Simultaneous Analysis of Xanthone Glycosides in *Halenia elliptica* by HPLC–DAD-ESI-MS

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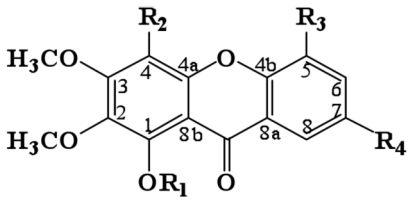
Abstract

A new, simple, and sensitive high-performance liquid chromatography–diode array detector (HPLC–DAD) method was developed for the simultaneous determination of six major xanthone glycosides in *Halenia elliptica*. The chemical profile of six xanthone glycosides, including 2,3,5-trimethoxy-1-O-primeverosyloxyxanthone (Analyte 1), 2,3,4,5-tetramethoxy-1-O-primeverosyloxyxanthone (Analyte 2), 2,3,5,7-tetramethoxy-1-O-primeverosyloxyxanthone (Analyte 3), 2,3,7-trimethoxy-1-O-primeverosyloxyxanthone (Analyte 4), 2,3,4,7-tetramethoxy-1-O-primeverosyloxy-xanthone (Analyte 5), and 2,3,4,5,7-pentamethoxy-1-O-primeverosyloxy-xanthone (Analyte 6) were acquired by using HPLC–DAD coupled to an electrospray ionization mass spectrometer. The analysis was performed on a Kromasil C_{18} column (5 µm, 250 ×4.6 mm i.d.), using acetonitrile–H_2O (25:75, v/v) as the mobile phase at a flow rate of 0.8 mL/min. Under UV detection at 260 nm, the recoveries of the analytes were in the range of 96.1–100.3%, the LODs were within 0.20 µg/mL, and all the xanthone glycosides showed good linearity ($r \geq 0.9990$) in a relatively wide concentration range; the intra-day relative standard deviations (%) ranged from 0.65 to 1.39%, and the inter-day RSDs% were not higher than 5%. The proposed method is suitable for quantitative and qualitative determination of the xanthone glycosides in *H. elliptica*.

Introduction

Species of the Gentianaceae family are known to contain secoiridoids and xanthones (1–5). Xanthones is a kind of natural products with polyphenolic structure and is of special interest for having many pharmacological effects, such as mono-aminooxidase (MAO) inhibition, antitumor, antibacterial, antioxidant, antifungal, and anti-inflammatory properties (6,7). Due to their strong bioactivities, those species in Gentianaceae containing xanthone compounds have been used as herbal medicines for the treatment of many diseases. For these reasons, the investigation of Gentianaceae has been one of many subjects in our laboratory (8,9). The genus *Halenia* (Gentianaceae) contains about 100 species, which are mainly distributed in America and are also found in Asia and Europe. *H. elliptica* is an important traditional Tibetan herbal medicine and has been used for the treatment of liver inflammations, stomach complaints, and fevers from contagious disease (10). Previous studies show that *H. elliptica* is rich in xanthone aglycones and glycosides (11–14). In our investigation of the plant, the methanol extract showed strong bioactive of antihepatitis. In order to trace the bioactive components in the methanol extract, the phyto-chemical study of this aerial part was carried out, and consequently six xanthone glycosides were isolated. Their structures (Figure 1) were elucidated by NMR spectroscopy together with high-resolution electrospray ionization-mass spectrometry (HR-ESI-MS) analysis and comparison of the spectroscopy data with those of corresponding compounds in the literature (5,15,16). Because of the outstanding effects of the xanthone compounds, developing the separation and determination methods for these constituents is of great significant for the crude drug's quality control.



Analyte	R_1	R_2	R_3	R_4
1	Primeverosyl	H	OCH_3	H
2	Primeverosyl	OCH_3	OCH_3	H
3	Primeverosyl	H	OCH_3	OCH_3
4	Primeverosyl	H	H	OCH_3
5	Primeverosyl	OCH_3	H	OCH_3
6	Primeverosyl	OCH_3	OCH_3	OCH_3

Figure 1. Molecular structures of analytes 1–6.

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Efficient detection and rapid characterization of natural products plays an important role as an analytical support in the work of natural products chemists. Existing methods for the analysis of xanthones involved the high-performance liquid chromatography (HPLC) (17,18), thin-layer chromatography (TLC) (19, 20), capillary electrophoresis (CE) (21, 22), and gas chromatography (GC) (23). Among several chromatographic methods, HPLC is the most widely used technique for both qualitative and quantitative analysis of phytochemical substance (24). During the past decade, HPLC and coupled techniques, especially dioda array detection (DAD) and mass spectrometry (MS), have been proved to be a powerful approach for the rapid identification of the constituents in natural materials because DAD and MS as a sensitive detector could provide abundant structural information and, thus, facilitate the structural identification of unknown compounds. There are several reports on the HPLC analysis of *H. elliptica* (25–27); however, to the best of our knowledge, no data have been reported on the simultaneous determination of these six xanthone glycosides in this plant.

In this paper, we described an HPLC method for identifying six major xanthone glycosides in *H. elliptica*. Using an HPLC–DAD-ESI-MS technique, the six xanthone glycosides were identified and characterized. The MS spectra and UV data obtained were applied to produce a library that allowed the complete identification of characteristic peaks in the chromatogram of *H. elliptica*. Their contents in *H. elliptica* also were determined by authentic standards and could be used to evaluate the crude drug's quality. In addition, a simple, rapid, and accurate analysis method is presented.

Experimental

Materials and reagents

H. elliptica was purchased from Xining city, Qinghai province, China. The reference xanthone glycosides, analytes 1, 2, 3, 4, 5, and 6 were isolated from the extracts of *H. elliptica* in our laboratory. Their chemical structures (Figure 1) were established on the basis of their HR-ESI-MS and NMR data and by comparing them with those of corresponding compounds in the literature. Purity analysis suggested that their purities were all > 98%.

HPLC-grade acetonitrile was purchased from J&K Chemical Ltd (Beijing, China). Water was distilled and deionized. All other organic solvents used in this study were of analytical-grade from Tianjing Chemical Reagent (Tianjing, China).

Apparatus

The HPLC system used for the xanthone glycosides separation was an Agilent 1100 HPLC system with DAD coupled with an LC–MSD Trap VL electrospray ionization mass spectrometer in series (Palo Alto, CA), equipped with quaternary pump, vacuum degasser, and autosampler. The chromatographic separation was performed on a Kromasil C_{18} column (5 µm, 250- × 4.6-mm i.d.) (Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian, China). The mobile phase consisted of acetonitrile–H_2O (25:75, v/v) at a flow rate of 0.8 mL/min. Analytes were injected via an auto-sampler, and eluate were monitored by a DAD detector set to scan between 200–400 nm. The effluent from the column passed through the DAD detector directly into the MS detector for analysis. Mass spectra were evaluated in negative mode, and parameters for acquisition of mass spectral data were the following: scan range, 100–1000 *m/z*; ESI needle voltage, 4 KV; nebulizing gas, N_2, 25 psi; dry gas, N_2, 8L/min; capillary temperature, 325°C; capillary offset voltage, –75 V; skimmer 1, –39 V; skimmer 2, –6 V. Chromatograms were processed based on Agilent ChemStation with Chemstation software. The temperature of the column during analysis was maintained at 25°C. The injection volume was 10 µL each time.

Standard solution preparation

To prepare the standard solutions, an accurately weighed amount of analyte 1, 2, 3, 4, 5, and 6 (3.0, 3.4, 1.7, 1.7, 2.6, and 2.4 mg, respectively) were dissolved in methanol (10 mL) for analysis. The stock solutions were stored at 4°C and brought to room temperature before their use. Calibration standard working solutions were freshly prepared by appropriate dilution of the stock solutions. Calibration graphs were plotted subsequently for linear regression analysis of the peak area with the concentration of the analytes injected.

Sample preparation

After being air dried and crushed into powder, 0.2 g of *H. elliptica* was accurately weighed and then ultrasonically extracted twice with 80% methanol (2 × 50 mL) for 30 min. The extract was combined, filtrated, then evaporated to dryness, and the residue was dissolved with 80% methanol and transferred to a 50-mL volumetric flask and diluted with 80% methanol to volume. The obtained solution was filtered through a membrane filter (0.45-µm pore size) prior to injection.

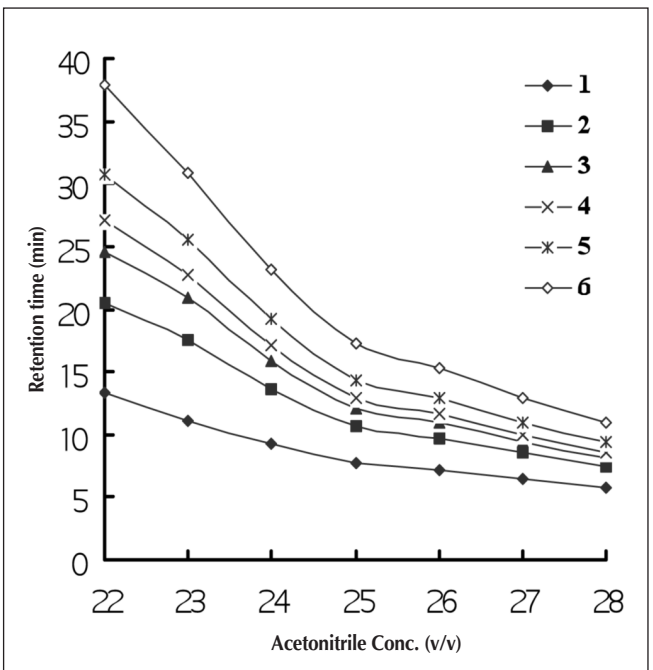

Figure 2. Effects of acetonitrile proportion on retention times of the analytes.

Results and Discussion

Optimization of chromatographic conditions

The choice of chromatographic conditions was guided by the need to obtain chromatograms with better resolution of adjacent peaks with a short analysis time, especially when numerous samples were to be analyzed. In the beginning, different proportional mixtures of water and methanol were used as the mobile phase, but the separation was not satisfactory. In an effort to achieve more rapid and efficient separation of six xanthone glycosides, much better resolution was achieved by the substitution of methanol with acetonitrile. The effects of the proportion of acetonitrile on the separation was investigated. Various proportions of acetonitrile, ranging from 22% to 28%, were tested for the separation of the six analytes. The retention time of these six xanthone glycosides was shortened with increasing acetonitrile proportion, while the resolution decreased (Figure 2). When the proportion of acetonitrile exceeded 25% (v:v), they could not be completely separated. Thus, 25% acetonitrile was selected to keep separation efficiency and shorten analytical time.

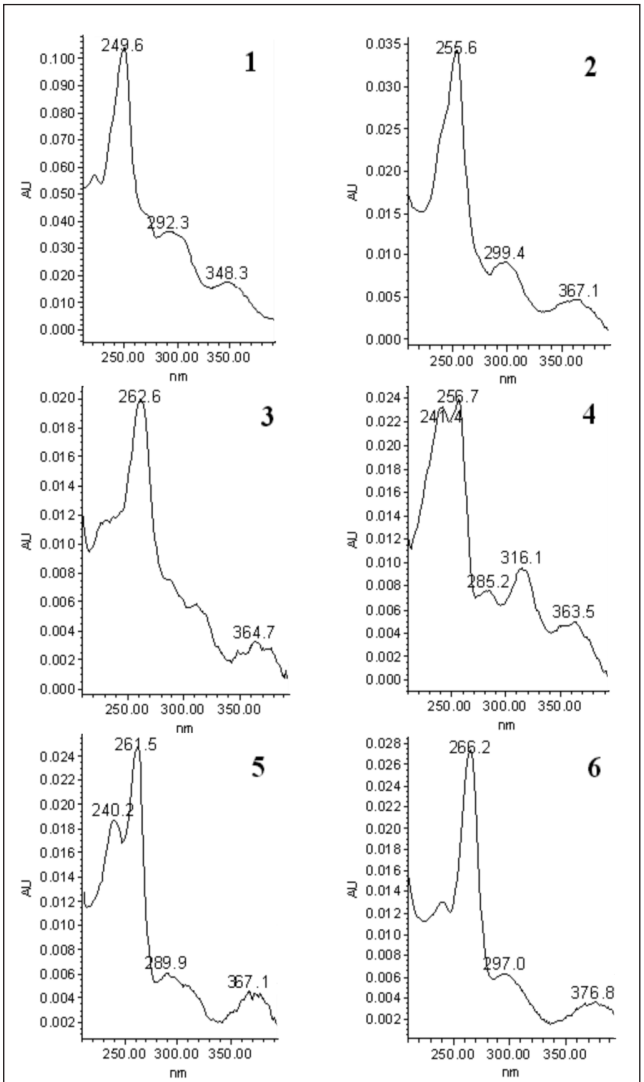

Figure 3. UV spectra of compounds 1–6.

DAD detection was employed at a wavelength range of 200–400 nm to investigate the UV spectra of the six xanthone glycosides. The attribution of the peaks to xanthones was unambiguous, as xanthones present characteristic UV spectra with four bands of decreasing intensity (15). These spectra are easily recorded during the LC separation of crude plant extract. UV spectra of analyte 1–6 are shown in Figure 3, and all the xanthone glycosides showed strong absorbance near 260 nm; thus, we select 260 nm as the detection wavelength. Figure 4 shows the chromatograms of xanthone glycosides and the 80% methanol extract of *H. elliptica*.

Identification of six xanthone glycosides in *H. elliptica*

In the present study, the effluent from the HPLC passed through the DAD detector directly into the MS detector for analysis. The MS spectra of major xanthone glycosides in *H. elliptica* were acquired in negative ion mode. Table I shows

Table I. HPLC–ESI-MS Data of the 80% Methanol Extract of *H. elliptica*

Peak	[M-H]⁻	[A-H]⁻	Fragments for MS^2 and MS^3
1	595	301	286, 271, 256, 243
Identification: 2,3,5-trimethoxy-1-O-primeverosyloxyxanthone			
2	625	331	316, 302, 301, 286, 271, 258, 215
Identification: 2,3,4,5-tetramethoxy-1-O-primeverosyloxyxanthone			
3	625	331	316, 301, 286, 273, 258, 245
Identification: 2,3,5,7-tetramethoxy-1-O-primeverosyloxyxanthone			
4	595	301	286, 271, 256, 243, 228, 200
Identification: 2,3,7-trimethoxy-1-O-primeverosyloxyxanthone			
5	625	331	316, 301, 286, 243, 230
Identification: 2,3,4,7-tetramethoxy-1-O- primeverosyloxyxanthone			
6	655	361	346, 316
Identification: 2,3,4,5,7-pentamethoxy-1-O-primeverosyloxyxanthone			

Table II. Results of Regression Analysis on Calibration Curves and the Detection Limits*

Analyte	Regression equation $y = ax + b$	r^2	Linear range (µg/mL)	LOD† (µg/mL)
1	$y = 8219x + 25109$	0.9995	4.69–300	0.17
2	$y = 19092x + 32960$	0.9997	5.06–340	0.12
3	$y = 9412x + 15160$	0.9996	2.66–170	0.18
4	$y = 15077x + 10430$	0.9995	2.66–170	0.14
5	$y = 17530x + 49342$	0.9995	4.06–260	0.15
6	$y = 5631x + 3533$	0.9995	7.50–120	0.20

* y = peak area; x = concentration of the analytes (µg/mL); r^2 = correlation coefficient.
† The detection limit was defined as the concentration where the signal-to-noise ratio is 3.

Table III. Contents of Desired Xanthone Glycosides in *H. elliptica* (n = 3)

Analyte	1	2	3	4	5	6
Content (%)	1.21	1.99	0.51	0.67	0.73	0.60

specific fragmentation behavior of the xanthone glycosides. For the six xanthone glycosides, clearly discernible $[M-H]^-$ ions were observed with the aglycone fragments $[A-H]^-$, which were the base peaks. The mass spectra of xanthone glycosides found in *H. elliptica* presented the loss of 294 corresponding to a primeverosyl residue, leading to the aglycone ion $[A-H]^-$. The fragments from the secondary order mass spectrum (MS^2) and third order mass spectrum (MS^3) indicated loss of one to five methyl groups and loss of carbon monoxide groups. As for analyte 1, six ions could be observed, *m/z* 595 $[M-H]^-$, *m/z* 301 $[A-H]^-$, *m/z* 286 $[A-CH_3-H]^-$, *m/z* 271 $[A-2 \times CH_3-H]^-$, *m/z* 256 $[A-3 \times CH_3-H]^-$, *m/z* 243 $[A-2 \times CH_3-CO-H]^-$. Again, the remaining xanthone glycosides (analyte 2–6) give similar mass spectral pattern. These results were in good agreement with those obtained for xanthone glycosides.

On the basis of the MS and UV spectra and comparison of the chromatographic retention times with those of authentic standards, the six xanthone glycosides were identified in *H. elliptica*.

Linear ranges and detection limits of the six xanthone glycosides

Table II lists the regression equation, correlation coefficients of linear calibration graphs, and the limits of detection (LOD) for the xanthone glycosides in optimum conditions. All the xanthone glycosides showed good linearity ($r \geq 0.9990$) in a relatively wide concentration range. In addition, the LODs for the six analytes were ranged from 0.12 to 0.20 µg/mL at a signal-to-noise ratio of 3.

Validation of the method

Precision test was carried out by replicated injections, and the results showed that relative standard deviation (RSD) of the peak area of the six xanthone glycosides were 0.79% for analyte 1, 0.88% for analyte 2, 1.04% for analyte 3, 0.86% for analyte 4, 0.65% for analyte 5, and 1.39% for analyte 6, respectively ($n = 5$).

The stability of the assay was evaluated by inter-day variability. The standard solution was analyzed on five consecutive days, and the RSDs of the xanthone glycosides were not higher than 5% ($n = 5$).

In order to verify the accuracy and precision of the analytical procedure, the recovery assays of the six xanthone glycosides were carried out by adding suitable amounts of the standards to the crude drug powder, which was treated according to the procedure described earlier. The average recoveries ($n = 5$) of the six xanthone glycosides were 98.4% for analyte 1, 96.1% for analyte 2, 97.6% for analyte 3, 100.0% for analyte 4, 96.5% for analyte 5, and 100.3% for analyte 6, respectively.

Evaluation of extraction efficiency

The dried plant material was extracted at room temperature using an ultrasonic bath. In order to obtain quantitative extraction, the influence of extraction solvent on the extraction efficiency was investigated. Pure and aqueous methanol (80%, 60%, 40%) was tried as the extraction solvents. The results showed that the best extraction solvent was found to be 80% methanol, which allowed extraction of all the xanthone glycosides in high yields while pure methanol and other aqueous methanol could not extract efficiently. The influence of extraction time on the extraction efficiency of the plant was also investigated. A 0.2 g sample of the plant was ultrasonically extracted once, twice, three times, and four times (each time for 30 min), respectively with 80% methanol. The results showed that all the xanthone glycosides were almost completely extracted after being ultrasonically extracted twice. Therefore, 80% methanol was selected as the extraction solvent, and extraction time was chosen as twice.

Quantification of xanthone glycosides in samples

The six predominant xanthone glycosides in *H. elliptica* were simultaneously determined by the proposed HPLC–DAD method. The quantitative analysis was performed by means of the external standard method. Typical chromatograms of the xanthone glycosides and the 80% methanol extract (ultrasonically extracted twice) of Tibetan herb *H. elliptica* were shown in Figure 4. The contents of the six xanthone glycosides in the samples were calculated and the results were shown in Table III with the mean values of the three replicated injections.

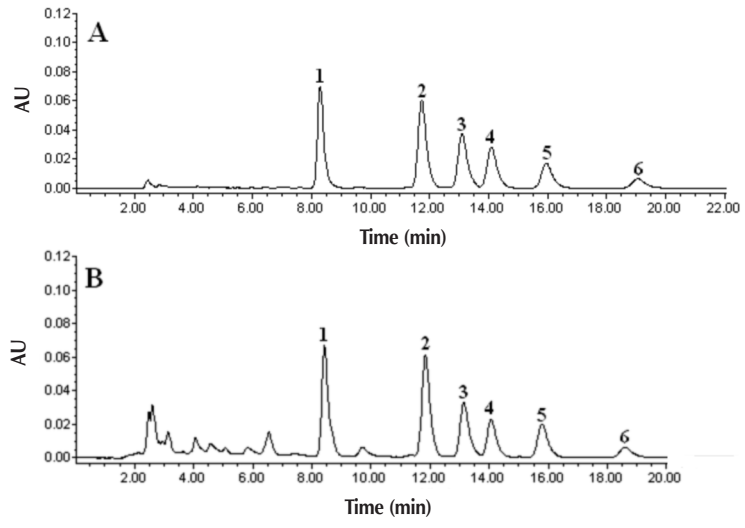

Figure 4. Chromatograms of standard (A) and the 80% methanol extract of the plant *H. elliptica* (B). Separation solution: acetonitrile–H_2O (25:75, v/v); Flow rate: 0.8 mL/ min; Detection wavelength: 260 nm.

Conclusion

In conclusion, we developed a new HPLC–DAD method for quantification of six xanthone glycosides in *H. elliptica* extracts and validated it. This method was fully compatible with MS detection and allowed us to quantify these six constituents in a sample of *H. elliptica*. Considering the bioactivities and amounts of these six xanthone glycosides in the plant, they can play an important role for the activity of *H. elliptica*. The HPLC method developed here represents an excellent technique for quality control of this Tibetan herbal medicine.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (NSFC No. 20475057) and National Key Technology Research and Development Program of China (No. 2007BAI37B05) and the Natural Science Foundation of Gansu Province (No. 3ZS051-A25-077).

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Manuscript received January 20, 2008;
revision received May 19, 2008.