Simultaneous Determination of Seven Compounds in Snow Lotus Herb Using High-Performance Liquid Chromatography

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Abstract

A reversed-phase high-performance liquid chromatographic method is developed for the quantitative determination of seven components of Snow Lotus Herb (*Saussurea tridactyla* Sch.-Bip. ex Hook.f.): umbelliferonglucoside, luteolin-7-*O*-β-D-glucoside, rutin, apigenin-7-*O*-β-D-glucoside, kaempferol-3-*O*-β-D-glucoside, apigenin-7-*O*-β-D-rutinoside, and luteolin. Samples are analyzed by means of a reverse-phase column (Diamonsil C_{18}) using methanol and water under gradient conditions as the mobile phase for 60 min. This method offers selectivity, accuracy, precision, linearity, and ruggedness, as well as efficiency and ease.

Introduction

Snow Lotus Herb, a common ethnomedicinal drug of Tibet, Mongolia, and Uygur, is used for treating traumatic bleeding, anthrax, rheumatoid arthritis, and other diseases (1,2). The botanical origin of this drug is the whole plants or the aerial parts of *Saussurea tridactyla* Sch.-Bip. ex Hook.f., *S. medusa* Maxim., *S. involucrate* (Kar. et Kir.) Sch.-Bip., and many other plants of the genus *Saussurea* family composite. The identification and quantitation of rutin has been carried out by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). Although the TLC method has been extensively employed in the quality assurance and quality control of Snow Lotus Herb and the products containing it (3), the method presents a challenge for sensitivity and selectivity.

The Snow Lotus Herb methanol extract is composed of many closely related flavones (quercetin, rutin, jaceosidin, apigenin, chrysoeriol-7-*O*-β-D-glucoside, and hispidulin). Because of the lack of absolute standards, the routine quantitation of Snow Lotus Herb is usually reported as being "total flavonoids". With the constraints of ease of use, thermal stability, and accuracy, our attention became focused on HPLC methods of analysis. Some promising work has been done with an HPLC method for rutin and syringin (4). However, after an initial evaluation, that method was not used because it did not separate seven compounds (umbelliferonglucoside, luteolin-7-*O*-β-D-glucoside, rutin, apigenin-7-*O*-β-D-glucoside, kaempferol-3-*O*-β-D-glucoside, apigenin-7-*O*-β-D-rutinoside, and luteolin) of Snow Lotus Herb methanol extract. The luteolin-7-*O*-β-D-glucoside and rutin peaks of both groups were usually merged. Our initial strategy for new method development was to adapt the reversed-phase procedure to allow for the separation of seven compounds.

The purpose of this study was to provide a validated analytical method that can be used to simultaneously quantitate each of the seven compounds: umbelliferonglucoside (Figure 1), luteolin-7-

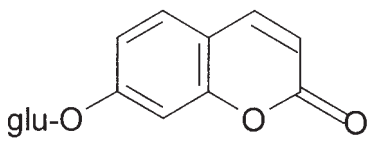

Figure 1. Structure of umbelliferonglucoside.

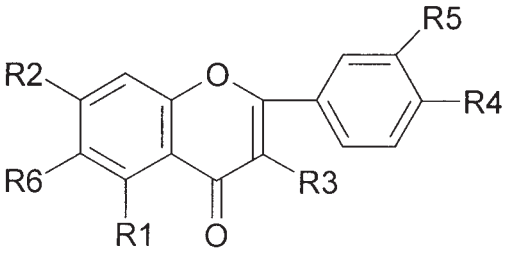

Figure 2. Structure of the six individual flavonoids. Luteolin-7-*O*-β-D-glucoside: R1 = R4 = R5 = OH, R2 = *O*-β-glu, R3 = R6 = H. Rutin: R1 = R2 = R4 = R5 = OH, R3 = *O*-β-rha-(2-1)-glu, R6 = H. Apigenin-7-*O*-β-D-glucoside: R1 = R4 = OH, R2 = *O*-β-glu, R3 = R5 = R6 = H. Kaempferol-3-*O*-β-D-glucoside: R1 = R2 = R4 = OH, R3 = *O*-β-glu, R5 = R6 = H. Apigenin-7-*O*-β-D-rutinoside: R1 = R4 = OH, R2 = *O*-α-rha-(2-1)-glu, R3 = R5 = R6 = H. Luteolin: R1 = R2 = OH, R4 = OCH3, R3 = R5 = R6 = H.

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O-β-D-glucoside, rutin, apigenin-7-*O*-β-D-glucoside, kaempferol-3-*O*-β-D-glucoside, apigenin-7-*O*-β-D-rutinoside, and luteolin (Figure 2).

Experimental

Materials and reagents

Snow Lotus Herb was obtained from the Institute of Medicinal Plant Development of the Chinese Academy of Medical Science (Beijing, China). All seven compounds as reference standards (99.0% purity) were purchased from the Medicinal Plant Development of the Chinese Academy of Medical Science. Methanol, phosphoric acid, and tetrahydrofuran were purchased from Fisher Scientific (Fair Lawn, NJ).

Apparatus

A Waters 2690 Alliance HPLC system (Waters, Milford, MA) equipped with a 996 photodiode-array UV detector, an online degasser, and an autosampler were used for solvent delivery and detection. The detector output was interfaced using a SATIN box for the Waters Millennium 32 chromatographic manager system that was loaded on a Digit computer for data handing and chromatogram generation.

Preparation of standard solution

In a clean and dry 10-mL volumetric flask, the following analytes of reference standards were dissolved in 10 mL of methanol to make stock resolution: the umbelliferonglucoside (0.2 mg), luteolin-7-*O*-β-D-glucoside (0.2 mg), rutin (0.8 mg), apigenin-7-*O*-β-D-glucoside (0.2 mg), kaempferol-3-*O*-β-D-glucoside (0.2 mg), apigenin-7-*O*-β-D-rutinoside (0.2 mg), and luteolin (0.2 mg). Calibration working standard solutions were prepared by diluting the stock solution with methanol in the appropriate quantities. All working solutions were stored at –20°C and brought to room temperature before use.

Preparation of sample solution

Finely pulverized (180 ± 7.6-µm particle size) Snow Lotus Herb was accurately weighed (0.5 g) in a polytetrafluroethylene-stopped 10-mL sample vial. A 10-mL HPLC-grade sample of methanol (Fisher) was added, and the mixture was shaken and then sonicated at room temperature for 30 min. After cooling, the mixture was filtered through filter paper into a 100-mL round-bottom flask, and the residue was returned to the sample vial. Another 10 mL of methanol was added, and the mixture was sonicated at room temperature for 30 min. The extract was filtered through filter paper into the same volumetric flask. The extraction procedure was repeated once more before washing the residue with methanol (3 × 10 mL) while on the filter. These combined methanol extracts were evaporated under reduced pressure at 35°C. The residue was redissolved and transferred with methanol to a 10-mL volumetric flask and diluted to volume with methanol. The sample solution was filtered through a 0.2-µm Waters membrane filter into an HPLC sample vial just before HPLC–diode-array-detection (DAD) analysis.

Chromatographic conditions

The chromatographic separations were carried out on a Diamonsil C_{18} column (150- × 4.6-mm i.d., 5-µm particle size) (Dikma, Beijing, China). The mobile phase used for the separation consisted of solvent A (methanol) and solvent B (water–phosphoric acid–tetrahydrofuran, 100:0.7:1, v/v/v). The elution

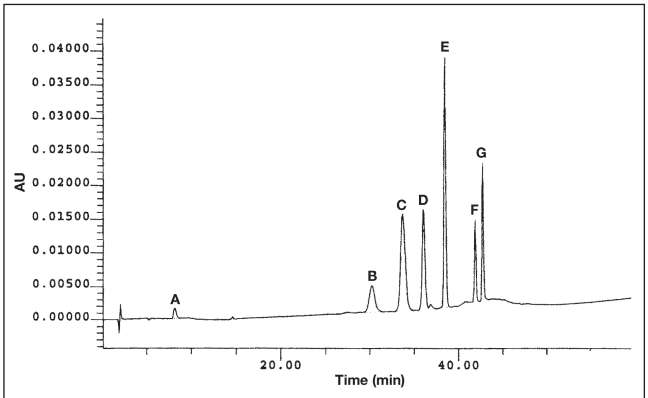

Figure 3. Chromatogram of the seven reference standards. The peaks represent umbelliferonglucoside (A), luteolin-7-*O*-β-D-glucoside (B), rutin (C), apigenin-7-*O*-β-D-glucoside (D), kaempferol-3-*O*-β-D-glucoside (E), apigenin-7-*O*-β-D-rutinoside (F), and luteolin (G).

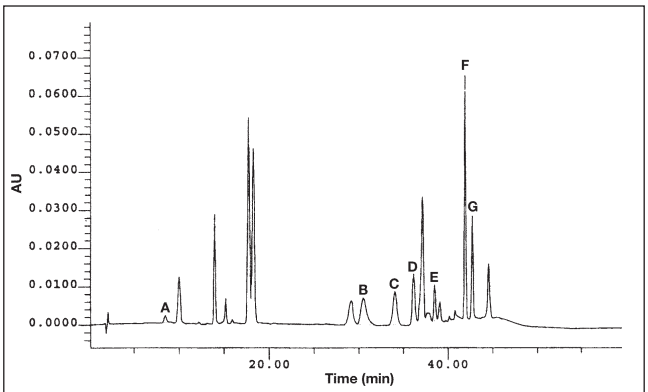

Figure 4. Chromatogram of the methanol extract of *Saussurea tridactyla* Sch.-Bip. ex Hook.f. The peaks represent umbelliferonglucoside (A), luteolin-7-*O*-β-D-glucoside (B), rutin (C), apigenin-7-*O*-β-D-glucoside (D), kaempferol-3-*O*-β-D-glucoside (E), apigenin-7-*O*-β-D-rutinoside (F), and luteolin (G).

Table I. Results of Snow Lotus Herb–Methanol Extract Samples Assayed for Accuracy

Compound	Spiked concentration (µg/mL)	Measured concentration* (µg/mL)	Recovery (%)	RSD (%)
Umbelliferonglucoside	60	59.89 ± 0.89	99.8	1.5
Luteolin-7-*O*-β-D-glucoside	27	26.78 ± 0.45	99.2	1.7
Rutin	20	19.77 ± 0.28	98.8	1.4
Apigenin-7-*O*-β-D-glucoside	10	9.83 ± 0.15	98.3	1.5
Kaempferol-3-*O*-β-D-glucoside	24	23.46 ± 0.42	97.8	1.8
Apigenin-7-*O*-β-D-rutinoside	36	36.54 ± 0.82	101.5	2.2
Luteolin	18	17.94 ± 0.47	99.7	2.6

* Mean ± standard deviation (n = 6).

profile was: 0–5 min, 85–80% B; 5–10 min, 80–65% B; 10–25 min, 65% B; 25–30 min, 65–60% B; 30–40 min, 60–30% B; 40–50 min, 30–60% B; and 50–60 min, 60–85% B (reconditioning). All gradient steps were linear. The flow rate was 1.0 mL/min, column temperature was 25°C, and injection volume was 10 µL. Peak identifications were based on retention time and comparison with the injected authentic reference standards (Figures 3 and 4). The peaks were detected with a Waters 996 photodiode-array UV detector. The detection wavelength was set at 350 nm. Prior to each run, the HPLC–DAD system was allowed to warm up for 30 min, and the pumps were primed using the protocol suggested by the manufacturer. Using a freshly prepared mobile phase, the baseline was monitored until it was stable before the samples were run.

Table II. Results of Six Replicate Analyses of Snow Lotus Herb–Methanol Extract and Individual Compounds for Precision

Compound	Average content (x 10^{-2}%)	Standard deviation (x 10^{-4})	%RSD
Umbelliferonglucoside	5.09	3.27	0.64
Luteolin-7-*O*-β-D-glucoside	3.38	3.56	1.05
Rutin	8.86	7.11	0.80
Apigenin-7-*O*-β-D-glucoside	7.48	4.91	0.66
Kaempferol-3-*O*-β-D-glucoside	7.41	4.40	0.59
Apigenin-7-*O*-β-D-rutinoside	2.44	1.83	0.75
Luteolin	2.62	5.73	0.22

Table III. Linear Regression Results

Compound	Regression analysis equation	Correlation coefficient
Umbelliferonglucoside	$Y = 272053.6988X - 2985.3487$	0.999227
Luteolin-7-*O*-β-D-glucoside	$Y = 3039370.2421X + 9269.0720$	0.999630
Rutin	$Y = 1398143.4911X + 28045.2103$	0.999929
Apigenin-7-*O*-β-D-glucoside	$Y = 1691198.3345X + 6273.3478$	0.999938
Kaempferol-3-*O*-β-D-glucoside	$Y = 856334.6667X + 6336.4205$	0.999950
Apigenin-7-*O*-β-D-rutinoside	$Y = 2277311.0788X + 2075.0272$	0.999775
Luteolin	$Y = 5889009.1649X + 15739.9654$	0.999711

Results and Discussion

Method validation

Validation of the developed method was carried out to assess the performance characteristics, which were for the following parameters: selectivity, accuracy, precision, linearity, and ruggedness.

Selectivity

Before the quantitation of each compound in Snow Lotus Herb–methanol extract can take place, a separation of each of the compounds from one another and from components of the sample matrix must be achieved. With the conditions outlined in the method, seven sample compounds in the sample were baseline separated (Figure 4).

Accuracy

The accuracy of the method was evaluated by injecting the six analyzed Snow Lotus Herb–methanol extracts. By substituting the peak area into the calibration curve equation from the same run, the measured concentrations were obtained.

The recovery was assessed by adding 600 µg umbelliferonglucoside, 270 µg luteolin-7-*O*-β-D-glucoside, 200 µg rutin, 100 µg apigenin-7-*O*-β-D-glucoside, 240µg kaempferol-3-*O*-β-D-glucoside, 360 µg apigenin-7-*O*-β-D-rutinoside, and 180 µg luteolin to 100 mg of Snow Lotus Herb–methanol samples and extracting them in a similar way as the previously mentioned sample. Table I shows that the recoveries of seven compounds of six assayed samples were 97.8–101.5% with relative standard deviations (RSDs) of 0.5–2.6%. These results indicated that the method was accurate.

Precision

The six Snow Lotus Herb extracts were employed for the validation of the method's precision or repeatability. Each sample was run with independently prepared standards. The analytical results (summarized in Table II) showed that the RSD of each compound for six runs was also within 2%.

Linearity

Linearity was tested by injecting a group of six

Table IV. Diode-Array Peak-Purity Assessments of Six Samples of Snow Lotus Herb–Methanol Extract

	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5		Sample 6	
Compound	Purity angle	Purity threshold	Purity angle	Purity threshold	Purity angle	Purity threshold	Purity angle	Purity threshold	Purity angle	Purity threshold	Purity angle	Purity threshold
Umbelliferonglucoside	0.621	1.165	0.613	1.144	1.091	1.139	1.072	1.134	1.059	1.140	0.471	1.102
Luteolin-7-*O*-β-D-glucoside	0.489	1.413	0.501	1.408	0.731	1.459	0.548	1.385	0.566	1.418	1.129	1.273
Rutin	0.355	1.099	0.681	1.096	0.728	1.112	0.917	1.090	0.586	1.102	0.362	1.063
Apigenin-7-*O*-β-D-glucoside	0.520	1.104	0.915	1.106	0.685	1.110	0.842	1.096	0.156	1.106	0.249	1.071
Kaempferol-3-*O*-β-D-glucoside	0.798	1.042	0.328	1.043	0.404	1.045	0.751	1.040	0.266	1.045	0.144	1.027
Apigenin-7-*O*-β-D-rutinoside	0.776	1.154	0.714	1.128	0.874	1.141	0.928	1.114	0.962	1.126	0.834	1.082
Luteolin	0.937	1.071	0.710	1.087	0.510	1.108	0.832	1.083	0.936	1.095	1.000	1.060

standard solutions. The linearity relationships were set up between the elution peak areas and the concentrations for each of the compounds. Correlation coefficients were then calculated by linear regression analysis (as listed in Table III).

Diode-array peak-purity analysis

A more quantitative assessment of selectivity was achieved using peak-purity reading from the diode-array detector. The six Snow Lotus Herb–methanol extracts used in the precision experiment were used to make a peak-purity analysis.

Typically, when using Waters Millennium 32 diode-array software, a component with a peak purity angle lower than its purity threshold would be considered most likely to be a pure substance, and the separation would be acceptable. The peak purity results for six samples are summarized in Table IV. All of the peak purity angles were lower than their respective purity thresholds.

Ruggedness

In order to check the ruggedness of the method, samples of the same Snow Lotus Herb–methanol extracts used in the precision section were analyzed by a different analyst on different days using a different HPLC system and serial number column. The results are expressed on Table V. Based on the average results obtained by chemist I and II in Table V, the RSDs were further calculated for the seven compounds, which were all below 3%.

Table V. Ruggedness Results

	Replicate 1		Replicate 2		Replicate 3	
Compound	Chemist I assay (%)	Chemist II assay (%)	Chemist I assay (%)	Chemist II assay (%)	Chemist I assay (%)	Chemist II assay (%)
Umbelliferon-glucoside	0.05090	0.05056	0.05075	0.5037	0.05127	0.05122
Luteolin-7-*O*-β-D-glucoside	0.03356	0.03397	0.03365	0.03360	0.03299	0.03227
Rutin	0.08840	0.08757	0.08839	0.08893	0.08849	0.08993
Apigenin-7-*O*-β-D-glucoside	0.07553	0.07499	0.07504	0.07395	0.07499	0.07484
Kaempferol-3-*O*-β-D-glucoside	0.07449	0.07475	0.07431	0.07367	0.07563	0.07598
Apigenin-7-*O*-β-D-rutinoside	0.2360	0.2318	0.2318	0.2333	0.2261	0.2330
Luteolin	0.02617	0.02631	0.02621	0.02639	0.02618	0.02635
	Replicate 4		**Replicate 5**		**Replicate 6**	
Compound	Chemist I assay (%)	Chemist II assay (%)	Chemist I assay (%)	Chemist II assay (%)	Chemist I assay (%)	Chemist II assay (%)
Umbelliferon-glucoside	0.05154	0.05158	0.05102	0.05079	0.05088	0.05073
Luteolin-7-*O*-β-D-glucoside	0.03451	0.03479	0.03389	0.03527	0.03278	0.03356
Rutin	0.08615	0.08537	0.08747	0.08862	0.08756	0.08782
Apigenin-7-*O*-β-D-glucoside	0.07652	0.07664	0.07643	0.07681	0.07667	0.07691
Kaempferol-3-*O*-β-D-glucoside	0.07475	0.07596	0.07377	0.07360	0.07568	0.07615
Apigenin-7-*O*-β-D-rutinoside	0.2273	0.2444	0.2251	0.2261	0.2238	0.2386
Luteolin	0.02631	0.02602	0.02612	0.02588	0.02605	0.02591
	Average		**Standard deviation**		**RSD(%)**	
Compound	Chemist I assay (%)	Chemist II assay (%)	Chemist I assay ($\times 10^{-4}$)	Chemist II assay ($\times 10^{-4}$)	Chemist I assay	Chemist II assay
Umbelliferon-glucoside	0.05104	0.05090	2.895	4.029	0.57	0.79
luteolin-7-*O*-β-D-glucoside	0.03356	0.03391	5.705	9.605	1.70	2.83
Rutin	0.08774	0.08804	8.220	14.37	0.94	1.61
Apigenin-7-*O*-β-D-glucoside	0.07586	0.07691	7.018	11.46	0.93	1.51
Kaempferol-3-*O*-β-D-glucoside	0.07477	0.07502	6.902	10.80	0.92	1.44
Apigenin-7-*O*-β-D-rutinoside	0.2284	0.2345	42.40	57.41	1.86	2.44
Luteolin	0.02617	0.02614	0.7972	2.123	0.30	0.81

Conclusion

An HPLC method has been developed for the simultaneous and quantitative determination of seven compounds in *Saussurea Tridactyla* Sch.-Bip. ex Hook.f. using a diode-array detector. Validation of the HPLC–DAD method included selectivity, accuracy, precision, linearity, and ruggedness. All of the validation parameters studied were found to have RSDs of less than 3% and did not show any bias in a single direction. The method was found to be rapid, relatively inexpensive, and reproducible.

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