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Simultaneous Determination of Four Neuritogenic Compounds in *Gentiana rigescens* from Different Regions by Ultra-Performance Liquid Chromatography Tandem Mass Spectrometry

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The roots and rhizomes of *Gentiana rigescens* are the original materials of Chinese traditional medicine "LongDan" which is used for treatment of hepatitis and cholecystitis for over a thousand years. In this study, the concentrations of four gentisides (gentiside A, B, J and K) were determined by UPLC-MS/MS in *Gentiana rigescens* from 2 different regions (Yunxian and Yongde) of southwest Yunnan in China. The maximum concentration of gentiside A, B, J and K were found in S5, S12, S5 and S3, respectively. Hierarchical clustering analysis showed the maximum total concentration of gentisides was in group IV, which indicated S5 could be regarded as optimizing original materials of *G. rigescens*. The cultivated samples collected from Yunxian showed the higher contents of gentisides. These results would provide information for sustainable utilization of *G. rigescens*.

Keywords: *Gentiana rigescens*, Gentisides, Different regions, UPLC-MS/MS.

INTRODUCTION

In Chinese Pharmacopoeia, *Gentiana rigescens* (Family: Gentianaceae) is recorded as one of the original materials of well-known traditional Chinese medicines "Longdan" which commonly used for treatment of hepatitis and cholecystitis for over a thousand years[1]. It mainly distributes in Yunnan, Sichuan, Guizhou, Hunan and Guangxi provinces in China and is usually found in slope grass with elevation of 1100-3000 m[2].

G. rigescens is rich sources of iridoids and triterpenoids which possess remarkable bioactivities, such as hepatoprotective, anti-inflammatory and cytotoxicity[3-7]. Gentiopicroside together with other iridoids such as swertiamarin and sweroside, the major chemical constituents in *G. rigescens*, are commonly regarded as the main index in evaluating the quality for *G. rigescens* and other plants from genus *Gentiana*[1,8-10].

Because of the remarkable medicinal functions, *G. rigescens* have been a hot topic in phytochemistry and pharmacology. Modern phytochemical and pharmacological studies reported that 11 compounds (gentiside A-K) with alkyl 2,3-dihydroxybenzoates nuclear derived from *G. rigescens* possessed significant neuritogenic activity against PC12 cells, which indicated that *G. rigescens* is an important source for obtaining neuritogenic activity constituents[11,12].

Early reported methods based on high-performance liquid chromatography (HPLC) coupled with UV detection have been published for the determination of gentiopicroside in *G. rigescens*[8,9]. However, to the best of our knowledge no report is available on quality control of *G. rigescens* for simultaneous determination gentisides by UPLC-MS/MS. In recent years, the LC-MS/MS has been widely used for quantification of drugs, which could provide molecular mass and structural information for identification of the target constituents in samples[13,14]. Furthermore, several reports indicated the UPLC system have significant advantages of reducing the chromatographic run time and enhancing response than HPLC system[15-17].

As a major original material of "LonDan", *G. rigescens* is widely cultivated in southwest Yunnan, China. The 15 sites in southwest Yunnan selected are the main cultivated sites which have significant influence on the supplement of *G. rigescens* sources. In order to evaluate *G. rigescens* from the 15 cultivated sites, we conducted to obtain the concentration information by ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) on four gentisides of *G. rigescens* collected from the 15 cultivated sites in two zones (Yunxian and Yongde) of southwest Yunnan, China.

EXPERIMENTAL

The HPLC grade solvent (methanol and formic acid) were purchased from Tedia and Dikmapure (USA), respectively. Other reagents and chemicals were analytical grade. The pure water was purified by a Milli-Q system from Millipore (USA). Gentiside A, B, J and K (Fig. 1) have been isolated from *G. rigescens* which are more than 90 % purity and are characterized on the basis of their spectroscopic data, which are in complete agreement with reported data[8,9]. Stock solutions of each marker prepared in 95 % ethanol by weighing accurately and separately were stored at 4 °C and used for calibration curves. *G. rigescens* samples with 3 years of growth were collected in November 2012 from 15 different sites in two zones (Yunxian and Yongde) of southwest Yunnan, China. The corresponding materials information was displayed in Table-1.

TABLE-1
SUMMARY FOR THE DIFFERENT REGIONS OF *G. rigescens*

No.	Growing regions	No.	Growing regions
S1	Dapinzhangshan, Yunxian	S9	Xinhuacun, Yunxian
S2	Zhanglongshan, Yunxian	S10	Daxueshan (a), Yongde
S3	Huangyandi, Yunxian	S11	Daxueshan (b), Yongde
S4	Xiaolongtan, Yunxian	S12	Xiangshuicun, Yunxian
S5	Fanshanlu, Yunxian	S13	Duoyicun, Yunxan
S6	Fuyanshan, Yunxian	S14	Xiajiaqing, Yunxian
S7	Aluodi, Yunxian	S15	Banka, Yongde
S8	Dawanshan, Yongde	–	–

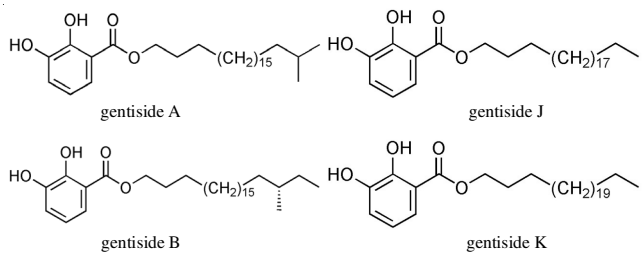

Fig. 1. Structure of the four gentisides

Sample preparation: The samples were dried at 60 °C until constant weight and then ground into powder and sieved through a 100 mesh stainless steel sieve before extraction. A total of 0.5 g of each sample powder was placed in test tube, while adding 10 mL 95 % ethanol at the room temperature for a whole night. The samples were extracted once with ultrasonication for 40 min. Then, 1 mL of filtrate was transferred by pipetting into a 25 mL volumetric flask and made up to the volume with 95 % ethanol. All extracts were stored at 4 °C and filtered through a 0.22 μm membrane filter before injection into the UPLC system.

Instruments and UPLC-MS-MS conditions: UPLC-MS/MS (Shimadzu, LCMS-8030, Japan) was equipped with auto-sample, binary gradient pumps, electrospray ionization interface (ESI) and triple quadrupole mass spectrometer detector. Chromatographic separation was performed on Shimpack XR-ODSIII (75 × 2 mm, 1.6 μm). The mobile phase was consisted of methanol: 0.1 % formic acid water (95:5, v/v) isocratic elution with the flow rate 0.45 mL/min. The column temperature was kept 40 °C and the injection volume was 5 μL.

The mass spectrometer parameters set as follows: Nebulizing gas and drying gas were nitrogen at a flow rate of 3 and 15 L/min, respectively. The interface voltage was set to 4.5 kV; desolvation line (DL) temperature was 250 °C and the heat block temperature was 400 °C. All the analytes were detected in negative ionization and detection was carried out within a mass range of 80-700 *m/z*. The multiple reaction monitor (MRM) acquisition mode was used for quantification purposes. All the MRM settings were auto-optimized (Table-2). Although gentiside A and J are the isomers, this problem is well solved by chromatograph.

TABLE 2
LC-MS/MS PARAMETERS (MRM) FOR THE SELECTED ANALYTES (DWELL TIMES, Q_1: PRECURSOR ION MASS, CE: COLLISION CELL ENERGY, AND Q_3: PRODUCT-ION MASS)

Analyte	Rt (min)	Q_1 (*m/z*) $[M-H]^-$	Q_3 (*m/z*)	Dwell time (msec)	CE (V)
Gentiside A	8.825	461.35	91.30	80	50
			109.30	80	50
Gentiside J	9.528	461.40	91.20	80	50
			109.20	80	50
Gentiside B	10.855	475.75	91.15	80	50
			109.15	80	50
Gentiside K	14.606	489.35	91.35	80	50
			135.20	80	40

Date analysis: The peak areas and concentration of all samples was calculated by Shimadzu lab solution software (Shimadzu, Japan). The hierarchical clustering analysis was performed based on the peaks' area of four gentisides from UPLC-MS/MS profiles of fifteen samples by SPSS 20.0 for Windows. The between groups linkage was applied and the squared Euclidean distance was selected to measure hierarchical clustering analysis.

RESULTS AND DISCUSSION

Method validation: The developed method for determination of the four gentisdes had been validated by our previous work, the result showed the linearity, precision and accuracy were all within the required limits[1,18]. All calculations were carried out using Shimadzu LabSolution software (Shimadzu, Japan).

The methanol stock solution of standards was prepare and diluted with methanol to appropriate concentrations for establishment of calibration curve. These calibration curves plotted with six different contents ranging from 0.26 to 6.67 μg/mL and the correlation coefficient was more than 0.9989. The limits of detection (LOD) and limits of quantification (LOQ) under the present chromatographic conditions were determined at S/N (of signal-to-noise ratio) of 3 and 10, respectively. Linearity data, the limits of detection (LODs) and limits of quantification (LOQs) are displayed in Table-3.

Intra-day and inter-day variation was chosen to determine the precision of the method. The known concentrations of four standard solutions were tested in six replicates. For intra-day variability test, the standard solutions were analyzed seven times within one day. For inter-day variability test, the standard solutions were analyzed within three successive days. The result showed % RSD of retention time and peak areas were less than 2 %.

TABLE-3
LINEAR REGRESSION DATA OF GENTISIDES

Analyte	Regression equation	Linearity range (μg/mL)	r	LOD (μg/mL)	LOQ (μg/mL)
Gentiside A	y=91855.74x-30185.66	0.39-4.44	0.9993	0.05	0.17
Gentiside J	y=91393.23x-35117.87	0.39-4.44	0.9990	0.11	0.33
Gentiside B	y=76030.71x-32415.63	0.59-6.67	0.9989	0.05	0.14
Gentiside K	y=125628.28x-29309.47	0.26-6.67	0.9994	0.08	0.25

Recovery test was used to evaluate the accuracy of the method. For the per cent recovery experiments, three different amounts *viz*. 50, 100 and 150 % addition of the four standard solutions were spiked to the S5. The spiked samples were extracted and analyzed by the proposed method. The recovery was calculated using the following equation and found to be in the range of 98.1-102.6 %. Precision and accuracy results are displayed in Table-4.

Recovery (%) = 100 × (amount found-original amount)/ amount added

Quantification of four gentisides by UPLC-MS-MS: The MRM acquisition mode was used for quantification purposes. All the MRM settings were auto-optimized (Table-2). The identification of four gentisides was confirmed by comparison of their retention times and mass dates with corresponding standard compounds.

The total contents of the four gentisides followed in the order: S5 > S12 > S13 > S4 > S14 > S3 > S7 > S1, S2 > S9 > S6 > S11, S15 > S10 > S8. The samples collected from Yunxian showed the higher contents of gentisides than samples from Yongde. The maximum concentration of gentiside A, B, J and K were found in S5, S12, S5 and S3, respectively. The contents of the four gentisides from different regions of *G. rigescens* are summarized in Table-5 and the total ions chromatogram (TIC) is shown in Fig. 2. However, according to the structures of gentisides, UPLC-MS/MS method coupled with atmospheric pressure chemical ionization (APCI) for determination of the four gentisides also should be performed.

Hierarchical clustering analysis: The hierarchical clustering analysis results displayed in Fig. 3 showed that these samples were divided into four groups. Among them, group IV contained only one sample (S5), group III included S3 and S13. S4, S12 as well as S14 were in group II and other samples belonged to group I. Hierarchical clustering analysis indicated that (1) the sample in group IV showed the maximum total contents of gentisides; (2) the rich gentiside B and K would be obtained from samples in group III and (3) the samples in group II could be consider as the optimizing materials for obtaining gentiside A and J. The investigation for gentisides should be continued to promote the further search for *G. rigescens*.

TABLE-5
AVERAGE CONTENTS (mg/g) OF GENTISIDE IN DIFFERENT REGIONS OF *G. rigescens* (n = 5)

No.	Gentiside A	Gentiside J	Gentiside B	Gentiside K	Total
S1	0.691	0.887	0.842	0.485	2.905
S2	0.679	0.883	0.848	0.495	2.905
S3	0.684	0.876	0.858	0.507	2.925
S4	0.691	0.934	0.854	0.479	2.958
S5	0.725	0.916	0.951	0.502	3.094
S6	0.682	0.879	0.836	0.478	2.875
S7	0.685	0.893	0.858	0.474	2.910
S8	0.667	0.863	0.845	0.473	2.848
S9	0.683	0.877	0.847	0.488	2.895
S10	0.669	0.868	0.856	0.477	2.870
S11	0.670	0.878	0.853	0.470	2.871
S12	0.688	0.948	0.848	0.481	2.965
S13	0.672	0.877	0.918	0.498	2.965
S14	0.710	0.912	0.856	0.466	2.944
S15	0.673	0.876	0.849	0.473	2.871

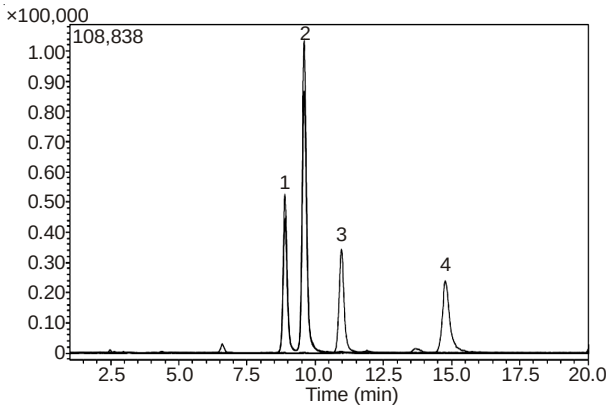

Fig. 2. A: TIC of sample (S5); 1:gentiside A; 2:gentiside J; 3: gentiside B; 4: gentiside K

Conclusion

In this study, a simple and reliable UPLC-MS/MS method was used for determination of four gentisides and finding the optimizing original materials of *G. rigescens*. Sample (S5) in

TABLE-4
PRECISION AND ACCURACY OF THE FOUR GENTISIDES

Analytes	Intra-day RSD (%)		Intra-day RSD (%)		Amount added (μg/mL)	Found (μg/mL)	Recovery (%)	RSD (%)
	Rt	Pa	Rt	Pa				
Gentiside A	0.58	0.71	0.59	1.35	0.8	0.816	102.0	0.96
					1.6	1.613	100.8	0.77
					2.4	2.354	98.1	1.32
Gentiside B	0.33	0.62	0.77	1.17	1	1.024	102.4	1.44
					2	1.968	98.4	1.56
					3	3.045	101.5	1.13
Gentiside J	0.69	0.54	0.66	1.47	2	2.052	102.6	1.65
					4	3.956	98.9	1.12
					6	5.946	99.1	1.03
Gentiside K	0.93	1.06	0.82	1.71	0.8	0.808	101	0.73
					1.6	1.587	99.2	0.68
					2.4	2.376	99	0.81

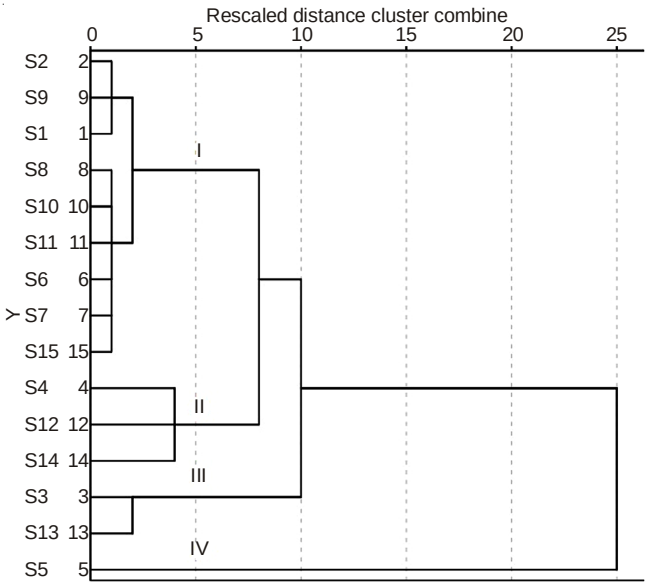

Fig. 3. Dendrograms resulting from peaks' area of four analytes on UPLC-MS-MS chromatograms of the tested 15 samples using between groups linkage of hierarchical cluster analysis

group IV was the optimizing gentisides sources of *G. rigescens*, the maximum contents of gentiside A and J was also found in samples (S4, S12 and S14) from group II and the maximum contents of gentiside B and K was found in samples (S3 and S13) from group III. These results would provide information for sustainable utilization of *G. rigescens*.

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