HPLC Fingerprint and LC-TOF-MS Analysis on Extract from Roots of *Gentiana macrophylla*

SU Qi[1], SHANG Ping-ping[1], ZHANG Yong-min[2], JIA Na[1], HE Jiao[1], ZHAO Wen-na[1], SUN Wen-ji[1*]

1. Biomedicine Key Laboratory of Shaanxi Province, Northwest University, Xi'an 710069, China

2. Institut Parisien de Chimie Moléculaire, Université Pierre et Marie Curie-Paris 6, 75005 Paris, France

Abstract: **Objective**   Establishing a fingerprint method to identify the characteristic chemicals in the roots of *Gentiana macrophylla* and evaluate their quality. **Methods**   RP-HPLC was developed for fingerprint analysis and determination of four ingredients in *G. macrophylla* roots from different sources. LC-ESI-TOF-MS was employed to identify the chromatographic peaks of the fingerprint. **Results**   Five common peaks were identified by comparing their retention time with reference secoiridoid glucosides. Eight major peaks in chromatographic fingerprint were analyzed by on-line LC-ESI-TOF-MS. Four secoiridoid glucosides were identified based on their MS data. **Conclusion**   The method is specific and could be served for the quality identification and comprehensive evaluation of *G. macrophylla*.

Introduction

Gentiana macrophylla Pall. is widely distributed and used in China. Its dry roots (*Gentianae Macrophyllae Radix*, Qinjiao in Chinese) possess various therapeutic activities, such as treating rheumatoid arthritis, liver protection, and choleretic effect, etc (Pharmacopeia Committee of P. R. China, 2010). During the past two decades, it has been revealed that the roots of *G. macrophylla* could generate a variety of secoiridiod glycosides, iridoid glycosides, dihydroflavone, triterpenes, sterols, and inorganic elements, etc (Chen, Shi, and Tu, 2005; Jiang, Liu, and Shang, 2010). Recently, HPLC method has been frequently used in analysis on secoiridoid glucosides and related compounds in *G. macrophylla* (Cao and Wang, 2010a; 2010b; Yu, 2010).

According to *Chinese Pharmacopoeia 2010*, *G. macrophylla*, *G. crassicaulis* Duthie. ex Burkill, *G. dahurica* Fisch. and *G. straminea* Maxim. were all used as *Gentianae Macrophyllae Radix*. However, the chemical constituents of these four species are significantly different (Wu, Chen, and Yu, 2008; Wang

et al, 2009; Cao, Li, and Wang, 2008). Our work focused on one of the four species, *G. macrophylla* (Daye Qinjiao in Chinese).

It is well known that the therapeutic effects of traditional Chinese medicines (TCM) are based on the synergic effect of their bioactive compounds, which are totally different from that of chemical drugs. Determination of a few components is not representative adequately (Kong, Zhao, and Xiao, 2009; Di, Chan, and Leung, 2003). Taking *G. macrophylla* as an example, the specified quality control method for detecting the total content of loganic acid and gentiopicroside in *G. macrophylla* could not completely reflect its quality (Pharmacopeia Committee of P. R. China, 2010). Moreover, the clarification of the main constituents lacks confirmation based on the retention time. Therefore, it is necessary to establish an effective method to clarify the main constituents of *G. macrophylla* and control the quality.

The HPLC fingerprint technique has been considered to be a useful method in identification and quality evaluation of herbs and their related finished products

in recent years, since it could systematically and comprehensively exhibit the types and quantification of the components in the herbal medicines (Wang *et al*, 2007; 2006). By now, HPLC coupled with electrospray ionization tandem mass spectrometry (ESI-MS) has become a powerful technique for analysis and identification of the chemical constituents in complex TCM systems (Kang *et al*, 2008; Han, Shen, and Chen, 2008; Guo *et al*, 2007; Yang *et al*, 2007; Liu *et al*, 2007; Han *et al*, 2007).

In previous studies, HPLC fingerprints of *G. crassicaulis* (Wang *et al*, 2009), and *G. macrophylla* (Cao, Li, and Wang, 2008; Ma, Deng, and Yang, 2010) were established, but they were deficient in objectives and comprehensive evaluations. The present study was carried out to explore a complete method which could be well utilized to control the quality of the roots of *G. macrophylla*. The chemical constituents in the fingerprint were identified based on liquid chromatography-electrospray ionization-time-of-flight mass spectrometry (LC-ESI-TOF-MS). Moreover, the contents of the main constituents in the roots of *G. macrophylla* were quantified by HPLC-UV, including loganic acid, swertiamarin, gentiopicroside, and sweroside.

Materials and methods

Instruments and chromatographic methods

The HPLC system consisted of a Waters 2695 Separation Module (USA) and a Waters 2487 Dual Absorbance Detector (USA). The output signal was recorded using Empower Data Acquisition and Analysis System. Chromatographic separation was achieved on a Welchrom-C_{18} column (250 mm × 4.6 mm, 5 μm).

HPLC analysis The mobile phase consisted of CH_3OH (25%) and H_2O acidulated with 0.1% phosphoric acid (75%). The flow rate was 1.0 mL/min and column temperature was 30 ℃. UV detection wavelength was set at 236 nm for acquiring chromatograms.

LC-ESI-TOF-MS analysis The mobile phase consisted of CH_3OH (A) and 0.1% aqueous formic acid (B). The elution program was as follows: 0－28 min, 31% A; 28－29 min, 31%－37% A; 29－40 min, 37% A; The flow rate was 0.3 mL/min and column temperature was 30 ℃. UV detection wavelength was

set at 236 nm for acquiring chromatograms.

The MS acquisition parameters were as follows: source type ESI, focus not active, scan from *m/z* 50 to 3000 *m/z*, ion polarity negative, set capillary 3500 V, dry heater 180 ℃ and dry gas 8.1 L/min. All data acquired were processed by Bruker Compass Data Analysis 4.0 software.

Solvents and reagents

CH_3OH (HPLC grade) was purchased from Baker Company (USA). Ultra high purity water was prepared by a Millipore-Q SAS 67120 Molsheim (France). Other chemicals were of analytical grade with purity over 99.5% and used without further purification. Sweroside was provided by Shenzhen Medherb Biotechnology Co., Ltd. Loganic acid, swertiamarin, and gentiopicroside were isolated from *G. macrophylla* in our laboratory. The purity of the isolated compounds was shown to be higher than 98% analyzed by HPLC, and their structures were identified by IR, ^{1}H-NMR, and ^{13}C-NMR.

Plant materials

Fourteen batches of *Gentiana macrophylla* Pall. (No. 1－14) were collected at different ages from Longxian of Shaanxi Province, China and were identified as the genuine medicinal herb by Professor WANG Ya-zhou, Northwest University. A voucher specimen (1007) was deposited at Biomedicine Key Laboratory of Shaanxi Province, Northwest University. Root powder (0.5 g) was accurately weighed and extracted with 20 mL of methanol in ultrasonic bath (500 W, 40 kHz) for 30 min at 30 ℃ and filtered. The continual filtrate (1 μL) was diluted to a 10 mL volumetric flask by water as sample solution and filtered through a 0.22 μm filter membrane before analysis. Sample solution (10 μL) was injected to HPLC column and separated under above-mentioned chromatographic conditions.

Preparation of reference solution

Accurately weighed reference substances, loganic acid, swertiamarin, gentiopicroside, and sweroside, dissolved in methanol. Mixed reference substance solutions contained loganic acid 0.081 mg/mL, swertiamarin 0.046 mg/mL, gentiopicroside 0.410 mg/mL, and sweroside 0.009 mg/mL, and were filtered through a 0.22 μm filter membrane before analysis.

Data analysis

Data analysis was performed by professional

software named Similarity Evaluation System for Chromatographic Fingerprint of TCM (Version 2004 A), which was developed and recommended by Chinese State Food and Drug Administration. In this study, the software was employed to synchronously do quantitative comparison among different samples, as well as to compute and generate the mean chromatogram as a representative standard fingerprint chromatogram for a group of chromatograms. Then the correlation coefficients of samples with mean chromatogram could be provided.

Furthermore, the relative retention time (RRT) and relative peak area (RPA) of each characteristic peak related to the reference peak were calculated for quantitative expression of the chemical properties in the chromatographic pattern of herbs.

Results

Selection of chromatographic condition

For the simultaneous determination of these four compounds, various mixtures of CH_3OH-H_2O and CH_3CN-H_2O were tested as mobile phase. Considering the presence of loganic acid in herbal extraction, a small amount of phosphoric acid was added into the mobile phase to reduce ionization of these compounds. The results showed that the best resolution and the shortest analysis time were achieved when methanol-water/phosphoric acid (100:0.1) system was used.

The results indicated that 30 ℃ was the optimum column temperature.

Selection of detection wavelength was one of the key factors for simultaneous determination of loganic acid, swertiamarin, gentiopicroside, and sweroside. It was observed that the maximum absorption wavelengths of these four compounds were 231, 236, 254, and 243 nm, respectively. It was observed that 236 nm should be selected as the detection wavelength where the four compounds in the chromatogram possessed strong UV absorbance (Fig. 1).

In LC-MS, because of the limitation of flow rate,

in isocratic elution mode, retention time (t_R) of some components in the sample was too long, therefore we used linear gradient elution. Taking into account the residual phosphate in mass spectrometer, we replaced phosphoric acid aqueous solution to formic acid aqueous solution.
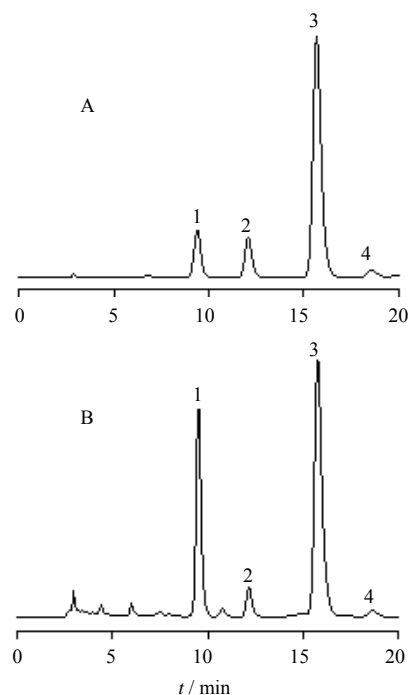

Fig. 1   HPLC chromatograms of mixed reference substances (A) and sample (B)
1: loganic acid   2: swetiamarin   3: gentiopicroside   4: sweroside

Linearity range

The four compounds were demonstrated to have good linear regression with high correlation coefficient values between peak area (Y) and amount (X, μg). All the analytes showed good linearity ($r^2 > 0.999$) in concentration ranges (Table 1).

Precision

Injection precision was assessed by repetitive injections of the same sample solution for six times in 1 d. The RSD of RPA was lower than 1.12%.

Repeatability

Repeatability was determined by analyzing six independently prepared samples of *G. macrophylla*

Table 1   Linearity of four ingredients in *G. macrophylla* (*n* = 6)

Compounds	Range / (μg·mL^{-1})	Regression equations	r^2
loganic acid	81－1296	$Y = 817\,021X － 23\,458$	0.9994
swertiamarin	46－736	$Y = 131.04X － 24999$	0.9994
gentiopicroside	410－6560	$Y = 105.29X － 159\,296$	0.9993
sweroside	8.9－142.4	$Y = 0.014\,392X － 2534.1$	0.9995

using the same method. RSD of RPA was less than 2.22%.

Sample stability

Stability was evaluated by analysis of six injections of the same sample solution every 3 h at 25 ℃. The RSD of RPA was found below 1.79%. The results indicated that the sample remained stable for 12 h.

Recovery

The recovery test was carried out by spiking certain known quantity of the four references with pulverized sample (0.25 g) of *G. macrophylla* and six samples were prepared for the test simultaneously. The recoveries of the quantitative compounds proved to be at the range from 99.3% to 103.23%, and the RSD value was less than 2.80%. It is an acceptable result for recovery analysis.

Sample determination

The valid method was employed to determine the four ingredients in each sample of 14 batches, and the contents of ingredients were shown in Table 2.

Table 2   Determination of 14 batches of *G. macrophylla* (μg·mL^{-1})

Sample No.	Loganic acid	Swertiamarin	Gentiopicroside	Sweroside
1	3.21	0.43	5.31	0.030
2	1.53	0.58	7.85	0.070
3	1.62	0.55	7.50	0.040
4	0.67	0.37	4.92	0.040
5	4.19	0.42	5.88	0.090
6	1.96	0.33	5.22	0.040
7	1.00	0.37	7.13	0.060
8	1.99	0.43	6.60	0.010
9	3.26	0.28	4.61	0.040
10	0.96	0.53	9.38	0.000
11	0.66	0.48	9.02	0.020
12	0.58	0.46	8.32	0.020
13	0.52	0.51	9.71	0.000
14	0.74	0.49	9.13	0.000
Average	1.635	0.445	7.184	0.003

Comparison of contents of four constituents from *G. macrophylla* at different ages

By comparing the contents of loganic acid, swertiamarin, gentiopicroside, sweroside, and the sum of these four constituents (Table 2, sample No. 2－4), we observed that different harvest time could influence the constituent contents of *G. macrophylla*. The differences between two-year and three-year were not obvious, but if harvested in four-year, the contents in most of the components decreased significantly. Thus, the best time of harvest should be two or three years.

The variation of accumulated ingredient contents from *G. macrophylla* at different ages could be ascribed to its stage of flowering, since the three-year *G. macrophylla* experienced the stage of flowering and fruiting, while the two-year flowering very few (Cao and Wang, 2010b).

Establishment of chromatographic fingerprint for cultured *G. macrophylla*

Selection of the samples   Fourteen batches of *G. macrophylla* samples were analyzed. There were five common peaks identified in the fingerprint of *G. macrophylla* (peaks 1 － 5, Fig. 2). Though the chromatograms of 14 batches of *G. macrophylla* samples were generally similar, the peak area ratios of the common peaks or some small peaks were distinctly different.

Selection of reference substance   To calculate the RRT and RPA, a reference substance should be chosen (Kang *et al*, 2008). There are two kinds of reference substances, one is an internal reference substance belonging to common peaks and the other is an external reference substance added to the sample. In this study, peak 5 (t_R = 15.7 min, gentiopicroside, Fig. 2) was chosen as the internal reference substance because this peak had maximum content, and existed in all chromatograms. The RRT and RPA of common peaks in simulative mean chromatograms of *G. macrophylla* from different sources were calculated. The RRT averages of peaks 1－5 were 0.380, 0.602, 0.682, 0.771, and 1.000, respectively; The averages of RPA were 0.0174, 0.2124, 0.0385, 0.0776, and 1.000, respectively.

Chromatograms similarities of 14 batches of *G. macrophylla*   The chromatograms similarities of 14 batches of *G. macrophylla* were 0.965, 0.999, 1.000, 0.997, 0.945, 0.994, 0.997, 0.995, 0.946, 0.995, 0.993, 0.992, 0.991, and 0.993, respectively.

HPLC-ESI-TOF-MS analysis

HPLC-ESI-TOF-MS was employed to analyze the components separated by HPLC. In ESI-TOF-MS experiment, accurate molecular mass of the components was obtained. Comparing MS results with the standard samples of loganic acid, 6′-*O*-β-*D*-glu-gentiopicroside, swertiamarin, and gentiopicroside, four compounds were
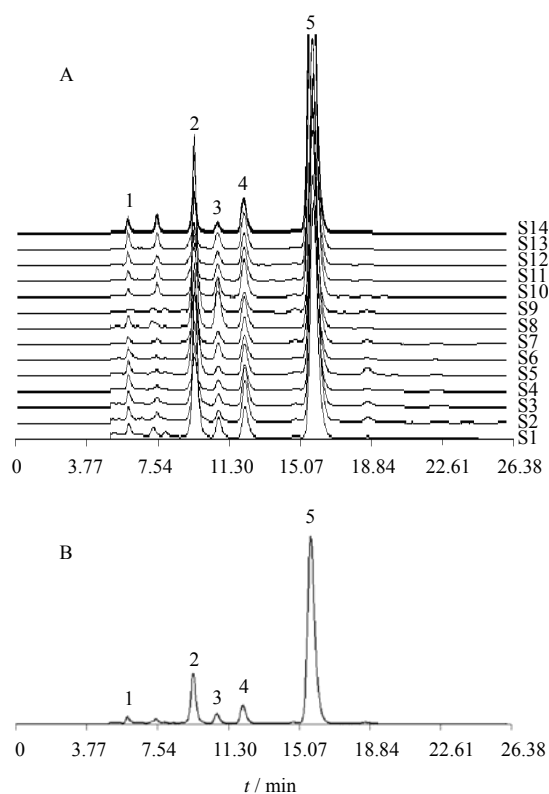
Fig. 2   Overlap of HPLC chromatograms (A) and HPLC chromatogram (B) for 14 batches of *G. macrophylla*

1: unknown   2: loganic acid   3: 6′-*O*-β-*D*-glu-gentiopicroside
4: swertiamarin   5: gentiopicroside

unambiguously identified based on their MS data. Since negative ionization ESI mode was used, most of the *m/z* data were [M－H]⁻ and [M + Cl]⁻. The mass data and the tentative assignment of the peaks are given in Table 3, TIC TOF-MS of the sample and TOF-MS of peaks 1－8 were shown in Fig. 3.

In ESI(−)-TOF-MS data, [M－H]⁻ is 375.1 ([M－H]⁻, calculated for $C_{16}H_{24}O_{10}$ 376.3), so peak 3 was deduced as loganic acid (Zhang *et al*, 2003). [M + Cl]⁻ is 553.1 ([M + Cl]⁻ calculated for $C_{22}H_{30}O_{14}$ 553.4), so peak 5 was positively identified as 6′-*O*-β-*D*-glu-gentiopicroside. [M + Cl]⁻ is 409.1 ([M + H]⁺, calculated for $C_{16}H_{22}O_{10}$ 374.3), so peak 6 was deduced as swertiamarin (Aberham *et al*, 2011). [M + Cl]⁻ is 391.1 ([M + Cl]⁻, calculated for $C_{16}H_{20}O_9$ 391.3), so peak 8 was deduced as gentiopicroside (Yang *et al*, 2009). Unfortunately, sweroside is not ionizable under the chosen MS conditions, probably due to its extremely low content.

Discussion

In this paper, we established an HPLC fingerprint analysis method to evaluate the quality of the roots of *G. macrophylla*, the method is faster and more convenient

Table 3   Mass data and compound names of peaks

Peak	*t* / min	Observed mass	Calculated mass	Assignment
1	8.5	539.1		unknown
2	9.0	683.2		unknown
3	15.5	537.2		unknown
4	19.2	375.1 [M－H]⁻	375.3 [M－H]⁻	loganic acid
5	20.9	553.1 [M + Cl]⁻	553.4 [M + Cl]⁻	6′-*O*-β-*D*-glu-gentiopicroside
6	24.4	409.1 [M + Cl]⁻	409.3 [M + Cl]⁻	swertiamarin
7	27.7	389.1		unknown
8	30.5	391.1 [M + Cl]⁻	391.3 [M + Cl]⁻	gentiopicroside

than previous reports (Wang *et al*, 2009; Cao, Li, and Wang, 2008). The contents of the four compounds were determined. Meanwhile, as one of the most powerful analytical techniques, LC-ESI-TOF-MS was utilized to confirm the structures of the main constituents in *G. macrophylla*, which provided a high level of sensitivity and resolution (Thurman, Ferrer, and Zweigenbaum, 2005; Han, Shen, and Chen, 2008).

An HPLC method was developed to obtain a fingerprint for the roots of *G. macrophylla*. The fingerprint showing five common peaks represented the characteristics of constituents in *G. macrophylla*.

The similarities of 14 batches of *G. macrophylla* samples were obtained with a standardized procedure. In order to further adequately and comprehensively assess the quality of *G. macrophylla* probably, more than one fingerprint would be needed due to the complexity of the constituents in herbal drugs. However, it could be acceptable as a first step to create a representative fingerprint, as presented here, and more fingerprints as new evidence will be studied later. In addition, by comparing the main component contents of *G. macrophylla* at different ages, two-year *G. macrophylla* showed the highest content. Thus, two or three years
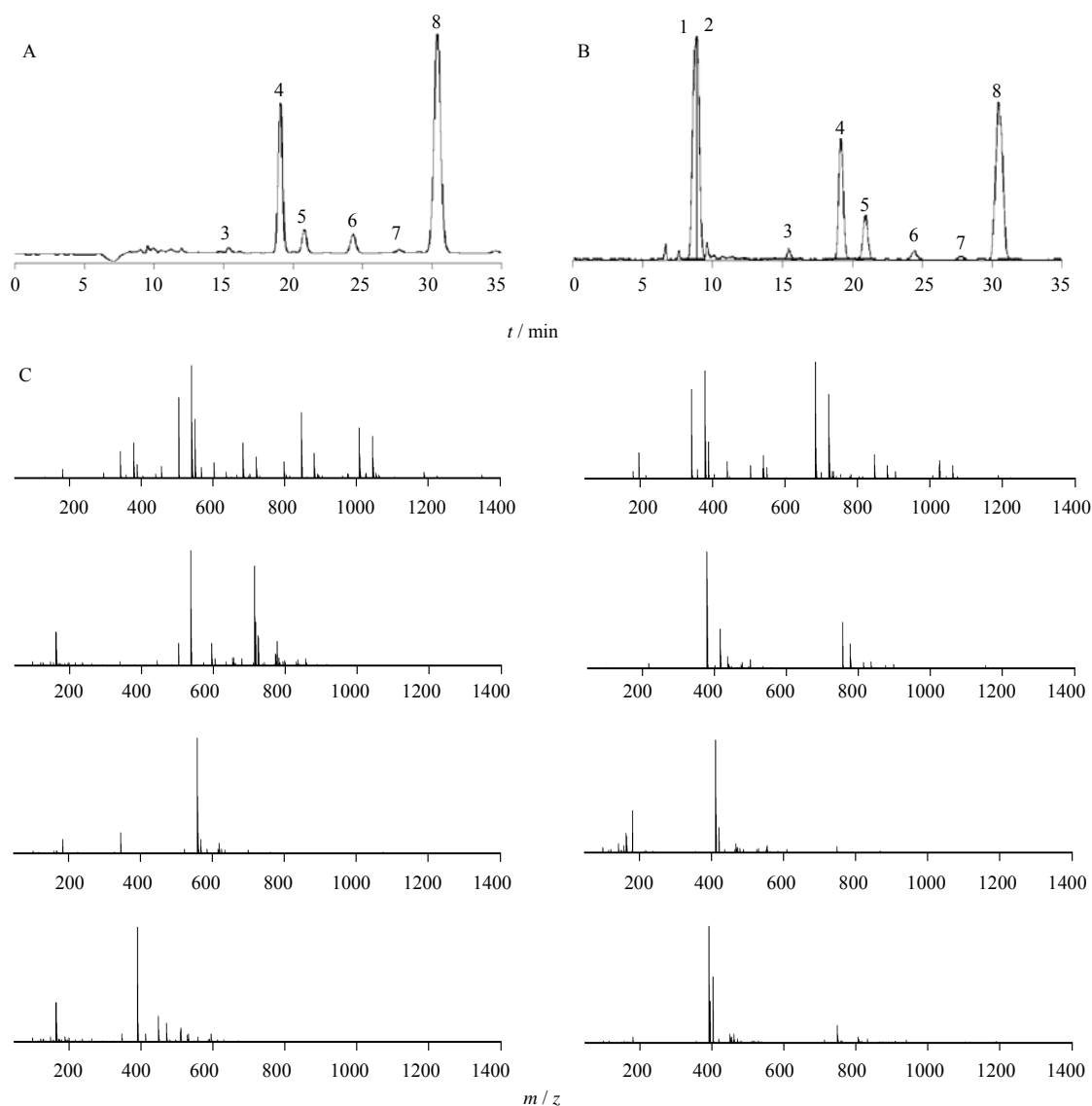
Fig. 3   HPLC chromatograms (A), TIC TOF-MS of sample 9 (B), and TOF-MS of peaks 1－8 (C)

1－3 and 7: unknown   4: loganic acid   5: 6′-*O*-β-*D*-glu-gentiopicroside   6: swertiamarin   8: gentiopicroside

should be the best time of harvest. To our knowledge, this is the first report on the analysis of *G. macrophylla* by LC-ESI-TOF-MS technique. The results indicated that this technique would be useful in comprehensive quality evaluation of *G. macrophylla*.

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