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Application of High-performance Liquid Chromatography to the Isolation of Ginsenoside-Rb_1, -Rb_2, -Rc, -Rd, -Re, and -Rg_1 from Ginseng Saponins

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The major components of ginseng saponins, ginsenoside-Rb_1, -Rb_2, -Rc, -Rd, -Re, and -Rg_1, were isolated from the crude saponin fraction by high-performance liquid chromatography (HPLC), using preparative HPLC on silica gel followed by semi-preparative HPLC on a column packing of Carbohydrate Analysis. This method was rapid and convenient. In addition, ginsenosides could be rapidly analyzed by HPLC on a Carbohydrate Analysis column, which gave excellent resolution.

Keywords——*Panax ginseng* C.A. MEYER; ginsenosides; high-performance liquid chromatography; fractionation; isolation; identification

The biochemical and pharmacological effects of ginsenosides, the purified saponins from *Panax ginseng* C.A. MEYER, were studied.[2,3] More than ten saponins are known and the structure of ginsenoside-Rx (x=0, b_1, b_2, b_3, c, d, e, f, 20-gluco-f, g_1, g_2, and h_1) have been established.[4–8] Separation, purification, and identification of ginsenosides are not easy, but chromatographic analyses of ginsenosides have been performed by thin-layer chromatography (TLC) on silica gel,[9,10] droplet counter-current chromatography,[11] gas liquid chromatography,[12] rod-thin-layer chromatography,[13] and high-performance liquid chromatography (HPLC).[14] The HPLC analysis resulted in the separation and determination of only ginsenoside-Rg_2, -Rg_1, -Rf, and -Re (whose aglycone is 20*S*-protopanaxatriol). The separation

1) Location: *2630 Sugitani, Toyama, 930-01, Japan.*
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and determination of ginsenoside-Rb_1, -Rb_2, -Rc, and -Rd (whose aglycone is 20*S*-protopanaxadiol) by HPLC has not yet been reported.

The isolation of ginsenosides have been accomplished by column chromatography on silica gel,[4] preparative-TLC on silica gel,[15] and semi-preparative HPLC on silica gel.[16] The semi-preparative HPLC was used in the isolation of ginsenoside-Re, -Rd, and -Rb_2. However, these isolation procedures are too time-consuming to allow the isolation of large quantities of major saponins, ginsenoside-Rb_1, -Rb_2, -Rc, -Rd, -Re, and -Rg_1, from ginseng root.

Therefore, in order to improve the separation and isolation efficiency of these ginseng saponins and to obtain large quantities of pure ginsenosides for biological studies, an improved HPLC procedure for the isolation of major components of ginseng saponins was developed.

Experimental

Apparatus and Column——Liquid chromatography was performed on the ALC 201 and PrepLC/System-500 (Waters Associates, Inc., Milford, Mass., U.S.A.), using a refractometer (RI detector). A stainless steel column (30 cm × 3.9 mm) packed with Carbohydrate Analysis (Waters Associates, Inc.), a stainless steel column (30 cm × 7.8 mm) with the same packing, or a PrepPAK-500/Silica cartridge (30 cm × 5.7 cm) for the PrepLC/System-500 (Waters Associates, Inc.) was used.

Materials——Ginsenoside standards used were provided by Professor Osamu Tanaka, Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine, Professor Junzo Shoji, School of Pharmaceutical Sciences, Showa University, and Dr. Teruaki Hayashi, Koshiro Co., Osaka.

The crude ginseng saponin fraction was prepared as follows: ginseng powder was first extracted with hot methanol (MeOH). After removal of the solvent by evaporation *in vacuo*, the extract was dissolved in water. This solution was partitioned between ether and the aqueous layer, and the aqueous layer was then extracted with butanol (BuOH) saturated with water. The BuOH layer was concentrated *in vacuo* and lyophilized to afford a crude saponin.

Identification Procedure——Each of the ginsenosides and crude ginseng saponins was dissolved in MeOH to *ca.* 20 mg/ml. All the samples or standard solutions were filtered through a TM-2P membrane filter (Toyo Roshi Co., Tokyo; pore size, 0.45 μm) before injection.

Fractionation Procedure——Crude ginseng saponin was dissolved in a carrier solvent (BuOH: EtOAc: H_2O=4: 1: 5, v/v, upper phase). The concentration of the solution was *ca.* 140 mg/ml. The sample was filtered through a TM-2P membrane filter before injection.

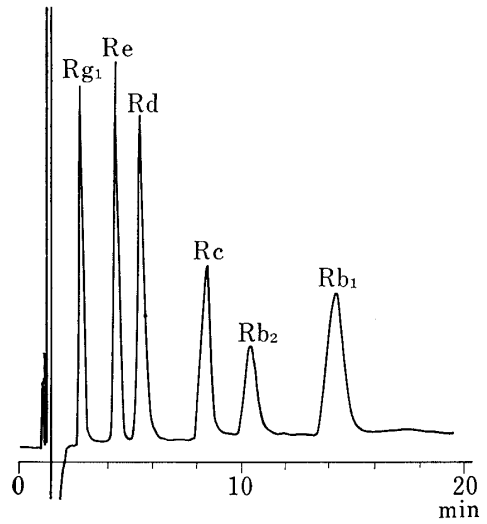

Fig. 1. Chromatogram of Ginsenoside–Rg_1, –Re, –Rd, –Rc, –Rb_2, and –Rb_1

Conditions: column, 30 cm × 3.9 mm; packing, Carbohydrate Analysis; mobile phase, AcCN: H_2O=80: 20 (v/v); flow rate, 2 ml/min; RI detector, attenuation 16×.

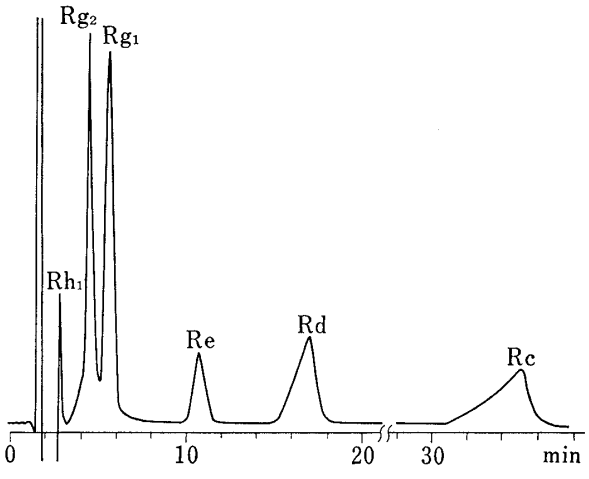

Fig. 2. Chromatogram of Ginsenoside–Rh_1, –Rg_2, –Rg_1, –Re, –Rd, and –Rc

Conditions: column, 30 cm × 3.9 mm; packing, Carbohydrate Analysis; mobile phase, AcCN: H_2O=86: 14 (v/v); flow rate, 2 ml/min; RI detector, attenuation 16×.

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Isolation Procedure——Each fractionated ginseng saponin was dissolved in a mixture of the carrier solvent and a little methanol. Sample solutions were filtered through a TM-2P membrane filter before injection.

HPLC Conditions for Identification——Liquid chromatography was performed on ALC 201. A stainless steel column (30 cm × 3.9 mm) packed with Carbohydrate Analysis was used. The mobile phase was a mixture of acetonitrile (AcCN) and water (H_2O) (80: 20 or 86: 14, v/v). The RI detector was used to determine each ginsenoside. The RI attenuation was set at 8× or 16× depending on the sample load, and the flow rate was 2 ml/min.

HPLC Conditions for Fractionation——Liquid chromatography was carried out with PrepLC/System-500. Two PrepPAK-500/Silica cartridges were used. The mobile phase was a mixture of BuOH: EtOAc: H_2O= 4: 1: 5 (v/v, upper phase). The flow rate was 50 ml/min.

HPLC Conditions for Isolation——Liquid chromatography was performed on ALC 201. A stainless steel column (30 cm × 7.8 mm) packed with Carbohydrate Analysis was used. The mobile phase was as specified in "Results and Discussion." The RI detector was used to determine each ginsenoside. The RI attenuation was set at 16× to 32× depending on the sample load, and the flow rate was 8 ml/min.

Results and Discussion

Analysis of Ginsenosides by HPLC

As illustrated in Fig. 1, ginsenoside-Rb_1, -Rb_2, -Rc, -Rd, -Re, and -Rg_1 were each separated from the mixture by HPLC employing a mixture of AcCN: H_2O=80: 20 (v/v) as a mobile phase. Resolution of ginsenoside-Rb_1, -Rb_2, -Rc, and -Rd (whose aglycone is 20*S*-protopanaxadiol) was satisfactory. Resolution of ginsenoside-Rh_1, -Rg_2, -Rg_1, and -Re (whose aglycone is 20*S*-protopanaxatriol) by HPLC employing a

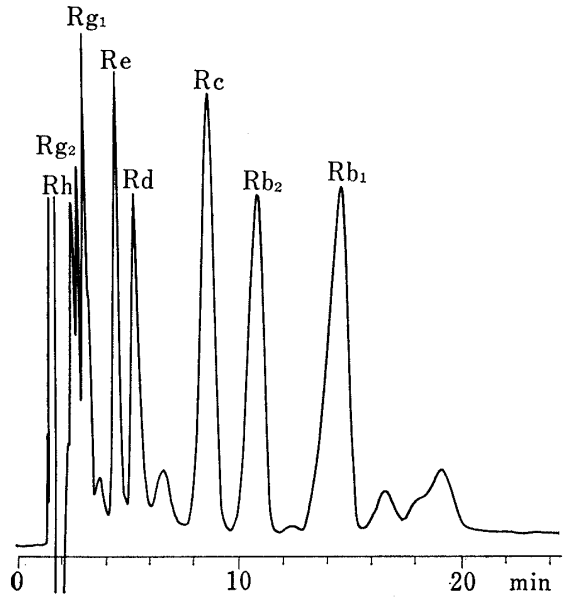

Fig. 3. Chromatogram of Crude Saponin from Ginseng Radix

Conditions: column, 30 cm × 3.9 mm; packing, Carbohydrate Analysis; mobile phase, AcCN: H_2O=80: 20 (v/v); flow rate, 2 ml/min; RI detector, attenuation 16×.

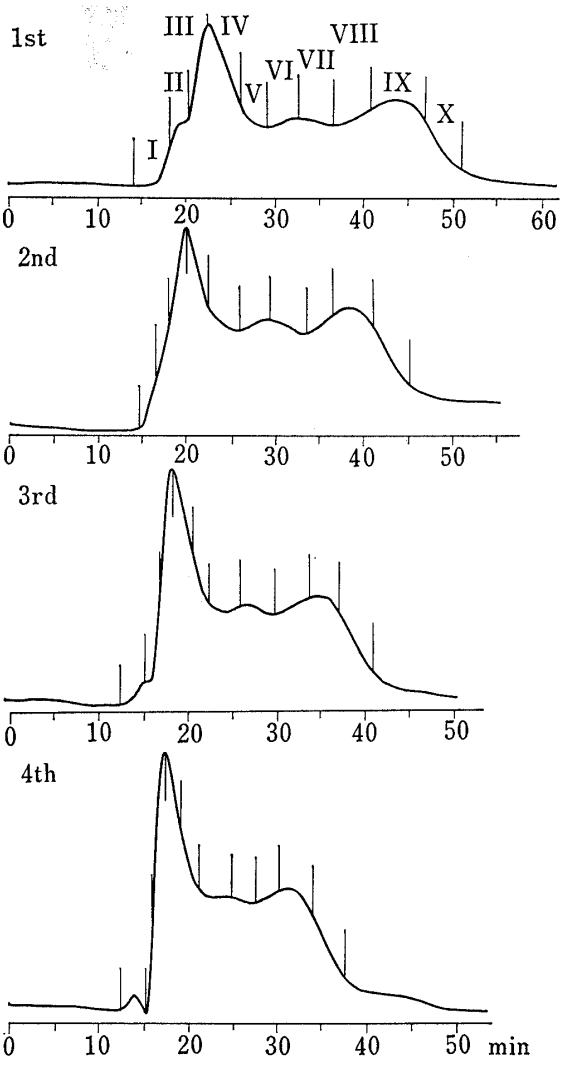

Fig. 4. Elution Profile of Crude Ginseng Saponins on Preparative HPLC

Conditions: instrument, PrepLC/System-500; column, PrepPAK-500/Silica cartridge × 2; mobile phase, BuOH: EtOAc: H_2O=4: 1: 5 (v/v, upper phase); flow rate, 50 ml/min; detector, RI detector (setting 5); sample load, 4.5 g/32 ml/injection (18 g in total).

mixture of AcCN: H_2O=86: 14 (v/v) as a mobile phase also produced a satisfactory result, as shown in Fig. 2.

The former method was adopted for the analysis of ginsenosides in crude ginseng saponins, and resulted in a satisfactory identification, as illustrated in Fig. 3. Therefore, HPLC employing the column packed with Carbohydrate Analysis was shown to be suitable for rapid analysis of major components of ginseng saponins.

Fractionation of Crude Saponins by Preparative HPLC

Fractionation of crude ginseng saponins had been done by column chromatography on silica gel, but this method was very time-consuming. Therefore, preparative HPLC was carried out 4 times successively for 6 hr with repeated use of the cartridges, applying 18 g of crude saponin in total. Fig. 4 shows the elution profiles. It was observed that the deactivation of silica packed in the cartridges by water in the carrier solvent affected the resolution

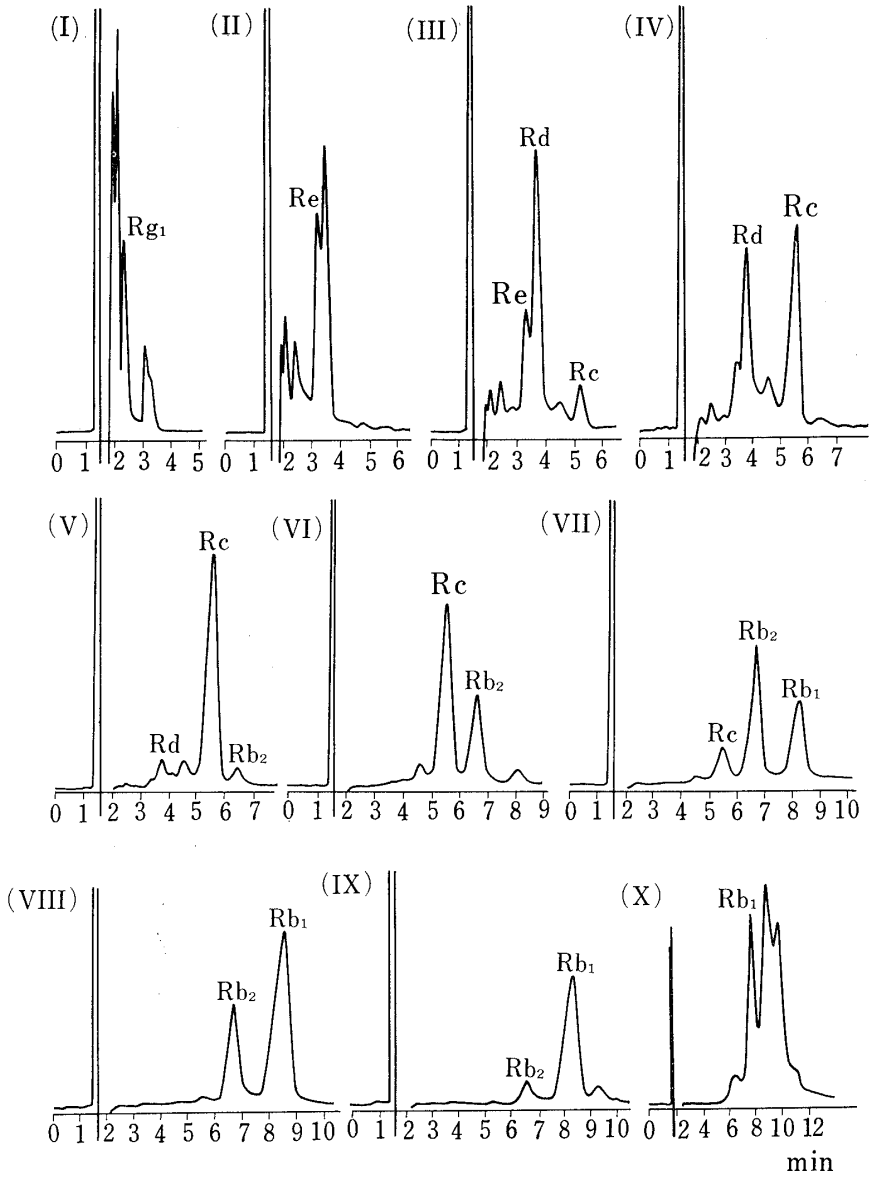

Fig. 5. Individual Chromatograms of Saponin Fractions obtained by Preparative HPLC

Conditions: column, 30 cm × 3.9 mm; packing, Carbohydrate Analysis; mobile phase, AcCN: H_2O=80: 20 (v/v); flow rate, 2 ml/min; RI detector, attenuation 16×.

and retention of saponins. In each HPLC, crude ginseng saponins were fractionated into 10 fractions, as illustrated in Fig. 4. Corresponding fractions from the preparative HPLC runs were pooled and concentrated *in vacuo*. The fractionated saponins were then identified by analytical HPLC, as shown in Fig. 5.

Isolation of Ginsenoside-Rb_1 and -Rb_2 by Semi-preparative HPLC

In preparative HPLC, it was observed that the crude saponin was separated into mixtures of 2 or 3 ginsenosides, and ginsenoside-Rb_1 and -Rb_2 were contained in fractions VII, VIII, and IX. Ginsenoside-Rb_1 and -Rb_2 were isolated from a mixture of fractions VII and VIII by semi-preparative HPLC, which was carried out with a semi-preparative column (30 cm × 7.8 mm) employing a mixture of AcCN: H_2O=81 : 19 (v/v) as a mobile phase at the flow rate of 8 ml/min by repeated fractionation. This semi-preparative HPLC could treat the ginsenosides mixture at a rate of *ca.* 40 mg/hr. No impurities in the isolated ginsenoside-Rb_1 and -Rb_2 were detected by analytical HPLC.

Isolation of Ginsenoside-Rc by Semi-preparative HPLC

Ginsenoside-Rc was isolated from fraction V by semi-preparative HPLC, eluting with AcCN: H_2O=82: 18 (v/v) at 8 ml/min, by repeated fractionation. This semi-preparative HPLC could treat fraction V at a rate of *ca.* 40 mg/hr. No impurities in the isolated ginsenoside-Rc were detected by analytical HPLC.

Isolation of Ginsenoside-Rd and -Re by Semi-preparative HPLC

Ginsenoside-Rd and -Re were isolated from a mixture of fractions II and III, which contained mainly these two components, using a semi-preparative column, employing a mixture of AcCN: H_2O=86: 14 (v/v) as a carrier solvent at 8 ml/min, by repeated fractionation. This semi-preparative HPLC could treat the ginsenosides mixture at a rate of *ca.* 45 mg/hr. No impurities in the isolated ginsenoside-Rd and -Re were detected by analytical HPLC.

Isolation of Ginsenoside-Rg_1 by Semi-preparative HPLC

In preparative HPLC, it was observed that ginsenoside-Rg_1 was contained in fraction I, as illustrated in Fig. 5. Fraction I was further fractionated by semi-preparative HPLC,

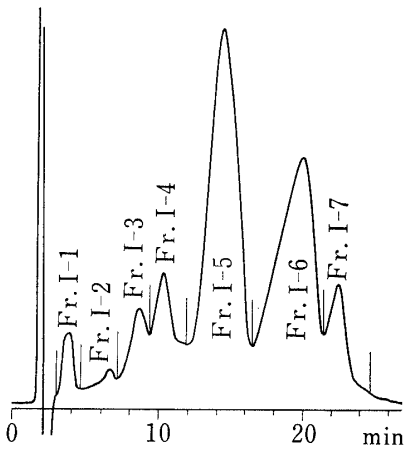

Fig. 6. Elution Profile of Fractionated Saponins (Fr. I) on Semi-preparative HPLC

Conditions: column, 30 cm × 7.8 mm; packing, Carbohydrate Analysis; mobile phase, AcCN: H_2O= 89: 11 (v/v); flow rate, 8 ml/min; RI detector, attenuation 16 × ; sample load, 30 mg/0.75 ml/injection.

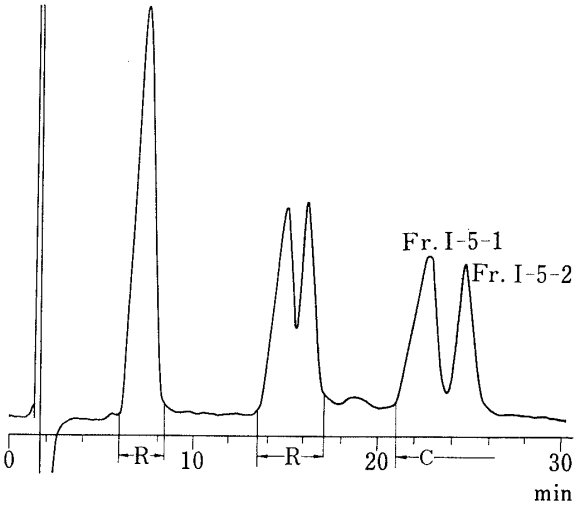

Fig. 7. Chromatogram of Fraction I-5 on Recycle Chromatography

Conditions: column, 30 cm × 7.8 mm; packing, Carbohydrate Analysis; mobile phase, AcCN: H_2O=87: 13 (v/v); flow rate, 8 ml/min; RI detector, attenuation 16 × ; sample load, 4 mg/0.2 ml/injection. R; recycle, C; collect.

eluting with a mixture of AcCN: H_2O=89: 11 (v/v) at 8 ml/min, by repeated fractionation. As shown in Fig. 6, fraction I was separated into 7 subfractions, and ginsenoside-Rg_1 was contained in fraction I-5. However, this fraction contained 2 components. Therefore, ginsenoside-Rg_1 was isolated from fraction I-5 by recycle chromatography employing a mixture of AcCN: H_2O=87: 13 (v/v) at a flow rate of 8 ml/min. Its elution profile is shown in Fig. 7. In recycle chromatography, fraction I-5 was separated into fractions I-5-1 and I-5-2. Fraction I-5-2 was identified as ginsenoside-Rg_1 by co-chromatography with an authentic sample and no impurities were detected by analytical HPLC. This semi-preparative HPLC could treat fraction I-5 at a rate of *ca.* 20 mg/hr.

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