Research Article

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Characterizing a full spectrum of physico-chemical properties of (20S)- and (20R)-ginsenoside Rg3 to be proposed as standard reference materials

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The authentication of the physico-chemical properties of ginsenosides reference materials as well as qualitative and quantitative batch analytical data based on validated analytical procedures is a prerequisite for certifying good manufacturing practice (GMP). Ginsenoside Rb1 and Rg1, representing protopanaxadiol and protopanaxatriol ginsenosides, respectively, are accepted as marker substances in quality control standards worldwide. However, the current analytical methods for these two compounds recommended by Korean, Chinese, European, and Japanese pharmacopoeia do not apply to red ginseng preparations, particularly the extract, because of the relatively low content of the two agents in red ginseng compared to white ginseng. In manufacturing fresh ginseng into red ginseng products, ginseng roots are exposed to a high temperature for many hours, and the naturally occurring ginsenoside Rb1 and Rg1 are converted to artifact ginsenosides such as Rg3, Rg5, Rh1, and Rh2 during the heating process. The analysis of ginsenosides in commercially available ginseng products in Korea led us to propose the inclusion of the (20S)- and (20R)-ginsenoside Rg3, including ginsenoside Rb1 and Rg1, as additional reference materials for ginseng preparations. (20S)- and (20R)-ginsenoside Rg3 were isolated by Diaion HP-20 adsorption chromatography, silica gel flash chromatography, recrystallization, and preparative HPLC. HPLC fractions corresponding to those two ginsenosides were recrystallized in appropriate solvents for the analysis of physico-chemical properties. Documentation of those isolated ginsenosides was achieved according to the method proposed by Gaedcke and Steinhoff. The ginsenosides were subjected to analyses of their general characteristics, identification, purity, content quantification, and mass balance tests. The isolated ginsenosides showed 100% purity when determined by the three HPLC systems. Also, the water content was found to be 0.534% for (20S)-Rg3 and 0.920% for (20R)-Rg3, meaning that the net mass balances for (20S)-Rg3 and (20R)-Rg3 were 99.466% and 99.080%, respectively. From these results, we could assess and propose a full spectrum of physico-chemical properties of (20S)- and (20R)-ginsenoside Rg3 as standard reference materials for GMP-based quality control.

Keywords: *Panax ginseng*, (20S)- and (20R)-ginsenoside Rg3, Physico-chemical property, Standard reference material, Documentation

INTRODUCTION

Panax ginseng has been used in traditional medicine in East Asian countries for over two thousand years. The

empirical, evidence-based clinical efficacies of ginseng have been elucidated by modern and multi-disciplinary

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experiments and thus enabled us to find a wide spectrum of pharmacological effects: antifatigue, immunomodulatory therapy, facilitation of blood circulation, spermatogenesis stimulation, and antistress effects are just a few of many [1]. To date, saponins (ginsenosides), polysaccharides, and polyacetylenes have been characterized from red or white *P. ginseng* as the active therapeutic ingredients [2,3]. However, ginsenosides are regarded as the major active components of ginseng among those bioactive agents and are therefore employed as the reference material for quality control. A total of 38 ginsenosides have been isolated and characterized from the ginseng plant [4].

Ginseng is classified as an herbal medicine or health food supplement depending on the country. The United States and Korea categorize it as a health food supplement, but the World Health Organization (WHO), European Union (EU), Middle East, and India consider it to be medicine. To market ginseng products in the EU, we need marketing authorization. Completion of the common technical document (CTD) is essential paper work for applying to the marketing authorization holder (MAH). Good manufacturing practice (GMP)-based quality control is an integral part of the CTD. An authenticity document of the physico-chemical properties for ginsenosides reference materials (RM) and both qualitative and quantitative batch analytical data based on validated analytical procedures are prerequisites to certify GMP. In this respect, it can be said that GMP will not be possible without an authenticity document for the full spectrum of physico-chemical properties of the reference materials. In addition, it is obligatory that every quality analytical method be validated according to guidelines issued by the International Conference on Harmonization (ICH) [5]. Japan, Korea, and European countries require quantitative analytical HPLC data for the ginsenoside Rb1 and Rg1 and representative reference materials for the protopanaxadiol and protopanaxatriol ginsenoside. However, the current analytical methods for these two compounds recommended by the Korean, Chinese, European, and Japanese pharmacopoeia are not applicable to red ginseng preparations, particularly the extract, because of the relatively lower content of these two agents in red ginseng compared to white ginseng. In the manufacturing process of fresh ginseng into red ginseng products, ginseng roots are exposed to high temperatures for many hours, and the naturally occurring Rb1 and Rg1 are converted to artifact ginsenosides such as Rg3, Rg5, Rh1, and Rh2, during the heating process. An analysis of ginseng extracts available in the Korean market led us

to include (20S)- and (20R)-ginsenoside Rg3, including ginsenoside Rb1 and Rg1, as additional reference materials for ginseng preparations. Moreover, methods for authenticity documentation of ginsenoside RM are not available from monographs of the WHO's selected medicinal plants and Japanese and Chinese pharmacopoeias. Therefore, the CTD and ICH guideline-based application is not strictly required for the application format of MAH in Japan and China. However, the EU requires a medicinal level of quality assurance data for ginseng preparations. In addition, regulation of quality control is gradually becoming stricter in China as well as in Korea due to adulterations and low-quality herbal medicinal products.

Germany has a well-established scientific and regulatory basis for development, quality assurance, and marketing authorization. We could find detailed authenticity documentation methods for RM from a textbook [6] written by German experts in drug regulatory affairs. We have thus far isolated 13 ginsenosides, including (20S)- and (20R)-ginsenoside Rg3, in their highest purity by employing adsorption chromatography, silica gel column chromatography, preparative HPLC and recrystallization; authenticity documentation for the isolated ginsenosides was achieved. Among those ginsenosides, documentation for the (20S)- and (20R)-ginsenoside Rg3 RM will be presented in this article. Authenticity certification of the RMs encompasses general characteristics in terms of appearance and solubility; identity in terms of IR, ¹H NMR, ¹³C NMR, mass, and UV spectra analyses; purity in terms of elementary analysis, three different HPLC methods, optical rotation, water content, melting point, residual organic solvents, and TLC; and content in terms of three different HPLC methods. Authenticity document for ginsenosides Rc, Rd, Re, Rf, (20S, 20R)-Rg2, (20E)-ginsenoside F4, and compound K and establishment of validated analytical procedure for those ginsenosides will be presented elsewhere.

MATERIALS AND METHODS

Chemicals

Methanol, acetonitrile, and isopropyl alcohol were HPLC grade and purchased from Burdick & Jackson (Honeywell International Inc., Muskegon, MI, USA). All other chemicals and solvents were of analytical grade, unless otherwise mentioned. TLC plates were purchased from Merck (HPTLC plate with silica gel 60 F254). Reference ginsenoside Rg3 was purchased from WaKo (Pure Chemical Industries, Osaka, Japan) for comparisons of purity and identity.

Isolation and purification of ginsenosides

Korean red ginseng extract, a water extract obtained from steamed and dried roots of *P. ginseng*, was employed for the isolation and purification of individual ginsenosides. Red ginseng water extract (1 kg) was dissolved in water to make approximately 20% to 25% (w/v) solution. The solution was passed through a Diaion HP-20 adsorption column (8 L), and the resin was washed with 4 wet resin volumes of 20% ethanol. Crude saponin adsorbed to the macro-reticular resin was eluted with 4 wet resin volumes of pure methanol. The methanol elute was then dried with silica gel powder *in vacuo* to obtain a pale brown powder. The powder was loaded on a glass column (15×100 cm) containing 3.5 L of silica gel and partitioned with solvent mixtures of CHCl₃-MeOH-H₂O (12:3:1, 9:3:1, 7:3:1, 4:3:1; lower phase, 4 bed volumes for each mixture). Fractions containing the ginsenosides Rg3 were dried *in vacuo* and recrystallized in water until a white powder was formed. The white crystalline amorphous powder was then subjected to preparative HPLC (20×250 mm, ODS YMC-Pack, 203 nm) with aqueous 45% CH₃CN as the mobile phase to purify single ginsenoside. The peak corresponding to each ginsenoside was collected and evaporated to dryness and further dried in a vacuum drier for 5 d. The resultant white powder was stored at -20°C until further use.

Determination of physico-chemical properties of isolated ginsenosides

Purified (20*S*)- and (20*R*)-ginsenoside Rg3 were subjected to authenticity tests to qualify the standard reference material (SRM) for quality control as suggested by Gaedcke et al. [6]. Authenticity of each ginsenosides was certified by determining general characteristics in terms of color, appearance, and solubility. The identities of the ginsenosides were certified by analyzing ¹H NMR, ¹³C NMR, IR, FAB/MS, and UV spectra. The identities of the compounds were verified by 2D NMR: ¹H-¹³C correlation spectroscopy (COSY), heteronuclear multiple spectroscopy (HMQC), heteronuclear multiple bond correlation (HMBC), and nuclear overhauser spectroscopy (NOSEY). The purity of the compounds was determined by measuring melting point, elemental composition, water content, residual solvent content, and TLC and HPLC fingerprints obtained by 3 different systems. The quantitation of these compounds was achieved by three different HPLC methods. The water content of ginsenosides was determined on a micro-scale by a Karl Fisher Titrator in accordance with the European pharmacopoeia method [7]. Residual solvent was analyzed by GC/MS

after preparing the sample by the headspace method. A UV spectrum was measured at a sample concentration of 0.5 mg/mL methanol. A 2 mg sample was introduced into the element analyzer. Optical rotation was determined with 5 replicates. Net mass balance of the ginsenosides was calculated as follows: [100%-water content (%)-residual solvent content (%)]×HPLC purity (%)/100. Finally, all the physico-chemical property data were compared with the values described in the literature.

Analytical instruments

NMR (JEOL Ltd., Tokyo, Japan; 500 MHz, TMS as the external reference, 20 mg/mL DMSO-*d*₆), FAB/MS (JMS-700 Mstation, JEOL Ltd.), IR (Mattson model Galaxy 7020A, Ge-coated KBr pellet, 3.0 mg/g), and UV (V-530; Jasco, Tokyo, Japan) spectrometers were used for determining identity. Melting point analyzer (Manstead/Electrothermal IA9100; Barnstead, Boston, MA, USA), element analyzer (Flash EA1112 series; CE Elantec, Lakewood, NJ, USA), Karl Fisher titrator (831KF; Metrohm, Hartmannsdorf, Germany), and polarimeter (Jasco P-1020, Jasco; filter 589 mm, cylindrical glass cell: 10×100 mm; c, 1.0 in MeOH) were used for the determination of purity. Two HPLC systems were employed to determine purity and content: 1) Hewlett-Packard (Wilmington, DE, USA) equipped with a G1313A automatic sample injector, a vacuum degasser unit, a G1311A quaternary pump, and a G1315A photo diode array detector; 2) Waters (Taunton, MA, USA) equipped with a 2707 automatic sample injector, 1525 binary HPLC pump, and an Alltech Model ELSD 2000 detector.

Preparation of standard solution

Five milligram of each (20*S*, 20*R*)-ginsenoside Rg3 were dissolved in 10 mL of HPLC grade methanol and diluted until the final concentration was 62.5 µg/mL. The diluted standard solution was kept at -20°C until use and stood at room temperature for at least 30 min prior to use.

Conditions for HPLC analysis

HPLC analysis of ginsenosides for content determination was performed in 3 different conditions: 1) Discovery HS C18 column (4.6×250 mm, 5 µm; Supelco, Bellefonte, PA, USA) at room temperature with a sample injection volume of 20 µL, mobile phase consisted of acetonitrile (solvent A) and water (solvent B), the following program was employed: equilibrated with A:B (20:80) for 20 min, linear gradient ranged from A:B (20:80) to (35:65) from 20 to 40 min, A:B (35:65) to (45:55) from 40 to 52 min, A:B (45:55) to (70:30) from

52 to 62 min, A:B (70:30) to (100:0) from 62 to 80 min and finally equilibrated with A:B (20:80) from 80 to 90 min. The flow rate of the mobile phase was 1.6 mL/min, and the detector wavelength was set to 203 nm; 2) Symmetry C18 column (250×4.6 mm, 5 µm; Waters) at room temperature with a sample injection volume of 20 µL, the mobile phase consisted of acetonitrile containing 0.1% formic acid (solvent A) and water containing 0.1% formic acid (solvent B). The following program was employed: equilibrated with A:B (20:80) for 20 min, linear gradient started with A:B (20:80) to (35:65) from 20 to 40 min, A:B (35:65) to (45:55) from 40 to 52 min, A:B (45:55) to (70:30) from 52 to 62 min, A:B (70:30) to (100:0) from 62 to 80 min and finally equilibrated with A:B (20:80) from 80 to 90 min. The flow rate of the mobile phase was 1.6 mL/min, and the detector wavelength was set to 203 nm; 3) Prevail carbohydrate ES column (250×4.6 mm, 5 µm; Alltech, Deerfield, IL, USA) at room temperature with a sample injection volume of 10 µL, the mobile phase consisted of acetonitrile/H₂O/isopropyl alcohol=80/5/15 (solvent A) and acetonitrile/H₂O/isopropyl alcohol=80/15/15 (solvent B), the following program was employed: linear gradient ranged from A:B (75:25) to (15:85) for 28 min, A:B (15:85) to (20:80) from 28 to 35 min, A:B (20:80) to (25:75) from 35 to 45 min, A:B (25:75) to (10:90) from 45 to 50 min, A:B (10:90) to (0:100) from 50 to 55 min and finally equilibrated with A:B (75:25) from 55 to 60 min. The flow rate of the mobile phase was 1.0 mL/min, and the detector was ELSD (gain 8, gas flow rate 2.0 SLPM drift tube temperature 90°C).

Statistical analyses

Data were presented as the means±SEM. Statistical analyses were performed using Student's *t*-test in Excel (Microsoft, Redmont, WA, USA). Differences were considered significant when *p*<0.05.

RESULTS

Isolation and purification of individual ginsenosides

We could isolate ten grams of (20S)- and (20R)-ginsenoside Rg3 via adsorption chromatography, silica gel partition chromatography, recrystallization, and preparative HPLC fractionation. Fractions obtained from CHCl₃-MeOH-H₂O (12:3:1) gave predominantly (20E)-ginsenoside F4 and that of CHCl₃-MeOH-H₂O (9:3:1) afforded mainly ginsenoside Rg3. The recrystallized white powder was purified by preparative HPLC (20×250 mm, ODS YMC-Pack, 203 nm) with 45% CH₃CN. A peak corresponding to (20S)- and (20R)-ginsenoside Rg3 was collected, dried *in vacuo*, and further dried in a vacuum drier for 5 d to obtain a white amorphous powder.

General characteristics of the isolated (20S)- and (20R)-ginsenoside Rg3

Isolated (20S)- and (20R)-ginsenoside Rg3 were subjected to various physico-chemical analyses, including general characteristics. As shown in Table 1, (20S)- and (20R)-ginsenoside Rg3 exhibited as white amorphous powders. (20S)-ginsenoside Rg3 was readily soluble in cold H₂O, EtOH, MeOH, and CH₃CN, whereas (20R)-ginsenoside Rg3 was readily soluble in DMSO and sparingly soluble in H₂O and CH₃CN.

Identification of the isolated (20S)- and (20R)-ginsenoside Rg3

Isolated (20S)- and (20R)-ginsenoside Rg3 were subjected to various spectrometric analyses for identification. Interpretation of ¹H and ¹³C NMR spectra together with ¹H-¹H COSY, HMQC, HMBC, and NOESY spectra allowed us to assign all the ¹H and ¹³C NMR signals (Table 2). Determining the chemical shift (ppm), multiplicity and coupling constant of each proton signal was also achieved by interpretation of NMR spectra. Comparing

Table 1. General characteristics of isolated (20S)- and (20R)-ginsenoside Rg3

	(20S)-ginsenoside Rg3	(20R)-ginsenoside Rg3
Biological source	<i>Panax ginseng</i> Meyer	<i>P. ginseng</i> Meyer
CAS registry no.	14197-60-5	38243-03-7
Empirical formula	C ₄₂ H ₇₂ O ₁₃	C ₄₂ H ₇₂ O ₁₃
Molar mass	785.023 g/molar	785.023 g/molar
Accurate mass	784.497g/molar	784.497g/molar
Appearance	White amorphous powder, solid at room temperature	White amorphous powder, solid at room temperature
Solubility (25°C)	Water, anhydrous ethanol and methanol	Sparingly soluble in water, anhydrous ethanol and methanol

CAS, Chemical Abstracts Service.

Table 2. NMR spectra assignment for (20S)- and (20R)-ginsenoside Rg3

Proton at C atom	(20S)-ginsenoside Rg3			(20R)-ginsenoside Rg3		
	Chemical shift (ppm)	Multiplicity, coupling constants (Hz)	Reference chemical shift (ppm) [8]	Chemical shift (ppm)	Multiplicity, coupling constants (Hz)	Reference chemical shift (ppm) [9]
1	1.54	m ; 1H	1.55	1.56	m ; 1H	1.54
	0.94	m ; 1H	0.93	0.94	m ; 1H	0.92
2	1.87	m ; 1H	1.87	1.87	m ; 1H	1.86
	1.55	m ; 1H	1.55	1.57	m ; 1H	1.55
3	3.02	m ; 1H	3.01	3.02	m ; 1H	3.00
5	0.69	d (J=11.5 Hz) ; 1H	0.69	0.70	d (J=11.5 Hz) ; 1H	0.69
6	1.46	m ; 1H	1.46	1.46	m ; 1H	1.46
	1.36	m ; 1H	1.36	1.36	m ; 1H	1.36
7	1.44	m ; 1H	1.43	1.44	m ; 1H	1.44
	1.17	m ; 1H	1.19	1.17	m ; 1H	1.16
9	1.33	m ; 1H	1.34	1.35	m ; 1H	1.34
11	1.69	m ; 1H	1.67	1.70	m ; 1H	1.67
	1.10	m ; 1H	1.11	1.13	m ; 1H	1.11
12	3.37	m ; 1H	3.37	3.37	m ; 1H	3.37
12-OH	6.05	s ; 1H		6.12	S ; 1H	5.81
13	1.57	m ; 1H	1.56	1.58	m ; 1H	1.56
15	1.41	m ; 1H	1.41	1.41	m ; 1H	1.40
	0.93	m ; 1H	0.93	0.93	m ; 1H	0.93
16	1.72	m ; 1H	1.73	1.72	m ; 1H	1.71
	1.21	m ; 1H	1.21	1.21	m ; 1H	1.20
17	1.89	m ; 1H	1.89	1.89	m ; 1H	1.88
18	0.91	s ; 3H	0.89	0.92	s ; 3H	0.89
19	0.82	s ; 3H	0.80	0.82	s ; 3H	0.80
20-OH	5.92	s ; 1H		5.95	s ; 1H	5.74
21	1.05	s ; 3H	1.01	1.00	s ; 3H	0.96
22	1.38	m ; 1H	1.39	1.35	m ; 1H	1.30
	1.28	m ; 1H	1.28	1.35	m ; 1H	1.30
23	2.07	m ; 1H	2.07	2.01	m ; 1H	1.99
	1.90	m ; 1H	1.90	2.01	m ; 1H	1.99
24	5.09	m ; 1H	5.09	5.08	m ; 1H	5.05
26	1.65	s ; 3H	1.63	1.63	s ; 3H	1.61
27	1.57	s ; 3H	1.55	1.57	s ; 3H	1.54
28	0.98	s ; 3H	0.97	0.98	s ; 3H	0.96
29	0.75	s ; 3H	0.73	0.75	s ; 3H	0.72
30	0.82	s ; 3H	0.81	0.82	s ; 3H	0.81

Table 2. (Continued)

Proton at C atom	(20 <i>S</i>)-ginsenoside Rg3			(20 <i>R</i>)-ginsenoside Rg3		
	Chemical shift (ppm)	Multiplicity, coupling constants (Hz)	Reference chemical shift (ppm) [8]	Chemical shift (ppm)	Multiplicity, coupling constants (Hz)	Reference chemical shift (ppm) [9]
Glucose 1 at C3						
1	4.29	d (J=7.0 Hz) ; 1H	4.26	4.29	d (J=7.0 Hz) ; 1H	4.24
2	3.37	m ; 1H	3.31	3.38	m ; 1H	3.28
3	3.38	m ; 1H	3.34	3.39	m ; 1H	3.33
	5.78	d ; 1H		5.77	d ; 1H	5.67
4	3.08	m ; 1H	3.07	3.08	m ; 1H	3.06
	5.22	d ; 1H		5.19	d ; 1H	4.98
5	3.08	m ; 1H	3.07	3.08	m ; 1H	3.06
6	3.64	m ; 1H	3.64	3.67	m ; 1H	3.63
	3.41	m ; 1H	3.41	3.47	m ; 1H	3.40
6-OH	4.71	t ; 1H		4.67	t ; 1H	4.37
Glucose 2 at C3						
1	4.48	d (J=7.5 Hz) ; 1H	4.42	4.48	d (J=7.5 Hz) ; 1H	4.40
2	3.00	m ; 1H	2.98	3.02	m ; 1H	2.97
3	5.54	d ; 1H		5.52	d ; 1H	5.40
3-OH	3.12	m ; 1H	3.12	3.12	m ; 1H	3.11
4	5.11	Overlapped with other signals		5.08	Overlapped	4.90
4-OH	3.11	m ; 1H	3.11	3.12	m ; 1H	3.10
5	5.11	Overlapped with other signals		5.08	Overlapped	4.83
	3.02	m ; 1H	3.02	3.03	m ; 1H	3.01
	3.64	m ; 1H	3.60	3.66	m ; 1H	3.58
6-OH	3.47	m ; 1H	3.47	3.47	m ; 1H	3.46
	4.61	t ; 1H		4.53	t ; 1H	4.10
C atom	(20 <i>S</i>)-ginsenoside Rg3 (ppm)		(20 <i>R</i>)-ginsenoside Rg3 (ppm)			
	Chemical shift	Reference chemical shift [8]	Chemical shift	Reference chemical shift [9]		
1	38.5 (overlapped with solvent)	38.5	Overlapped with solvent (49.0)	38.4		
2	25.6	25.7	25.5	25.8		
3	88.4	88.1	88.4	88.0		
4	38.7 (overlapped with solvent)	38.6	Overlapped with solvent (49.0)	38.2		
5	55.4	55.4	55.5	55.4		
6	17.6	17.6	17.6	17.7		
7	34.2	34.3	34.3	34.3		
8	39 (overlapped with solvent)	36.2	Overlapped with solvent (49.0)	36.2		
9	49.3	49.3	49.1	49.3		
10	36.1	38.8	36.1	38.6		
11	30.7	30.8	30.7	31.0		
12	69.8	69.7	69.7	69.5		
13	47.2	47.3	47.8	47.8		
14	50.8	50.4	50.9	50.1		
15	30.4	30.5	30.5	30.4		
16	25.8	25.8	25.5	25.5		
17	53.2	53.4	49.3	49.4		
18	15.3	15.4	15.2	16.4		
19	15.8	15.8	15.8	15.8		
20	72.2	71.8	72.2	71.4		

Table 2. (Continued)

C atom	(20S)-ginsenoside Rg3 (ppm)		(20R)-ginsenoside Rg3 (ppm)	
	Chemical shift	Reference chemical shift [8]	Chemical shift	Reference chemical shift [9]
21	26.0	26.3	21.9	22.0
22	34.8	34.8	41.8	41.9
23	21.8	21.8	21.3	21.4
24	125.2	125.4	124.9	124.9
25	130.3	129.8	130.3	129.5
26	25.4	25.4	25.4	25.4
27	17.4	17.5	17.3	17.5
28	27.3	27.4	27.3	27.4
29	15.7	15.7	15.7	15.7
30	16.3	16.5	16.6	15.4
Glucose 1 at C3				
1	103.2	103.5	103.3	103.5
2	80.4	81.2	80.5	81.0
3	76.5	76.4	76.5	76.4
4	69.8	69.9	69.8	69.7
5	76.1	76.3	76.1	76.2
6	60.9	60.9	60.9	60.9
Glucose 2 at C3				
1	103.4	103.8	103.4	103.8
2	74.9	75.2	74.9	75.0
3	76.0	76.0	76.0	75.7
4	69.8	69.8	69.8	69.6
5	76.7	76.7	76.7	76.7
6	60.9	60.7	60.9	60.7

^1H and ^{13}C NMR spectra assignments with data in the literature cited suggest that the reference ginsenosides isolated and analyzed by our laboratory are identical. IR spectra were in accordance with the structure of (20S)- and (20R)-ginsenoside Rg3 and corresponded to the reference spectra. Other IR wavenumbers were not significant to the structure (Table 3). Fragmentation ion of m/z 807.6 in positive FAB/MS mode of (20S)- and (20R)-ginsenoside Rg3 revealed $[\text{M}+\text{Na}]^+$ and that of m/z 783.5 in negative mode peak indicated $[\text{M}-\text{H}]^-$. Fragmentation ions of m/z 783.5, 621.4, and 459.3 demonstrated $[\text{M}-\text{H}]^-$, $[\text{M}-\text{H}-\text{C}_6\text{H}_{10}\text{O}_5]^-$, and $[\text{M}-\text{H}-\text{C}_6\text{H}_{10}\text{O}_5-\text{C}_6\text{H}_{10}\text{O}_5]^-$, respectively. The UV spectra of (20S)- and (20R)-ginsenoside Rg3 showed no characteristic absorption patterns. The two compounds demonstrated end absorption with absorption maximum at the vicinity of 203 nm. Identification also took place within the framework of the HPLC (Hewlett Packard) analyses with a photodiode array

detector at 203 nm, demonstrating the corresponding UV spectra for those peaks of (20S)- and (20R)-ginsenoside Rg3. There was absolutely no difference in UV spectra between the two compounds. Based on those spectroscopic data, we could identify authenticity and chemical structures of the two ginsenosides (Fig. 1).

Purity of isolated (20S)- and (20R)-ginsenoside Rg3

Purity was determined by analyzing melting point, elemental composition, and HPLC purity in 3 different conditions, water and residual solvent content, optical rotation, and TLC. The melting points of the (20S)- and (20R)-ginsenoside Rg3 were determined to be 295.47°C and 248.55°C, respectively (Table 4). These values correspond with the data in the literature [8], indicating a valid temperature range between 293°C to 303°C, which certifies the purity of the (20S)- and (20R)-ginsenoside Rg3. Elemental analysis was tested in triplicate, and the result

Table 3. Spectrophotometric data for the isolated (20*S*)- and (20*R*)-ginsenoside Rg3

Parameter	(20 <i>S</i>)-ginsenoside Rg3		(20 <i>R</i>)-ginsenoside Rg3	
	Isolated	Reference [8]	Isolated	Reference [8]
IR	3413, 2947, 1636, 1385, 1076	3400, 2930, 1635, 1390, 1080	3413, 2947, 1636, 1385, 1076	3400, 2930, 1635, 1390, 1080
UV	203	203	203	203
FAB/MS	[M+Na] ⁺ 807.6	807.4	[M+Na] ⁺ 807.6	807.4
	[M-H] ⁻ 783.8	783.5	[M-H] ⁻ 783.8	783.5

IR, infrared spectroscopy; FAB/MS, fast atom bombardment mass spectrometry.

Table 4. Major Purity data for (20*S*)- and (20*R*)-ginsenoside Rg3

	(20 <i>S</i>)-ginsenoside Rg3		(20 <i>R</i>)-ginsenoside Rg3	
	Isolated	Reference	Isolated	Reference
Melting point	295.47°C	293°C-303°C [8]	248.55°C	236°C-238°C
Optical rotation	+0.382 (c, 0.9 in DMSO)	+8.5, +13.2 [10, 11]	-0.88 (c, 1.0 in DMSO)	+8.5 [12,13]
Water content		0.534%		0.920%
TLC (R _f)		0.48 ¹⁾		0.48 ¹⁾
HPLC (rt min)	Method 1	55.620		56.812
	Method 2	59.603		60.449
	Method 3	18.920		18.920

rt, retention time.

¹⁾ Silica gel 60 F₂₅₄, CHCl₃:MeOH:H₂O/9:3:1, lower phase.

Fig. 1. Chemical structures of (20*S*)-ginsenosides Rg3 (A) and (20*R*)-ginsenosides Rg3 (B).

was expressed as a mean value. The elemental composition of (20*S*)- and (20*R*)-ginsenoside Rg3 was 63.28% carbon, 9.64% hydrogen, and 24.31% oxygen. These values coincided with the theoretical values: C₄₂H₇₂O₁₃; 42×12.011 (64.26%)+72×1.0079 (9.24%)+13×15.999 (24.31%).

The optical rotation of the isolated (20*S*)-ginsenoside Rg3 was +0.382 (c, 0.9 in DMSO), and there was a slight difference between the value obtained with our isolate and the reference value. The optical rotation of the isolated (20*R*)-ginsenoside Rg3 was -0.88 (c, 0.9 in DMSO), and there was a slight difference between the value ob-

tained with our isolate and the reference value (Table 4).

The 1st HPLC (reverse phase, aqueous CH₃CN) fingerprint for the (20*S*)-ginsenoside Rg3 showed no peak derived from other organic contaminants or ginsenosides except the peak of (20*S*)-ginsenoside Rg3. The peak purity was also determined by means of spectral analysis. The calculated peak purity value (purity angle, 10.070; 0.400) was much lower than the threshold (16.412, 0.457), which means that the peak can be regarded as being spectrally homogeneous. The 2nd HPLC (normal phase, aqueous CH₃CN) fingerprint demonstrated the same result as that in the first HPLC condition. The peak

purity was also determined by means of spectral analysis. The calculated peak purity value (purity angle, 10.070; 0.400) is lower than the threshold (16.412, 0.457), which means that the peak could be regarded as being spectrally homogeneous. The 3rd HPLC fingerprint for ginsenoside Rg3 was exactly same as that in the 1st and 2nd. In addition, the three HPLC fingerprints for ginsenoside Rg3 also demonstrated the same purity properties (Table 4). Residual organic solvents, such as CHCl₃, MeOH and BuOH used in the process of isolation and purification, were not detected by gas chromatography. The water content of (20*S*)- and (20*R*)-ginsenoside Rg3 reference material was determined on a micro-scale by means of a Karl Fisher titration in accordance with the method in European pharmacopoeia. The water content of (20*S*)- and (20*R*)-ginsenoside Rg3 equaled 0.534% and 0.920%, respectively. On a normal phase silica gel TLC plate, the R_f value for (20*S*)- and (20*R*)-ginsenoside Rg3 reference material accounted for 0.48 in the given chromatographic conditions. No other band attributed to contamination of other ginsenosides was detected. The small peaks at approximately 5 min were derived from the methanol solvent used to dissolve the ginsenosides.

Content quantification of isolated (20*S*)- and (20*R*)-ginsenoside Rg3

Content of isolated (20*S*)- and (20*R*)-ginsenoside Rg3 accounted for 100% when measured solely by three different HPLC systems. When the same amount of those ginsenosides was injected 6 times to determine reproducibility, HPLC method 1 exhibited the smallest deviation in peak area.

Mass balance of the isolated (20*S*)- and (20*R*)-ginsenoside Rg3

Mass balance could be calculated after obtaining the water content, content of residual solvent, and HPLC purity in three different systems. Residual solvent was not detected by GC/MS in those two isolated ginsenosides. The HPLC purity of those two compounds was 100%. Therefore, the only impurity we have to take into account when calculating net mass balance was water. The water contents of (20*S*)- and (20*R*)-ginsenoside Rg3 were 0.534% and 0.920%, respectively. Taking these data together, the mass balance for (20*S*)- and (20*R*)-ginsenoside Rg3 amounted to 99.466% and 99.080%, respectively.

DISCUSSION

As previously mentioned, ginseng is classified either

as a medicine or health food supplement depending on the country. Therefore, quality control should be GMP-based, which requires ICH-guidelines conforming to validated analytical procedures in those countries in which ginseng is categorized as a medicine. In addition, validated quantitative analytical procedures should be constructed before initiating pharmacodynamic and clinical studies to certify batch analysis data for the employed samples. Otherwise, the pharmacodynamic and clinical results will not be accepted by the competent authority of the relevant government. Furthermore, validated analytical procedures cannot be fulfilled without the standard reference materials. In a majority of countries, including EU and Korea, ginsenoside Rb1 and Rg1 are accepted as the reference materials for ginseng preparations. In contrast, China requires additional quantitative analysis of Re, and the EU requires qualitative analysis of Rf. In cases of qualitative analysis, a high-purity standard reference material is not required. However, high purity standard reference materials of ginsenoside Rb1 and Rg1 are essential for the registration of ginseng products as a medicine in the EU. Currently, the analytical method for those two compounds recommended by Korean, Chinese, European, and Japanese pharmacopoeia is biased against red ginseng preparations, particularly the extract, because of the relatively lower content of the two agents in red ginseng compared to white ginseng. In manufacturing fresh ginseng into red ginseng products, ginseng roots are exposed to high temperatures for many hours, and the naturally occurring ginsenoside Rb1 and Rg1 are converted to artifact ginsenosides such as Rg3, Rg5, Rh1, and Rh2 during the heating process. Analytical results of ginsenosides led us to set (20*S*)- and (20*R*)-ginsenoside Rg3, which are commercially available in the Korean market, as additional reference materials for ginseng preparations. In this respect, this study's purpose was to isolate high purity (20*S*)- and (20*R*)-ginsenoside Rg3 and develop those ginsenosides as standard reference materials by analyzing the full spectrum of physico-chemical properties of those two agents before establishing a validated analytical procedure.

We used red ginseng water extract to isolate artifact ginsenosides together with naturally occurring ginsenosides. Diaion HP-20 adsorption chromatography was employed for the preparation of crude saponin (CS). As we have reported previously [14], CS prepared by Diaion HP-20 demonstrated higher purity than obtained by conventional butanol partition chromatography. Then, we adopted silica gel flash chromatography for the crude fraction of ginsenosides. In that process we could

roughly divide ginsenosides according to polarity. In the 2nd step of purification, a recrystallization technique was employed until we could obtain a white powder from (20S)- and (20R)-ginsenoside Rg3. Recrystallization technique plays a key role in the isolation and purification procedure for single ginsenosides as is the case with other natural compounds. In general, recrystallization is enough for the isolation of a single compound. However, in the case of ginsenosides, recrystallization does not guarantee its highest purity because in most cases diverse ginsenosides with similar chemical structures crystallize together. HPLC purification procedure is time-, labor-, and money-consuming, however, a preparative HPLC isolation procedure seems essential to obtain highest purity single ginsenosides unless alternative methods are devised. UV spectra of ginsenosides from other sources exhibited absorption pattern wavelengths other than 203 nm, indicating poor purity. The commercially available ginsenosides showed a purity value of 92.5% when determined solely by HPLC method. European pharmacopoeia and Herbal Medicinal Products define that SRM employed for the quality control should be higher than 95% in purity when determined solely by HPLC. Therefore, those commercially available ginsenosides cannot meet the qualification as the SRM. In addition, those commercially available ginsenosides are not provided with the full spectrum of physico-chemical properties as an appendix. (20S)- and (20R)-ginsenoside Rg3 isolated from our laboratory revealed purity of 100% within the limits of HPLC. In addition, we prepared a full spectrum of physico-chemical data for (20S)- and (20R)-ginsenoside Rg3 as the SRM. Therefore, those ginsenosides can be employed as the SRM in GMP-based quality analysis.

Optical rotation for (20S)- and (20R)-ginsenoside Rg3 was analyzed in DMSO, pyridine and MeOH due to differences in solubility of the epimers. In addition, optical rotation values available from data base were found to be wrong. In the data base, optical rotation for (20R)-ginsenoside Rg3 (c, 1.0 in MeOH) was +8.5. Actually, (20R)-ginsenoside Rg3 is not soluble in MeOH at that concentration. We found the source of the value of +8.5 by searching the original reference. They proved the stereo-structure of (20S)-ginsenoside Rg3 by an organic synthesis method and reported the optical rotation for the compound as +8.5 (c, 1.0 in MeOH). Our data for structure determination confirmed the stereochemistry of the two epimers. Although values of optical rotation for those epimers vary greatly depending upon the solvent, absolute values of plus or minus were not changed in the

three organic solvents used in this experiment. The gap between +8.5 (value from reference) and +11.25 could be attributed to differences in sample purity and mechanical deviation. We are confident in the purity of the compounds that we isolated. Other spectrometric and purity data demonstrated no significant differences between ours and the reference values.

Recently, certified reference materials (CRM) of ginsenoside Rb1 and Rg1 were developed by the Korea Ginseng Corporation (Daejeon, Korea) in collaboration with the Korea Standard Research Institute (Daejeon, Korea). For a natural product, a CRM long term (3 yr) study period is required in close collaboration with qualified national standard institutes and traceability, stability, ash content, etc. should be investigated. Furthermore approximately 30 g of high purity single ginsenosides are required to establish the compound as CRM. It would be extremely time-, labor-, and money-consuming to obtain 30 g of natural reference material with 100% HPLC purity. The samples for spectrometric measurement can be recovered, but the samples for the determination of ash content and elemental analysis are not. Approximately 10 g of high purity ginsenosides would be burned to investigate ash content. Therefore, it may not be cost-effective to do quality analysis with CRM ginsenosides. Fortunately, reference materials for quality control analysis are not severely defined. According to Gaedcke et al. [6], GMP-based reference materials for quality control analysis general characteristics, identity, purity, and quantitation should be verified. In addition, the reference material should show HPLC purity higher than 95%.

Taking all these circumstances into consideration, the (20S)- and (20R)-ginsenoside Rg3 standard reference materials isolated and documented by our research group meet all the requirements for a standard reference material for ICH-conforming GMP-based quality control. Validated analytical procedures established with those two standard reference materials together with (20S)- and (20R)-ginsenoside Rg3 will be presented elsewhere.

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