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Short communication

Development of a LC–MS/MS method for the determination of antrodin B and antrodin C from *Antrodia camphorata* extract in rat plasma for pharmacokinetic study

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ABSTRACT

A selective and sensitive liquid chromatography–tandem mass spectrometry (LC–MS/MS) method was developed and validated for the determination of antrodin B and antrodin C in rat plasma. Both target compounds, together with the internal standard (diazepam), were extracted from rat plasma samples by liquid–liquid extraction with ethyl acetate. Chromatographic separation was carried out on an Agilent XDB-C_8 column with an isocratic mobile phase consisting of acetonitrile and water (70:30, *V*/*V*) at a flow rate of 0.5 mL/min. The mass spectrometric detection was performed by selected reaction monitoring (SRM) mode *via* atmospheric pressure chemical ionization (APCI) source operating in positive ionization mode. The assay exhibited a linear dynamic range of 47.6–4760 ng/mL for antrodin B and 56.6–5660 ng/mL for antrodin C. The intra- and inter-day precision was less than 5.3% and the accuracy was less than 2.7% for both analytes. The validated method has been applied to the pharmacokinetic study of antrodin B and antrodin C in rats following oral administration of *Antrodia camphorata* extract.

1. Introduction

Antrodia camphorata is a rare medicinal fungus of the family Polyporaceae that historically used as a folk remedy for the treatment of food and drug intoxication, hypertension, itchy skin and liver diseases [1,2]. Previous studies have shown that the aqueous extracts of *A. camphorata* possess hepatoprotective effects, anti-tumor, antioxidant and immunomodulatory activities [3–5]. Some researchers have also demonstrated that the alcohol extracts of *A. camphorata* possess antioxidant, anti-tumor and immunomodulatory activities. These biological activities have been attributed to the constituents such as total phenols, triterpenoids, carbohydrates and adenosine [6–8]. Recently it was reported that the lipophilic extracts of *A. camphorata* had potential cytotoxic and hepatoprotective effects, anti-tumor and antioxidant activities [9,10]. To date, nine maleimide and maleic anhydride derivatives (i.e. antrocinnamonims A, B, C, D and antrodin A, B, C, D, E) have been isolated from the lipophilic extracts of the mycelia of *A. camphorata*, among which antrodin B and antrodin C exhibited significant cytotoxic effects on Lewis lung carcinoma (LLC) tumor cell [11,12].

Although the biological activities of different extracts of *A. camphorata* have been extensively studied, very little attention has been devoted to the pharmacokinetic studies of bioactive components in *A. camphorata* extract. The present paper reports, for the first time, the development and validation of a selective and sensitive liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for the determination of antrodin B and antrodin C in rat plasma and a study of their pharmacokinetics after oral administration of *A. camphorata* extract to rats.

2. Experimental

2.1. Chemicals and materials

Mycelial *A. camphorata* was supplied by GeneFerm Biotechnology Co., Ltd., Taiwan. Reference standards of antrodin B and antrodin C were isolated from mycelial *A. camphorata*. On the basis of UV, MS, NMR and HPLC analysis, the isolated reference standards are confirmed with ≥96% purity. Diazepam (purity >99%) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) and was used as an internal standard (I.S.). HPLC-grade acetonitrile and methanol were purchased from Fisher Scientific (Pittsburgh, PA, USA) and Concord Technology Co., Ltd. (Tianjin, China), respectively. All other reagents were of analytical grade. Double distilled water was used throughout the study.

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2.2. Instrumentation and analytical conditions

A Shimadzu liquid chromatography system (Kyoto, Japan), consisting of a LC-10ADvp pump and a Shimadzu SIL-HTA autosampler, coupled with a Thermo Finnigan TSQ Quantum Ultra triple-quadrupole mass spectrometer (San Jose, CA, USA) equipped with an atmospheric pressure chemical ionization (APCI) interface were used for all LC–MS/MS analyses. Xcalibur 1.4 data system and LCquan 2.0 quantitation software were used for data acquisition and processing. Chromatographic separation was carried out on an Agilent XDB-C_8 column (4.6 mm × 150 mm, 5 μm) with a C_{18} guard cartridge (8 mm × 4 mm I.D, 5 μm) at 20 °C. The mobile phase consisted of acetonitrile and water (70:30, *V*/*V*) with at a flow rate of 0.5 mL/min. The APCI source was performed in positive ion mode. The corona discharge current was set at 4 μA. The capillary temperature was maintained at 270 °C and the vaporizer temperature at 450 °C. Nitrogen was used as the sheath gas (35 Arb) and auxiliary gas (5 Arb) for nebulization and desolvation. Argon was used as the collision gas (1.0 mTorr) for collision-induced dissociation (CID). Quantitation was performed using selected reaction monitoring (SRM) of precursor-product ion transitions at *m*/*z* 314 → 246 for antrodin B, *m*/*z* 330 → 262 for antrodin C and *m*/*z* 285 → 193 for I.S. The collision energies for antrodin B, antrodin C and I.S. were 15, 15 and 37 eV, respectively.

2.3. Preparation of calibration standards and QC samples

A mixed stock solution containing 95.2 μg/mL of antrodin B and 113.2 μg/mL of antrodin C was prepared in methanol. A series of working standard solutions were prepared by successive dilution of the mixed stock solution with methanol. A 25.0 ng/mL I.S. working solution was similarly prepared by diluting a stock standard solution of diazepam with methanol. Calibration standards were prepared by spiking 50 μL of the appropriate standard working solutions into 100 μL blank plasma to yield calibration concentrations of 47.6, 95.2, 142.8, 238.0, 476.0, 952.0, 2380, 4760 ng/mL for antrodin B and 56.6, 113.2, 169.8, 283.0, 566.0, 1132, 2830, 5660 ng/mL for antrodin C. QC samples were prepared at 119.0, 952.0, 3880 ng/mL for antrodin B and 141.5, 1132.0, 4528 ng/mL for antrodin C.

2.4. Sample preparation

A 100 μL volume of plasma sample was pipetted into a 10 mL glass test tube with 50 μL of I.S. working solution. The mixture was vortex-mixed for 1 min and extracted with 2 mL of ethyl acetate on an orbital shaker for 10 min. After centrifugation at 4000 rpm for 10 min, the upper organic phase was transferred into a clean tube and evaporated to dryness under a gentle stream of nitrogen at 45 °C. The residue was reconstituted in 50 μL mobile phase and vortex-mixed for 30 s. A 20 μL aliquot of the solution was injected into the LC–MS/MS system for analysis.

2.5. Method validation

Method linearity was evaluated by analyzing calibration standards in duplicate at each concentration level over three consecutive days. The accuracy and precision were assessed by analyzing QC samples in six replicates at three concentration levels on three validation days. The extraction recovery was evaluated at three concentration levels and for the I.S. at one concentration level by comparing the peak areas of the analytes obtained from six plasma samples with the analytes spiked before and after extraction. Matrix effect was evaluated by comparing the peak areas of the analytes obtained from six plasma samples with the analytes spiked after extraction, at three concentration levels, to those for the neat standard solutions at the same concentrations. The stability of antrodin B and antrodin C in rat plasma at low and high concentration levels was evaluated under a variety of storage and process conditions.

2.6. Pharmacokinetic application

Six Sprague–Dawley rats weighing 220–250 g were fasted for 12 h. Each rat was administered an oral dose of 9.096 g/kg ethyl acetate extract from mycelial *A. camphorata* suspended in an aqueous solution containing 0.5% carboxymethyl cellulose sodium (equivalent to 22.8 mg/kg of antrodin B and 50.2 mg/kg of antrodin C). 500 μL blood samples were collected in heparinized Eppendorf tubes *via* the oculi chorioideae vein before dosing (0 min) and subsequently at 5, 15, 30, 60, 120, 180, 240, 360, 480, 600 and 1440 min after administration. The heparinized blood was immediately centrifuged for 10 min at 4000 rpm, and the plasma obtained was stored at −70 °C until analysis. The pharmacokinetic parameters were calculated using DAS 2.0 software (Mathematical Pharmacology Professional Committee of China, Shanghai, China).

3. Results and discussion

3.1. Method development

Antrodin B and antrodin C were strongly retained on C_{18} columns. In order to reduce their retention times, an Agilent XDB-C_8 column (4.6 mm × 150 mm, 5 μm) was used for chromatographic separation. Different mobile phases consisting of methanol–water or acetonitrile–water were attempted. It was found that acetonitrile provided higher mass spectral signal and lower background noise than methanol. However, the addition of acidic modifiers to the mobile phase had no significant effect on the ionization efficiency of the analytes under APCI conditions. Finally, a mobile phase consisting of acetonitrile–water (70:30, *V*/*V*) was chosen to achieve symmetrical peak shapes and short chromatographic run time, and to minimize the matrix effect.

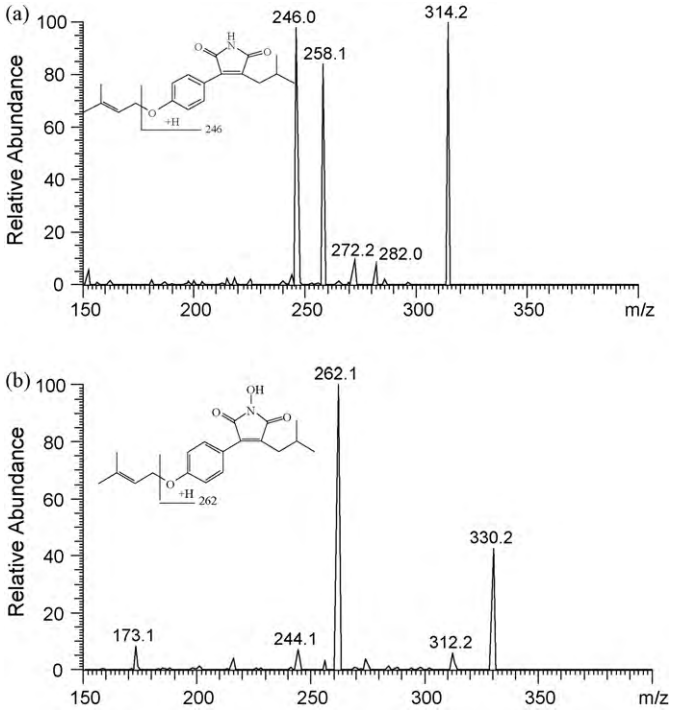

Fig. 1. (a) Product ion spectra of antrodin B and (b) product ion spectra of antrodin C.

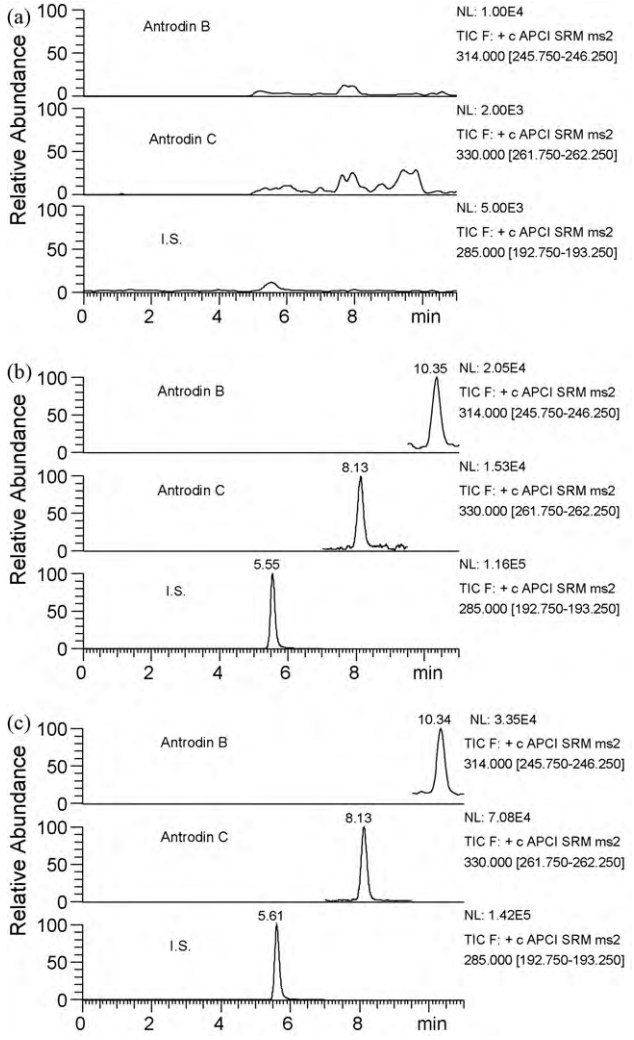

Fig. 2. Total ion chromatograms (TIC) of (a) blank plasma and (b) plasma spiked with antrodin B (47.6 ng/mL) and antrodin C (56.6 ng/mL) and (c) a plasma sample collected 4 h after oral administration of an ethyl acetate extract of mycelial *Antrodia camphorata*. The retention time of antrodin B, antrodin C and internal standard are 10.3, 8.1 and 5.5 min, respectively.

The MS/MS detection of antrodin B and antrodin C was optimized by infusing a standard solution of each analyte into the mobile phase using a syringe pump. Fig. 1 displays the product ion mass spectra of antrodin B and antrodin C.

3.2. Method validation

Fig. 2 shows the typical SRM chromatograms of a blank plasma, a plasma spiked with antrodin B (47.6 ng/mL), antrodin C (56.6 ng/mL) and the I.S. (25.0 ng/mL), and a plasma sample collected 4 h after an oral administration of *A. camphorata* extract to a rat. No interference from endogenous substances was observed at the retention times of the analytes and the I.S. Calibration curves showed good linearity over the range of 47.6–4760 ng/mL for antrodin B and 56.6–5660 ng/mL for antrodin C. The LLOQs of antrodin B and antrodin C were 47.6 and 56.6 ng/mL, respectively.

Table 1 presents the precision and accuracy for antrodin B and antrodin C by analyzing QC samples. The intra- and inter-day precision was less than 5.3% and the accuracy was less than 2.7% for both analytes. The mean extraction recoveries were 78.8 ± 3.7%, 69.4 ± 10.3% and 76.1 ± 12.3% for antrodin B and 73.9 ± 10.0%, 74.9 ± 7.2% and 78.3 ± 11.6% for antrodin C. The mean recovery of the I.S. was 75.8 ± 3.0%. With regard to matrix effect, all the calculated values were between 85% and 115%, which indicated that the co-eluting matrix components had little or no effect on the ionization of the analytes and the I.S.

The stability study showed that antrodin B and antrodin C were stable in plasma at room temperature (25 °C) for 2 h (RE < 3.0%), at −70 °C for 15 days (RE < 3.1%) and after three freeze–thaw cycles (RE < 4.0%). The two analytes were also shown to be stable after the reconstitution at 25 °C for 24 h (RE < 4.2%).

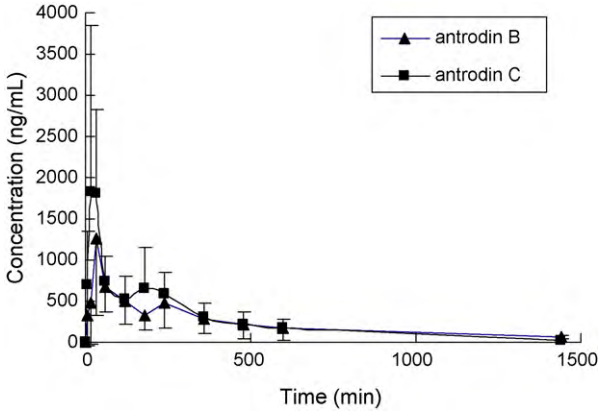

Fig. 3. The mean plasma concentration versus time profiles of antrodin B and antrodin C after single oral administration of ethyl acetate extract of mycelial *Antrodia camphorata* to rats.

3.3. Pharmacokinetic application

The validated method was applied to the pharmacokinetic study of antrodin B and antrodin C after oral administration of *A. camphorata* extract at a dose of 9.096 g/kg (equivalent to 22.8 mg/kg of antrodin B and 50.2 mg/kg of antrodin C) to six rats. The mean plasma concentrations of antrodin B and antrodin C versus time profile are presented in Fig. 3. The two analytes exhibited consistent tendency in plasma concentration–time profiles. Both the concentration–time profiles fitted a two-compartment model. C_{max}, T_{max}, AUC_{0-t} and $t_{1/2}$ of antrodin B were

Table 1
Precision and accuracy data for the determination of antrodin B and antrodin C in rat plasma samples (intra-day: $n = 6$; inter-day: $n = 6$; $x \pm SD$).

Compound	Added (ng/mL)	Found (ng/mL)	Intra-day RSD (%)	Inter-day RSD (%)	Relative error (%)
Antrodin B	47.6	46.7	1.9	2.5	1.9
	119.0	119.5	3.1	5.3	0.4
	952.0	956.3	2.4	3.6	0.5
	3808.0	3836.3	3.0	4.2	0.7
Antrodin C	56.6	55.1	1.2	2.7	2.7
	141.2	141.9	3.0	1.2	0.5
	1132.0	1119.6	3.1	4.3	1.1
	4528.0	4598.6	3.0	3.5	1.6

1277 ± 944 ng/mL, 35.0 ± 12.2 min, $326{,}981 \pm 166{,}403$ ng min/mL and 263.7 ± 123.1 min, respectively. C_{max}, T_{max}, AUC_{0-t} and $t_{1/2}$ of antrodin C were 2425 ± 1688 ng/mL, 27.5 ± 6.1 min, $327{,}432 \pm 136{,}971$ ng min/mL and 251.4 ± 168.0 min, respectively.

4. Conclusion

This is the first report on the development of a LC–MS/MS method for the determination of antrodin B and C in rat plasma. The method is sensitive and selective and has been successfully applied to the pharmacokinetic study of antrodin B and antrodin C in rats following oral dose of *A. camphorata* extracts.

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