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Quantitative determination of spinosin in rat plasma by liquid chromatography-tandem mass spectrometry method

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ABSTRACT

A sensitive method for the quantitative determination of spinosin in rat plasma was developed and validated using high-performance liquid chromatographic separation with tandem mass spectrometric detection. The analytes of interest were extracted from rat plasma samples by methyl *tert*-butyl ether (MTBE) after acidification with 1.0% acetic acid aqueous solution. Chromatographic separation was achieved on an Agilent Zorbax SB-C_{18} (50 mm × 4.6 mm, 5 μm) using a isocratic mobile phase consisting of acetonitrile–water (30:70, v/v) with 1% isopropyl alcohol and 0.01% heptafluorobutyric acid. The flow rate was 0.2 ml/min. The column temperature was maintained at 25 °C. Detection was performed on a triple quadrupole tandem mass spectrometer by selected reaction monitoring (SRM) mode via electrospray ionization (ESI). The calibration curve was linear over the range of 1.00–400 ng/ml in rat plasma, with 1.00 ng/ml of the lower limit of quantification (LLOQ). The inter- and intra-day precisions and accuracy for all samples were satisfactory. The validated method was successfully applied for the pharmacokinetic study of spinosin in rat. After oral administration of 20 mg/kg spinosin to rats, the main pharmacokinetic parameters of T_{max}, C_{max}, $T_{0.5}$ and AUC_{0-T} were 5.33 ± 0.58 h, 132.2 ± 10.6 ng/ml, 4.89 ± 0.37 h, 1.02 ± 0.09 μg h/l, respectively.

1. Introduction

Suanzaoren (*Semen Ziziphi Spinosae*) is one traditional Chinese medicinal (TCM) herb for treating insomnia [1,2]. It has been officially listed in the Chinese Pharmacopoeia (2005) [2]. Suanzaoren contains a series of saponins and flavones. Spinosin (one flavone-C-glycoside in Suanzaoren) is one of the major active components in Suanzaoren. It has been widely reported that it plays an important role in sedation and hypnosis [3–6]. Our previous research also confirmed that spinosin was one therapeutic component. It could inhibit spontaneous motion and extend the sleeping duration of mice induced by pentobarbital sodium (super threshold dose) [7,8]. HPLC method with UV detection for determination of spinosin (from Suanzaoren crude herb or TCM compound preparations containing Suanzaoren, not used as a single component) in biological samples have been found in literatures [9,10]. While, our previous research showed that, as a single component, spinosin was poorly absorbed in rats after oral administration [7]. Detectable spinosin was not found by HPLC-UV method in rat plasma and further pharmacokinetic study could not be performed. The lack of sensitive and selective methods limits *in vivo* studies of spinosin as a single active component.

Herein, a sensitive method for the quantitative determination of spinosin in rat plasma was developed and validated using high-performance liquid chromatographic separation with tandem mass spectrometric detection for the first time. The validated LC/MS/MS method was successfully applied for pharmacokinetic study of single spinosin (20 mg/kg) after oral administration to Wistar rats.

2. Experimental

2.1. Chemicals and reagents

Spinosin was kindly supplied by Shenyang Pharmaceutical University (Shenyang, China). Propranolol (used as an internal standard) was provided by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Their structures were shown in Fig. 1. Acetonitrile of HPLC grade was purchased from Yuwang Chemical Co. (Shandong, China). Distilled water, prepared by a Milli-Q water purification system from Milli-

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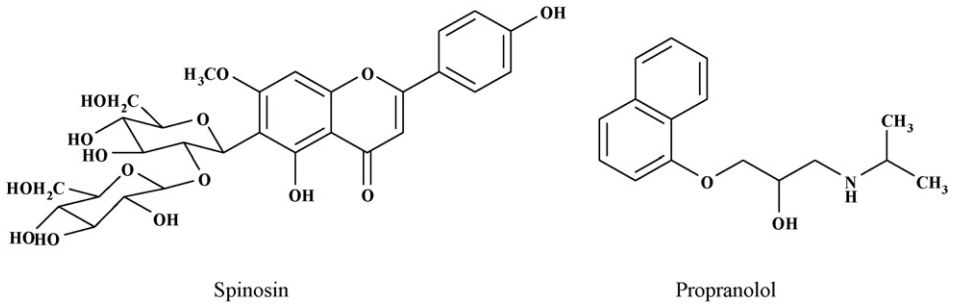

Spinosin Propranolol

Fig. 1. Chemical structures of spinosin and propranolol.

pore (Molsheim, France), was used throughout the study. All other chemicals were of analytical grade.

2.2. Instrumentation

The LC/MS/MS system consisted of a Shimadzu series LC-10AD pump and SIL-HTA autosampler (Kyoto, Japan) and a Thermo Finnigan TSQ Quantum Ultra triple quadrupole mass spectrometer (San Jose, CA, USA) equipped with electrospray ionization (ESI) and atmosphere pressure chemical ionization (APCI) sources. Data acquisition was performed with Xcalibur 1.3 software (Thermo Finnigan, USA). Peak integration and calibration were performed using LCQuan software (Thermo Finnigan, USA). Injection volume was 20 μl.

2.3. LC–MS conditions

Chromatographic separation was achieved on an Agilent Zorbax SB-C_{18} (4.6 mm × 50 mm, 5 μm) using a isocratic mobile phase consisting of acetonitrile–water (30:70, v/v) with 1% isopropyl alcohol and 0.01% heptafluorobutyric acid. The flow rate was 0.2 ml/min. The column temperature was maintained at 25 °C.

The mass spectrometer was operated in the positive ion mode. Quantification was performed using selected reaction monitoring (SRM) of the transitions m/z 609 → m/z 447 for spinosin and m/z 260 → m/z 183 for propranolol, with a scan time of 0.3 s per transition. The tuning MS parameters were optimized for spinosin and propranolol as follows: The spray voltage and the temperature of the heated capillary were 4000 V and 280 °C, respectively. Tube lens offsets for spinosin and propranolol were 177 and 125 V, respectively. Nitrogen was used as the sheath (30 a.u.) and auxiliary (20 a.u.) gas. Argon was used as the collision gas at a pressure of approximately 1.0 mTorr. The optimized collision energies chosen for spinosin and propranolol were both 25 eV.

2.4. Preparation of standard and quality control samples

Standard stock solutions of spinosin and propranolol were prepared individually in acetonitrile at 40.0 and 50.0 μg/ml, respectively. The stock solution of spinosin was serially diluted with acetonitrile to obtain the desired concentrations. The internal standard working solution (50.0 ng/ml) was prepared by diluting the 50.0 μg/ml stock solution of propranolol with acetonitrile. All solutions were stored at 4 °C and brought to 20 °C before use.

Calibration curves were prepared by spiking 50 μl of the appropriate standard solution to 100 μl of blank rat plasma to concentrations of 1.00, 2.00, 4.00, 10.0, 40.0, 100, and 400 ng/ml. The quality control (QC) samples used in the validation and in the plasma level determinations were prepared in the same manner as the calibration standards. The nominal plasma concentrations of QC samples were 2.00, 40.0, and 360 ng/ml. The spiked plasma samples (standards and quality controls) were extracted on each analytical batch along with the unknown samples.

2.5. Sample preparation

To a 100 μl aliquot of rat plasma sample, 50 μl of internal standard (50.0 ng/ml propranolol), 50 μl of acetonitrile, and 100 μl of 1.0% acetic acid solution were added. The samples were briefly mixed and 1 ml of MTBE was added. The mixture was vortex-mixed for 1 min and then shaken on a mechanical shaker for 10 min. After centrifugation at 5000 × g for 5 min, the upper organic layer was removed and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residue was reconstituted in 100 μl of mobile phase, then vortex-mixed. A 20 μl aliquot of the resulting solution was injected into the LC/MS/MS system for analysis. Those plasma samples whose concentrations were greater than the upper limit of quantification (ULOQ) were diluted appropriately with blank plasma to adjust the concentration to within the range of the standard curve before sample preparation, and then reanalyzed.

2.6. Method validation

The method was validated for linearity, lower limit of quantification (LLOQ), accuracy, and precision. Plasma samples were quantified using the ratio of the peak area of spinosin to that of propranolol as the assay response. The peak area ratio (y) and concentration of spinosin (x) were subjected to a weighted ($1/x^2$) least squares linear regression analysis to calculate calibration equation and correlation coefficients. Selectivity was assessed by comparing the chromatograms of six different batches of blank rat plasma with the corresponding spiked plasma. To evaluate linearity, plasma calibration curves were prepared and assayed in duplicate on three separate days. Accuracy and precision were also assessed by analysis of QC samples using six replicate preparations of plasma samples at three different concentrations (2.00, 40.0, and 360 ng/ml) for spinosin on three validation days. Accuracy was expressed by relative error (RE) and precision by relative standard deviation (R.S.D.).

The LLOQ, defined as the lowest concentration at which both precision and accuracy were less than or equal to 20%, was evaluated by analyzing six replicate samples that were prepared by spiking 100 μl of blank rat plasma with 50 μl of the standard solution containing 2.00 ng/ml spinosin.

The extraction recoveries of spinosin and propranolol were calculated by comparing the analytical results of extracted QC samples with samples at the same analyte concentrations obtained by spiking extracted blank rat plasma samples with analyte working standard solutions.

The stability of spinosin in rat plasma was investigated under a variety of storage and process conditions. The storage stability

at −20 °C was evaluated for at 30 days. The freeze–thaw stability of spinosin was assessed by analyzing QC samples at three concentrations (2.00, 40.0, and 360 ng/ml) subject to three freeze (−20 °C)–thaw (20 °C) cycles. The stability of the reconstituted solution was investigated by placing QC samples at three concentrations under ambient conditions for 24 h. The results were compared with results for freshly prepared QC samples, and the percentage concentration deviation was calculated.

2.7. Pharmacokinetic study

Six Wistar rats (200 ± 20 g, three males and three females, Experimental Animal Institute of the Medical Science Academy, Beijing, China) were used in pharmacokinetic studies. The rats were housed under standard conditions and had *ad libitum* access to water and standard laboratory diet throughout the experiments. Two days before the experiment, polyethylene cannulae were implanted in the femoral vein of rats anesthetized with pentobarbital. The cannulae were externalized at the back of the neck and filled with heparinized saline. The rats were orally dosed with spinosin at 20 mg/kg through oral gavages. Serial blood samples (0.3 ml) were collected at 0, 0.5, 1, 2, 5, 6, 8, 10, 12, 15, and 24 h post-dose. Plasma was separated by centrifugation at 5000 × *g* for 5 min and stored at −20 °C until analysis.

3. Results and discussion

3.1. Mass spectrometry

The MS parameters were optimized for spinosin and propranolol. Quantification was performed in the positive ion mode using selected reaction monitoring (SRM) of the transitions *m*/*z* 609 → *m*/*z* 447 for spinosin and *m*/*z* 260 → *m*/*z* 183 for propranolol (Fig. 2).

An LC/MS/MS method for the determination of spinosin and propranolol in rat plasma was developed. Firstly, the possibility of using positive or negative ion detection mode was investigated during the early stage of assay development. Positive ion detection mode offered greater sensitivity for the analytes than negative ion detection mode. Secondly, the influence of ESI or APCI source on the sensitivity of the analytes under positive ion detection mode was evaluated. ESI offered greater sensitivity for the analytes than APCI. Thus, the analytes of interest were determined using an ESI source under positive ion detection mode.

3.2. Chromatography

The mobile phase was chosen with acetonitrile–water in various ratios in order to obtain good responses and separation of spinosin and propranolol. The mobile phase of (30:70, v/v) with 1% isopropyl alcohol and 0.01% heptafluorobutyric acid provided low background noise, rapid separation, and good peak shape. Under the present chromatographic conditions, the run time of each sample was 6 min. The retention times were 3.07 min for spinosin and 3.30 min for propranolol, respectively.

Both protein precipitation and liquid–liquid extraction sample preparation methods were investigated in present study. However, after precipitation of protein using acetonitrile, plasma samples contain endogenous ionic substances that cause strong ion suppression when using LC/MS/MS detection. This problem was overcome by extracting the analytes of interest using a liquid–liquid extraction sample preparation method. Sample acidification was necessary to inhibit ionization and to increase the extraction recovery of spinosin and propranolol, because both analytes of interest are weak acids. After several trials, 1% acetic acid solution was chosen. Ethyl acetate and MTBE were then evaluated as extraction solvents. The recoveries of spinosin and propranolol were both greater (81.8% and 82.1%) when using MTBE as the extraction solvent than when using ethyl acetate (63.3% for spinosin). The R.S.D. was within 15% throughout the entire standard concentration range, demonstrating good consistency for recovery.

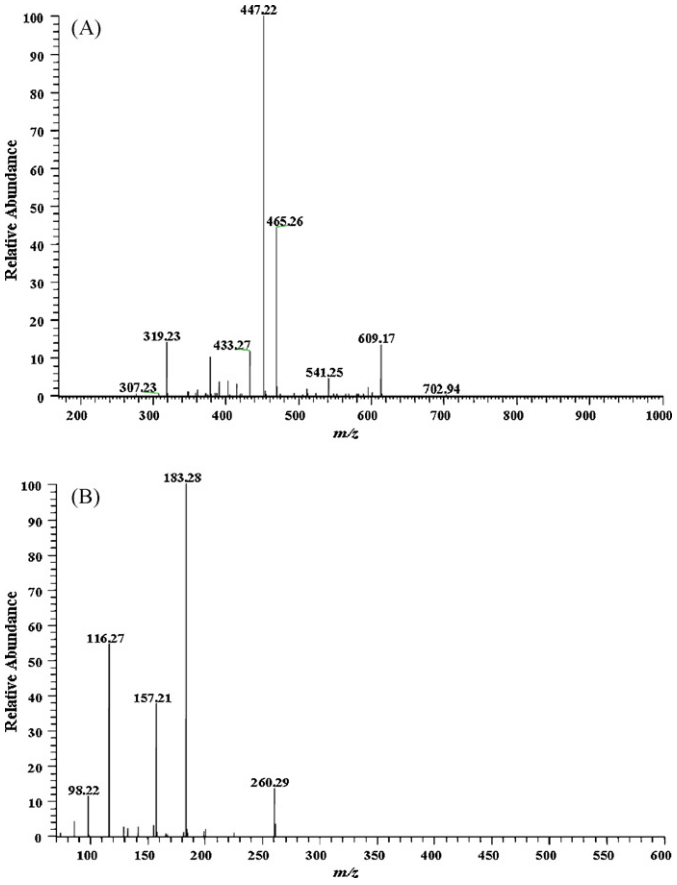

Fig. 2. Full-scan product ion mass spectra of spinosin (A) and propranolol (B).

3.3. Method validation

3.3.1. Selectivity

Selectivity was assessed by comparing the chromatograms of six different batches of blank rat plasma with the corresponding spiked plasma. No significant interference or ion suppression from endogenous substances was observed at the retention times of the analytes, as demonstrated by the typical chromatograms of a blank, a spiked rat plasma sample with spinosin at the LLOQ concentration of 1.00 ng/ml and propranolol of 25.0 ng/ml, and a rat plasma sample collected at 2 h after oral administration of 20 mg/kg spinosin (Fig. 3).

3.3.2. Linearity of calibration curves and lower limits of quantification (LLOQ)

The linear regression of the peak area ratio versus concentration was fitted over the concentration range of 1.00–400.0 ng/ml for spinosin in rat plasma. A typical equation of the calibration curve was as follows: $y = 0.0059x - 0.0004$, $r^2 = 0.9982$, where y represents the ratio of spinosin peak area to that of propranolol and x represents the plasma concentration of spinosin. The present assay offered an LLOQ of 1.00 ng/ml in rat plasma with an accuracy of 14.2% and a precision of 7.6% ($n = 6$).

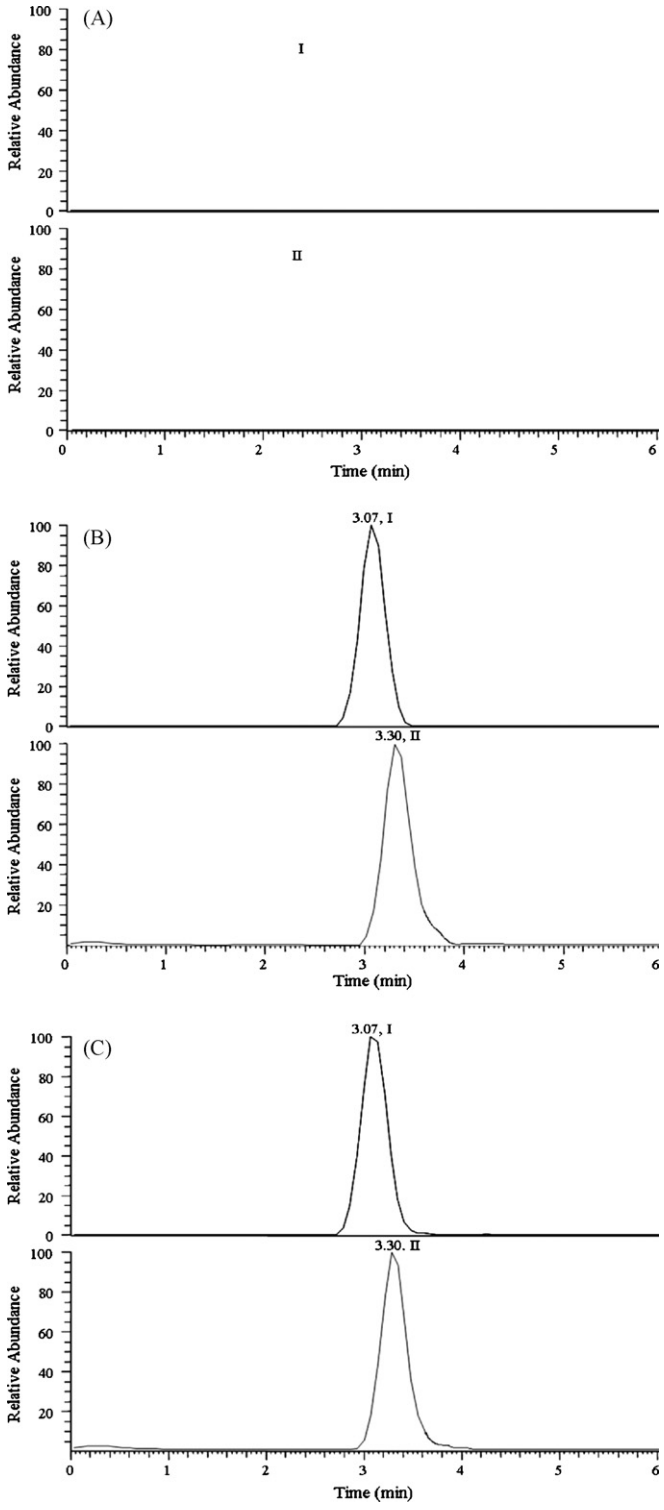

Fig. 3. Representative SRM chromatograms of spinosin (I) and propranolol (II) in rat plasma. (A) Blank rat plasma sample; (B) blank rat plasma sample spiked with spinosin (1.0 ng/ml) and propranolol (25.0 ng/ml); (C) rat plasma sample collected at 2 h after an oral dose of 20 mg/kg spinosin.

3.3.3. Precision and accuracy

In the assay of three QC concentrations of spinosin in rat plasma, the intra-day precision ranged from 2.87% to 10.7% and the inter-day precision ranged from 5.72% to 13.1% (Table 1). The accuracy ranged from −0.31% to 5.50%. The results indicated that the values were within the acceptable range, thus the method is accurate and precise [11].

Table 1
Precision and accuracy of LC/MS/MS method for determining spinosin in rat plasma ($n = 6$)

Concentration (ng/ml)		R.S.D. (%)		RE (%)
Added	Found	Intra-day	Inter-day	
2.00	2.20	10.7	13.1	5.50
40.0	42.3	6.15	8.43	4.75
360.0	357.9	2.87	5.72	−0.31

Table 2
The extraction recoveries of spinosin and propranolol in rat plasma by LC/MS/MS method ($n = 6$)

Compound	Added concentration (ng/ml)	Extract recovery (%)	R.S.D. (%)
Propranolol	25.0	82.1	1.93
Spinosin	2.00	78.9	8.41
	40.0	82.3	4.77
	360	84.1	5.80

3.3.4. Extraction recovery

A simple one-step extraction was introduced to extract analytes from plasma. The acidified low polar organic solvent facilitated good recoveries for spinosin and propranolol. The recovery results were shown in Table 2. Mean extraction recoveries for spinosin at 2.00, 40.0, and 360 ng/ml were 78.9%, 82.3%, and 84.1%, respectively. Mean recovery for the internal standard (50.0 ng/ml) was 82.1% ($n = 6$). The R.S.D. for each concentration was within 15% throughout the entire standard concentration range.

3.3.5. Stability

Results of stability experiments were presented in Table 3. Spinosin in rat plasma was stable for 30 days when stored at −20 °C. The relative error (RE%) of spinosin in rat plasma between the initial concentrations and the concentrations following three freeze–thaw cycles ranged from −9.76% to 10.4%. Processed samples were also stable in the reconstituted solution for at least 24 h at 20 °C.

3.4. Pharmacokinetics of spinosin in rats

The plasma concentration of spinosin at scheduled intervals were determined by above method and the plasma concentration–time curve of spinosin was shown in Fig. 4. The main pharmacokinetic parameters were analyzed using DAS program (Drug and Statistics 2.0, Mathematical Pharmacology Professional Committee of China) and shown in Table 4. T_{max} (the time to reach peak concentration) and C_{max} (the peak concen-

Table 3
Stability of spinosin in rat plasma at different conditions determined by LC/MS/MS method ($n = 6$)

Conditions	Spiked concentration (ng/ml)		
	2.00	40.0	360
Freeze–thaw stability (relative error, %)			
0 cycle	5.72	−9.76	10.4
3 cycles	−4.11	−8.94	1.73
Storage stability at −20 °C (relative error, %)			
0 day	−4.60	−3.24	−4.13
30 days	7.95	0.73	−1.98
Processed plasma samples at 20 °C (relative error, %)			
0 h	3.84	−3.75	−0.77
24 h	1.67	−2.88	1.89

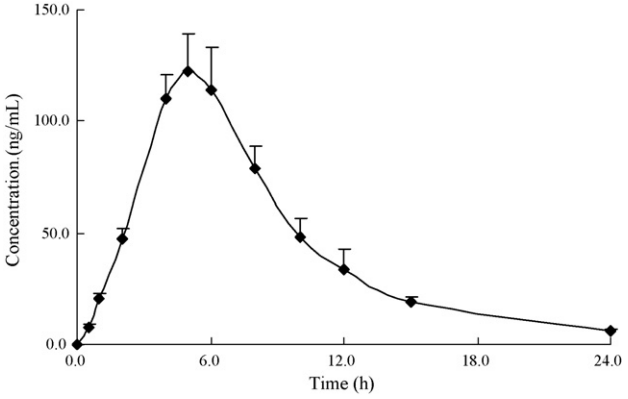

Fig. 4. Mean plasma concentration–time profile of spinosin determined by LC/MS/MS method after oral administration of 20 mg/kg spinosin to rats. Each point represents the mean ± S.D. (n = 6).

Table 4
Pharmacokinetic parameters of spinosin (20 mg/kg) after oral administration to rats (n = 6, mean ± S.D.)

Parameters	Values
K_e (h^{-1})	0.142 ± 0.01
$T_{0.5}$ (h)	4.89 ± 0.37
C_{max} (ng/ml)	132.2 ± 10.6
T_{max} (h)	5.33 ± 0.58
AUC_{0-T} (μg h/l)	1.02 ± 0.09
$AUC_{0-\infty}$ (μg h/l)	1.06 ± 0.09

tration) were 5.33 ± 0.58 h and 132.2 ± 10.6 ng/ml, respectively. $T_{0.5}$ (apparent elimination half-life), k_e (apparent elimination rate constant), AUC_{0-T} (area under concentration–time curve) and $AUC_{0-\infty}$ were 4.89 ± 0.37 h, 0.142 ± 0.01 h^{-1}, 1.02 ± 0.09 μg h/l, and 1.06 ± 0.09 μg h/l, respectively. According to the previous research [7], it was calculated that the absolute bioavailability of spinosin in rat was 2.2%. These parameters indicated that spinosin was cleared slowly from rats.

4. Conclusions

A sensitive LC/MS/MS method with ESI interface was developed and validated for the quantitative determination of spinosin in rat plasma for the first time. The developed LC/MS/MS method for the determination of spinosin in rat plasma offers sufficient selectivity, linearity, accuracy, precision, and recovery. This method has been successfully applied for pharmacokinetic study of spinosin as a single component (20 mg/kg) after oral administration to Wistar rats.

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