International Journal of Mass Spectrometry 341–342 (2013) 7–17

Contents lists available at SciVerse ScienceDirect

International Journal of Mass Spectrometry

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

Structure characterization and identification of steroidal saponins from the rhizomes of *Anemarrhena asphodeloides* by ultra performance liquid chromatography and hybrid quadrupole time-of-flight mass spectrometry

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ARTICLE INFO

Article history:
Received 31 October 2012
Received in revised form 1 March 2013
Accepted 4 March 2013
Available online 15 March 2013

Keywords:
Identification
Anemarrhena asphodeloides
UPLC/Qtof MS^E
Steroidal saponins

ABSTRACT

In this work, ultra performance liquid chromatography (UPLC) coupled with a hybrid quadrupole time-of-flight mass spectrometry (Q-TOF MS^E) was used to separate and identify steroidal saponins from the crude extracts of *Anemarrhena asphodeloides.* The fragmentation patterns of seven known steroidal saponins, which could be classified into six types, were investigated by UPLC/Qtof MS^E technique in both negative and positive ion modes. By using this strategy, both intact precursor and fragment ion information were obtained from a single injection. Based on the exact mass, fragment ions, and retention times of peaks, and comparison with reference standards and known compounds in the literature, sixty-seven steroidal saponins were identified or tentatively identified from *A. asphodeloides*. This included sixteen groups of isomers. Fourteen of these saponins were identified as new compounds. This study provides a good example for the rapid identification of chemical constituents in complex samples such as TCM by UPLC/Qtof MS^E, in the mean time, avoiding the time-consuming and tedious purification of compounds from the crude extracts.

1. Introduction

The rhizome of *Anemarrhena asphodeloides* Bunge (Liliaceae) is an antipyretic in traditional Chinese medicine (TCM), known as “zhimu” in China. It was found to have anti-microbial activity, decreases the blood glucose level, inhibits platelet aggregation, inhibits carcinoma decreases radiation injury, and anti-dementia activity [1–5]. Steroidal saponins are the most important bioactive compounds in *A. asphodeloides*. Sarsasapogenin and its proto structures are considered the major aglycones of the steroidal saponins and typical sugars present in steroidal saponins are xylose, galactose and glucose [1,6–9]. Because of structural complexity and low abundance, the isolation and purification of steroidal saponins from *A. asphodeloides* using the traditional methods was often tedious and labor intensive. In recent years, the liquid chromatography coupled with mass spectrometry (LC–MS) has been increasingly applied for research in natural products [10–15]. There are published reports shown the characterization of steroidal saponins from *A. asphodeloides* using HPLC-MS^n, only less than 30 saponins had been identified [16–18].

UPLC/Qtof MS^E, combining the advantages of UPLC (high resolution, high sensitivity and high speed separation) with the exact mass measurement offered by TOF mass spectrometry, is an effective analytical technique to rapidly screen and identify compounds of interest. In the present work, the UPLC/Qtof MS^E technique was used in both negative and positive ion modes to separate and identify the steroidal saponins from *A. asphodeloides* extract. Seven saponins from *A. asphodeloides*, divided into six classes based on their aglycones (Fig. 1), were investigated by the UPLC/Qtof MS^E. Based on the fragmentation behaviors and retention times of reference steroidal saponins, a total of sixty-seven steroidal saponins (represented by sixty-one peaks) were identified or tentatively identified, including sixteen groups of isomers. In addition, fourteen saponins are new compounds.

2. Experimental

2.1. Chemicals and herb materials

Acetonitrile (HPLC grade) was purchased from Fisher Scientific Co. (Loughborough, UK). Deionized water (18.2 MΩ) was further purified using a Milli-Q system (Millipore, Billerica, USA). Formic

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http://dx.doi.org/10.1016/j.ijms.2013.03.005

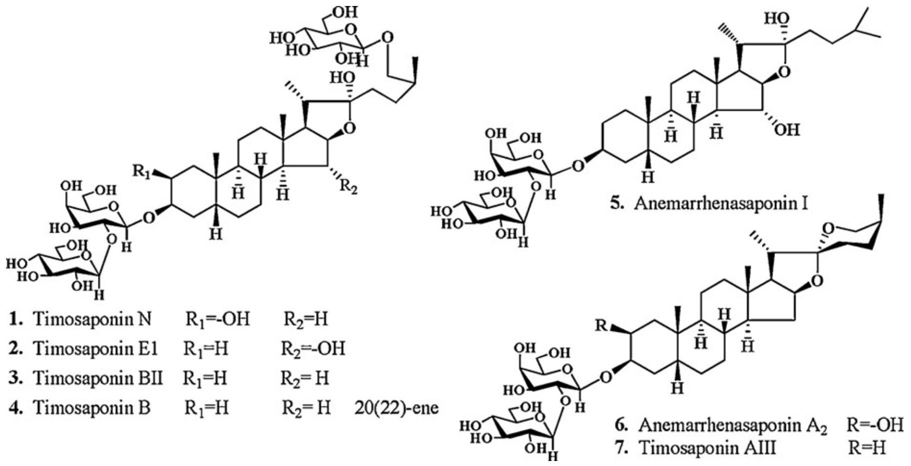

Fig. 1. Structures of seven reference standards.

acid (HPLC grade) was purchased from Acros Co. Ltd. (NJ, USA). Other reagents were obtained commercially in analytical purity (Beijing, China). SPE columns were purchased from Agela Technologies (Tianjin, China).

The rhizomes of *A. asphodeloides* were collected from the Anguo region of Hebei Province, China. The plant was identified by Prof. Li-juan Zhang of Tianjin University of Traditional Chinese Medicine.

Seven saponin standards (Fig. 1), timosaponins N, E1, BII, B, and AIII, and anemarrhenasaponins I and A_2 were isolated previously and their structures were confirmed by MS as well as 1D and 2D NMR spectra [9].

2.2. Preparation of herbal extract

A. asphodeloides (500 mg) were extracted three times with hot 60% aqueous EtOH for 1 h per extraction. The combined extracts were filtered and concentrated at reduced pressure. The extract was subjected to a SPE column, and then eluted with 80% aqueous acetonitrile to yield fraction A (52.5 mg). 10 mg Fr. A was dissolved in 1.5 mL of 60% ethanol and filtered using a 0.22 μm syringe filter, with 1 μL injected for UPLC/Qtof MS^E analysis.

2.3. UPLC/Qtof MS^E analysis

UPLC/Qtof MS^E analysis was performed on an Acquity UPLC^{TM} system (Waters Corp., Milford, MA, USA) coupled with a Synapt MS system (Waters Corp., Manchester, UK). A Waters Acquity UPLC HSS T3 column (100 × 2.1 mm, 1.8 μm) was used for the analysis, with the column temperature was set at 45 °C. Mobile phases were water with 0.1% formic acid (A) and acetonitrile (B). The gradient used was as follows: (0–0.5) min, 5% B; (0.5–1.0) min, 5% → 20% B; (1.0–5.0) min, 20% → 22% B; (5.0–8.5) min, 22%B; (8.5–12.5) min, 22% → 38% B; (12.5–16.0) min, 38% → 50% B; (16.0–17.0) min, 50% B; (17.0–18.5) min, 50% → 85% B; (18.5–20.5) min, 85% B. The flow rate was 600 μL/min. The injection volume of sample was 2 μL. The data acquisition mode was MS^E. Each extract was injected twice: once for ESI+ analysis, and once for ESI− analysis Data were acquired from 50 to 1500 Da. The source temperature was 100 °C, and the desolvation temperature was 450 °C, with desolvation gas flow of 800 L/h. Leucine enkephaline was used as lock mass. The capillary voltage was 3 kV. At low CE scan, the cone voltage was 30 V for ESI+ and 40 V for ESI−, and the collision energy was 6 eV (trap) and 4 eV (transfer). At high CE scan, the cone voltage was 30 V for ESI+ and 50 V for ESI−, and the collision energy was 30–50 eV ramp (trap) and 12 eV (transfer) for ESI+, and 50–65 eV ramp (trap) and 12 eV (transfer) for ESI−. The instrument was controlled by Masslynx 4.1 software (Waters Corporation).

3. Results and discussion

3.1. Investigation of the fragmentation patterns and retention times of reference standards

To obtain more information about precursor ions and characteristic fragment ions of steroidal saponins, both the positive and negative ion modes were tested with the same LC mobile phase. Because the reference standards have different aglycones and showed different fragment ions, they were classified into six types: Type I (timosaponins E1 and N), Type II (timosaponin BII), Type III (timosaponin B), Type IV (Anemarrhenasaponin I), Type V (Anemarrhenasaponin A_2), and Type VI (timosaponin AIII). The fragmentation patterns for these reference standards were discussed in detail below.

In ESI−, timosaponin N produced a deprotonated molecular ion at *m/z* 935.4888 $[M-H]^-$ and an adduct ion at *m/z* 981.4917 $[M+HCOO]^-$ at low CE (Fig. 2a); at high CE, timosaponin N produced fragment ions at *m/z* 935.4869 $[M-H]^-$, 773.4352 $[M-H-Glc]^-$, 611.3783 $[M-H-2Glc]^-$, 449.3292 $[M-H-2Glc-Gal]^-$, and 431.3188 $[M-H-2Glc-Gal-H_2O]^-$ (Fig. 2b).

In ESI+, at low CE, timosaponin N produced major fragment ions at *m/z* 919.4917, 757.4395, 595.3835, and 433.3368 (Fig. 2c); at high CE, the fragment ions at *m/z* 919.4941, 757.4382, 595.3880, 433.3352, and 415.3197 were attributed to the sequential loss of one molecule of water, two glucosyls and one galactosyl residues, and one molecule of water from the $[M+Na]^+$ ion (*m/z* 959.4797). The characteristic fragment ions at *m/z* 289.2197, 271.2100, and 253.2013 resulted from consecutive losses of 144 (formula $C_8H_{16}O_2$) and two 18 Da from the fragment ion at *m/z* 433.3352 (Fig. 2d). The proposed fragmentation pathways of timosaponin N are shown in Fig. 3.

In ESI−, timosaponin BII produced $[M-H]^-$ (*m/z* 919.4923) and $[M+HCOO]^-$ (*m/z* 965.4998) ions at low CE; at high CE, the major fragment ions were observed at *m/z* 919.4887 $[M-H]^-$, 757.4403 $[M-H-Glc]^-$, 595.3839 $[M-H-2Glc]^-$, and 433.3331 $[M-H-2Glc-Gal]^-$. In ESI+, timosaponin BII produced major ions at *m/z* 943.4899 $[M+Na]^+$, 903.4943 $[M+H-H_2O]^+$, 741.4469 $[903.4943-Glc]^+$, 579.3918 $[741.4469-Glc]^+$, 417.3412 $[579.3918-Gal]^+$, 273.2271 $[417.3412-C_8H_{16}O_2]^+$, and 255.2178 $[273.2271-H_2O]^+$. The ions at *m/z* 417, 273, and 255, together with the ion $[M+H-H_2O]^+$ in high CE ESI+ can be considered as the diagnostic ions for this type of steroidal saponin.

Timosaponin B produced the major ions at *m/z* 901.4828 $[M-H]^-$, 739.4210 $[M-H-Glc]^-$, and 577.3769 $[M-H-2Glc]^-$ in ESI−. In high CE ESI+, timosaponin B produced the fragment ions at *m/z* 925.4760 $[M+Na]^+$, 903.4972 $[M+H]^+$, 741.4468 $[M+H-Glc]^+$,