Simultaneous Determination of Triterpene Saponins in Ginseng Drugs by High-Performance Liquid Chromatography

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A HPLC method for the simultaneous determination of 11 triterpene saponins with four-type aglycones (protopanaxadiol, protopanaxatriol, ocotillol and oleanolic acid types) in Ginseng drugs was developed and validated. Using a gradient of acetonitrile and 10 mM K-phosphate buffer (pH 5.80) as the mobile phase and UV detection at 196 nm, more than 18 ginsenosides with different aglycones were separated satisfactorily within 60 min. The detection limits (signal/noise≥3) were 0.1 μg for ginsenosides Rb_1, Rc, Rd, Re and Rg_1, chikusetsusaponin III, and notoginsenoside R_2, 0.2 μg for gisenoside Ro and chikusetsusaponin IVa, 0.3 μg for chikusetsusaponin IV, and 3 μg for majonoside R_2. The calibration curve of each saponin had a correlation coefficient close to 1. Intra- and interday precisions were less than 2.1% (n=5) and 3.3% (n=15), respectively. The recovery rates of extraction were in the range of 96.4—102.7% for all ginsenosides. By adopting this method, the determinations of 11 ginsenosides in three Ginseng drugs derived from *Panax ginseng*, *Panax vietnamensis* var. *fuscidiscus* and *Panax japonicus* (Japan) were achieved.

Key words ginsenoside; HPLC method; Ginseng drug; *Panax* spp.; quantitative comparison

Ginseng drugs, the roots and/or rhizomes of *Panax* spp. (Araliaceae), are a group of the most important herbal medicines in the Orient. More than 10 *Panax* taxa reported worldwide are available as medicinal resources in traditional Chinese medicine as well as in folk medicine, such as Ginseng, American Ginseng, Notoginseng, Chikusetsu-ninjin (Japanese Ginseng), Vietnamese Ginseng, *etc.* The main bioactive constituents of Ginseng drugs are considered to be triterpene saponins, generally referred to as ginsenosides. To date, more than 80 ginsenosides have been isolated from these drugs and most of them possess four types of aglycones, *i.e.*, protopanaxadiol, protopanaxatriol, ocotillol and oleanolic acid types.[1—3] Pharmacologic studies showed that the bioactivities of ginsenosides vary depending on the type of aglycone and the sugar moiety.[4—6] Phytochemical studies revealed that the composition and relative abundance of various ginsenosides in these drugs are different.[7—9] Comparative studies of the protopanaxadiol type [ginsenoside Rb_1 (**1**), Rb_2, Rc (**2**), and Rd (**3**)] and protopanaxatriol type [ginsenoside Re (**5**), Rg_1 (**6**), and Rf] in Ginseng, American Ginseng and Notoginseng have been reported.[10—12] However, a systematic comparison of the four-type ginsenosides, including the ocotillol and oleanolic acid types, has not been investigated so far. Recently, majonoside R_2 (**8**), a typical ocotillol type saponin, has attracted much attention for its high medicinal value as an antistress and antitumor active agent.[13,14] The oleanolic acid saponins, ginsenoside Ro (**9**) and chikusetsusaponin IV (**10**) have also been reported to have an antiinflammatory effect and to protect against stress ulcers.[15] Therefore a convenient and efficient method that allows simultaneous determination of the four-type ginsenosides is desirable for chemical and biological evaluation of different Ginseng drugs.

In the present study, 11 ginsenosides (**1—11**) (Fig. 1) representing the four types of saponins were chosen as standards based on extensive existence of relatively high concentration in different Ginseng drugs, which also have biological potential and phytotaxonomic significance. Here, we report the investigation and validation of a convenient HPLC method for the simultaneous determination of 11 ginsenosides with four-type aglycones and its utilization in the quantitative analysis of three Ginseng drugs with completely different compositions of ginsenosides,[7,8,16] which are Ginseng, Ye-Sanchi, and Satsuma-ninjin derived from *Panax ginseng*, *Panax vietnamensis* var. *fuscidiscus*, and *Panax japonicus* (Japan), respectively.

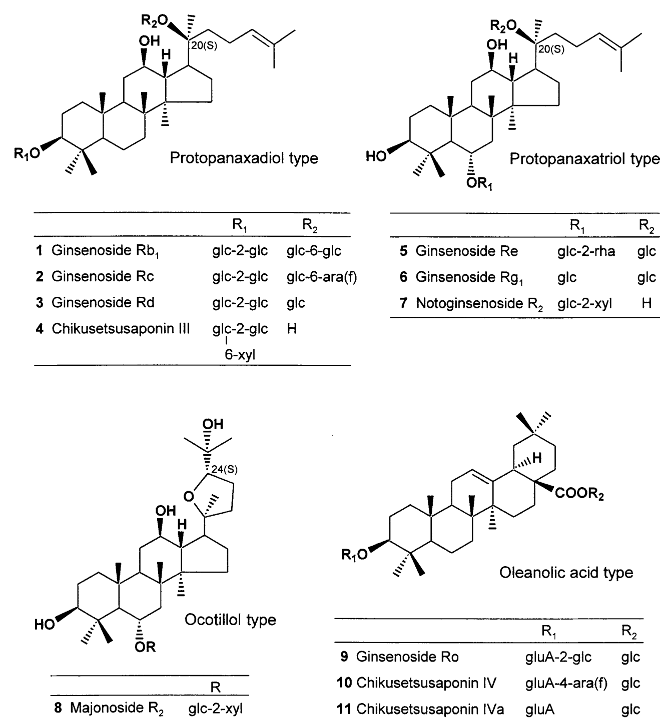

Fig. 1. Structures of 11 Standard Ginsenosides Used for Quantitative Determination

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Results and Discussion

It has been reported that the presence of K-phosphate buffer in the mobile phase can give a satisfactory separation of acidic as well as neutral ginsenosides.[17,18] A low concentration of the buffer was also recommended to avoid column contamination.[17] Based on preliminary trials, we applied a binary mixture of 10 mM K-phosphate buffer (pH 5.80) and acetonitrile as a mobile phase and an appropriate gradient was developed to achieve good resolution of 11 standard saponins. As shown in Fig. 2A, a baseline separation of 11 standard saponins with four different types of aglycones was achieved within 60 min by adopting the gradient system shown in Table 1. In particular, good resolution of **5** and **6** was obtained (Fig. 2D), and the other nine saponins were evenly distributed within the 60-min chromatogram, which facilitated the subsequent quantitative determination. Under the chromatographic conditions used, the protopanaxatriol derivatives were eluted first (**5**, **6**), followed by the ocotillol derivative (**8**) and oleanolic acid derivatives (**9**—**11**), while the protopanaxadiol ones (**1**—**4**) were eluted last (Fig. 2A). Furthermore, as shown in Fig. 3, satisfactory separation of additional seven saponins was also achieved with this gradient system in sample solution of Ye-Sanchi (TMPW no. 19759) which has been demonstrated to contain a variety of saponins.[16,19] The peaks in the chromatogram of Ye-Sanchi were identified by direct comparison of retention times and UV spectra with those of authentic saponins as well as by cochromatography of the sample solution spiked with authentic saponins (isolated in our laboratories[16,19]). These seven saponins, however, were not included in the subsequent quantitative determination due to their relatively limited distribution in Ginseng drugs. After each injection, the column was completely washed for 12 min (from 58 to 70 min in Table 1) and reequilibrated with the initial eluent for 13 min to ensure the efficiency and reproducibility of the column.

Quantitative studies on ginsenosides (protopanaxadiol, protopanaxatriol, and oleanolic acid types) by UV detection at wavelengths of 203 nm, 202 nm, and 198 nm have been reported.[12,17,18] However, detection of ocotillol type saponins in Ginseng drugs using a UV detector has not been conducted since the lack of a chromophore in ocotillol type saponins results in insufficient signal intensity at the wavelengths mentioned above. Using a photodiode array detector, we compared the absorption intensities of 11 standard saponins with four-type aglycones ranging from 195 to 400 nm. It was found that majonoside R_2 (**8**), an ocotillol type saponin exhibited a relatively intensive signal at the detection wavelength of 196 nm, which was 4- or 14-fold stronger than that at 198 nm and 203 nm, respectively (Fig. 2, peak **8**). The intensity of the peaks for other types of ginsenosides was obviously increased (Figs. 2A—C) and good linearity of analytes was also observed at this wavelength

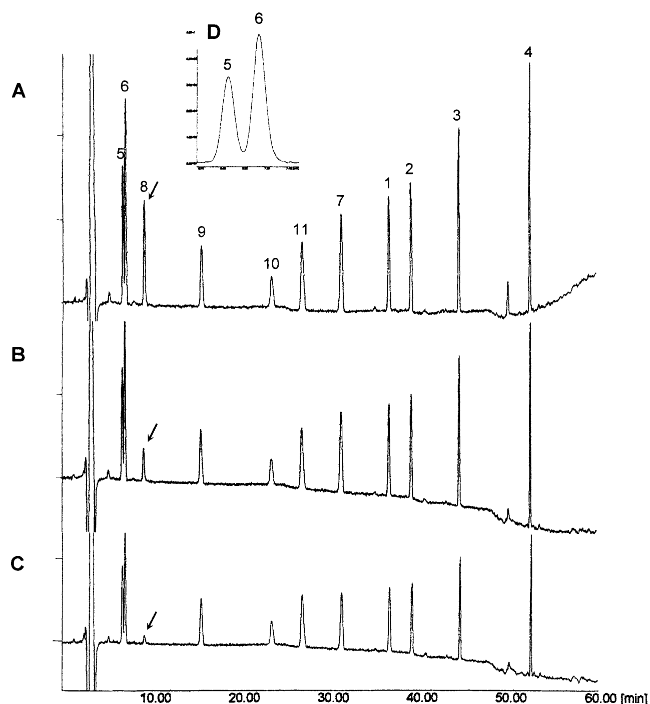

Fig. 2. HPLC Chromatograms of 11 Standard Ginsenosides in Ginseng Drugs

A, detected at 196 nm; B, detected at 198 nm; C, detected at 203 nm; D, dilated chromatogram from 6.40 to 7.20 min of Fig. 2A. Arrow highlights the peak of majonoside R_2, an ocotillol type saponin, which shows an intense signal at 196 nm, 4- or 14-fold stronger than that at 198 nm and 203 nm, respectively. Peaks 1—11 are as follows: **1**, ginsenoside Rb_1; **2**, ginsenoside Rc; **3**, ginsenoside Rd; **4**, chikusetsusaponin III; **5**, ginsenoside Re; **6**, ginsenoside Rg_1; **7**, notoginsenoside R_2; **8**, majonoside R_2; **9**, ginsenoside Ro; **10**, chikusetsusaponin IV; **11**, chikusetsusaponin IVa.

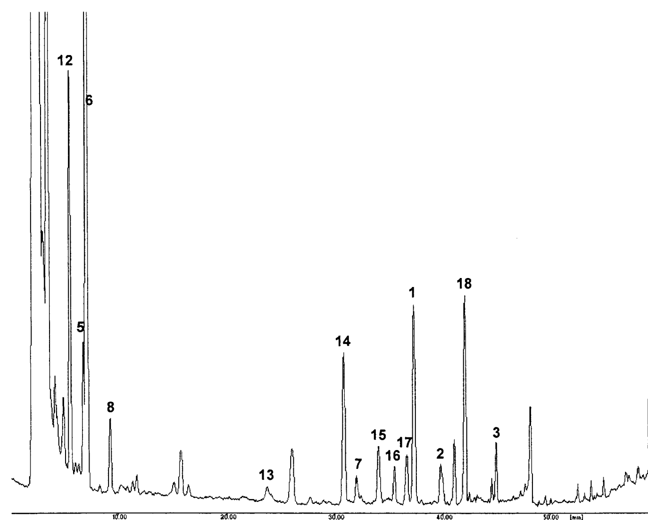

Fig. 3. HPLC Chromatogram of Drug Sample Ye-Sanchi (TMPW no. 19759)

Peaks are as follows: **1**, ginsenoside Rb_1; **2**, ginsenoside Rc; **3**, ginsenoside Rd; **5**, ginsenoside Re; **6**, ginsenoside Rg_1; **7**, notoginsenoside R_2; **8**, majonoside R_2; **12**, notoginsenoside R_1; **13**, vina-ginsenoside R_2; **14**, notoginsenoside R_4; **15**, notoginsenoside Fa; **16**, ginsenoside Rg_2; **17**, ginsenoside Rh_1; **18**, ginsenoside Rb_3.

Table 1. Gradient Condition of Mobile Phase

Time	A	B	C
0	73	27	0
20	72	28	0
34	66	34	0
44	57	43	0
57.5	15	85	0
58	0	85	15
65	0	85	15
70	80	20	0
72	73	27	0
85	73	27	0

A: 10 mM K-phosphate buffer (pH 5.80), B: acetonitrile, C: distilled water.

(data presented below). The detection limits (signal/noise≥3) of 11 ginsenosides were 0.1 μg for **1**—**7**, 0.2 μg for **9** and **11**, 0.3 μg for **10**, and 3 μg for **8**. Therefore, data collection was conducted at 196 nm in the present study, which enabled simultaneous determination of 11 ginsenosides with four-type aglycones.

The calibration curve of each standard saponin was investigated between the peak area (y) and the quantity of each saponin (x, μg) using prepared standard working solution. Triplicate injections were performed to obtain the absorption plots ranged from 0.2 to 40 μg for each of the 11 saponins. As shown in Table 2, the results of regression analysis revealed that the calibration curve of each saponin had a correlation coefficient very close to 1.

Intra- and interday precisions were evaluated by replicate injection of a mixture of 11 standard saponins and a sample solution of Ginseng (TMPW no. 19899). Five injections per day were conducted on days 1, 2 and 7 after sample preparation to determine reproducibility (after measurement, the solution was stored at 3 °C). The intraday precision of the 11 saponins in the mixture solution was found to be 0.1—2.1% (n=5), and the interday precision was 0.9—2.1% (n=15). Similarly, the peak areas of **1**, **2**, **5**, **6**, and **9** were measured and the intra- and interday precisions in sample solution were found to be 0.9—1.8% (n=5) and 2.1—3.3% (n=15), respectively. This result shows that the sample solution was stable for at least 1 week when stored at 3 °C.

To examine the accuracy of the method as well as the recovery rate of extraction, a standard addition test was performed. Certain amounts of standard ginsenosides were spiked into three Ginseng drugs (Ginseng, Ye-Sanchi, and Satsuma-ninjin), and the mixtures were subjected to the extraction procedure described in preparation of the sample solution. For comparison, three blank samples (without spiking with standard saponin) were prepared and similarly analyzed. As shown in Table 3, extraction recoveries in the range of 96.4 to 102.7% were obtained.

By adopting the established method, quantitative determinations of the 11 ginsenosides in Ginseng, Ye-Sanchi, and Satsuma-ninjin were conducted. The results (Table 4) showed that the ginsenosides were varied considerably in composition and quantity among the three Ginseng drugs. The total saponin content of Ginseng was 10-fold lower than that of Ye-Sanchi and Satsuma-ninjin. The markedly high content of majonoside R_2 (**8**) and only trace amount of oleanolic acid saponin (**9**) in Ye-Sanchi differentiated it from the other two. Satsuma-ninjin, a special form of Chikusetsu-ninjin produced in southern Kyushu, Japan, was characterized by a high content of oleanolic acid saponins (**9**—**11**), as well as relative rich amount of dammarane saponins (**1**, **2**, **4**, **5**—**7**). The constituent pattern of each Ginseng drug was clearly characterized through quantitative comparison of the 11 major saponins.

In conclusion, the present HPLC method coupled with a photodiode array detector is useful for simultaneous quanti-

Table 2. Calibration Equation, Correlation Coefficient and Detection Limit of 11 Standard Saponins

Aglycone type	Saponin	Calibration equation[a]	Correlation coefficient
Protopanaxadiol	**1**	y=322313x+13735	r=1.00000
	2	y=340302x+13943	r=0.99997
	3	y=368200x+16195	r=0.99996
	4	y=328166x+89463	r=0.99836
Protopanaxatriol	**5**	y=360182x+8584	r=0.99998
	6	y=354833x+62156	r=0.99998
	7	y=453599x+11149	r=1.00000
Ocotillol	**8**	y=11482x+5141	r=0.99985
Oleanolic acid	**9**	y=247993x+15659	r=0.99998
	10	y=235987x+5485	r=0.99994
	11	y=324456x+41952	r=0.99995

a) y is the peak area and x is the quantity of injected analyte (μg).

Table 4. Contents (mg/g) of 11 Saponins in Three Ginseng Drugs (±S.D., n=3)

	Ginseng	Ye-Sanchi	Satsuma-ninjin
Protopanaxadiol type			
1	2.03±0.06	6.44±0.08	3.38±0.11
2	1.20±0.02	0.83±0.00	1.01±0.04
3	0.22±0.01	0.95±0.01	Trace
4	N.D.[a]	N.D.	2.38±0.08
Protopanaxatriol type			
5	0.74±0.01	2.42±0.00	2.44±0.09
6	3.15±0.11	23.05±0.22	4.97±0.14
7	Trace	0.66±0.01	1.97±0.05
Ocotillol type			
8	N.D.	57.80±0.54	N.D.
Dammarane subtotal	7.34±0.21	92.15±0.86	16.15±0.51
Oleanolic acid type			
9	1.77±0.08	Trace	37.94±1.34
10	N.D.	N.D.	38.19±1.14
11	N.D.	N.D.	2.29±0.10
Oleanolic acid subtotal	1.77±0.08	—	78.42±2.58
Total	9.11±0.29	92.15±0.86	94.57±3.09

a) N.D.: not detected.

Table 3. Recovery of 11 Triterpene Saponins

Sample	weight	Saponin	Amount in sample (mg)	Spiked weight (mg)	Measured weight (mg)	Recovery (%)
Ginseng	1.00 g	**1**	1.96	1.00	2.96	100.4
		2	1.21	0.90	2.08	97.1
		6	3.09	0.95	4.04	99.9
		9	1.76	0.90	2.66	99.7
Ye-Sanchi	0.50 g	**3**	0.43	0.50	0.91	96.4
		5	1.25	0.70	1.96	102.7
		7	0.33	0.50	0.78	101.8
		8	28.22	5.20	33.29	97.5
Satsuma-ninjin	0.20 g	**4**	0.52	1.00	1.49	96.9
		10	7.69	3.00	10.64	98.5
	1.00 g	**11**	2.30	2.00	4.33	101.4

tative determination of 11 ginsenosides, possessing four types of aglycones, which are major constituents in various Ginseng drugs. The appropriate linearity, intra- and interday precisions, accuracy, and recovery rates of extraction were validated. Using the method, three Ginseng drugs derived from three different *Panax* taxa were analyzed. Furthermore, we have subsequently conducted a comparative study on the 11 ginsenosides of 47 samples of Ginseng drugs derived from 12 *Panax* taxa using the established method to characterize the chemical constituent pattern of each Ginseng drug and to investigate relationships among genetic varieties[20] and chemical constituent pattern, and the results will be reported in our future paper.[21]

Experimental

Materials The following three Ginseng drugs were quantitatively examined: Ginseng (TMPW no. 19899), Ye-Sanchi (TMPW no. 19759), and Satsuma-ninjin (a special form of Chikusetsu-ninjin, TMPW no. 12022), derived from the underground parts of *P. ginseng*, *P. vietnamensis* var. *fuscidiscus*, and *P. japonicus* (Japan), respectively. A sample of Chikusetsu-ninjin (TMPW no. 12187) was used to isolate compounds of chikusetsusaponin III (**4**) and IV (**10**). Voucher specimens have been deposited in the Museum of Materia Medica, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University, Toyama (TMPW).

Isolation and Identification of Chikusetsusaponin III and IV The powdered sample (100 g) of Chikusetsu-ninjin (TMPW no. 12187) was extracted four times with methanol (300 ml, 2 h) under reflux. The combined methanol extract was filtered and evaporated under reduced pressure to give 47 g of dry residue. This residue was subjected to column chromatography on Diaion HP-20 and eluted successively with H_2O (1.5 l), 50% MeOH (2.0 l), and MeOH (1.5 l) to give fractions I (discarded), II (10.5 g) and III (14.0 g), respectively. Using HPLC analysis, compounds **9**, **10**, and **11** were found to be the major components in fraction II, while compound **4** was the major component in fraction III. From fraction II, 100 mg was submitted to normal-phase preparative TLC, developed by $CHCl_3$–MeOH–H_2O= 65 : 35 : 10 (lower phase) to give **10** (19.5 mg). Similarly, compound **4** (43.0 mg) was obtained from 100 mg of fraction III.

The structures of **4** and **10** were identified by comparison of their ^{1}H- and ^{13}C-NMR spectral data (recorded on a JEOL JMN-LA400MB-FT spectrometer; ^{1}H, 400 MHz; ^{13}C, 100 MHz) with those reported in the literatures.[1,22]

Standard Saponins and Reagents Of the 11 standards, **1**, **3**, **5**—**9**, and **11** were isolated and identified in our laboratories,[16,19,23] compounds **4** and **10** were obtained as mentioned above, and ginsenoside Rc (**2**) was purchased from Wako Pure Chemical Industries, Ltd. (Japan). Reagents for HPLC analysis including acetonitrile and distilled water (HPLC grade) were purchased from Kanto Chemical Co., Inc. (Japan). Potassium dihydrogenphosphate and dipotassium hydrophosphate (analytical grade) were obtained from Wako Pure Chemical Industries, Ltd.

Apparatus and Analytical Conditions The JASCO HPLC system (Jasco Co., Ltd., Japan) is composed of a CO-1580 intelligent HPLC pump, a DG-1580-53 3-line degasser, a LG-1580-02 ternary gradient unit, a CO-1565 intelligent column oven, an AS-2057 plus intelligent sampler, and a MD-1510 diode array detector. Comparative analysis was carried out using a YMC-Pack ODS-AQ303 column (S-5 μm, 12 nm, 250 mm×4.6 mm i.d.) with column temperature at 40 °C. The mobile phase was a binary eluent of (A) 10 mM K-phosphate buffer (pH 5.80, adjusted with 1 M K_2HPO_4 solution) and (B) CH_3CN under gradient conditions (Table 1). The flow rate was 1.0 ml/min and detection wavelength was 196 nm. The chromatographic data were collected and processed using BORWIN-PDA APPLICATION and BORWIN CHROMATOGRAPHY Software (version 1.5, Jasco Co., Ltd., Japan)

Preparation of Standard Solution and Samples Each standard saponin (10.0 mg) was accurately weighed into a 10.0 ml volumetric flask and dissolved in 70% EtOH to make a stock solution of 1.0 mg/ml. To draw calibration curves, a series of standard solutions were prepared from the stock solution and filtrated through a 0.2 μm Millipore filter unit (Advantec, Japan). Typical calibration curves containing 0.2, 1.0, 2.0, 5.0, 10.0, 20.0, and 40.0 μg of analytes were prepared, plotting peak area against injection amount.

Samples were pulverized and the powder was screened through a 150 μm sieve. 1.0 g of the fine powder was accurately weighed and extracted with 70% EtOH (7 ml, 6 ml, 6 ml, 6 ml) by ultrasonication at room temperature for 30 min, mixed periodically by vortex to obtain fuller extraction. Then the supernatant was obtained by centrifugation at 2500 rpm (H-103N Centrifuge, Kokusan, Japan) for 10 min. Supernatants were transferred into volumetric flasks, and 70% EtOH was added to obtain a final volume of 25.0 ml. After filtration through a 0.2 μm Millipore filter unit (Advantec), 10.0 μl of this solution was injected into the HPLC system for analysis.

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