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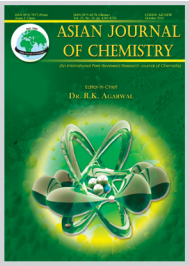

Simultaneous Quantitative Determination of *Gentiana scabra* by HPLC-MS/MS

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An approach for simultaneous quantitative determination of bioactive components in traditional Chinese medicine, using HPLC-MS/MS technique was described in this paper. *Gentiana scabra*, a well-known traditional Chinese medicine, was studied using the established method as for an application. The column was Kromasil C18 and the gradient mobile phase was optimized for a better resolution. As a result, the linearities and recoveries of the 6 components were suitable and good for the detection. This work could provide a simple, rapid and relatively comprehensive quality control method for the clinical safety of *G. scabra*.

Key Words: *Gentiana scabra*, HPLC-MS/MS, Simultaneous, Quantitative determination.

INTRODUCTION

In recent years, traditional Chinese medicine has been given increasing popularity worldwide for their complementary therapeutic effects to the Western drugs but with minimum side effects[1,2]. The effects of traditional Chinese medicine are brought about by its chemical constituents. Thus, the chemical analysis of traditional Chinese medicine is especially important because it helps to understand which chemical components exist inside and which ingredients are the real bioactive ones for certain therapeutic effects and then to establish scientific and rational quality control methods. Each traditional Chinese herb comprises hundreds of different constituents, therefore, systematical and comprehensive analysis of traditional Chinese medicine is an urgent task.

HPLC-UV method is widely used for the detection of components in traditional Chinese medicine, while it also has some shortages. An HPLC-UV method could not detect the compounds without conjugated groups for the lack of the generation of UV absorbance. At the same time, the Superposition Principle of UV suggested that the multiple targets must have good resolution, otherwise the quantitation could not be achieved simultaneously. HPLC-MS technology has been shown to be a useful tool to solve the above problems and becoming more and more widely accepted in the field of pharmaceutical analysis[3-5].

G. scabra distributed in China, Japan, Korea and southeast Asia and was widely used for the treatment of type-B encephalitis, jaundice and convulsion, *etc.*[6-9]. Although several literatures had reported its content detection[10-12], to the best of our knowledge, there was no determination of more than 3 bioactive components in *G. scabra* simultaneously. The aim of this paper is to provide a simultaneous determination method for a comprehensive analysis and quality control of *G. scabra* for future clinical use.

EXPERIMENTAL

Dried plants of *G. scabra* were collected in Liaoning Province, China. Voucher specimen had been identified by Pharmacognosist Zengxi Guo and also been kept under certain conditions for future identification.

The dried powder of *G. scabra* (5 g) was refluxed with 70 % ethanol for 1 h, then the extract was evaporated to dryness at 40 ºC under a stream of nitrogen. The residue was dissolved to a 5 mL volumetric flask with methanol. The solution was ready for chromatographic analysis after passing through a 0.45 μm membrane filter.

HPLC-MS/MS conditions: An Agilent 1200 series LC system was employed in this research, which consisted of a

TABLE-1
RESULTS OF LINEARITIES, LIMIT OF DETECTION AND LIMIT OF QUANTIFICATION

Compounds	Standard curve[a]	R^2	Linear range (mg)	Limit of detection (μg)	Limit of quantification (μg)
1	$Y = 2.014 \times 10^4 + 246.53$	0.999 1	0.42-5.77	0.15	1.56
2	$Y = 4.491 \times 10^4 + 156.31$	0.999 2	0.58-5.13	0.34	1.69
3	$Y = 2.231 \times 10^4 + 331.77$	0.999 6	0.15-3.46	0.32	2.21
4	$Y = 3.564 \times 10^3 + 159.26$	0.999 9	0.54-4.86	0.12	1.79
5	$Y = 6.325 \times 10^4 + 125.23$	0.999 4	0.18-3.12	0.12	2.36
6	$Y = 3.456 \times 10^4 + 326.12$	0.999 2	0.35-1.98	0.38	2.69

[a]Y was the peak area in HPLC chromatograms, X was the compound amount injected, and Y, X were the logarithmic values of area and amount injected in HPLC chromatograms.

TABLE-2
RESULTS OF PRECISION AND REPEATABILITY

Compounds	Precision				Repeatability (n = 5)	
	Intra-day (n = 6)		Inter-day (n = 3)			
	Mean (mg g^{-1})	RSD (%)	Mean (mg g^{-1})	RSD (%)	Mean (mg g^{-1})	RSD (%)
1	2.13 ± 0.01	1.3	2.12 ± 0.36	2.9	2.17 ± 0.22	1.9
2	2.16 ± 0.05	2.0	2.20 ± 0.27	2.3	2.17 ± 0.12	1.7
3	3.10 ± 0.22	1.9	3.08 ± 0.19	2.4	3.14 ± 0.12	1.7
4	3.05 ± 0.04	1.5	3.03 ± 0.45	2.3	3.05 ± 0.18	1.3
5	1.56 ± 0.10	1.2	1.51 ± 0.33	3.1	1.61 ± 0.13	1.4
6	2.07 ± 0.11	1.9	2.01 ± 0.36	2.9	2.03 ± 0.21	1.7

G1376A Cap Pump, a G1379B Degasser, a G1376B Autosampler and a Hystar PP work station.

An Agilent 6460 QQQ MS was employed in the analysis. The relative parameters were optimized as the following: positive mode, capillary voltage 3500 V; drying gas 4 L/min; nebulizer 1.5 psi; gas temp. 250 ºC; fragmentor voltage 145 V, CE 20 eV. Through the manner of multiple reaction monitor (MRM), the ion pairs for the quantitative detection had been established.

The analysis was carried out on a Kromasil C18 (250 × 4.6 mm, 5 μm), which was protected by a RP18 guard column. The solvents used for HPLC separation were acetonitrile as solvent A and water as solvent B at a flow rate of 1.0 mL min^{-1}. The mobile phase was as the following: 0 min 10 % A, 15 min 30 % A, 25 min 60 % A and then maintained for 10 min. The column temperature was 35 ºC and the sample injection volume was 10 μL.

Method validation: Standard stock solutions of 6 components were prepared by dissolving each compound in methanol to obtain a concentration *ca.* 1 mg mL^{-1}. Ten addition calibration levels were prepared by diluting each solution with methanol. Limit of detection (LOD) was defined as 3 times of signal to noise and limit of quantitation (LOQ) as 10 times (Table-1). The intra- and inter-day variabilities of each compound were assayed at each mass concentration (n = 6) on the same day and on three sequential days, respectively. The accuracy was calculated from the nominal mass concentration (C_{nom}) and the mean value of the observed concentration (C_{obs}) as follows: Accuracy = $[(C_{obs} - C_{nom})/(C_{nom})] \times 100$ %. The relative standard deviation (RSD) was calculated from the observed mass concentrations as follows: RSD = [standard deviation (SD)/C_{obs}] × 100 % (Table-2). The recoveries of 6 compounds were investigated using the standard addition method. Accurate amounts of mixed standards were added to approximate 1 g of sample with determined content. The sample was then extracted and analyzed as described above. Three parallel samples were prepared and the assay was repeated 3 times (Table-3).

TABLE-3
RECOVERIES OF THE 6 ANALYTES (n = 3)

Compounds	Amount added (mg)	Amount found (mg)	Recovery (%)	RSD (%)
1	5.71	5.65 ± 0.42	99.13	1.7
2	5.15	5.24 ± 0.16	100.26	2.0
3	3.37	3.35 ± 0.12	99.60	1.7
4	7.11	7.15 ± 0.12	100.15	1.9
5	4.28	4.13 ± 0.24	97.56	1.9
6	4.22	4.28 ± 0.36	101.46	2.0

RESULTS AND DISCUSSION

A good separation of the 6 compounds, luteolin-7-O-glucoside (**1**), apigenin-7-O-glucoside (**2**), isoorientoside (**3**), luteolin (**4**), apigenin (**5**) and 1,4,8-trihydroxy-5,6-dimethoxyxanthone (**6**), was identified by the optimized mobile program, with the retention times at 15.7, 16.8, 22.7, 27.1, 28.5 and 32.1 min, respectively (Fig. 1). Then, with the help of Agilent optimizer software and comparison with the references, ion pairs of the 6 components for the multiple reaction monitor quantification were established as the following (m/z): 1: 449.1-383.1; 2: 433.1-357.1; 3: 449.3-287.3; 4: 287.2-217.2; 5: 271.3-217.2; 6: 305.3-227.2. All the quantitative ions showed the best respond to the detection conditions, which guaranteed sensitive and accurate detection of the targets (Fig. 2).

Good linearities between peak areas and concentrations were also obtained for all compounds over the tested concentration ranges with a correlation coefficient > 0.999. The recoveries of the tested constituents were all evaluated within the range of 97.56-101.46 % with RSD below 2 % by the analysis of spiked samples. The validated HPLC-MS/MS method was applied to the determination of 6 compounds in

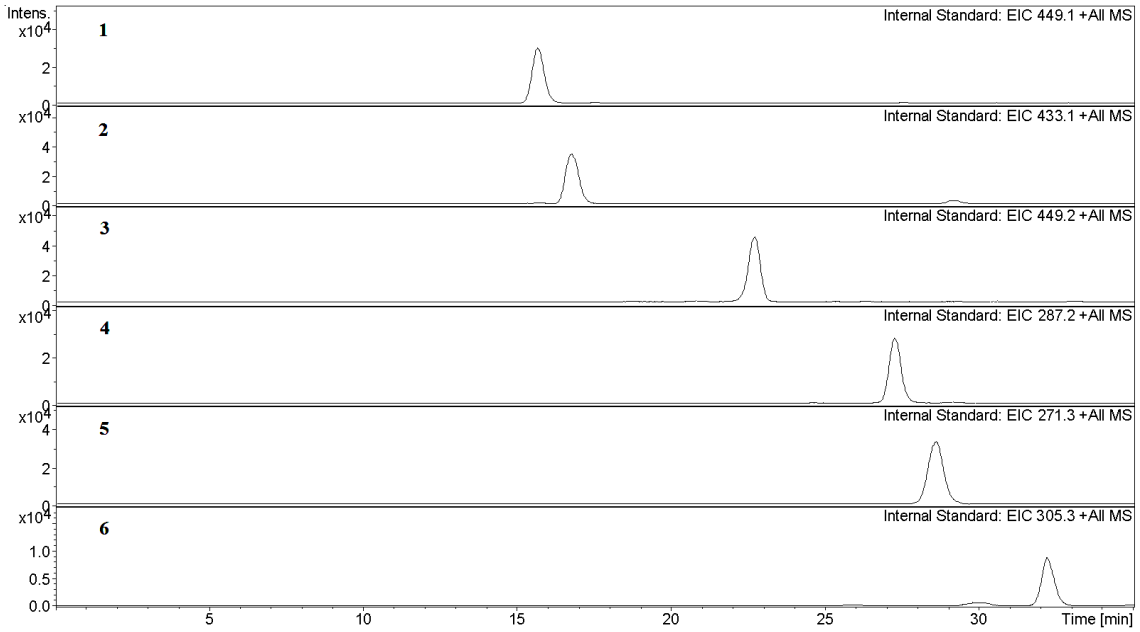

Fig. 1. EIC of the six components in *G. scabra*

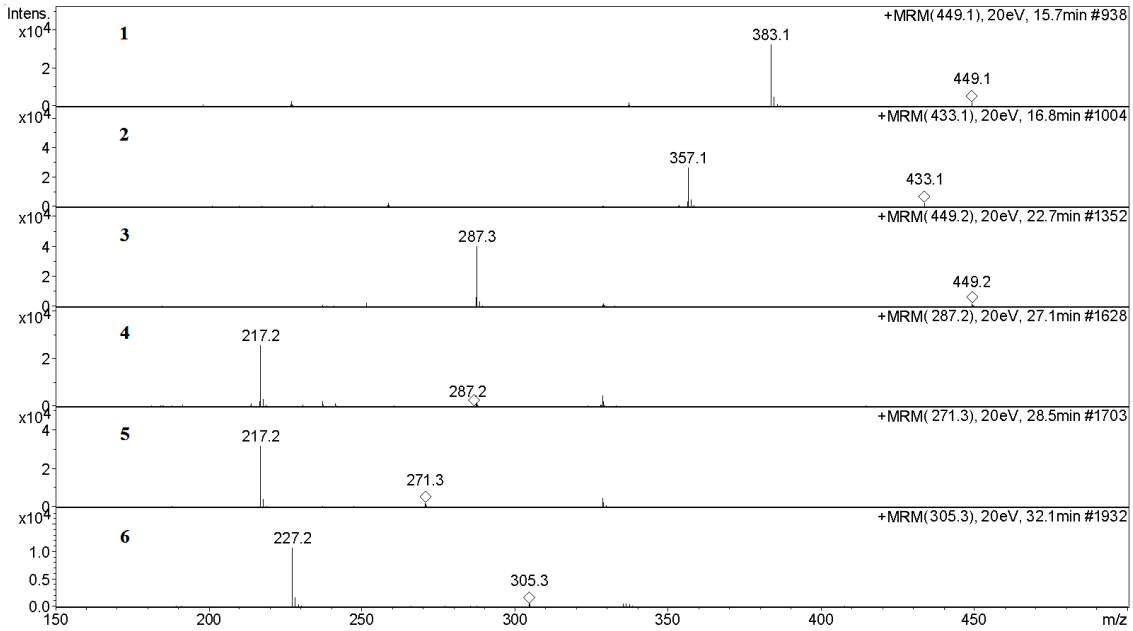

Fig. 2. MRM of the six components in *G. scabra*

commercial samples of *G. scabra*. Each sample was determined triplicately, which also gained good results. These results demonstrated that the method was simple, rapid and relatively comprehensive for the quality control of *G. scabra*.

In this paper, an HPLC-MS/MS method has been developed for the simultaneous determination of 6 bioactivity components in *G. scabra*. The separation of the 6 compounds with tandem mass spectrometric detection could provide an accurate and reproducible quantification method, then significantly improve the assay performance.

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