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Short communication

Quantitation of Armillarisin A in human plasma by liquid chromatography–electrospray tandem mass spectrometry

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Abstract

A rapid and sensitive LC–MS/MS method for quantifying Armillarisin A in human plasma after a single oral dose (40 mg) has been developed and validated. Sample preparation used liquid–liquid extraction with a mixture of diethyl ether–dichloromethane (60:40, v/v) in an acidic environment. The retention times of Armillarisin A and the internal standard, probenecid, were 1.63 and 1.78 min, respectively. The calibration curve was linear over the range 0.15–50 ng/mL with a limit of quantitation of 0.15 ng/mL. The coefficient of variation as a measure of intra- and inter-day precision was <9.3% and the accuracy was in the range 92.5–108.0%. The Armillarisin A concentration–time profile in human plasma was determined after an oral dose of a 40 mg tablet.

Keywords: Armillarisin A; Probenecid; LC–MS/MS

1. Introduction

Armillarisin A (3-acetyl-5-hydroxymethyl-7-hydroxycoumarin) is a new coumarin derivative extracted from the fungus *Armillariella tabescens* (Scop. ex Fr.) Sing. It is used as a choleretic to improve bile secretion and regulate the pressure of the bile duct to ease inflammation and adjust liver function. Armillarisin A is an important component of traditional Chinese medicine for the treatment of infection of the biliary system, gastritis and hepatitis [1,2].

Previous pharmacokinetic studies of Armillarisin A in rat have been based on assay of radiolabelled drug [3,4] but such methods suffer from low specificity and are ethically undesirable. In the last decade, LC–MS/MS techniques have become popular in the determination of drug compounds in biological matrices because of its excellent specificity, speed, and sensitivity [5,6]. This paper reports on the development and validation of a fast, specific and sensitive LC–MS/MS method for the direct determination of Armillarisin A in human plasma. The assay uses a small sample volume (0.2 mL) and has been successfully applied to a pharmacokinetic study in healthy volunteers given an oral 40 mg dose of Armillarisin A.

2. Experimental

2.1. Materials and reagents

Armillarisin A (purity > 99%) and probenecid (purity > 99%) for use as internal standard were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, PR China). HPLC grade methanol and other solvents were of analytical grade. Distilled water, prepared from demineralized water, was used throughout the study.

2.2. Sample preparation

Whole blood samples were collected into heparinized tubes. Plasma samples were obtained by centrifugation at 2000 × *g* for 5 min and stored at −20 °C prior to analysis. Liquid–liquid extraction was carried out as follows: internal standard solution (100 μL of 200 ng/mL probenecid in methanol:water (50:50, v/v)) was added to 200 μL of plasma in a 10 mL capped

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test-tube. After addition of 100 μL methanol: water (50:50, v/v), 100 μL glacial acetic acid and 500 μL water, extraction was performed with 3 mL diethyl ether and dichloromethane (60:40, v/v) by vortex-mixing for 1 min followed by centrifugation for 5 min at 2000 × *g*. The upper organic layer was transferred to another 10 mL test-tube and evaporated to dryness by heating at 40 ± 0.5 °C under a stream of nitrogen. Residues were reconstituted in 150 μL mobile phase and 20 μL injected into the LC–MS system.

2.3. LC–MS/MS conditions

The LC–MS system consisted of an Agilent 1100 series HPLC (Agilent Technologies, Palo Alto, CA, USA) coupled to an Applied Biosystems Sciex API4000 Mass Spectrometer (Applied Biosystems Sciex, Ontario, Canada). Separation was achieved on a C_{18} column (150 mm × 4.6 mm, 5 μm, Zorbax Extend) using a mobile phase of 10 mM ammonium acetate–methanol–acetonitrile (30:35:35, v/v/v) at a flow-rate of 0.9 mL/min. The mass spectrometer was equipped with an ion-spray source and operated in the positive ion mode. The LC–MS/MS system was optimized for Armillarisin A and internal standard by syringe pump infusion of a constant flow (20 μL/min) of a solution of the two dissolved in mobile phase into the stream of mobile phase eluting from the column. Optimum ion source parameters were: curtain gas = 15 psi; collision gas = 3 psi; ion-spray voltage = 5000 V; ion source gas1 = 50 psi; ion source gas 2 = 40 psi; temperature = 500 °C. The interface heater was set to on mode. Hydrophilic impurities were diverted to waste for 60 s after an injection using a 10-way switching valve. Data acquisition was carried out by Analyst 1.3 software on a *DELL* computer.

2.4. Preparation of standard solutions

A stock solution of Armillarisin A (500 μg/mL) was prepared in methanol:water (50:50, v/v) and stored at −20 °C. Standard solutions were prepared by dilution with methanol and water to obtain concentrations of 0.30, 0.60, 2.00, 4.00, 10.00, 30.00 and 100.00 ng/mL. Effective concentrations of Armillarisin A in plasma samples were 0.15, 0.30, 1.00, 2.00, 50.00, 15.00 and 50.00 ng/mL. A working internal standard solution was prepared at 200 ng/mL. Low, medium and high quality control (QC) samples with effective concentrations in plasma of 0.30, 5.00 and 40.0 ng/mL were similarly prepared.

2.5. Validation

Calibration standards and QC samples ($n = 6$) were analyzed on three separate days. Three independent calibration curves of Armillarisin A were prepared on each day to validate the linearity of the method. Linear regression of calibration curves based on peak area ratios of Armillarisin A against internal standard obtained from LC–MS/MS was weighted according to $1/x$ ($x = (\text{concentration})^2$). Intra- and inter-day precisions were assessed in terms of the coefficient of variation (CV) and accuracy was assessed as relative error. The limit of quantitation

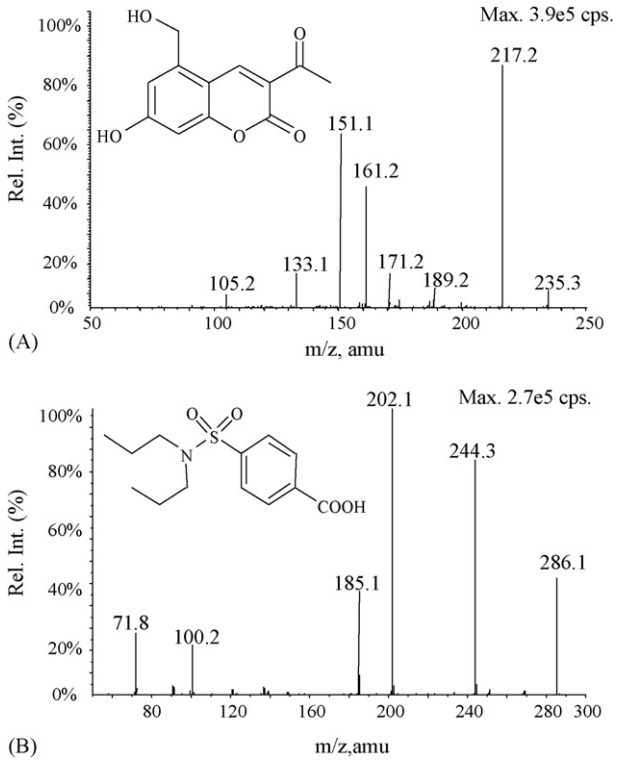

Fig. 1. Full-scan product mass spectra and the structures for (A) Armillarisin A and (B) probenecid.

(LOQ) was defined as the concentration below which the inter-day CV exceeded 20% and the limit of detection (LOD) was defined as the lowest concentration that the analytical assay can reliably differentiate from background levels (signal-to-noise (S/N) > 3). The extraction recoveries of Armillarisin A and the internal standard were evaluated by comparing the peak areas of extracted QC samples and internal standard with those of reference solutions reconstituted in blank plasma extracts. Long-term stability was assessed using low, medium and high quality control samples stored at −20 °C for 1 month. Stability in plasma in the autosampler at room temperature for 6 h was also assessed as was the effect of three freeze–thaw cycles.

2.6. Application of method

Armillarisin A levels in plasma of 20 healthy volunteers were measured in a pharmacokinetic study of a single oral administration of a 40 mg tablet. Blood samples (1 mL) were collected at 0.00, 0.08, 0.17, 0.33, 0.50, 0.67, 0.83, 1.00, 1.17, 1.33, 1.50, 2.00 and 2.50 h after the oral administration.

3. Results and discussion

3.1. LC–MS

The structures and full-scan product mass spectra of Armillarisin A and probenecid are shown in Fig. 1. Multiple reaction monitoring (MRM) was performed at unit resolution using the mass transition ion-pairs *m/z* 235.3 → *m/z* 217.2 for Armillar-

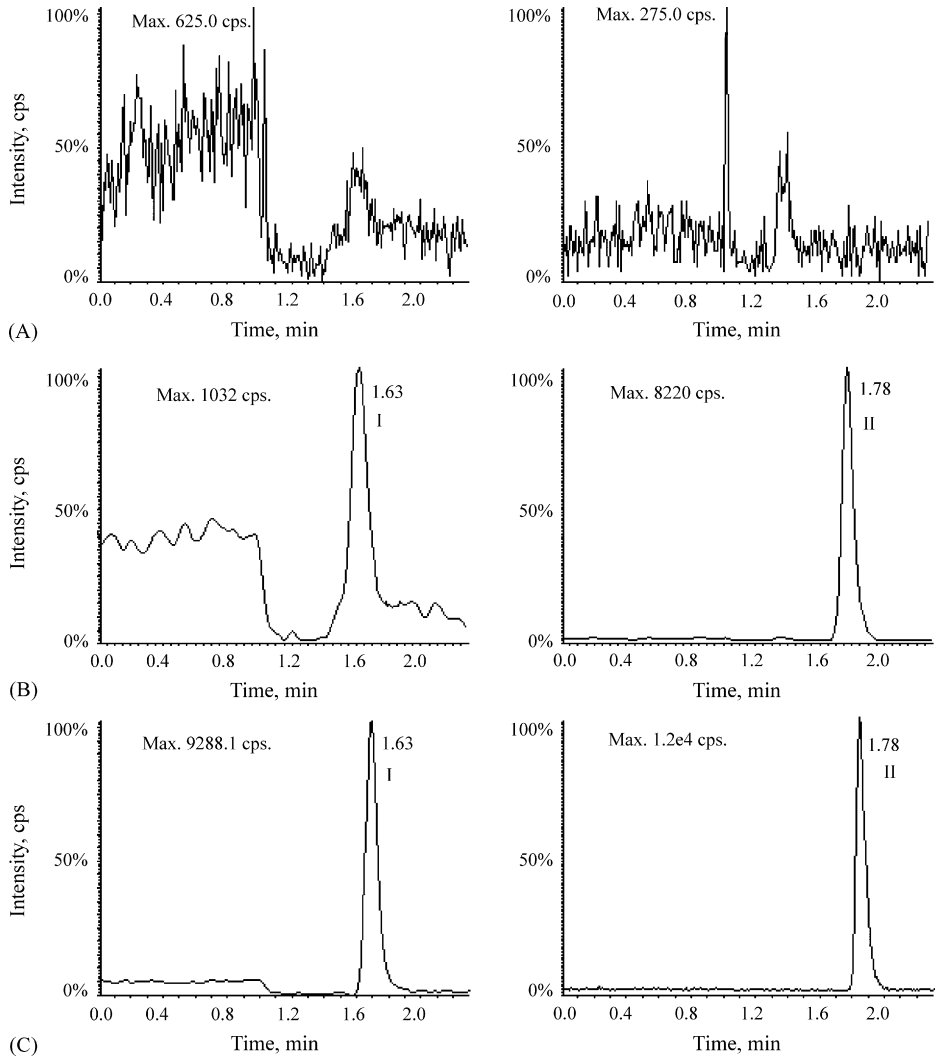

Fig. 2. Representative single reaction monitoring chromatograms of (A) blank plasma; (B) plasma spiked with Armillarisin A at the limit of quantitation (0.15 ng/mL) and probenecid (200 ng/mL); (C) a plasma sample at 0.5 h after an oral administration (40 mg/day) of Armillarisin A to healthy volunteers (0.943 ng/mL). Peak I, Armillarisin A; Peak II, probenecid.

isin A (declustering potential (DP) 55 V; collision energy (CE) 27 eV) and *m/z* 286.1 → *m/z* 202.1 for probenecid (DP 60 eV; CE 23 eV).

Various combinations of acetonitrile, methanol, acetic acid and formic acid were investigated with a view to optimizing the mobile phase for sensitivity, speed and peak shape. Addition of acid reduced base line and increased signal-to-noise, but decreased response. However, the inclusion of 10 mM ammonium acetate instead of pure water increased signal-to-noise without decreasing response. Of a number of C_{18} columns investigated (Nova-Pak, Nucleosil and Hypersil), Zorbax Extend C_{18} gave the best chromatography. With a flow rate of 0.9 mL/min, the cycle time was 2.3 min allowing a sample throughput of 200–240 samples per day.

As shown in Fig. 2A, no endogenous peaks were observed in the chromatogram of blank plasma. The chromatogram of a standard sample at the LOQ is shown in Fig. 2B. The retention time for Armillarisin A and internal standard were 1.63 and 1.78 min, respectively. A low level of background noise and a stable baseline were maintained throughout the study.

3.2. Selection of internal standard

It is necessary to use an internal standard to get high accuracy when a mass spectrometer is used as the HPLC detector. Probenecid was adopted in the end as internal standard because of its similarity of retention action and ionization as well as extraction efficiency.

3.3. Assay validation

The calibration curve showed good linearity (correlation coefficient $r > 0.997$) over the concentration range 0.15–50.0 ng/mL. The LOQ and LOD for Armillarisin A in human plasma were 0.15 and 0.04 ng/mL, respectively. Precision and accuracy at three concentrations are given in Table 1. The CV as a measure of intra- and inter-day precision was <9.3% and the accuracy was in the range 92.5–108.0%.

The extraction recoveries for low, medium and high QC samples were 74.7%, 74.0% and 79.3%, respectively. The recovery of the internal standard was 78.5%. Glacial acetic acid gave a

Table 1
Precision and accuracy for the determination of Armillarisin A in human plasma (data are based on assay of six replicates on three different days)

Added concentration (ng/mL)	Found concentration (ng/mL)	Intra-day R.S.D. (%)	Inter-day R.S.D. (%)	Relative error (%)
0.300	0.299	5.09	3.12	−0.42
5.00	5.08	6.31	8.34	1.67
40.00	40.32	6.14	9.27	0.81

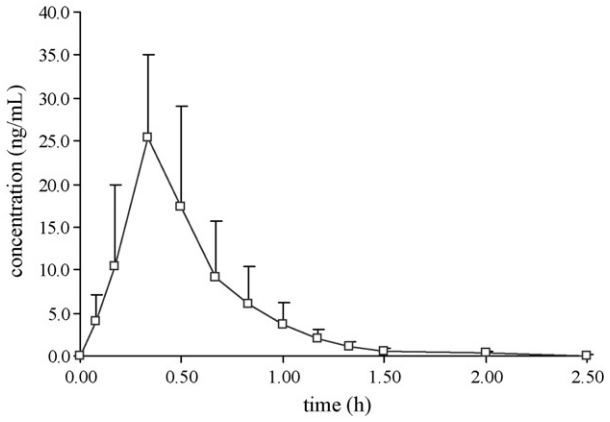

Fig. 3. Plasma concentration–time profile for Armillarisin A after oral administration of Armillarisin A. Data is mean ± S.D. for 20 healthy volunteers.

better extraction recovery for both Armillarisin A and internal standard than hydrochloric acid. The addition of water was necessary to obtain good separation of the organic phase from the aqueous phase.

Matrix effects were evaluated by comparing peak areas of QC and internal standard solutions reconstituted in extracts of blank plasma from four different individuals with those of the same solutions injected directly into the LC–MS system. The results indicate that no co-eluting endogenous substances significantly influenced the ionization of Armillarisin A and internal standard. Armillarisin A was stable under all the conditions tested with mean recoveries of 94.3–105.6% of the nominal concentrations (0.30, 5.00 and 40.0 ng/mL).

3.4. Method application

The applicability of the method was successfully demonstrated in a human pharmacokinetic study. Fig. 3 shows the plasma concentration–time profile for Armillarisin A after an oral dose of a 40 mg tablet. The mean maximum concentration (C_{max}) was 26.6 ± 9.5 ng/mL occurring at 0.35 ± 0.09 h and the mean plasma elimination half-life ($t_{1/2}$) was 0.42 ± 0.19 h.

4. Conclusions

A highly selective, sensitive and rapid method for the determination of Armillarisin A in human plasma is reported using high-performance liquid chromatography with detection by tandem mass spectrometry. The precision and accuracy were acceptable within the concentration range 0.15–50.0 ng/mL. The coefficient of variation as a measure of intra- and inter-day precision was <9.3% and the accuracy was in the range 92.5–108.0%. The method allows high sample throughput (200–240 samples per day) and is suitable for clinical pharmacokinetic studies.

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