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Sensitive high-performance liquid chromatography method of non-polar ginsenosides by alkaline-enhanced pulsed amperometric detection

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ABSTRACT

We determined the minute amount of non-polar ginsenosides in red ginseng with a reversed-phase high-performance liquid chromatography-pulsed amperometric detection (RP-HPLC-PAD) method. Non-polar ginsenosides efficiently extracted by ethyl acetate were well separated in 40 min using a water–acetonitrile gradient eluent and detected by PAD under NaOH alkaline conditions. The ginsenoside detection limits (S/N = 3) were 0.03–0.10 ng. The coefficients of linear regression were 0.9972–0.9990. Intra- and inter-day precision (RSDs) was less than 8.34% and average recovery was 98.06–102.73%. The total amount of non-polar ginsenosides in hairy root of red ginseng was slightly higher than in the main root.

1. Introduction

Ginseng, the root of *Panax ginseng*, has been widely used in Chinese traditional medicine, and contains ginsenosides as medicinal components. Red ginseng is made through additional process of steaming and drying from ginseng. During the steaming process, some of the polar ginsenosides are transformed into non-polar ginsenosides by hydrolysis of sugar moiety. Consequently, non-polar ginsenosides are produced in small quantities in red ginseng. Therefore, red ginseng contains both polar and non-polar ginsenosides, whereas ginseng contains only polar ginsenosides. In Korea Pharmacopoeia (VIII), the pharmacopoeia names for ginseng and red ginseng is differently described as Ginseng Radix Alba and Ginseng Radix Rubra, respectively. Typical red ginseng has non-polar ginsenosides, such as ginsenoside Rh_1 (G-Rh_1), ginsenoside Rh_2 (G-Rh_2), ginsenoside Rg_2 (G-Rg_2), ginsenoside Rg_3 (G-Rg_3), ginsenoside Rg_5 (G-Rg_5), ginsenoside Rk_1 (G-Rk_1) as well as polar ginsenosides, such as ginsenoside Rb_1(G-Rb_1), ginsenoside Rb_2 (G-Rb_2), ginsenoside Rb_3 (G-Rb_3), ginsenoside Rc (G-Rc), ginsenoside Rd (G-Rd), ginsenoside Rf (G-Rf), ginsenoside Rg_1 (G-Rg_1), and ginsenoside Re (G-Re) [1,2]. The chemical structures of ginsenosides are shown in Fig. 1. The non-polar ginsenosides in red ginseng have shown diverse biologically beneficial activities, such as anticancer effects (G-Rg_3, G-Rg_5, G-Rh_2) [3–6], antioxidant activities (G-Rg_3, G-Rg_3, G-Rh_1, G-Rh_2, G-Rk_1) [7–9], a neuroprotective effect (G-Rg_2, G-Rg_3) [10,11], a vasodilating effect (G-Rg_3) [12], and hepatoprotective effects (G-Rg_3, G-Rh_2) [13].

The various methods for the analysis of polar ginsenosides in ginseng have widely been used without any problem [14–17], while small amount of non-polar ginsenosides in red ginseng was not easily detected because of lack of detector sensitivity [18,19]. The HPLC-evaporate light scattering detection (ELSD) method has been a general method for the analysis of ginsenosides in red ginseng, but could not detect small amount of non-polar ginsenosides [18]. Therefore, it is desirable to develop sensitive detection methods for non-polar ginsenosides in red ginseng.

High-performance anion-exchange chromatography-pulsed amperometric detection (HPAEC-PAD) has been used for the quantitation of carbohydrates in plant resources [20–22], because of its strong anion-exchange properties, that efficiently separate carbohydrates. PAD, an electrochemical detector that measures the positive potential produced by carbohydrate oxidation on a gold electrode, allows the direct detection of carbohydrates at low pico-mole levels [23].

Although PAD has been used for the analysis of sugars, it has not been applied for the analysis of glycosides because of the difficulty in separating glycosides from sugars and glycosides on an anion-exchange column. We have developed a reversed-phase method

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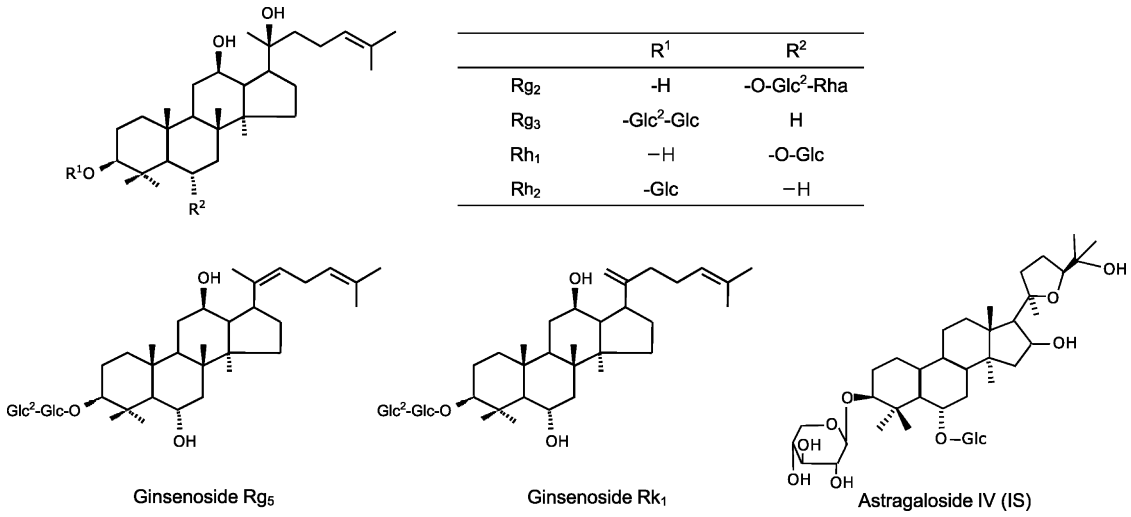

	R^1	R^2
Rg_2	-H	-O-Glc^2-Rha
Rg_3	-Glc^2-Glc	H
Rh_1	−H	-O-Glc
Rh_2	-Glc	−H

Fig. 1. Chemical structures of ginsenosides and astragaloside IV (I.S.).

of high-performance liquid chromatography-pulsed amperometric detection (RP-HPLC-PAD) for glycoside analysis. Previously, we demonstrated the analysis of several polar ginsenosides in ginseng [24]. We demonstrated highly efficient separation of polar ginsenosides from sugars on a reversed-phase column and their detection by PAD. Here, we established a noble method for determining non-polar ginsenosides in red ginseng by modified extraction protocol and optimized separation conditions. By this new method, we successfully analyzed non-polar ginsenosides in 6-year-old red ginseng (MR-6), 4-year-old red ginseng (MR-4), and hairy root of 4-year-old red ginseng (HR-4).

We also compared the limits of detection (LODs) and limits of quantitation (LOQs) by PAD to those reported for the ELSD method. The robustness of this method was also evaluated by intra- and inter-day validation. The six non-polar ginsenosides (G-Rh_1, G-Rh_2, G-Rg_2,G-Rg_3, G-Rg_5 and G-Rk_1) in red ginseng were successfully separated with high recovery.

2. Experimental

2.1. Materials

Crude drugs were purchased from the *Kyungdong* Market (Seoul, South Korea), in accordance with the standards stipulated in Korea Pharmacopoeia (VIII). The main roots of 6-year-old red ginseng (MR-6) and 4-year-old red ginseng (MR-4), as well as the hairy root of 4-year-old red ginseng (HR-4), were purchased from a local drug store. HPLC-grade acetonitrile and 50% sodium hydroxide were purchased from Fisher Scientific (Fairlawn, NJ, USA). All other reagents and solvents used were of guaranteed or analytical grade. G-Rg_3, G-Rh_2, G-Rg_2, G-Rh_1, G-Rb_2, G-Rb_3, G-Rf and astragaloside IV (AST IV) were purchased from ChromaDex (Santa Ana, CA, USA). G-Rk_1 (95.2%) and G-Rg_5 (96.1%) were working standards obtained from Seoul National University [25]. G-Rb_1, G-Rc, G-Rd, G-Re, and G-Rg_1 were purchased from Wako (Tokyo, Japan). A Millipore membrane filter (type HA, pore size 0.45 μm) was used for solvent filtration. All samples were filtered through disposable syringe filters (Hydrophobic PTFE, pore size 0.20 μm, Advantec MFS, Tokyo, Japan) before injection. To prepare the standard solutions, sample solutions, and mobile phase, we used 18 MΩ purified water produced by our laboratory's water purification system, Automatic Aquarius AW-1001 (Top Trading, Seoul, South Korea). The weight of each sample was measured on a Mettler Toledo AX 105 DeltaRange (Greifensee, Switzerland). Mass spectra were obtained with a JEOL AccuTOF TLC JMS-T100TD (Tokyo, Japan).

2.2. Apparatus and high-performance liquid chromatography

The PAD system from the ICS-3000 series Dionex (Sunnyvale, CA, USA) was equipped with an Au-Flow cell containing a gold working electrode and a solvent compatible cell containing an Ag/AgCl reference electrode. We cleaned up the gold electrode by rubbing the surface twice a week with the pink eraser (Dionex P/N. 049721) provided in the polishing kit. HPLC equipment, consisting of a Model Nanospace SI-2/3201 pump and a 3004 column oven, was purchased from Shiseido (Tokyo, Japan). Nanospace SI-2/3201 pump has metal-free head made of polyetheretherketone (PEEK) resin, which resist aggressive chemicals such as alkaline solutions.

Chromatographic separation was performed by using a Unison UK-C-18 column (150 mm × 2.0 mm I.D.; 3 μm, Imtakt, Kyoto, Japan). The potential waveform was as follows: E1 = −0.2 V (from 0.00 to 0.04 s); E2 = 0 V (from 0.05 to 0.21 s); E3 = +0.22 V (from 0.22 to 0.46 s); E4 = 0 V (from 0.47 to 0.56 s); E5 = −2 V (from 0.57 to 0.58 s); and E6 = +0.6 V (0.59 s). The mobile phase consisted of 30% (v/v) acetonitrile (solvent A) and 80% (v/v) acetonitrile (solvent B). The following procedure was employed: isocratic elution with A:B (92:8) for 8 min, linear gradient elution from A:B (92:8) to (68:32) from 8 to 10 min, and isocratic elution with A:B (68:32) from 10 to 45 min. The column was then washed with 100% B for 10 min. The flow rate was 0.2 mL/min, and the separation temperature was 30 or 40 °C. A post-column delivery system of 200 mM sodium hydroxide with a flow rate of 0.8 mL/min was added to the RP-HPLC-PAD system. The mobile phase was made through being degassed by vacuum filtration after the mixture of water with acetonitrile on a daily basis, followed by sonication for 20 min before use. A post-column delivery system was purged to remove carbonate from the water with helium throughout the experiment. The data were controlled on a computer running the Chromeleon client program supplied by Dionex. The injection volume was 10 μL.

2.3. Standard preparation

Stock solutions were prepared by dissolving 1 mg of each standard (G-Rg_2 G-Rg_3, G-Rh_1, G-Rh_2, G-Rg_5, and G-Rk_1) in 1 mL of 50% (v/v) acetonitrile/water in an Eppendorf tube. Each stock solution was diluted to create six calibration points (1, 2, 5, 10, 25, and