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Isolation of Ginsenoside-Rh_1 and -Rh_2 by High Performance Liquid Chromatography

Jin Ho Choi, Woo Jung Kim, Soon Keun Hong, Sung Ki Oh* and Hikokichi Oura**

*Korea Ginseng Research Institue, Seoul 110, Korea, *Department of Food Technology, Kyunghee University, Seoul 131, Korea, **Research Institute for WAKAN-YAKU, Toyama Medical and Pharmaceutical University, Toyama, Japan*

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高速液體 크로마토그래피에 依한 Ginsenoside -Rh_1 및 -Rh_2의 分離

崔 鎭浩 · 金 友政 · 洪 淳根 · 吳 成基* · 大浦 彦吉**

高麗人蔘硏究所, *慶熙大學校 食品加工學科, **富山醫科藥科大學 和漢藥硏究所

(1981년 1월 23일 수리)

Abstract

An effective method for isolation of the major components of ginseng saponin such as ginsenoside-Rb_1, -Rb_2, -Rc, -Rd, -Re and -Rg_1 and the minor components such as ginsenoside-Rf, -Rg_2 and -Rh_1 was developed and reported in previous papers (*J. Korean Agr. Chem. Soc.*, 23(4), 199 and 206(1980) The conditions and procedures used for isolation and identification for ginsenosides described in the previous papers were not sufficient enough for clean separation of minor components, ginsenoside-Rh_1 and -Rh_2.

In this work, modifications in extraction method and in mobile phase for HPLC were attempted. It was found that application of ethyl acetate extraction at 60°C for 3 hr on crude saponin resulted in a removal of diol group saponin from crude saponin which made it possible for using higher portion of acetonitrile in mobile phase. The mixed solvents of acetonitrile : water (92 : 8 and 94 : 6) gave excellent resolution of ginsenoside-Rh_1 and -Rh_2.

Introduction

A number of works concerning isolation and identificattn of ginseng saponin have been reported since the saponin was proved to be one of the most active principles for ginseng effect. Chromatographic methods such as thin layer[1,2], droplet countercurrent [3] and gas liquid phase chromatographies [4,5] have been frequently used for the isolation of ginseng saponin. These methods appear to be time consuming and sometimes inapplicable for quantitative analysis for minor component of saponin. Recently, high performance liquid chromatography

(HPLC) has been emerged as a powerful tool for the isolation of large quantities of ginseng components and also for the rapid analysis.

An application of HPLC [6~12] attracted great attention to many workers in ginseng research for obtaining pure saponin and ginsenosides. As reported in our previous papers [9~10], we also used analytical and preparative HPLC very efficiently for the isolation of ginseng components in large quantities. The saponins isolated were ginsenoside-Rb_1, -Rb_2, -Rc, -Rd, -Re, -Rf, -Rg_1, -Rg_2 and -Rh_1. However, the previous HPLC method for the isolation of ginsenoside-Rh_1 and -Rh_2 was found to de impratical.

In this investigation, attempts were made to improve the previous HPLC isolation technique for separation of ginsenoside -Rh_1 and -Rh_2 in ginseng by the modification in extraction method and mobile phase.

Materials and Methods

Materials

The material used was 6 year old red ginseng which was manufactured and supplied by the Korea Ginseng Factory of the Office of Monopoly, Buyo, Korea. Authentic sample used for ginsenosides identification was donated from Department of Biochemistry, Research Institute for WAKAN-YAKU, Toyama Medical and Pharmaceutical University, Toyama, Japan. The mobile phases used for HPLC were mixed solvents of acetonitrile and water (89 : 11, 90 : 10, 92 : 8, 94 : 6). All of the samples were filtered through 0.45 μm TM-2P filter(Waters Associates, Inc., Milford, Mass., U.S.A.) before injection.

Liquid chromatographs used were preparative HPLC (prep LC/system-500), analytical HPLC and semipreparative HPLC (ALC-201, Waters Associates, Inc., Milford, Mass., U.S.A.) equipped with a Refractometer R-401 (RI detector). A prep PAK-500/silica cartridge (57 *mm* ID×30 *cm*), μ Bondapak C_{18} column (7.8 *mm* ID×30 *cm*) and carbohydrate analysis column (3.9 *mm* ID×30 *cm*) and carbohydrate analysis column (3.9 *mm* ID×30 *cm*) were used for prep LC/system-500, semipreparative and analytical identification, respectively.

Extraction and fractionation

As shown in Fig. 1, 10 *kg* red ginseng which was extracted with 70% ethanol for 8 hr at 80~90°C resulted in 4 *kg* of the concentrated extract (40% yield from the 74° Bx material). The extract was then diluted with water, and was again extracted with *n*-butanol saturated with water. After the *n*-butanol layer was washed with water and evaporated in vacuum, it was finally lyophilized to give a crude butanol extract, and obtained 580 *g* of the crude butanol extract (5.8% yield of the dried material). This crude butanol extract was extracted with 6 *l* of ethyl acetate for 3 hr at room temperature and then the residue (Ⅰ) was extracted with 6 *l* of ethyl acetate for 3 hr at 60°C to separate ethyl acetate layer (Ⅱ). As a result, 29 g of ethyl acetate layer (Ⅱ) were obtained from 4 *kg* of the ethanol extract. This ethyl acetate extract was dissolved in carrier solvent containing approximately 10% methanol, followed by filtration through a membrane filter TM-2P before injection. The ethyl acetate layer (Ⅱ) was fractionated into 25 g fractions by prep LC/System-500 equiped with a prep PAK-500/silica cartridge. The cartridges were eluted with a mixed solvent of *n*-butanol : ethyl acetate : water (4 : 1 : 2, upper phase) at a flow rate of 50 *ml*/min.

Isolation and identification of ginsenosides

The isolation and identification of ginsenosides were performed by analytical HPLC. A partition and reverse phase system with a μ Bondapak C_1 column using a series of mixed solvents of acetonitrile : water (89 : 11, 92 : 8 and 94 : 6) at a flow rate of 8 *ml*/min was used for the isolation of ginsenoside-Rh_1, and -Rh_2 Before the isolation each fraction was occasionally fractionated once more by semipreparative HPLC if necessary, evaporated in vacuum, dissolved in carrier solvent

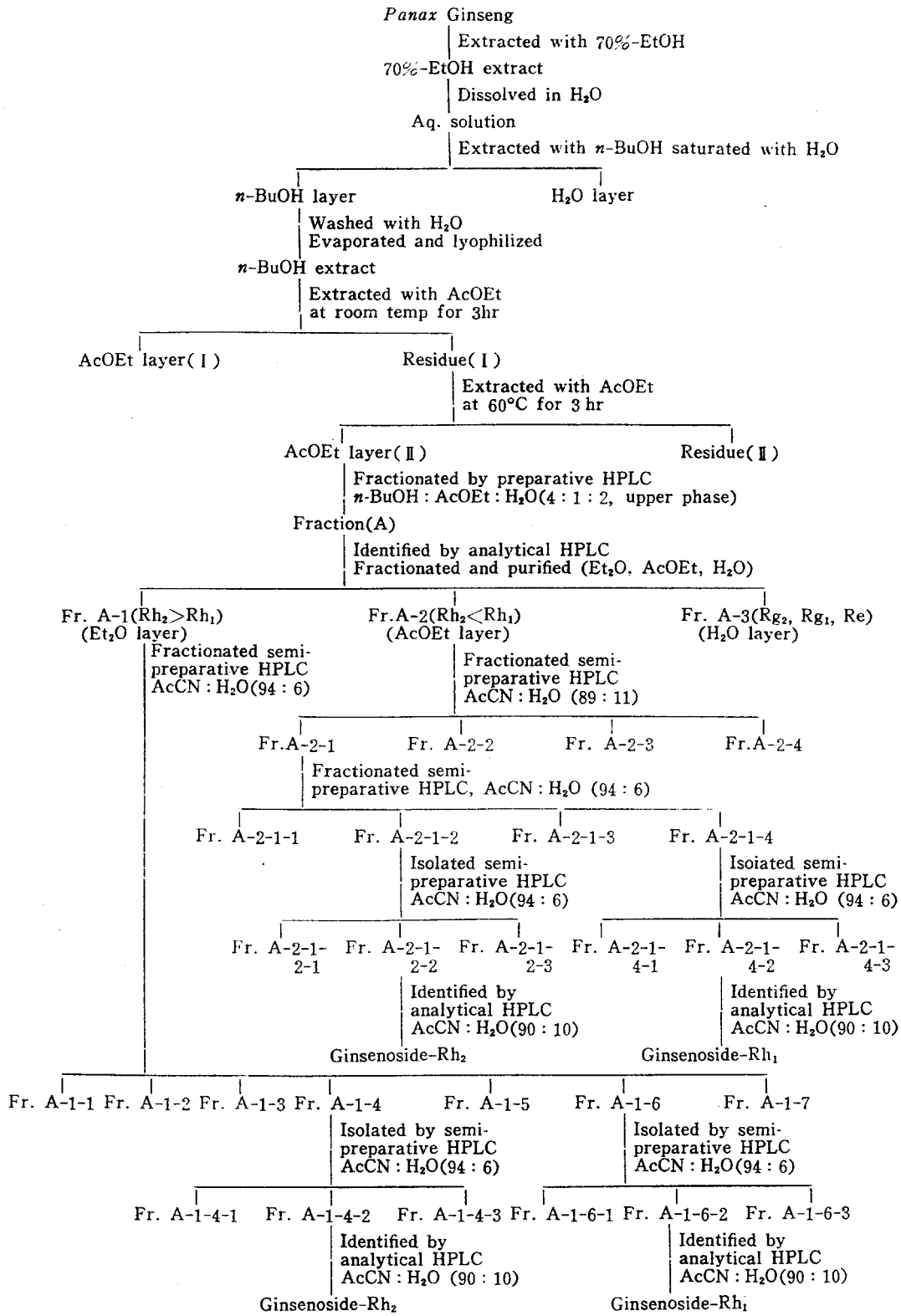

Fig. 1. Flow sheet of extraction and isolation of ginseng saponin

dentify the isolated fraction for ginsenoside-Rh_1 and -Rh_2, a special analysis system with a carbohydrate analysis column using a mixed solvent of acetonitrile : water (90 : 10) at a flow rate of 2 *ml*/min was employed.

Results and Discussion

Analysis of ethyl acetate layer (II)

Fig.2. shows the HPLC chromatogram of ginsenosides-Rd, -Re, -Rg_1, -Rg_2 and -Rh_1 which were obtained by using a mobile phase of acetonitite : water (86 : 14) at the flow rate of 2 *ml*/min following the same method described in the previous papers[9,10]. These ginsenosides were used as an authentic material for analysis of ethyl acetate layer. Before fractionation of the ethyl acetate layer, it was analyzed for its overall content of ginsenosides by using analytical HPLC.

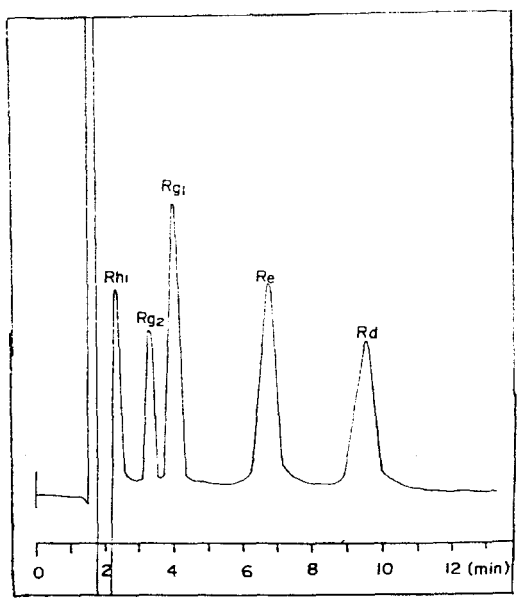

Fig. 2 Chromatogram of standard ginsenoside-Rh_1, -Rg_2, -Rg_1, -Re and -Rd by analytical HPLC
Conditions :
Packing: Carbohydrate analysis
Column: 3.9 *mm* ID×30 *cm*
Mobile phase: AcCN: H_2O (86:14),
Flow rate: 2 *ml*/min
RI detector: Attenuation 8×

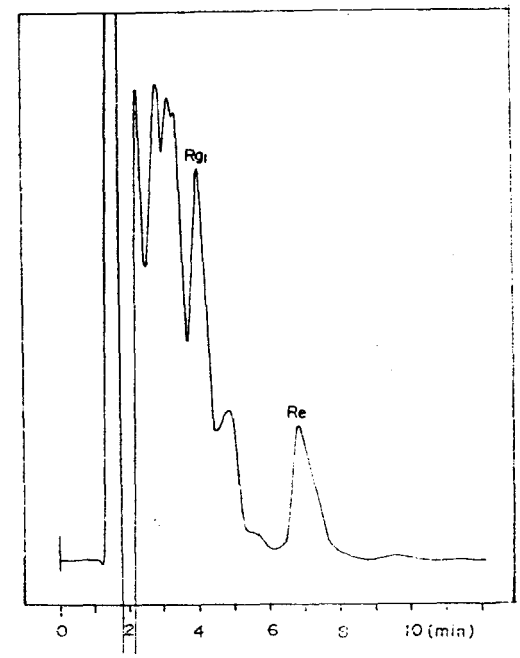

Fig. 3 Chromatogram of extracted crude saponin from *Panax ginseng* by analytical HPLC
Conditions :
Packing: Carbohydrate analysis
Column: 3.9 *mm* ID×30 *cm*
Mobile phase: AcCN : H_2O (86 : 14)
Flow rate: 2 *ml*/min
RI detector: Attenuation 8×

By using the same method, ginsenoside-Rh_1 and -Rh_2 were analyzed from ethyl acetate layer (Ⅱ) as shown in Fig. 3. It was found that those ginsenosides present in ethyl acetate layer (Ⅱ) consisted of only triol group saponin, ginsenoside-Rf, -Re, -Rg_1, -Rg_2 and -Rh_1, whose genuine sapogenin is 20(s)-protopanaxatriol. The residue (Ⅱ) resulted from extraction with ethyl acetate was found to contain a small amount of ginsenoside-Rg_1, -Rg_2 and Re. Howerer, there was no detection of ginsenoside Rh group in this residue. Therefore, a 14 ~15 *g*/60~80 *ml* of ethyl acetate layer (Ⅱ) was injected into prep LC/System-500 for the isolation of ginsenoside Rh group and was fractionated into 25 fractions according to their retention time (Fig. 4.). All of the 25 fractions were separately evaporated in vacuum, dissolved in carrier solvent containing small amount of methanol and identified by analytical HPLC. Fig. 5 shows HPLC chroma-

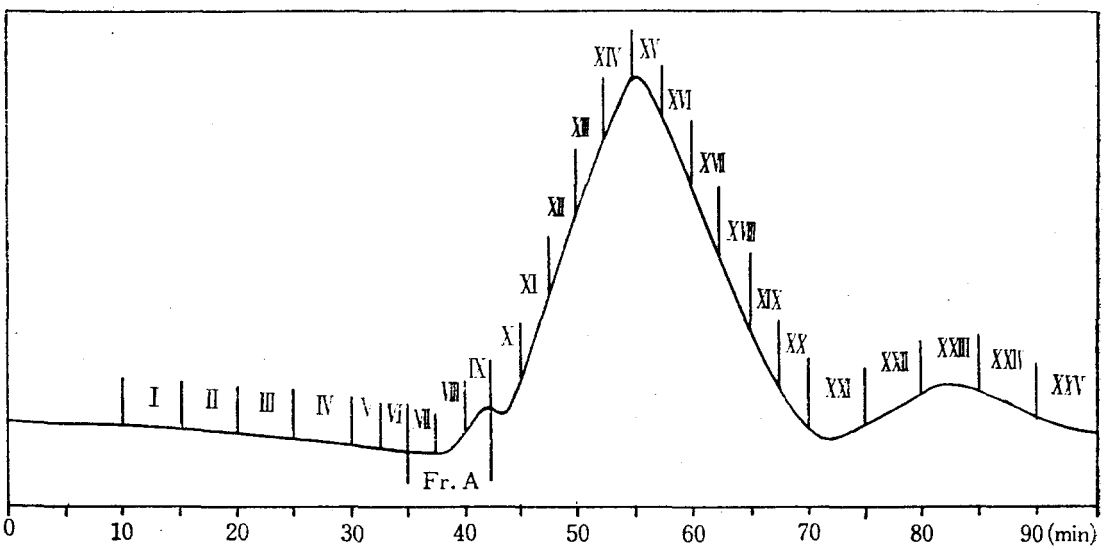

Fig. 4 Elution profile of extracted ethyl acetate layer (Ⅱ) from *Panax ginseng* by preparative HPLC

Conditions :

Instrument: Prep LC/System-500
Packing column: Prep. PAK-500/silica cartridge (57 *mm* ID×30 *cm*)
Mobile phase: *n*-BuOH : AcOEt : H_2O (4 : 1 : 2, upper phase)
Flow rate: 50 *ml*/min
Detector: RI detector
Sample load: 14~15 *g*/60-80 *ml*/injection

tograms of several fractions which contained ginsenoside Rh group. Among these fractions, only those fractions of Fr Ⅶ, Ⅷ and Ⅸ which had ginsenoside Rh as a major group were pooled and named fraction A in order to mininize contamination from other ginsenosides.

In order to ensure the separability of ginsenoside Rh_1 and Rh_2 from Fraction A, authentic ginsenoside -Re, -Rg_1, -Rg_2 and -Rh_1 were chromatographed on analytical HPLC dy using a mixed solvent of acetonitrile : water (92 : 8) and then was compared to those of fraction Ⅶ and Ⅷ (Fig. 6 and 7). It was found that those conditions used for separation were good enough for identification of ginsenoside-Rh_1 and -Rh_2

Solvent partition

As illustrated in Fig 8, fraction A, the major fraction for ginsenoside Rh group was dissolved in water, and then partitioned with ether. The ether layer washed with water and ether layer was named fraction A-1. The water layer resulted from ether partition was further partitioned with ethyl acetate. After the ethyl acetate layer was washed with water, it was named fraction A-2. This partition procedure was repeated several times and the combined water layer was named fraction A-3.

Each fraction of A-1, A-2, and A-3 was dissolved in carrier solvent containing small amount of methanol and chromatographed on analytical HPLC using a mixed solvent of acetonitrile : water (92 : 8) (Fig. 9A, 9B and 9C). It was found that fraction A-1 contained smaller amount of ginsenoside -Rh_1 than the amount of ginsenoside-Rh_2. On the contrary, fraction A-2 showed greater quantities of ginsenoside -Rh_1. However, ginsenoside -Rg_2 and -Re was the major ginsenosides in Fr. A-3 rather than ginsenoside-Rh group. Therefore, Fraction A-3 was not used for isolation of ginsenoside Rh group.

Isolation and identification

To obtain ginsenoside-Rh_1 and -Rh_2, Fraction A-1 was further subfractionated into 7 parts (Fig. 10) by semipreparative HPLC using a mixed solvent of

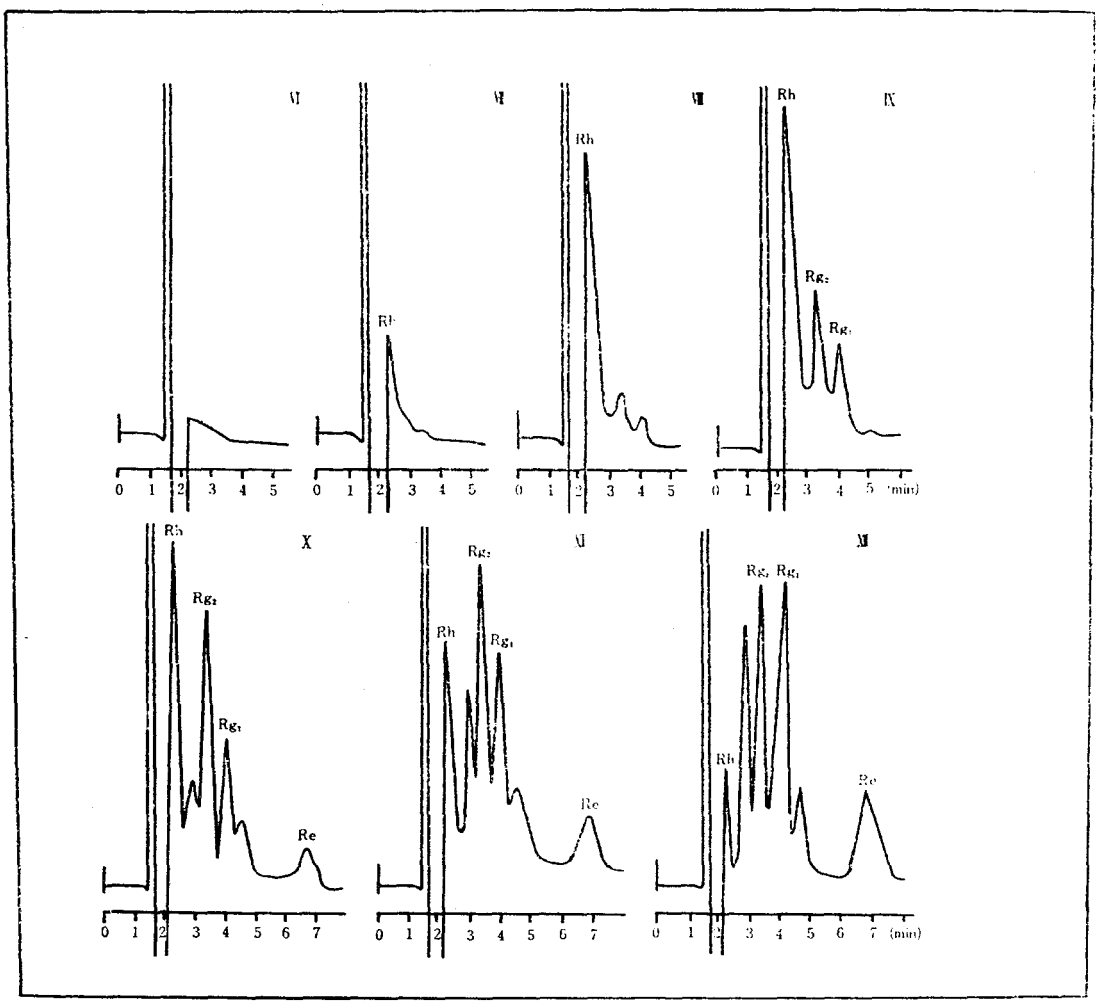

Fig. 5 Chromatograms of each fraction fractionated from ethyl acetate layer (Ⅱ) by preparative HPLC

Conditions :

Packing: Carbohydrate analysis
Column: 3.9 *mm* ID×30 *cm*
Mobile phase: AcCN : H_2O (86 : 14)
Flow rate: 2 *mm*/min
RI detector: Attenuation 8×

acetonitrile : water (94 : 6). It was found that fractions A-1-6 and A-1-4 came respectively under ginsenoside-Rh_1 and -Rh_2, and the homogeneities were examined with authentic sample. Ginsenoside -Rh_1 (Fr. A-1-6-2) and -Rh_2 (Fr. A-1-4-2) were respectively isolated from fractions A-1-6 and A-1-4 by semipreparative HPLC using acetonitrile : water (94 : 6) as shown in Fig. 11.

Fig. 12 shows the subfractionation of Fraction A-2 by semipreparative HPLC using a mixed solvent of acetonitrile : water (89 : 11) (Fig. 12). Subfraction A-2-1 was identified as ginsenoside Rf group. Ginsenoside-Rh_1 and -Rh_2 were isolated from fractions A-2-1-4 and A-2-1-2, respectively by semipreparative HPLC using a mixed solven of acetonitrile : water (94 : 6) (Fig. 13).

Identification was carried out for all these sub fractions dy using authentic samples. Homogen eities were proved for Fr. A-1-6-2 and A-2-1-4 a ginsenoside-Rh_1 and for Fr. A-1-4-2 and A-2-1-

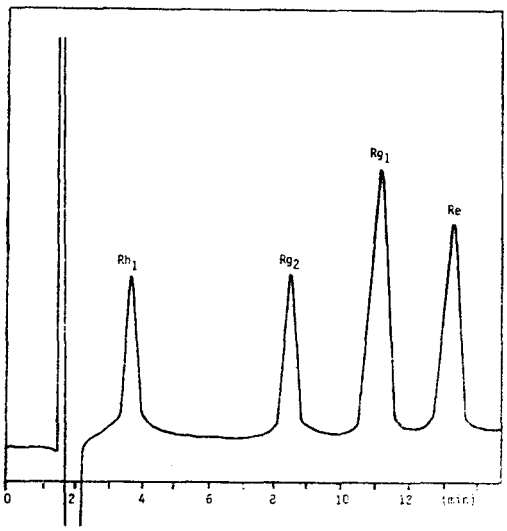

Fig. 6 Chromatogarm of standard ginsenoside -Rh_1, -Rg_2, -Rg_1 and -Re by analytical HPLC

Conditions :

Packing: Carbohydrate analysis
Column: 3.9 *mm* ID×30 *cm*
Mobile phase: AcCN : H_2O (92 : 8)
Flow rate: 2 *ml*/min
RI detector: Attenuation 8×

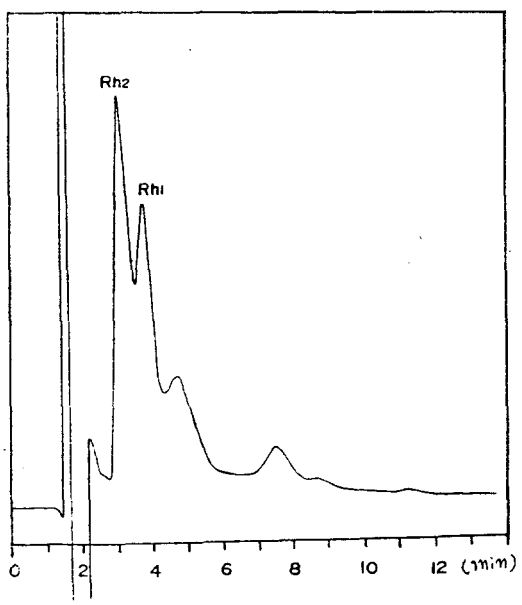

Fig. 7 Chromatograms of Fraction A fractionated from ethglacetate layer (I) by preparative HPLC

Conditions :

Packing: Carbohydrate analysis
Column: 3.9 *mm* ID×30 *cm*
Mobile phase: AcCN : H_2O (92 : 8)
Flow rate: 2 *ml*/min
RI detector: Attenuation 8×

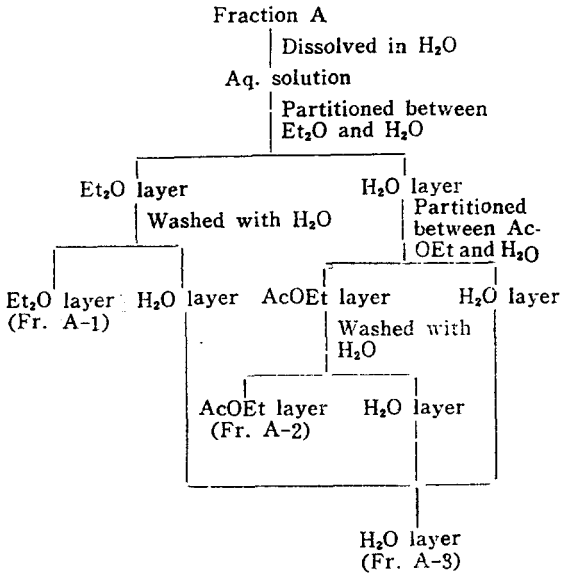

Fig. 8 Further fractionation and purification of Fraction A

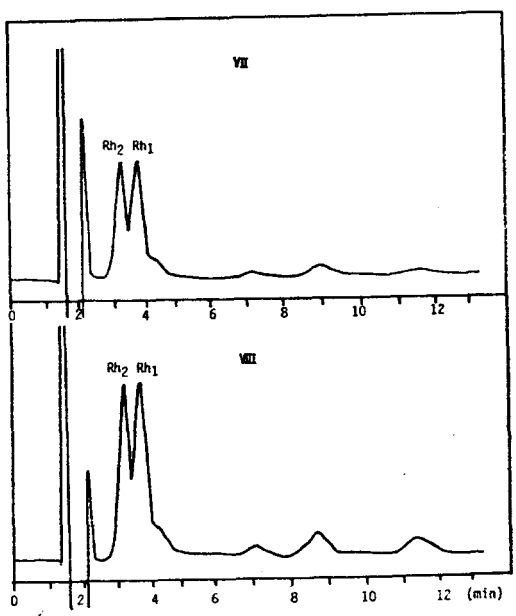

Fig. 9A Chromatogram of Fraction A-1 by analytical HPLC

Conditions :

Packing: Carbohydrate analysis
Column: 3.9 *mm* ID×30 *cm*
Mobile phase: AcCN : H_2O (92 : 8)
Flow rate: 2 *ml*/min
RI detector: Attenuation 8×

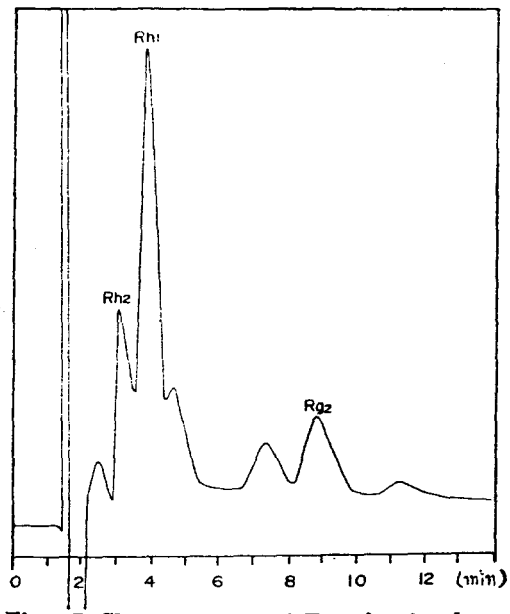

Fig. 9B Chromatogram of Fraction A-2 by analytical HPLC

Cenditions :

Packing: Carbohydrate analysis
Column: 3.9 *mm* ID×30 *cm*
Mobile phase: AcCN : H_2O (92 : 8)
Flow rate: 2 *ml*/min
RI detector: Attenuation 8×

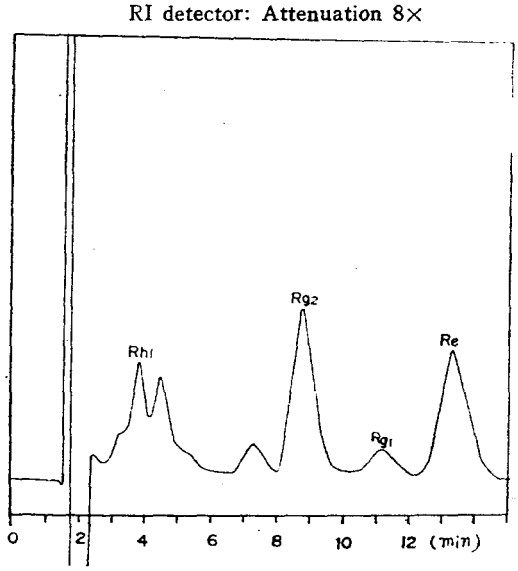

Fig. 9C Chromatogram of Fraction A-3 by analytical HPLC

Conditions :

Packing: Carbohydrate analysis
Column: 3.9 *mm* ID×30 *cm*
Mobile phase: AcCN : H_2O (92 : 8)
Flow rate: 2 *ml*/min
RI detector: Attenuation 8×

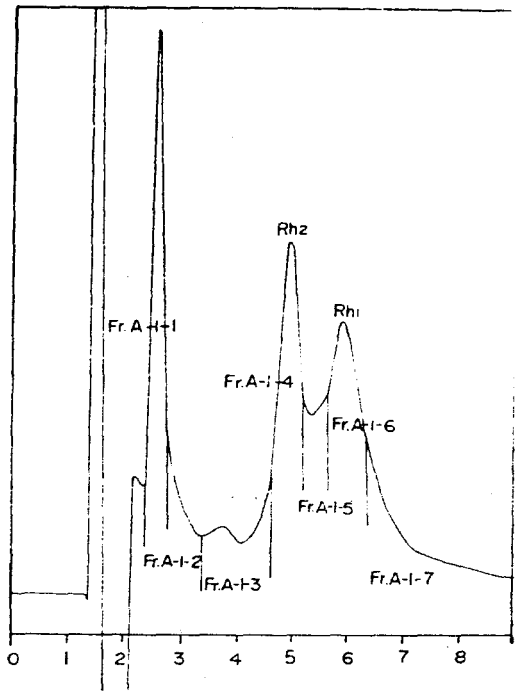

Fig. 10 Elution profile of Fraction A-1 by semipreparative HPLC

Conditions :

Packing: μ Bondapak C_{18}
Column: 7.8 *mm* ID×30 *cm*
Mobile phase: AcCN : H_2O (94 : 6)
Flow rate: 8 *ml*/min
RI detector: Attenuation 8×

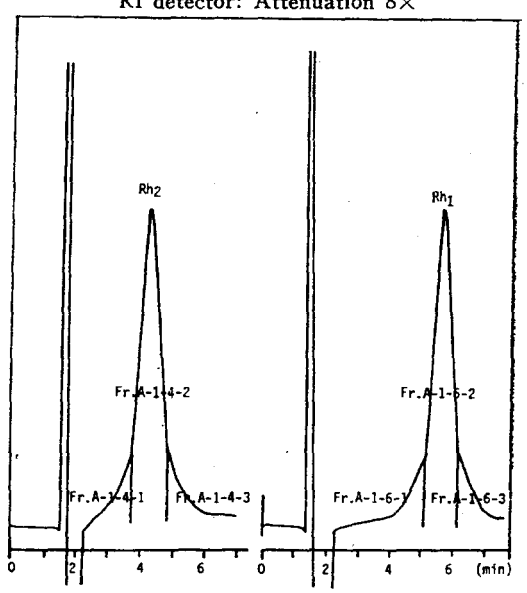

Fig. 11 Elution profiles of Fraction A-1-4 and A-1-6 by semipreparative HPLC

Conditions :

Packing: μ Bondapak C_{18}
Column: 7.8 *mm* ID×30 *cm*
Mobile phase: AcCN : H_2O (94 : 6)
Flow rate: 8 *ml*/min
RI detector: Attenuation 8×

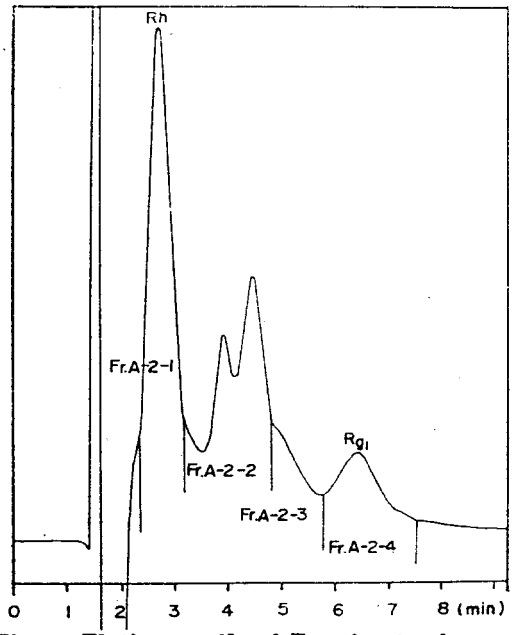

Fig. 12 Elution profile of Fraction A-2 by semi-preparative HPLC
Conbitions :
Packing: μ Bondapak C_{18}
Column: 7.8 *mm* ID×30 *cm*
Mobile phase: AcCN: H_2O (89 : 11)
Flow rate: 8 *ml*/min
RI detector: Attenuation 8×

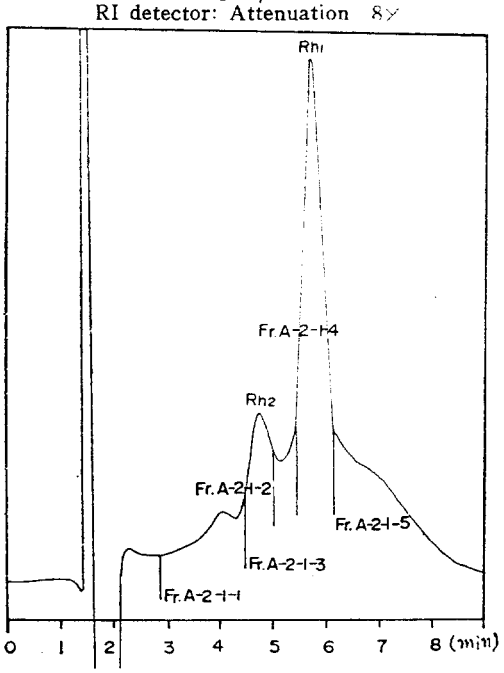

Fig. 13 Elution profile of Fraction A-2-1 by semipreparative HPLC
Conditions :
Packing: μ Bondapak C_{18}
Column: 7.8 *mm* ID×30 *cm*
Mobile phase: AcCN : H_2O (94 : 6)
Flow rate: 8 *ml*/min
RI detector: Attenuation 8×

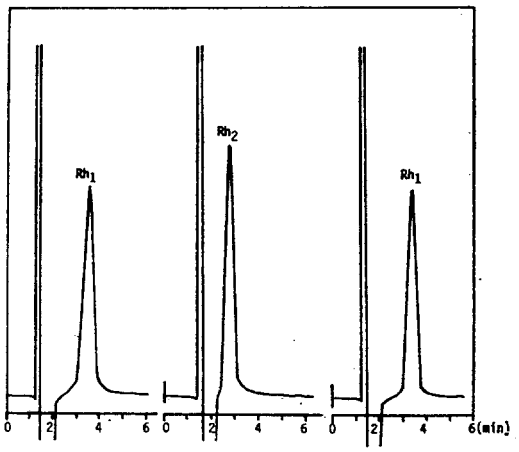

Fig. 14 Chromatogrms of standard ginsenoside -Rh_1 and isolated ginsenoside-Rh_1 and -Rh_2 by analytical HPLC
Conditions :
Packing: Carbohydrate analysis
Column: 3.9 *mm* ID×30 *cm*
Mobile phase: AcCN: H_2O (90 : 10)
Flow rate: 2 *ml*/min
RI detector: Attenuation 8×

as ginsenoside-Rh_2 (Fig. 14) by analytical HPLC using a mixed solvent of acetonitrile : water (90 : 10) at a flow rate of 2 *ml*/min.

As a result, ginsenoside-Rh_1 and -Rh_2 isolated were 80 *mg* and 150 *mg*, respectively. Accordingly, yield of ginsenoside-Rh_1 and -Rh_2 corresponded to 0.0008% and 0.0015% in red ginseng in dry weight basis, respectively.

요 약

인삼주성분 사포닌 및 미량성분 사포닌의 단리법에 대하여 HPLC의 응용을 검토하여 이미 보고한 바 있다. 따라서 인삼미량성분인 Ginsenoside-Rh_1 및 미지성분인 Ginsenoside-Rh_2의 단리법을 검토하였다. 저자등의 방법에 따라 홍삼의 70% 에탄올추출액(4 *kg*)에서 부탄올추출액을 얻어 에틸 아세테이트로 처리 (실온에서 3시간 및 60°C에서 3시간)하여 에틸 아세테이트 획분(29 *g*)을 preparative HPLC인 prep LC/System-500을 사용하여 부분분획하여 각 획분을 analytical HPLC/ALC-201로 동정, ginsenoside-Rh group을 다시 에테르, 에틸 아세테이트 및 물로 정제한 후, semipreparative HPLC/ALC-201로 ginsenoside -Rh_1 및 -Rh_2를 단리했다. 미지성분단리에 HPLC의

응용이 효과적임이 판명되었다.

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