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Validation and application of an LC–ESI-MS method for simultaneous determination of astilbin and its major metabolite 3′-O-methylastilbin in rat plasma

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ABSTRACT

We herein describe the development of an LC–MS method for simultaneous determination of astilbin and 3′-O-methylastilbin in rat plasma. A simple liquid–liquid extraction procedure was followed by injection of the extracts on to a Shim-pack C_{18} column (150 mm × 2.0 mm I.D., 5 μm) with gradient elution and detection in negative ionization mode. Initially, the method was validated regarding linearity, accuracy and precision. The correlation coefficients of all the calibration curves showed good linearity ($r > 0.999$) within test ranges, and the relative deviation was less than 10% for intra- and inter-day assays. Besides, this method was also validated for its stability, extraction efficiency, matrix effect and so on. Finally, this proposed method was successfully applied to rat pharmacokinetic study and yielded the most comprehensive data on systemic exposure of them to date.

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1. Introduction

In recent years, there has been an increasing application of herbal medicines and dietary supplements to treat various chronic diseases and to promote health in both developing and developed countries [1]. *Smilax glabra Rox B* (Family *Liliaceae*), a well-known herbal medicine, had been widely used in China for more than 500 years for the treatment of brucellosis, syphilis, furunculosis, eczema, dermatitis, nephritis, and cystitis [2,3]. As a flavonoid isolated from the rhizome of *Smilax glabra Rox B*, astilbin (Fig. 1) is a selective immunodepressant and is beneficial for the treatment of human immune diseases [4–8]. In addition, just as many other flavonoids had been reported, astilbin may undergo extensive biotransformation including oxidation, reduction, methylation, sulfation and glucuronidation in vivo [9]. Among these processes, methylation is much more significant than others because the enzyme (catechol-O-methyltransferase) responsible for this metabolism is a kind of intracellular enzyme which distributes in mammalian organs widely [10]. In 2007, 3′-O-methylastilbin (Fig. 1), a major metabolite of astilbin, was reported to play an important role in selective inhibition against activated T lymphocytes [11].

As compared to the comprehensive research on the pharmacological activities of astilbin, few studies have been done on its pharmacokinetics. In 2004, Guo et al. [12] reported an HPLC–UV method to determine astilbin in rabbit plasma, but it showed poor sensitivity with a limit of quantification (LOQ) about 160 ng/mL when 1.0 mL plasma was used. The maximum plasma concentration (C_{max}) of astilbin was expected to be lower than 100 ng/mL after a single intragastric administration at the dose of 40 mg/kg. That is to say, the method mentioned above was not sensitive enough in the preclinical or clinical pharmacokinetic studies for astilbin or its metabolites. Therefore, it is necessary to develop and validate an assay with appropriate sensitivity, selectivity, accuracy, precision and feasibility. In support of large numbers of samples in preclinical studies, an ideal method should have simple sample preparation, fast on-column separation, and specific detection.

In the present work, we developed an LC–MS method for simultaneous determination of astilbin and 3′-O-methylastilbin in rat plasma using a single-step liquid–liquid extraction (LLE) with ethyl acetate. An electrospray ionization (ESI) interface was used because it provided more sensitivity and better reproducibility for astilbin and 3′-O-methylastilbin than atmospheric pressure chemical ionization (APCI). Importantly, this assay was fully validated and successfully used to assess the pharmacokinetics of astilbin and 3′-O-methylastilbin in rats.

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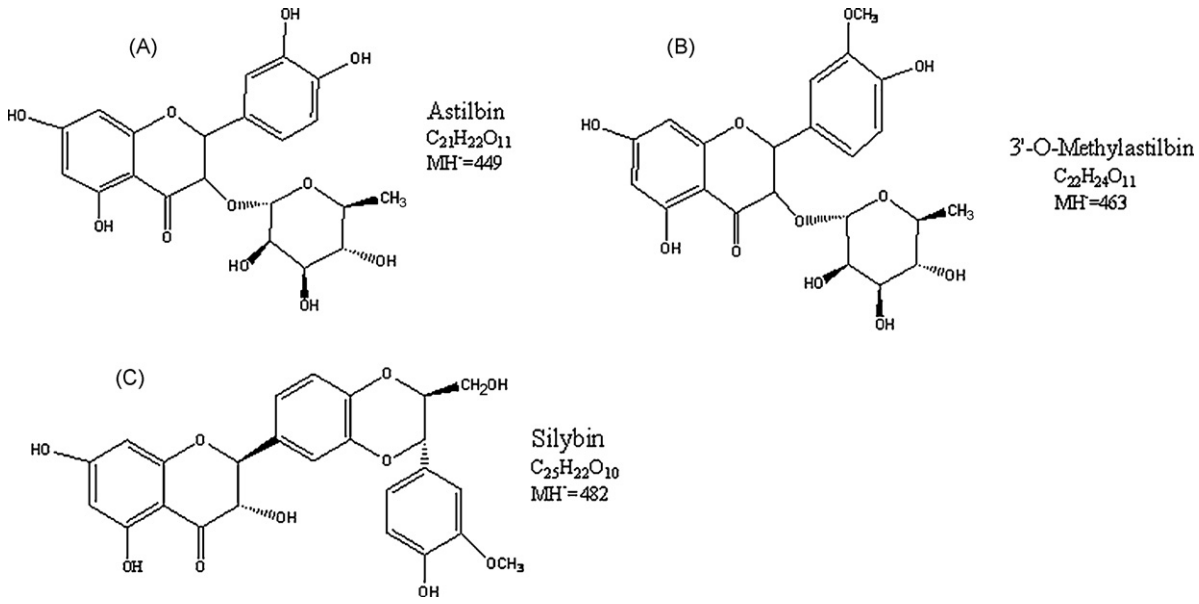

Fig. 1. Molecular structures of (A) astilbin, (B) 3′-O-methylastilbin, and (C) silybin (IS).

2. Experimental

2.1. Reference standards

Astilbin and 3′-O-methylastilbin (purity >99.5%) were isolated and purified by State Key Laboratory of Pharmaceutical Biotechnology of Nanjing University (Nanjing, China). Reference standard of silybin (Fig. 1, purity >98.0%) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Analytical grade formic acid was purchased from Sigma Chemicals (St. Louis, MO). HPLC-grade methanol was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Water was collected from a Milli-Q Ultrapure water system with the water outlet operating at 18.2 MΩ (Millipore, Bedford, USA). Other chemicals and solvents were all of analytical grade.

2.2. Chromatographic and mass spectrometer conditions

The HPLC system was a Shimadzu LC-10AD model (Shimadzu, Kyoto, Japan). This system consisted of Shimadzu LC-10AD pumps, DGU-14AM degasser, SIL-HTc autosampler and CTO-10ADvp column oven. Chromatographic separation was achieved on a Shim-pack C_{18} column (150 mm × 2.0 mm I.D., 5 μm, Shimadzu Co.) at 40 °C. The mobile phase (delivered at 0.2 mL/min) consisted of solvent A, CH_3OH/H_2O (5:95, v/v, containing 0.01% HCOOH) and solvent B, CH_3OH/H_2O (95:5, v/v, containing 0.01% HCOOH). A binary gradient elution was performed: initial 30% B for 0.2 min, linear gradient 30–80% B from 0.2 to 3.5 min, isocratic 80% B from 3.5 to 6.0 min, then quickly returned to initial 30% B and maintained for 5 min for column equilibration.

MS analyses were performed on a quadrupole mass spectrometer (LC-MS-2010A, Shimadzu, Kyoto, Japan) plus with an ESI probe using selected ion monitoring (SIM) mode. For all analytes and internal standard, SIM setting was as follows: spray gas and drying gas flow rate were set at 1.5 and 2.5 L/min, respectively; curved desolvation line and heat block temperature were set at 250 and 200 °C, respectively; detector voltage was 1.6 kV; probe voltage was 4.0 kV; interval time was 0.2 s. The extracted ions were *m/z* 449.1 $[M-H]^-$ for astilbin, *m/z* 463.1 $[M-H]^-$ for 3′-O-methylastilbin, and *m/z* $[M-H]^-$ 481.2 for silybin. The LC–MS system was controlled by the LC–MS solution version 2.4, and the data were collected with the same software.

2.3. Preparation of standards

The primary stock solutions of astilbin and 3′-O-methylastilbin were prepared by dissolving 10.0 mg of astilbin and 4.0 mg of 3′-O-methylastilbin in 10 mL methanol, and were stored at 4 °C until analysis. Working solutions of the analytes were prepared by appropriate dilution of the primary stock solutions in methanol. Then, 5 μL of astilbin and 5 μL of 3′-O-methylastilbin working solutions were spiked into 90 μL blank rat plasma in an Eppendorf 1.5 mL microcentrifuge polypropylene tube with a snap top to make a calibration standard curve. The final concentrations of astilbin and 3′-O-methylastilbin were as follows: 1.0, 2.0, 5.0, 10.0, 20.0, 50.0, 100.0, 200.0, and 500.0 ng/mL for astilbin and 0.8, 1.6, 3.1, 6.3, 12.5, 25.0, 50.0, 100.0, and 200.0 ng/mL for 3′-O-methylastilbin.

The quality control (QC) samples were made at the beginning of the project initialization. 100 μL of astilbin and 100 μL of 3′-O-methylastilbin working solution were added into 1.8 mL rat plasma for homogenization. The theoretical concentrations of astilbin and

Table 1
Recovery and matrix effect of astilbin and 3′-O-methylastilbin in rat plasma by LC–MS.

	QC sample (ng/mL)	Astilbin	3′-O-methylastilbin	Silybin (IS)
Recovery, *n* = 5 (%)	2.0	97.2 ± 1.5	95.7 ± 2.8	95.3 ± 8.4
	50.0	92.4 ± 2.2	90.5 ± 3.8	94.8 ± 5.1
	400.0	91.1 ± 3.2	90.7 ± 2.3	94.6 ± 3.6
Matrix effect, *n* = 5 (%)	2.0	103.5 ± 5.8	105.0 ± 5.7	104.7 ± 6.7
	12.5	103.1 ± 3.4	95.7 ± 3.1	99.8 ± 3.1
	100.0	96.8 ± 2.5	95.5 ± 3.3	98.5 ± 3.1

3′-O-methylastilbin in rat plasma were listed in Table 1. The QC samples were divided into 100 μL and stored at −70 °C in Eppendorf 1.5 mL microcentrifuge polypropylene tubes. Only one tube of QC standard at each concentration level was taken out to be melted for use at each run.

Silybin was used as the internal standard (IS). The initial stock solution was prepared by dissolving 10.0 mg of silybin in 10 mL methanol to produce a primary stock solution, and the final silybin concentration was 2.0 μg/mL, prepared by serial dilution in methanol.

2.4. Sample clean-up

QC samples, calibration standards, and plasma samples were extracted by LLE technique. To each tube containing 100 μL rat plasma, 5 μL of internal standard solution (2.0 μg/mL) and 1.0 mL of acetic ether were added, respectively. The sample was vortexed for 2 min and centrifuged at 40,000 × *g* for 10 min. Then 0.75 μL of the supernatant was transferred into 1.5 mL microcentrifuge polypropylene tube and evaporated to dryness in rotary evaporator (Thermo Fisher Scientific, NJ, USA) at 45 °C. The residue was dissolved in 200 μL methanol and transferred into disposable glass autosampler vials. Finally, 5 μL methanol solution was injected into the LC–MS system.

2.5. Method validation

The validation was done according to US FDA guidance for industry on bioanalytical method validation [13].

The specificity of the method was investigated by analyzing six different batches of drug-free rat plasma (without IS nor analytes) for the exclusion of any endogenous co-eluting interferences at the peak region of each analyte. The linearity of the method was determined by analysis of calibration standard samples prepared above, and the calibration curves were plotted using weighted linear least-squares regression analysis with a $1/x$ weighting factor to compensate for heteroscedasticity. The precision and accuracy of the developed method was determined by analysis of QC samples at three concentrations. Intra-day variation of the assay was assessed by injecting five samples at three concentrations on the same day, and five samples of each concentration were injected on 4 days to assess inter-day variation. The precision of the method was expressed in terms of CV (%).

Matrix effect on the ionization efficiency of analytes was assessed by comparing analytes peak areas in six unique blank extracted rat plasma samples spiked with three levels of working solution with the same nominal concentrations, prepared in methanol. The values >115% indicated ionization enhancement in plasma vs. neat standards, whereas values <85% indicated ionization suppression. Extraction recovery of astilbin, 3′-O-methylastilbin and IS from rat plasma was determined by comparing peak areas of analytes obtained from injection of extracted QC samples with those obtained from direct injection of the same concentrations of astilbin, 3′-O-methylastilbin or IS dissolved in methanol.

Finally, the room temperature matrix stability, the matrix freeze/thaw stability, the post-preparative stability and a 10-day −20 °C storage stability for QC standards were evaluated.

3. Preclinical study design

Sprague–Dawley rats weighing 200 ± 20 g were obtained from the Laboratory Animal Center of Peking University Health Science Center (Beijing, PR China) and kept in an environmentally controlled breeding room for at least 3 days before experimentation, fed with standard laboratory food and water and fasted overnight but with free access to water before the test. Animal welfare and experimental procedures were strictly in accordance with the guide for the care and use of laboratory animals (National Research Council of USA, 1996) and the related ethical regulations of our university. After orally administrating astilbin at 40 mg/kg, heparinized blood samples of 250 μL were collected at 10, 20, 40 min, 1, 2, 3, 4, 5, 6, 8 and 12 h from the ophthalmic veins by sterile capillary tube under anesthesia, then, shaken up and centrifuged at 2000 × *g* for 10 min. The supernatants were decanted, and immediately frozen at −20 °C until analysis. In addition, 6 rats were killed under anesthesia to gain drug-free plasma.

4. Data analysis

All LC–MS data were acquired and analyzed using LC–MS solution version 2.4 software (Shimadzu, Kyoto, Japan). Pharmacokinetic parameters in rats were estimated by a noncompartmental method using DAS 2.0 software package (purchased from Wannan Medical College). The maximum plasma concentration (C_{max}) and the time taken to achieve the peak concentration (T_{max}) were observed values with no interpolation. The area under concentration–time curve up to the last measured time point (AUC_{0-t}) was calculated by the trapezoidal rule. The AUC_{0-t} extrapolated to infinity ($AUC_{0-\infty}$) was generated by extrapolating the AUC_{0-t} to infinity using the elimination rate constant and the last measured concentration.

5. Results and discussion

5.1. Method development

Selection of IS. It is necessary to use an IS to obtain high accuracy when a mass spectrometer is used as the HPLC detector. Silybin was chosen for quantification as the IS due to its similarity with astilbin and 3′-O-methylastilbin in structure, ionization characteristics, extraction recovery and stability (data not shown).

Sample clean-up. LLE was advantageous because this technique not only extracted the analytes and IS with sufficient efficiency and specificity, but also minimized the experimental cost. Acetic ether, diethyl ether, *n*-hexane and trichlormethane were all tested as extraction solvent, and acetic ether was finally adopted for its high extraction and low toxicity.

Separation and relative retention time. In this study, one major concern was about whether the benefit of the low concentration of mobile phase additive (ammonium acetate, trifluoroacetic acid, ammonia water, and formic acid) might be offset by an undesired chromatographic performance and/or increasing matrix effects. Fortunately, addition of 0.01% HCOOH was found to be an important factor for amelioration on the chromatographic peak widths, peak asymmetry, ionization efficiency and peak retention times. When raising the concentration of formic acid in mobile phase under the same organic solvent percentage, ionization efficiency and signal/noise decreased rapidly although the retention times and chromatographic peak widths of the analytes did not change significantly. Contradictory, peak shape would not be improved efficiently when decreasing the concentration of HCOOH. Besides, higher methanol ratios had been tried for shortening the analysis time, the analytes could not be totally separated from the endogenous plasma components.

Mass spectrometry. During the full scan of astilbin and 3′-O-methylastilbin, the ESI-MS system revealed the presence of $[M-H]^-$ as well as $[M+Cl]^-$ ion. The chromatographic intensity of $[M-H]^-$ was about five times higher than that of $[M+Cl]^-$ under the same concentration. Thus, $[M-H]^-$ ions (m/z 449.1 for astilbin, m/z 463.1 for 3′-O-methylastilbin, and m/z 481.2 for silybin) were employed

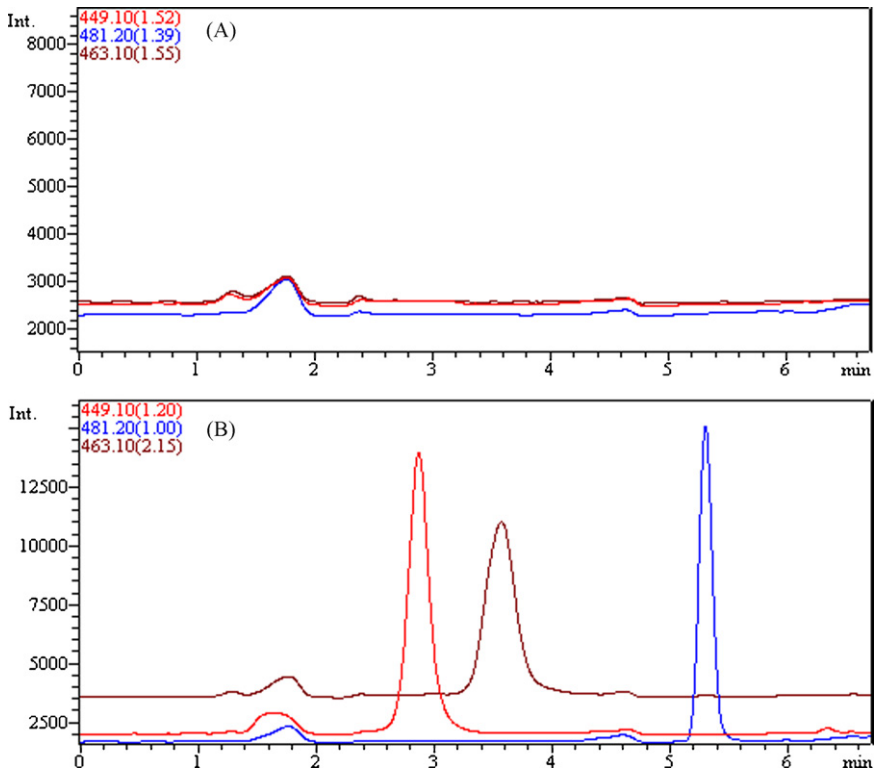

Fig. 2. Chromatograms of rat plasma samples: (A) blank plasma; (B) plasma sample at 20 min after intragastric administration of 40 mg/kg astilbin. The retention times of astilbin, 3′-O-methylastilbin and the silybin (IS) were 2.8, 3.5 and 5.4 min, respectively. The concentrations of astilbin, 3′-O-methylastilbin and the IS were 48.6, 6.4 and 200.0 ng/mL, respectively.

Table 2
Method calibration data for astilbin and 3′-O-methylastilbin in rat plasma by LC–MS.

Analyte	Astilbin	3′-O-methylastilbin
Dynamic range	1.0–500.0 ng/mL	0.8–200.0 ng/mL
Slope ± SE (n = 5)	0.0125 ± 0.001	0.0181 ± 0.002
Intercept ± SE (n = 5)	0.0015 ± 0.0070	0.0037 ± 0.0014
Coefficient of determination ± SE (R^2) (n = 5)	0.9999 ± 0.0003	0.9998 ± 0.0010
Retention time ± SD (min) (n = 45)	2.8 ± 0.01	3.5 ± 0.02

as the detection ions for the quantitation analysis in the selected ion mode.

5.2. Validation

We present the first validated LC–MS method for the simultaneous analysis of astilbin and 3′-O-methylastilbin in rat plasma to ensure adequate specificity, sensitivity, reproducibility, and accuracy prior to analysis of preclinical specimens.

The total LC–MS analysis time was 11.0 min per sample. The SIM chromatograms obtained from extracted rat plasma sample of blank and a specimen at 1 h after oral administration of 40 mg/kg astilbin are depicted in Fig. 2. Due to the high specificity of the SIM mode, no interferences were observed for astilbin, 3′-O-methylastilbin and IS in blank plasma samples. In addition, recovery and matrix effect were evaluated at three concentration levels (Table 1). Recovery ranged from 90% to 97%. No ionization suppression effects were found under the developed sample preparation and chromatographic conditions because the peak area ratios for the plasma extracts vs. clean methanol solutions were between 90% and 110%.

Calibration results for astilbin and 3′-O-methylastilbin can be found in Table 2. LOQ was 1.0 ng/mL for astilbin and 0.8 ng/mL for 3′-O-methylastilbin (judged from the fact that the precision was less than 15%, accuracy was more than 85% and the S/N ratios were much more than 10, Fig. 3). The linear range was more than 200-fold for both analytes with correlation coefficient (R^2) >0.99. Retention times for astilbin and 3′-O-methylastilbin during the whole analytical run varied within the range of ±2%.

Accuracy and precision were evaluated across the dynamic range of the assay using controls at three concentrations (see Table 3). Intra-assay precision was less than 5% for each of the three batches, and inter-assay precision was less than 10% for all analytes at all concentrations. Accuracy, calculated as percent of target value, was greater than 90% across the analytical range.

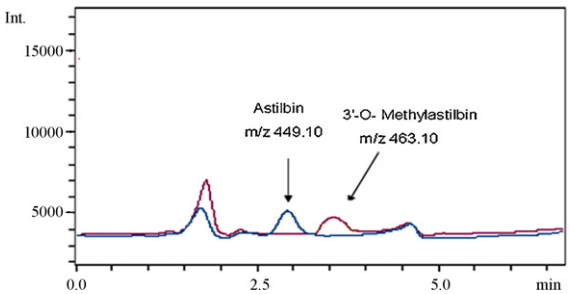

Fig. 3. Chromatograms of astilbin and 3′-O-methylastilbin at LOQ (rat plasma fortified with 1.0 ng/mL astilbin and 0.8 ng/mL 3′-O-methylastilbin).

Table 3
Precision and accuracy results for astilbin and 3′-O-methylastilbin in rat plasma by LC–MS.

	Concentration (ng/mL)	Intra-day (n = 5)		Inter-day (n = 20)	
		Precision (CV, %)	Accuracy (%)	Precision (CV, %)	Accuracy (%)
Astilbin	2.0	3.54	97.5	5.24	97.7
	50.0	2.35	98.2	4.28	97.5
	400.0	1.63	99.4	3.49	97.1
3′-O-methylastilbin	2.0	3.42	91.1	6.79	101.2
	12.5	4.27	97.7	2.45	99.2
	100.0	2.57	99.5	0.60	97.7

Table 4
Stability of astilbin and 3′-O-methylastilbin in rat plasma by LC–MS.

	Low		Medium		High	
	Astilbin recovery (%)	3′-O-methylastilbin recovery (%)	Astilbin recovery (%)	3′-O-methylastilbin recovery (%)	Astilbin recovery (%)	3′-O-methylastilbin recovery (%)
Keep at room temperature for 6 h	93.2 ± 9.8	97.1 ± 9.1	96.4 ± 4.1	90.5 ± 6.1	90.6 ± 5.7	91.7 ± 3.9
Freeze and thaw for three times	90.1 ± 9.5	93.5 ± 5.8	93.8 ± 3.2	91.3 ± 8.1	89.1 ± 4.5	93.1 ± 8.5
Freeze at −20 °C for 10 days	89.1 ± 7.4	90.4 ± 2.4	88.5 ± 3.3	90.1 ± 3.8	90.5 ± 4.2	91.5 ± 2.4
Keep at 10 °C in the autosampler for 24 h	94.9 ± 6.4	90.9 ± 7.5	99.7 ± 7.3	96.2 ± 4.1	97.9 ± 6.7	94.7 ± 6.8

Stability of the analytes in rat plasma was tested under a variety of conditions to mimic those encountered in the laboratory. No significant degradation of astilbin and 3′-O-methylastilbin was observed during all of the sample preparation, storage and analysis periods. Table 4 summarizes the data from the short-term, freeze–thaw, and long-term stability, as well as for the post-preparative test of astilbin and 3′-O-methylastilbin. As a result, no stability-related problems occurred during the routine analyses for the preclinical pharmacokinetic studies.

5.3. Application to a pharmacokinetic study

To demonstrate the applicability of the validated method described above, we applied it to analyze plasma samples obtained from 6 rats administrated with astilbin at a single dose of 40 mg/kg. The mean plasma concentration (log scale)–time profiles of astilbin and 3′-O-methylastilbin are shown in Fig. 4, and both of them could be detected in plasma up to 12 h after administration of astilbin. Thus, the assay can be used to assess systemic exposure of rats to astilbin and 3′-O-methylastilbin.

The main pharmacokinetic parameters calculated by DAS 2.0 are represented in Table 5. The mean C_{max} of astilbin and 3′-O-methylastilbin was 37.7 ± 14.7 and 17.8 ± 8.5 ng/mL, respectively. The mean T_{max} was 25.8 ± 34.3 min for astilbin and 101.7 ± 50.0 min for 3′-O-methylastilbin. The terminal elimination half-life was similar and about 2.5 h for both of them. The AUC_{0-t} was calculated using trapezoidal rule and the results have revealed that the exposure of 3′-O-methylastilbin was slightly lower than that of unchanged drug.

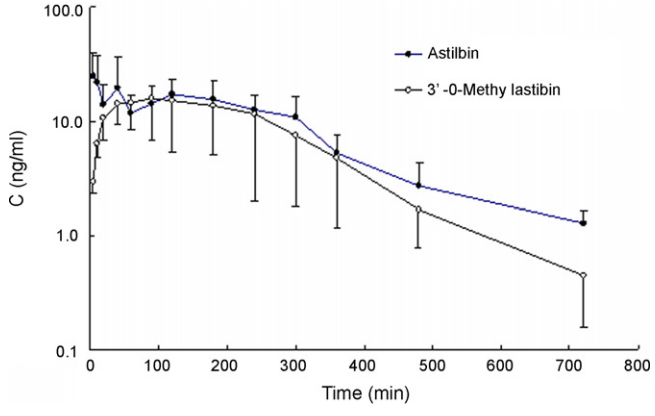

Fig. 4. Mean plasma concentration (log scale)–time profiles of astilbin and 3′-O-methylastilbin after intragastric administration of 40 mg/kg astilbin to 6 rats.

Table 5
Pharmocokinetic parameters of astilbin and 3′-O-methylastilbin in after intragastric administration of 40 mg/kg astilbin to 6 rats.

Parameters	Astilbin	3′-O-methylastilbin
C_{max} (ng/mL)	37.7 ± 14.7	17.8 ± 8.5
T_{max} (min)	25.8 ± 34.3	101.7 ± 50.0
$t_{1/2}$ (min)	161.6 ± 44.1	139.2 ± 88.2
AUC_{0-t} (ng min/mL)	5353.4 ± 1456.3	4120.6 ± 2407.9
$AUC_{0-\infty}$ (ng min/mL)	5741.2 ± 1567.0	5131.7 ± 3012.9

6. Conclusion

By the present study, a robust and sensitive LC–ESI-MS method for simultaneous determination of astilbin and 3′-O-methylastilbin in rat plasma has been successfully developed and validated. The analysis requires only 100 μL of rat plasma, which has an advantage in preclinical pharmacokinetic studies. The data for the validation indicated that the method is accurate, specific, reproducible and sensitive to support the assay of astilbin and 3′-O-methylastilbin in rat plasma samples.

In addition, this LC–ESI-MS assay was successfully applied to the detailed pharmacokinetic studies of astilbin and 3′-O-methylastilbin in rats. It is the first study, as far as we know, of LC–MS method for simultaneous determination of astilbin and 3′-O-methylastilbin in vivo.

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