JPP Journal of Pharmacy and Pharmacology

JPP 2001, 53: 1637–1643

Received May 18, 2001
Accepted July 24, 2001
ISSN 0022-3573

A validated method for quantitative determination of saponins in notoginseng (*Panax notoginseng*) using high-performance liquid chromatography with evaporative light-scattering detection

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Abstract

A gradient high-performance liquid chromatographic (HPLC) method with evaporative light-scattering detection (ELSD) for the determination of major saponins in a Chinese traditional herbal medicine, *Panax notoginseng*, is described. Samples were analysed by means of a reverse-phase column (Waters Spherisorb ODS-2, C-18) using acetonitrile and water under gradient conditions as the mobile phase over 60 min. The ELSD used was set at an evaporating temperature of 35°C and pressurized air pressure of 3.4 bars. The detection limit (signal/noise > 3) of the saponins was 50 ng. The method was validated by inter- and intra-day assays and recovery tests.

Introduction

Ginseng – Asian ginseng (*Panax ginseng*) and North American ginseng (*Panax quinquefolius*) – is known worldwide for its tonic value. Notoginseng is prepared by various processing methods from the main root with a small rhizome of *Panax notoginseng* (Burk.) F. H. Chen and has similar medical effects in Chinese traditional medicines, but appears to be generally unrecognized in the West. Notoginseng, also called Sanqi ginseng (or Sanchi ginseng) and Tianqi ginseng (or Tienchi ginseng) in Chinese, was first recorded in the book of "Ben-Cao-Gang-Mu" in 1590s (during the Ming Dynasty in China) (Li 1982). For years, it has been used as a creditable drug for the treatment of haemoptysis, haematemesis and haematoma in Chinese traditional medicine because of its haemostatic and anti-inflammatory properties (Jiangsu Medical College 1977; Li & Chu 1999; White et al 2000), and is prescribed in several famous Chinese traditional formulae including "Yun-Nan-Bai-Yao", which is used for the treatment of trauma and bleeding after internal and external injury, and "Pian-Zai-Huang", which is known as a specific medicine against hepatitis. In addition, the herb is also prescribed for the treatment of coronary heart disease and the sequelae of cerebrovascular disease in combination with other herbs. Pharmacological research revealed that notoginseng possesses protective effects on cardiovascular and cerebrovascular systems (Xiong & Sun 1989; Li & Shi 1990; Li et al 1990; Shi et al 1990; Chen et al 1992, 1994; Guan et al 1994; Zhang et al 1994; Jiang & Qian 1995; Wu et al 1995), as well as anticarcinogenic (Konoshima et al 1999) and hepatoprotective (Liu et al 1994; Prasain et al 1995) properties. More recently, the herb was reported to enhance

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sperm motility as well as progression of sperm with inferior motility (Chen et al 1998, 1999).

Chemically, dammarane-type saponins are the major constituents of notoginseng, which might contribute to its pharmacological and therapeutic activity. More than 30 saponins have been isolated and characterized from the roots of notoginseng (Yang et al 1983; Zhao et al 1996; Yoshikawa et al 1997a, b; Ma et al 1999). Among them, ginsenosides Rg_1, Re, Rb_1, Rc, Rb_2 and Rd count for more than 85% of total saponins in the notoginseng roots (Yoshikawa 1997a, b).

Liquid chromatographic determination of saponins (ginsenosides) in Asian and North American ginsengs has been widely studied over the past 20 years. Although the major constituents of notoginseng (i.e. damarane-type saponins) are very similar to those of Asian and North American ginseng, and the methods used to determine the ginsenosides in Asian and North American ginseng might be also suitable for the analysis of notoginseng, there have been only a few reports describing the methodology of the quality assurance and quality control of notoginseng (Yamaguchi et al 1988; Lang et al 1993; Chuang et al 1995; Ma et al 1996). Moreover, the saponins in notoginseng, as in Asian and North American ginseng, show only very weak UV absorption (detection is limited between 200 and 205 nm) and is often characterized by high level of baseline noise and poor sensitivity. Other techniques have been proposed, such as capillary supercritical fluid chromatography of rearranged aglycones, panaxatriol and panaxadiol (produced by acidic hydrolysis of extract with H_2SO_4) (Li et al 1991), and HPLC coupled with NIR spectroscopy (Chen & Sorensen 2000). However, the repeatability and sensitivity of these methods present a challenge.

The evaporative light-scattering detector (ELSD) was mainly used for the determination of biological molecules such as triglycerides, fatty acids esters, steroids and sugars, which possess poor chromophores. ELSD includes nebulization and evaporation of the mobile phase using a stream of nitrogen or air. The ELSD first nebulizes the chromatographic effluent into droplets, from which the solvent can be easily evaporated. In the nebulization chamber, a narrow droplet size distribution is created by eliminating the larger droplets, which condense on the sides of the glass walls of the chamber and flow outside through a siphon overflow. A constant nebulization process is needed for satisfactory repeatability of the analysis. The average diameter of the droplets and their distribution are influenced by several factors, such as density, viscosity, and liquid surface tension, and they vary in diameter from about 4 to 400 μm. The droplets are carried by the nebulizing gas towards the evaporator, where evaporation occurs and the more volatile mobile phase is converted to gas, while the analyte remains as particles. Finally, the analyte particles emerging from the evaporator enter the light cell where they are directed toward a polychromatic light beam. The light, scattered by the analyte particles of non-volatile material, is measured by a photomultiplier or a photodiode. The intensity of the signal is related to the concentration of the analyte in the effluent and thus allows its determination.

It has been reported that ELSD can be used with most solvents, including water, and is able to detect all types of analytes regardless of their molecular construction. Unlike electrochemical detection, ELSD is insensitive to the composition of the mobile phase and could create a flat baseline with a solvent gradient program that covers a wide range of solvent polarities (Avery et al 1999).

Although HPLC-ELSD has been applied to the determination of ginsenosides in Asian ginseng (Park et al 1996; Fuzzatti et al 2000), the methods reported have not been fully validated, especially the intra- and inter-day assay and recovery. The purpose of the present investigation was to develop and validate a quick, accurate, sensitive analytical method to quantify the saponins in notoginseng. The method employed reverse-phase HPLC coupled to an ELSD to quantify six saponins (Rg_1, Re, Rb_1, Rc, Rb_2 and Rd).

Materials and Methods

Chemicals and materials

Methanol and acetonitrile (HPLC grade) were obtained from Fisher Scientific Co. (Fair Lawn, NJ, USA). Deionized water was obtained using an in-house Nanopure water system (Barnstead, Newton, MA). The ginsenoside standards (Rg_1, Re, Rb_1, Rc, Rb_2 and Rd) were isolated, purified and identified in the Program for Collaborative Research in the Pharmaceutical Sciences (PCRPS) (College of Pharmacy, University of Illinois at Chicago). Camellia notoginseng (Tienchi) powder was exported by Yunnan Medicines and Health Products Import and Export Corporation, China.

HPLC system

A Waters 2690 Alliance HPLC system (Milford, MA), equipped with a 996 photodiode array UV detector, an on-line degasser and an autosampler, was used for solvent delivery and detection. The measurements were

Table 1 The gradient mobile phase composition.

Time (min)	Solvent A (%)	Solvent B (%)	Flow-rate (mL min^{-1})
0	80	20	1.6
20	80	20	1.6
60	58	42	1.6
61	10	90	1.6
70	10	90	1.6
71	80	20	1.6
80	80	20	1.6

carried out on a Waters Spherisorb ODS-2 RP-18 column (250 × 4.6 mm, 5 μm particle size, serial no. 0123391941L) protected by a Waters Delta-Pak RP-18 guard column (Waters Technology Ireland, Ltd, Wexford, Ireland) and set at room temperature (20°C). The solvents used for separation were water (solvent A) and acetonitrile (solvent B). Solvent gradient conditions are reported in Table 1. All injections were 10 μL in volume and 60 min in run-time. After the UV detector, the column effluent was directed to a Sedex 75 ELSD (Cedex 94141; Alfortville, France). Nebulization of the effluent in the ELSD was provided by a stream of pressurized air at 3.4 bars. The nebulization was performed at room temperature, and the nebulized effluents were evaporated at 35°C. The detector output was interfaced, using a SATIN box, to the Waters Millennium 2000 chromatographic manager system (Waters, Milford, MA) loaded on a Compaq 6400X/10000/CDS computer (Houston, TX) for data handling and chromatogram generation.

Before each assay, the HPLC-UV-ELSD system was allowed to warm up for 20–30 min and the pumps were primed using the protocol suggested by the manufacturer. Using freshly prepared mobile phase, the baseline was monitored until stable before the samples were run.

Sample preparation

Standard solutions

The ginsenosides Rg_1, Re, Rb_1, Rc, Rb_2 and Rd were dissolved in methanol to make a stock solution. This stock solution was found to be stable for more than two weeks when stored at 4°C. Calibration standard working solutions were prepared by diluting the stock solution with methanol in appropriate quantities. A series of calibration standard solutions (concn range 25–300 μg mL^{-1}) were made for each ginsenoside. Three controls were prepared at 75, 125 and 250 μg mL^{-1} for ginsenosides Rg_1, Re and Rb_1, respectively. Recovery standard working solutions were also prepared at concentrations of 0.3, 0.5 and 1.0 mg mL^{-1} for Rg_1, Re and Rb_1, respectively. In ELSD, the calibration was generated by plotting log-transformed peak area versus log-transformed concentration of the standards. The equation of the regression line was used to quantify control, recovery and unknown samples.

Sample solutions

A portion of the sample powder (0.5g) was accurately weighed into a tared 50-mL flask. Methanol (15 mL) was added and the resulting mixture was shaken for a short time before sonication at 25–30°C for 30 min. After cooling, the resulting mixture was filtered through a filter paper (Whatman 40) into a 250-mL round-bottomed flask, and the residue was returned to the flask. Methanol (15 mL) was added and the mixture was sonicated at 25–30°C for 30 min. The extract was filtered into the same flask, and the residue on the filter was washed with methanol (3 × 15 mL). The combined methanol extracts were evaporated to dryness under vacuum at 45–50°C. The residue was dissolved with methanol (4 × 2 mL) and transferred to a 10-mL volumetric flask, and made up to the volume with methanol. The solution was filtered through a 0.2-μm nylon cartridge just before HPLC analysis. For each sample, triplicate analyses were conducted in the same day.

Reproducibility

Intra-day assay

The intra-day accuracy and precision was evaluated by establishing calibration curves using freshly prepared calibration standard working solutions. After the calibration curve was generated, a set of thirteen freshly prepared control solutions was run on the same day. The concentrations of the control solutions were determined using the equations derived from the calibration curves. The percentage deviation from the theoretical concentration was calculated and used as the parameter to evaluate intra-day accuracy. The variation within controls was calculated and used to evaluate intra-day precision.

Inter-day assay

The inter-day accuracy and precision was evaluated by generating calibration curves using freshly prepared calibration standard working solutions on each of three

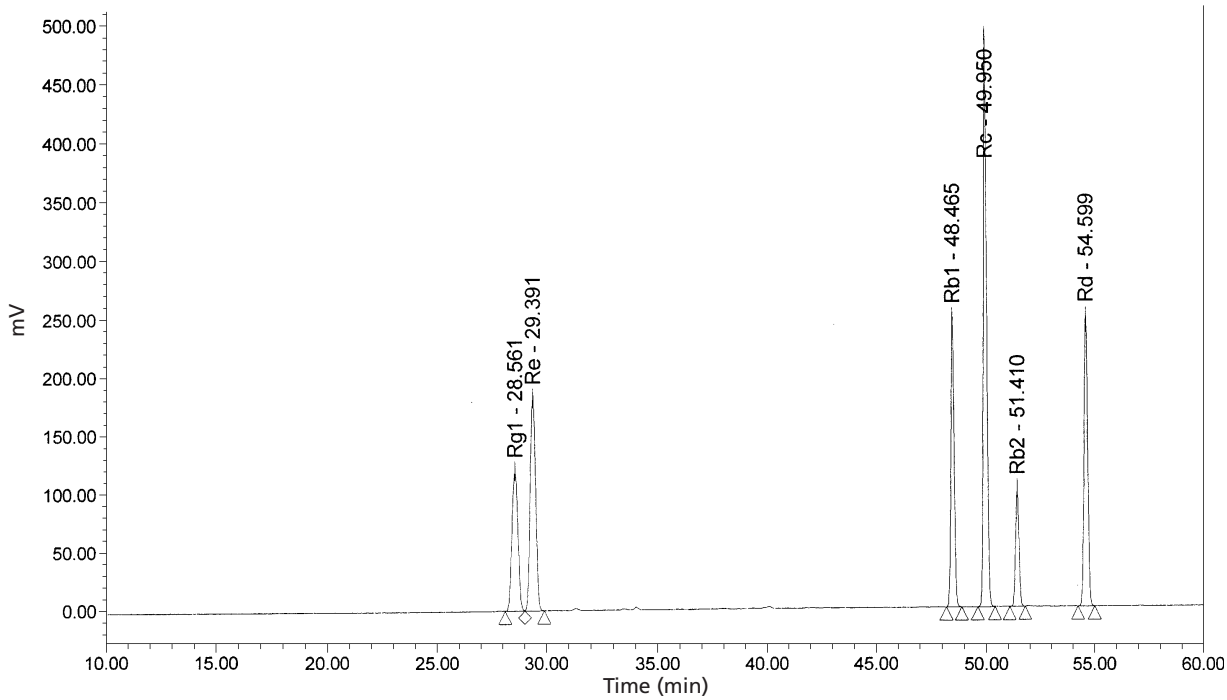

Figure 1 Typical HPLC-ELSD chromatogram of saponin standards of notoginseng.

days. The controls were freshly prepared on the first day and used on the two subsequent days. The controls were quantified using the equation derived from the calibration curves constructed on each of the three days. The percentage deviation from the theoretical value was calculated for each control on each of the three days and used to evaluate the inter-day accuracy. The variation within replicate injections was also calculated and used to evaluate intra-day precision.

Recovery

Notoginseng powder ($\sim$ 5.0 g) was extracted in a 50-mL flask with 25 mL methanol by sonication for 30 min. After filtration, the residue was returned to the same flask and 25 mL fresh methanol was added. The flask was sonicated for another 30 min before filtration. The above extraction procedure was repeated until no saponin peaks were detected in the filtrate by HPLC-ELSD, as described above. The residue was dried before use. A portion of dried residue powder (0.5 g) was accurately weighed into a tared 50-mL flask. To the flask, 1 mL standard recovery working solution (containing 0.3, 0.5 and 1.0 mg mL^{-1} of Rg_1, Re and Rb_1, respectively) and 15 mL methanol were added. The recovery sample was prepared as described above for sample preparation. Meanwhile, a blank recovery sample (without adding Rg_1, Re and Rb_1) was prepared and analysed for comparison.

Results and Discussion

Several HPLC methods have been described for the analysis of notoginseng. However, these methods either used a time-consuming HPLC program (more than 60 min) (Chuang et al 1995), or failed to achieve baseline separation of ginsenosides tested (Yamaguchi et al 1988; Lang et al 1993; Ma et al 1996). In the present study, several combinations of acetonitrile and water were evaluated as mobile phase with a Waters Spherisorb ODS-2 C-18 column. After optimization of the chromatographic conditions, the solvent system was determined because not only could the ginsenosides be simultaneously determined in the 60-min program, but the different ginsenosides in each group (especially ginsenosides Rg_1 and Re) were also well separated. The typical HPLC-ELSD chromatograms of ginsenoside standards and the methanolic extract of notoginseng are shown in Figures 1 and 2. Baseline separation of the ginsenosides tested was observed with retention times of 28.6, 29.4, 48.4, 49.8, 51.3 and 54.4 min for ginsenosides Rg_1, Re, Rb_1, Rc, Rb_2 and Rd, respectively. Obviously,

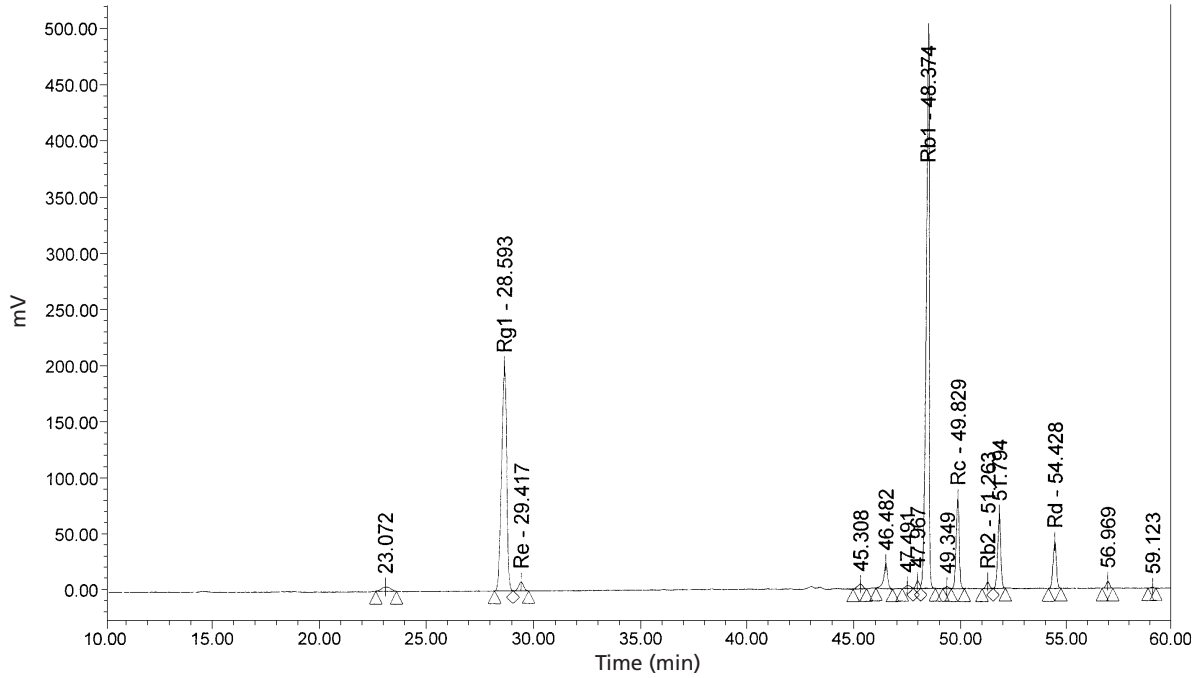

Figure 2 Typical HPLC-ELSD chromatogram of methanolic extract of notoginseng.

the ginsenoside with the retention time of 51.8 min has a higher content than that of ginsenosides Rb_2 and Re in the methanolic extract of the sample (Figure 2). It was not quantified in the current assay because of the shortage of authentic standard.

The nebulizer gas flow-rate is an important adjustable parameter in ELSD and significantly affects the signal response. When the gas flow-rate is too low, large droplets are formed, resulting in spikes and random noise. However, when the gas flow-rate is too high, the size of droplets decreases, resulting in a decreased detector response. The evaporating temperature is also an important adjustable parameter affecting the signal response. Low temperatures result in incomplete evaporation of the solvent. However, at high temperatures, improper evaporation of the nebulized analytes in the evaporating chamber occurs resulting in a decrease in particle size, and thus, a decrease in the detector response. The optimum nebulizer gas (air) pressure and evaporating temperature in the present work were determined to be 3.4 bar and 35°C, respectively.

Table 2 Determination of intra-day accuracy and precision.

Injection	Rg_1	Re	Rb_1
1	778.64	1274.74	2705.34
2	864.90	1322.30	2692.02
3	761.36	1292.04	2631.08
4	711.69	1251.88	2619.44
5	745.89	1236.07	2616.86
6	721.04	1224.87	2636.27
7	692.39	1200.89	2663.07
8	678.80	1183.47	2662.56
9	733.51	1213.29	2712.82
10	707.94	1210.76	2655.56
11	725.29	1239.25	2709.12
12	725.56	1257.65	2682.60
13	705.36	1228.93	2631.58
Mean	734.80	1241.24	2662.95
s.d.	47.52	38.49	34.68
r.s.d.%	6.47	3.10	1.30
Theoretical amount (ng/injection)	750	1250	2500
r.e.%	−2.03	−0.70	6.52

Method validation

Linearity

The linearity was examined by applying different calibration standard working solutions (Rg_1, Re, Rb_1, Rc, Rb_2 and Rd, 0.25–3.0 μg/injection) to the HPLC-ELSD system. The calibration curve, log-transformed peak area versus log-transformed concentration (ng on column), was calculated according to the least-squares

Table 3 Determination of inter-day accuracy and precision.

Controls	Day 1			Day 2			Day 3		
	Rg_1	Re	Rb_1	Rg_1	Re	Rb_1	Rg_1	Re	Rb_1
Theoretical concn (ng/injection)	750	1250	2500	750	1250	2500	750	1250	2500
Calculated concn (ng/injection)	722.11	1310.19	2610.62	732.90	1333.56	2646.52	703.71	1198.08	2561.91
r.s.d. (%)	5.71	4.02	3.00	5.10	0.70	1.68	7.26	0.15	0.91
r.e. (%)	−3.72	4.82	4.42	−2.28	6.68	5.86	−6.17	−4.15	2.48

Table 4 Determination of recovery.

	Rg_1	Re	Rb_1
Batch 1	314.01	497.47	1038.45
Batch 2	275.12	493.63	953.34
Batch 3	308.03	486.95	999.78
Mean	299.05	492.68	997.19
s.d.	20.95	5.33	42.62
r.s.d. (%)	7.00	1.08	4.27
Theoretical amount (ng/injection)	300	500	1000
r.e. (%)	−0.32	−1.46	−0.28

method ($y = a + bx$) for different ginsenosides tested with a regression of greater than 0.997 for all of them.

Detection limits

The lowest detection limit determined (signal/noise > 3) was 50 ng/injection for Rg_1, Re, Rb_1, Rc, Rb_2 and Rd (Vial & Jardy 1999).

Intra-day accuracy and precision

The intra-day accuracy and precision was accessed by injecting a set of 13 control solutions in a single day and calculating the concentrations of each control based on the calibration curves. It was observed that controls were quantified as 97.97, 99.30 and 106.52% of the actual values for ginsenosides Rg_1, Re and Rb_1, respectively. The variation within controls was found to be 6.47, 3.10 and 1.30% for ginsenosides Rg_1, Re and Rb_1, respectively (Table 2).

Inter-day precision and accuracy

Variation within replicate injections (precision) of the controls on all three days was found to be within 7.26% ($n = 3$; Table 3). The quantitation of controls (accuracy) fell between 93.83 and 106.68% ($n = 3$) and did not deviate with a bias on either the negative or positive side.

Recovery

The results of the recovery test are summarized in Table 4, with recoveries of 99.68, 98.54 and 99.72% for Rg_1, Re and Rb_1, respectively.

Sample analysis

As shown in Figures 2, three sets of samples were analysed according to the method described above. The average content of ginsenosides Rg_1, Re, Rb_1, Rc, Rb_2 and Rd in notoginseng powder was found to be 0.14 ± 0.002, 0.026 ± 0.001, 0.17 ± 0.004, 0.067 ± 0.001, 0.012 ± 0.001 and 0.053 ± 0.0004%, respectively.

Conclusion

An HPLC method has been developed and validated for the determination and quantitation of saponins in notoginseng using ELSD. Saponins were successfully quantified, using the calibration curves, with detection limits of five of the saponins at 50 ng. Although the detection limits for individual saponins were not as low as for some analytical methods reported, the HPLC-ELSD method described here had the advantage of quantifying all the saponins tested proportionally. Validation of the HPLC-ELSD method for the analysis of saponins in notoginseng included inter- and intra-day precision and accuracy and recovery. All of the validation parameters studied were found to have an r.s.d. of less than 7.5% and did not show a bias in any single direction. This assay could be applied to the analysis of notoginseng preparations. The HPLC-ELSD method was found to be rapid, relatively inexpensive, straightforward and reproducible. Moreover, in principle, it could be used to quantify any saponin of interest.

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