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On-line strategies for the identification of unknown flavone glycosides in *Dracocephalum tanguticum* Maxim

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ABSTRACT

In this text we present a series of LC–MS/UV-based strategies for on-line structure identification of unknown flavone glycosides in *Dracocephalum tanguticum*. The aglycone portion, the glycosylation position, the order of sugars and the identity of sugars of each glycoside were unambiguously identified. The new strategy—identifying the partial structure of a molecule by comparison with a different compound possessing the same assumed partial structure—is quite effective for structure identification of the aglycone of **1**–**4**; the unusual non-radical $[Y_0–H–CH_2]^-$ ion observed for the methoxylated flavonoid glycoside **8** is quite useful for structure elucidation of the aglycone of **5**–**8**; the UV data obtained on-line with post-column addition of UV-shift reagents are powerful for determination of the glycosylation and methoxylation positions for each flavone glycoside. Finally, isolation and subsequent individual analysis by NMR were performed to provide complementary information for the configurations of each sugar and the linkage types of each disaccharide. Thus, the structures of the eight detected flavone glycosides, which all have never been reported in *D. tanguticum* before, were positively identified.

1. Introduction

Flavonoids have been considered as an important part of human diet and the active principles of many medicinal plants. They exist in almost all green plants, and are mainly present as O- or C-glycosides. All flavonoids contain a $C_6–C_3–C_6$ skeleton, but present numerous forms because of the differences in the number and the position of hydroxyl, methoxyl, glycosyl and other substitutes. Modern epidemiological and pharmacological studies have shown that flavonoids exhibit diverse biological activities and potentially beneficial effects. In particular, flavonoids have been known for a long time acting as effective natural antioxidants to scavenge reactive oxygen species [1–3].

Our main interest in this text is in the flavonoids in *Dracocephalum tanguticum* Maxim (*D. tanguticum*), a perennial herb of the Labiatae family, which is an endemic species distributing on the Qinghai-Tibet Plateau of China. *D. tanguticum* is widely used in traditional Tibetan medicine for the treatment of hepatitis, gastritis, dizziness, arthritis and ulcer, under the name “zhi yangge” in Tibetan. However, to the authors’ knowledge, there are only three papers concerning phytochemical investigation of this plant, resulting in the isolation of several triterpenoids, steroids, and flavonoids [4–6].

For conventional phytochemical investigation, a process including isolation, purification, and individual analysis is required. This process is time-consuming, laborious and costly, and it is usually hard to achieve a comprehensive investigation of the plant studied. Thus, on-line structure identification of active substances in complex biological matrixes including plants, based on chromatographic separation-hyphenated techniques, such as liquid chromatography–mass spectrometry (LC–MS) and liquid chromatography–diode array UV detection (HPLC–UV), have developed rapidly in the past two decades. The LC–MS technique provides important advantages because of the combination of the separation capability of LC and the power of MS as an identification and confirmation tool. Careri et al. [7], Stobiecki [8], de Rijke et al. [9] and Marcha et al. [10] have reviewed the use of MS for structure identification of flavonoids. For this use, an ion trap MS is usually selected due to its property of providing multiple-stage MS spectra. The fragmentation patterns of protonated or deprotonated flavonoid glycosides can be used to determine the number, the linked order and the tentative identity of the saccharide moieties [11–13]. Furthermore, the well-known retro Diels-Alder (RDA) MS fragmentation of flavonoids is very useful as it yields information about the distribution of substituents between the A ring and B ring [14–16]. LC–UV is quite informative for the sub-

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stitution patterns of flavonoids. In some cases, especially with the addition of UV-shift reagents by a post-column derivatization strategy, glycosylation and methoxylation patterns of flavonoids can be determined positively [17–20]. However, in most of the previous LC–MS/UV-based identification studies, complete structural information generally cannot be obtained, thus positive identification of the target flavonoid is usually carried out with the help of standards or previous literature data. Of note, given that many standards are not commercially available and many plants lack sufficient scientific researches before, such as *D. tanguticum*, the unequivocal on-line identification of active substances in phytochemical research is still an analytical challenge.

In this text, on-line structure identification of unknown flavonoids in *D. tanguticum* was carried out. Such a study has two purposes, one to conduct a phytochemical investigation of *D. tanguticum*, one to propose a series of strategies for on-line structure identification of unknown flavonoids in a complex sample based on LC–MS/UV techniques. Several strategies are classical ones and their efficiencies have been proved before, but some are new and are quite effective in this study. By integrating all the information obtained, the structure of eight flavone glycosides in *D. tanguticum* were preliminary identified. Finally, the accuracy of the on-line results was demonstrated by the off-line analysis of pure substances by NMR after a preparative isolation process. The eight flavone glycosides are all reported in *D. tanguticum* for the first time.

2. Experimental

2.1. Plant and chemical materials

D. tanguticum (the aerial parts) was provided by Tibet Autonomous Region Institute for Food and Drug Control, and was identified by Professor Jianzhong Li, Department of Botany, Hunan Normal University, China. Caffeic acid, resorcinol, hydroquinone and catechol were purchased from Shanghai Usea Biotech Company (Shanghai, China). HPLC-grade acetonitrile (ACN), purchased from Tedia Company (Fairfield, Ohio, USA), and ultrapure water, prepared by a Millipore Milli-Q purification system (Millipore Corp., Bedford, MA), were used to prepare the mobile phase. Other reagents including formic acid, aluminium chloride ($AlCl_3$), sodium acetate (NaOAc) and hydrochloric acid (HCl) were all analytical grade.

2.2. Sample preparation

The aerial parts of *D. tanguticum* were powdered. 1 g of the powders was mixed with 50 mL of methanol and sonicated for 60 min. Then the supernatant was filtered through a 0.45 μm filter for HPLC analysis.

2.3. HPLC–UV/MS(MS^n) analysis

Chromatographic separation was performed on a Waters Alliance 2695 HPLC system equipped with a photodiode array 2996 PDA detector (Milford, MA, USA), using a 5 μm Ultimate XB C_{18} column (250 mm × 4.6 mm) (Welch Materials, Ellicott, USA). A two-component linear gradient method with ACN–H_2O (+0.05% formic acid) (20:80 during 5 min, 20:80–35:65 in 15 min and 35:65–60:40 in 10 min) using a flow rate of 1.0 mL/min was used to separate the flavone glycosides in *D. tanguticum*. The injection volume was 10 μL. The on-line MS and MS^n data were obtained based on a Finnigan LTQ linear ion trap mass spectrometer with an ESI source (San Jose, CA, USA) connected to HPLC. The outlet of the UV detector was split, only 0.2 mL/min of the effluent was delivered into the Finnigan LTQ MS. The LTQ was used as following condition: in positive ESI mode, spray voltage, 4.5 kV, sheath gas flow rate, 40 arbitrary units, auxiliary gas flow rate, 15 units, heated capillary temperature, 300 °C, capillary voltage, 15 V; in negative ESI mode, spray voltage, 3.5 kV, sheath gas flow rate, 40 units, auxiliary gas flow rate, 15 units, heated capillary temperature, 300 °C, capillary voltage, 10 V. For full scan MS analysis, the spectra were recorded in the range of *m*/*z* 100–1000. To obtain MS^n data, the isolation width of precursor ions was set at 2.0 U and the relative collision energy was adjusted to 40% of maximum.

2.4. HPLC–UV analysis with post-column addition of UV-shift reagents

The HPLC separation condition was similar to Section 2.3 except for no addition of formic acid in the aqueous phase. A Shimadzu LC-6A pump (Shimadzu, Kyoto, Japan) was used for post-column addition of UV-shift reagents with a flow rate of 0.3 mL/min. Derivatization reactions of aqueous aluminium chloride (4%) with and without addition of hydrochloric acid (0.05%) were both performed with a reaction coil (10 m × 0.25 mm) at 90 °C, while this of aqueous sodium acetate (4%) was performed without a reaction coil at ambient temperature.

2.5. Fraction collection and GC–MS analysis

20 μL of the extract solution was injected into the HPLC, and the separation condition was the same as Section 2.3. The eight flavone glycosides detected were all fractionated. Fractions from three runs were combined and the eluent was evaporated to dryness in an 80 °C water bath under nitrogen.

Methanol (100 μL), deionized water (80 μL) and 6 M HCl (20 μL) were added to the residue (final concentrations, 0.6 M HCl in 50% methanol). Then the mixture was hydrolyzed in an 80 °C water bath block for 30 min. The hydrolyzed solution was evaporated to dryness in an 80 °C water bath under nitrogen and the residue was kept in the evaporator overnight. Derivatization of the sugars in the residue was performed with 50 μL of 1-(trimethylsilyl)-imidazole and 50 μL of pyridine by stirring at 60 °C water bath for 10 min. After drying the solution with a stream of N_2, the residue was partitioned between H_2O and CH_2Cl_2 (200 μL, 1:1, v/v), and the CH_2Cl_2 layer was injected into an Agilent 6890 gas chromatograph with a 5795 mass spectrometer (Agilent, CA, USA) using a L-Chirasil-Val column (dimethylsiloxane with L-valine buid into the siloxane chain, 25 m × 0.32 mm). Temperature of the injector was 200 °C. A temperature gradient system was used for the oven, starting at 110 °C for 1 min and increasing up to 200 °C at a rate of 5 °C/min. Helium was used as the carrier gas. The electron impact (EI) MS was operated in scan mode (scan range, 50–800 *m*/*z*) with the ionization energy set at 70 eV, and the MS interface and source temperatures were maintained at 250 and 200 °C, respectively. Identification of each sugar was based on retention times in GC–MS and on comparison with mass spectra of authentic sugar standards.

2.6. Preparative isolation and individual NMR analysis

The aerial parts of the plants (1 kg) were air-dried, powdered and refluxed at 80 °C for three times with methanol for 2 h each. After filtration, the methanol extract solutions were evaporated to dryness under reduced pressure, and the residues were partitioned between chloroform and water. The resulted aqueous layer was subjected to a polyamide column (80–100 mesh, 4 cm × 55 cm) eluting with a step gradient of ethanol–water (0:10, 2:8, 3:7 each 3 L) to afford three fractions (**a-1**, **a-2**, and **a-3**). HPLC analysis of the three fractions revealed that fraction **a-3** is a mixture of compound **4** and **8**, while fraction **a-2** contains the other six flavone glycosides. Frac-