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Systematic identification of shikonins and shikonofurans in medicinal Zicao species using ultra-high performance liquid chromatography quadrupole time of flight tandem mass spectrometry combined with a data mining strategy

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ABSTRACT

Shikonin, shikonofuran and their derivatives are the main bioactive components of Zicao, a traditional Chinese herbal medicine prepared with the dried roots of *Lithospermum erythrorhizon*, *Arnebia euchroma* or *Arnebia guttata*. However, approaches on the systematic discovery and identification of shikonins and shikonofurans, especially unknown ones, are still not available. To address this issue, the gas-phase CID-fragmentation routes for the shikonins and shikonofurans were established by using ESI-QTOF-MS in the negative ion mode and low-energy collision induced dissociation tandem mass spectrometry (CID-MS/MS) in this study using seventeen standards. As a result, diagnostic product ions for rapid discovery and classification of shikonins and shikonofurans were determined. In addition, various mobile phase compositions and UHPLC elution programs were evaluated to achieve optimal separation efficiency and detection response of these types of analytes. Based on these findings, an integral approach using UHPLC-ESI-QTOF-MS and CID-MS/MS analyses together with a novel two steps data mining strategy was developed for systematic analysis of shikonins and shikonofurans in complex samples. Consequently, 58 compounds including 32 novel ones were efficiently discovered and identified from the crude extract of Zicao. Moreover, comparative analyses of the 58 chemical components in three Zicao species including *Lithospermum erythrorhizon*, *Arnebia euchroma* and *Arnebia guttata* samples were conducted using the established analytical method, which can be instructive for future utilization of Zicao and its related medicinal products.

1. Introduction

Shikonins, including shikonin, its enantiomer alkannin, and their numerous ester derivatives, are classified as naphthoquinones, most commonly found in the root epidermis of at least 150 species belonging to the genera *Arnebia*, *Alkanna*, *Lithospermum*, *Echium*, *Onosma*, *Anchusa* and *Cynoglossum* of the Boraginaceae family [1]. Shikonofurans are furylhydroquinone derivatives and are closely related to shikonin derivatives with regard to biosynthetic pathways. Shikonins and shikonofurans are of particular abundance in the three species including *Lithospermum erythrorhizon* Sieb. et Zucc (LE), *Arnebia euchroma* (Royle) Johnst. (AE), and *Arnebia guttata* Bunge (AG), used interchangeably known as 'Zicao' in the Chinese Materia Medica. Zicao have been used for thousands of years for the treatment of macular eruptions, measles, sore throat, carbuncles, burns, skin ulcers, inflammations [2–4] and recently for the treatment of cancer [5,6]. In addition, shikonins and shikonofurans have exhibited extensive antimicrobial, anti-inflammatory and antitumor activities [7]. Because of the importance of the shikonins and shikonofurans in the pharmaceutical, cosmetic and food industry [8,9], it is crucial to establish a reliable analytical approach for efficient discovery of these

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compounds in Zicao from various sources. Meanwhile, this method can be applied to distinguish different Zicao species and to assess the quality of raw materials of Zicao from various sources.

A variety of methods including semi-preparative TLC, HPLC-VIS, HPLC-ESI-MS, High-speed Counter-current Chromatography approaches were used to analyze the chemical components in Zicao species, with a focus on the separation and isolation [10,11], quantitative analysis [1,12–14], and pharmaceutical activity evaluation [15–18] of a few shikonin compounds. So far, there are no reports available on systematic characterization of shikonins and shikonofurans and on comparative analyses of these bioactive components among the three different Zicao species.

In this study, the characterization of shikonins and shikonofurans was carried out using ultra-high liquid performance chromatography coupled to electrospray ionization source (in the negative ion mode) mounted on a quadrupole-time-of-flight hybrid mass spectrometer instrument operating in CID-MS/MS mode, which provided accurate mass measurement of both precursor and product ions for individual components. The gas-phase CID-MS/MS fragmentation routes of seventeen shikonins and shikonofurans were systematically studied through direct infusion and LC-ESI-MS analysis. The LC-ESI-MS and CID-MS/MS conditions were optimized for the improvement of separation efficiency and detection sensitivity. Based on the CID-MS/MS fragmentation routes and comprehensive analyses of these compounds, a novel two-step data mining strategy was developed for the rapid discovery, classification, and identification of shikonins and shikonofurans. A pooled sample was prepared with Zicao from twenty-two sources. Then, this pooled sample was used to establish the analytical method for systematic screening and characterization of shikonins and shikonofurans in crude extract of Zicao samples. As a result, 58 compounds including 32 novel ones were efficiently discovered and identified from the pooled Zicao sample. In addition, the individual Zicao samples from three different Zicao species were compared with regard to the 58 chemical components identified.

2. Experimental

2.1. Chemicals and reagents

Acetonitrile and methanol of HPLC grade were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Deionized water was produced by a Milli-Q water system (Millipore, Bedford, MA, USA). Formic acid (≥96%) and ammonium formate of analytical grade were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). The seventeen reference standards of shikonins and shikonofurans including shikonofuran A (**3**), shikonin (**6**), shikonofuran D (**13**), shikonofuran E (**18**), 1-methoxyacetylshikonin (**21**), shikonofuran B (**22**), shikonofuran C (**23**), β-hydroxyisovalerylshikonin (**24**), acetylshikonin (**28**), propionylshikonin (**35**), deoxyshikonin (**37**), β-acetoxy-isovalerylshikonin (**41**), isobutyrylshikonin (**45**), β,β-dimethylacrylshikonin (**51**), α-methylbutyrylshikonin (**52**), isovalerylshikonin (**53**), and 6-(11′-deoxyalkannin)-alkannin/shikonin acetate (**56**) (Fig. 1) were in-house isolated from the crude extract of AE, AG and LE. Their chemical structures were elucidated on the basis of comprehensive spectral analysis of HR-ESI-MS, ^{1}H NMR and ^{13}C NMR data, and by comparison with the data previously published [19–22]. Their absolute configurations were not determined in this study. The purities of these standards were above 98% according to HPLC-UV analysis. Standard solutions were prepared in methanol at a concentration of 1.00 μg/mL, respectively.

Table 1
Three different chromatographic conditions.

No.	HPLC gradient conditions
(1)	30% B (0–0.1 min), 30–60% B (0.1–15.0 min), 60–95% B (15.0–25.0 min), 95% B (25.0–30.0 min), 95–30% B (30.0–30.1 min), 30% B (30.1–35.0 min)
(2)	20% B (0–0.1 min), 20–50% B (0.1–10.0 min), 50–60% B (10.0–30.0 min), 60–95% B (30.0–35.0 min), 95% B (35.0–40.0 min), 95–20% B (40.0–40.1 min), 20% B (40.1–45.0 min)
(3)	30% B (0–0.1 min), 30–50% B (0.1–15.0 min), 50–70% B (15.0–45.0 min), 70–95% B (45.0–50.0 min), 95% B (50.0–55.0 min), 95–30% B (55.0–55.1 min), 30% B (55.1–60.0 min)

A Welch Ultimate UHPLC C18 column (100 mm × 2.1 mm, 1.7 μm) was evaluated under various HPLC gradient conditions (Table 1) with a flow rate of 0.3 mL/min. The mobile phase consisted of water containing 0.01% formic acid with 5 mM ammonium formate as mobile phase A (A) and acetonitrile as mobile phase B (B).

2.2. Plant materials and sample preparation

A total of twenty-two Zicao herbs grown in different geographic areas of China, Pakistan, Afghanistan and Russia were obtained from the herbal medicine markets in Bozhou city, Anhui province and Anguo City, Hebei province, China and authenticated by the authors (Table S1). The voucher specimens were deposited at the herbarium of Huazhong University of Science and Technology. The raw materials of the dried Zicao root from each location were separately pulverized into powders and sieved through a No. 40 mesh. 2.0 g powder from each sample was extracted with 20.0 mL of methanol by sonication for 45 min at room temperature. The supernatant of these samples was individually filtered and centrifuged at 10,000 rpm for 10 min. A pooled Zicao sample combined with 200 μL of supernatant from each of these twenty-two samples was used for establishing an analytical method for systematic identification of shikonins. The supernatant obtained from three individual Zicao species was used for comparative analyses. 10.0 μL of the final supernatant was injected for UHPLC-ESI-QTOF-MS and CID-MS/MS analyses.

2.3. LC-ESI-MS and CID-MS/MS analyses conditions

The LC analyses were conducted on a Shimadzu UHPLC system (Kyoto, Japan), equipped with a LC-30AD solvent delivery system, a SIL-30AC autosampler, a CTO-30A column oven, a DGU-20A$_3$ degasser, a CBM-20A controller, and a Welch Ultimate UHPLC C18 column (100 mm × 2.1 mm, 1.7 μm). The column oven temperature was set at 40 °C. The mobile phase consisted of water containing 0.01% formic acid and 5 mM ammonium formate as mobile phase A (A) and acetonitrile as mobile phase B (B). As listed in Table 1, three HPLC gradient programs were evaluated to optimize chromatographic separation. The flow rate was set at 0.3 mL/min.

Mass spectrometric detection was performed in negative electrospray ionization (ESI) mode on a Triple TOF™ 5600 system with a Duo Spray source (AB Sciex, Foster City, CA, USA). The ESI-MS conditions were as follows: ion spray voltage, −4.5 kV; ion source temperature, 500 °C; curtain gas, 30 psi; nebulizer gas (GS 1), 50 psi; heater gas (GS 2), 50 psi; declustering potential (DP), −60 V. The mass ranges were set at *m/z* 100–800 for ESI-TOF-MS scan, 50–800 for CID-MS/MS experiments. In the ESI-TOF-MS-IDA-9-CID-MS/MS experiments, the most intensive 8 ions from each ESI-TOF-MS scan were selected for CID-MS/MS scan. Dynamic background subtraction (DBS) was used to match the information dependent acquisition (IDA) criteria. The collision energy was set at −30 eV with a collision energy spread (CES) of 15 eV for CID-MS/MS experiments. The data was analyzed by Peak View Software™ 1.2 (AB Sciex, Foster City, CA, USA).