

Characterizing a Full Spectrum of Physico-Chemical Properties of Ginsenosides Rb₁ and Rg₁ to Be Proposed as Standard Reference Materials

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Good manufacturing practice (GMP)-based quality control is an integral component of the common technical document, a formal documentation process for applying a marketing authorization holder to those countries where ginseng is classified as a medicine. In addition, authentication of the physico-chemical properties of ginsenoside reference materials, and qualitative and quantitative batch analytical data based on validated analytical procedures are prerequisites for certifying GMP. Therefore, the aim of this study was to propose an authentication process for isolated ginsenosides Rb₁ and Rg₁ as reference materials (RM) and for these compounds to be designated as RMs for ginseng preparations throughout the world. Ginsenoside Rb₁ and Rg₁ 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 the isolated ginsenosides was made according to the method proposed by Gaedcke and Steinhoff. The ginsenosides were subjected to analyses of their general characteristics, identification, purity, content quantitation, and mass balance tests. The isolated ginsenosides were proven to be a single compound when analyzed by three different HPLC systems. Also, the water content was found to be 0.940% for Rb₁ and 0.485% for Rg₁, meaning that the net mass balance for ginsenoside Rb₁ and Rg₁ were 99.060% and 99.515%, respectively. From these results, we could assess and propose a full spectrum of physico-chemical properties for the ginsenosides Rb₁ and Rg₁ as standard reference materials for GMP-based quality control.

Keywords: *Panax ginseng*, Ginsenoside Rb₁, Ginsenoside Rg₁, Physico-chemical property, Standard reference material, Documentation

INTRODUCTION

Panax ginseng has been used as a traditional medicine for treating various disorders in East Asian countries for more than two thousand years. Modern multi-disciplinary approaches have enabled us to describe a wide spectrum of pharmacological effects of ginseng including anti-fatigue, immunomodulation, facilitation of blood circulation, spermatogenesis stimulation, and anti-stress effects

to name just a few of many uses for ginseng [1]. The active ingredients of red and white *P. ginseng* are known to be saponin (ginsenosides) and certain polysaccharides and polyacetylenes [2,3]. However, the ginsenosides are regarded as the major active ingredient of ginseng among these bioactive agents and, therefore, are employed as reference materials for quality control purposes. A total

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of 38 ginsenosides have been isolated and characterized from the ginseng plant [4].

Ginseng is classified as an herbal medicine or a health food supplement depending on the countries. The United States and Korea categorize ginseng as a health food supplement but the World Health Organization (WHO), the European Union (EU), several Middle Eastern countries, and India classify ginseng as a medicine. To market ginseng products in the EU, a marketing authorization is required. Documentation of the common technical document (CTD) is an essential process in applying for the marketing authorization holder (MAH). Good manufacturing practice (GMP)-based quality control is an integral part of CTD and the authentication of the physico-chemical properties of the ginsenoside reference materials (RM), and qualitative and quantitative batch analytical data based on validated analytical procedures are a prerequisite to receive GMP certification. Therefore, obtaining GMP certification is not possible without authenticating a full spectrum of the physico-chemical properties of the reference material. In addition, it is obligatory that every quality analysis method be validated according to guidelines issued by the International Conference on Harmonization (ICH) [5]. Korea, Japan, and European countries require quantitative and analytical HPLC data for ginsenosides Rb₁ and Rg₁, the representative reference materials for protopanaxadiol and protopanaxatriol ginsenosides, respectively. However, a method for authenticating ginsenoside RM is not available from the monographs of WHO's selected medicinal plants, and Japanese and Chinese pharmacopoeias. Therefore, CTD- and ICH guideline-based application is not strictly required to obtain an MAH in Japan and China. In fact, the quality control regulation for ginseng preparations is less stringent in many East Asian countries, even though ginseng is well established as a traditional medicine. However, the EU requires a medicinal level of quality assurance data for ginseng preparations. In addition, regulation of quality control is becoming stricter in China and Korea because of the prevalence of product adulteration and low-quality herbal medicinal products. Thus, our aim was to propose an authentication process for isolated ginsenosides Rb₁ and Rg₁ to be used as standardized reference materials.

Germany has well-established scientific and regulatory protocols for the development, quality assurance, and marketing authorization of ginseng and detailed authenticity documentation methods for RM can be found in a textbook [6] written by German experts in drug regulatory affairs (DRA). Thus far, we have isolated 13 ginsenosides, including ginsenosides Rb₁ and Rg₁, in pure form

by employing adsorption chromatography, silica gel flash chromatography, preparative HPLC and recrystallization. Methods for documenting the authenticity of the isolated ginsenosides Rb₁ and Rg₁ RM will be presented in this article. Authentication 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. Protocols for authenticating ginsenosides Rc, Rd, Re, Rf, (20*S*, 20*R*)-Rg₂, (20*S*, 20*R*)-Rg₃, (20*E*)-ginsenoside F₄, and compound K, and the establishment of validated analytical procedures for those ginsenosides will be presented elsewhere.

MATERIALS AND METHODS

Chemicals

Methanol, acetonitrile and isopropyl alcohol were of HPLC grade and from Burdick & Jackson (Honeywell International Inc., Muskegon, MI, USA). All other chemicals and solvents were of analytical grade, unless otherwise mentioned. The TLC plates were from Merck (HPTLC plate with silica gel 60 F₂₅₄). Reference ginsenosides Rb₁ and Rg₁ were from WaKo (Pure Chemical Industries, Ltd., Osaka, Japan) for comparing the purity and identity of the ginsenosides.

Isolation and purification of ginsenosides

Korean red ginseng extract, a water extract obtained from steamed and dried root of *P. ginseng*, was used to isolate and purify the individual ginsenosides. Red ginseng water extract (10 kg) was dissolved in water to make an approximately 20% to 25% (w/v) solution. The solution was passed through Diaion HP-20 adsorption chromatography (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 absolute methanol. The methanol eluate was then dried with silica gel powder *in vacuo* to obtain a pale brown powder. The powder was loaded onto a glass column (15×100 cm) containing 3.5 L of silica gel and partitioned with a mixture of solvents including acetochloroform (CHCl₃), methanol (MeOH), and water (H₂O) at various ratios (12:3:1, 9:3:1, 7:3:1, 4:3:1; lower phase, 4 bed volumes for each mixture). Each fraction was tested for ginsenoside composition, dried *in vacuo*, and recrystallized in ethanol or water until the appearance of

a white powder. White crystalline amorphous powder was then subject to preparative HPLC (ODS, 20×250 mm YMC-Pack, 203 nm) with aqueous CH₃CN (35% for Rb₁ and 25% for Rg₁) as the mobile phase to obtain the individual ginsenosides. The peak corresponding to each ginsenoside was collected, evaporated to dryness, and crystallized again in an appropriate solvent. Finally, the crystallized ginsenosides were dried in a vacuum dryer for 5 d. The resultant white crystalline powder was stored at -20°C until use.

Determination of physico-chemical properties of isolated ginsenosides

The purified ginsenosides, Rb₁ and Rg₁, were authenticated for qualification as standard reference materials (SRM) for quality control purposes as suggested by Blasius *et al.* [6]. The authenticity of each ginsenoside was certified by determining their general characteristics in terms of color, appearance and solubility. The identity of the ginsenosides was certified by analyzing ¹H NMR, ¹³C NMR, IR, FAB/MS and UV spectra. The identity of these compounds was further supported by 2D NMR: ¹H-¹³C COSY, HMQC, HMBC, and NOSEY. The purity of the compounds was determined by measuring the melting point, elemental composition, water content, residual solvent content, and TLC and HPLC fingerprints obtained by three different systems. Content quantitation of those compounds was achieved by three different HPLC methods. The water content of ginsenosides was determined on micro-scale by means of Karl Fisher Titrator in accordance with the European pharmacopoeia (Ph Eur) method [7]. Residual solvent was analyzed by GC/MS after preparing the sample by the head space method. The UV spectrum was measured at a sample concentration of 0.5 mg/mL of methanol. A total of 2 mg sample was introduced into the element analyzer and measured at a temperature of 1,100°C. Optical rotation was determined with 5 replicates. The 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), FAB/MS (JMS-700 Mstation, JEOL Ltd.), IR (Mattson model Galaxy 7020A, Ge-coated KBr pellet, 3.0 mg/g), and UV (Jasco V-530; Jasco, Tokyo, Japan) spectrometers were used for the determination of identity. Melting point analyzer (Man-

stead/Electrothermal IA9100; Barnstead, Dubuque, IA, USA), element analyzer (Flash EA1112 series; CE Lantech, 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 for the determination of purity and content: 1) Hewlett-Packard (Wilmington, DE, USA) equipped with a G1313A automatic sample injector, a vacuum degasser unit, a G1311A quatpump and a G1315A photo diode array detector; 2) Waters (Taunton, MA, USA) equipped with a 2707 automatic sample injector, 1525 binary HPLC pump and Alltech Model ELSD 2000 detector.

Preparation of standard solution

Ten mg of ginsenoside Rb₁ or Rg₁ was dissolved in 10 mL of HPLC grade methanol and diluted until the final concentration of those compounds was 62.5 µg/mL. The diluted standard solution was kept at -20°C until use and was allowed to adjust to room temperature for at least 30 min prior to use.

Conditions for HPLC analysis

HPLC analysis of ginsenosides for content determination was performed under three different conditions: 1) Discovery HS C18 material (Supelco, 250×4.6 mm, 5 µm) 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: eluted with A:B (20:80) for 20 min, linear gradient eluted from A:B (20:80) to (35:65) from 20 to 40 min, linear gradient eluted with A:B (35:65) to (45:55) from 40 to 52 min, linear gradient eluted with A:B (45:55) to (70:30) from 52 to 62 min, linear gradient eluted with 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 material (Waters, 250×4.6 mm, 5 µm) 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: eluted with A:B (20:80) for 20 min, linear gradient eluted with A:B (20:80) to (35:65) from 20 to 40 min, linear gradient eluted with A:B (35:65) to (45:55) from 40 to 52 min, linear gradient eluted with A:B (45:55) to (70:30) from 52 to 62 min, linear gradient eluted with 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) Carbohydrate ES column (250×4.6 mm, 5 μm; Alltech Prevail, 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 eluted from A:B (75:25) to (15:85) for 28 min, linear gradient eluted with A:B (15:85) to (20:80) from 28 to 35 min, linear gradient eluted with A:B (20:80) to (25:75) from 35 to 45 min, linear gradient eluted with A:B (25:75) to (10:90) from 45 to 50 min, linear gradient eluted with 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 mean±SEM. Statistical analyses were performed using Student's *t*-test in Microsoft Excel. Differences were considered significant when *p*<0.05.

RESULTS

Isolation and purification of individual ginsenosides

We isolated five grams of ginsenoside Rb₁ and Rg₁, respectively, via adsorption chromatography, silica gel flash chromatography, recrystallization, and preparative HPLC fractionation. Fractionation with a CHCl₃:MeOH:H₂O solvent at a ratio of 12:3:1 resulted in predominantly (20*E*)-ginsenoside F₄ while the fractionation with a similar solvent at a ratio of 9:3:1 yielded mainly ginsenoside Rg₃. The CHCl₃:MeOH:H₂O solvent at a 7:3:1 ratio

yielded Rg₁, Rg₂ and Rf, while the solvent at a 4:3:1 ratio resulted in Re, Rd, Rc, Rb₂ and Rb₁. The 4:3:1 solvent fraction was more finely sub-fractionated by eluting with solvent mixtures at 6:3:1 (Re and Rd) and 4:3:1 (Rc, Rb₁, Rb₂) ratios. The ginsenoside Rb₁-rich fractions were recrystallized in EtOH and the recrystallized white powder was purified by preparative HPLC (20×250 mm, ODS YMC-Pack, 203 nm) with 35% CH₃CN. A peak corresponding to Rb₁ was collected, dried *in vacuo*, recrystallized in H₂O, and dried in a vacuum dryer for 7 d to obtain a white amorphous powder. Similarly, the Rg₁-rich fraction was subjected to preparative HPLC with 25% CH₃CN, dried *in vacuo*, recrystallized in H₂O and dried in a vacuum dryer for 7 d to obtain a white amorphous powder.

General characteristics of the isolated ginsenosides Rb₁ and Rg₁

Isolated ginsenosides Rb₁ and Rg₁ were subjected to various physico-chemical analyses to identify their general characteristics. As shown in Table 1, ginsenosides Rb₁ and Rg₁ can be physically characterized as an odorless white amorphous powder. Ginsenoside Rb₁ was readily soluble in H₂O, EtOH, MeOH, and CH₃CN, whereas ginsenoside Rg₁ was readily soluble in MeOH and slightly soluble in H₂O, EtOH, and CH₃CN.

Identification of the isolated ginsenoside Rb₁ and Rg₁

Isolated ginsenosides Rb₁ and Rg₁ were subjected to various spectrometric analyses for identification. Interpretation of the ¹H and ¹³C NMR spectra, together with the ¹³C, ¹H-¹H COSY, HMQC, HMBC, and NOESY spectra, allowed us to assign all the ¹H and ¹³C NMR signals (Table 2). Determination of the chemical shift (ppm), multiplicity, and coupling constant of each proton signal was also achieved by the interpretation of those NMR spectra. A comparison of the ¹H and ¹³C NMR spectra

Table 1. General characteristics of isolated ginsenosides Rb₁ and Rg₁

| | Ginsenoside Rb ₁ | Ginsenoside Rg ₁ |
|-------------------|---|---|
| Biological source | <i>Panax ginseng</i> Meyer | <i>P. ginseng</i> Meyer |
| CAS registry no. | 41753-43-9 | 22427-39-0 |
| Empirical formula | C ₃₄ H ₉₂ O ₂₃ | C ₄₂ H ₇₂ O ₁₄ |
| Molar mass | 1109.307 g/molar | 801.022 g/molar |
| Accurate mass | 1109.298 g/molar | 800.49 g/molar |
| Appearance | White amorphous powder, solid at room temperature | White amorphous powder, solid at room temperature |
| Solubility (25°C) | Water, anhydrous ethanol and methanol | Water, anhydrous ethanol and methanol |

CAS, Chemical Abstracts Service.

Table 2. NMR spectra assignment for ginsenosides Rb₁ and Rg₁

| Proton at C atom | Ginsenoside Rb ₁ | | | Ginsenoside Rg ₁ | | |
|------------------|-----------------------------|---------------------------------------|------------------------------------|-----------------------------|---------------------------------------|------------------------------------|
| | 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 | 0.71 | t (J=11.5 Hz); 1H | 0.74 | 1.01 | t (J=13.0 Hz); 1H | 0.99 |
| | 1.51 | m; 1H | 1.55 | 1.73 | m; 1H | 1.71 |
| 2 | 2.17 | m; 1H | 2.20 | 1.86 | m; 1H | 1.85 |
| | 1.81 | m; 1H | 1.83 | 1.97 | m; 1H | 1.93 |
| 3 | 3.24 | dd (J=4.0 Hz; 4.0 Hz); 1H | 3.27 | 3.52 | brd (J=8.5 Hz); 1H | 3.51 |
| 5 | 0.64 | d (J=11.5 Hz); 1H | 0.67 | 1.43 | d (J=10.5 Hz); 1H | 1.41 |
| | 1.44 | m; 1H | 1.48 | 4.43 | m; 1H | 4.41 |
| 6 | 1.34 | m; 1H | 1.34 | - | - | - |
| | 1.16 | m; 1H | 1.20 | 1.94 | m; 1H | 1.93 |
| 7 | 1.44 | m; 1H | 1.48 | 2.51 | m; 1H | 2.47 |
| | 1.34 | m; 1H | 1.36 | 1.53 | m; 1H | 1.52 |
| 9 | 1.95 | m; 1H | 1.97 | 2.08 | m; 1H | 2.07 |
| | 1.51 | m; 1H | 1.52 | 1.53 | m; 1H | 1.51 |
| 12 | 4.14 | m; 1H | 4.17 | 4.13 | m; 1H | 4.12 |
| | 1.99 | m; 1H | 2.00 | 1.99 | m; 1H | 1.98 |
| 15 | 0.97 | m; 1H | 1.00 | 1.07 | m; 1H | 1.07 |
| | 1.53 | m; 1H | 1.57 | 1.62 | m; 1H | 1.64 |
| 16 | 1.81 | m; 1H | 1.84 | 1.75 | m; 1H | 1.74 |
| | 1.34 | m; 1H | 1.36 | 1.29 | m; 1H | 1.28 |
| 17 | 2.57 | m; 1H | 2.60 | 2.49 | m; 1H | 2.49 |
| 18 | 0.94 | S; 3H | 0.97 | 1.17 | S; 3H | 1.16 |
| 19 | 0.79 | S; 3H | 0.82 | 1.04 | S; 3H | 1.03 |
| 21 | 1.63 | S; 3H | 1.66 | 1.60 | S; 3H | 1.59 |
| 22 | 2.37 | m; 1H | 2.39 | 2.39 | m; 1H | 2.38 |
| | 1.81 | m; 1H | 1.84 | 1.81 | m; 1H | 1.80 |
| 23 | 2.57 | m; 1H | 2.59 | 2.49 | m; 1H | 2.48 |
| | 2.37 | m; 1H | 2.38 | 2.23 | m; 1H | 2.23 |
| 24 | 5.29 | m; 1H | 5.32 | 5.26 | m; 1H | 5.26 |
| 26 | 1.58 | S; 1H | 1.61 | 1.60 | S; 3H | 1.60 |
| 27 | 1.63 | S; 1H | 1.66 | 1.60 | S; 3H | 1.60 |
| 28 | 1.26 | S; 1H | 1.29 | 2.08 | S; 3H | 2.06 |
| 29 | 1.08 | S; 1H | 1.11 | 1.60 | S; 3H | 1.60 |
| 30 | 0.94 | S; 1H | 0.97 | 0.82 | S; 3H | 0.81 |
| Glucose 1 at C3 | | | Glucose at C6 (6-O-Glc) | | | |
| 1 | 4.89 | d (J=7.5 Hz); 1H | 4.92 | 5.04 | d (J=8.0 Hz); 1H | 4.98 |
| 2-6 | 3.87-4.72 | m; 5H | 3.92-4.56 | 3.95-4.52 | m; 5H | 3.94-4.98 |
| Glucose 2 at C3 | | | Glucose at C20 (20-O-Glc) | | | |
| 1 | 5.35 | d (J=7.5 Hz); 1H | 5.40 | 5.18 | d (J=8.0 Hz); 1H | 5.12 |
| 2-6 | 3.87-4.72 | m; 5H | 3.93-4.50 | 3.82-4.49 | m; 5H | 3.88-4.45 |
| Glucose 1 at C20 | | | | | | |
| 1 | 5.11 | d (J=7.5 Hz); 1H | 5.13 | | | |
| 2-6 | 3.87-4.72 | m; 5H | 3.94-4.73 | | | |
| Glucose 2 at C20 | | | | | | |
| 1 | 5.07 | d (J=7.5 Hz); 1H | 5.10 | | | |
| 2-6 | 3.87-4.72 | m; 5H | 3.93-4.52 | | | |

| C atom | Ginsenoside Rb ₁ (ppm) | | Ginsenoside Rg ₁ (ppm) | |
|------------------|-----------------------------------|------------------------------|-----------------------------------|------------------------------|
| | Chemical shift | Reference chemical shift [8] | Chemical shift | Reference chemical shift [9] |
| 1 | 39.2 | 39.1 | 39.4 | 39.5 |
| 2 | 26.6 | 26.6 | 28.0 | 28.0 |
| 3 | 88.9 | 89.3 | 78.7 | 78.8 |
| 4 | 39.2 | 39.6 | 40.4 | 40.4 |
| 5 | 56.4 | 56.3 | 61.4 | 61.5 |
| 6 | 18.4 | 18.6 | 80.2 | 80.2 |
| 7 | 35.1 | 35.1 | 45.1 | 45.2 |
| 8 | 39.6 | 39.9 | 41.1 | 41.2 |
| 9 | 50.2 | 50.1 | 50.0 | 50.1 |
| 10 | 36.9 | 36.8 | 39.7 | 39.8 |
| 11 | 30.7 | 30.8 | 31.0 | 31.0 |
| 12 | 79.2 | 70.1 | 70.2 | 70.4 |
| 13 | 49.6 | 49.3 | 49.2 | 49.2 |
| 14 | 51.6 | 51.3 | 51.4 | 51.5 |
| 15 | 30.8 | 30.8 | 30.7 | 30.8 |
| 16 | 26.7 | 26.6 | 26.6 | 26.7 |
| 17 | 51.4 | 51.6 | 51.6 | 51.8 |
| 18 | 16.0 | 16.2 | 17.6 | 17.6 |
| 19 | 16.2 | 15.9 | 17.6 | 17.6 |
| 20 | 83.4 | 83.5 | 83.3 | 83.5 |
| 21 | 22.4 | 22.6 | 22.3 | 22.5 |
| 22 | 36.2 | 36.1 | 36.1 | 36.2 |
| 23 | 23.2 | 23.1 | 23.2 | 23.4 |
| 24 | 125.9 | 125.8 | 126.0 | 126.0 |
| 25 | 131.0 | 131.0 | 130.9 | 131.0 |
| 26 | 25.8 | 25.8 | 25.8 | 25.9 |
| 27 | 17.9 | 17.9 | 17.8 | 17.9 |
| 28 | 28.1 | 28.0 | 31.8 | 31.8 |
| 29 | 16.6 | 16.5 | 16.4 | 16.5 |
| 30 | 17.4 | 17.3 | 17.2 | 17.2 |
| Glucose 1 at C3 | | | Glucose at C6 (6-O-Glc) | |
| 1 | 105.1 | 105.0 | | |
| 2-6 | 62.7-83.5 | 62.6-82.9 | | |
| Glucose 2 at C3 | | | Glucose at C20 (20-O-Glc) | |
| 1 | 106.1 | 105.6 | | |
| 2-6 | 62.7-78.4 | 62.6-78.8 | | |
| Glucose 1 at C20 | | | | |
| 1 | 98.0 | 97.9 | | |
| 2-6 | 71.6-78.1 | 571.5-78.0 | | |
| Glucose 2 at C20 | | | | |
| 1 | 105.3 | 105.0 | | |
| 2-6 | 62.7-78.1 | 62.8-78.0 | | |

Table 3. Other spectrometric identity data for the isolated ginsenosides Rb₁ and Rg₁

| Parameter | Ginsenoside Rb ₁ | | Ginsenoside Rg ₁ | |
|-----------|------------------------------|------------------------------|------------------------------|------------------------------|
| | Isolated | Reference | Isolated | Reference |
| IR | 3419, 2943, 1644, 1387, 1078 | 3400, 2930, 1635, 1390, 1080 | 3424, 2930, 1641, 1456, 1076 | 3400, 2930, 1635, 1390, 1080 |
| UV | 203 | 203 | 203 | 203 |
| FAB/MS | [M+Na] ⁺ 1131.6 | 1131.6 | [M+Na] ⁺ 823.48 | 823 |
| | [M-H] ⁻ 1107.6 | 1107.6 | [M-H] ⁻ 801.53 | - |

with data from the literature suggests that the reference ginsenosides isolated and analyzed by our laboratory are identical. IR spectra were in accordance with the structure of ginsenosides Rb₁ and Rg₁ and corresponded to the reference spectra. Other IR wavenumbers were not significant to the structure (Table 3). Fragmentation ion of *m/z* 1131 in positive FAB/MS mode of ginsenoside Rb₁ revealed [M+Na]⁺, and that of *m/z* 1107.6 in negative mode peak indicated [M-H]⁻. Fragmentation ions of *m/z* 1107.6, 945.5, 783.4 and 765.4 demonstrated [M-H]⁻, [M-H-C₆H₁₀O₅]⁻, [M-H-C₆H₁₀O₅-C₆H₁₀O₅]⁻, and [C₆H₁₀O₅-H]⁻, respectively. On the other hand, fragmentation ions of *m/z* 823.48 [M+Na]⁺, 801.05 [M-H]⁻, 643.46 [M-H-C₆H₁₀O₅]⁻, 489.39 [M-H-C₆H₁₀O₅-C₆H₁₀O₅]⁻, were observed in the mass spectrum of ginsenoside Rg₁.

The UV spectra of ginsenosides Rb₁ and Rg₁ showed no characteristic absorption patterns with an absorption maximum at the vicinity of 203 nm. Identification of the ginsenosides was also performed by HPLC (Hewlett Packard) analyses with a photodiode array detector at 203 nm, demonstrating the corresponding UV spectra for those peaks of ginsenoside Rb₁ and Rg₁. There was absolutely no difference in the UV spectra of the two compounds. However, UV absorption intensity at 203 nm was higher in Rg₁ than in Rb₁. Based on these spectrometric data, we authenticated and identified the chemical structures of the two ginsenosides (Fig. 1).

Purity of isolated ginsenoside Rb₁ and Rg₁

Purity was determined by analyzing the melting point, elemental composition, HPLC purity under three different conditions, water and residual solvent content, optical rotation, and TLC. The melting points of the ginsenosides Rb₁ and Rg₁ were 197°C and 194°C, respectively (Table 4). These values are in agreement with the data in the literature [8] that suggests a melting temperature range of 197 to 198°C, which certifies the purity of the ginsenoside Rb₁. The melting point of Rg₁ was found to be variable depending on the analyst. However, our data correspond exactly with one of the values reported previously [10]. Elemental analysis was performed in

triplicate and the result was expressed as a mean of the three experiments. The elemental composition for ginsenoside Rb₁ was 58.89% carbon, 8.39% hydrogen, and 33.24% oxygen. These values correlate well with the theoretical elemental composition of Rb₁ of C₅₄H₉₂O₂₃ [54×12.011 (58.46%)+92×1.0079 (8.36%)+23×15.999 (33.17%)]. Similarly, the elemental composition for ginsenoside Rg₁ was 60.28% carbon, 9.14% hydrogen, and 30.31% oxygen. We could not find a reference standard associated with the elemental analysis of Rg₁; however, the measured values were well in accordance with the composition calculated from the empirical formula, C₄₂H₇₂O₁₄ [42×12.011 (60.13%)+72×1.0079 (9.16%)+14×15.999 (30.74%)].

The optical rotation of the isolated ginsenoside Rb₁ was +12.4 (c, 0.9 in MeOH) and there was a slight difference between the values obtained with our isolate and the reference value (Table 4). The optical rotation of the isolated ginsenoside Rg₁ was +19.5 (c, 1.0 in MeOH) and there was absolutely no difference between the value obtained with our isolate and the reference value. However, the optical rotation data by Baek's group was very different from our data [11].

The first HPLC (reverse phase, aqueous CH₃CN) fingerprint for ginsenoside Rb₁ shows no peak derived from other organic contaminants or ginsenosides except for the peak corresponding to ginsenoside Rb₁. The peak purity was also determined by means of spectral analysis. The calculated peak purity value (purity angle=10.070; 0.400) was lower than the threshold (=16.412; 0.457), which means that the peak can be regarded as being spectrally homogeneous. The second HPLC (normal phase, aqueous CH₃CN) fingerprint demonstrated the same result as the first HPLC fingerprint. The peak purity was also determined by means of spectral analysis. The calculated peak purity value (purity factor 999.946) was lower than the threshold (0.036), which means that the peak is spectrally homogeneous. The third HPLC fingerprint for ginsenoside Rb₁ was exactly the same as the first two fingerprints. In addition, the three HPLC fingerprints for ginsenoside Rg₁ also exhibited the same purity (Table 4).

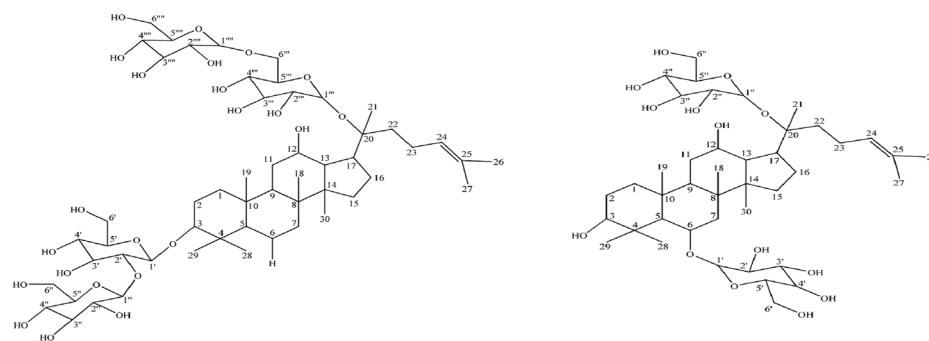


Fig. 1. Chemical structures of ginsenosides Rb₁ and ginsenoside Rg₁.

Table 4. Major purity data for ginsenosides Rb₁ and Rg₁

| | Ginsenoside Rb ₁ | | Ginsenoside Rg ₁ | |
|-----------------------|-----------------------------|--|-----------------------------|--------------------|
| | Isolated | Reference | Isolated | Reference |
| Melting point | 197°C | 197-198°C [8] 171°C [11] | 194°C | 194-196°C [10] |
| Optical rotation | +12.4 (c, 0.9 in MeOH) | +6.49 [11] +13.2 [12] +11.5 [13] | +19.5 (c, 1.0 in MeOH) | +19.5 [13] |
| Water content | | 0.940% | | 0.485% |
| TLC (R _f) | | 0.32 ¹⁾ | | 0.38 ²⁾ |
| HPLC (r.t. min) | Method 1 | 42.5 | 17.9 | |
| | Method 2 | 41.5 | 22.1 | |
| | Method 3 | 43.6 | 16.8 | |

¹⁾Silica gel 60 F₂₅₄; CHCl₃:MeOH:H₂O/65:35:10, lower phase; ²⁾9:3:1, lower phase.

Residual organic solvents such as chloroform, methanol, and butanol used in the process of isolation and purification were not detected by gas chromatography. Actually, recrystallized ginsenoside Rb₁ was lyophilized after being dissolved in H₂O. The water content of ginsenoside Rb₁ reference material was determined on a micro-scale by means of Karl Fisher titration in accordance with the method in Ph Eur. The water content of ginsenoside Rb₁ amounted to 0.940%. On a normal phase silica gel TLC plate, R_f value for ginsenoside Rb₁ reference material was 0.32 and that for ginsenoside Rg₁ was R_f 0.38 under the given chromatographic conditions. No other bands indicative of contamination with the other ginsenosides could be detected. The multiple small peaks that appeared after approximately 5 min was found to be derived from the methanol solvent used to dissolve the ginsenosides.

Content quantitation of isolated ginsenoside Rb₁ and Rg₁

The isolated ginsenosides Rb₁ and Rg₁ accounted for 100% of the total content when quantitated by three different HPLC systems. The HPLC fingerprints for ginsenoside Rb₁ and Rg₁ measured under three different conditions are not presented here due to limited space. When equivalent amounts of the ginsenosides were injected, in 6 separate experiments, to validate the repeatability of the first HPLC fingerprinting method, this method exhibited the least deviation in peak area leading us to build a validated quantitative analytical procedure using HPLC system 1.

Mass balance of the isolated ginsenoside Rb₁ and Rg₁

Mass balance was calculated after obtaining water content, residual solvent content, and HPLC purity

using three different systems. Residual solvent was not detected by GC/MS in the two isolated ginsenosides. HPLC purity of the two compounds was 100%. The only impurity that must be taken into account when calculating net mass balance is water content. The water content for ginsenoside Rb₁ and Rg₁ was 0.940% and 0.485%, respectively. Therefore, the mass balance for ginsenosides Rb₁ and Rg₁ amounted 99.060% and 99.515%, respectively.

DISCUSSION

As mentioned above, ginseng is classified as a medicine or health food supplement depending on the country. Therefore, quality control should be GMP-based and GMP-based quality control requires ICH-guidelines confirming validated analytical procedures in those countries where ginseng is categorized as a medicine. In addition, validated quantitative analytical procedures should be in place 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 authorizing agencies. Moreover, validated analytical procedures cannot be performed without standardized reference materials. In many countries, including the European Union and the Republic of Korea, ginsenosides Rb₁ and Rg₁ are accepted as standardized reference materials for certifying ginseng preparations. China requires additional quantitative analysis of ginsenoside Re, while the EU requires additional qualitative analysis of ginsenoside Rf. If performing a qualitative analysis of the ginsenosides, a highly pure standard reference material is not required. However, high-purity standard reference materials of ginsenosides Rb₁ and Rg₁ are essential for the certification of ginseng products as a medicine in the EU. Therefore, the purpose of this study was to isolate highly pure ginsenosides, especially Rb₁ and Rg₁, and to propose these ginsenosides as standardized reference materials by analyzing a full spectrum of their physico-chemical properties.

The content of ginsenosides Rb₁ and Rg₁ is markedly lower in red ginseng extracts compared to white or fresh ginseng because the two naturally occurring compounds are degraded by heat during processing. However, we used red ginseng 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 reported previously, CS prepared by Diaion HP-20 demonstrated

higher purity than CS obtained by conventional butanol partition extraction [14]. Additionally, we adopted a silica gel flash chromatography method for the crude fraction of ginsenosides. Using this process, we could roughly partition the ginsenosides according to their polarity. In the second step of purification, a recrystallization technique was employed to obtain absolute white powder. Recrystallization plays a key role in the isolation and purification procedure for single ginsenosides as is the case with other natural compounds. Fractions rich in ginsenoside Rb₁ or Rg₁ were recrystallized in ethanol to obtain a white amorphous powder. In general, recrystallization is sufficient for the isolation of a single compound. However, in case of ginsenosides, recrystallization does not guarantee the highest purity because diverse ginsenosides with similar chemical structures can crystallize together. The HPLC-based purification method is time-, labor- and money-consuming; however, this method seems to be essential for obtaining the highest purity single ginsenoside until an alternative method is devised. We included the NMR spectra to provide more detailed information for those who are interested in isolating and purifying the ginsenosides, or for those preparing their ginseng for MAH approval. We also show the UV spectra for ginsenoside Rb₁ and Rg₁ to assist the peer reviewers in comparing purity. The UV spectra of ginsenosides from other sources demonstrate an absorption pattern in other wavelength than 203 nm, thus indicating poor purity. Therefore, the UV spectra data are very important for certifying the purity of the ginsenosides. The commercially available ginsenosides showed 92.5% purity when determined solely by HPLC method. Ph Eur and Herbal Medicinal Products define that SRM employed for the quality control should be greater than 95% pure when determined solely by HPLC. Therefore, those commercially available ginsenosides cannot qualify as a SRM. In addition, the commercially available ginsenosides are not provided with a full spectrum of physico-chemical analyses. Ginsenosides Rb₁ and Rg₁ isolated from our laboratory were 100% pure as determined by HPLC. In addition, we prepared a full spectrum of physico-chemical data for ginsenosides Rb₁ and Rg₁ as the SRM. Therefore, these ginsenosides can be employed as the SRM in GMP-based quality analysis.

According to Cho *et al.* [11], the melting point of ginsenoside Rb₁ was 170°C to 171°C. However, we believe that the melting point of ginsenoside Rb₁ is most probably around 197°C. Another difference between the data presented by Cho *et al.* and our laboratory is optical rotation. The optical rotation of ginsenoside Rb₁ was +6.49

but the value reported by Cho *et al.* was +12.4. We cannot account for these differences, but experimentally, the only difference was concentration (0.5 mg/mL by Cho *et al.* and 0.9 mg/mL in our report) of sample and equipment employed. However, the difference in optical rotation can result from impurities and not from concentration, temperature, or solvent. As we are confident of our purity measurements, the differences in optical rotation might be due to differences in ginsenoside purity. Other spectrometric and purity data demonstrated no significant differences between isolated ginsenosides and the reference materials.

The most recently certified reference materials (CRM) of the ginsenosides Rb₁ and Rg₁ were produced by Korea Ginseng Corporation in collaboration with Korea Research Institute of Standards and Science [15]. To qualify as a natural product CRM, a long term (3 yr) study period is required and the reference material must be validated by a national metrology institute accounting for traceability, stability, ash content, etc. Furthermore, approximately 30 g of highly pure ginsenosides are required to establish the compound as CRM. It would be extremely time-, labor- and money-consuming to obtain 30 g of a 100% pure natural reference material validated by HPLC. The sample 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 ginsenoside should be combusted 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 strictly defined. According to Blasius *et al.* [6], GMP-based reference material for quality control analysis requires the verification of general characteristics, identity, purity, and quantitation. Under these circumstances, the ginsenoside Rb₁ and Rg₁ standard reference materials isolated and documented by us meet all the requirements to be designated as a standard reference material for ICH-conforming GMP-based quality control. A validated analytical procedure established with those two standard reference materials together with (20S & 20R) ginsenosides Rg₃ will be presented elsewhere.

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