Journal of Pharmaceutical and Biomedical Analysis 55 (2011) 1163–1169

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

Determination of chamaechromone in rat plasma by liquid chromatography–tandem mass spectrometry: Application to pharmacokinetic study

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ARTICLE INFO

Article history:
Received 6 February 2011
Received in revised form 7 April 2011
Accepted 10 April 2011
Available online 15 April 2011

Keywords:
Chamaechromone
Rat plasma
Method validation
LC–MS/MS
Pharmacokinetics

ABSTRACT

A rapid, simple and accurate method was developed for the determination of chamaechromone in rat plasma using liquid chromatography tandem mass spectrometry (LC–MS–MS). Rosuvastatin was used as the internal standard. The plasma samples were extracted by liquid–liquid extraction with ethyl acetate. Chromatographic separation was performed on Xbridge™ C_{18} column (2.1 mm × 50 mm, 3.5 μm) with linear gradient elution using water and methanol, both of which were acidified with 0.1% aqueous formic acid. The flow rate was 0.4 mL/min and the total run time was 6 min. Detection was performed on a triple-quadrupole tandem mass spectrometer using positive ion mode electrospray ionization (ESI) in the multiple reaction monitoring (MRM) mode. The MS/MS ion transitions monitored were m/z 543.3 → 198.9 and 481.9 → 258.3 for chamaechromone and rosuvastatin, respectively. Good linearity was observed over the concentration range of 8–6400 ng/mL in 0.1 mL of rat plasma. The lowest concentration (8 ng/mL) in the calibration curve was estimated as LLOQ with both deviation of accuracy and RSD of precision <20% (n = 6). Intra-assay and inter-assay variability were less than 11% in plasma. This method was successfully applied to a pharmacokinetic study of chamaechromone in rats after intravenous (5 mg/kg) and oral (100 mg/kg) administration. Following oral administration the concentration–time curve of chamaechromone exhibited a biphasic absorption profile. The maximum mean concentration in plasma (C_{max}, 795.9 ± 14.6 ng/L) was achieved at 11.3 ± 0.8 h (T_{max}) and the area under curve (AUC_{0-60}) was 6976.7 ± 1026.9 ng h/L. After single intravenously administration of chamaechromone, the essential pharmacokinetic parameters C_{max}, AUC_{0-48} were 4300.7 ± 113.6 ng/L and 3672.1 ± 225.4 ng h/L, respectively. The result showed that the compound was poorly absorbed with an absolute bioavailability being approximately 8.9%.

1. Introduction

Stellera chamaejasme L. (Thymelaeaceae) is widely distributed in Mongolia, Tibet and the northern parts of China. Its roots are commonly used as "Langdu" which was embodied in the Pharmacopoeia of the P.R. China (2010) as a toxic TCM [1]. It has been used for the treatment of scabies, tinea, stubborn skin ulcers, chronic tracheitis, and tuberculosis in China and other Asian countries [2,3]. In recent years, "Langdu" has also been used for the treatment of intestinal, gastric and pulmonary cancers [4]. The antitumor activities have been mainly focused on the presence of biflavones and flavonoids in the extracts from the roots. It was reported that biflavonoids had anti-inflammatory, antiviral, cytotoxic, and antioxidative activity [5–7]. Chamaechromone, a biflavone constituent, was a major component in dried roots of *S. chamaejasme* [8], and it was found that it showed anti-HBV effect against HBsAg secretion [9].

Several methods for identifying and quantifying chamaechromone in raw herbs have been reported. Most of them were based on liquid chromatography–mass spectrometry (LC–MS) [8], rapid-resolution liquid chromatography–diode array detection (RRLC–DAD) and electrospray ionization time-of-flight mass spectrometry (ESI-TOF/MS) [10], high performance liquid chromatography with ultraviolet detection (HPLC–UV) [11], high-resolution ESMS [11] and NMR spectrometry [11,12] for detection and identification of the constituents. Until now, no method has been reported on pharmacokinetic study. So it is highly important to develop a sensitive method to detect chamaechromone biological fluids for its clinical application and further development. In this paper, a liquid chromatography–mass spectrometry (LC–MS) method was developed for the determination of chamaechromone in rat plasma for the first time, and it was applied to investigate the pharmacokinetics and absolute bioavailability of chamaechromone in rats. The results of the pharmacokinetic study provided a mean-

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doi:10.1016/j.jpba.2011.04.009

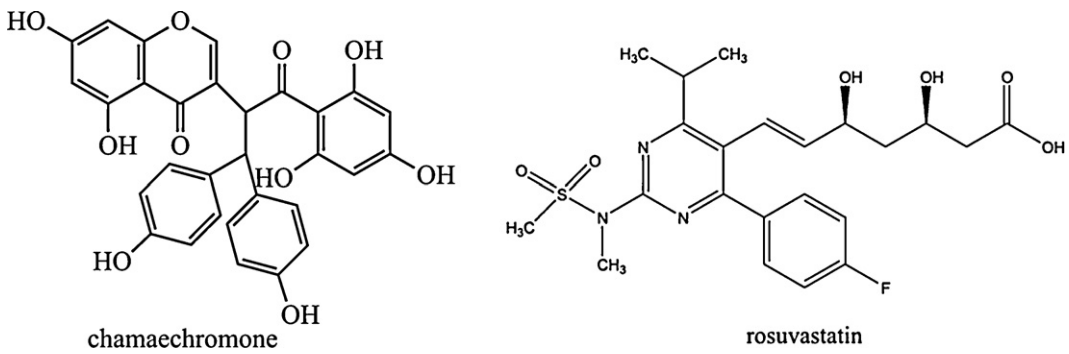

Fig. 1. Chemical structures of chamaechromone and rosuvastatin.

ingful basis for evaluating the clinical applications of the herbal medicine.

2. Experimental

2.1. Chemicals and reagents

The dried rhizomes of *S. chamaejasme* L. were purchased from Nanjing Qingze Medical Technological Development Co. Ltd (Nanjing, China). Chamaechromone was isolated and purified from dried roots of *S. chamaejasme* in our laboratory, and the structure was characterized by spectral methods, including MS, ^{1}H and ^{13}C NMR spectra. The data were consistent with previous literatures [2].

Rosuvastatin (internal standard, IS) was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The purity (>99.0%) was determined by HPLC–PDA–MS. Chemical structures of both compounds are shown in Fig. 1.

HPLC grade methanol and formic acid were purchased from TEDIA Inc. (Fairfield, USA). Ultra-pure water (18.2 MΩ) was obtained from an ELGA-purelab Ultra system (High Wycombe, UK). All other reagents were analytical grade and obtained from the chemical reagent company of Ludu, Shanghai.

2.2. Animals

Male Sprague-Dawley rats (200–220 g) were obtained from the Animal Center of Zhejiang Academy of Medical Sciences (Hangzhou, China). Animals were bred in a breeding room with temperature at 25 °C, humidity of 50 ± 10%, and a 12 h dark–light cycle. They had free access to water and rodent chow all the time. All the experiment animals were housed under the above conditions for one week for acclimation, and were fasted overnight before the experiments. The study was approved by the Animal Ethics Committee of Zhejiang University.

2.3. Instrumentation and chromatographic conditions

A Waters ACQUITYTM TQD with an ultra performance liquid chromatography (Waters, Milford, MA, USA) was used. Chromatographic separation was achieved by using an Xbridge^{TM} C_{18} column (2.1 mm × 50 mm, 3.5 μm; Waters). The mobile phase consisted of methanol–water containing 0.1% formic acid with a gradient elution starting at 5% methanol and progressing linearly to 90% methanol over 5.0 min then returning to 5% methanol. Total runtime was 6 min. Mobile phase was delivered at a flow rate of 0.4 mL/min and introduced into ESI source with no split, and effluent of 0–0.5 min from chromatographic column was switched to waste before it flew into ion source.

Analytes were detected by MS/MS with an electrospray ionization (ESI)-interface in positive multiple reaction monitoring (MRM)-mode. Mass transitions of chamaechromone (m/z 543.3 → 198.9) and rosuvastatin (m/z 481.9 → 258.3) were optimized. The ESI-MS/MS operating parameters used in this study were as follows: nitrogen was used as the desolvation and cone gas with a flow rate of 550 and 50 L/h, respectively. The source and desolvation gas temperature were set at 118 and 350 °C, respectively. Capillary voltage was set at 1.8 kV, and cone voltage, extractor and RF lens voltage were 27, 3 and 0.1 V, respectively. Collision energy was 10 eV (chamaechromone) and 30 eV (IS). Data acquisition and processing were performed using Masslynx 4.1 software and Quanlynx V4.1 (Micromass, Manchester, UK).

2.4. Preparation of calibration standard and quality control (QC) samples

The stock solutions of chamaechromone (1.60 mg/mL) and the internal standard (25.0 mg/mL) were separately prepared with methanol. Standard solutions of chamaechromone at concentrations of 6400, 3200, 640, 320, 64, 32, 16, and 8 ng/mL were obtained by a further dilution of the stock solution with methanol for the preparation of calibration and quality control (QC) samples. A solution containing 12.5 ng/mL of rosuvastatin was also obtained by dilution of the internal standard stock solution with methanol. All standard solutions were kept at −20 °C.

Each drug-free rat plasma sample (90 μL) was spiked with IS solution (10 μL) and different concentrations of chamaechromone (10 μL) to prepare calibration standards in the concentration ranging from 8 to 6400 ng/mL. QC samples with 12.5 ng/mL rosuvastatin and concentrations of chamaechromone at high (5120 ng/mL), medium (640 ng/mL), and low (12 ng/mL) were freshly prepared.

2.5. Sample preparation

In this study, a conventional liquid–liquid extraction (LLE) method was applied to extract the analyte and IS from rat plasma. All samples were thawed at room temperature before analysis. After 100 μL of plasma sample was transferred into a 1.5 mL centrifuge tube, 10 μL of IS work solution (125 ng/mL) was added. The mixture was vortex-mixed for 2 min and extracted with 1 mL of ethyl acetate by vortex-mixing for 5 min. Then the sample was centrifuged at 13,000 rcf for 20 min. The supernatant was transferred into another test tube and evaporated to dryness with vacuum at room temperature. Finally, 7 μL supernatant was injected for HPLC–MS analysis after the residue was reconstituted in 100 μL incipient mobile phase by vortex-mixing for 4 min and centrifuged at 13,000 rcf for 20 min.

2.6. Method validation

A thorough and complete method validation of chamaechromone in rat plasma was done following the FDA guidelines [13]. The validation parameters included specificity, sensitivity, linearity, accuracy and precision, recovery, matrix effect and stability.

2.6.1. Specificity

The specificity of the method was assessed by comparing the chromatograms of six different batches of blank rat plasma. Each blank plasma sample was tested under chromatographic conditions described above to ensure no interference of the analyte from plasma.

2.6.2. Linearity and sensitivity

Plasma samples were quantified by using the ratio of the peak area of analyte to that of IS as the assay parameter. A standard curve was constructed using peak area ratios versus analyte concentrations at 8, 16, 32, 64, 320, 640, 3200 and 6400 ng/mL, and described in the form of $y = a + bx$.

The lower limit of quantification (LLOQ) was determined as the lowest concentration of the standard curve (S/N ratio > 10). The lower limit of detection (LLOD) was defined as the amount of chamaechromone which caused a signal three times to noise.

2.6.3. Accuracy and precision

The accuracy and precision were calculated by determining QC samples at high, middle and low concentration levels on three different validation days. The accuracy was expressed by the relative error (RE) and the precision by relative standard deviation (RSD). The acceptance values used for validation of RSD and RE were within 15%, except LLOQ (within 20%).

2.6.4. Recovery

The recovery of analyte and IS was determined by comparing the responses of the analyte from QC samples with the responses of analyte spiked in post-extracted blank rat plasma at equivalent concentrations. The recovery of IS was determined at a single concentration of 12.5 ng/mL.

2.6.5. Matrix effect

The absolute matrix effect was determined by comparing the peak areas obtained from mobile phase spiked with low, middle and high concentrations of analyte (12, 640, 5120 ng/mL, $n = 6$, respectively) and IS (12.5 ng/mL, $n = 6$) with post-extraction blank rat plasma spiked samples.

2.6.6. Stability

The stability of analyte in rat plasma was evaluated by using QC samples (12, 640 and 5120 ng/mL) with five samples for each concentration. The stability of chamaechromone was tested under the following conditions: (1) freeze–thaw stability of chamaechromone in rat plasma through three freeze–thaw cycles; (2) short-term stability of chamaechromone in rat plasma at room temperature for 8 h; (3) long-term stability of chamaechromone in rat plasma stored at −20 °C for 30 days; (4) post-preparative stability of chamaechromone during storage in the auto-sampler at 4 °C for 24 h.

2.7. Pharmacokinetic study

The validated method was applied to the pharmacokinetic study of chamaechromone. Twelve rats were divided into two groups at random. Rats were administered 100 mg/kg chamaechromone by oral administration after an overnight fasting period or intravenous injection of 5 mg/kg to rats via the tail vein. The drug was formulated by dissolving chamaechromone with a dimethyl sulfoxide–PEG 400–physiologic saline (1:6:13, v:v:v) and mixed well for intravenous administration. And the powder of chamaechromone (200 mg) was dissolved in 1 mL anhydrous ethanol (5%, v/v) and then diluted to 20 mL water containing 5% glucose and 20% β-cyclodextrin to get 10 mg/mL solution for oral administration. The rats were fasted for the first 2 h with free access to water after dosing. About 200 μL blood samples were collected by scissoring rat tails into heparinized tubes. This was performed at 0 (pre-dose), 0.5, 1, 2, 4, 7, 8, 9, 10, 11, 12, 14, 16, 20, 24, 36, 48 and 60 h after administration orally and at 0 (pre-dose), 0.083, 0.17, 0.25, 0.5, 1, 1.5, 2, 3, 5, 7, 9, 12, 24 and 48 h after i.v. administration via the tail vein. After plasma collection, each blood sample was immediately centrifuged at 3500 rcf for 5 min at 4 °C, and 100 μL plasma was transferred into 0.5 mL centrifuge tube and then stored at −20 °C until analysis. During routine analysis, each analytical run included six different batches of blank rat plasma, a set of calibration samples, a set of QC samples and unknowns.

2.8. Statistical analysis

To determine the pharmacokinetic parameters of chamaechromone, the concentration–time data were analyzed by DAS Software (ver.2.0, China State Drug Administration). Data were expressed as mean ± SD.

3. Results and discussion

3.1. Method development

3.1.1. Optimization of extraction conditions

Protein precipitation and liquid–liquid extraction (LLE) were compared during sample preparation. The former was discarded because of its high noise level and interference by endogenous substances. The latter offered a very clean sample that made the method more robust and scalable. Consequently, the LLE method was used for sample preparation, and optimized by testing the influence of different kinds of organic reagents (cyclohexane–isopropanol, diethyl ether, chloroform, ethyl acetate) on the recovery. Ethyl acetate was the best owing to its low background noise, ease of sample preparation, and relatively high extraction recovery for analyte and the IS. The optimal conditions were presented above.

3.1.2. Optimization of LC conditions

To improve peak shape, enhance analyte signal response, and decrease run time, different analytical columns and mobile phase compositions were tried to achieve good resolution and symmetric peak shapes for each analyte and the IS. Compared Xbridge™ C_{18} (2.1 mm × 50 mm, 3.5 μm; Waters) column with Acquity UPLCHSS T3 column (2.1 mm × 100 mm, 1.8 μm; Waters) column, the former had a better peak shape of the analyte. As a result, the Xbridge™ C_{18} (2.1 mm × 50 mm, 3.5 μm; Waters) was selected for analysis. The most critical chromatographic parameter was the composition of the mobile phase. The mobile phase had been tried with acetonitrile/water and methanol/water binary solvent system using different buffers such as ammonium formate and formic acid. As a result, the mobile phase consisted of methanol–water containing 0.1% formic acid with a gradient elution could improve symmetry of peak shape and enhance the signal response. And flow rates (in the range of 0.2–0.5 mL/min) were also tested to identify the optimal mobile phase. Consequently, a flow rate of 0.4 mL/min was chosen, and the run time were 3.78 and 4.06 min for chamaechromone and IS, respectively.

Table 1
Intra- and inter-batch precision and accuracy for determination of chamaechromone in rat plasma.

Spiked (ng/mL)	Intra-batch precision and accuracy ($n=5$)			Inter-batch precision and accuracy ($n=3$)		
	Measured (mean ± SD)	RSD (%)	RE (%)	Measured (mean ± SD)	RSD (%)	RE (%)
12	11.7 ± 1.1	9.6	−2.2	12.3 ± 1.3	10.8	2.6
640	605.5 ± 31.9	5.3	−5.4	604.2 ± 45.2	7.5	−5.6
5120	5193.7 ± 219.8	4.2	1.4	5189.2 ± 308.2	5.9	1.4

Abbreviation—RE: relative error.

3.1.3. Optimization of MS conditions

ESI was employed to obtain good sensitivity and fragmentation. To optimize ESI conditions for detection chamaechromone and IS, positive or negative ion detection mode was tried. It was found that the two compounds had good responses in positive ion detection mode with low background noise level. Detection was finally operated in positive ion mode in this study. At first, auto-tune was used to get initial optimum MS parameters, but the sensitivity for the analyte was not good enough. Fine tuning was done to collision energy, cone voltage, ion source temperature, and desolvation gas temperature by manual manipulation, considering that these parameters played an important role on the ion response of chamaechromone.

3.2. Method validation

3.2.1. Specificity and sensitivity

Typical chromatograms of the blank and spiked plasma are given in Fig. 2. Under the described chromatographic conditions, a good separation was achieved and no obvious interferences from endogenous plasma substances were observed.

3.2.2. Linearity and calibration curve

The calibration curve of chamaechromone with a $1/x^2$ weighted regression was linear over the concentration range of 8–6400 ng/mL. Typical standard curve was $y = 0.002639x - 0.006234$, and the square regression coefficient (r^2, $n=5$) was found to be ≥0.995. The LLOQ of chamaechromone was 8 ng/mL (RSD% = 4.0%, $n=5$, S/N ratio > 10). The LOD of chamaechromone in rat plasma was evaluated to be 2.0 ng/mL ($n=5$, S/N ratio > 3) in this study.

3.2.3. Accuracy and precision

Accuracy and precision data for intra- and inter-day plasma samples are listed in Table 1. The intra-day accuracy (expressed as percent of nominal values) ranged from 94.6% to 101.4% and the inter-day accuracy ranged from 94.4% to 102.6%. The intra-day precision ranged from 4.2% to 9.6%, and the inter-day precision ranged from 5.9% to 10.8%. The assay values on both intra- and inter-day were found to be within the acceptance criteria.

3.2.4. Recovery

The results of the comparison of pre-extracted standards versus post-extracted plasma standards were evaluated for chamaechromone at 12, 640 and 5120 ng/mL, and the absolute recovery ranged from 81.2% to 94.1%. The absolute recovery of IS at 12.5 ng/mL was 107.5% (Table 2).

Table 2
Matrix effects, recovery and process efficiency for the assay of chamaechromone and IS in plasma ($n=6$).

Concentration (ng/mL)	RE (mean ± SD (%))	ME (mean ± SD (%))	PE (mean ± SD (%))
Chamaechromone			
12	86.7 ± 0.32	90.5 ± 1.2	78.5
640	81.2 ± 4.9	99.1 ± 2.2	80.5
5120	94.1 ± 1.3	97.4 ± 1.1	91.7
IS			
12.5	107.5 ± 6.3	109.1 ± 5.3	116.6

Abbreviations—ME: matrix effects; RE: recovery; PE: process efficiency.

3.2.5. Matrix effect

The ratios of the peak area resolved in the post-extraction blank sample with that resolved in the mobile phase of chamaechromone and IS are shown in Table 2. The results showed that there were no significant matrix effects.

3.2.6. Stability

The results indicated that chamaechromone was stable in auto-sampler (24 h) at 4 °C, bench-top (8 h) at room temperature, repeated three freeze/thaw cycles and frozen condition at −20 °C for 30 days (Table 3) and it would satisfy a routine pharmacokinetic study.

3.3. Application to pharmacokinetic study

This new developed method was applied to determine the plasma concentration of chamaechromone in rats following i.v. (5 mg/kg) and oral (100 mg/kg) administration. The mean plasma concentration–time profiles of chamaechromone after the two doses in rats are illustrated in Fig. 3 and its estimated pharmacokinetic parameters calculated by DAS 2.0 software are presented in Table 4.

It was found that the best fit pharmacokinetic model to estimate the pharmacokinetic parameters was single compartment model with weight of $1/C^2$ for oral administration, and two compartments model with weight of $1/C^2$ for intravenous administration by using DAS software. The concentration–time curve of chamaechromone in rat plasma after oral administration showed significant double-peak phenomenon, which often occurred in the pharmacokinetic studies of biflavones [14–16]. This phenomenon might be caused by the following reasons: (1) the absorption varied within different regions of the gut; (2) enterohepatic circulation might exist and the analyte was rapidly resorbed from stomach or upper small intestine (duodenum), then the intermediate disappeared into liver and gall-

Table 3
Stability of chamaechromone in rat plasma ($n=5$).

Spiked (ng/mL)	24 h (auto-sampler)		8 h (bench-top)		3rd freeze–thaw		40 days at −20 °C	
	Mean ± SD (ng/mL)	Accuracy (%)	Mean ± SD (ng/mL)	Accuracy (%)	Mean ± SD (ng/mL)	Accuracy (%)	Mean ± SD (ng/mL)	Accuracy (%)
12	11.5 ± 0.7	95.7	12.4 ± 0.5	103.7	11.6 ± 0.4	96.8	11.8 ± 0.6	98.3
640	626.4 ± 9.2	97.9	627.6 ± 9.8	98.1	618.4 ± 11.6	96.6	607 ± 10.8	94.8
5120	5174.4 ± 166.0	101.1	5204.4 ± 88.4	101.6	5089.4 ± 147.3	99.4	5061.4 ± 140.4	98.9

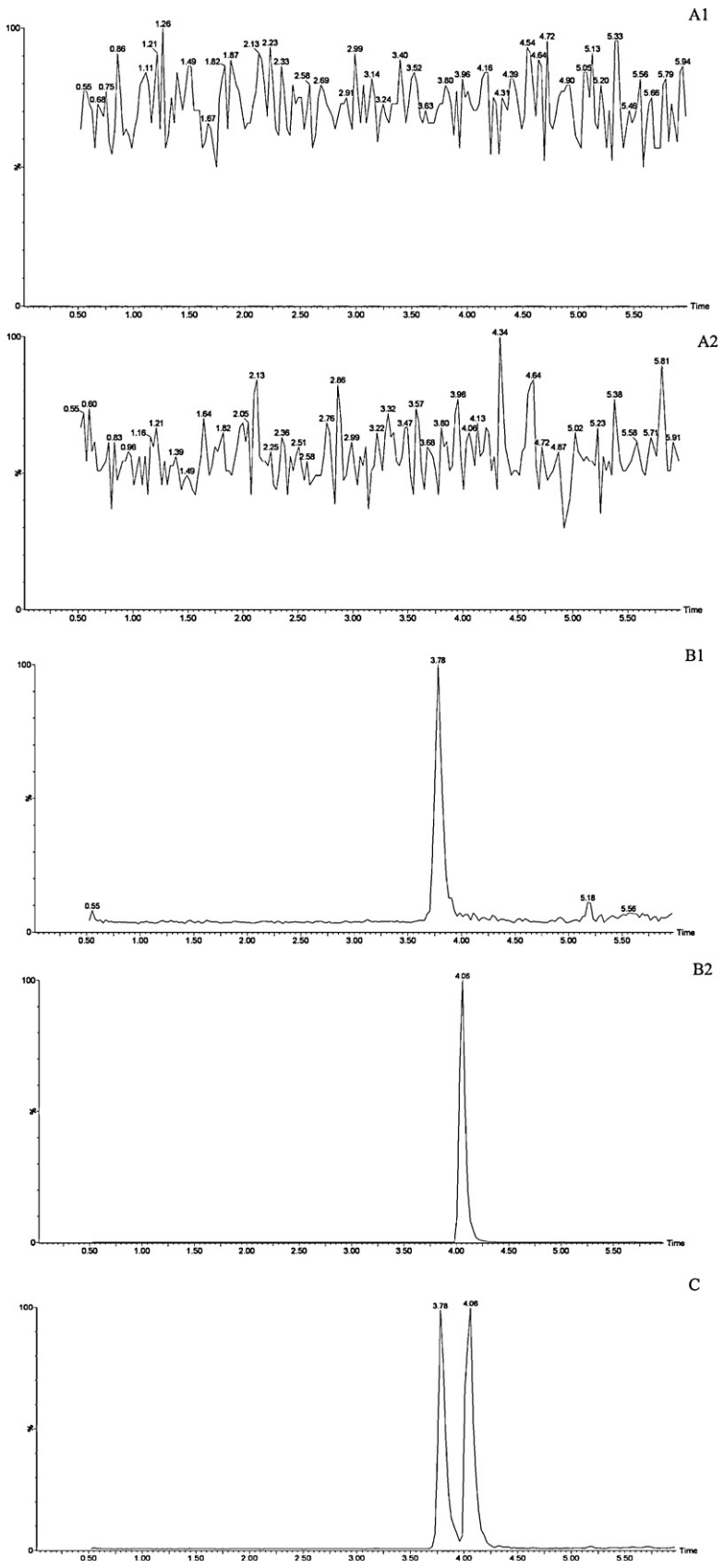

Fig. 2. MRM chromatograms of (A1, A2) blank plasma, (B1, B2) blank plasma spiked with chamaechromone at LLOQ and rosuvastatin at 12.5 ng/mL and (C) real sample 2 h after oral administration of chamaechromone.

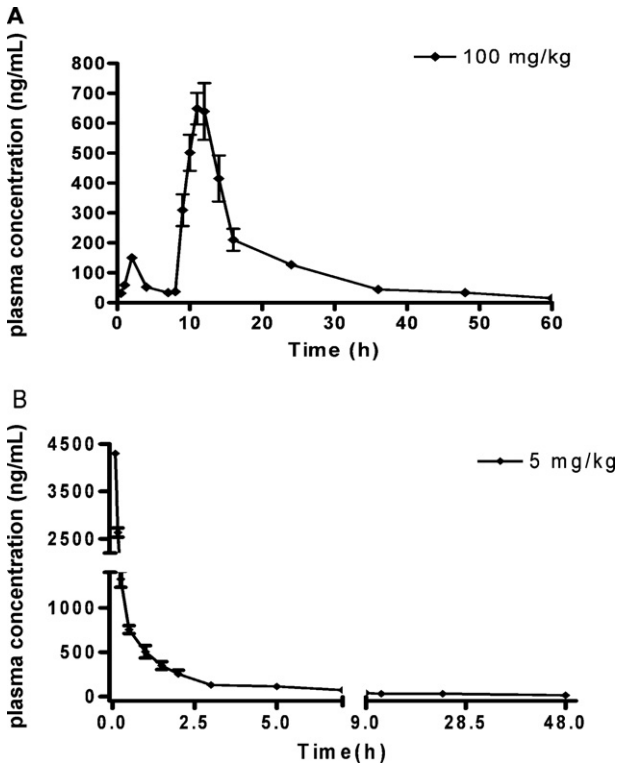

Fig. 3. Mean plasma concentration of chamaechromone vs. time in rat plasma. (A) Oral administration of 100 mg/kg, and (B) intravenous administration of 5 mg/kg. Each point and bar represent the mean ± SD (n = 6).

bladder and was slowly absorbed in lower small intestine (ileum) or colon. The identification of enterohepatic recirculation may be required by a comparison of AUC obtained after oral administration of the compound in normal and bile duct cannulated rats. Further studies are needed to investigate the exact mechanism underlying the double-peak phenomenon for chamaechromone. The half-life ($t_{1/2}$, 30.0 ± 19.3 h) is calculated based on the second peak. The C_{max} (795.9 ± 14.6 ng/L) and T_{max} (11.3 ± 0.8 h) were obtained from the observed data directly based on the second peak. The ratio of mean value of AUC_{0-60} (6976.7 ± 1026.9 ng h/L) to that of $AUC_{0-\infty}$ (7388.6 ± 940.0 ng h/L) was 94.4%.

As for intravenous administration, the disposition of chamaechromone in rat plasma was biphasic, subdivided into a fast distribution and a slow elimination phase. The half-life of the distribution phase was 0.47 ± 0.22 h, and that of elimination phase was 19.5 ± 9.5 h, indicating a high tissue binding. The mean ± SD values of C_{max} and clearance (CL) were 4300.7 ± 113.6 ng/L and 1202.1 ± 121.8 L/(h kg), respectively. And the ratio of the area under the curve (AUC_{0-48}) (3672.1 ± 225.4 ng h/L) versus $AUC_{0-\infty}$ (4129.2 ± 231.8 ng h/L) was 88.9%.

Absolute bioavailability (F) was calculated based on the $AUC_{0-\infty}$ obtained after oral and i.v. administration. And its absolute bioavailability was low with a value being 8.9%. Poor permeability through the intestinal epithelial membrane and efficient metabolism in intestine might be responsible for the low bioavailability of flavanoid compounds [17,18]. Whether the low bioavailability was caused by either low permeability or efficient metabolism, further experiments are required to prove these assumptions.

Table 4
Pharmacokinetic parameters of chamaechromone in rats following intravenous (i.v., 5 mg/kg) and oral (100 mg/kg) administration. All data are expressed as mean ± SD (n = 6).

Oral		Intravenous	
Parameters	Mean ± SD	Parameters	Mean ± SD
C_{max} (ng/L)	795.9 ± 14.6	C_{max} (ng/L)	4300.7 ± 113.6
T_{max} (h)	11.3 ± 0.8	$t_{1/2\alpha}$ (h)	0.47 ± 0.22
$t_{1/2}$ (h)	30.0 ± 19.3	$t_{1/2\beta}$ (h)	19.5 ± 9.5
AUC_{0-60} (ng h/L)	6976.7 ± 1026.9	CL (L/(h kg))	1202.1 ± 121.8
$AUC_{0-\infty}$ (ng h/L)	7388.6 ± 940.0	AUC_{0-48} (ng h/L)	3672.1 ± 225.4
CLz/F (L/(h kg))	13,731.8 ± 1871.6	$AUC_{0-\infty}$(ng h/L)	4129.2 ± 231.8
Vz/F (L/kg)	277,476.2 ± 132,126.1	K_{10} (1/h)	0.72 ± 0.66
MRT_{0-t} (h)	18.5 ± 0.675	K_{12} (1/h)	1.67 ± 2.34
$MRT_{0-\infty}$ (h)	22.3 ± 4.9	K_{21} (1/h)	0.185 ± 0.261
–	–	MRT_{0-t} (h)	8.1 ± 0.67
F (%)	8.9	$MRT_{0-\infty}$ (h)	16.1 ± 3.6

4. Conclusions

A LC–MS/MS method has been established for the first time to determine chamaechromone in rat plasma using liquid–liquid extraction as sample clean-up procedure. This method showed excellent sensitivity, linearity, precision and accuracy, and was successfully applied to evaluate the pharmacokinetics and oral bioavailability of chamaechromone in rats.

Acknowledgements

This project was supported by National Major Special Project for Science and Technology Development of Ministry of Science and Technology of China (No. 2009ZX09304-003) and the National Science Foundation for Post-doctoral Scientists of China (No. 20090461391).

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