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A new validated analytical method for the quality control of red ginseng products

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The main active components of *Panax ginseng* are ginsenosides. Ginsenoside Rb1 and Rg1 are accepted as marker substances for quality control worldwide. The analytical methods currently used to detect these two compounds unfairly penalize steamed and dried (red) *P. ginseng* preparations, because it has a lower content of those ginsenosides than white ginseng. To manufacture red ginseng products from fresh ginseng, the ginseng roots are exposed to high temperatures for many hours. This heating process converts the naturally occurring ginsenoside Rb1 and Rg1 into artifact ginsenosides such as ginsenoside Rg3, Rg5, Rh1, and Rh2, among others. This study highlights the absurdity of the current analytical practice by investigating the time-dependent changes in the crude saponin and the major natural and artifact ginsenosides contents during simmering. The results lead us to recommend (20*S*)- and (20*R*)-ginsenoside Rg3 as new reference materials to complement the current *P. ginseng* preparation reference materials ginsenoside Rb1 and Rg1. An attempt has also been made to establish validated qualitative and quantitative analytical procedures for these four compounds that meet International Conference of Harmonization (ICH) guidelines for specificity, linearity, range, accuracy, precision, detection limit, quantitation limit, robustness and system suitability. Based on these results, we suggest a validated analytical procedure which conforms to ICH guidelines and equally values the contents of ginsenosides in white and red ginseng preparations.

Keywords: *Panax ginseng*, Reference material, Quality control, Quantitative analytical method, Validation

INTRODUCTION

In Oriental countries, *Panax ginseng* has been used as a traditional medicine for various illnesses for more than two thousand years. Accumulated scientific findings indicate that ginseng is beneficial for fatigue, blood circulation, immune dysfunction, carcinogenesis, hepatotoxicity, nephrotoxicity, diabetes, atherosclerosis, sexual dysfunction, and other symptoms and illnesses [1]. *P. ginseng* is classified as either an herbal medicine or a health food supplement depending on the country or continent. The United States categorizes ginseng as a health food supplement but the European community, the Middle East, and India categorize it as an herbal medicine. China and Korea categorize ginseng either as a medicine or a health food supplement depending on how it is administered. Bioassay-guided chemical research has elucidated various active compounds in ginseng such as ginsenosides, polysaccharides, polyacetylenes and peptides [2,3]. Ginsenosides, however, are regarded as the principle active compounds because they are naturally occurring triterpenoids unique to the plants of the ginseng genus. Since the chemical structure of ginsenosides was first determined, 24 different naturally occurring ginsenosides

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have been isolated from *P. ginseng* and their chemical structures have been identified [4,5]. Studies on the pharmacological characteristics of ginsenosides generated by pyrolysis have been performed, and these heat-generated ginsenosides were found to have interesting bioactivities applicable to: anti-inflammation, antitumorigenesis, anticancer, antihypertension, antidiabetes, anti-nociception, anti-stress, and anti-oxidant effects [6].

The European Union and the World Health Organization placed *P. ginseng* in the "Monograph on selected medicinal plants," thereby requiring GMP (good manufacturing practice)-based batch analysis data. In addition, every quality analysis must be validated according to the International Conference of Harmonization (ICH) guidelines [7]. For example, ginsenoside Rb1 and Rg1 are standard reference materials used for quality control and must be characterized to specific identity, purity, content, and mass balance standards. Furthermore, the analytical procedures used on the reference materials must be validated for specificity, linearity, range, accuracy, precision, detection limit, quantitation limit, robustness, and system suitability. It is reasonable to determine the quantity of the two naturally occurring ginsenoside Rb1 and Rg1, known as protopanaxadiol and protopanaxatriol saponins, respectively, for quality control [8]. However, this quality control method unfairly penalizes heat-treated products because ginsenoside Rb1 and Rg1 degrade to artifact ginsenosides during the production of red ginseng products or ginseng extract concentrate. Current regulations therefore are unfairly biased towards white ginseng products and alcohol (EtOH or MeOH) extracts to the detriment of red ginseng preparations. Traditionally, herbal medicine has been prepared by decoction at a low flame for many hours. The analysis of commercially available *P. ginseng* extracts in the Korean market shows low or negligible amounts of the ginsenoside Rb1 or Rg1 and high amounts of (20*S*)-ginsenoside Rg3 and (20*R*)-ginsenoside Rg3. Analytical procedures that encompass both heat-treated and non heat-treated ginseng preparations are therefore needed.

In this study, we determined the ginsenoside Rb1 and Rg1 content of commercially available ginseng extract. We also measured the crude saponin content, the concentration change of the major naturally occurring ginsenoside Rb1 or Rg1 and the generation of artifact ginsenosides as a function of time at 80°C. Based on these results, we propose a more valid and ICH-guideline compliant quality analytical procedure for *P. ginseng* preparations.

MATERIALS AND METHODS

Chemicals

HPLC grade methanol, acetonitrile and isopropyl alcohol were obtained from Burdick & Jackson (Honeywell International, Muskegon, MI, USA). All other chemicals were of analytical grade, unless otherwise noted. The TLC plate was purchased from Merck (HPTLC plate with silica gel 60 F254; Frankfurt, Germany). Ginsenoside Rb1, Rg1, (20*S*)-ginsenoside Rg3 and (20*R*)-ginsenoside Rg3, used as standard reference material, were prepared from steamed and dried *P. ginseng* (red ginseng) root. Crude red ginseng saponin (CS) obtained by adsorption chromatography with Diaion HP-20 columns was further subjected to silica gel flash chromatography using a $CHCl_3$-MeOH-H_2O (12:3:1, 9:3:1, 7:3:1, 4:3:1) solvent system [8]. Each fraction was re-crystallized in ethanol or water, and the resulting crystallized white amorphous powder was subjected to preparative HPLC (20×250 mm, ODS YMC-Pack, 203 nm) to obtain single ginsenosides. The physico-chemical properties of the four ginsenosides were analyzed as recommended by Gaedcke and Steinfoff [9] to insure that they qualified as a standard reference material for herbal medicine. The general characteristics were determined, including identity, purity, content quantitation and mass balance. The four reference ginsenosides employed for this validation study were 100% pure as determined by HPLC (ODS, 4.6×200 mm, 203 nm, aqueous CH_3CN gradient). Documentation of the physico-chemical properties of these four compounds has been published [8]. Devil's claw root (*Harpagophytum procumbens*) was purchased from an herbal medicine shop in the Kyungdong Botanical Market of Seoul, Korea.

Analytical instruments

Two HPLC systems were employed to determine purity and content: 1) A Hewlett-Packard HPLC (Wilmington, DE, USA) equipped with an automatic sample injector (G1313A), a vacuum degasser unit, a quaternary pump (G1311A) and a photo diode array detector (G1315A); 2) a Waters HPLC (Taunton, MA, USA) equipped with an automatic sample injector (2707), a binary HPLC pump (1525) and an Alltech Model ELSD 2000 detector. A wet-type red ginseng maker (GF-1601; Hongwon, Seoul, Korea) was used to simmer the red ginseng root.

Analysis of the crude saponin and ginsenosides content while simmering

A glass vessel containing 250 g of fresh ginseng root

and 2,500 mL of water was simmered (approximately 80°C) for 72 h. Aliquots (sample A, 100 mL) of the simmering ginseng root were removed at 3, 6, 12, 24, 36, 48, 60, and 72 h, and the samples were subjected to gel adsorption chromatography (MCI; Supelco, Bellefonte, PA, USA) or butanol partition to determine the crude saponin content. The crude saponin fraction was dissolved to a concentration of 20 mg/mL, and a HPLC quantitative analysis of the 11 ginsenosides was performed. The content of individual ginsenosides was expressed in mg% (w/v). This experiment was independently performed 3 times, and the data were expressed as mean±SEM. The ginsenosides content of commercially available *P. ginseng* extracts was analyzed using the same method.

Preparation of the crude ginseng saponin fraction

The CS fraction was prepared by butanol partition and MCI gel adsorption chromatography. First, 50 mL of 10% (w/v) ginseng extract was prepared (sample A). An aliquot of 25 mL was used in butanol partition and the remaining 25 mL was used in MCI gel column chromatography. For the butanol partition, the solution was placed in a 50 mL Falcon tube with 20 mL of *n*-butanol. The tube was then vortexed vigorously for 30 s and centrifuged at 3,000 rpm for 15 min. The lower aqueous layer was removed carefully with a Pasteur pipette and subjected to a second and third partition using the same process. The butanol layer was then washed with 25 mL of distilled water to eliminate water-soluble impurities. The washed butanol layer was dried *in vacuo*, producing a light brown powder. A separate 25 mL of the solution was passed through a glass column (20.0×4.5 mm) containing 5 mL of MCI gel. The resin was then washed with 4 bed-volumes of distilled water, and the CS was obtained through elution with 20 mL of absolute methanol. The methanol eluted was then dried to a pale brown powder. The methanol extract of devil's claw root (*H. procumbens*) was prepared using the same method as in the preparation of the lipophilic ingredients, and the resulting lipophilic fraction was used as the matrix for the accuracy (recovery) test.

Preparation of standard solution

Ten milligrams of each ginsenoside Rb1, Rg1, (20*S*)-ginsenoside Rg3, and (20*R*)-ginsenoside Rg3 were dissolved in 5 mL of HPLC grade methanol, and a two-fold dilution was performed to a final concentration of 62.5 ng/mL. This solution was used as the reference. The diluted standard solution was maintained at -20°C until 30 min before use and placed at room temperature to thermally equilibrate the solution.

Conditions for HPLC analysis

Two HPLC systems were employed to determine purity and content: The HPLC analysis of the ginsenosides for preliminary validation tests was performed using 3 different HPLC configurations. The 1st HPLC setup: Discovery HS C18 material (Supelco, Bellefonte, PA, USA; 250×4.6 mm, 5 μm) at room temperature (25°C) with a sample injection volume of 20 μL and a mobile phase consisting of acetonitrile (solvent A) and water (solvent B). The gradient program used was: A:B (20:80) for 20 min, a linear gradient from A:B (20:80) to (35:65) from 20 to 40 min, a linear gradient from A:B (35:65) to (45:55) from 40 to 52 min, a linear gradient from A:B (45:55) to (70:30) from 52 to 62 min, a linear gradient from A:B (70:30) to (100:0) from 62 to 80 min and finally equilibration 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 at 203 nm.

The 2nd HPLC configuration: Symmetry C18 material (Waters; 250×4.6 mm, 5 μm) at room temperature (25°C) 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 gradient program was employed: A:B (20:80) for 20 min, a linear gradient from A:B (20:80) to (35:65) from 20 to 40 min, a linear gradient from A:B (35:65) to (45:55) from 40 to 52 min, a linear gradient from A:B (45:55) to (70:30) from 52 to 62 min, a linear gradient from A:B (70:30) to (100:0) from 62 to 80 min and finally equilibration 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 at 203 nm.

The 3rd HPLC configuration: Carbohydrate ES column (Alltech Prevail, Waukegan, IL, USA; 50×4.6 mm, 5 μm) at room temperature (25°C) with a sample injection volume of 10 μL. The mobile phase consisted of acetonitrile/H_2O/isopropyl alcohol (80/5/15, solvent A) and acetonitrile/H_2O/isopropyl alcohol (80/15/15, solvent B). The following gradient program was employed: a linear gradient from A:B (75:25) to (15:85) for 28 min, a linear gradient from A:B (15:85) to (20:80) from 28 to 35 min, a linear gradient from A:B (20:80) to (25:75) from 35 to 45 min, a linear gradient from A:B (25:75) to (10:90) from 45 to 50 min, a linear gradient from A:B (10:90) to (0:100) from 50 to 55 min and finally equilibration 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 an ELSD (gain 8, gas flow rate 2.0 SLPM drift tube temperature 90°C).

Validation of the analytical procedure for ginsenosides

The 1st HPLC configuration (reverse phase, gradient aqueous CH_3CN) was employed for the validation of the ginsenoside quantitative analytical procedure. The HPLC quantitative analytical procedure for the four reference ginsenosides followed ICH guidelines [7] and Japanese pharmacopoeia [10]. The specificity, linearity, range, accuracy, precision, detection limit, quantitation limit, robustness and system suitability were all briefly determined. The specificity of the employed analytical procedure was investigated by determining the resolution of the four ginsenosides in the HPLC fingerprint and the presence or absence of impurities in the corresponding peaks using LC/MS. The linear relationship was evaluated for the full range of the analytical procedure by visual inspection of the signals as a function of analyzed concentration. Five sample concentrations were employed to establish linearity, and the correlation coefficient, y-intercept, and the slope of the regression line were measured to quantify the same. The range was derived from both the content of the four naturally occurring ginsenosides in the red ginseng sample and the linearity data. The accuracy was assessed using devil's claw root (*H. procumbens*) powder spiked with 3 known amounts of the reference materials after the precision, linearity and specificity had been established. Nine samples at 3 concentration levels (3 concentrations with 3 replicates each) were assessed to test the recovery. The precision was determined using repeatability. Nine samples at the three given concentrations were assessed for the repeatability measurement. The relative standard deviation of the peak areas in the HPLC fingerprint was calculated. The detection and quantitation limits were obtained visually. The robustness was determined using different columns from different suppliers at different temperatures and flow rates. The system suitability was determined by measuring the system's performance and reproducibility as described in the Japanese pharmacopoeia [10] and was determined by measuring system performance and repeatability. For the system performance study, 10 mg of the ginsenoside Rg1 and Re, ginsenoside Rb1 and Rc or (20*S*)-ginsenoside Rg3, (20*R*)-ginsenoside Rg3 isomer standard reference materials were dissolved separately in 100 mL of methanol. When the HPLC procedure was run with 10 μL of this solution in the 1st HPLC operating configuration, the resolution between the two peaks in each mixture was calculated. System repeatability was determined with 10 μL of the standard solution containing the four standard reference ginsenosides. The relative standard deviation of the peak area in the HPLC fingerprint was determined after six repetitions with the 10 μL of aliquot.

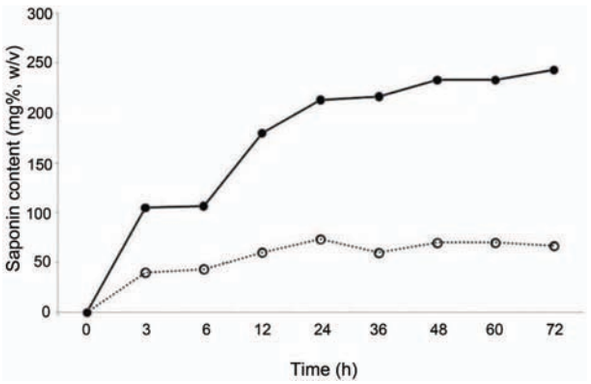

Fig. 1. Change in crude saponin content during simmering at 80°C (mg%, w/v). ● Crude saponin prepared by butanol partition. ○ Crude saponin prepared by MCI gel adsorption chromatography.

Statistical analysis

Data were presented as mean±SEM. Statistical analyses were performed using Student's *t*-test in Excel (Microsoft office professional plus 2010; Microsoft, Seattle, WA, USA).

RESULTS

The change in crude saponin content during simmering

The CS fraction content in the aliquots, as determined by MCI gel adsorption column chromatography, increased steadily for the first 24 h of simmering and remained constant afterwards (Fig. 1). However, the content of the CS fraction prepared by butanol partition increased for 72 h. In addition, there are marked differences in the CS content depending on the CS preparation method. The butanol partition CS content was approximately 3 times higher than that prepared by MCI gel adsorption chromatography.

The change in the marker ginsenoside content during simmering

Before simmering, the content of ginsenosides Rb1, Rg1, (20*S*)-Rg3 and (20*R*)-Rg3 in fresh ginseng root was 1.51 mg% (w/w), 1.27 mg% (w/w),0.00 mg% and 0.00 mg% (w/w), respectively. As Figs. 2 and 3 show, the ginsenosides Rb1 and Rg1 peaks were observed until 3 h but disappeared rapidly thereafter, and no more was detected after 24 h. However, content of (20*S*)-ginsenoside Rg3 and (20*R*)-ginsenoside Rg3 increased markedly until either 48 or 60 h, depending on the compound.

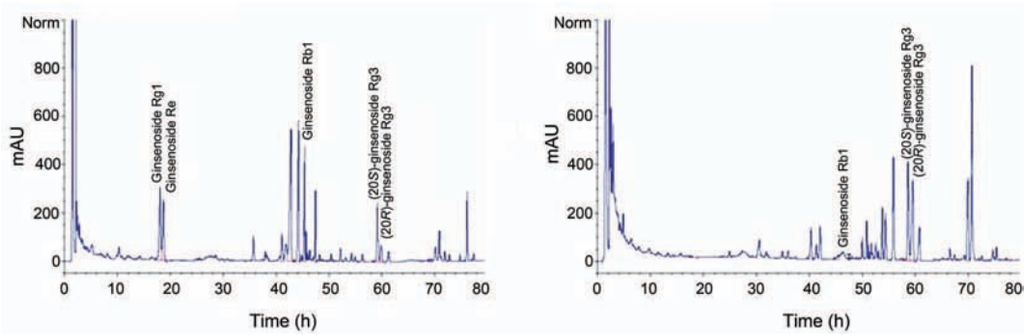

Fig. 2. HPLC fingerprints of fresh ginseng extract simmered for 3 and 24 h. The HPLC setup: Discovery HS C18 material (Supelco; 250×4.6 mm, 5 μm) at room temperature (25°C) with a sample injection volume of 20 μL (20 mg/mL); mobile phase: acetonitrile and water gradient.

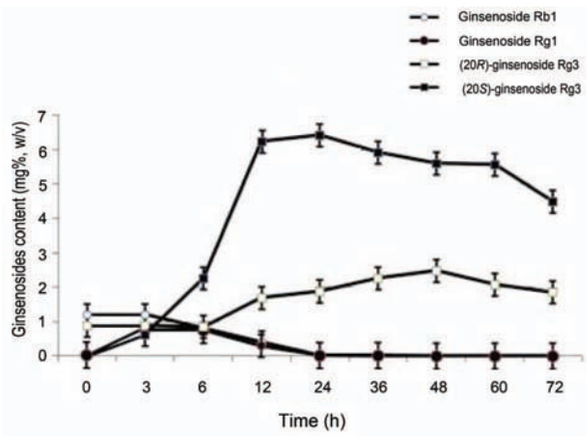

Fig. 3. Change in ginsenosides content during simmering at 80°C (mg%, w/v). Aliquots (100 mL) of the simmering ginseng root were sampled at 3, 6, 12, 24, 36, 48, 60 and 72 h, and the samples were subjected to MCI gel adsorption chromatography to determine the crude saponin content. The content of the (20*S*)-ginsenoside Rg3 and (20*R*)-ginsenoside Rg3 increased to a greater extent.

System suitability testing

The four marker ginsenosides were well separated with retention times of 18.4, 45.4, 59.4, and 60.4 min for ginsenoside Rg1, ginsenoside Rb1, (20*S*)-ginsenoside Rg3 and (20*R*)-ginsenoside Rg3, respectively, in the given HPLC conditions (Fig. 2). The resolution values by pairing were ginsenoside Rg1 and ginsenoside Re ≤2.0, ginsenoside Rb1 and ginsenoside Rc ≥2.0, and (20*S*)-ginsenoside Rg3 and (20*R*)-ginsenoside Rg3 ≥2.0.

Robustness

The robustness was determined by comparing different columns from different suppliers at different temperatures, flow rates, pHs and mobile phases. The variation of the quantitative analytical data due to HPLC equipment differences was less than 1%. The mobile phase containing formic acid (pH 4.0) caused downgrading of the baseline in the gradient system, though no significant variation in the quantification data for the 4 reference materials was observed. The ELSD system showed a stable baseline but poor linearity. The deviation between analysts was less than 0.2%. The correlation coefficients (r^2) of the UV detector and ELSD were 0.9999 and 0.9951, respectively.

Selectivity

The HPLC peaks obtained from the lipophilic fraction of the devil's claw did not interfere with the *P. ginseng* ginsenosides signals (Fig. 4). In addition, the ginsenoside Rb1, Rg1, (20*S*)-ginsenoside Rg3, and (20*R*)-ginsenoside Rg3 did not overlap.

Range and linearity

The concentration range under investigation was 2.0 mg/mL to 15.625 ng/mL, and linearity was investigated in the concentration range 1.0 mg/mL to 62.5 ng/mL by two-fold dilution. Standard curves (linearity) obtained with the 4 ginsenosides reference materials demonstrated r^2>0.999 in the concentration range of 1.0 mg/mL to 62.5 ng/mL. The standard curve (relative standard deviation, RSD) was less than 1.5% based on triplicate measurements.

Precision and accuracy

The precision was verified through the repeatability and reproducibility, and the accuracy was verified through the measurement of known amounts of reference material. The repeatability data, using concentrations of 0.25, 0.5, and 1 mg/mL, are shown in Table 1. The recovery of the 4 compounds by the proposed pretreatment and HPLC quantitation ranged from 92% to 100%.

Limit of detection and quantitation

According to ICH guidelines, limit of quantitation

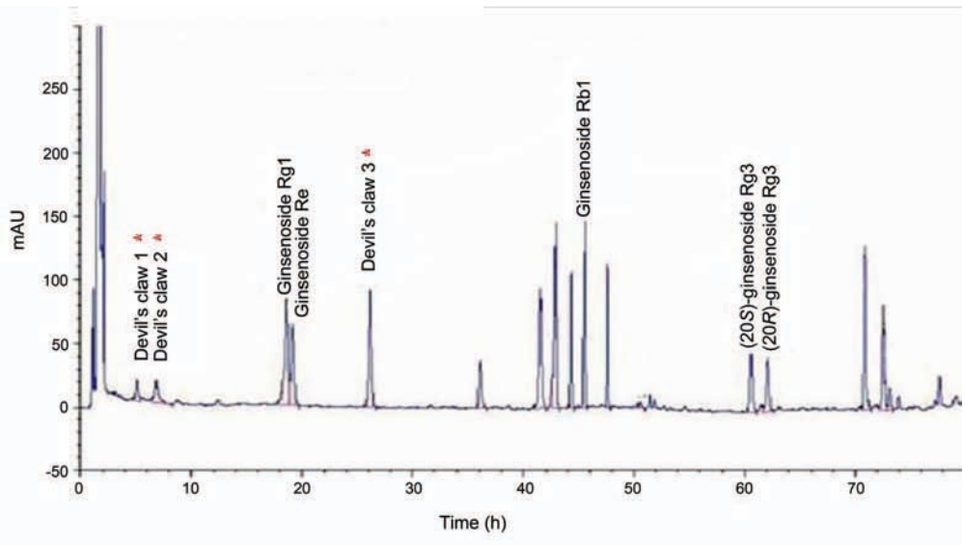

Fig. 4. HPLC fingerprint of ginsenosides standards in a devil's claw* matrix. The HPLC setup: Discovery HS C18 material (SUPELCO, 250×4.6 mm, 5 μm) at room temperature (25°C) with a sample injection volume of 20 μL (20 mg/mL); mobile phase: acetonitrile and water gradient.

Table 1. Method validation for the HPLC analysis of ginsenoside Rb1, Rg1, (20*S*)-ginsenoside Rg3, and (20*R*)-ginsenoside Rg3

	Ginsenosides Rb1			Ginsenosides Rg1			(20*S*)-ginsenosides Rg3			(20*R*)-ginsenosides Rg3		
Correlation coefficient (r^2)	0.99993			0.99986			0.99988			0.99960		
Regression curve with Y	Y=3365.22424X+18.9929			Y=6085.42275A+54.4736			Y=5874.7157A+63.08576			Y=1441.85004A–4.79027		
Range	16.5 ng/mL to 1.0 mg/mL			16.5 ng/mL to 1.0 mg/mL			16.5 ng/mL to 1.0 mg/mL			16.5 ng/mL to 1.0 mg/mL		
Precision (peak area) [1)]	3,348.999±26.437			1,579.21±40.968			3,348.999±26.437			1,536.31±12.430		
LC/MS $[M+Na]^+$	1,131.6			823.48			807.5			807.5		
Limit of quantitation	30 μg/L			30 μg/L			30 μg/L			45 μg/L		
Limit of detection	20 μg/L			20 μg/L			20 μg/L			20 μg/L		
Recovery Spiked (mg/mL)	1	0.5	0.25	1	0.5	0.25	1	0.5	0.25	1	0.5	0.25
BuOH (%)	98±0.57	97±0.48	98±0.27	98±0.46	97±0.42	95±0.68	100±0.25	97±0.47	92±0.57	99±0.17	95±0.36	92±0.11
MCI gel (%)	98±0.56	99±0.37	99±0.59	98±0.43	99±0.46	96±0.42	100±0.24	96±0.56	96±0.48	1,001±0.24	98±0.56	91±0.39

[1)] Concentration of all samples was 0.5 mg/mL.

(LOQ) is defined as the lowest concentration of analyte in a standard that can be reproducibly measured with acceptable accuracy and precision (RSD≤2%). LOQ for ginsenosides Rb1, Rg1, (20*S*)-ginsenoside Rg3, and (20*R*)-ginsenoside Rg3 were 30 μg/L, 30 μg/L, and 45 μg/L. The limit of detection (LOD) is determined by the analysis of standard with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected. LOD for ginsenosides Rb1, Rg1, (20*S*)-ginsenoside Rg3, and (20*R*)-ginsenoside Rg3 were 20 μg/L for all samples (Table 1).

DISCUSSION

The polarity of ginsenosides varies depending on the number of sugars attached to the dammarane nucleus. The Ro, Ra, and Rb ginsenosides are very polar and are readily soluble in warm water. Thus, butanol is conventionally employed for the preparation of crude ginseng saponin fractions because it partitions almost all the ginsenosides in the organic solvent phase. However, butanol holds a significant amount of water due to its strong polarity. The butanol partition may therefore transfer high amounts of water-soluble impurities such as free sugars. The crude saponin content was higher when prepared through butanol partition than through MCI gel chromatography. This result might be due to an increase in the butanol soluble ingredients and not due to the increase in the ginsenosides content in the decoction soup.

Protopanaxadiol ginsenosides are degraded into

ginsenoside Rg3 and from there to Rh2, while protopanaxatriol ones degrades into ginsenoside Rh1 and then into sapogenin. The content of the (20*S*)- and (20*R*)-ginsenosides Rg3 increased to a greater extent. However, there was not a one-sided increase in ginsenoside Rg3 or Rh1. In other words, analysis of either the protopanaxadiol or protopanaxatriol artifact ginsenosides is enough to determine the heat-generated artifact concentration. In practice, *P. ginseng* root is boiled 4 times at 85°C for 8 h. The extract is further concentrated at 65°C for 24 h. Thus, focusing the quantitative analysis on ginsenoside Rb1 and Rg1, which are known as protopanaxadiol and protopanaxatriol ginsenosides, respectively, is not appropriate for concentrated extract, which has been heated for many hours during production. During the production of *P. ginseg* (steamed and dried, red ginseng) and ginseng extract, the majority of ginsenoside Rb1 and other protopanaxadiol ginsenosides are converted into (20*S*)- and (20*R*)-ginsenoside Rg3 [2]. Analytical investigations of ginseng products collected from the market show that many of the ginsenosides have been converted to (20*S*)- and (20*R*)-ginsenoside Rg3.

The content of ginsenoside Rb1 and Rg1 decreased rapidly during simmering. This result strongly suggests that the quantitative analysis of ginsenoside Rb1 and Rg1 is not enough to analyze the quality of *P. ginseng* extract concentrate or *P. ginseg* (steamed and dried, red ginseng). Thus, the adulteration of white ginseng with red ginseng sometimes occurs. The HPLC-based analytical procedures for ginsenoside Rb1 and Rg1 cannot determine whether the product is derived from red or white ginseng. Adulteration is therefore undetectable. European Pharmacopoeia recommends the use of microscope examination for the detection of white or red ginseng to detect adulteration. In addition, the quality analytical method recommended by the Korea Food and Drug Agency is not appropriate for the detection of white and red ginseng extract. The European Union requires a dossier for standard reference materials and validated analytical procedures based on ICH guidelines [7].

The Chinese government recommends measuring the content of ginsenoside Rb1, Rg1, and Re. As shown in Figs. 2 and 3, ginsenoside Rb1 and Rg1 disappear rapidly and are no longer detected after 24 h of simmering. The increase in the amount of these ginsenosides in the soup during the first several hours might be due to the increased dissolution of ginsenosides. The decrease in the amount of ginsenosides after 3 h is not due to a decrease in dissolution but due to degradation. Generally, the number of reference materials and the number of sample preparation steps used in the standardized quality analysis methods should be minimized as much as efficiency and accuracy permit.

European countries require quantitative data for ginsenoside Rb1 and Rg1 and qualitative data for Rf. However, we suggest that, due to traditional *P. ginseng* preparation methods, (20*S*)-ginsenoside Rg3 and (20*R*)-ginsenoside Rg3 should be added to the conventional reference list as a quality control marker for *P. ginseng* preparations. We recommend including (20*S*)-ginsenoside Rg3 and (20*R*)-ginsenoside Rg3 because there are no ginsenosides that interfere with ginsenoside Rg3. Ginsenoside Rb1 or Rg1 should not be eliminated as quality control reference agents because the majority of countries use these two compounds as the reference materials in *P. ginseng* preparations.

We employed ginsenoside Rb1, Rg1, (20*S*)-ginsenoside Rg3, and (20*R*)-ginsenoside Rg3 as the standard references for establishing validated analytical procedures. When employed in validation tests, the four ginsenosides showed an HPLC purity of 100%. Therefore, these ginsenosides meet the standard reference material qualifications as defined in "Herbal medicinal products" [9] and "European pharmacopeia" [11]. We attempted method validation with 3 HPLC systems. Among the three methods, the 1st HPLC configuration (reverse phase, gradient aqueous CH_3CN) gave the best repeatability when measured 9 times with 4 concentrations. The peak area deviation in the HPLC fingerprints of the four reference ginsenosides were 0.8%, 0.9%, 0.7%, and 1.2%, respectively, for ginsenoside Rb1, Rg1, (20*S*)-ginsenoside Rg3, and (20*R*)-ginsenoside Rg3 when precision (repeatability) was determined in sextuplet [12]. The 2nd HPLC configuration (ODS, formic acid with CH_3CN) was not inferior to those in the 1st HPLC configuration in terms of specificity, linearity, or sensitivity. However, this configuration was inferior in system repeatability. In addition, the formic acid employed in the mobile phase can erode the steel column. The 3rd HPLC configuration had poor linearity and relatively lower correlation coefficients compared to the 1st HPLC configuration.

The linearity determined by the carbohydrate column and ELSD was unsatisfactory due to an exponentially increasing sensitivity. Moreover, it did not demonstrate good resolution for isomer type ginsenosides. If we spike ginsenosides in *P. ginseng* powder or extract for the evaluation of accuracy, the intrinsic content of ginsenosides in the matrix produces deviations, thereby reducing the accuracy of the data. Accordingly, we employed devil's claw root as a matrix for the determination of accuracy.

Lipophilic components derived from devil's claw root do not interfere with ginsenosides in HPLC fingerprint. Furthermore, the peaks tagged with red asterisks did not increase when the amount of spiked ginsenoside reference was increased by two or three times, thus further supporting the conclusion that the non-saponin peaks do not originate in *P. ginseng*.

We determined system suitability with ginsenosides pairs Rb1 and Re, and Rg1 and Rc because they are closest together on the ODS HPLC fingerprint. The main disadvantage of this HPLC method was the long operational recycling time. If the recycling time is shortened, the resolution of ginsenoside Rb1 and Re or Rg1 and Rc is not satisfactory. In addition, the separation of ginsenoside Rg3 isomer would not be possible. Recently, UPLC has been recommended for the quantitative analysis of ginsenosides. UPLC equipment reduces recycling time and increases resolution, but the equipment is not prevalent. Consequently, the authors recommend a newly validated HPLC analytical method for ginsenoside Rb1 and Rg1 that includes (20*S*)-ginsenoside Rg3 and (20*R*)-ginsenoside Rg3 for the qualitative and quantitative determination of reference materials for *P. ginseng* preparations.

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