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Determination of five major iridoid glucosides in *Flos Lonicerae* by high-performance liquid chromatography coupled with evaporative light scattering detection

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Abstract

This study presents a new HPLC method using evaporative light scattering detection for the simultaneous determination of five major iridoid glucosides, namely 7-*epi*-loganin, sweroside, loganin, 7-*epi*-vogeloside, and secoxyloganin in *Flos Lonicerae*, an important traditional Chinese medicinal herb. The optimal conditions of separation and detection were achieved on a C_{18} analytical column with an isocratic mobile phase consisting of methanol–water (30:70, v/v) containing 0.5% acetic acid at the flow-rate of 1.0 ml/min, temperature for the detector drift tube set at 90 °C and the nitrogen flow-rate of 2.6 l/min. The limit of detection ($S/N=3$) is less than 35.1 μg/ml and the limit of quantification ($S/N=10$) is less than 140.1 μg/ml. All calibration curves show good linear regression ($r^2>0.996$) within test ranges. This method provides good reproducibility for the quantification of the major iridoid glucosides in four *Lonicera* species with overall intra- and inter-day variation of less than 5% and 9%, respectively. The assay was successfully applied to quantify the main iridoid glucosides in the herb and to identify the botanical origin of *Flos Lonicerae*.
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Keywords: Pharmaceutical analysis; *Lonicera* spp.; Plant materials; Iridoid glucosides; Glucosides

1. Introduction

Flos Lonicerae, derived from the flower buds of several species of the genus *Lonicera* (*Caprifoliaceae*), has been used for the treatment of affection by exopathogenic wind-heat or epidemic febrile diseases at the early stage, sores, carbuncles, furuncles and swelling in traditional Chinese medicine for centuries [1]. Chemical investigations on the genus *Lonicera* result in the isolation of several kinds of metabolites: chlorogenic acid and its analogues, iridoid glucosides, flavonoids, triterpenoid saponins [2].

Previous quality control and evaluation of *Flos Lonicerae* were generally concerned with chlorogenic acid considering its high content (no less than 1.5%) and antipyretic property [3–5]. However, chlorogenic acid alone could not be responsible for the overall pharmacological activities of *Flos Lonicerae* and synergistic effects between various constituents may exist [6]. Also, as another group of metabolites, iridoid glucosides show a number of

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activities such as antimicrobial, antitumoral, hypotensive, sedative and hepatoprotective [7,8]. Consequently, it is necessary to determine the major iridoid glucosides in *Flos Lonicerae* in order to comprehensively evaluate this herbal drug and to ensure its effective clinical use.

The UV absorption maximum of iridoid glucosides is in the range of 210–230 nm, this low wavelength may be detrimental to the sensitivity of a UV detector and limits the use of some ordinary solvents in the mobile phase. Fortunately, evaporative light scattering detection (ELSD) is a powerful technique that can be used to monitor a solute as it is less volatile than the mobile phase, no matter if the analyte is chromophoric or not. Recently, ELSD is increasingly employed as a quasi-universal detector for the analysis of non-chromophoric compounds [9]. Although some researchers have determined iridoids by HPLC with UV detection at low wavelength, there have been no HPLC–ELSD methods developed for the determination of any iridoid glucosides in medicinal plants [10,11]. The aim of the present study is to develop for the first time a simple HPLC analytical method using ELSD for the simultaneous determination of the major iridoid glucosides, i.e. 7-*epi*-loganin, sweroside, loganin, 7-*epi*-vogeloside, and secoxyloganin (Fig. 1) in *Flos Lonicerae*.

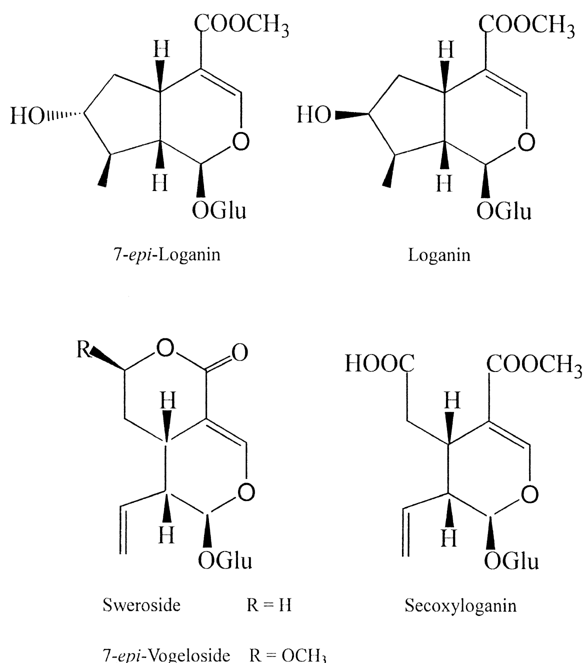

Fig. 1. Structures of 7-*epi*-loganin, sweroside, loganin, 7-*epi*-vogeloside and secoxyloganin.

2. Experimental

2.1. Samples, chemicals and reagents

Four samples of the flower buds of *Lonicera japonica*, *L. dasystyla*, *L. hypoglauca*, *L. confusa* were collected in China. Plants were identified by Professor Ping Li. The voucher specimens were deposited in the Department of Pharmacognosy, China Pharmaceutical University, Nanjing, China.

Five iridoid glucosides: 7-*epi*-loganin, sweroside, loganin, 7-*epi*-vogeloside, and secoxyloganin were isolated from the ethyl acetate extract of the flower buds of *L. japonica* by repeated silica gel column chromatography and their structures were established by comparison of their spectral data (UV, IR, mass, ^{1}H- and ^{13}C-NMR) with the literature data [12–15].

The HPLC-grade methanol was purchased from Hanbang Science & Technology (Nanjing, China), and the deionized water from Robust (Guangzhou, China). All other solvents were of analytical grade from Nanjing Chemical Factory (Nanjing, China).

2.2. Apparatus and chromatographic conditions

The HPLC system consisted of a Shimadzu LC-6A pump (Kyoto, Japan) and an Alltech ELSD 2000 (Alltech, USA). An Alltima C_{18} analytical column (250×4.6 mm I.D., 5 μm) protected by an Alltima C_{18} guard column (12.5×4.6 mm I.D., 5 μm) at a column temperature of 26 °C was used for all analyses. A Rheodyne 7125 sampling valve (Cotati, USA) equipped with a sample loop of 20 μl was used for sample injection. The analog signal from ELSD was transmitted to a HP Chemstation for processing through an Agilent 35900E (Agilent Technologies, USA).

The mobile phase consisted of methanol–water (30:70, v/v) acidified with 0.5% acetic acid (pH 5.7) with a flow-rate of 1.0 ml/min. Temperature for the detector drift tube was set at 90 °C and the nitrogen

Table 1
Calibration curves for five analytes

Analyte	Calibration curve[a]	r^2	Test range (μg/ml)	LOD (μg/ml)	LOQ (μg/ml)
7-*epi*-Loganin	$y=2135.5x-73.3$	0.9968	25.2–302.3	12.6	50.2
Sweroside	$y=2262.1x-167.7$	0.9986	50.3–633.5	17.6	55.4
Loganin	$y=1943.6x-142.3$	0.9979	62.1–744.5	20.7	70.3
7-*epi*-Vogeloside	$y=3129.3x-314.6$	0.9967	66.8–821.7	15.0	60.8
Secoxyloganin	$y=2443.3x-276.3$	0.9965	70.2–842.6	35.1	140.1

[a] y, Peak area count; x, concentration of standard (μg/μl).

flow-rate was 2.6 l/min. Chromatographic peaks were identified by comparing retention time (t_R). In addition, spiking samples with the standards further confirmed the identities of peaks.

2.3. Limits of detection and quantification

The limits of detection (LOD) and quantification (LOQ) under the chromatographic conditions were separately determined in six replicate determinations at a signal-to-noise ratio (S/N) of 3 and 10, respectively. LOD and LOQ are reported in Table 1 for each compound.

2.4. Linearity range and calibration curves

Methanol stock mixed solution containing five iridoid glucosides was prepared and diluted to appropriate concentration ranges for the construction of calibration curves. A calibration curve for each compound was performed with six different concentrations in triplicate by plotting the peak area versus concentration. The results are presented in Table 1.

2.5. Repeatability, reproducibility and recovery

The measurements of intra- and inter-day variability were utilized to assess the repeatability and reproducibility of the developed assay. The intra-day variability was performed under optimal conditions by means of six replicates of a mixed standard solution. The relative standard deviation (RSD) values for t_R and peak area of each compound were calculated. The inter-day variability was examined over 3 days by performing six replicates each day. The results are given in Table 2.

In a recovery test, the proposed method was applied to the flower buds of *L. japonica* spiked with the mixed standard solution at levels of 2.0 and 4.0 mg/g. The mean recoveries ($n=3$) of the test iridoid glucosides, 7-*epi*-loganin, sweroside, loganin, 7-*epi*-vogeloside, and secoxyloganin were 96.7%, 99.3%, 103.5%, 98.2% and 101.4%, respectively, and the RSD values were 2.53%, 3.81%, 4.68%, 3.64%, and 2.95%, respectively.

Table 2
Repeatability and reproducibility of the five analytes

Analyte	Intra-day variability		Inter-day variability	
	RSD of t_R (%)	RSD of peak area (%)	RSD of t_R (%)	RSD of peak area (%)
7-*epi*-Loganin	1.6	4.4	2.8	8.7
Sweroside	1.1	2.9	2.0	5.4
Loganin	1.5	3.3	1.9	6.2
7-*epi*-Vogeloside	1.3	3.7	2.4	4.8
Secoxyloganin	0.9	1.8	1.9	3.6

2.6. Sample pretreatment

Sample preparation was as follows. The flower buds of *Lonicera* spp. were collected in the middle of May in China, dried until constant weight at 50 °C. Approximately 2.5 g of the flower buds were pulverized, accurately weighed, and then defatted with petroleum ether before it was extracted exhaustively with methanol in a Soxhlet extractor for 6 h. The extract was evaporated to dryness and the residue dissolved with methanol into a 10-ml flask. It was then filtered through No.1 Xinhua filter paper (Hangzhou Paper Factory, China) and 1 ml of the succeeding filtrate was transferred accurately to a 2-ml volumetric flask and filled with methanol to the mark. The resultant solution was filtered through a 0.45 μm syringe membrane filter (Type Millex-HA, Millipore, USA) prior to HPLC. The contents of the analytes were determined from the corresponding calibration curves.

3. Results and discussion

It has been stated that the drift tube temperature and the gas flow-rate are the two most important adjustable parameters for ELSD which play a prominent role in an analyte's response [16]. Theoretically, the response of an analyte should increase with a decreasing temperature and flow-rate, but low temperature and slow flow-rate do not lead to desirable LOD while considering the increase in noise. Consequently, variations of the temperature ranging from 80 to 100 °C and of the flow-rate from 2.2 to 3.0 l/min were examined systematically. 7-*epi*-Loganin was selected as the testing iridoid glucoside for optimizing ELSD conditions, as all the samples contained it. Eventually, the temperature of 90 °C and flow-rate of 2.6 l/min were preferred to detect the analytes, and these two exact experimental parameters should be strictly controlled in the analytical procedure.

Optimized chromatographic conditions were achieved after several trials with methanol, water, and acetic acid in various proportions. It was found that the presence of acetic acid in the mobile phase had a significant effect on the retention behavior of secoxyloganin. When acetic acid was absent in the methanol–water (30:70, v/v) system, the t_R value was near 55 min. Once 0.5% acetic acid was added, the t_R value shortened dramatically to 22.5 min. Considering the existence of a carboxylic acid function in the molecular structure of secoxyloganin, this change was recognizable. For the same reason, the t_R values of other analytes were not pH-dependent since they had no carboxylic acid group. Subsequently, the optimal mobile phase consisting of methanol–water (30:70, v/v) containing 0.5% acetic acid was employed leading to well-separated resolution, satisfactory peak shape as well as relatively short time for analysis. The typical chromatograms of the extracts of different *Lonicera* spp. are shown in Fig. 2, and the quantities of individual iridoid glucoside are summarized in Table 3.

The four species selected for the present study are documented in the China Pharmacopoeia as the plant sources for *Flos Lonicerae* [1]. In fact, *Flos Lonicerae* has been very complicated with respect to its sources on the market. Traditionally, the identification of the specific species of *Flos Lonicerae* is carried out by a sophisticated botanist through careful inspection of the morphological and histological characteristics [17–19]. However, it is difficult to make a decision when the crude herb is processed and its appearance or texture is damaged. Therefore, it is important to establish a simple and reliable technique to unambiguously distinguish one species from another. By comparing the overall HPLC profiles of the different samples from *Lonicera* spp., we found it possible to distinguish clearly the four species in terms of the occurrence and/or relative concentration of iridoid glucosides. Analysis results showed that the species *L. japonica* contained all five iridoid glucosides, with sweroside (4.245 mg/g), 7-*epi*-vogeloside (3.215 mg/g) and secoxyloganin (3.578 mg/g) being of highest content, respectively. Conversely, in *L. dasystyla* the first, second and third constituents with highest content were 7-*epi*-vogeloside (4.965 mg/g), 7-*epi*-loganin (1.857 mg/g) and sweroside (1.401 mg/g), respectively, and secoxyloganin and loganin were absent. In contrast, in *L. hypoglauca* and *L. confusa*, the most eminent iridoid glucoside was secoxyloganin whose amounts were up to 14 and 6.6 mg/g, respectively, while other compounds were relatively low or not even detectable. Thus, by means of the chemical

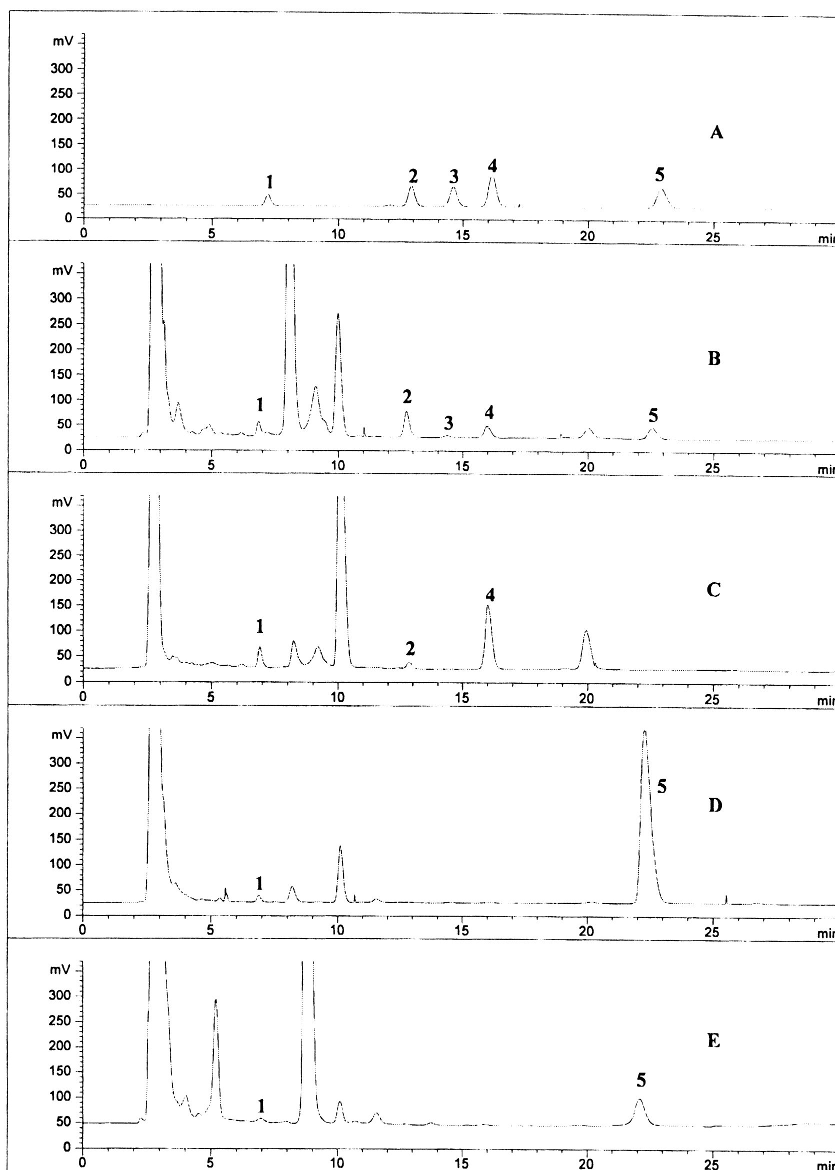

Fig. 2. HPLC chromatograms of mixed standards and methanol extracts of *Flos Lonicerae*. Column, Alltima C_{18} (250×4.6 mm I.D., 5 μm); column temperature, 26 °C; mobile phase, methanol–water (30:70, v/v) containing 0.5% acetic acid; flow-rate, 1.0 ml/min; drift tube temperature, 90 °C; gas flow-rate, 2.6 l/min. (A) Mixed standards (1, 0.10 mg/ml; 2, 0.21 mg/ml; 2, 0.25 mg/ml; 2, 0.27 mg/ml; 2, 0.28 g/ml), (B) *L. japonica*, (C) *L. dasystyla*, (D) *L. hypoglauca*, (E) *L. confusa*; 1, 7-*epi*-loganin; 2, sweroside; 3, loganin; 4, 7-*epi*-vogeloside; 5, secoxyloganin.

evidence we could rapidly clarify the botanical origin of these crude herbs.

To the best of our knowledge, no references concerning the determination of iridoid glucosides in the genus *Lonicera* can be found in publications except a report on *L. japonica* cell suspension cultures [20]. Moreover, in China the genus *Lonicera* consists of about 98 species of which 47 species are used in indigenous systems of medicine as an anti-inflammatory, antipyretic, diuretic, hepato-protective

Table 3
Concentration of five major iridoid glucosides in *Lonicera* spp.

Original plant	Location[a]	Content (mg/g, $n=3$)[b]				
		1	2	3	4	5
L. japonica	Pingyi City, Shandong Prov.	2.358	4.245	0.6572	3.215	3.578
L. dasystyla	Guilin City, Guangxi Prov.	1.857	1.401	nd	4.965	nd
L. hypoglauca	Jiujiang City, Jiangxi Prov.	0.7256	nd	nd	tr	14.00
L. confusa	Xupu City, Hunan Prov.	tr	nd	nd	nd	6.573

[a] All samples were collected in China.
[b] tr, Content was below the LOQ; nd, not detected. 1, 7-*epi*-loganin; 2, sweroside; 3, loganin; 4, 7-*epi*-vogeloside; 5, secoxyloganin.

or stomachic and so on [21]. This newly validated HPLC–ELSD method not only facilitates quality control and identification of *Flos Lonicerae*, but could advance systematic investigation into the distribution of iridoid glucosides in the genus *Lonicera* and other iridoid-containing plants so as to utilize the medicinal plant resources.

4. Conclusions

In conclusion, this is the first report on qualification and quantification of major iridoid glucosides in *Flos Lonicerae*. It documents that the verified HPLC–ELSD method can be successfully applied to quantification of 7-*epi*-loganin, sweroside, loganin, 7-*epi*-vogeloside, and secoxyloganin in *Flos Lonicerae* with good sensitivity, repeatability and reproducibility. In addition, this method is very suitable for the identification of *L. japonica*, *L. dasystyla*, *L. hypoglauca* and *L. confusa* when the five iridoid glucosides are chosen as chemical markers. Furthermore, it can be hopefully employed to screen iridoid glucosides in the genus *Lonicera* and other medicinal plants.

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