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Short communication

A rapid method for the simultaneous determination of 11 saponins in *Panax notoginseng* using ultra performance liquid chromatography

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Abstract

A rapid ultra performance liquid chromatography coupled with photo diode array detection method (UPLC-PDA) was developed for the simultaneous determination of 11 saponins, namely notoginsenoside R1, ginsenoside Rg1, Re, Rf, Rb1, Rg2, Rc, Rb2, Rb3, Rd and Rg3 in *Panax notoginseng*. The analysis was performed on Acquity UPLC system with Acquity UPLC BEH C_{18} column and gradient elution of water and acetonitrile in 12 min. The high correlation coefficient ($r^2 > 0.9968$) values indicated good correlations between the investigated compounds' concentrations and their peak areas within the test ranges. The LOQ and LOD were lower to 0.2–2.4 and 0.1–1.8 ng on column, respectively. The overall intra- and inter-day variations (R.S.D.) of 11 saponins were lower than 3.1%. The developed method was successfully used for the analysis of saponins in *P. notoginseng* with overall recovery of 93.0–101.6% for the analytes. The results show that UPLC is a powerful tool for analysis of components in Chinese medicines.
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Keywords: Ultra performance liquid chromatography; *Panax notoginseng*; Ginsenoside; Notoginsenoside; Pressurized liquid extraction

1. Introduction

The root of *Panax notoginseng* (Burk.) F.H. Chen. (Sanqi in Chinese), one of the valued traditional Chinese medicines, is widely used for the treatment of cardiovascular diseases [1–5]. Triterpene saponins are considered as its major active components [6–10]. Up to date, there are over 50 saponins have been isolated from *P. notoginseng*, which mainly belong to two chemical types, i.e. 20(S)-protopanaxatriol (e.g. ginsenoside Rg1, Rg2, etc.) and 20(S)-protopanaxadiol (e.g. ginsenoside Rb1, Rd, etc.) type. These two types of saponins showed different pharmacological effects [11]. Therefore, they are usually used as the markers for quality control of *P. notoginseng*. Besides the methods of colorimetry [12], thin layer chromatography [13] and gas chromatography [14]. HPLC coupled with UV [15–18], ELSD [19–21], fluorescence detection (FD) [22] and MS [23–25] detection have been extensively used for analysis of saponins in *P. notoginseng*. Unfortunately, these methods suffered from either long analysis time of more than 45 min [15,17,18,20,21] or expensive equipment [22–25].

Ultra performance liquid chromatography (UPLC), which utilizes silica particles 1.7 μm, makes it possible to perform efficient separations in short periods of time [26–28]. It has the advantages of the fast analysis, high peak capacity, great resolution and good sensitivity [29]. But the small particles also create operating pressures that are very high (in the range of 6000–15,000 psi). Recently, Acquity UPLC was introduced as commercially available instrument, which has been applied for the pharmaceutical, toxicological and biochemical analysis [30–35].

In this paper, a rapid UPLC method for the simultaneous determination of 11 saponins, namely notoginsenoside R1, ginsenoside Rg1, Re, Rf, Rb1, Rg2, Rc, Rb2, Rb3, Rd and Rg3 in *P. notoginseng* was developed, which was also compared with the conventional HPLC method [16–18,20].

2. Experimental

2.1. Materials and chemicals

Eleven batches root of *P. notoginseng* (PN1–PN11) were obtained from Wenshan (PN1–PN5), Xichou, Yanshan, Mengzi, Maguan, Qiubei and Guangnan, Yunnan Province of China,

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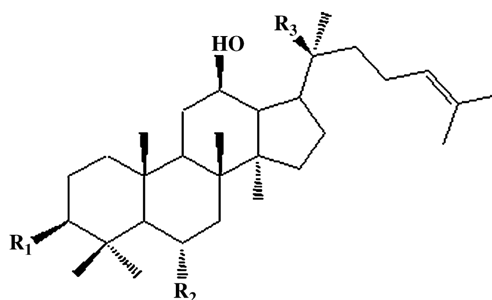

No.	Saponins	R1	R2	R3
1	NG-R1	-OH	-Oglc(2-1)xyl	-Oglc
2	G-Rg1	-OH	-Oglc	-Oglc
3	G-Re	-OH	-Oglc(2-1)rha	-Oglc
4	G-Rf	-OH	-Oglc(2-1)glc	-OH
5	G-Rb1	-Oglc(2-1)glc	-H	-Oglc(6-1)glc
6	G-Rg2	-OH	-Oglc(2-1)rha	-OH
7	G-Rc	-Oglc(2-1)glc	-H	-Oglc(6-1)araf
8	G-Rb2	-Oglc(2-1)glc	-H	-Oglc(6-1)arap
9	G-Rb3	-Oglc(2-1)glc	-H	-Oglc(6-1)xyl
10	G-Rd	-Oglc(2-1)glc	-H	-Oglc
11	G-Rg3	-Oglc(2-1)glc	-H	-OH

Fig. 1. Chemical structures of 11 saponins in *Panax notoginseng*. NG: notoginsenoside; G: ginsenoside; Glc: β-D-glucose; Rha: α-L-rhamnose; Arap: α -L-arabinose (pyranose); Araf: α-L-arabinose (furanose); Xyl: β-D-xylose.

respectively. The botanical origin of material was identified and the voucher specimens of *P. notoginseng* were deposited at the Institute of Chinese Medical Sciences, University of Macau, Macao, China.

Notoginsenoside R1 (1) (Fig. 1) was supplied by National Institute for the Control of Pharmaceutical and Biological Products (Beijing, PR China). Ginsenoside Rg1 (2), Re (3), Rg2 (6), Rc (7), Rb2 (8), Rd (10) and Rg3 (11) were purchased from International Laboratory (San Bruno, CA, USA) and ginsenoside Rf (4), Rb1 (5), Rb3 (9) were obtained from Chromadex Company (Santa Anna, CA, USA). Methanol and acetonitrile for HPLC were purchased from Merck (Darmstadt, Germany). Deionized water was prepared using a Millipore Milli Q-Plus system (Millipore, Bedford, MA, USA).

2.2. Sample preparation

Pressurized liquid extraction (PLE) was performed on a Dionex ASE 200 system (Dionex Corp., Sunnyvale, CA, USA) under optimized conditions. In brief, dried powder of *P. notoginseng* (0.5 g) was placed into an 11 ml stainless steel extraction cell. Then PLE was performed as previous described method [21]. The extract was transferred into a 50 ml volumetric flask which was made up to its volume with methanol, and filtered through a 0.22 μm MILLEX GV syringe filter (Millipore, MA, USA) prior to injection into the UPLC system.

2.3. UPLC analysis

All analyses were performed on Waters Acquity UPLC system (Waters, MA, USA), including binary solvent manager, sampler manager, column compartment and photo diode array (PDA) detector, connected with Waters Empower 2 software. An Acquity UPLC BEH C_{18} column (50 mm × 2.1 mm i.d, 1.7 μm) also from Waters was used. The column temperature was maintained at 45 °C. The standards and samples were separated using a gradient mobile phase consisting of water (A) and acetonitrile (B). The gradient condition is: 0–5.5 min, 18–19% B; 5.5–6.0 min, 19–31% B; 6.0–9.5 min, 31–35% B; 9.5–12.0 min, 35–56% B, and finally, reconditioning the column with 18% B

isocratic for 2 min after washing column with 100% B for 3 min. The flow rate was set at 0.35 ml min^{-1} and the injection volume was 1 μl. The detection wavelength was set at 203 nm.

2.4. HPLC analysis

HPLC analysis was performed as previous report [21]. In brief, it was performed on an Agilent 1100 series HPLC system with Zorbax ODS C_{18} column (250 mm × 4.6 mm i.d., 5 μm) and gradient elution of water (A) and acetonitrile (B). The column temperature was set at 40 °C, and the gradient program: 0–30 min, 18–19% B; 30–40 min, 19–31% B; 40–60 min, 31–56% B. The flow rate was at 1.5 ml/min and sample injection volume was 10 μl. The detection wavelength was set at 203 nm.

2.5. Calibration curves

Methanol stock solution of mixed standards containing ginsenoside Rg1, Re, Rf, Rb1, Rg2, Rc, Rb2, Rb3, Rd, Rg3 and notoginsenoside R1 was prepared and diluted to appropriate concentrations for the establishment of calibration curves. Each concentration of the mixed-standard solution was injected in triplicate, and then the calibration curves were constructed by plotting the peak areas versus the concentrations of each analyte.

2.6. Limits of detection and quantification

The stock solutions containing reference compounds were diluted with methanol to appropriate concentrations, and an aliquot of the diluted solutions was injected into UPLC for analysis. The limits of detection (LOD) and quantification (LOQ) for each analyte were determined at a signal-to-noise ratio (S/N) of about 3 and 10, respectively.

2.7. Precision and accuracy

Intra- and inter-day variations were chosen to determine the precision of the method. A known concentration solution of 11 standards was prepared. For intra-day variability test, the standard solution was analyzed for five times within 1 day, while for inter-day variability test, the sample was examined each day for consecutive 5 days. Variations were expressed by the relative standard deviations. For every calibration curve, the calibration concentration was back calculated (concentration measurement of the standards with know concentration based on their peaks area and calibration curve) from the peak area of the analytes.

Recovery was used to evaluate the accuracy of the method. Known amounts of 11 saponins were added to approximate 50 mg the root of *P. notoginseng* (PN1), and then extracted in duplicate using PLE as described in Section 2.2. After cooling, partial methanol was removed in vacuo, and the residue was transferred into a 10 ml volumetric flask, which was made up to its volume with methanol. The final solution was filtered through a 0.22 μm filter before analysis. The quantity of each analyte was subsequently obtained from the corresponding calibration curve.

3. Results and discussions

3.1. Optimization of UPLC conditions

For the separation of saponins in *P. notoginseng*, it is the key to obtain good resolution between ginsenoside Rg1 (2) and Re (3), as well as ginsenoside Rb2 (8) and Rb3 (9), which are the main factors increasing the analysis time [20,21]. With the great resolution of UPLC, the problem was resolved under appropriate gradient elution. Higher column temperature was used so as to decrease the pressure at higher flow rate, which could improve the separation and peak shape. Finally, under the optimized UPLC conditions, the resolutions between ginsenoside Rg1 (2) and Re (3), as well as ginsenoside Rb2 (8) and Rb3 (9) were 1.53 and 1.58, respectively. The investigated saponins were well separated within 12 min (Fig. 2). The peaks were identified by comparing the retention times of the peaks with those of the reference compounds eluted under the same conditions and spiking the sample with stock standard solutions of saponins.

3.2. Validation of the developed method

The linearity, regression and precision of 11 saponins were performed using the developed UPLC method. The high correlation coefficient ($r^2 > 0.9968$) values indicated good correlations between investigated compounds concentrations and their peak areas within the test ranges. The LOQ and LOD were lower to 0.2–2.4 and 0.1–1.8 ng on column, respectively (Table 1). The overall intra- and inter-day variations of 11 saponins were less than 3.1% (R.S.D.), and the developed method had the accuracy with the overall recovery of 93.0–101.6% for the analytes (Table 2). The results indicated that this UPLC method was rapid, precise, accurate and sensitive for quantitative determination of saponins in *P. notoginseng*.

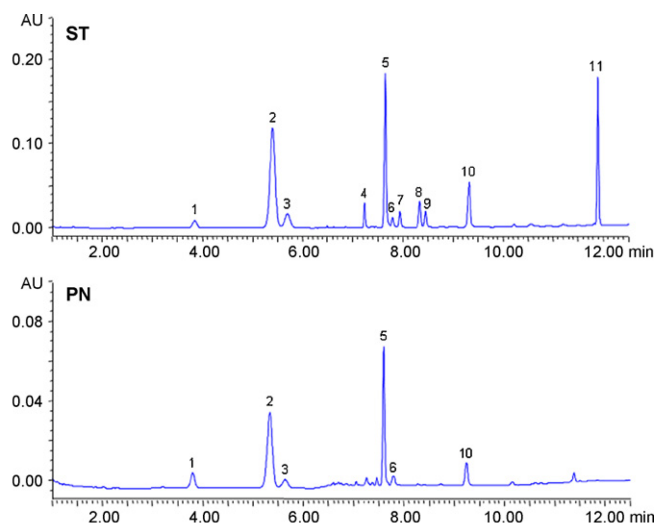

Fig. 2. Typical UPLC chromatograms of mixed standards (ST) and PLE extracts of *P. notoginseng* (PN). Notoginsenoside R1 (1); ginsenoside Rg1 (2), Re (3), Rf (4), Rb1 (5), Rg2 (6), Rc (7), Rb2 (8), Rb3 (9), Rd (10) and Rg3 (11).

Table 1
Calibration data and LOQ, LOD of 11 investigated saponins in *Panax notoginseng*

Analytes	Linear regression data			LOQ (ng)	LOD(ng)
	Regressive equation	r^2	Test range (mg ml^{-1})		
NG-R1	$y = 438453x - 3592$	0.9994	0.016–0.240	2.4	1.6
G-Rg1	$y = 1000000x - 30355$	0.9991	0.073–1.090	2.4	1.8
G-Re	$y = 1000000x - 7988$	0.9990	0.018–0.270	1.8	1.4
G-Rf	$y = 568003x - 3259$	0.9990	0.017–0.250	0.6	0.3
G-Rb1	$y = 980419x - 20869$	0.9992	0.067–1.010	0.5	0.2
G-Rg2	$y = 339524x - 2943$	0.9975	0.016–0.240	1.6	1.2
G-Rc	$y = 434271x - 3005$	0.9994	0.017–0.250	1.7	0.6
G-Rb2	$y = 719512x - 4434$	0.9992	0.017–0.250	1.3	0.4
G-Rb3	$y = 511485x - 3293$	0.9991	0.015–0.230	2.3	0.4
G-Rd	$y = 842675x - 5868$	0.9968	0.019–0.470	0.9	0.4
G-Rg3	$y = 3000000x - 6956$	0.9975	0.011–0.280	0.2	0.1

NG: notoginsenoside; G: ginsenoside.

3.3. Quantitation of saponins in P. notoginseng

The developed UPLC method was applied to analyze saponins in 11 samples of *P. notoginseng*. The typical UPLC profile of PLE extract of *P. notoginseng* was shown in Fig. 2. The data were summarized in Table 3. Generally, the contents of the quantitated saponins, including ginsenoside Rg1, Re, Rb1, Rg2, Rd and notoginsenoside R1 had no significant difference among the 11 samples collected in different places (R.S.D. < 15%, $n = 11$), which is in accordance with the previous reports [16–18,20]. The low variation of saponins contents in *P. notoginseng* may attribute to its long time cultivation and the performance of good agriculture practice (GAP) in China recent years. It was also noticed that the contents of notoginsenoside R1 in samples of PN7 (39%) and PN2 (33%), while ginsenoside Rd in samples of PN7 (32%) were higher than those in PN1. The difference may drive from the locations and/or cultivation.

Table 3
The content (mg g^{-1}) of six detected saponins in *P. notoginseng* from different locations

No.	Locations	NG-R1	G-Rg1	G-Re	G-Rb1	G-Rg2	G-Rd
PN1	Wenshan, Yunnan	10.38[a]	31.53	4.06	20.54	6.43	5.93
PN2	Wenshan, Yunnan	15.46	35.11	4.24	23.84	6.84	7.13
PN3	Wenshan, Yunnan	13.57	32.91	4.27	21.20	6.11	6.89
PN4	Wenshan, Yunnan	13.80	31.46	3.42	21.43	5.34	7.08
PN5	Wenshan, Yunnan	12.35	31.71	3.35	19.56	5.26	6.24
PN6	Xichou, Yunnan	12.52	31.22	3.66	20.79	5.84	6.07
PN7	Yanshan, Yunnan	17.07	39.71	5.28	25.34	7.82	8.77
PN8	Mengzi, Yunnan	13.53	31.92	3.79	23.87	5.49	8.58
PN9	Maguan, Yunnan	13.13	31.85	4.02	18.44	6.46	6.61
PN10	Qiubei, Yunnan	14.10	31.58	4.40	23.25	5.71	6.27
PN11	Guangnan, Yunnan	13.90	34.88	4.77	23.06	6.23	8.36

NG: notoginsenoside; G: ginsenoside.
[a] The data are the average of duplicates.

3.4. Comparison of UPLC and HPLC

UPLC offers higher peak capacity, greater resolution, good sensitivity and high speed of analysis [30]. Comparing with conventional HPLC [21], UPLC only need 12 min for good separation of 11 saponins in *P. notoginseng*, which is only 1/5 of analysis time of HPLC with better resolutions of ginsenoside Rg1 and Re as well as ginsenoside Rb2 and Rb3 (Fig. 3). Therefore, UPLC is a powerful tool for analysis of complex system

Table 2
Repeatability and recovery of 11 investigated saponins

Saponins	Intra-day ($n = 5$)		Inter-day ($n = 5$)		Recovery (%)[b]	
	R.S.D. (%)	Accuracy (%)[a]	R.S.D. (%)	Accuracy (%)	Mean[c]	R.S.D. (%)[c]
NG-R1	0.2	101.5	1.2	102.8	96.1	1.8
G-Rg1	0.4	99.3	0.9	98.5	93.0	1.6
G-Re	1.3	100.9	1.3	102.7	95.2	2.4
G-Rf	0.1	99.0	1.5	101.2	ND	ND
G-Rb1	0.5	100.5	0.7	101.3	95.1	3.6
G-Rg2	1.7	100.2	2.9	104.3	95.0	5.0
G-Rc	1.6	103.6	1.5	103.1	ND	ND
G-Rb2	0.3	101.4	1.0	102.4	ND	ND
G-Rb3	0.6	102.2	0. 9	103.5	ND	ND
G-Rd	0.5	90.4	3.1	91.8	101.6	2.0
G-Rg3	0.5	98.2	1.5	100.4	ND	ND

NG: notoginsenoside; G: ginsenoside; ND: not determined because there is none in the samples.
[a] Accuracy (%) = 100 × mean of measured concentration/nominal concentration.
[b] Recovery (%) = 100 × (amount found-original amount)/amount spiked.
[c] Mean value for two different concentrations.

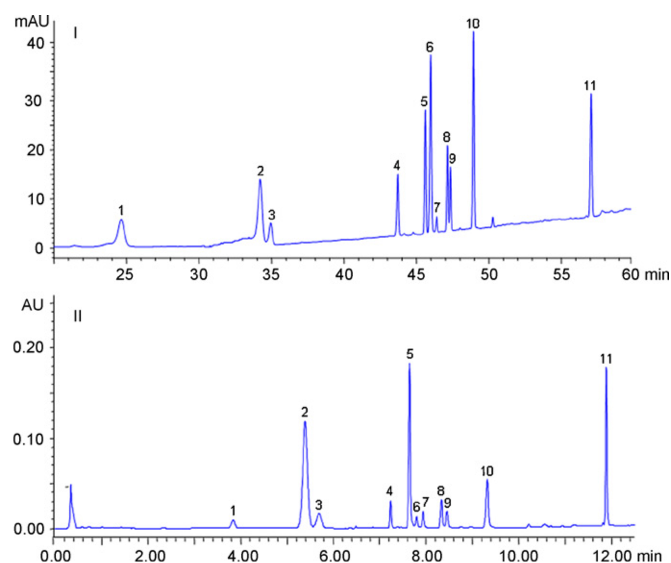

Fig. 3. Chromatograms of 11 saponins analyzed by HPLC-DAD (I) and UPLC-PDA (II). Notoginsenoside R1 (1); ginsenoside Rg1 (2), Re (3), Rf (4), Rb1 (5), Rg2 (6), Rc (7), Rb2 (8), Rb3 (9), Rd (10) and Rg3 (11).

such as traditional Chinese medicines. With the lower flow rate and much shorter analysis time, solvent consumption of UPLC was also obviously reduced, which is friendly to environment and financial expense.

It was noteworthy that only six saponins, namely notoginsenoside R1, ginsenoside Rg1, Re, Rb1, Rg2 and Rd were detected in *P. notoginseng*, while eight saponins including ginsenoside Rc and Rf were quantitated by HPLC [18,21]. The difference may attribute to the high resolution of UPLC, which well separated the interferents and analytes to obtain accurate identification of analytes. The contents of ginsenoside Rg1, Re and Rd determined by HPLC and UPLC were similar. But the content of notoginsenoside R1 and ginsenoside Rg2 determined by HPLC were only a half and a quarter of those of UPLC, respectively. Furthermore, the content of ginsenoside Rb1 by HPLC was as 1.5-folds high as that of UPLC. The difference may derive from the different samples and/or long time storage. The details will be further investigated.

4. Conclusion

The developed UPLC method is rapid, sensitive and accurate for simultaneous determination of ginsenoside Rg1, Re, Rf, Rb1, Rg2, Rc, Rb2, Rb3, Rd, Rg3 and notoginsenoside R1 in *P. notoginseng*, which suggests UPLC is a powerful tool for analysis of components in Chinese medicines.

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