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Quantitative determination of atractylenolide III in rat plasma by liquid chromatography electrospray ionization mass spectrometry

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Abstract

Atractylenolide III is a major active component in *Atractylodes macrocephala*. This paper describes a simple, rapid, specific and sensitive method for the quantification of atractylenolide III in rat plasma using a liquid–liquid extraction procedure followed by liquid chromatography mass spectrometric (LC-MS) analysis. A Kromasil 3.5 μm C_{18} column (150 mm × 2.00 mm) was used as the analytical column. Linear detection responses were obtained for atractylenolide III concentration ranging from 5 to 500 ng mL^{-1}. The precision and accuracy data, based on intra-day and inter-day variations over 5 days were within 10.29%. The lower limit of quantitation for atractylenolide III was 5 ng mL^{-1}, using 0.1 mL plasma for extraction and its recoveries were greater than 85% at the low, medium and high concentrations. The method has been successfully applied to a pharmacokinetic study in rats after an oral administration of atractylenolide III with a dose of 20.0 mg kg^{-1}. With the lower limits of quantification at 5 ng mL^{-1} for atractylenolide III, this method was proved to be sensitive enough for the pharmacokinetics study of atractylenolide III.

Keywords: Atractylenolide III; LC-MS; Pharmacokinetics

1. Introduction

Atractylenolide III (molecular structure seen in Fig. 1a), a marker substance in *Atractylodes macrocephala*, possesses well-documented anti-inflammatory [1] and anticancer activity [2]. *A. macrocephala* is the dried root of compositae plant *A. macrocephala Koidz*, which has been widely used in China as a herbal medicine, and reported as a nutrient for energy and for the treatment of dyspepsia and anorexia [3]. *A. macrocephala* was also contained in many known Chinese compounds such as Danggui-Shaoyao-San (DSS) which has attracted much attention from the researchers in China and worldwide, due to its potential therapeutic efficacy for senile dementia [4–6]. In the procedures of screening and locating the effective compounds contained in DSS, Gu W and his colleagues found that the volatile oil fraction extracted from DSS contributed most to the prevention and treatment of senile dementia [7]. Atractylenolide III was later found to be one of the main constituents present in the effective volatile oil fraction, which indicates that atractylenolide III may be a potential effective compound of treating senile dementia. Related pharmacological research has now being undertaken by our co-workers.

As an important part in the procedures of new drug development and research, pharmacokinetics screening and evaluation entering into the early stage of new drug development has received more and more attention from the companies and researchers related. Developing and validating a satisfactory bioanalytical method are a preliminary step for the followed pharmacokinetics studies. The main purpose of this study was, thus, to develop and validate a simple, rapid, sensitive and reproducible quantification method for the followed pharmacokinetics evaluation of atractylenolide III.

There are many analytical methods including micellar electrokinetic chromatography (MEKC) [8] and HPLC [9–12] been reported for determining atractylenolide III contained in the plants or medical preparations. However, no bioanalyitical method is developed for atractylenolide III quantification up to now. Since, the plasma or other biosamples' concentrations were always much lower than that in the plants or pharmaceutical preparations, all of the previously developed methods with

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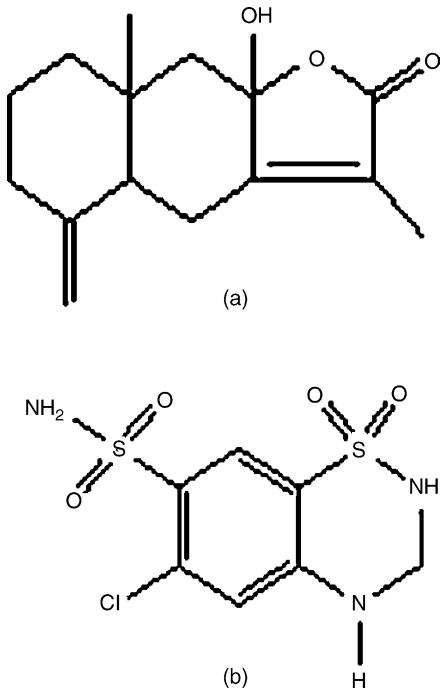

Fig. 1. Chemical structures of (a) atractylenolide III and (b) hydrochlorothiazide.

the best sensitivity (LLOQ) at 20.5 μg mL^{-1}, cannot be used in pharmacokinetics study of atractylenolide III.

Here, we report a simple and sensitive HPLC-MS method to determine the concentration of atractylenolide III in rat plasma and apply it to the subsequent pharmacokinetic study. This method was fully validated for its specificity, accuracy, precision, and sensitivity, and was successfully applied to the pharmacokinetic study of atractylenolide III in rats.

2. Experimental

2.1. Chemicals and reagents

Atractylenolide III (>99% purity) was kindly provided by Department of Chinese medicinal prescription of China pharmaceutical university. Hydrochlorothiazide was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Purified water was obtained from a Milli-Q (Millipore, Milford, MA, USA) water purification system. HPLC grade of methanol was supplied from Merck (Darmstadt, Germany). All other chemicals were of analytical reagent grade.

2.2. Instruments and analytical conditions

The HPLC system consisted of a LC-10AD pump, a Shimadzu 10ATvp autosampler, and a CTO-10 Avp column oven (Shimadzu, Kyoto, Japan). A Shimadzu 2010 liquid chromatograph-mass spectrometer (Shimadzu, Kyoto, Japan) equipped with an ESI (Electrospray Ionization) probe and a QoQ system (Q-array-Octapole-Quadrupole mass analyzer) was used in this study. Separation was carried out by a Kromasil 3.5 μm C_{18} column (150 mm × 2.00 mm) protected by a C_{18} guard column maintained at 40 °C. A mobile phase composed of water and methanol (15:85, v/v) was used throughout the analysis at a flow rate of 0.2 mL min^{-1}.

2.3. Mass spectrometric conditions

All measurements were carried out with the mass spectrometer operated under the negative ESI mode. Mass spectrometer conditions were optimized to obtain maximal sensitivity. The curve dissolution line (CDL) temperature was maintained at 250 °C and the block temperature was at 200 °C. The detector voltage 1.65 kV and probe voltage 4.5 kV were fixed as in tuning method. Mass Vacuum was obtained by Turbo molecular pump (Edwards 28, England). Liquid nitrogen (99.995%, from Gas Supplier Center of Nanjing University, China) was used as the nebulizer gas (1.5 L min^{-1}) and curtain gas (2.0 L min^{-1}) source. LC-MS Solution Version 2.04 working on Windows 2000 operating system was used for data processing. All analytes were assayed by quantifying the $[M-H]^-$ ions, with atractylenolide IIIdetected at *m*/*z* 247 and internal standard (molecular structure seen in Fig. 1b) at *m*/*z* 296.

2.4. Preparation of calibration samples and quality control samples.

The standard stock solution of 2.0 mg mL^{-1} of atractylenolide III was prepared in purified deionised water. A series of standard working solutions at 50, 100, 250, 500, 1000, 2500 and 5000 ng mL^{-1} was obtained by further dilution of the standard stock solution with methanol. The internal standard stock solution of 1.0 mg mL^{-1} of hydrochlorothiazide was prepared in methanol. Internal standard working solution (5.0 μg mL^{-1}) was prepared by diluting the internal standard stock solution with methanol. All solutions were stored at 4 °C. To prepare the calibration samples, 10 μL of working solutions were diluted each day with 100 μL blank plasma to span a calibration standard range of 5–500 ng mL^{-1} (5, 10, 25, 50, 100, 250, 500 ng mL^{-1}). The final concentration of the internal standard was 500 ng mL^{-1} in all samples. Quality control (QC) samples (5, 50, 500 ng mL^{-1}) were prepared in the same way and were stored at −20 °C until analysis.

2.5. Sample preparation

Each collected blood sample was immediately centrifuged at 800 × *g* for 5 min and plasma was transferred into a clean Eppendorf tube. The plasma samples were stored at −20 °C until analysis. Aliquots (100 μL) of plasma were spiked with internal standard working solution (10 μL), vortex-mixed (Scientific industries Inc., USA) for 30 s and extracted with ethylacetate (1 mL) using a vortex mixer for 3 min. Then the tubes were centrifuged at 10,000 × *g* for 10 min (Micromax RF, Thermo Electron Corporation, USA). The upper organic phase (900 μL) was transferred into a clean tube and evaporated to dry using the Thermo Savant SPD 2010 SpeedVac System (Thermo Elec-

tron Corporation, USA). The residue was then reconstituted in 100 μL methanol immediately before LC/MS analysis and 10 μL was injected.

2.6. Method validation

2.6.1. Linearity

Linearity of calibration was tested and assayed in consecutive 5 days. Calibration curves in the concentration range of 5–500 ng mL^{-1} for atractylenolide III were constructed by plotting the peak-area ratios of analyte/internal standard to the spiked concentrations. The linearity was determined from linear regression analysis on the calibration curves.

2.6.2. Recovery

To determine the recovery of atractylenolide III by the liquid–liquid extraction method, plasma samples were spiked with atractylenolide III at concentrations of 5, 50 and 500 ng mL^{-1}. The resulting peak-area ratios (analyte:internal standard) were compared with that of the standards prepared in mobile phase to provide the recovery values.

2.6.3. Precision and accuracy

Intra-day accuracy and precision (each, $n = 5$) were evaluated by analysis of QC samples at different times during the same day. Inter-day accuracy and precision were determined by repeated analysis of QC samples over 5 consecutive days ($n = 1$ series per day). The concentration of each sample was determined using calibration standards prepared on the same day.

2.6.4. Stability

The effects of three freeze–thaw cycles and at room temperature in plasma for 4 h on the compound stability were evaluated by repeated analysis ($n = 3$) of QC samples. Long-term stability in plasma was also tested by assaying frozen QC samples after storage at −20 °C for 6 months. The amount of atractylenolide III in these plasma samples were determined using a newly prepared calibration curve. Stability was expressed as a percentage of nominal concentration.

2.7. Application

Sprague-Dawley rats (210–240 g) were obtained from experiment animal breeding center of China Pharmaceutical University and housed six to a cage with unlimited access to food and water except for 12 h before and during the experiment. The experimental protocol was approved by the animal care committee of China Pharmaceutical University. Each rat was given atractylenolide III at a single dose of 20.0 mg kg^{-1} by oral administration. Blood samples (about 250 μL) were collected in heparinized 1.5 mL polythene tubes immediately before and 0.5, 1, 1.5, 2, 4, 8, 12 and 24 h after dosing, and were at once centrifuged at 800 × *g* for 10 min at 4 °C. A 100-μL volume of plasma was finally obtained, and stored at −20 °C until analysis.

3. Results and discussion

3.1. Chromatography and mass spectrum

The mass spectra of atractylenolide III and internal standard (IS) were shown in Fig. 2a and b, respectively. It is clear that the analyte and IS both formed predominantly deprotonated molecules $[M-H]^-$ in the full scan spectra, with *m*/*z* at 247 for atractylenolide III and 296 for IS. As shown in Fig. 3, the retention times of atractylenolide III and hydrochlorothiazide (IS) were approximately 3.3 and 1.9 min, respectively. There were no endogenous plasma components interfering with them. The overall chromatographic run time was finished within 5 min.

3.2. Linearity

The linear regression analysis of atractylenolide III was constructed by plotting the peak-area ratio of atractylenolide III to the internal standard (*y*) versus analyte concentration (ng mL^{-1}) in spiked plasma samples (*x*). The calibration curves were constructed in the range 5–500 ng mL^{-1} for atractylenolide III. The regression equation of the curves and the correlation coefficients (*r*) were calculated as follows: $y = 0.0023x - 0.0016$ ($r = 0.9991$). It showed good liner relationships between the peak areas and the concentrations.

3.3. Recovery and matrix effect

The recovery was assessed by comparing the peak-area ratios (analyte:internal standard) obtained from spiked plasma samples of different analyte concentrations to the peak-area ratios for the samples containing the equivalent amounts of the analyte and internal standard directly dissolved in mobile phase. The recoveries of atractylenolide III from rat plasma were

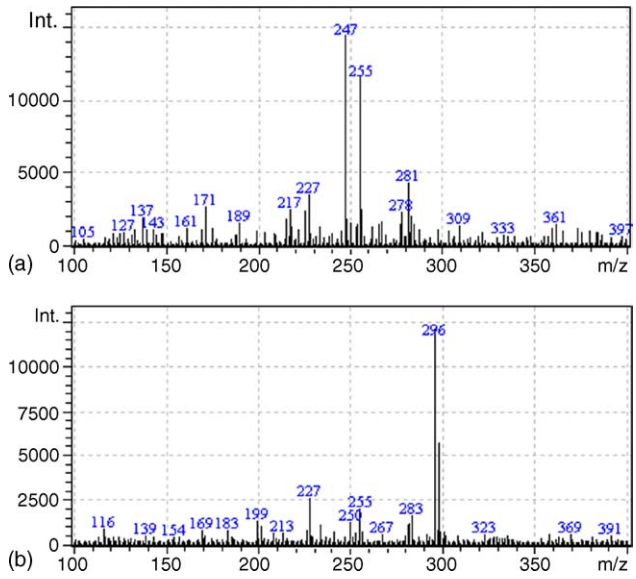

Fig. 2. Mass spectra of (a) atractylenolide III and (b) hydrochlorothiazide (internal standard).

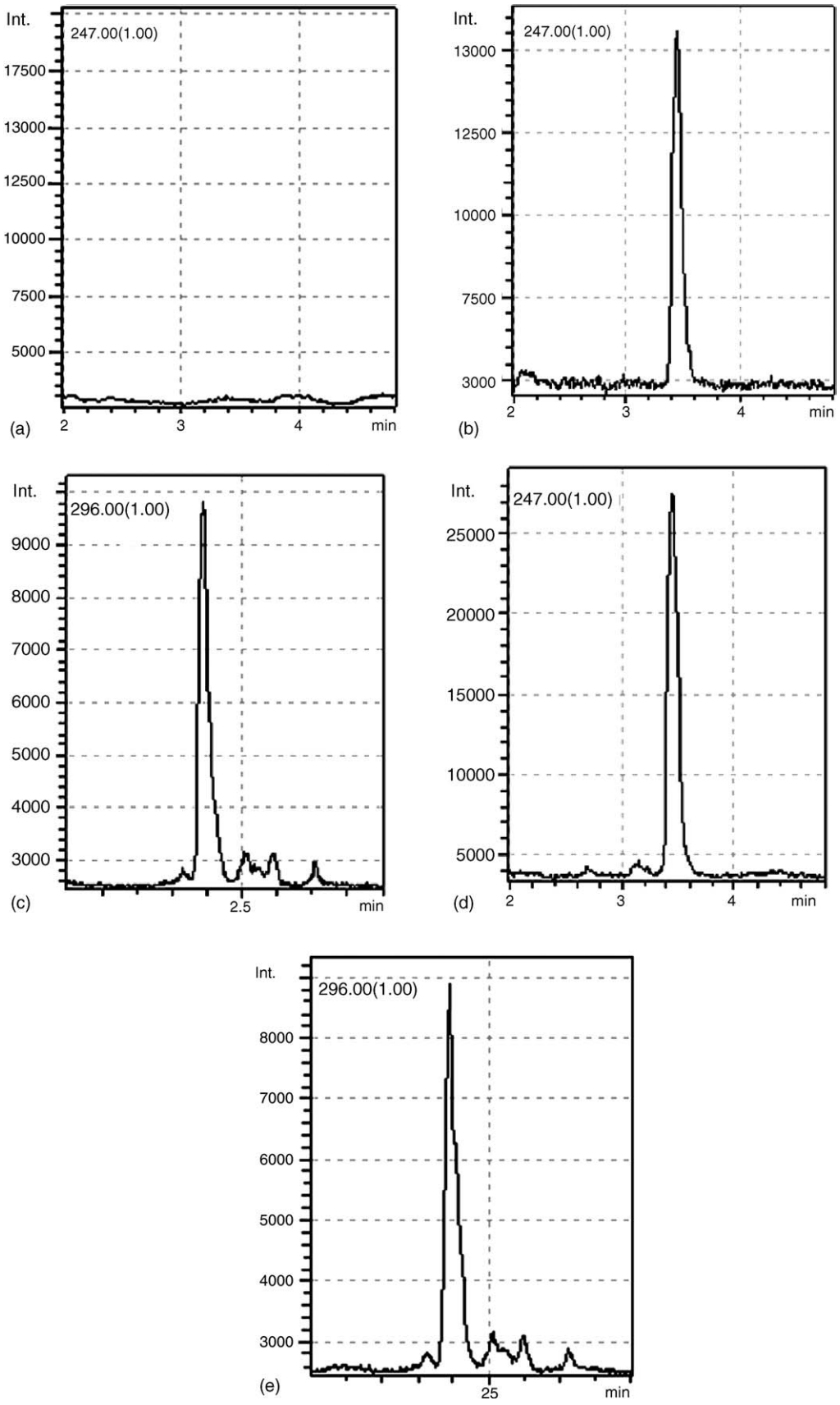

Fig. 3. Chromatograms of atractylenolide III in rat plasma: (a) blank rat plasma; (b) blank plasma spiked with 500 ng mL^{-1} atractylenolide III, (c) 5000 ng mL^{-1} IS and monitored at *m/z* 247.00 and 296.00, respectively; (d) a rat plasma sample 0.5 h after oral administration of 20.0 mg kg^{-1} atractylenolide III, (e) 5000 ng mL^{-1} internal standard and monitored at *m/z* 247.00 and 296.00, respectively.

Table 1
Recovery of atractylenolide III from rat plasma ($n = 5$)

	Spiked concentration ($ng\,mL^{-1}$)	Measured concentration ($ng\,mL^{-1}$)	Recovery (%)
Atractylenolide III	5	4.18 ± 0.13	85.56 ± 2.57
	50	43.05 ± 1.00	86.10 ± 2.00
	500	425.98 ± 13.37	85.20 ± 2.67

shown in Table 1. The mean recoveries of atractylenolide III were more than 85% ($n = 5$). The possibility of a matrix effect caused by ionization competition occurred between the analytes and the endogenous co-eluents was evaluated at three concentrations. Results from comparing the peak responses of the post-extraction spiked samples with that of the pure standards prepared in methanol suggested negligible matrix effect occurred in this method.

3.4. Accuracy and reproducibility

Analytical accuracy and precision data were shown in Table 2. The intra-day precision shown a relative standard deviation (R.S.D.%) was less than 10%. The inter-day precision was evaluated at the above concentration levels for 5 days. The inter-day R.S.D.% was also less than 15%. The data proved good precision and accuracy of the developed HPLC-MS method.

3.5. Stability

All stability results are shown in Table 3. Atractylenolide III was stable for at least 4 h at room temperature in plasma samples; the mean recoveries from the nominal concentration were 106.05, 99.55 and 101.58%, respectively, at 5, 50 and 500 $ng\,mL^{-1}$. Atractylenolide III were stable in plasma samples when stored at −20 °C for a 6-month period, and following three freeze–thaw cycles. Mean recoveries from nominal concentration were more than 90%.

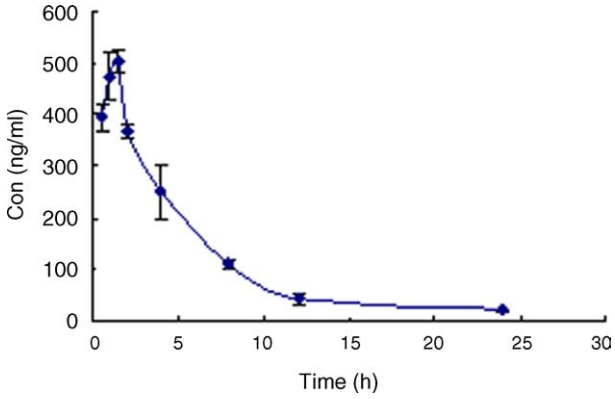

Fig. 4. Mean plasma concentration–time curve in rats after a single oral dose of atractylenolide III (20.0 $mg\,kg^{-1}$). Each point represents the mean ± S.E. of the mean for four rats.

3.6. Application

In order to test whether this method can satisfy the need of pharmacokinetics study, and to estimate the pharmacokinetic characteristics of atractylenolide III in rat, the plasma concentrations of atractylenolide III following oral administration of 20.0 $mg\,kg^{-1}$ were analyzed and the time to plasma concentration curve was profiled. The chromatograms of plasma obtained from pre- and post-doses rats showed that no significant interfering peak was detected at the retention times of atractylenolide III and internal standard, indicating the method was specific enough for its pharmacokinetics study. The plasma concentration–time profiles of atractylenolide III were shown in Fig. 4. The plasma concentration maximum (C_{max}) of atractylenolide III was 502.5 ± 21.10 $ng\,mL^{-1}$ and decreased to be about 20.43 ± 1.57 $ng\,mL^{-1}$ at 24 h post-dosing. Obviously, the previously reported method cannot satisfy the requirements of the pharmacokinetics study on atractylenolide III followed oral administration, whereas the present method based on LC-MS with a LLOQ of 5 $ng\,mL^{-1}$ was sensitive enough for the

Table 2
Intra- and inter-day assay variations of atractylenolide III in rat plasma ($n = 5$)

	Spiked concentration ($ng\,mL^{-1}$)	Intra-day		Inter-day	
		Measured concentration ($ng\,mL^{-1}$)	R.S.D. (%)	Measured concentration ($ng\,mL^{-1}$)	R.S.D. (%)
Atractylenolide III	5	5.11 ± 0.45	8.77	5.30 ± 0.55	10.29
	50	47.58 ± 4.03	8.47	48.54 ± 4.72	9.73
	500	495.26 ± 41.11	8.30	499.18 ± 46.47	9.31

Table 3
Stability of atractylenolide III in rat plasma

	Nominal concentration ($ng\,mL^{-1}$)	Remaining (%)		
		Freeze–thaw (three cycles)	Room temperature (for 4 h)	Stored at −20 °C (for 6 months)
Atractylenolide III	5	94.20	106.05	97.24
	50	90.85	99.55	92.29
	500	97.69	101.58	99.50

pharmacokinetics research of atractylenolide III and further studies are still underway.

4. Conclusion

The established HPLC-MS method of determination of atractylenolide III was fully validated in terms of sensitivity, accuracy and specificity. Selected ion monitoring at *m/z* 247.00 provides a lower limit of quantification of 5 ng mL^{-1} in plasma with no interference from endogenous substances. A preliminary pharmacokinetics application proved further the developed method based on LC/MS detection was sensitive enough and reproducible for the pharmacokinetics study of atractylenolide III.

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