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Liquid chromatography-tandem mass spectrometry for the determination of jaceosidin in rat plasma

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ABSTRACT

Jaceosidin (4′,5,7-trihydroxy–3′,6-dimethoxyflavone), isolated from Artemisia species as well as Eupatorium species, has antiallergic, anticancer, anti-inflammatory and antioxidant activity. A rapid, sensitive and selective liquid chromatography-tandem mass spectrometric (LC/MS/MS) method for the quantification of jaceosidin in rat plasma was developed to characterize the pharmacokinetics of jaceosidin. Jaceosidin and the internal standard, linezolid, were extracted from rat plasma with ethyl acetate at acidic pH and analyzed on a Luna phenyl-hexyl column using the mixture of acetonitrile and 0.1% formic acid (45:55, v/v) as a mobile phase. The analytes were determined using an electrospray ionization tandem mass spectrometry in the multiple-reaction-monitoring mode. The calibration curve was linear (r^2 = 0.9973) over the concentration range of 2.00–500 ng/ml. The lower limit of quantification for jaceosidin was 2.0 ng/ml using 50 μl of plasma sample. The coefficients of variation of intra- and inter-assay at four QC levels were 2.4–9.6% and the relative errors were −9.1 to 10.0%. The matrix effects for jaceosidin and linezolid were practically absent. The recoveries of jaceosidin and linezolid were 87.0 and 87.7%, respectively. This method was successfully applied to the pharmacokinetic study of jaceosidin in rats.

1. Introduction

Jaceosidin (4′,5,7-trihydroxy–3′,6-dimethoxyflavone) (Fig. 1) is isolated from Artemisia species as well as Eupatorium species. Jaceosidin showed potent peroxynitrile ($ONOO^-$) and 1,1-diphenyl-2-picryl-hydrazyl radical scavenging activities and antioxidant activity in low-density lipoprotein oxidation [1,2]. Jaceosidin potently inhibited a passive cutaneous anaphylaxis reaction and scratching behaviors induced by IgE-antigen complex and compound 48/80, respectively in mice [3]. Jaceosidin showed topical anti-inflammatory activity in the TPA mouse ear edema [4] based on the inhibition of nitric oxide production, prostaglandin E2 production and cyclooxygenas-2 (COX-2) activity [5]. Jaceosidin exhibited strong antimutagenic activity against 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole, 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole, 2-amino-3-methylimidazo[4,5-*f*]quinoline, 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline and 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline in *Salmonella typhimurium* TA98 [6]. Jaceosidin might be used as a potential cancer chemopreventive agent via functional inhibition of E6 and E7 oncoproteins of human papillomavirus, inhibition of TPA-induced upregulation of COX-2 and matrix metalloproteinase-9 in human breast epithelial cells or inhibition of the proliferation of MCF10A-ras cells [7–9].

The evaluation of the pharmacokinetics and bioavailability of botanical drugs can link data from pharmacological assays to clinical effects and also help the design of rational dosage regimens [10]. The pharmacokinetic data of jaceosidin in the rats and human as well as the bioanalytical method for the determination of jaceosidin in biological fluids were not reported to the best of our knowledge. A selective, sensitive and reliable analytical method for the determination of jaceosidin in rat plasma is required in order to evaluate the pharmacokinetics of jaceosidin in rats. LC-tandem mass spectrometry (LC/MS/MS) is recognized as a powerful tool for the quantitative determination of the active compound of a herbal drug in biological samples due to the selectivity, sensitivity, robustness and sample throughput.

The purpose of this paper was to develop the selective, sensitive, rapid and reliable LC/MS/MS assay using liquid–liquid extraction for the quantification of jaceosidin in rat plasma. The present method was successfully applied to the pharmacokinetic study of jaceosidin in the rats.

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2. Experimental

2.1. Materials and reagents

Jaceosidin was synthesized in research laboratories of Dong-A Pharm. Co. (Yongin, Korea) with 99.4% purity and linezolid (internal standard) was obtained from SynFine Research, Inc. (Ontario, Canada). Acetonitrile and ethyl acetate (HPLC grade) were obtained from Burdick & Jackson, Inc. (Muskegon, MI, USA) and the other chemicals were of HPLC grade or the highest quality available. Drug-free rat plasma containing sodium heparin as the anticoagulant was obtained from rats.

2.2. Preparation of calibration standards and quality control samples

Primary stock solutions of jaceosidin and linezolid (1 mg/ml) were prepared in dimethylsulfoxide. Working standard solutions of jaceosidin were prepared by diluting each primary solution with 50% acetonitrile. The working solution for linezolid (1 μg/ml) was prepared by diluting an aliquot of stock solution with acetonitrile. Jaceosidin and linezolid solutions were stored at 4 °C in polypropylene tubes in the dark for 3 weeks.

Rat plasma calibration standards of jaceosidin (2.0, 4.0, 10.0, 50.0, 200, 400 and 500 ng/ml) were prepared by spiking the working standard solutions into a pool of 10 lots of drug-free rat plasma. Quality control (QC) samples at 2.0, 5.0, 100 and 350 ng/ml were prepared in bulk by adding 75 μl of appropriate working standard solutions (80, 200, 4000 and 14,000 ng/ml) to drug-free rat plasma (2925 μl). The bulk QC samples were aliquoted (50 μl) into polypropylene tubes and stored at −20 °C until analysis.

2.3. Sample preparation

Fifty microliters of blank rat plasma, rat plasma standard and QC samples were vortex-mixed with 200 μl of 50 mM hydrochloric acid, 10 μl of linezolid in acetonitrile solution and 1000 μl of ethyl acetate in 1.5 ml-polypropylene tubes. The mixtures were centrifuged at 10,000 × *g* for 5 min. The organic layer was pipette-transferred and evaporated to the dryness using a vacuum concentrator. The residues were dissolved in 40 μl of 45% acetonitrile by sonicating for 3 min and centrifuged. The aliquots (10 μl) were injected onto LC/MS/MS system.

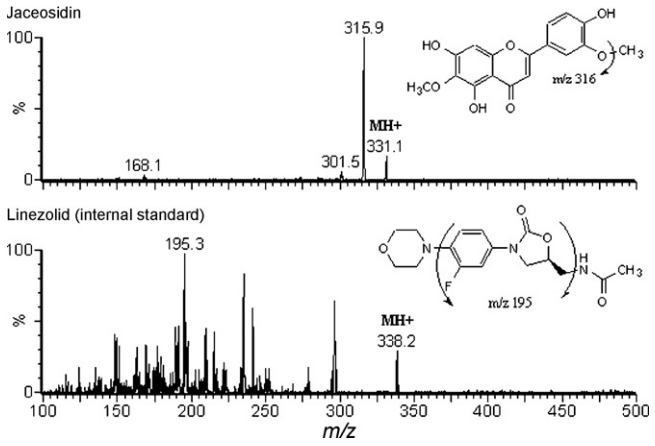

Fig. 1. Product ion spectra of jaceosidin and linezolid (internal standard).

2.4. LC/MS/MS analysis

For LC/MS/MS analysis, the chromatographic system consisted of a Nanospace SI-2 pump, a SI-2 autosampler and an S-MC system controller (Shiseido, Tokyo, Japan). The separation was performed on a Luna phenyl-hexyl column (3 μm, 2 mm i.d. × 100 mm, Phenomenex, Torrance, CA, USA) using a mixture of acetonitrile-0.1% formic acid (45:55, v/v) at a flow rate of 0.2 ml/min. The column and the autosampler tray temperatures were 40 °C and 4 °C, respectively. The analytical run time was 3.5 min. The eluent was introduced directly into the positive ionization electrospray source of the tandem quadrupole mass spectrometer (Quattro LC, Micromass UK Ltd, UK). The ion source and desolvation temperatures were held at 120 °C and 350 °C, respectively. The optimum cone voltages were 45 V for jaceosidin and 35 V for linezolid. The molecular ions of jaceosidin and linezolid were fragmented at collision energy of 26 eV and 24 eV using argon as collision gas. Multiple reaction monitoring (MRM) mode using specific precursor/product ion transitions was employed for the quantification. Detection of the ions was performed by monitoring the transitions of *m*/*z* 331.1 to *m*/*z* 315.9 for jaceosidin and *m*/*z* 338.2 to *m*/*z* 195.3 for linezolid. Peak areas for all components were automatically integrated using MassLynx version 3.5 (Micromass UK Ltd).

2.5. Method validation

Batches, consisting of triplicate calibration standards at each concentration, were analyzed on three different days to complete the method validation. In each batch, QC samples at 2.0, 5.0, 100 and 350 ng/ml were assayed in sets of five replicates to evaluate the intra- and inter-day precision and accuracy. The percentage deviation of the mean from true values, expressed as relative error (RE), and the coefficient of variation (CV) serve as the measure of accuracy and precision, respectively. To investigate the effect of diluting over-range samples into the calibration range, the accuracy and precision of dilution control samples at 3500 ng/ml were assessed by performing a 10-fold dilution ($n = 5$).

The absolute and relative matrix effects and recoveries of jaceosidin and linezolid were assessed by analyzing three sets of the standards at four concentrations (2.0, 5.0, 100 and 350 ng/ml) according to the approach of Matuszewski et al. [11]. The absolute matrix effect for jaceosidin and linezolid was assessed by comparing mean peak areas of the analyte spiked at four concentrations into the extracts originating from five different lots of blank plasma samples (set 2) to mean peak areas for neat solutions of the analytes in 45% acetonitrile (set 1). The variability in the peak areas of analytes spiked after liquid–liquid extraction into five different plasma extracts (set 2) expressed as CVs (%), was considered as a measure of the relative matrix effect.

Recovery of jaceosidin was determined by comparing mean peak areas of jaceosidin spiked before liquid–liquid extraction into the same five different sources as set 2 (set 3) with those of the jaceosidin spiked post-extraction into different blank plasma extracts (set 2) at four concentrations.

To evaluate the freeze/thaw stability and room temperature storage stability, five replicates of QC samples at each of the low and high concentrations (5.0 and 350 ng/ml) were subjected to three freeze/thaw cycles or the storage at room temperature for 4 h before processing, respectively. Five replicates of QC samples at each of the low and high concentrations were processed and stored under autosampler conditions for 24 h were assayed to assess post-preparative stability.

2.6. Application study

The developed LC/MS/MS assay method was used in a pharmacokinetic disposition study after an intravenous administration of jaceosidin to male Sprague–Dawley rats (body weight 297 ± 16 g, Orient ENG, Inc., Sungnam, Korea). Animals were kept in plastic cages with free access to standard rat diet (Samyang Co., Seoul, Korea) and water. The animals were maintained at a temperature of 23 ± 2 °C with a 12 h light/dark cycle and relative humidity of 50 ± 10%.

The rats were anesthetized by i.p. injection of ketamine (90 mg/kg) and cannulated with polyethylene tubing (0.58 mm i.d. and 0.96 mm o.d., Clay Adams Co., Parsippany, NJ, USA) in the carotid artery and jugular vein. Each rat was housed individually in a rat metabolic cage and allowed to recover from anesthesia for 1 day before the study began. The rats were not restrained at any time during the study. A heparinized 0.9% NaCl-injectable solution (20 U/ml) was used to flush each cannula to prevent blood clotting. Jaceosidin was dissolved in a mixture of *N*,*N*-dimethyl acetamide: propylene glycol: saline (2:4:1, v/v) and administered to rats by a bolus injection over 1 min via the jugular vein at dose of 2 mg/ml/kg (n = 6). Arterial blood samples were collected prior to and at 1, 5, 15, 30, 45, 60, 90, 120, 240 and 360 min after drug administration. Plasma samples were harvested by centrifugation at 3000 × g for 10 min and stored at −20 °C until analysis. At 24 h, the metabolic cage was rinsed with 10 ml distilled water, and the rinsed solutions were combined with the pooled urine samples collected for 24 h. After measuring the exact volume of the combined urine samples, two 100-μl aliquots of each sample were taken and kept at −20 °C.

The plasma concentration-time data were analyzed by a non-compartmental method using the nonlinear least squares regression program WinNonlin (Pharsight Co., Cary, NC, USA). The area under the plasma concentration-time curve (AUC) was calculated using the trapezoidal rule extrapolated to infinite time. The terminal elimination half-life ($t_{1/2\lambda z}$), systemic clearance (Cl), mean residence time (MRT) and volume of distribution at steady state (V_{ss}) were calculated. Pharmacokinetic parameter values were expressed as mean ± S.D.

3. Results and discussion

3.1. LC/MS/MS

The electrospray ionization of jaceosidin and linezolid produced the abundant protonated molecular ions (MH^+) at m/z 331.1 and 338.2, respectively under positive ionization conditions, without any evidence of fragmentation and adduct formation.

MH^+ ions from jaceosidin and linezolid were selected as the precursor ion and subsequently fragmented in product scan mode to obtain the product ion spectra, yielding useful structural information (Fig. 1). Jaceosidin produced the prominent product ion at m/z 315.9 (the loss of a methyl group from MH^+) and the major product ion for linezolid was m/z 195.3 (the loss of morpholinyl and acetamide group from MH^+). The quantification of the analytes was performed using the MRM mode due to the high selectivity and sensitivity of MRM data acquisition, where the transition of the precursor ion to product ion is monitored. Two pairs of MRM transitions were selected: m/z 331.1 → 315.9 for jaceosidin and m/z 338.2 → 195.3 for linezolid (internal standard).

The peak tailing of jaceosidin was severe in nonpolar columns such as Atlantis C_{18}, Xbridge C_{18} and Luna2 C_{18}, whereas the use of a Luna phenyl-hexyl column resulted in good peak shape of jaceosidin. The compositions of mobile phase may have a strong impact on the chromatographic resolution and the ionization efficiency of the analytes in LC/MS. The use of 0.1% formic acid in the mobile phase gave the good peak shape and ionization efficiency of jaceosidin compared to the use of ammonium formate buffers at pH 3, 4.5 and 6.5.

The analysis of blank plasma samples from 10 different sources did not show any interference at the retention times of jaceosidin

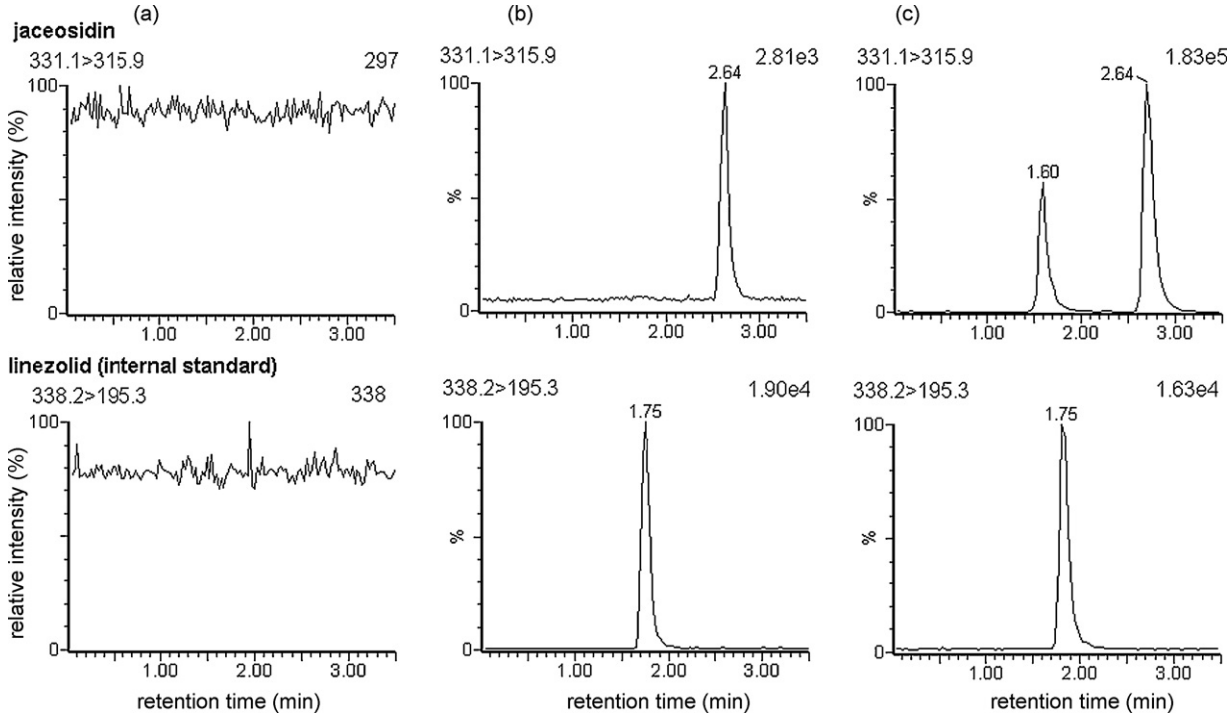

Fig. 2. MRM LC-MS/MS chromatograms of (a) blank rat plasma, (b) rat plasma sample spiked with 2.00 ng/ml of jaceosidin and (c) a plasma sample obtained at 5 min after intravenous administration of jaceosidin at a dose of 2 mg/kg in rat.

Table 1
Calculated concentrations of jaceosidin in calibration standards prepared in rat plasma ($n = 3$).

	Theoretical concentration (ng/ml)									
	2.00	4.00	10.0	50.0	200	400	500	Slope	Intercept	r^2
Mean (ng/ml)	2.03	4.11	10.8	51.3	202	380	515	0.0594	−0.0261	0.9973
CV (%)	13.9	8.7	1.6	4.8	1.4	2.5	1.4	6.0		0.2
RE (%)	1.5	2.8	8.0	2.6	1.0	−5.0	3.0			

Table 2
Precision and accuracy of jaceosidin in quality control samples.

	Intra-day ($n = 5$)				Inter-day ($n = 3$)			
QC (ng/ml)	2.00	5.00	100	350	2.00	5.00	100	350
Mean (ng/ml)	2.18	5.50	98.4	318	2.10	5.24	105	345
CV (%)	9.6	2.4	2.8	3.3	6.5	5.7	5.6	7.7
RE (%)	9.0	10.0	−1.6	−9.1	5.0	4.8	5.0	−1.4

(2.7 min) and linezolid (1.8 min), confirming the selectivity of the present method (Fig. 2). Sample carryover effect was not observed.

3.2. Method validation

Calibration curves were obtained over the concentration range of 2.00–500 ng/ml of jaceosidin in rat plasma. Linear regression analysis with a weighting of 1/concentration^2 gave the optimum precision (CV, 1.4–13.9%) and accuracy (RE, −5.0 to 8.0%) of the corresponding calculated concentrations at each level (Table 1). The low CV value (6.0%) for the slope indicated the repeatability of the method (Table 1).

Table 2 shows a summary of intra- and inter-day precision and accuracy data for QC samples containing jaceosidin. Both intra- and inter-assay CV values ranged from 2.4 to 9.6% at four QC levels. The intra- and inter-assay RE values for jaceosidin were −9.1 to 10.0% at four QC levels. These results indicated that the present method has the acceptable accuracy and precision. The lower limit of quantification (LLOQ) was set at 2.00 ng/ml for jaceosidin using 50 μl of rat plasma. Representative chromatogram of an LLOQ is shown in Fig. 2b and the signal-to-noise ratio for jaceosidin is about 20. The back-calculated concentrations for dilution control samples (3500 ng/ml) were in good agreement with the theoretical concentrations. After 10-fold dilution of dilution control samples, the CV and RE for jaceosidin were 9.6 and −6.4%, respectively, indicating the acceptability of 10-fold dilution prior to analysis.

The mean absolute matrix effect, the ratio of mean peak areas of set 2 to those of set 1 multiplied by 100, was 101.8 and 101.5% for jaceosidin and linezolid, respectively (Table 3). A value of 100% indicates that the response in the solvent and in the extracts originating from five different lots of blank plasma samples were the same and no absolute matrix effect was observed. A value of <100% indicates an ionization suppression and a value of >100% indicates an ionization enhancement. There was little absolute matrix effect for jaceosidin and linezolid.

The assessment of relative matrix effect was made based on the direct comparison of the peak areas of jaceosidin and linezolid spiked after liquid–liquid extraction into the organic layers originating from five different lots of rat plasma (set 2). The CVs of determination of set 2 at four different concentrations ranged from 4.6 to 8.0% for jaceosidin and 3.4 to 8.2% for linezolid (Table 4). This variability seemed to be comparable to the precision of determination of standards injected directly in 45% acetonitrile (set 1) (5.0–6.5% for jaceosidin and 3.1–9.5% for linezolid, Table 4). These data confirm that the relative matrix effect for jaceosidin and linezolid was practically absent. The CV of the ratio of jaceosidin/linezolid for samples spiked post-extraction into organic layers from five different lots of plasma varied from 4.4 to 8.7% at four concentrations and was similar to the CV of the ratio of jaceosidin/linezolid injected directly in 45% acetonitrile (4.0 to 6.5%, set 1 in Table 4), confirming that the absolute and relative matrix effects for ratio of jaceosidin and linezolid have practically no effect on the determination of jaceosidin spiked into five different lots of plasma using liquid–liquid extraction for sample preparation.

Ethyl acetate at acidic pH gave the best recovery of jaceosidin compared to the use of methyl-*tert*-butyl ether and dichloromethane at acidic and neutral pH. As shown in Table 3, the overall extraction recovery of jaceosidin was 87.0%, which was consistent over the concentration range of 2.0–350 ng/ml. The recovery of linezolid was 87.7%. The one-step liquid–liquid extraction with

Table 3
Matrix effect and recovery data for jaceosidin and linezolid in five different lots of rat plasma.

Nominal concentration (ng/ml)	Mean peak area[a]						Matrix effect[e] (%)		Recovery[f] (%)	
	Jaceosidin			linezolid						
	Set 1[b]	Set 2[c]	Set 3[d]	Set 1	Set 2	Set 3	Jaceosidin	I.S.	Jaceosidin	I.S.
2.00	374	380	328	2430	255	2250	101.6	105.0	86.3	88.2
5.00	763	779	679	2226	2250	1925	102.1	101.1	87.2	85.6
100	13,273	13,457	11,805	1969	2023	1760	101.4	102.7	87.7	87.0
350	41,520	42,395	36,839	1985	1927	1733	102.1	97.1	86.9	89.9
Mean							101.8	101.5	87.0	87.7

[a] In arbitary units, $n = 5$.
[b] Jaceosidin and linezolid standards in 45% acetonitrile.
[c] Jaceosidin and linezolid spiked into the plasma extracts after liquid–liquid extraction of five different plasma lots.
[d] Jaceosidin and linezolid spiked into five different lots of plasma before liquid–liquid extraction.
[e] Matrix effect expressed as the ratio of mean peak area of set 2 to the mean peak area of set 1 multiplied by 100.
[f] Recovery calculated as the ratio of the mean peak area of set 3 to the mean peak area of set 2 multiplied by 100.

Table 4
Precision[a] (CV, %) of determination of peak areas of jaceosidin and linezolid (I.S.), and peak area ratios (jaceosidin/linezolid) in sets 1[b], 2[c] and 3[d].

Nominal concentration (ng/ml)	Precision (CV, %)								
	Peak area of jaceosidin			Peak area of linezolid			Peak area ratio		
	Set 1	Set 2	Set 3	Set 1	Set 2	Set 3	Set 1	Set 2	Set 3
2.00	6.3	4.6	9.0	9.5	6.2	3.7	6.5	8.6	5.9
5.00	6.5	7.4	8.2	6.5	3.4	6.6	5.5	4.4	6.6
100	5.1	6.3	6.4	3.1	6.0	5.6	4.0	6.5	5.5
350	5.0	8.0	6.5	7.3	8.2	5.8	4.1	8.7	3.6

[a] $n = 5$.
[b] Jaceosidin and linezolid standards in 45% acetonitrile.
[c] Jaceosidin and linezolid spiked into the plasma extracts after liquid–liquid extraction of five different plasma lots.
[d] Jaceosidin and linezolid spiked into five different lots of plasma before liquid–liquid extraction.

ethyl acetate at acidic pH has been successfully applied to the extraction of jaceosidin from rat plasma.

Stabilities of processing (freeze–thaw and short-term room temperature storage) and chromatography (re-injection) were evaluated and shown to be of insignificant effect (Table 5). QC samples that went through three freeze–thaw cycles or that were exposed to room temperature for 4 h showed the acceptable accuracy and precision. The reanalysis of the reconstituted extracts stored for 24 h at 4 °C showed the acceptable accuracy and precision for QC samples.

3.3. Pharmacokinetics of jaceosidin in rats

The suitability of the present method was proved in the pharmacokinetic study of jaceosidin after intravenous injection of jaceosidin at a dose of 2 mg/kg to six male SD rats. Fig. 2c shows the representative MRM chromatograms obtained from the analysis of a plasma sample obtained at 5 min after intravenous administration of jaceosidin to a rat. After intravenous injection of jaceosidin in the rats, the mean plasma concentration-time profile of jaceosidin is shown in Fig. 3 and the concentration of 240 and 360 min plasma samples were below LOQ. A peak of 1.6 min shown in Fig. 2c was identified as jaceosidin glucuronide, a major metabolite of jaceosidin.

The area under the plasma concentration-time curve (AUC) and volume of distribution (V_{ss}) of jaceosidin were 10.1 ± 3.2 μg min/ml and 1382 ± 1036 ml/kg, respectively, after intravenous administration of jaceosidin (2 mg/kg) to male SD rats. Jaceosidin exhibited a high systemic clearance (Cl = 219.9 ± 84.7 ml/min/kg), the short terminal elimination half-life ($t_{1/2\lambda z}$ = 35.9 ± 12.0 min) and mean residence time (MRT = 5.7 ± 2.0 min). The percentages of jaceosidin excreted in the urine for 24 h as an intact drug (Ae_{0-24h}) were 2.3 ± 2.3% of the dose and the renal clearance of jaceosidin was 4.0 ± 3.4 ml/min/kg. Nonrenal clearance of jaceosidin was 215.9 ± 85.6 ml/min/kg, suggesting that jaceosidin may be extensively metabolized in rats.

Table 5
Stability of jaceosidin in samples ($n = 5$).

Statistical variable	Theoretical concentration (ng/ml)	
	5.00	350
Three freeze and thaw cycles		
Mean	5.16	340
CV (%)	5.4	4.6
RE (%)	3.2	−2.9
4 h at room temperature		
Mean	5.21	329
CV (%)	6.2	5.0
RE (%)	4.2	−6.0
Post-preparative stability (24 h at 4 °C)		
Mean	4.77	363
CV (%)	13.2	9.9
RE (%)	−4.6	3.7

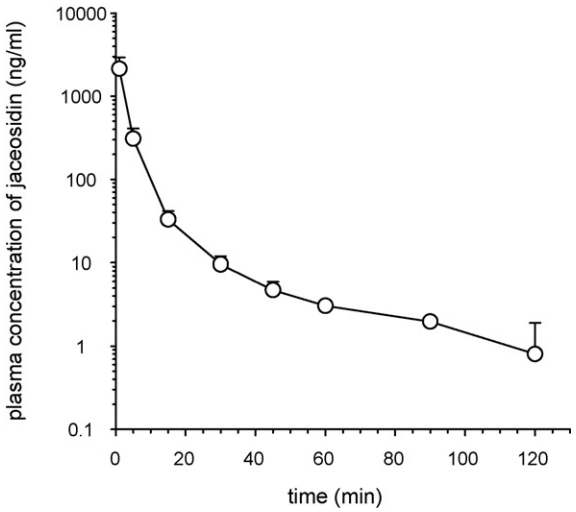

Fig. 3. Mean plasma concentration-time plot of jaceosidin after intravenous administration of jaceosidin at a dose of 2 mg/kg to male SD rats. Each point represents the mean ± S.D. ($n = 6$).

4. Conclusion

A rapid, sensitive and selective LC/MS/MS method for the analysis of jaceosidin in rat plasma has been developed and validated. Jaceosidin and linezolid were extracted from plasma samples with ethyl acetate at acidic pH. This method demonstrated the selectivity, sensitivity, precision, accuracy and stability with less matrix effect. The present method was successfully applied to the pharmacokinetic study of jaceosidin after intravenous administration of jaceosidin in rats.

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