APPLICATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY TO THE ISOLATION OF GINSENOSIDES FROM GINSENG SAPONINS

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Introduction

The biochemical and pharmacological effects of ginsenosides, the purified saponins from *Panax ginseng* C.A.Meyer, were studied. 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. Separation, purification, and identification are not easy, but chromatographic analyses of ginsenosides have been performed by thin-layer chromatography (TLC) on silica gel, droplet counter-current chromatography, gas liquid chromatography, and rod thin-layer chromatography. High-performance liquid chromatography (HPLC) was done by Drs. Sticher and Soldati.

The isolation of ginsenosides has been accomplished by column chromatography on silica gel, preparative-TLC. Semi-preparative HPLC on silica gel was done by Drs. Chen and Staba. However, these isolation procedures are too time-comsuming to allow the isolation of large quantities of major and minor saponins from ginseng root.

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

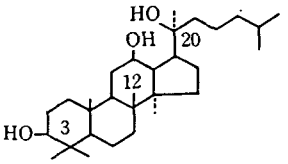
With ginsenoside-Rb_1, -Rb_2, -Rc, and -Rd, the aglycone is 20–S-protopanaxadiol, while on the other hand, with ginsenoside-Rh_1, -Rg_2, -Rg_1, and Re, the aglycone is 20–S-protopanaxatriol. The sugar moieties are different from each other. Therefore, it would be possible to employ the Carbohydrate Analysis column for the separation of ginsenosides.

Materials and Method

Materials: Materials used were four-year-old white ginseng (dried roots of *Panax ginseng* C.A. Meyer) whcih cultivated at Kum-San, Korea, were donated from Korea Ginseng Research Institute, Seoul, Korea.

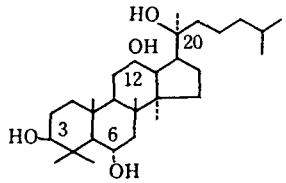
Ginsenosides standard used were donated from professor Osamu Tanaka, Institute of pharmaceutical Sciences, Hiroshima University School of Medicine, professor Junzo Shoji, school of pharmaceutical sciences, Showa University.

Apparatus and Column: Liquid chromatography was performed on the ALC 201 and PrepLC/System-500 (Waters Associates, Inc., U. S.A.), using a refractometer. A stainless steel



20-S-Protopanaxadiol

Ginsenoside	20–P	3–P
Rb_1	D-Glcβ(1→6)D-Glc	D-Glcβ(1→2)D-Glc
Rb_2	L-Ara(pyr)α(1→6)D-Glc	D-Glcβ(1→2)D-Glc
Rc	L-Ara(fur)α(1→6)D-Glc	D-Glcβ(1→2)D-Glc
Rd	D-Glc	D-Glcβ(1→2)D-Glc
(Rb_3)	D-Xylβ(1→6) D-Glc	D-Glcβ(1→2)D-Glc



20-S-Protopanaxatriol

Ginsenoside	20–P	6–P
Re	D-Glc	L-Rhaα(1→2)D-Glc
Rf	—	D-Glcβ(1→2)D-Glc
Rg_1	D-Glc	D-Glc
Rg_2	—	L-Rhaα(1→2)D-Glc
(20-Gluco-Rf)	D-Glc	D-Glcβ(1→2)D-Glc
Rh_1	—	D-Glc

Fig. 1. The structure of ginsenosides

column (30 cm × 3.9mm) packed with Carbohydrate Analysis (Waters Associates, Inc.) for analysis, a stainless steel column(30 cm × 7.8 mm) with the same packing for semi-preparative HPLC, and a PrepPAK-500/Silica cartridge (30 cm × 5.7 cm) for the preparative HPLC were used.

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

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

Also the crude ginseng saponin fraction for the isolation of ginsenoside-Rh_1 is noted in. Fig. 2

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

HPLC Conditions for Identification: Liquid chromatography was performed on ALC 201. A stainless steel column (30 cm × 3.9 mm) packed

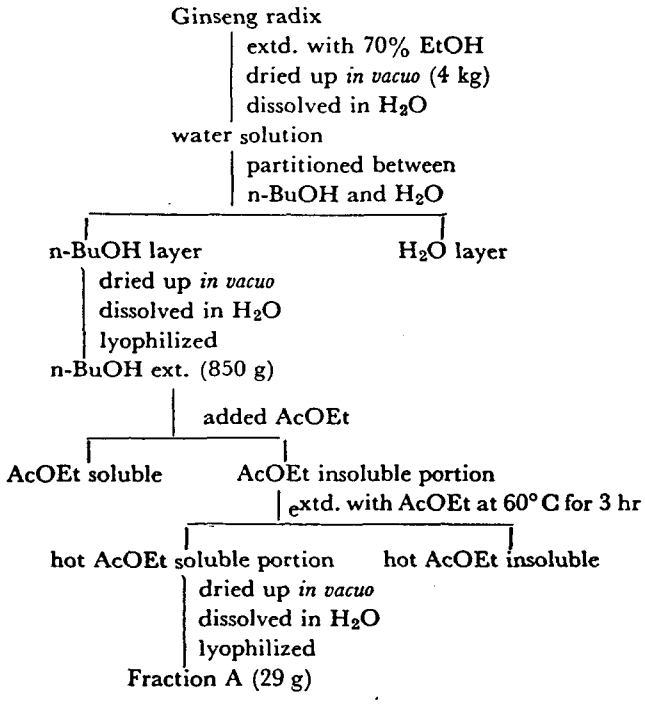


Fig. 2. Extraction and fractionation procedure of crude ginseng saponins for the isolation of ginsenoside-Rh_1.

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 8x or 16x depending on a 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 **b** 4:1:5 (v/v, upper layer). 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 specified in the results. The RI detector was used to determine each ginsenoside. The RI attenuation was set at 16x to 32x depending on the sample load, and the flow rate was 8 ml/min.

Results and Discussion

Analysis of Ginsenosides by HPLC

Ginsenoside-Rb_1, -Rb_2, -Rc, -Rd, -Re, and -Rg_1 were each separated from the mixture by HPLC employing a mixture of acetonitrile (AcCN): H_2O = 80:20 (v/v) as a mobile phase. Resolution of ginsenoside-Rb_1, -Rb_2, -Rc, and -Rd (whose aglycone is 20S-protopanaxadiol) was satisfactory. Resolution of ginsenoside-Rh_1, -Rg_2, -Rg_1, and Re (whose aglycone is 20S-protopanaxatriol) by HPLC employing a mixture of AcCN: H_2O = 86:14 (v/v) as a mobile phase also produced a satisfactory result.

Fractionation of Crude Saponins by Preparative HPLC

Rapid and convenient method for the identification of ginsenosides was developed. So, to reduce isolation time, preparative HPLC was used for the fractionation of crude saponins. The instrument used was prepLC System-500 (Water Associates Inc.) applying two PrepPAK-500/Silica cartridges.

The preparative HPLC conditions were as follows: Mobile phase, n-Butanol: Ethyl-acetate: water = 4:1:5 (v/v, upper phase); Flow rate, 50 ml/min.

The preparative HPLC was carried out 4 times successively for 6 hr with repeated use of the cartridges, and was applied to 18 grams of crude

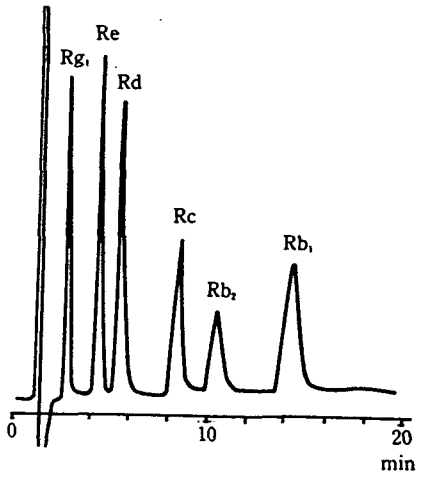


Fig. 3. Chromatogram of ginsenoside—Rg_1, -Re, -Rd, -Rc, -Rb_2, and -Rb_1

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

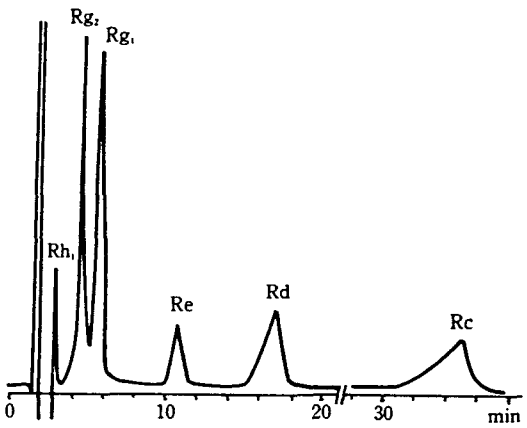


Fig. 4. Chromatogram of ginsenoside-Rh_1, -Rg_2, -Rg_1, -Re, -Rd, and -Rc

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×.

saponins in total. In each HPLC, crude ginseng saponins were fractionated into ten fractions. Corresponding fractions from the preparative HPLC runs were pooled and concentrated. The fractionated saponins were then identified by analytical HPLC. (Fig. 4)

Fig. 6 shows the individual chromatograms of fraction I-X. The HPLC conditions were as follows; mobile phase, AcCN:H_2O = 80:20 (v/v); Flow rate, 2 ml/min.

In fraction I, the peak is Rg_1; in Fr.II, Re, and in Fr. III, Re, Rd, and -Rc; in Fr. IV, Rd,

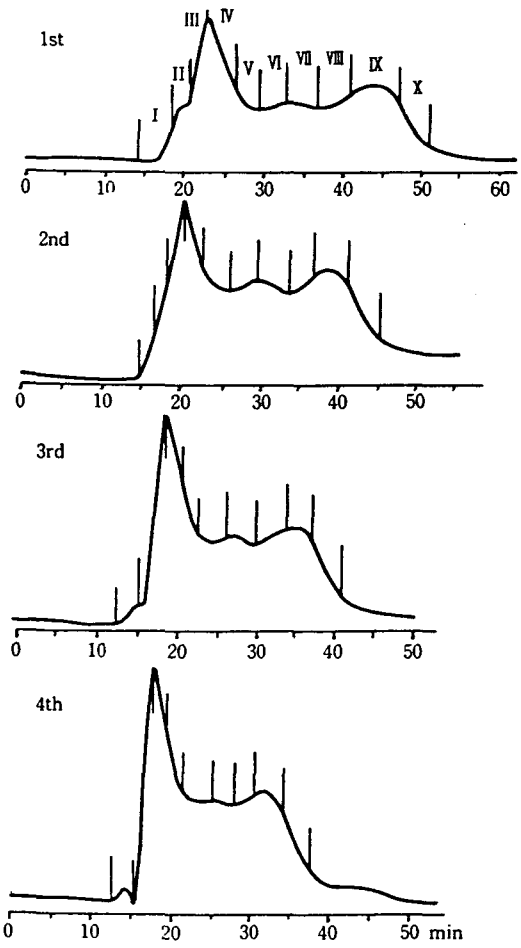


Fig. 5. Elution profile of crude ginseng saponins on Preparative HPLC

Conditions: instrument, PrepLC/System-500; colunm, Prep PAK-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).

and Rc; in Fr. V Rc; in Fr. VI, Rc, Rb_2; in Fr. VII, Rc, Rb_2, Rb_1; in Fr. VIII, Rb_2 Rb_1; in in Fr. IX, Rb_1.

Therefore, HPLC employing the column packed with Carbohydrate Analysis was shown to be suitable for rapid analysis of major components of ginseng saponins.

In order to reduce the isolation time of ginsenoside-Rh_1 and gain a large quantity of the component, preparative HPLC was used for the fractionation of fraction A. The instrument used was PrepLC/System 500 applying two PrepPAK-500/Silica cartridges. The preparative HPLC conditions were as follows: mobile phase, n-Butanol:Ethyl-acetate: water = 4:1:2 (v/v, upper phase), flow rate, 50 ml/min.

The preparative HPLC was carried out twice successively for 4 hr with repeated use of the cartridges, and was applied to 28 grams of fraction A in total. The elution profiles are illustrated. Fraction A-1 was contained mainly ginsenoside-Rh_1. (Fig. 6)

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 that ginsenoside-Rb_1

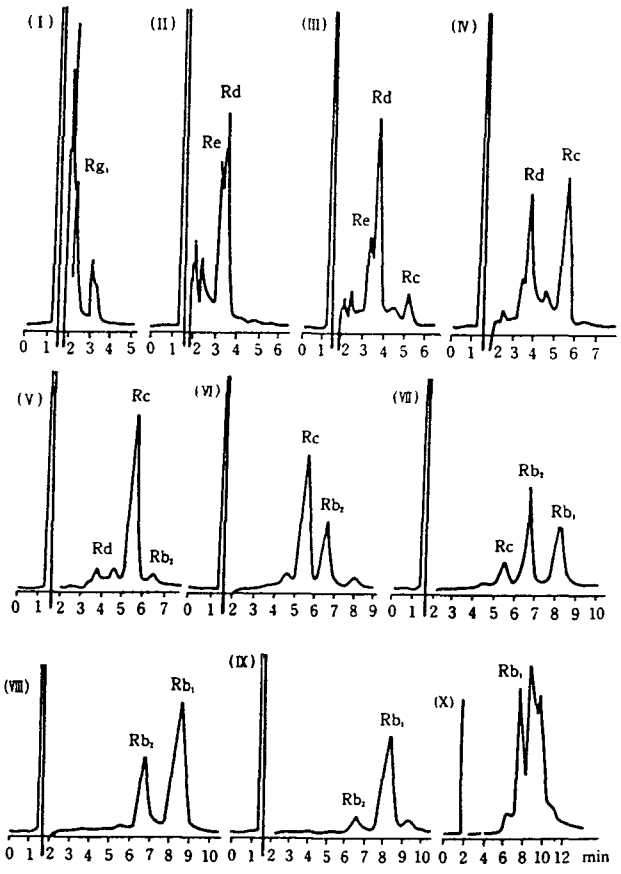


Fig. 6. Individual chromatograms of saponin fractions obtained by preparative HPLC

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 -Rb_2 were contained in fractions VII, VIII, and IX.

Accordingly, ginsenoside-Rb_1 and -Rb_2 were isolated from a mixture of Fr. VII and Fr. VIII by semi-preparative HPLC. The semi-preparative HPLC conditions are as follows: column; length, 30 cm, diameter 7.8 mm; packing, Carbohydrate Analysis, mobile phase, AcCN:H_2O = 81:19(v/v), flow rate, 8 ml/min.

No impurities in the isolated ginsenoside-Rb_1 and -Rb_2 were detected by analytical HPLC.

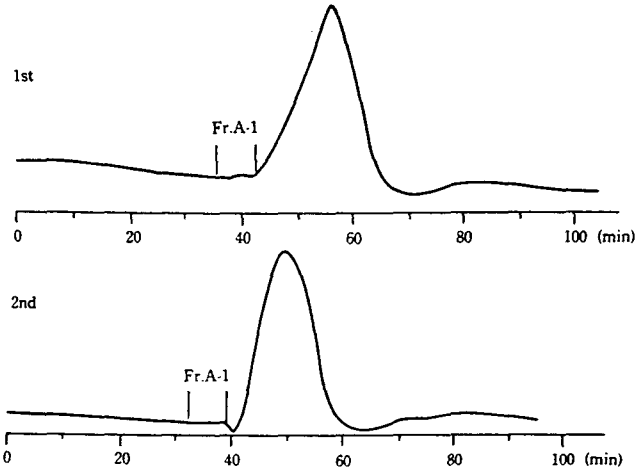


Fig. 7. Elution profiles of fraction A on preparative HPLC

Conditions: instrument, PrepLC/System-500; column, PrepPAK-500/Silica cartridge × 2; mobile phase, BuOH: AcOEt:H_2O = 4:1:2 (v/v, upper phase); flow rate, 50 ml/min; Detector, RI detector (setting 2); sample load, 14 g/60 ml/injection (28 g in total).

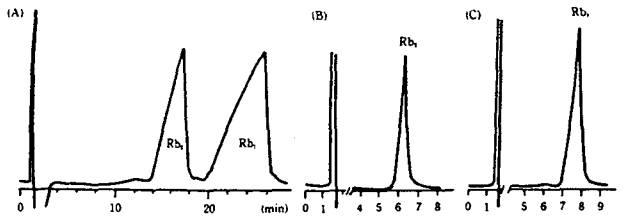


Fig. 8. (A) Elution profile of fractionated saponin (Fr. VII + VIII) on Semi-preparative HPLC, (B, C) Chromatograms of Isolated Ginsenoside-Rb_1 and Rb_2

Conditions: (A) column, 30cm × 7.8 mm; packing, Carbohydrate Analysis; mobile phase, AcCN:H_2O = 81:19 (v/v); flow rate 8 ml/min; RI detector, attenuation 16×; sample load, 17mg/2ml/injection; (B, C) column, 30cm × 3.9mm; packing, Carbohydrate Analysis; mobile phase, AcCN:H_2O = 80:20 (v/v); flow rate, 2ml/min; RI detector, attenuation 8×

Isolation of Ginsenoside-Rc by Semi-preparative HPLC

Ginsenoside-Rc was isolated from Fr. V by semi-preparative HPLC. The HPLC conditions were as follows: mobile phase, AcCN:H_2O = 82:18 (v/v), flow rate, 8 ml/min.

No impurities in the isolated ginsenoside-Rc were detected by analytical HPLC. (Fig. 9)

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

Ginsenoside-Rd and -Re were isolated from a mixture of Fr. II and Fr. III. The preparative HPLC conditions were as follows: mobile phase, AcCN:H_2O = 86:14 (v/v), flow rate, 8 ml/min.

No impurities were detected in the isolated ginsenosides by analytical HPLC. (Fig. 10)

Isolation of Ginsenoside-Rg_1 and -Rg_2 by Semi-preparative HPLC

In preparative HPLC, ginsenoside-Rg_1 was contained in Fr. I. This fraction was further fractionated by semi-preparative HPLC. The HPLC conditions were as follows: mobile phase, AcCN:H_2O = 89:11 (v/v), flow rate, 8 ml/min.

As shown in Fig. 11 Fr. I was fractionated into 7 subfractions. Judging from retention time, Fr. I-5 may be ginsenoside-Rg_1. However, this

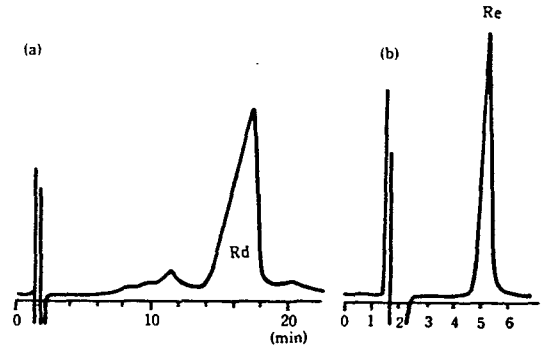


Fig. 9. (A) Elution profile of fractionated saponin (Fr. V) on semi-preparative HPLC. (B) chromatogram of Isolated Ginsenoside-Rc

Conditions: (A) column, 30 cm × 7.8 mm; packing, Carbohydrate Analysis; mobile phase, AcCN:H_2O = 82:18 (v/v); flow rate, 8 ml/min; RI detector, attenuation 16×; sample load, 16 mg/ml/injection; (B) 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×

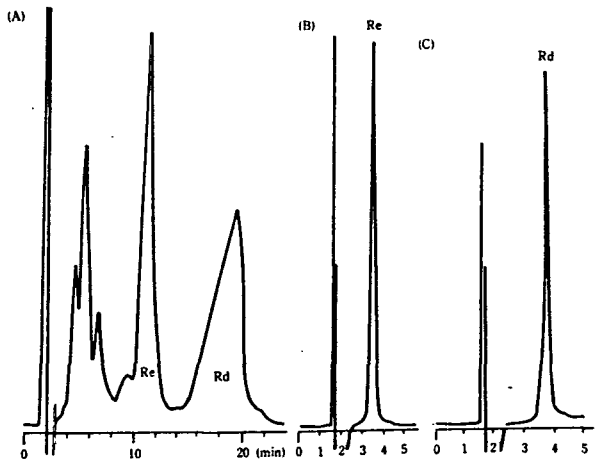
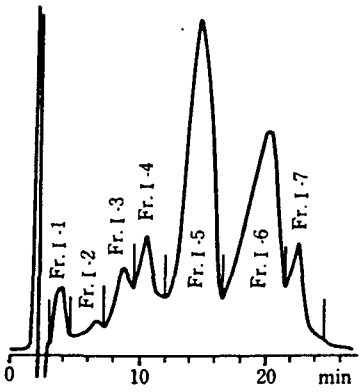


Fig. 10. (A) Elution profile of fractionated saponin (Fr. II + III) on Semi preparative HPLC, (B, C) Chromatograms of Isolated Ginsenoside-Re and -Rd

Conditions: (A) column, 30 cm × 7.8 mm; packing, carbohydrate analysis; mobile phase, AcCN:H_2O = 84:16 (v/v); flow rate, 8ml/min; RI detector, attenuation 16 ×; sample load, 15 mg/ml/injection; (B, C) 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 ×



Elution profile of fractionated

Fig. 11. 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.

subfraction contained 2 components.

Therefore, ginsenoside-Rg_1 was isolated from fraction I-5 by re-cycle chromatography.

The re-cycle chromatography conditons were as follows: mobile phase, AcCN:H_2O = 87:13 (v/v), flow rate, 8 ml/min.

In re-cycle chromatography, Fr. I-5 was separated into fractions I-5–1 and I-5–2. (Fig 12)

This Fig. 13 shows the chromatograms of authentic sample and isolated ginsenoside-Rg_1, and the chromatogram of co-chromatography.

Ginsenoside-Rg_2 was isolated from Fr. I-4 by HPLC. The HPLC conditions were as follows: column, 30 cm × 3.9 mm, flow rate. 2 ml/min, mobile phase, AcCN:H_2O= 86:14 (v/v).

As shown in Fig. 14, Fr. I-4 was further separated into 2 fractions.

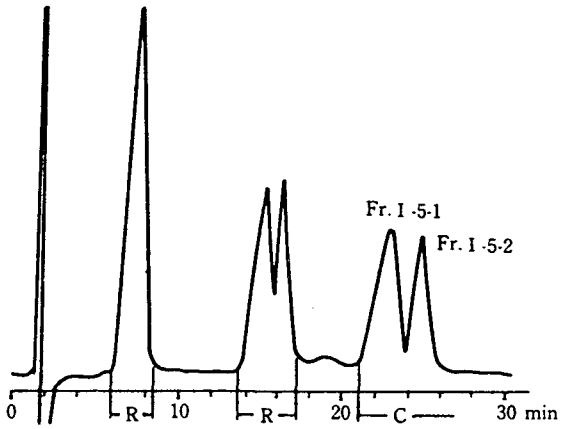


Fig. 12. Chromatogram of fraction I–5 on recycle chromatography

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.

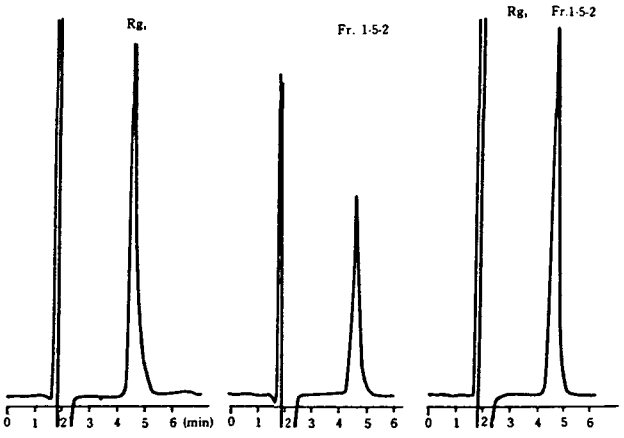


Fig. 13. Chromatograms and co-chromatogram of ginsenoside-Rg_1 and isolated ginsenoside-Rg_1(fraction I-5-2)

Conditions: column, 30 cm × 3.9 mm; packing, Carbohydrate Analysis; mobile phase, AcCN:H_2O = 85:15 (v/v); flow rate, 2 ml/min; RI detector, attenuation 16 ×

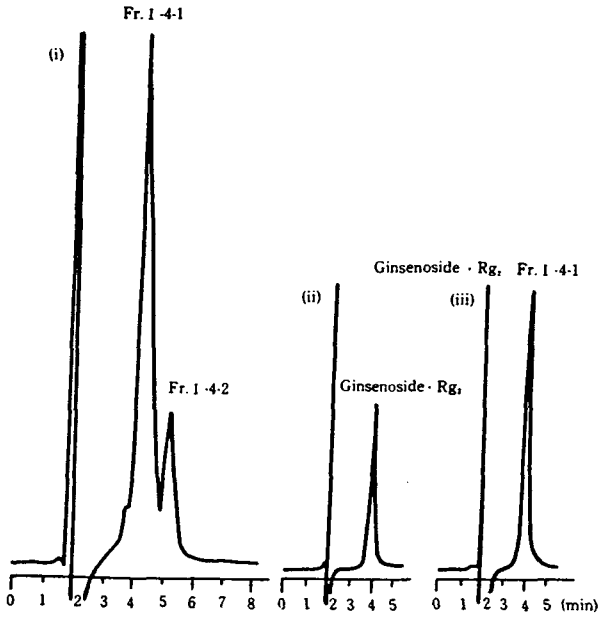


Fig. 14. Elution profiles of fraction I-4[1] and chromatograms of ginsenoside-Rg_2 and isolated ginsenoside-Rg_2 (Fr. I–4–1)

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×.
(i); fraction I–4
(ii); authentic sample
(iii); authentic sample and isolated ginsenoside-Rg_2 (Fr. (I–4–1)

In co-chromatography with an authentic sample, Fr. I-4-1 was identified as ginsenoside-Rg_2.

This slide illustrates the chromatograms of ginsenoside-Rg_2 and Fr. I-4–1, and the co-chromatogram of an authentic sample and isolated ginsenoside-Rg_2.

Isolation of Ginsenoside-Rf by Semi-preparative HPLC

The minor components of ginseng saponin, ginsenoside-Rf was contained in fraction I-6. So, ginsenoside-Rf was isolated from fraction I-6 by semi-preparative HPLC. The HPLC conditions as follows: mobile phase, AcCN:H_2O = 86:14 (v/v), flow rate, 8 ml/min.

This slide shows the chromatograms of Fr. I-6 and authentic samples, and the co-chromatogram of authentic sample and isolated ginsenoside-Rf.

Isolation of Ginsenoside-Rh_1 by Semi-preparative HPLC

Fraction A-1 obtained by the preparative HPLC was further fractionated for the enrichment of ginsenoside-Rh_1 by the method of partition between ether, ethyl-acetate and water, as shown in Fig. 16

Fig. 17 shows the individual chromatograms of fractions A-1–1, A-1–2, and A-1–3 by analytical HPLC. The HPLC conditions were as follows: mobile phase, AcCN:H_2O = 92:8, flow rate, 2 ml/min

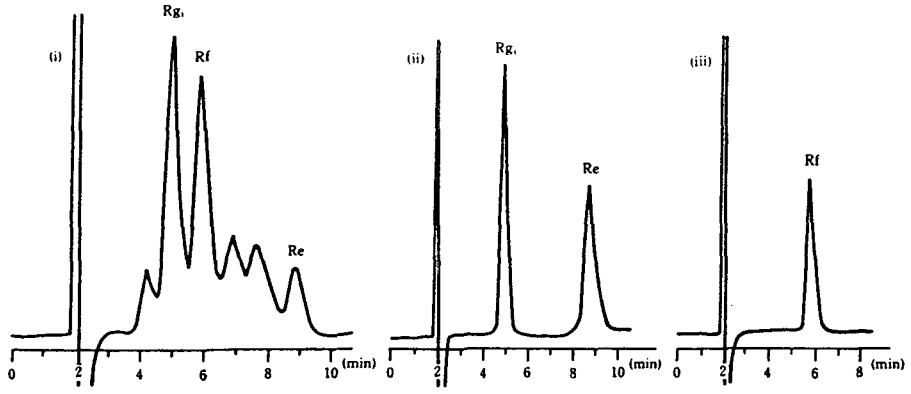


Fig. 15. Chromatograms of fraction I-6[1], ginsenoside-Rg_1, ginsenoside-Rf, and ginsenoside-Re

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×.
(i); fraction I–6
(ii); ginsenoside-Rg_1 and ginsenoside-Re
(iii); authentic sample and isolated ginsenoside-Rf

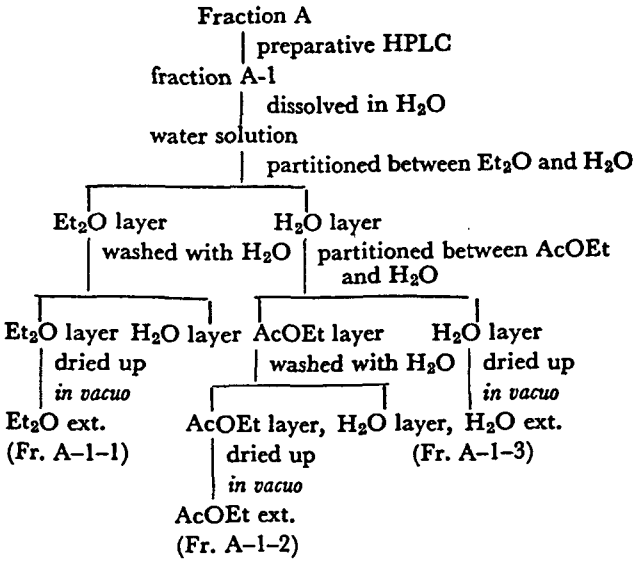


Fig. 16. Further fractionation procedure from fraction A-1

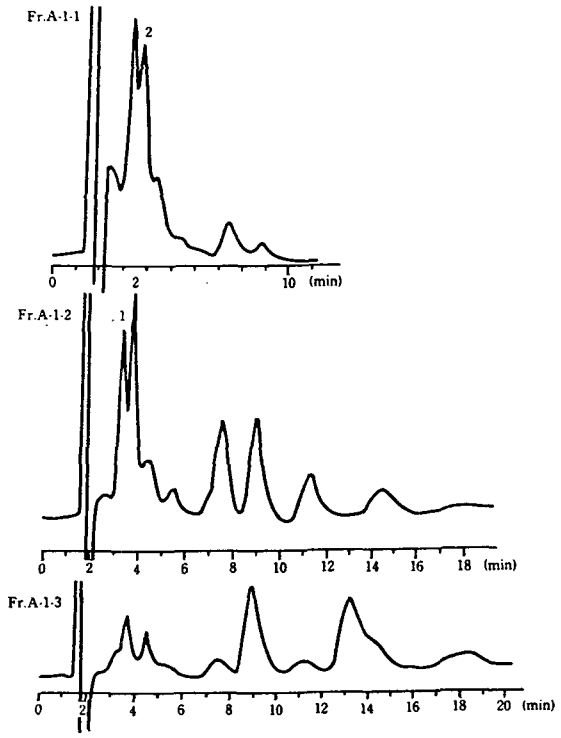


Fig. 17. Elution profiles of fractions A-1-1, A-1-2, and A-1-3

Conditions: column, 30 cm × 3.9 mm; packing, carbohydrate Analysis; mobile phase, AcCN:H_2O = 92.8 (v/v); flow rate, 2 ml/min; RI detector, attenuation, 16×.
peak 2; ginsenoside-Rh_1

This peak (peak 2) may be ginsenoside-Rh_1, judging from the retention time and co-chromatography with an authentic sample.

Therefore, ginsenoside-Rh_1 was isolated from fraction A-1-2.

The fraction A-1-2 was further fractionated by semi-preparative HPLC, eluting with a mixture of AcCN:H_2O = 89:11 (v/v) at 8 ml/min.

The chromatogram is shown in Fig. 18. Ginsenoside-Rh_1 was enriched this fraction.

In the previous procedure, ginsenoside-Rh_1 was enriched in the subfraction A-1-2a. Accordingly, it was isolated from the fraction by semi-preparative HPLC, eluting with a mixture of AcCN:H_2O = 94:6, at the flow rate of 8 ml/min.

Fig. 19 shows the elution profile. This Fr. I-1-2a-1 fraction was identified as ginsenoside-Rh_1.

The major components of saponin-ginsenoside -Rb_1, -Rb_2, -Rc, -Rd, Re and -Rg_1 and the minor components of saponin-ginsenoside -Rf, -Rg_2 and -Rh_1 were isolated from *Panax ginseng* C.A. Meyer by high performance Liguid chro-

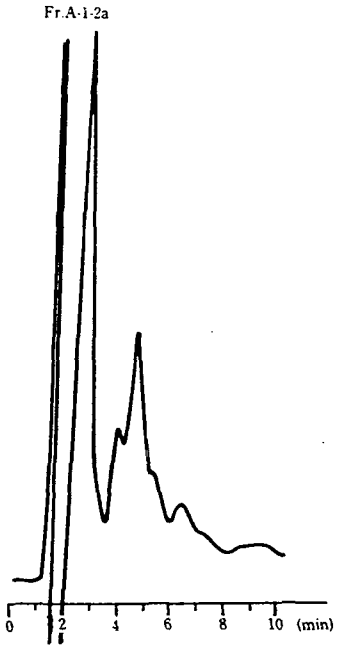


Fig. 18. Elution profile of fraction A-1-2 on semi-preparative HPLC

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×.

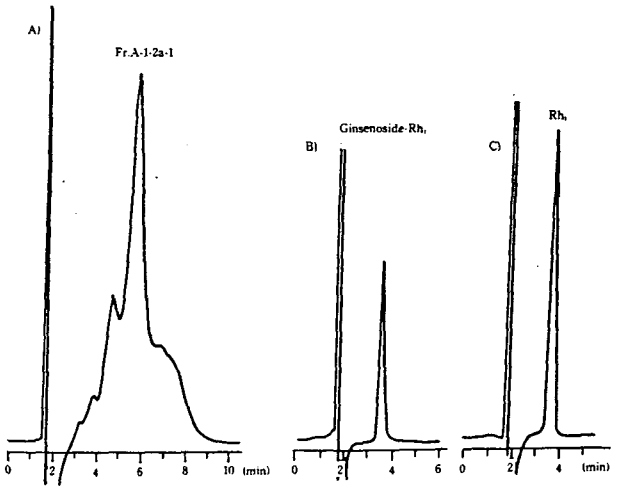


Fig. 19. A) Elution profile of fraction A-1-2a on semi-preparative HPLC B,C) chromatograms of Ginsenoside-Rh_1 and fraction A-1-2a-1 (isolated ginsenoside-Rh_1) on analytical HPLC

Conditions: A) column, 30 cm × 7.8 mm; packing, carbohydrate Analysis; mobile phase, AcCN:H_2O = 94:6 (v/v); flow rate, 8 ml/min; RI detector, attenuation 16×, B, C) column, 30 cm × 3.9 mm; packing, carbohydrate analysis; mobile phase, AcCN:H_2O = 90:10 (v/v); flow rate, 2 ml/min; RI detector, attenuation 8×.

A) fraction A-1-2a

B) authentic sample

C) isolated ginsenoside-Rh_1 (Fr. A-1-2a-1) and authentic sample

matography, that is, preparative, semi-preparative and analytical HPLC. The application of HPLC to the isolation of ginsenosides was not only available for rapid analysis but also reduced the isolation time. This work was very efficient and to be useful for further studies in biological and pharmacological aspects at ginsenoside Level

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