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Ultrahigh-performance liquid chromatography-ion trap mass spectrometry characterization of the steroidal saponins of *Dioscorea panthaica* Prain et Burkill and its application for accelerating the isolation and structural elucidation of steroidal saponins

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ABSTRACT

Dioscorea panthaica is a traditional Chinese medicinal herb used in the treatment of various physiological conditions, including cardiovascular disease, gastropathy and hypertension. Steroidal saponins (SS) are the main active ingredients of this herb and have effects on myocardial ischemia and cancer. The phytochemical evaluation of SS is both time-consuming and laborious, and the isolation and structural determination steps can be especially demanding. For this reason, the development of new methods to accelerate the processes involved in the identification, isolation and structural elucidation of SS is highly desirable. In this study, a new ultrahigh performance liquid chromatography-ion trap mass spectrometry (UHPLC-IT/MS^n) method has been developed for the identification of the SS in *D. panthaica* Prain et Burkill. Notably, the current method can distinguish between spirostanol and furostanol-type compounds based on the fragmentation patterns observed by electrospray ionization-ion trap mass spectrometry (ESI-IT/MS^n) analysis. UHPLC-IT/MS^n was used to conduct a detailed investigation of the number, structural class and order of the sugar moieties in the sugar chains of the SS present in *D. panthaica*. The established fragmentation features were used to analyze the compounds found in the 65% ethanol fraction of the water extracts of *D. panthaica*. Twenty-three SS were identified, including 11 potential new compounds and six groups of isomers. Two of these newly identified SS were selected as representative examples, and their chemical structures were confirmed by ^{1}H and ^{13}C NMR analyses. This newly developed UHPLC-IT/MS^n method therefore allowed for the efficient identification, isolation and structural determination of the SS in *D. panthaica*.

1. Introduction

Dioscorea panthaica Prain et Burkill is a traditional Chinese medicine, which is grown in Sichuan, Yunnan, Guizhou and Hunan provinces. The dried rhizome of *D. panthaica* is widely used in traditional medicine for the treatment of numerous ailments, including cardiovascular and gastric diseases, bones injuries, rheumatic arthritis and traumatic tuberculosis injuries. Steroidal saponins (SS) are the main constituents of this *D. panthaica*, and over 30 SS compounds have been isolated from this plant to date. Many of these SS display a variety of interesting bioactivities, such as improving myocardial ischemia and dilating blood vessels [1–4]. Notably, some of the SS isolated from the rhizome of *D. panthaica* have been reported to exhibit anticancer effects [5]. Based on the wide range of attractive therapeutic applications for the SS found in *D. panthaica*, we decided to conduct a comprehensive study of this plant and its SS.

The process of isolating and determining the structural characteristics of SS is generally very labor intensive. The major difficulty associated with the isolation of these compounds is that they are high polar because of the long sugar chains attached to their aglycone unit. In terms of their characterization of SS, it can also be difficult to determine the structure of their aglycone moiety and the attached sugars. LC–MS^n has been proven to be one of the most effective methods for the rapid identification of trace amounts of known and unknown compounds from the extracts of medicinal herbs [6–10].

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Although several reports have been published in the literature pertaining to the MS/MS analysis of SS, studies pertaining to the MS^n ($n \geqslant 3$) analysis of SS are rare. In this study, UHPLC-ESI-IT/MS^n spectrometry has been used to identify the SS from *D. panthaica*. Ten reference SS were used to study the fragmentation features of SS in both the positive and negative ionization modes. Using these data, 23 SS were identified from the water extract *D. panthaica*, including 11 potential new compounds and six groups of isomers. Notably, these compounds were identified within a 30-min run time from a fraction eluted with 65% ethanol from the original water extract of *D. panthaica*. Among the identified compounds, two compounds were selected as representative examples of the different structural classes and subsequently isolated to assess the effectiveness of our newly developed analytical technique. The chemical structures of these two compounds were confirmed by ^{1}H and ^{13}C NMR spectroscopic analysis and represent the first two compounds to be isolated from *D. panthaica*. Furthermore, a method to distinguish between furostane- and spirostane-type compounds was established during this study. Taken together, the results of the current study show UHPLC-IT/MS^n can be an efficient technique to accelerate the identification, isolation and structure determination of SS from plant material.

2. Experimental

2.1. Reagents and materials

The plant material was collected from Linquan County, Anhui province, China. The material was then authenticated and voucher specimens were deposited at the Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences.

HPLC grade acetonitrile and methanol used for UHPLC-IT/MS analysis were obtained from Fisher Scientific Co. (Fair lawn, New Jersey, USA). Formic acid was purchased from Anaqua Chemical Supply (UHPLC grade, Houston, TX, USA). Purified water was provided by Wahaha Co. (Hangzhou, Zhejiang, China). Dichloromethane and all of the other analytical-grade reagents were purchased from Sinopharm Chemical Reagent Beijing. Co., Ltd. (Beijing, China).

Ten reference steroid saponins were isolated, purified and fully characterized in our laboratory from dried *D. panthaica* rhizomes. Eight kilograms of dried rhizomes was ground into a fine power, which was extracted three times by refluxing with water. The combined extracts were filtered to remove any solids and then purified on a resin column, which was eluted in a step-wise manner with a mixture of ethanol and water (10:90, 65:35, 95:5 – v/v). The 65% ethanol fraction was collected and distilled to dryness to give a residue (110 g), which was purified by column chromatography over silica gel eluting with a mixture of chloroform and methanol (5:1, 3:1, 2:1 – v/v) to give Fr.1–3. Fr.4 was obtained by eluting the column with pure methanol. All four fractions were then individually purified over a silica gel column eluting with a mixture of chloroform/methanol or chloroform/methanol/water to give 10 subfractions. The subfractions were then further purified by preparative HPLC eluting with a mixture of methanol and water to give ten reference substances, including trillin, dioscin, gracillin, progenin III, progenin II, dioscoreside E, 26-O-β-D-glucopyranosyl-3β,20,26-triol-25(R)-furosta-5,22(23)-dien-3-O-α-L-rhamnopyranosyl(1 → 4)-[α-L-rhamnopyranosyl-(1 → 2)]-β-D-glucopyranoside (hsy-14), protodioscin, pseudoprotodioscin and dioscoreside D, respectively. The structures of these compounds were confirmed by MS and ^{1}H and ^{13}C NMR spectroscopic analyses, as well as a comparison with the literature data [12–20]. The purities of all of these compounds were determined to be >95% by HPLC analysis using UV detection.

2.2. Preparation of reference standards

The reference solutions were prepared by accurately weighing the above reference substances and dissolving them in HPLC grade methanol. All of the reference standards were made up at a concentration of 0.1 mg/mL.

2.3. Preparation of samples for UHPLC-IT/MS analysis

Dried *D. panthaica* rhizome power (8000 g) was extracted by refluxing with water (3 × 64 L for 2, 1 and 1 h) and the combined extracts were cooled to room temperature and then filtered to remove and solids. The filtrate was then loaded on to a column of AD-8 macroporous resin, which was washed with water, 10% ethanol, 65% ethanol and 90% ethanol. The fraction eluted with 65% ethanol was collected and evaporated to dryness to give a residue (see Fig. 1). A portion of the residue was accurately weighed into a volumetric flask and dissolved with 75% methanol in water to give a 1.0 mg/mL sample solution. The solution was centrifuged at 3000×*g* for 10 min at room temperature and the supernatant was sampled for analysis by UHPLC-IT/MS^n. The remaining residue was used for the isolation of novel compounds.

2.4. UHPLC-IT/MS^n analysis

UHPLC-IT/MS^n analyses were performed on an Agilent 1200 series UHPLC system (Agilent Company, USA) equipped with a binary solvent delivery manager and an autosampler. The samples were

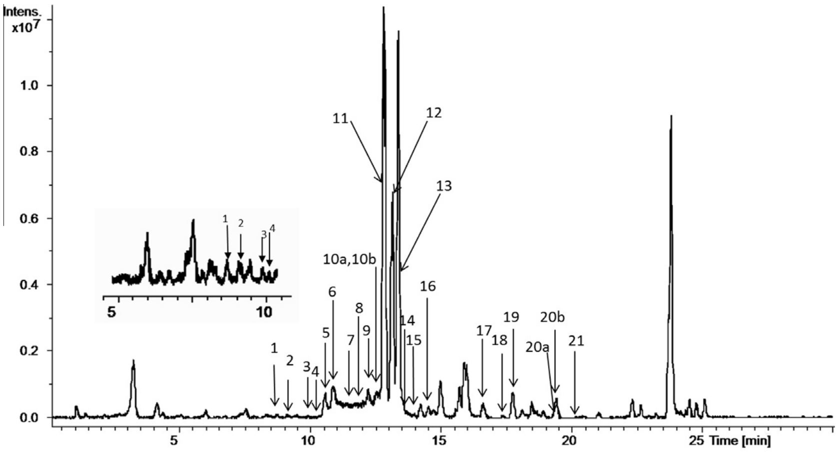

Fig. 1. The TIC (total ion chromatography) spectrum of 65% ethanol from the ethanol extract.