Journal of Pharmaceutical and Biomedical Analysis 48 (2008) 205–211

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

Short communication

Analysis of iridoid glucosides in *Hedyotis diffusa* by high-performance liquid chromatography/electrospray ionization tandem mass spectrometry

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ARTICLE INFO

Article history:
Received 9 January 2008
Received in revised form 6 May 2008
Accepted 6 May 2008
Available online 20 May 2008

Keywords:
HPLC/MS
Tandem MS
Hedyotis diffusa
Iridoid glucosides

ABSTRACT

An HPLC-DAD-ESI-MS/MS method was developed for analysis of iridoid glucosides (IGs) from *Hedyotis diffusa* Willd. The optimized separation condition was achieved with the Complex Sample Analysis Software System (CSASS) software, under which the whole analytes were achieved complete resolution especially for some isomeric IGs. Based on the UV and fragmentations, eleven IGs were detected. According to the fragmentation patterns of the three standard IGs, especially those of the isomeric standards, seven IGs including three pairs of isomers were unambiguous/tentatively identified. For the isomeric IGs with methyl ester or carboxyl group at C-4, the extents of the losses of CH_3OH and/or H_2O from their molecular and/or the aglycone adducts are useful for the differentiation of the stereoisomers in positive ion (PI) mode, which depends on the stereochemistry of the hydroxyl group on the cyclopentanoid unit.

1. Introduction

Hedyotis diffusa Willd has been known as a traditional Chinese medicine (TCM) for a long time, and used as a treatment for cancer and inflammations such as appendicitis, urethritis, and bronchitis, due to its antibacterial activity [1–4]. Recently, this herb has gained increasingly attention to further effectively utilize its usage as a treatment for tumours [5]. Up to now, three major classes of compounds, including iridoid glucosides (IGs), flavonoids, and anthraquinones, have been reported as bioactive compounds from this herb [6,7].

IGs are one of the most important classes of natural products with bioactivities of anti-oxidation [8], neuroprotective activity [7], anti-inflammatory and immunomodulatory [9], etc., and some of them have entered clinical trials [10]. Up to now, the characterization of IGs from *H. diffusa* is almost using the NMR spectra of the pure compounds obtained by preparative isolation and purification [5,7,11,12]. But this method wastes time and energy, and some of the minor compounds cannot be purified. Recently, LC–MS has become a very convenient technique for the identification of plant secondary metabolites. The method has advantages of higher sensitivity and providing molecular weight (MW) and structural information on-line. It, however, has not been greatly used for the analysis of IGs from *H. diffusa*.

In this work, we studied the application of HPLC-DAD-ESI-MS/MS for the analysis of IGs from *H. diffusa*. As some of IGs are isomeric, it is very important to achieve completely spectral resolution of them for acquiring apart their UV and mass spectra. The optimized elution curve was achieved with five different gradient programs using the CSASS software (Complex Sample Analysis Software System, Dalian Institute of Chemical Physics, Chinese Academy of Science, Dalian, China). Referred to the fragmentation patterns of the isolated standards, the structures of the IGs detected from *H. diffusa* were discussed.

2. Experimental

2.1. Chemicals and reagents

HPLC-grade acetonitrile and methanol were purchased from Fisher Scientific (Loughborough, Great Britain). Water used for liquid extraction was deioned water and Milli-Q water was used for LC/MS analysis. Formic acid (HPLC grade) was purchased from TEDIA (USA). Deacetylasperuloside acid methyl ester (DAME), scandoside methyl ester (SME) and scandoside (SD) were used as standards, isolated from the extract of *H. diffusa* by our research group, and identified by ^{1}H-NMR, ^{13}C-NMR, MS, UV spectra and comparison with those reported in the literatures [8,13,14]. Macro-

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doi:10.1016/j.jpba.2008.05.013

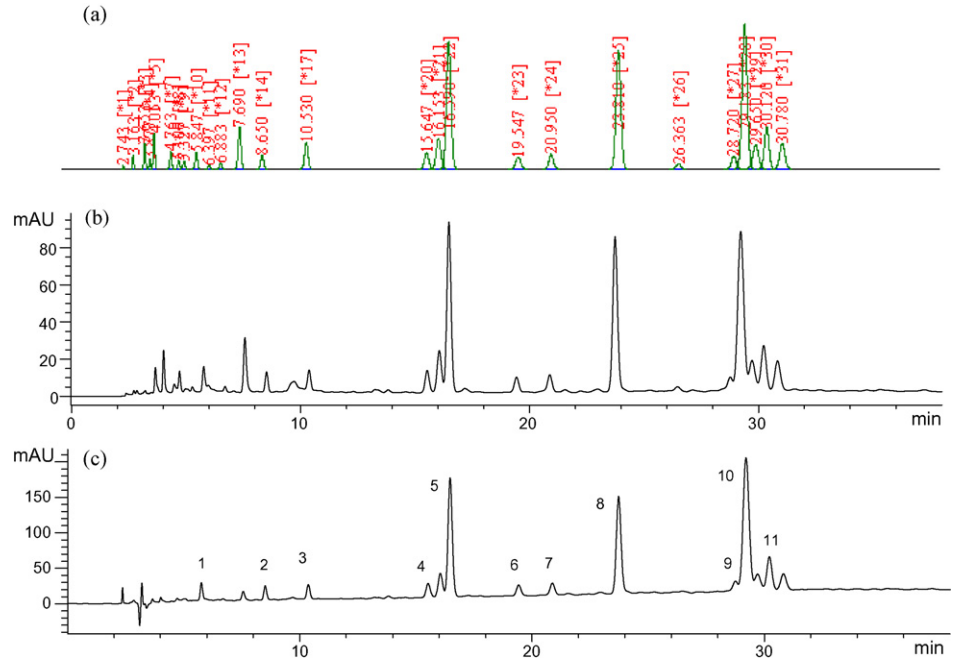

Fig. 1. Chromatograms of the extract of *H. diffusa* (a) simulated, at 254 nm; experimental: (b) at 254 nm, (c) at 235 nm.

porous resins D4020 used were purchased from Chemical Plant of Nankai University (China).

2.2. Materials

The aerial parts of *H. diffusa* (Willd.) were collected from Hengdong County, Hunan province (China) in May 2005. The herb was authenticated by Institute of Traditional Chinese Medicine, China Academy of Chinese Medical Sciences. The voucher specimens were deposited at Dalian Institute of Chemical Physics, Chinese Academy of Sciences, China.

The CSASS software was applied for optimizing the chromatographic condition.

2.3. Sample preparation

Dried and powdered samples (2 kg) of *H. diffusa* were extracted with tenfold water under reflux for twice, and each reflux time was 2 h. After filtration of the aqueous fraction, the filtrate was concentrated. An aliquot (50 g) of the extract was dissolved in 70% EtOH (v/v, 500 mL). The supernate was filtered and evaporated under reduced pressure. The residual aqueous solution was partitioned with EtOAC, *n*-BuOH, 70% EtOH in turns. The 70% EtOH fractions were subjected to macroporous resin D4020 and successfully eluted with H_2O and different concentrations of EtOH. The 35% EtOH fraction was concentrated to dryness under reduced pressure. In this work, an aliquot (5 mg) of the dry sample of 35% EtOH fraction was dissolved in 10 mL of water and filtered through a membrane filter (0.22 μm) prior to HPLC/MS analysis.

2.4. HPLC-DAD-ESI-MS analysis

2.4.1. Mass spectrometry analysis

All MS experiments were conducted on a MSD Trap XCT mass spectrometer equipped with electrospray ionization (ESI) interface (Agilent Technologies, USA). Both the auxiliary and sheath gases were nitrogen with a flow rate of 8 L/min. The dry gas temperature was set at 325 °C, and the nebulizer pressure was set at 35 psi. Spectra were recorded in positive and negative ion (PI and NI) modes at a spray voltage of +4.5 and −4.5 kV, respectively. The mass scan range was between *m*/*z* 100 and 1000.

2.4.2. Chromatographic conditions

HPLC-DAD measurements were carried out using Agilent 1100 liquid chromatography (Agilent Technologies), consisting of an HPLC quaternary pump, an autosampler, a column oven, and a diode array detector. Chromatograms were monitored at wavelengths of 235 and 254 nm, and UV spectra were recorded between 200 and 400 nm. Separation was performed on a C18 column (250 mm × 4.6 mm i.d., 5 μm, Chrom expert company, USA) and the column temperature was set at 30 °C. On the basis of the eluent system with optimized ionization efficiency, the linear gradient consisted of A (0.1% (v/v) formic acid in aqueous phase) and B (0.1% (v/v) formic acid in methanol) with a flow rate of 1.0 mL/min. Five linear gradient program conditions were investigated in (a)–(e) to obtain the optimized separation with the CSASS software.

(a) 5–25% B in 50 min, then to 95% B in 10 min;
(b) 7–25% B in 50 min, then to 95% B in 10 min;
(c) 8–25% B in 50 min, then to 95% B in 10 min;
(d) 9–25% B in 50 min, then to 95% B in 10 min;
(e) 10–25% B in 50 min, then to 95% B in 10 min.

2.5. Preparation the IGs from the extract of H. diffusa

In order to validate the structures of the IGs tentatively identified from the extract of *H. diffusa*, the isolation of the IGs was performed by AutoPurification System (Waters, USA) using a four-channel sample manager with four XTerra MS C18 columns (250 mm × 19 mm i.d., 5 μm, Waters, USA). The elution system consisted of A (0.1% (v/v) formic acid in aqueous phase) and B (0.1% (v/v) formic acid in methanol) with a flow rate of 16.4 mL/min. The linear gradient program was: 3% B, holding for 10 min, then to 10% B in 20 min, holding for 20 min, and finally to 95% B in 3 min. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a Bruker DRX-400 spectrometer.