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Short communication

Quality evaluation of *Polygala japonica* through simultaneous determination of six bioactive triterpenoid saponins by HPLC-ELSD

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Abstract

Polygala japonica Houtt (Polygalaceae), a traditional Chinese herb, has been used as expectorant, anti-inflammatory, antibacterial and antidepressant agent. To evaluate the quality of *P. japonica*, a high performance liquid chromatography (HPLC) with evaporative light scattering detector (ELSD) method was developed for the simultaneous determination of six active triterpenoid saponins. With a Discovery C_{18} analytical column, the analytes were separated efficiently using acetonitrile–methanol–0.05% trifluoroacetic acid (TFA) as mobile phase in a gradient program. The evaporator tube temperature of ELSD was set at 105 °C, and with the nebulizing gas flow-rate of 2.6 l/min. All calibration curves showed good linear regression ($\gamma > 0.9991$) within the tested range. Additionally, reproducibility for the quantification of six saponins in *P. japonica* with intra- and inter-day variations of less than 5.0% was observed. Quantification of the six active saponins in *P. japonica* from different locations was performed by this newly developed method, which provides a new tool for the assessment of quality of *P. japonica*.

Keywords: *Polygala japonica* (Polygalaceae); Triterpenoid saponins; Quantification; HPLC-ELSD

1. Introduction

The whole plant of *Polygala japonica* has long been used as an expectorant, anti-inflammatory, antibacterial and antidepressant agent in traditional Chinese medicine [1]. *P. japonica* is a perennial plant that was collected at autumn in the third year of growth. Phytochemical studies on *P. japonica* have led to the discovery of a number of triterpenoid saponins with different structures [2–9]. Among identified saponins, some bayogenin types from this plant were found to exert significant anti-inflammatory activity by inhibiting carrageenan-induced acute paw edema in mice in our previous studies, and antibacterial activity of bayogenin and medicagenic acid type saponins in other plants was reported [10]. Moreover, medicagenic acid type saponins in *P. japonica* possess antidepressant activity [9]. Therefore, triterpenoid saponins with bayogenin and medicagenic acid glycosides should be considered as useful markers relevant to biological activities for the quality control of *P. japonica*.

In clinic, tablet and granule preparations from the aqueous extract of *P. japonica* have showed the effectiveness against pharyngitis, nephritis and so on [11,12]. Unfortunately, the lack of a reliable method to control the quality of *P. japonica* is problematic for these preparations to exhibit clinical effects consistently. Thus, it is important to develop a method to evaluate the quality of *P. japonica* for the purpose of ensuring clinical efficacy of this Chinese herbal medicine.

Because triterpenoid saponins with bayogenin and medicagenic acid in *P. japonica* represent the majority of the clinical benefits of this herbal medicine, it is reasonable and logical to determine the concentrations of the six saponins (Fig. 1) as chemical makers for quality control. However, the absence of a chromophore in these saponins hampers their detection with a UV detector. With high level of sensitivity and selectivity,

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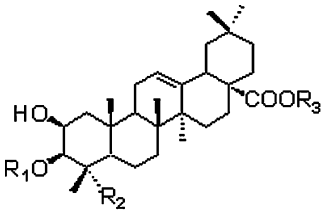

Saponins	R_1	R_2	R_3
1	Glc^{2}---^{1}Glc	CH_2OH	Glc^{2}---$^{1}Rha^{4}$---^{1}Xyl
2	Glc	CH_2OH	Glc^{2}---$^{1}Rha^{4}$---^{1}Xyl ($^{3}_{1}$Api)
3	Glc	CH_2OH	Glc^{2}---$^{1}Rha^{4}$---^{1}Xyl
4	Glc	COOH	Glc^{2}---$^{1}Rha^{4}$---^{1}Xyl ($^{3}_{1}$Api)
5	Glc	COOH	Glc^{2}---$^{1}Rha^{4}$---^{1}Xyl
6	Glc	COOH	Glc^{2}---$^{1}Rha^{4}$---^{1}Xyl ($^{3}_{1}$Api)

Fig. 1. Structures of six triterpenoid saponins in *Polygala japonica*. Glc: glucoside; Rha: rhamnose; Xyl: xylose; Api: apiose.

mass spectrometry is a good choice for quantification of these compounds, but the expensive running costs do not permit its application for routine analyses [13]. On the other hand, evaporative light scattering detector (ELSD) has been successfully applied for saponins profiling and quantification [14]. Thus, an ELSD coupled with HPLC was employed in this study to overcome the hurdle. In present report, a HPLC-ELSD method has been developed for simultaneous determination of six active triterpenoid glycosides in the Chinese herbal medicine *P. japonica*, namely 3-*O*-β-D-glucopyranosyl (1 → 2)-β-D-glucopyranosyl bayogenin 28-*O*-[β-D-xylopyranosyl(1 → 4)-α-L-rhamnopyranosyl(1 → 2)-β-D-glucopyranosyl] ester (**1**), 3-*O*-β-D-glucopyranosyl bayogenin 28-*O*-{β-D-xylopyranosyl (1 → 4)-[β-D-apiofuranosyl (1 → 3)]-α-L-rhamnopyranosyl (1 → 2)-β-D-glucopyranosyl} ester (**2**), 3-*O*-β-D-glucopyranosyl bayogenin 28-*O*-[β-D-xylopyranosyl(1 → 4)-α-L-rhamnopyranosyl(1 → 2)-β-D-glucopyranosyl] ester (**3**), 3-*O*-β-D-glucopyranosyl medicagenic acid 28-*O*-{β-D-xylopyranosyl (1 → 4)-[β-D-apiofuranosyl(1 → 3)]-α-L-rhamnopyranosyl (1 → 2)-β-D-glucopyranosyl} ester (**4**), 3-*O*-β-D-glucopyranosyl medicagenic acid 28-*O*-[β-D-xylopyranosyl (1 → 4)-α-L-rhamnopyranosyl(1 → 2)-β-D-glucopyranosyl] ester (**5**), 3-*O*-β-D-glucopyranosyl medicagenic acid 28-*O*-{β-D-xylopyranosyl(1 → 4)-α-L-rhamnopyranosyl(1 → 2)-[β-D-apiofuranosyl(1 → 3)]-β-D-glucopyranosyl} ester (**6**) (Fig. 1). In addition, this method was fully validated and also employed to quantify these active saponins in *P. japonica* growing from different locations.

2. Experimental

2.1. Chemicals and materials

Methanol (HPLC-grade) and acetonitrile (HPLC-grade) were purchased from Hanbon Scientific (Jiangsu, China) and Merck (Darmstadt, Germany), respectively, and deionized water was from Robust (Guangzhou, China). Trifluoroacetic acid (TFA) was purchased from Shanghai Chemical Reagent Company (Shanghai, China). Other solvents from Nanjing Chemical Factory (Nanjing, China) were of analytical grade.

The reference standards of triterpenoid saponins **1**–**6** were isolated previously from the methanol extract of *P. japonica* in our laboratory. Their structures were established based on spectroscopic analyses compared with the reference data [5,7,9]. The purities of these saponins were determined to be greater than 98% by normalization of the peak areas detected by HPLC-ELSD and confirmed by LC–MS, NMR spectroscopy.

Samples of the herb *P. japonica* were collected from provinces of Jiangsu, Anhui, Jiangxi, Guangdong, Guangxi, Yunnan, Hunan, Hubei and Henan in China. The voucher specimens, identified by Dr. Zenglai Xu (Jiangsu Zhongshan Arboretum, Nanjing, China), have been deposited at the Herbarium of China Pharmaceutical University, Nanjing, China.

2.2. Apparatus and chromatographic conditions

Shimadzu series HPLC apparatus was used including DGU-12A degasser, LC-10ATvp pumps, SCL-10Avp system controller programmed by a Classvp Chemstation. The chromatography was carried out on a Discovery C_{18} column (5 μm, 250 mm × 4.6 mm) at a column temperature of 30 °C and flow rate of 1 ml/min using (A) acetonitrile–methanol (90:10) and (B) 0.05% aqueous TFA as mobile phase with a linear gradient: 0–30 min (30% A), 30–40 min (30% A → 40% A), 40–50 min (40% A → 50% A). The LC system was connected to an Alltech ELSD 2000ES, and evaporator temperature for the ELSD was set at 105 °C with the nebulizing gas flow-rate of 2.6 l/min.

2.3. Sample preparation

Approximately 1.0 g of dried herb was milled into powder, accurately weighed, and added to a round-bottom flask containing 50 ml methanol and the mixture was heated under reflux for 6 h. After evaporating methanol to dryness by a rotary evaporator, residue was dissolved in methanol in a 25 ml flask, and then filtrated through a 0.45 μm millipore filter. Three aliquots of the solution (20 μl) were injected to RP-HPLC-ELSD system.

3. Validation of the method

3.1. Calibration curves

A stock solution containing six analytes was prepared by dissolving the reference compounds in 95% methanol and then was diluted with methanol to appropriate concentrations for

establishing calibration curves. Solutions containing different concentrations of the six analytes were injected in triplicate. Calibration curves were plotted logarithm using peak area versus concentration for each analyte.

3.2. Limits of detection and quantitation

Stock solution containing six reference compounds was diluted to a series of appropriate concentrations with methanol, and an aliquot of the diluted solutions was injected into HPLC for analysis. The limit of detection (LOD) and limit of quantification (LOQ) for each analyte was calculated with corresponding standard solution on the basis of a signal-to-noise ratio (S/N) of 3 and 10, respectively.

3.3. Precision, stability and accuracy

Intra- and inter-day variations were determined for precision of the developed method. Certain concentrations of standard and sample were tested. For intra-day variability test, the standard solution was analyzed for six times within 1 day, and the inter-day reproducibility was determined with six individual sample solutions for three consecutive days. Stability study was performed with sample solution on two consecutive days ($n = 8$). Variations were expressed by relative standard deviations (R.S.D.).

To determine the recovery, the contents of the six analytes in a sample were calculated according to their respective calibration curves. Different amounts of standard compounds were spiked into the sample a ca. 0.8, 1.0, 1.2 times of the estimated mass of each analyte presented in the sample in triplicate. Then, the fortified samples were extracted, disposed as described above, and analyzed with the procedure, each in triplicate. The average recoveries were estimated by the formula: recovery (%) = (amount found − original amount)/amount spiked × 100%, and R.S.D. (%) = (S.D./mean) × 100%.

4. Results and discussion

4.1. Optimization of separation conditions

Several mobile phases including methanol–water and acetonitrile–water in combination with acetic acid were examined. Finally, it was found that acetonitrile–methanol–0.05% trifluoroacetic acid (TFA) system gave the best separation of six triterpenoid saponins. Representative chromatograms for the six standard analytes and for a herb sample were shown in Fig. 2. Fig. 2A displayed that the six standard analytes were well separated and the resolution between any two compounds was greater than 1.5. Other compounds in the sample do not interfere with the analysis of the six saponins, as shown in Fig. 2B. The chromatographic peaks were identified by comparing their retention time with that of each reference compound, which was eluted in parallel with a series of mobile phases. In addition, spiking samples with the reference compounds showed no additional peaks, which further confirmed the identities of the analytes' peaks.

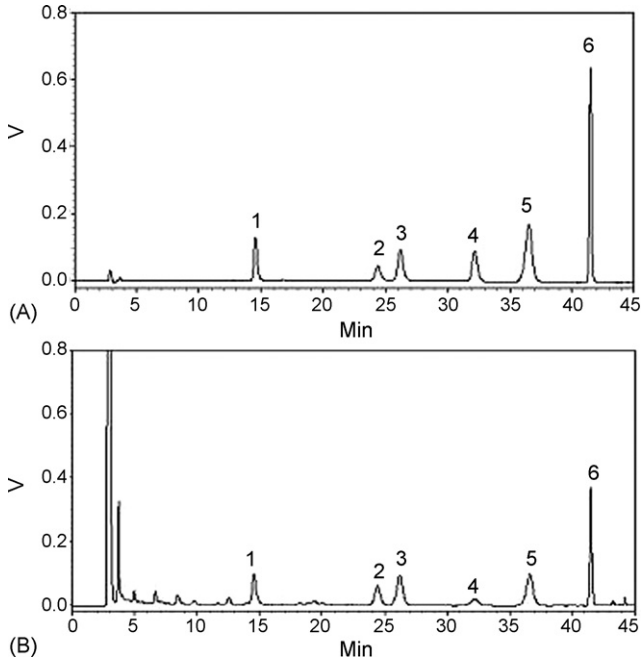

Fig. 2. Reprehensive HPLC chromatograms of mixed standards and methanol extract of *P. japonica*. Column: Discovery C_{18} column (250 mm × 4.6 mm, 5.0 μm), temperature of 30 °C; detector: ELSD, evaporator tube temperature 105 °C, nebulizing gas flow-rate 2.6 l/min. (A) Mixed standards; (B) samples of *P. japonica*.

4.2. Optimization of ELSD parameters

The quantitation of investigated saponins was achieved by using an ELSD 2000ES (Alltech, USA). The parameters of ELSD including evaporator tube temperature and nebulizing gas flow-rate were optimized to obtain the best signals when ratio of signal to noise (S/N) was taken as a measurement. Compound **6** was selected as a model saponin for optimizing ELSD conditions because it was contained in most samples. Temperature and flow rate of the gas for the detector was evaluated systematically from 90 to 110 °C, and from 2.2 to 3.0 l/min, respectively. The effect of evaporator tube temperature of ELSD based on S/N of **6** showed that the optimal temperature was identified as 105 °C. In general, the highest signal should be obtained at the lowest nebulizing gas flow-rate, but in the present study the baseline was unstable when gas flow-rate was decreased to 2.4 l/min. Therefore, the optimal gas flow-rate was 2.6 l/min according to the effect of nebulizing gas flow-rate of ELSD on S/N of the analyte. Accordingly, the optimized parameters of ELSD were 105 °C for evaporator tube temperature and 2.6 l/min for nebulizing gas flow-rate. Controlling these two parameters is important in the analytical procedure for the accuracy and reproducibility according to the literature [15].

4.3. Validation of the method

It has been generally observed that the detector response, as measured by peak area, varies exponentially with the mass of analyte [16], and this behavior can be mathematically expressed in logarithmic form. Present experimental results showed that

Table 1
Linear regression, precision and recovery data of the six analytes in *Polygala japonica*

Analytes	Linear regression				Precision		Recovery	
	Calibration curves	γ	Linear range (μg)	LOD (μg)	Intra-day R.S.D. (%)	Inter-day R.S.D. (%)	Mean (%)	R.S.D. (%)
1	$Y = 1.3563x + 7.4775$	0.9998	0.52–20.70	0.12	1.43	2.38	95.09	3.81
2	$Y = 1.2503x + 8.0111$	0.9994	0.35–13.90	0.17	1.14	1.81	102.02	1.45
3	$Y = 1.3278x + 7.8385$	0.9993	0.51–20.40	0.11	0.70	2.29	97.61	1.71
4	$Y = 1.0740x + 8.9233$	0.9991	0.18–7.30	0.09	2.75	3.97	101.32	3.62
5	$Y = 1.3275x + 8.0522$	0.9996	0.51–20.35	0.06	0.88	2.89	95.50	2.40
6	$Y = 1.3308x + 8.0919$	0.9996	0.41–16.35	0.05	0.72	1.97	101.90	2.02

In the calibration curves, $Y = \lg A$, $x = \lg C$ (A: peak area, C: μg/ml; lg: logarithm) and γ is the correlation coefficient. LOD refers to limit of detection. R.S.D. refers to relative standard deviation.

Table 2
Contents of six analytes in samples of *P. japonica* (mg/g)

Sample origin	1 (mg/g)	2 (mg/g)	3 (mg/g)	4 (mg/g)	5 (mg/g)	6 (mg/g)	Sum total (mg/g)
Anhui	4.78	2.19	5.11	3.64	5.22	5.19	26.13
Jiangxi	5.33	2.32	5.04	1.49	6.32	0.58	21.08
Jiangsu	4.75	3.93	5.47	2.01	5.88	5.28	27.32
Guangdong	3.82	3.66	4.73	2.20	4.65	4.15	23.21
Guangxi	5.02	3.09	1.62	1.68	5.47	6.26	23.14
Yunnan	1.62	1.09	nd[a]	0.62	0.60	0.52	4.45
Hunan	2.17	1.89	3.74	1.40	3.56	tr[b]	12.76
Hubei	2.77	1.72	nd	tr	nd	3.13	7.62
Henan	3.28	2.09	nd	tr	nd	3.02	8.39

[a] Not detected.
[b] Trace.

the logarithm of peak area of each standard was linearly correlated to the logarithm of injected concentration within a particular range (Table 1). As shown in Table 1, all calibration curves showed good linear regression ($\gamma > 0.9991$) and the LOD was less than 0.17 μg, indicating that this HPLC-ELSD method is precise and sensitive for the quantitative evaluation of major active saponins in *P. japonica*. Validation studies of the method proved that this assay had good reproducibility, and the overall intra-day and inter-day variations were less than 5.0% for all analytes (R.S.D., 0.70–3.97%). It was also found that the analytes in the sample solution stable in 2 days with a relative standard deviation of 1.30–2.97%. As illustrated in Table 1, this newly developed analytical method was accurate with the overall recovery of more than 95%, which indicated that there was negligible loss of the six triterpenoid saponins during the extraction process.

4.4. Quantitative determination of P. japonica

Bayogenin type saponins in *P. japonica* possess significant anti-inflammation effect, and medicagenic acid type saponins also have antidepressant activity. Therefore, these six triterpenoid saponins containing both types at the form of aglycones were adopted as chemical markers to establish a method for the quality control of *P. japonica*.

The current method was utilized to analyze the six triterpenoid saponins in nine samples collected from different locations (Table 2). It was found that there were remarkable differences, in terms of concentrations of the six saponins among different places. All the six saponins could be detected in raw materials of *P. japonica* from Provinces of Anhui, Jiangsu, Jiangxi, Guangdong and Guangxi in China. Among the six saponins, **6** was found to be most abundant in most samples, but with greatest variations. The total content of the six saponins ranged from 4.45 to 27.32 mg/g. Samples from the five main producing places (Anhui, Jiangsu, Jiangxi, Guangdong and Guangxi) had the total content over 20 mg/g, while samples from provinces of Hubei and Henan only contained saponins **1**, **2** and **6**.

5. Conclusions

A validated analytical method for qualification and quantification of effective triterpenoid saponins in *P. japonica* is reported in the present study. The new method was evaluated to be precise and accurate. Nine raw materials of *P. japonica* were assayed with this method, and the chromatographic results demonstrated that the six saponins accounted for most of the constituents of the samples. Experimental data of nine samples suggested that the quality of *P. japonica* is correlated to the origins of the samples. Thus, it can conclude that the total content of the six saponins could be used as a chemical marker to assess the quality of *P. japonica*.

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