Journal of Medicinal Plants Research Vol. 5(16), pp. 3968-3974, 18 August, 2011
Available online at http://www.academicjournals.org/JMPR
ISSN 1996-0875 ©2011 Academic Journals

Full Length Research Paper

Simultaneous quantification of schisandrin and gomisin A in rat plasma by liquid chromatography–mass spectrometry (LC–MS): Application to a pharmacokinetic study

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Accepted 4 July, 2011

A simple, accurate and sensitive liquid chromatography–mass spectrometry (LC–MS) method was established and validated for the simultaneous determination of schisandrin and gomisin A in rat plasma. The analytes of interest were extracted from rat plasma samples by diethyl ether and performed on Hypersil-C_{18} (150 mm × 4.6 mm, 5 μm) with the mobile phase of methanol –water (72:28). The analytes were monitored with positive atmospheric pressure chemical ionization (APCI) by selected ion monitoring (SIM) mode. The target ions were m/z 415.00 for schisandrin, m/z 399.00 for gomisin A and m/z 284.90 for internal standard (IS) diazepam. The calibration curves were linear over the range 5.0 to 2000 ng·mL^{-1} for schisandrin and 5.0 to 250 ng·mL^{-1} for gomisin A. The lower limit of quantifications (LLOQs) for the two lignans were 5.0 ng·mL^{-1}. The validated method was successfully applied to the pharmacokinetic study of schisandrin and gomisin A in rat plasma after oral administration of Zaoren-an-shen granule.

Key words: Column liquid chromatography-mass spectrometry, pharmacokinetic study, schisandrin and gomisin A, Zaoren-an-shen granule.

INTRODUCTION

Zaoren-an-shen granule is a traditional Chinese medicine (TCM) formulation recorded in the ministerial standards of Ministry of Health (Chinese Pharmacopoeia Commission, 1998), which has been clinically used for the treatment of agrypnia, morbid forgetfulness and cardiopalmus caused by nervous exhaustion. The formula, on which the preparation is based, is composed of three Chinese medicinal herbs, Suanzaoren (Semen Ziziphi Spinosae), Danshen (Radix et Rhizoma Salviae Miltiorrhizae) and Wuweizi (Fructus Schisandrae Chinensis) which are all listed in the Chinese pharmacopoeia and have been commonly used for treating insomnia diseases for a long time (Peng et al., 2000; Fang et al., 2010; Huang et al., 2007). Our recent studies (Zhang et al., 2010) showed that schisandrin and gomisin A were selected as therapeutic components in Zaoren-an-shen granule. Pharmacokinetic studies of active ingredients in Chinese herbs are useful to explain and predict a variety of events related to the efficacy and toxicity. Schisandrin and gomisin A, therapeutic components in Zaoren-an-shen granule, have not been reported in pharmacokinetic studies because of the low concentration of gomisin A in plasma. While the high performance liquid chromatography coupled with mass spectrometry (LC–MS) is widely used in recent years, the studies of simultaneous analysis of schisandrin and gomisin A *in vivo* become possible (Wei et al., 2010). In this paper, an LC–APCI-MS method was developed and validated for determination of the concentration of schisandrin and

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gomisin A in rat plasma and for pharmacokinetic studies of the compounds after oral administration of Zaoren-an-shen granule.

MATERIALS AND METHODS

Materials, chemicals and reagents

Schisandrin and gomisin A (Figure 1a, b) were supplied by Shenyang Pharmaceutical University. Diazepam (IS) was purchased from the National Institute. The purity of the compounds was above 98%. Methanol (HPLC grade) was obtained from Tedia Company (Tedia Fairfield, OH, USA). All other reagents were of analytical grade. Distilled water was purified by a Millipore system Milli Q (Molsheim, France).

Animals

Male wistar rats (190 to 250 g) were provided by the Experimental Animal Center of Shenyang Pharmaceutical University, Shenyang, China. They were housed to a cage with a standard laboratory food and water ad libitum for 3 days before the start of the experiments. Animal study was carried out in accordance with the guidelines for animal experimentation of Shenyang Pharmaceutical University and the protocol was approved by the animal ethics committee of the institution.

LC-MS instruments and analytical conditions

LC analytical procedures were performed using a system consisted of a DGU-14AM degasser, two Shimadzu 10ADvp Pumps, a high pressure mixer, an automatic thermostatic column compartment, a Shimadzu 10ATvp Auto-injector (Shimadzu, Kyoto, Japan) and a quadruple mass spectrometer with an atmospheric pressure chemical lionization (LC/MS-2010). Data acquisition and processing software LC–MS solution version3.0 was from Shimadzu.

Chromatographic separations were carried out on Hypersil-C_{18} (150 × 4.6 mm id，5 μm particle size), preceded by a C_{18} guard column (12.5× 4.6 mm id) from Zhonghuida Co. (Dalian, China) at 25ºC. The mobile phase was a methanol–water (72:28, v/v) at a flow rate of 0.8 ml·min^{-1}. The injection volume was 20 μL.

The analytes and IS were ionized by APCI source in positive ion mode under the following source conditions: capillary voltage at 1.7 kV, nebulizing gas, 2.5L·min^{-1}; drying gas, 2.0L·min^{-1}; CDL temperature, 250ºC; heat block temperature, 200ºC; interface temperature, 400ºC; the other parameters were fixed as the tuning file. Analysis was carried out by SIM for schisandrin $[M+H-H_2O]^+$ m/z 415.00, gomisin A $[M+H-H_2O]^+$ m/z 399.00 and IS $[M+H]^+$ m/z 284.90.

Preparation of Zaoren-an-shen granule

Amounts of crude drugs (Suanzaoren 250 g, Danshen 50 g and Wuweizi 50 g) equivalent to the dose of Zaoren-an-shen granules were mixed. A 10-fold mass of water was added and the mixtures were boiled on an electric heater for two h. The extraction was repeated twice; then, the extracted solutions were combined together and concentrated to get the Zaoren-an-shen granule with the concentration of schisandrin and gomisin A of 2.0 and 0.38 mg·mL^{-1}, respectively.

Preparation of standard and quality control samples

A stock solution containing schisandrin and gomisin A was prepared in methanol at the concentration of 20 and 2.0 μg·mL^{-1}, respectively. Internal standard diazepam was prepared in methanol at a concentration of 1000 ng·mL^{-1}. All stock solutions were kept at 4℃ before use. Calibration curve samples (5.0, 10, 25, 100, 500, 1000, 2000 ng·mL^{-1}) for schisandrin and (5.0, 10, 25, 40, 80, 160, 250 ng·mL^{-1}) for gomisin A were prepared by spiking control rat plasma with appropriate amounts of the standard solution. Quality control (QC) solutions of schisandrin (12.5, 250 and 1600 ng·mL^{-1}) and gomisin A (12.5, 50, and 160 ng·mL^{-1}) were independently prepared. All samples were stored at -20ºC until analyzed.

Plasma sample preparation

The internal standard diazepam 50 μL, diethyl ether 3mL and methanol 50 μL (volume of the corresponding standard solution for calibration curve and QC samples) were added to 200 μL of plasma sample. The mixture was vortex-mixed for 1min and centrifuged at 4,000 rpm for 5 min. The organic layer was transferred to another clean test tube and evaporated to dryness in a water bath at 40ºC under a stream of nitrogen. The residue was reconstituted in 100 μL mobile phase, with vortex mixing for 1 min and then the centrifugation procedure was repeated. A 20μL aliquot was injected into the LC–MS system for analysis.

Method validation

The method validation was conducted by the guidance for industry bioanalytical method validation. The specificity of the method was evaluated by analyzing blank rat plasma extracts and extracts spiked with schisandrin, gomisin A and IS. The working solutions of schisandrin, gomisin A were prepared together in methanol. A series of standard solution were added to 200 μL blank plasma to yield final concentrations of 5.0, 10, 25, 100, 500, 1000 and 2000 ng·mL^{-1} for schisandrin, and 5.0, 10, 25, 40, 80, 160 and 250 ng·mL^{-1} for gomisin A. The samples were dealt with the method “Plasma sample preparation” and injected into HPLC. Daily calibration curves were performed by least-squares linear regression of the peak area ratios of the two drugs to the IS versus the nominal standard concentration with a weighted (1/square of concentration) factor. The unknown sample concentrations were calculated from the calibration curve. QC samples at three concentrations were analyzed to validate the accuracy and precision of the described method. Six replicates were analyzed in each of three analytical runs. The precision was evaluated by the relative standard deviation (RSD), while the accuracy was expressed as the relative error (R.E.). Recovery of internal standard diazepam, schisandrin and gomisin A was determined by comparison of the response of the analytes extracted from replicating QC samples (n=18) with the response of the analytes from non-extracted standard solutions at the same concentration. Stability of schisandrin and gomisin A in rat plasma was studied under different storage conditions: the stability of processing (3 freeze-thaw cycles), sample storage (at room temperature for 4 h, at –20ºC for 14 days) and post-treatment (in the reconstituted extract at 4ºC for 8 h).

Pharmacokinetic study

All the rats were deprived of food but free access to water for 12h before and during the experiment. After i.g.administration of 20 mL·kg^{-1} of Zaoren-an-shen granule (containing 2.0 mg·mL^{-1} schisandrin and 0.38 mg·mL^{-1} gomisin A) to each rat. Blood samples (0.5 mL) were collected in heparinized tubes from the ophthalmic vein at 0, 0.5, 1, 3, 5, 6, 7, 8, 9, 10, 12, 14, 16 and 24 h after drug

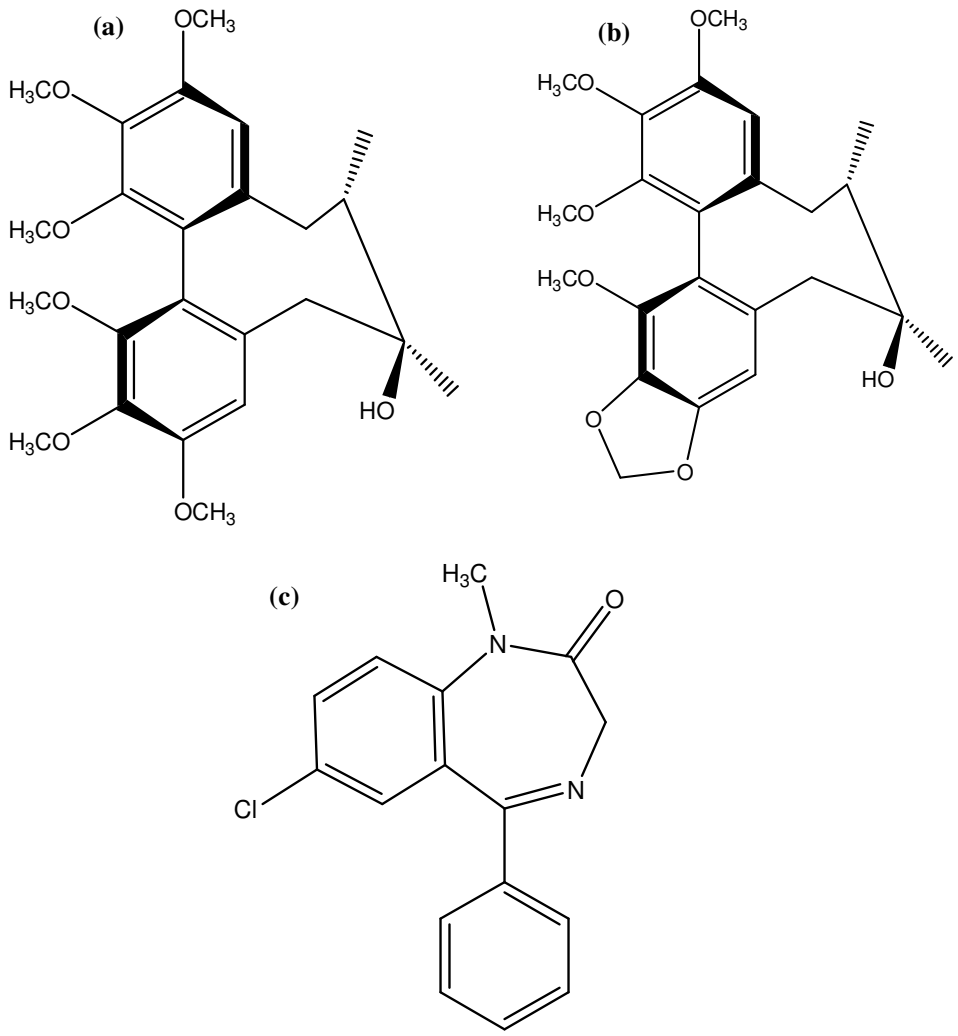

Figure 1. Chemical structures of schisandrin (a) gomisin A (b) and IS (c).

given, then separated by centrifugation at 15,000×g for 5 min, finally stored at -20°C until analysis.

The plasma concentrations of the analytes at different times were expressed as mean±SD and the mean concentration-time curves were plotted, respectively. All the pharmacokinetic` parameters were determined by non compartmental analysis using DAS2.0 pharmacokinetic program (Chinese Pharmacological Society).

RESULTS AND DISCUSSION

Mass spectra analysis

The high specificity and sensitivity of the established LC–APCI–MS method has made it possible to simultaneously determine the two active lignanoids in rat plasma. The lignanoids and IS were analyzed by MS in APCI positive ion mode. The full scan mass spectrum of schisandrin, gomisin A and IS after direct injection in mobile phase were shown in Figure 2.

The most sensitive ions were $[M+H-H_2O]^+$ for the lignanoids and $[M+H]^+$ for IS, so the quantitative analysis was carried out by SIM for schisandrin at m/z 415.00, gomisin A at m/z 399.00 and IS at m/z 284.90. The analytes were identified by comparing their retention times and mass spectra with the standards. Typical chromatograms of rat plasma samples showed well-shaped and baseline separated peaks, without any interference (Figure 3).

ESI interface was also tested, but the sensitivity was lower and some endogenous substances interfered with schisandrin.

Specificity

The chromatograms for blank plasma, blank plasma spiked with schisandrin , gomisin A and IS, as well as the rat plasma samples 1h after administration of Zaoren-an-shen granule were presented in Figure 3 and

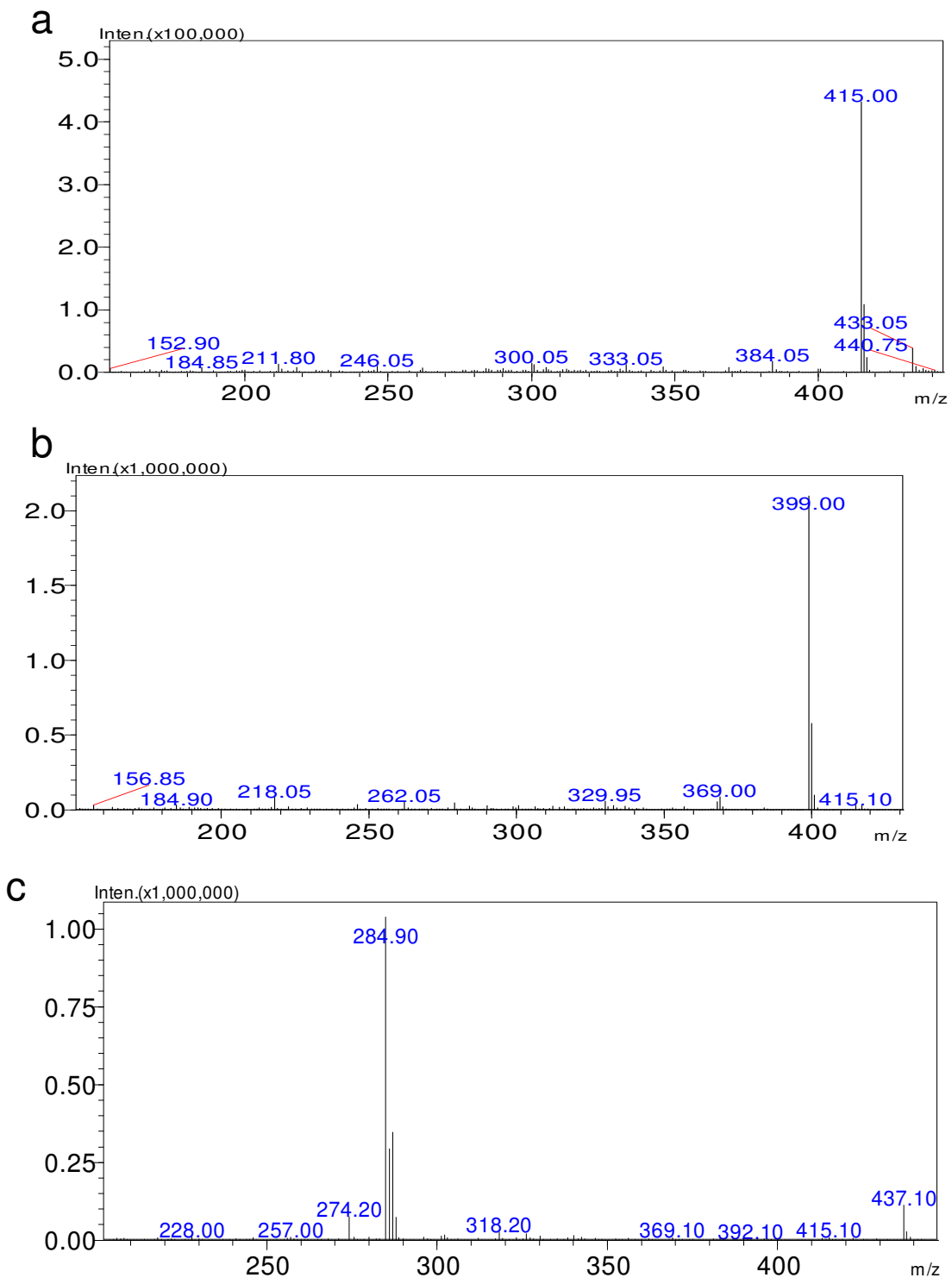

Figure 2. Positive scan mass spectra of schisandrin (a) gomisin A (b) and IS (c).

no interference was observed at the retention time of the analyte 6.5 , 9.6 and IS 7.7 min due to endogenous substances in drug-free rat plasma. The method was specific for schisandrin and gomisin A.

Calibration and validation

The representative regression equations of the calibration curve were as follows: $Y = 3.777 \times 10^{-3} x - 2.642 \times 10^{-3}$

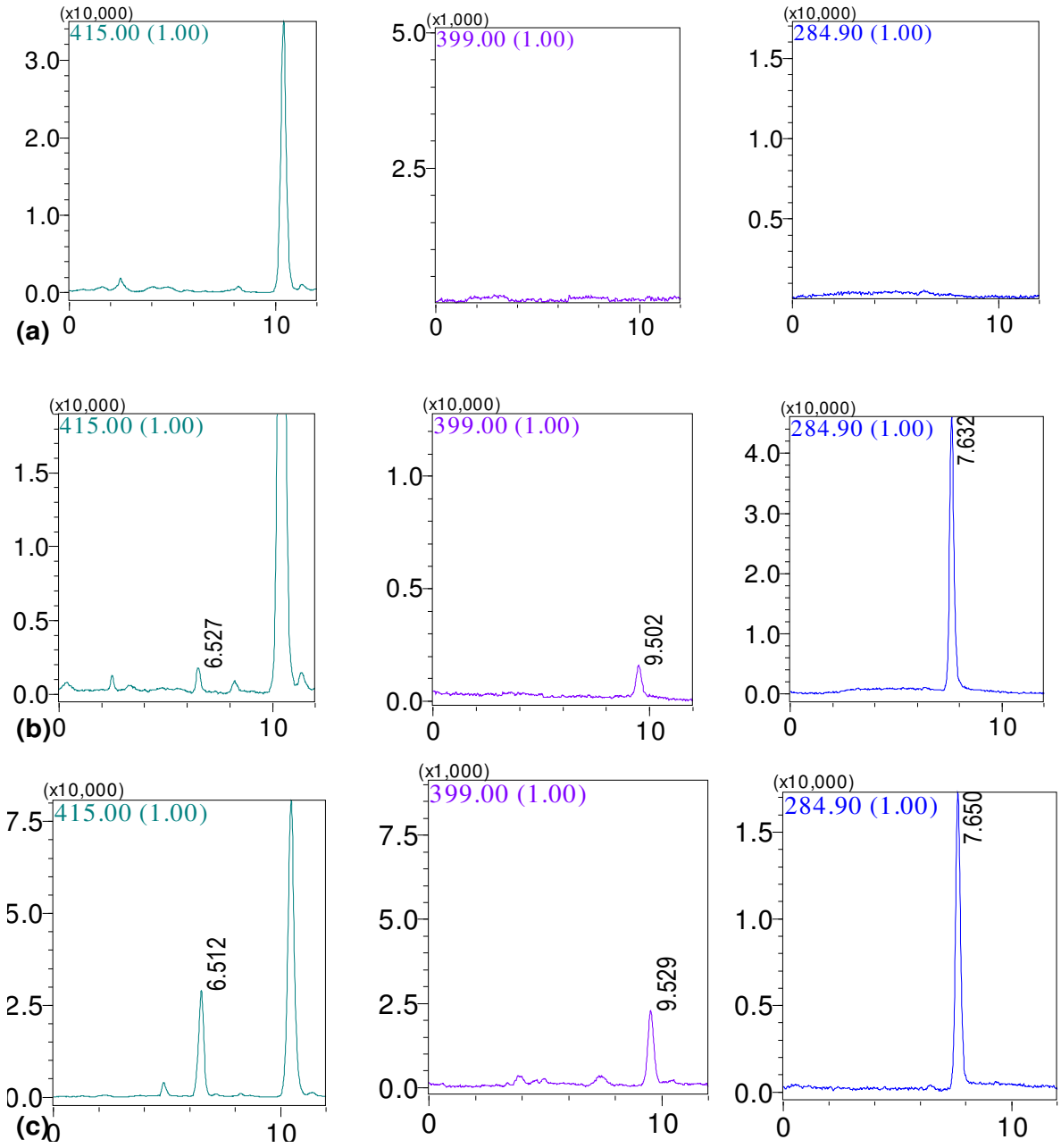

Figure 3. SIM chromatograms of blank rat plasma (a), blank rat plasma spiked with schisandrin, gomisin A (5 ng/mL, LLOQ) and IS (b), A rat plasma sample 1 h after oral administration of Zaoren-an-shen granule sample (c), schisandrin: RT=6.5 min, gomisin A: RT=9.5 min, IS: RT=7.7 min.

(R^2= 0.994) for schisandrin, Y = 4.142× 10^{-3} x – 2.952 × 10^{-3} (R^2 = 0.991)for gomisin A. The linear ranges in rat plasma were from 5.0 to 2000 ng·mL^{-1} for schisandrin and from 5.0 to 250 ng·mL^{-1} for gomisin A. The LLOQs of schisandrin and gomisin A were both 5.0 ng·mL^{-1} in plasma. The $1/x^2$ weighting yielded better accuracy at lower standard levels. The intra and inter-day precision and accuracy data were shown in Table 1 and met the requirements of biological analysis.

The recoveries of schisandrin and gomisin A at three concentration levels were 77.32 to 91.5 and 78.80 to 88.4%, respectively (Table 1). The mean extraction recovery of internal standard diazepam was 88.6%.

The stability study showed that the two active lignanoids were stable within three freeze–thaw cycles and showed no significant degradation for sample storage (at room temperature for 4 h, at –20ºC for 14 days,) and post-treatment (in the reconstituted extract at 4ºC for 8 h)

Table 1. Precision and accuracy for analysis of schisandrin and gomisin A in rat plasma (n=3 day, six replicates per day).

	Concentration spiked (n g·mL^{-1})	Inter-day R.S.D. (%)	Intra-day R.S.D. (%)	Accuracy R.E. (%)	Recovery (%)
Schisandrin	12.5	5.2	6.3	3.6	77.3
	250	2.7	3.1	-2.4	91.5
	1600	3.3	3.4	-0.5	87.2
Gomisin A	12.5	4.9	0.9	4.4	78.8
	50	2.9	3.6	-0.6	88.4
	200	3.8	3.1	-1.4	84.7

Table 2. Stability for analysis of schisandrin and gomisin A in rat plasma (n=3).

	Concentration spiked (n g·mL^{-1})	Three freeze-thaw cycles, RE (%)	8 h in auto sampler condition, RE (%)	4h at ambient temperature, RE (%)	14 days at –20ºC , RE (%)
Schisandrin	12.5	0.1	0.3	8.0	6.9
	250	- 5.0	-6.3	-1.0	-4.8
	1600	0.2	-2.5	-0.2	3.2
Gomisin A	12.5	5.1	0.3	5.6	4.3
	50	-10.0	-4.0	-0.5	-8.7
	200	0.25	-0.5	-2.5	1.2

(Table 2). The results showed that the procedures described above were satisfactory with respect to accuracy, precision and stability.

Application of the method in pharmacokinetic studies

The developed and validated LC–MS method for the analysis of two lignans in rats in support of *in vivo* studies was successfully demonstrated. The pharmacokinetic parameters are listed in Table 3. After a single oral administration of the Zaoren-an-shen granule at a dose of 20 mL·kg^{-1} (containing 20 mg·mL^{-1} of schisandrin and 0.38 mg·mL^{-1} of gomisin A) in rats, the concentrations of schisandrin and gomisin A in plasma increased quickly during the 0.5 h which suggested the entrance of the two compounds into plasma was fast (Wang et al., 2008). In addition, a double-peak profile occurred in gomisin A, the reason may be hepatoenteral circulation, transformation from other lignans or other reasons.

Conclusion

A simple, sensitive and accurate analytical LC–MS method was developed for simultaneous determination of the schisandrin and gomisin A from Zaoren-an-shen granule in rat plasma and it was successfully applied to a pharmacokinetics study in rats after oral administration of Zaoren-an-shen granule. With opportunely modification, this LC–MS method could also be developed and validated for analysis of schisandrin and gomisin A in other biological matrixes, for example serum, urine and tissue homogenates. This is the first report on simultaneous quantitation of the two lignanoids compounds in Zaoren-an-shen granule in biological samples. The pharmacokinetic results could be useful for further studies of the clinical

Table 3. Pharmacokinetic parameters of schisandrin and gomisin A in rats after oral administration of Zaoren-an-shen granule.

Parameter	Schisandrin (mean ± SD)	Gomisin A (mean ± SD)
$T_{1/2}$ (h)	3.1 ± 0.7	3.0 ± 0.9
T_{max} (h)	7.2 ± 0.4	7.6 ± 0.5
C_{max} (ng/mL)	778.9 ± 271.0	83.3± 38.9
AUC_{0-t} (ng·h/mL)	5673 ±1153	338.5 ± 72.3
$AUC_{0-\infty}$ (ng·h/mL)	5851 ± 1288	409.8 ± 46.1

applications of Zaoren-an-shen granule.

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