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Isolation of Ginsenosides Rb1, Rb2, Rc, Rd, Re, Rf and Rg1 from Ginseng Root by High Performance Liquid Chromatography

Nam Ho Paik, Man Ki Park, Kang Ju Choi* and Yung Hyun Cho

*College of Pharmacy, Seoul National University, Seoul 151 and *Korea Ginseng and Tobacco Research Institute, Seoul 110, Korea*

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Abstract □ Ginsenosides Rb1, Rb2, Rc, Rd, Re, Rf and Rg1 were effectively isolated from ginseng root by preparative liquid chromatography (LC) on two PrepPAK-500/C18 cartridges in series and semi-preparative LC on a μBondapak cabohydrate analysis column, a μBondapak C18 column or a μPorasil column. The identities of the isolated ginsenosides were confirmed by analytical high-performance liquid chromatography (HPLC) and infrared spectrophotometry. By this method large scale isolation of pure ginsenosides was efficiently accomplished.

Keywords □ Ginseng root—ginsenoside—preparative and semi-preparative liquid chromatography—fractionation—isolation—identification

Parts of ginseng medicinal effects were successfully proved by many scholars, but their most studies were achieved only at the level of ginseng extract, because large quantities of pure ginsenosides contained in ginseng root alone could not easily isolated. Recently ginsenosides have been isolated from ginseng root by column chromatography on silicagel[1,2], alumina[3] and gel filtration[4], preparative thin-layer chromatography on silicagel[5,6] semi-preparative LC on silicagel[7], and preparative LC on silicagel[8,9]. Some HPLC methods for separation and determination of ginsenosides in ginseng root have been already known.[10-13]

This paper describes the method that large quantities of pure ginsenosides Rb1, Rb2, Rc, Rd, Re, Rf and Rg1 were isolated from ginseng root by LC, using preparative LC on two PrepPAK-500/C18 cartridges in series followed by semi-preparative LC on a μBondapak carbohydrate analysis column, μBondapak C18 column or μPorasil column. We determined the purites and identities of the isolated ginsenosides by analytical HPLC on a μBondapak carbohydrate analysis column using mixtures of acetonitrile-water as mobile phases.

EXPERIMENTAL METHODS

Equipment

Liquid chromatography was performed with ALC-244 and PrepLC/system-500 (Waters Associates, Inc., Milford, Mass., USA), using a differential refractometer (RI detector).

In analytical HPLC, ALC-244 was equipped with a stainless steel column (3.9mmφ×30cm) packed with μBondapak carbohydrate analysis (Waters Associates, Inc.).

In semi-preparative LC, ALC-244 was equipped with a stainless steel column (7.8mmφ×30cm) packed with μBondapak carbohydrate analysis, μBondapak C18 or μPorasil (Waters Associates, Inc.).

In preparative LC PrepLC/system-500 was equipped with two PrepPAK-500/C18 cartridges

(5.7cmϕ×30cm, Waters Associates, Inc.) in series. Identities of the isolated ginsenosides were carried out by Perkin-Elmer 599B infrared spectrophotometer (KBr pellet).

Chemicals

Extra pure acetonitrile (Kanto Co., Japan) was used in preparative LC. Acetonitrile LiChrosolv and n-butanol LiChrosolv (Merck Co.) were used in analytical or semi-preparative LC. Water with an 18-megaohm resistivity level by ion exchange was prepared at our laboratories by the Milli-Q system. All solvents were filtered through Millipore FH-type membrane filter (pore size 0.45μm) before use.

Materials

Ginseng crude saponin was prepared as follows; dried ginseng root was extracted with hot methanol. The methanol extract was well mixed with a small portion of sillicagel washed with methanol, filtered through Toyo No. 5A filter paper, and then concentrated *in vacuo*. Water was added to the extract and then the suspension was extracted with ethyl ether. The aqueous layer was extracted with water-saturated n-butanol three times. The n-butanol layers were comined and finally washed with water. The n-butanol layer was evaporated to afford the crude saponin. Authentic ginsenosides were provided by J.H. Choi, Korea Ginseng and Tobacco Research Institute, Seoul, Korea.

Fractionation of Crude Saponin by Preparative LC

12.5g of ginseng crude saponin were dissolved in 50mls of carrier solvent (acetonitrile: water =40 : 60), and the solution was filtered through Millipore FH-type membrane filter before loading. Ten mls of the sample solution were loaded into PrepPAK-500/C18 cartridges and eluted with a mixture of acetonitrile-water (40 : 60) at a flow rate of 12ml/min.

Isolation of Each Ginsenoside by Semi-preparative LC

After removal of the solvent by evaporation *in vacuo*, each fractionated saponin was dissolved in 70% methanol. Sample solutions were filtered through Millipore FH-type membrane filter before loading. In semi-preparative LC with ALC-244, a reversed-phase system with μBondapak carbohydrate analysis column or μBondapak C18 column using acetonitrile-water as eluent was employed; or a normal phase system with μPorasil using the lower phase of chloroform - methanol - water (65 : 35 : 10) as eluent was employed. The eluent was specified in "Results and Discussion".

Identification of Ginsenosides

All solutions of the fractionated saponins and isolated ginsenosides were, if necessary, filtered through Millipore FH-type membrane filters before injection. Identification of ginsenosides was carried out on a μBondapak carbohydrate analysis column. The mobile phase was composed of acetonitrile and water. The differential refractometer was used to detect each ginsenoside, and its attenuation was set at 8×. These identities were further confirmed by their infrared spectra.

RESULTS AND DISCUSSION

Separation and Identification of Ginsenosides

Liquid chromatograms of crude saponin are in Fig. 1. We assigned each peak to the known ginsenoside, judging from the retention time of an authentic sample. We could separate the ginsenosides Rb1, Rb2, Rc, Rd, Re, Rf, Rg1 and Rg2 on a μBondapak C18 column with mixtures of acetonitrile-water (Fig. 1, Fig. 4). Also on a μBondapak carbohydrate analysis column the ginsenosides could be separated. As can be seen

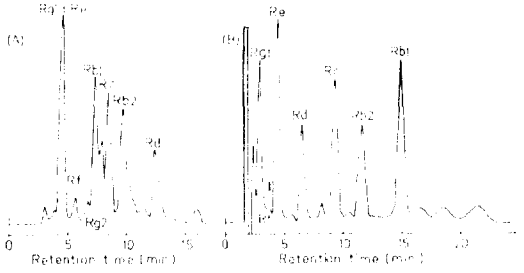

Fig. 1: HPLC chromatograms of ginseng crude saponins.
(A): column, μBondapak C18 (3.9mmφ×30 cm); eluent, acetonitrile-water (40:60); flow rate, 0.7ml/min; detector, RI 8×
(B): column, μBondapak carbohydrate analysis (3.9mmφ×30cm); eluent, acetonitrile-water (80:20); flow rate, 1.5ml/min; detector, RI 8×

from Fig. 1 the liquid chromatogram on a μBondapak C18 column is different from that on a μBondapak carbohydrate analysis column in the elution sequence of each ginsenoside. So PrepPAK-500/C18 cartridge was applied in the preparative LC and a μBondapak carbohydrate analysis column was mainly applied in the semi-preparative LC. A μBondapak carbohydrate analysis column was more suitable for the identification of each ginsenoside, because of the better resolution for ginsenosides as compared with a μBondapak C18 column.

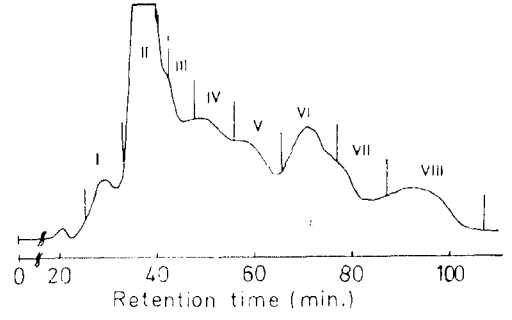

Fig. 2: Fractionation profile of crude saponins on preparative liquid chromatography. Column, PrepPak-500/C18 cartridge (5.7cmφ×30cm)×2; eluent, acetonitrile-water (40:60); flow rate, 12ml/min; detector, RI (set at 20); sample load, 2.5g/10ml.

Fractionation of Crude Saponin

Fig. 2 shows a fractionation profile. Single loading amount of crude saponin was 2.5g/10 ml. Crude saponin was eluted for 100 minutes and fractionated into 8 fractions as shown in Fig. 2. Each fraction was evaporated *in vacuo* and then its constituents were identified by analytical HPLC, resulting with mixtures of 3 or 4 ginsenosides (Fig. 3).

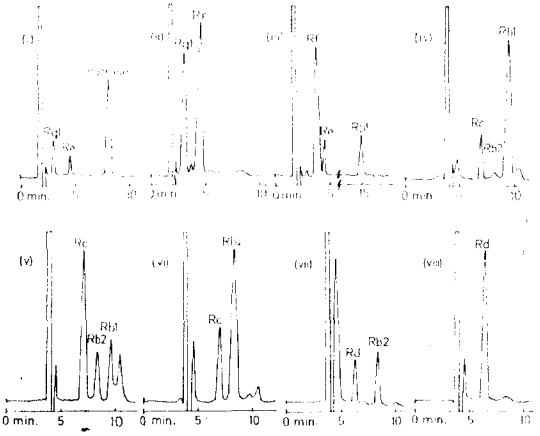

Fig. 3: Individual chromatograms of each fraction obtained by preparative liquid chromatography.
(I)-(III): Conditions same as in the chromatogram (B) of Fig. 1.
(IV)-(VIII): Column, μBondapak carbohydrate analysis (3.9mmφ×30cm); eluent, acetonitrile-water (75:25); flow rate, 1.0 ml/min; detector, RI 8×

Isolation of Ginsenosides Rg1 and Re

Ginsenosides Rg1 and Re were contained in fraction II and isolated by semi-preparative LC. A reversed-phase system with a μBondapak C18 column using acetonitrile-water (21 : 79) 5ml/min was adopted for the isolation of ginsenosides Rg1 and Re from fraction II by repeated runs. Single loading amount of fraction II was appro-

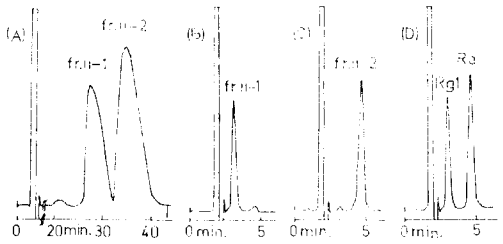

Fig. 4: Elution profile of fraction II by semi-preparative LC(A) and chromatograms of isolated fr. II-1, fr. II-2 and authentic samples (B, C,D)
(A): column, μBondapak C18 (7.8mmφ×30 cm); eluent, acetonitrile-water (21:79); flow rate, 5.0ml/min; detector, RI 64×; sample load, 10mg/1.2ml
(B,C,D): conditions same as in the chromatogram (B) of Fig. 1

ximately 10mg/1.2ml. As shown in Fig. 4, fraction II was separated into fraction II-1 and fraction II-2. Fraction II-1 and fraction II-2 were, respectively, identified as ginsenosides Rg1 and Re by analytical co-chromatography with an authentic sample. The productivities of ginsenosides Rg1 and Re were about 6.2mg/hr and 8.7mg/hr, respectively. Also fraction II could be further seperated into fraction II-3 and fraction II-4 by recycle chromatography

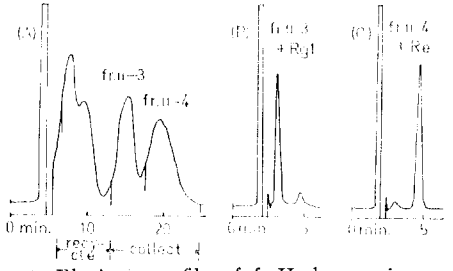

Fig. 5: Elution profile of fr.II by semi-preparative LC(A) and co-chromatorgrams of authentic samples and isolated fractions(B,C)
(A): column, μPorasil (7.8mmφ×30cm); eluent, chloroform-methanol-water(65:35:10, lower phase); flow rate, 4.0ml/min; detector, RI 64×; sample load, 6.7mg/0.8ml
(B,C): conditions same as in the chromatogram (B) of Fig. 1

with a μPorasil column (7.8mmφ×30cm) employing the lower phase of chloroform-methanol-water (65 : 35 : 10). Judging from the retention time and co-chromatography with an authentic sample, fraction II-3 and fraction II-4 were identified as ginsenosides Rgl and Re, respectively (Fig. 5). A loading amount of fraction II was about 6.0mg, and the yields of ginsenosides Rgl and Re were 2.3mg and 3.0mg, respectively. No impurities in the isolated ginsenosides Rgl and Re were detected by analytical HPLC. Infrared spectra of the isolated ginsenosides Rgl and Re were identical to those reported by Han et al.[14] and Shibata et al.[2]

Isolation of Ginsenoside Rf

Ginsenoside Rf was isolated from fraction III with a μBondapak carbohydrate analysis column, eluting with a mixture of acetonitrile-water (81:19) at a flow rate 5ml/min by repeated runs. A loading amount of fraction III was approximately 9.0mg/1.0ml. The productivity was about 20mg/hr. The identity of the isolated ginsenoside Rf was confirmed by analytical co-chromatography with an authentic sample and infrared spectrum. Infrared spectrum of the isolated ginsenoside Rf was identical to that reported by Shibata et al.[2] No impurities in the isolated ginsenoside Rf were detected by analytical HPLC.

Isolation of Ginsenosides Rb1, Rb2 and Rc

Ginsenoside Rb1 was mainly present in fraction IV; ginsenoside Rb2, in fraction VI; ginsenoside Rc, in fraction V. By the semi-preparative LC on a μBondapak carbohydrate analysis column with eluent acetonitrile-water(80:20), we seperated ginsenosides Rb1, Rb2 and Rc from these fractions by repeated runs. The eluent was flowed at 6ml/min. A loading amount of fraction IV was about 11.6mg/1.0 ml; fraction V, about 12.1mg/1.2ml; and

fraction VI, about 10.8mg/1.0ml The productivity of ginsenoside Rb1 from fraction IV was about 17mg/hr; ginsenoside Rb2 from fraction VI, about 17mg/hr; and ginsenoside Rc from fraction V about 15mg/hr. The identities of the isolated ginsenoside Rb1, Rb2 or Rc were confirmed by co-chromatography with an authentic sample and infrared spectra. Their infrared spectra were identical to those reported by Han et al.[14] and by Shibata et al.[1] No impurities in the isolated ginsenosides Rb1, Rb2 or Rc were detected by analytical HPLC.

Also on a μBondapak C18 column (7.8mmφ ×30cm) with eluent acetonitrile-water-methanol (32:68:0.5), ginsenosides Rb1 and Rc could be purely isolated from a mixture of fraction IV and V. A loading amount was approximately 8mg, the yield was about 2.6mg ginsenoside Rb1 and about 3.2mg ginsenoside Rc (Fig. 6).

Isolation of Ginsenoside Rd

Ginsenoside Rd occurred in fraction VIII as a major component and was isolated on a semi-preparative μBondapak carbohydrate analysis column by repeated runs. A mixture of acetonitrile-water (82:18) as a mobile phase was flowed at a rate of 6ml/min. A loading amount of fraction VIII was *ca.* 10mg/1.4ml, and the productivity of ginsenoside Rd was about 25mg/hr. The identity of the isolated ginsenoside Rd was confirmed by analytical co-chromatography and infrared spectrum. The infrared spectrum was identical to that reported by Shibata et al.[1] No impurities in the isolated ginsenoside Rd were detected by analytical HPLC.

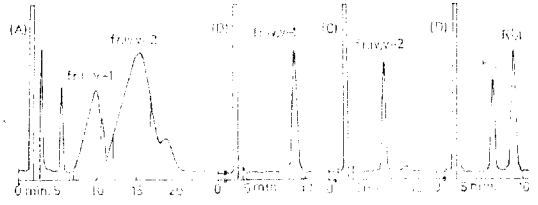

Fig. 6: Elution profile of a mixture of fraction IV and V(A) and chromatograms of isolated fractions and authentic samples (B,C,D)
(A): column, μBondapak C18 (7.8mmφ× 30cm); eluent, acetonitrile-water-methanol (32:68:0.5); flow rate, 3.2ml/min; detector, RI 32×; sample load, 8.0mg/1.0ml
(B,C,D): conditions same as in the chromatogram (IV) of Fig. 3

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