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Short communication

HPLC determination and NMR structural elucidation of sesquiterpene lactones in *Inula helenium*

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ABSTRACT

A high performance liquid chromatography (HPLC) method was developed for the simultaneous quantification of three bioactive sesquiterpene lactones in *Inula helenium* L., namely igalane (**1**), isoalantolactone (**2**) and alantolactone (**3**). The HPLC separation was performed on an Agilent Zorbax XDB-C_{18} column (250 mm × 4.6 mm, 5 μm) with a mobile phase consisting of 55% acetonitrile and 45% water, at a flow rate of 1.0 ml/min, and detected at 210 nm. All three regression equations indicated good linear relationships ($r^2 \geq 0.9994$) between the peak area of each compound and its respective concentration. The HPLC assay was reproducible with overall intra- and inter-day variation of less than 2.85%. The recoveries, measured at three concentrations, varied from 96.60 to 104.43%. This assay was successfully applied to the determination of three bioactive sesquiterpene lactones in eleven samples. The results demonstrated that the assay developed herein was rapid, accurate, reliable and could be readily utilized as a quality control method for *I. helenium*. In addition, two minor isomers in *I. helenium* were isolated by semi-preparative HPLC. Their structures were elucidated on the basis of NMR analysis.

1. Introduction

Inula helenium L. (Compositae) is a widely distributed perennial herb in Europe, North American and East Asia [1]. Its roots have been used in the folk medicine against a variety of ailments including asthma, cough, bronchitis, lung disorders, tuberculosis, indigestion, chronic enterogastritis, infectious and helminthic diseases [2–5]. Recent pharmacological studies have demonstrated that the extract of *I. helenium* possesses anti-tumor, anti-bacterial and insecticidal activities [4–9]. The roots of *I. helenium* have been shown to contain high levels of eudesmanolide-type sesquiterpene lactones such as alantolactone and isoalantolactone [10]. In recent years, extensive investigations on constituents obtained from *I. helenium* demonstrated that sesquiterpene lactones are responsible for its various biological activities [4,5,8,11–13]. Therefore, the analysis of sesquiterpene lactones in *I. helenium* is of great significance to evaluate the quality of this medicinal plant.

Up to now, a number of techniques including gas chromatography (GC) [14–16], capillary electrophoresis (CE) [17,18], thin layer chromatography (TLC) [19] were applied to the analysis of *I. helenium*. However, these methods often suffer from limitations such as quantitative analysis on a few constituents, or poor resolution. Recently, Stojakowska et al. [20] reported a high performance liquid chromatography (HPLC) method detecting alantolactone and isoalantolactone in *I. helenium*, but this method was not applicable for quantitative determination due to a poor separation between two isomers. Furthermore, few papers on identification of two hardly separated minor isomers in *I. helenium* have been published. In this study, a simple and feasible method has been developed for the simultaneous quantification of three bioactive sesquiterpene lactones from *I. helenium* and for the quality control of this well-known herbal medicine.

2. Experimental

2.1. Materials and chemicals

Crude materials were collected from different provinces of China, and authenticated as *Inula helenium* L. by one of the authors (Prof. Xiaobo Li).

HPLC grade acetonitrile and methanol (Xingke Biochemistry Co., Ltd., Shanghai, China) were used for the HPLC analysis and sample preparation. Deionized water was obtained from a Milli-Q Water purification system (Millipore, MA, USA). All the other organic solvents used in this study were of analytical grade.

Igalane (**1**, 100 mg), isoalantolactone (**2**, 900 mg) and alantolactone (**3**, 800 mg) were isolated from *I. helenium* in our group. The purities of isolated compounds were all above 98% by HPLC analy-

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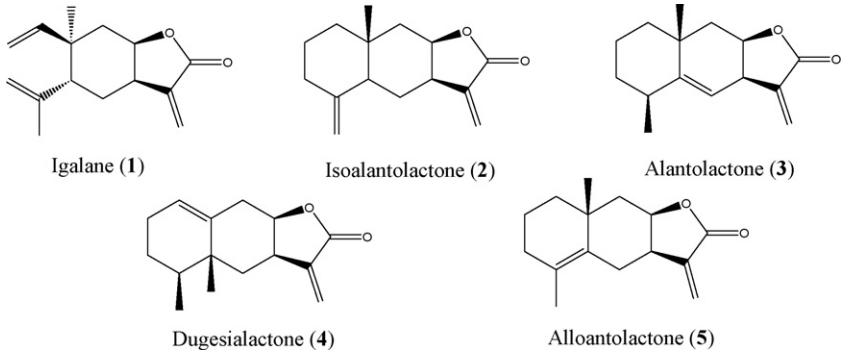

Fig. 1. Chemical structures of five investigated sesquiterpene lactones in *I. helenium*.

sis. The chemical structures of all three standards were confirmed by ^{1}H NMR and ^{13}C NMR [21,22], and shown in Fig. 1.

2.2. Apparatus and chromatographic conditions

An Agilent 1200 liquid chromatography system (Agilent Technologies Deutschland, Waldbronn, Germany), equipped with a quaternary solvent delivery system, an auto-sampler and a DAD detector, was used for quantitative analysis. The quantitative analysis was carried out on an Agilent Zorbax XDB-C_{18} column (250 mm × 4.6 mm, 5 μm). The mobile phase consists of 55% acetonitrile and 45% water. UV absorption was monitored at 210 nm. The column temperature was set at 40 °C. The flow rate was 1.0 ml/min and sample injection volume was 10 μl. The semi-preparation was carried out on a Waters Nova-Pak HR C_{18} column (300 mm × 7.8 mm, 6 μm). The mobile phase for compound **4** consists of 47% MeOH with 53% water, for compound **5** consists of 52% MeOH with 48% water. All chromatographic conditions were identical to the quantitative experiments, except that the flow rate was set to 2.0 ml/min.

The ^{1}H, ^{13}C and 2D NMR spectra were recorded on a Bruker AM-500 spectrometer (500 MHz) equipped with a 5-mm probe, using $CDCl_3$ as solvent and TMS as internal standard.

2.3. Preparation of standard solutions

Igalane (**1**), isoalantolactone (**2**) and alantolactone (**3**) are stable in methanol and can be stored at 4 °C for at least 6 months. The standard solution mixture was prepared by dissolving the reference standards in methanol to final concentration of 1740.0 μg/ml for igalane (**1**), 2120.0 μg/ml for isoalantolactone (**2**), 2040.0 μg/ml for alantolactone (**3**). This solution was further diluted to appropriate concentrations for establishing calibration curves. The limit of detection (LOD) and limit of quantification (LOQ) values under present chromatographic conditions were determined on the basis of response at a signal-to-noise ratio (S/N) of 3 or 10, respectively.

2.4. Preparation of sample solutions

About 10 g of dried *I. helenium* roots were milled into powder and passed through 40 mesh. An accurately weighed mass of the powder (1.0 g) was transferred into a 25-ml volumetric flask adjusted with methanol and sonicated for 45 min. The mixture was allowed to cool for 15 min and was adjusted to the original volume. The supernatant of sample solution was filtered through a 0.22-μm membrane before HPLC analysis.

2.5. Isolation of two minor isomers for NMR analysis

After an isolation of extract (15 g) by silica gel chromatography eluting with petroleum ether–acetone (100:2, v/v), a fraction containing two minor isomers (80 mg) was dissolved in 6 ml methanol, the solution was transferred to an auto-sampler, and 10 μl injections were made. Isolation was carried out by using the conditions mentioned in Section 2.2. After ensuring that each isolated compound was >90% pure by re-chromatography, their chemical structures were examined by the NMR analysis.

3. Results and discussion

3.1. Optimization of extraction conditions

To obtain quantitative extraction of the investigated compounds, variables such as solvent and extraction time were optimized. Two extraction procedures, refluxing and ultrasonic extractions, were compared. The results suggested that ultrasonic extraction was simpler and more effective for extraction of sesquiterpene lactones. Hence, ultrasonication was chosen as the preferred method. The extraction time were examined by ultrasonication in methanol. The extraction time was set as 15, 30, 45 and 60 min. It was found that three investigated compounds were extracted almost completely within 45 min. Therefore, later samples were extracted for 45 min.

3.2. Optimization of chromatographic conditions

The chromatographic conditions were optimized to obtain better resolution of adjacent peaks within shorter time period. Different chromatographic columns were tested including Waters XTerra MS C_{18}, Merck Purospher Star C_{18}, Agilent Zorbax XDB-C_{18} and Shimadzu VP-ODS C_{18} column; the Agilent Zorbax XDB-C_{18} column was proved to offer better resolution. The optimization

Table 1
Calibration curves of compounds **1**–**3** in *I. helenium* (n = 3).

Compounds	Regression equation	r^2	Linear range (μg/ml)	LOQ (μg/ml)	LOD (μg/ml)
1	y = 20.0466x + 56.88	0.9999	17.4–1740.0	0.187	0.087
2	y = 21.6602x + 238.64	0.9994	21.2–2120.0	0.336	0.042
3	y = 23.5797x + 177.59	0.9998	20.4–2040.0	0.123	0.041

y, peak area; x, concentration of compounds (μg/ml).

Table 2
Intra-, inter-day variations and accuracy for compounds **1**–**3**.

Compounds	Reproducibility			Accuracy (n = 3)			
	Concentration (μg/ml)	R.S.D. of Intra-day (n = 5)	R.S.D. of Inter-day (n = 3)	Added (μg/ml)	Measured (μg/ml)	Recovery (%)	R.S.D. (%)
1	348.0	2.65%	1.21%	45.4	47.3	104.13	0.22
	696.0	0.87%	1.55%	90.8	94.0	103.55	1.20
	1392.0	0.09%	1.31%	181.6	189.4	104.31	0.16
2	424.0	2.85%	1.16%	243.5	249.0	102.26	0.21
	848.0	0.81%	1.82%	487.0	508.6	104.43	0.48
	1696.0	0.12%	1.39%	974.0	994.8	102.14	0.07
3	408.0	2.50%	0.97%	219.0	215.6	98.45	0.21
	816.0	0.81%	1.33%	438.0	438.5	100.11	0.91
	1632.0	0.09%	1.12%	876.0	846.2	96.60	0.09

of solvent systems was aimed at obtaining chromatograms with the best possible resolution within a reasonable run time. Two mobile phase systems, methanol–water and acetonitrile–water, were tested; the acetonitrile–water system resulted in the best separation of the investigated compounds. Furthermore, addition of acid in mobile phase was found to have no significant improvement on the separation. The most suitable temperature and flow rate were chosen as 40 °C and 1.0 ml/min, respectively. On the basis of the 3D UV spectra plot, the detection wavelength was set at 210 nm.

3.3. Method validation

The linearity, regression, and linear ranges of the HPLC method were determined (Table 1). The correlation coefficient ($r^2 \geq 0.9994$) values indicated excellent correlations between concentrations of investigated compounds and their peak areas within the test ranges. The LOD values were less than 0.087 μg/ml and the LOQ values ranged from 0.123 to 0.336 μg/ml. As shown in Table 2, the HPLC method has good reproducibility for the quantification of the three sesquiterpene lactones, with intra- and inter-day variations less than 2.85 and 1.82%, respectively. The developed method also has good accuracy with the overall recovery of 96.60–104.43%, and the R.S.D. ranging from 0.07 to 1.20% (Table 2). These results indicated that the HPLC method is precise, accurate, and sensitive for quantitative determination of the three sesquiterpene lactones in *I. helenium* samples. For the system stability test, the same sample solution was analyzed at different time points within 48 h at room temperature. The R.S.D. values of the peak areas were all lower than 1.36%, suggesting that the system is stable to analyze the sample within 2 days.

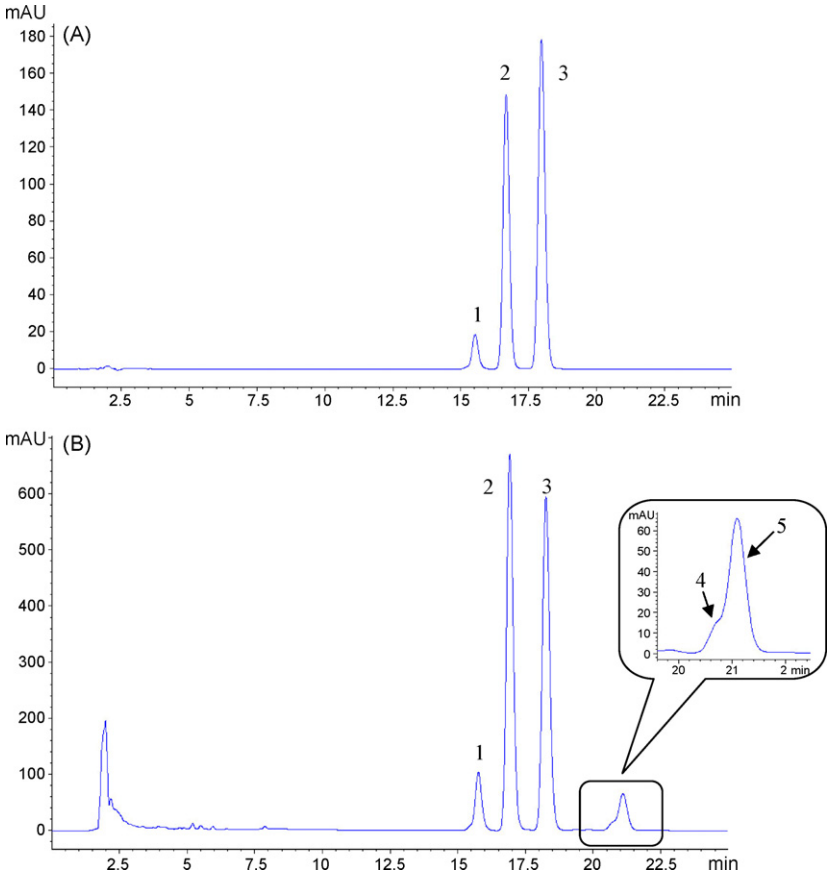

Fig. 2. HPLC chromatograms of standard mixture (A) and *I. helenium* (B).

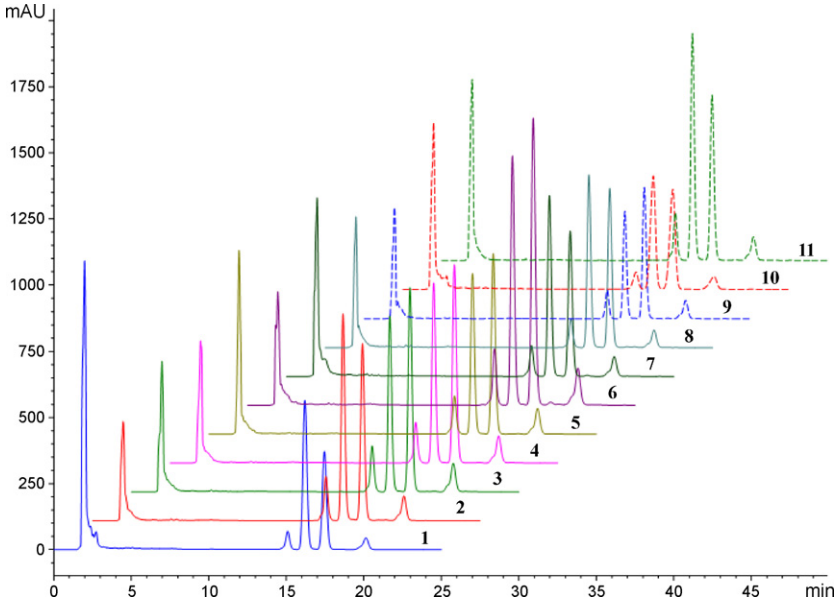

Fig. 3. HPLC chromatograms of the 11 batches of samples.

3.4. Sample analysis

The developed analytical method was then applied to the simultaneous quantification of three sesquiterpene lactones from 11 batches of *I. helenium* samples obtained from different locations. Representative chromatogram is shown in Fig. 2. The content of each individual compound in 11 samples was determined according to the calibration curve (HPLC chromatograms shown in Fig. 3) (Table 3). It was found that the isoalantolactone and alantolactone were the main compounds in all herbal samples, whose contents ranged from 10.72 to 18.01 mg/g, 6.96 to 20.70 mg/g respectively. On the other hand, it was recognized that the contents of the three compounds varied greatly among samples. We reasoned that the variations are probably due to the difference of plant origin, the effect of environment and some other factors, such as season of collection, drying process and storage conditions, etc. Because variations of the bioactive compounds may influence the quality and potency of the medicinal herb, it was necessary to develop a qualitative and quantitative method to evaluate the quality of *I. helenium*.

3.5. Isolation and identification of two minor isomers

A typical chromatogram of *I. helenium* (Fig. 2B) shows two partially overlapped peaks, a minor (**4**) and major (**5**) with retention times of 20.71 and 21.09 min, respectively. However, several attempts with various conditions (column, solvent system, temperature, and flow rate) failed to separate them (HPLC chromatogram not shown), indicating that these two compounds are not suitable for quantitative analysis. Semi-preparative HPLC isolation was then conducted using methanol–water as eluant. After multiple injections with early-cut fraction collection, 10 mg of **4** and 40 mg of **5** pure compounds were obtained, and their structures were elucidated by NMR analysis as dugesialactone [23,24] and alloantolactone [21], respectively (Fig. 1). Dugesialactone (**4**) was firstly isolated from genus *Inula*, and its ^{13}C NMR data had never been reported. By the aid of various spectral techniques, its NMR data were unequivocally assigned as: ^{1}H NMR(500 MHz, $CDCl_3$) δ: 5.49(1H, s, H-1), 2.05(2H, m, H-2), 1.54(2H, m, H-3), 1.58(1H, m, H-4), 1.71(1H, dd, J = 5.5, 5.3 Hz, H-6β), 1.62(1H, m, H-6α), 2.92(1H, m, H-7), 4.55(1H, m, H-8), 2.59(1H,dd, J = 7.7, 7.6 Hz, H-9α), 2.32(1H, m, H-9β), 5.58(1H, d, J = 1.9 Hz, H-13a), 6.22(1H, d, J = 2.3 Hz, H-13b), 0.92(3H, d, J = 3.2 Hz, 14-CH_3), 0.93(3H, s, 15-CH_3). ^{13}C NMR(125 MHz, $CDCl_3$) δ: 124.6(C-1), 25.1(C-2), 26.8(C-3), 35.0(C-4), 37.1(C-5), 38.8(C-6), 36.7(C-7), 79.0(C-8), 34.8(C-9), 138.4(C-10), 139.5(C-11), 170.9(C-12), 121.8(C-13), 20.6(C-14), 16.1(C-15).

Table 3
Contents of compounds **1**–**3** in different *I. helenium* samples[a].

No.	Samples	Content[b] (mg/g)		
		1	**2**	**3**
1	Rikaze, Tibet	3.65 ± 0.00	12.38 ± 0.00	14.28 ± 0.00
2	Xi'an, Shaanxi	3.42 ± 0.01	13.32 ± 0.01	14.60 ± 0.00
3	Anguo, Hebei	3.19 ± 0.00	11.59 ± 0.03	13.02 ± 0.01
4	Chengdu, Sichuan	4.50 ± 0.00	18.01 ± 0.04	20.70 ± 0.06
5	Bozhou, Anhui	2.46 ± 0.08	12.44 ± 0.53	9.93 ± 0.35
6	Hebei	2.33 ± 0.00	12.41 ± 0.01	11.41 ± 0.01
7	Henan	2.04 ± 0.00	10.72 ± 0.01	11.10 ± 0.01
8	Sichuan	1.73 ± 0.00	10.79 ± 0.01	9.51 ± 0.02
9	Henan	3.77 ± 0.07	17.58 ± 0.04	12.78 ± 0.48
10	Hebei	3.95 ± 0.01	16.21 ± 0.08	11.68 ± 0.06
11	Authentic drug	1.42 ± 0.00	10.72 ± 0.01	6.96 ± 0.01

[a] Sample 11 was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, PR China).
[b] Content = mean ± S.D., n = 3.

4. Conclusion

A simple, rapid and accurate HPLC method was developed for the determination of bioactive constituents in *I. helenium*. This was the first report on the simultaneous quantification of the three sesquiterpene lactones in *I. helenium*. Our results suggest that this method could be applied to the quality control of *I. helenium*.

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