Steroids 96 (2015) 21–29

Contents lists available at ScienceDirect

Steroids

journal homepage: www.elsevier.com/locate/steroids

Development and validation of liquid chromatography–tandem mass spectrometry method for simultaneous determination of six steroidal saponins in rat plasma and its application to a pharmacokinetics study

Zhirui Liu [a,1], Wenxing Qin [b,1], Zhenyu Zhu [c], Yao Liu [a], Fengjun Sun [a], Yifeng Chai [c,*], Peiyuan Xia [a,*]

[a] Department of Pharmacy, Southwest Hospital, Third Military Medical University, Chongqing 400038, PR China
[b] Department of Medical Oncology, Changzheng Hospital, Second Military Medical University, Shanghai 200070, PR China
[c] School of Pharmacy, Second Military Medical University, Shanghai 200433, PR China

ARTICLE INFO

Article history:
Received 21 July 2014
Received in revised form 6 January 2015
Accepted 8 January 2015
Available online 21 January 2015

Keywords:
Steroidal saponins
UHPLC–MS/MS
Rat plasma
Pharmacokinetic study

ABSTRACT

A specific and reliable liquid chromatography–electrospray ionization-tandem mass spectrometry method was developed for the simultaneous determination of timosaponin H1 (TH1), timosaponin E1 (TE1), timosaponin E (TE), timosaponin B-II (TB-II), timosaponin B-III (TB-III) and anemarrhenasaponin I (AS-I) in rat plasma. After addition of internal standard (IS) ginsenoside Rh1, plasma samples were pretreated by protein precipitation with acetonitrile. Chromatographic separation was performed on a reverse phase ACQUITY™ BEH C_{18} column (100 mm × 2.1 mm i.d., 1.7 μm) using a gradient mobile phase system of acetonitrile–water containing 0.05% formic acid and 5 mM ammonium formate. The triple quadruple mass spectrometer was set in negative electrospray ionization mode and multiple reaction monitoring (MRM) was used for six steroidal saponins quantification. The precursors to produce ion transitions monitored for TH1, TE1, TE, TB-II, TB-III, AS-I and IS were *m/z* 1211.5 > 1079.6, 935.5 > 773.4, 935.4 > 773.5, 919.6 > 757.4, 901.5 > 739.3, 757.4 > 595.3 and 637.3 > 475.3, respectively. The method validation was conducted over the curve range of 0.5–400 ng/mL for the six saponins. The intra- and inter-day precisions (RSD%) were less than 9.4% and the average extraction recoveries ranged from 82.5% to 97.8% for each analyte. Six steroidal saponins were proved to be stable during sample storage, preparation and analytical procedures. The validated method was successfully applied for the first time to determine the concentrations of six main steroidal saponins in incurred rat plasma samples, after intragastric administration of the extract of *Anemarrhena asphodeloides* Bge. for a rat pharmacokinetic study.

1. Introduction

Anemarrhena asphodeloides (Zhi Mu in Chinese), the rhizome of *A. asphodeloides* Bunge (Fam. Liliaceae), has been commonly used in Chinese Material Medica for thousands of years. As a typical herbal medicine, *A. asphodeloides* frequently used to treat febrile diseases, cough, hematochezia and hemoptysis in clinical practice [1]. Phytochemical studies on *A. asphodeloides* revealed that it contained steroidal saponins, xanthones, anthraquinones, phenylpropanoids, alkaloids and so on [2]. Furthermore, it has been reported that steroidal saponins are the most important active constituents that contribute to the pharmacological efficacy of *A. asphodeloides* [3]. Timosaponin H1 (TH1), timosaponin E1 (TE1), timosaponin E (TE), timosaponin B-II (TB-II), timosaponin B-III (TB-III) and anemarrhenasaponin I (AS-I) are six major steroidal saponins that have found to be active. They have shown a variety of biological effects such as anti-platelet aggregation [4], anti-inflammatory [5], anti-tumor [6] and cognition-enhancing effects [7]. Therefore, an integral investigation into the pharmacokinetic evaluation of the six steroidal saponins in *A. asphodeloides* could better understand its pharmacological and clinical effects.

Several analytical methods have been proposed for the determination of steroidal saponins in *A. asphodeloides*, including thin-layer chromatography (TLC), gas chromatography (GC) and high-performance liquid chromatography (HPLC) with different detectors [8–10]. However, these methods suffer from long analysis time and/or low sensitivity and thus are not suitable for the determination of steroidal saponins in biological fluids after

* Corresponding authors. Tel./fax: +86 023 68754438 (P. Xia). Tel./fax: +86 021 81871201 (Y. Chai).
E-mail addresses: yfchai@smmu.edu.cn (Y. Chai), peiyuan_xia2013@163.com (P. Xia).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.steroids.2015.01.006
0039-128X/

administration of *A. asphodeloides* extract. In recent years, liquid chromatography (LC) coupled with mass spectrometry (MS) has been widely used for the analyses of various types of steroidal saponins [11]. In our previous study, HPLC coupled with single quadrupole mass spectrometry (HPLC–MS) in selected ion monitoring (SIM) mode has been developed to determine timosaponin B-II and timosaponin A-III in rat plasma [12]. Due to the low sensitivity and selectivity of single quadrupole MS detection, it is not applicable for pharmacokinetic study of multiple active saponins after administration of *A. asphodeloides* extract. Ultra-high pressure liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) is a very powerful and reliable tool for the determination of chemical constituents, and MS detection in multiple reaction monitoring (MRM) has the advantage in high sensitivity and specificity [13]. Nowadays, it is increasingly utilized for the determination of multiple targeted components in complex biological matrices by official and routine laboratories. To the best of our knowledge, the use of UHPLC–MS/MS for the simultaneous determination of multiple steroidal saponins from *A. asphodeloides* in biological fluids has not yet been demonstrated.

In this paper, a fully validated UHPLC–MS/MS method was developed for the simultaneous determination of TH1, TE1, TE, TB-II, TB-III and AS-I in rat plasma. LC separation was performed on a 1.7 μm particles column to ensure its high resolution and speed. The MS parameters under the negative electrospray ionization conditions were optimized for achieving good sensitivity for the six steroidal saponins in one single analytical run. Their structures are listed in Fig. 1. Using the method presented, pharmacokinetic profiles of these six components were further investigated after a single oral administration of *A. asphodeloides* extract.

2. Experimental

2.1. Chemicals and reagents

Reference standards of TH1, TE1, TE, TB-II, TB-III, AS-I and internal standard of ginsenoside Rh1 (GRh1) were all purchased from Dingrui Pharmaceutical Co., Ltd. (Shanghai, China). Their purities were determined to be over 98% by normalization of the peak areas detected by LC–MS/MS. An ethanol extract of *A. asphodeloides* was prepared in Key Laboratory of Drug Metabolism, Xinan Hospital (Chongqing, China). The contents of TH1, TE1, TE, TB-II, TB-III and AS-I were 3.1, 7.1, 2.8, 46.2, 2.2 and 0.7 mg/g dry extract, respectively. Acetonitrile, ammonium formate and formic acid of MS-grade were purchased from Fisher Scientific (Tustin, CA, USA). Ultra-pure water was prepared by Milli-Q System (Millipore, Bedford, MA, USA). Other reagents used here were of analytical grade.

2.2. Instrumentation and conditions

Chromatographic analysis was performed using an Agilent 1200 series liquid chromatography system (Agilent Technologies, Palo Alto, CA, USA), including a binary solvent delivery system, an online vacuum degasser, an autosampler and a thermostatically controlled column apartment. Chemical ingredients were separated on an ACQUITY™ BEH C_{18} column (100 mm × 2.1 mm i.d., 1.7 μm) with an VanGuard™ Pre-column (5 mm × 2.1 mm i.d., 1.7 μm). The mobile phase consisted of 0.05% formic acid in water with 5 mM ammonium formate (mobile phase A) and 0.05% formic acid in acetonitrile with 5 mM ammonium formate (mobile phase B). The linear gradient conditions were as follows: 0 min 25% B, 0.5 min 28% B, 4 min 30% B, 4.5 min 100% B, 7 min 100% B. The composition was then held at 100% B for 2 min and returned to initial conditions and maintained for three column volumes for equilibration. The flow rate was 0.3 mL/min and the injection volume was 5 μL. The column was kept constant at 40 °C throughout the running time. An injector rinse solvent consisting of acetonitrile–water (50:50, v/v) was used.

Mass spectrometric detection was carried out on an Agilent 6410 triple–quadrupole mass spectrometer (Palo Alto, CA, USA) equipped with electrospray ionization (ESI) source and operating in negative ion mode. The drying gas temperature was maintained at 350 °C at a flow rate of 10 L/min, and the nebulizing gas (N_2) pressure and capillary voltage was set at 40 psi and −3500 V. The dwell time was 200 ms and mass analyzers Q1 and Q3 operated at unit mass resolution were used for each MRM transition. The precursor-to-product ion pair, the optimized fragmentor voltage (Frag) and collision energy (CE) for each analyte are described in Fig. 1. Agilent MassHunter B3.0 software was used for the control of equipment, data acquisition and analysis.

2.3. Preparation of calibration standards, quality control and internal standard

The stock solutions of TH1, TE1, TE and TB-II were prepared in acetonitrile–water mixture 70:30 (v/v), and the stock solutions of TB-III, AS-I and IS (GRh1) were prepared in methanol. All the individual stocks were stored at −40 °C and were stable for 3 months. The stock solutions of the standards were further diluted in acetonitrile–water mixture 70:30 (v/v) to produce combined standard working solutions at concentrations of 20, 40, 100, 200, 400 ,1000, 2000, 4000 and 8000 ng/mL for TB-II and 10, 20, 50, 100, 200, 500, 1000, 2000 and 4000 ng/mL for TH1, TE1, TE, TB-III and AS-I. Calibration samples were prepared by a 1:20 dilution of the corresponding combined standard working solutions with pooled blank rat plasma to obtain final concentrations in the range of 1.0–400 ng/mL for TB-II and 0.5–200 ng/mL for TH1, TE1, TE, TB-III and AS-I. Quality control (QC) samples including 3.0, 30 and 300 ng/mL for TB-II and 1.5, 15 and 150 ng/mL for TH1, TE1, TE, TB-III and AS-I were also prepared as the same procedure as the calibration standards.

2.4. Preparation of plasma sample

Rat plasma samples were taken from −40 °C storage and thawed under the ambient condition. To each 200 μL plasma, 100 μL IS working solution (30 ng/mL) and 400 μL acetonitrile were added. After thorough vortex for 2 min, the mixture was centrifuged at 10,400×*g* for 10 min at 4 °C, and the supernatant was transferred to another eppendorf tube and dried under a Thermo Savant SpeedVac concentrator at low temperature (ambient temperature). The residue was added in 100 μL acetonitrile–water (1:1, v/v), a 5 μL aliquot of the supernatant was injected into the UHPLC–MS/MS for analysis after centrifugation at 10,400×*g* for 10 min at 4 °C.

2.5. Method validation

The method was validated for specificity, linearity, sensitivity, precision, accuracy, matrix effect, extraction recovery and stability according to the FDA Guidance for Industry on Bioanalytical Method Validation [14].

2.5.1. Assay specificity

The specificity of the method was assessed by preparing and analyzing six different batches of drug-free rat plasma. Each blank sample was tested for endogenous interference using the proposed extraction procedure and LC–MS/MS conditions. The chromatogram of a blank plasma sample was compared with those obtained with a solution at the concentration of lower limit of quantification