ($T_{1/2}$) was 8.51 ± 1.85 h, while the area under the curve (AUC_{0-t}) was 981.95 ± 106.71 ng/mL h, indicating DSS could not only be absorbed into blood from gastrointestinal tract, but also exhibited PK characteristics as rapid oral absorption.

The PK profile of FA has been investigated by published studies, whereas most of them showed limitations of insufficient selectivity, sensitivity and trace concentrations in biological fluids due to limited analytical condition or the tiny amount in dosing volumes [32,42]. Thus, LC–ESI-MS/MS method with high selectivity and sensitivity was chosen in the present study. The T_{max} (0.08 h) observed was a little faster than that of published studies, which indicated rapid absorption of FA following a single oral administration of JTT, also it may attribute to the interference of other constituents in JTT.

SAA was found to be rapidly absorbed from the gastrointestinal tract following a single oral dose of JTT. The C_{max} achieved at 0.45 ± 0.19 h at 136.57 ± 75.26 ng/mL. After 12 h, the concentration level of SAA in plasma decreased significantly. The AUC_{0-t} of SAA in rats was 103.39 ± 90.58 ng/mL h and the elimination half life ($T_{1/2}$) was 2.79 ± 1.53 h.

The chemical structure of SAB was constructed by two ester functional groups for the connection of polyphenolic complex. Enzymatic biodegradation and phase II biotransformation could be performed regularly in the polyphenolic complex, which implied great challenge in establishing bioanalytical method to support its PK study [43,44]. In the present study, sample pre–treatment was optimized as the ultimate sample preparation method with vitamin C and formic acid added to prevent the possible degradation. SAB reached the maximum concentration within 1 h and could be monitored up to 8 h after oral administration of JTT, which was similar to the results from published studies. Following oral administration of JTT, multiple peaks were observed in both individual and mean plasma concentration curves of SAB. It was reported that the result might be presumably due to the distribution, re–absorption and/or enterohepatic circulation in SAB absorption [45]. However, elucidation of the mechanism of the phenomenon in PK studies needs further detailed studies. Maximum mean plasma concentrations (C_{max}) of the SAB were exhibited as 100.21 ± 52.48 ng/mL at approximately 0.39 h post dose (T_{max}), indicating a rapid oral absorption. The AUC_{0-t} of SAB was 359.44 ± 146.37 ng/mL h, while the terminal elimination half-life ($T_{1/2}$) was 12.88 h and the mean residence time (MRT) for SAB was 18.55 h.

3.4. Marker compound selection and dosage design

In view of the uncertainty of marker compounds in TCMs and the direct influence of dosage on the PK parameters, increasing concerns have been expressed to the dosage design in PK study of TCMs. For instance, which marker compounds are the representative agents of the whole formula? How to determine the oral dosage of TCMs in PK study? etc. Different from the development of western medicines, TCMs are achievements of the accumulated experience of several TCM pioneers, the dosage of TCMs are also derived from their clinical usage to obtain synergistic effects or diminish potential adverse reactions. Therefore, the marker compound of a typical formula is not merely of one or partial chemical markers that with the highest contents in the crude drugs, but the compounds with these characters, including therapeutic, bioactive, characteristic, main, synergistic, correlative, toxic and analytical [46]. However, due to the extreme complexity and diversity of the co–existing components in TCMs, it is difficult to quantify the trace but with interesting biological activities maker compounds in vivo. Therefore, a rational dosage design, which not only within the window of therapeutic dosage, but also let the marker compounds determined by the high tech products such as LC–MS, LC–NMR, etc. is a plausible solution for the PK study of TCMs. In the present study, the oral dosage of JTT was set at 15 g/kg, though twenty folds of the dosage recommended by the manufacturers, it was still an acceptable value, which not only within the window of therapeutic dosage (0.1–30 g/kg) resulted from the pharmacodynamic study on SD rats, but also can give typical plasma concentration–time profiles of the six compounds. Consequently, the PK evaluation of the target compounds in the present study may provide a solid basis for marker compound selection and dosage design for the therapeutic drug monitoring and PK study of JTT in its clinical applications.

4. Conclusions

An LC–ESI-MS/MS method has been developed and validated for the simultaneous determination of ADL, DSS, FA, HSYA, SAA and SAB in rat plasma. Sample pretreatment procedures for plasma were optimized and carried out by easy–to–use and high throughput methods. Subsequently, no interference from endogenous

materials or other source was found at the same retention time as the analytes with a single run in 7 min, and the PK properties of the analytes showed significant differences due to the different physicochemical properties of each compound or the PK interaction of the prescribed chemical constituents. In conclusion, the established LC–ESI-MS/MS method demonstrated good performance in terms of linearity, specificity, detection and quantification limits, precision and accuracy, and was successfully utilized to quantify hydrophilic components in rat plasma to support the PK studies.

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