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RESEARCH NOTE

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Novel Purification Method of Two Monoterpene Glucosides, Paeoniflorin, and Albiflorin, from Peony

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Abstract Two monoterpene glucosides, paeoniflorin and albiflorin, in peony (*Paeonia lactiflora*) were purified from 70% ethanol extract of Paeoniae Radix by diethyl ether washing and *n*-butanol partition, acetone dissolution, and gradient preparative HPLC. After the whole course of purification, yield of paeoniflorin, albiflorin, and the sum of them were 75.0, 38.8, and 68.7%, respectively, together with the corresponding purity of 96.2, 93.8, and 96.0%.

Keywords: monoterpene glucoside, peony, gradient preparative HPLC

Introduction

An Oriental medicinal herb, peony (*Paeonia lactiflora*), contains monoterpene glycosides such as paeoniflorin, albiflorin, and benzoyloxypaeoniflorin (1, 2). From traditional herb medicine, solvent extracts of peony have been used for cleansing heat, cooling blood, invigorating blood circulation, and so on (3). Recently, some functional activities like inhibitory effect on the change of extracellular calcium concentration and cardiovascular vasodilator effect have been reported for the purified compounds and isolated fractions from peony (1, 4, 5). Cardiovascular protective, platelet aggregation inhibitory activity was also found by our group for the herbal extract from peony (data not shown). Out of the active compounds in peony, paeoniflorin and albiflorin have been reportedly known to be present in large amount (2). Especially, paeoniflorin content is considered to be an important criterion for the quality evaluation of marketed Paeoniae Radix (5).

Paeoniflorin and albiflorin contain glucose as a constituent sugar bound to monoterpene moiety, which endows them with amphiphatic property (2). Until now, some purification studies for these compounds have been performed using this property as follows. Wen *et al.* (6) have separated paeoniflorin in herbal extracts by an isocratic high performance liquid chromatography (HPLC), making use of Lichrospher RP-18 column, and the eluent which has the composition of water-acetonitrile-methanol-acetic acid (80:15:5:1, v/v/v/v). Other reversed-phase HPLC separations, employing Zorbax CN, μBondapak C_{18}, and Symmetry C_{18} column, have also been reported for the active compounds in the herbal extracts, callus cultures, and pharmaceutical preparations from peony (7-10). Okamoto and Noguchi (11) have obtained paeoniflorin by a thin layer chromatography (TLC) separation, making use of a 3-cyanopropyltrichlorosilane-coated plate and a mobile phase of methanol-water (10:90, v/v). A combining method comprising capillary zone electrophoresis and electrokinetic chromatography has been developed to separate peony components (3).

The aim of this study is to develop a novel purification method, comprising diethyl ether washing and *n*-butanol partition, acetone dissolution, and gradient preparative HPLC, for paeoniflorin and albiflorin in peony.

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Materials and Methods

Reagents Standard samples of paeoniflorin (Mw 480.47) and albiflorin (Mw 480.46) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). HPLC-grade acetonitrile, methanol, and distilled water were obtained from Honeywell Burdick & Jackson (Morristown, NJ, USA), and were used for the analysis and purification of the active compounds in peony. Solvents for the extraction of the powdered sample of Paeoniae Radix were of guaranteed reagent grade from various suppliers.

Preparation of the herbal extract A type of Paeoniae Radix, purchased at Gyungdong Market (Seoul, Korea), was crushed with FM-681 Hanil Food Mixer (Hanil Electrical Co., Seoul, Korea) after drying in a convection oven at 40°C. Five g of the resulting powder and 300 mL of 70% ethanol were added into an Erlenmeyer flask. The flask was then installed inside a water bath incubator at 45°C, followed by the extraction overnight in a static mode. During extraction, however, the flask was shaken gently for 1 min every 30 min during initial 2 hr. The resulting slurry was filtered through Whatman #1 filter paper (Whatman International Ltd., Maidstone, UK). The residue in the flask was added with a small portion of 70% ethanol and filtered again. The combined filtrate was transferred into a round-bottomed flask and concentrated under reduced pressure with a rotary evaporator (RE 121: Büchi Labortechnik AG, Flawil, Switzerland).

Analytical HPLC for the active compounds At each step of the purification procedure for paeoniflorin and albiflorin, an analytical HPLC was conducted at the conditions described below (4).

A gradient HPLC system consisting of intelligent pumps (PV-980; Jasco Inc., Hachioji, Japan), a variable

wavelength ultraviolet detector (UV-975; Jasco Inc.) having a wavelength setting of 230 nm, an intelligent column thermostat (CO-966; Jasco Inc.) maintained at 35°C, and an autosampler (AS-950-10; Jasco Inc.) was used. The column used was Inertsil ODS-3 C_{18} (250×4.6 mm i.d., GL Sciences Inc., Tokyo, Japan) having a particle size of 5 μm. A guard column (GL Sciences Inc.) containing the same C_{18} packing was placed in front of the analytical column. Data was acquired with Borwin chromatography software (revision 1.2150, Jasco Inc.). The flow rate and the injection volume of the system were 1.0 mL/min and 10 μL, respectively. Prior to analysis, analytical column was equilibrated with the initial eluent composition for 1 hr at room temperature and then for 30 min at the operating temperature of 35°C. Gradient elution was performed as follows with acetonitrile and water containing 0.1% trifluoroacetic acid. An isocratic elution of 15:85 acetonitrile-water was conducted for initial 15 min, followed by the first gradient elution from 15:85 acetonitrile-water to 40:60 (v/v) acetonitrile-water from 15 to 30 min. Elution was maintained at this eluent composition until 50 min mark, followed by the second gradient elution back to 15:85 (v/v) acetonitrile-water until 55 min mark. In this method, column re-equilibration for 10 min was employed before starting the next measurement.

Purification procedure for the active compounds The total purification procedure consisted of partial purification comprising diethyl ether washing of the herbal extract, *n*-butanol partition, and acetone dissolution, and gradient preparative HPLC. For partial purification, 150 mL of diethyl ether and 50 mL of distilled water were mixed with the herbal extract, and the mixture was shaken vigorously. The resulting suspension was transferred into a separatory funnel. The upper diethyl ether layer was discarded after water layer was retained. This washing process was repeated twice more. One-hundred mL of *n*-butanol were mixed with the finally obtained water layer from the previous washing step and the mixture was shaken vigorously. After setting still, the resulting *n*-butanol layer was collected. This partition process was performed again and the combined *n*-butanol fraction was concentrated *in vacuo*. Acetone was added to the concentrate of *n*-butanol fraction. The resulting slurry was shaken vigorously and centrifuged at 1,250×g. The supernatant obtained was concentrated *in vacuo*.

Gradient preparative HPLC was undertaken for acetone fraction as follows. The concentrate of acetone fraction was dissolved in 5 mL of methanol and then passed through 0.45 μm PVDF syringe filter membrane (Gelman Sciences Inc., Ann Arbor, MI, USA). For the preparative separation of paeoniflorin and albiflorin in the filtrate, Inertsil ODS-2 C_{18} column (250×10.0 mm i.d., GL Sciences Inc.) was used and the solvent programming for gradient formation was the same as that used in the above analytical HPLC. In this case, however, flow rate and injection volume were separately optimized.

Determination of yield and purity according to the purification procedure Each step of the purification procedure was performed once. Yield was defined as the percentage of total or individual content of the active compounds at each step of the purification procedure against that in the herbal extract and purity was calculated as the percentage of total or individual content of the active compounds against the dry weight of each fraction or purified active compound (12).

Results and Discussion

Partition of the active compounds between fractionating solvents To establish a novel purification procedure for the active compounds in a herb, it is essentially required to undergo a partition procedure using solvents differing in polarity before instrumental isolation (13-15). To select fractionating solvents, the relative partition of the active compounds in peony was measured during the liquid-liquid extraction making use of each organic solvent and the co-solvent, distilled water. When organic solvent was diethyl ether, most of paeoniflorin and albiflorin over 90% in the herbal extract were partitioned into water layer. On the other hand, 93.4 and 96.3% of paeonifloirin and albiflorin were present in *n*-butanol layer after liquid-liquid extraction, respectively. When organic solvent was ethyl acetate, albiflorin was almost partitioned into water layer. However, 38.4% of paeoniflorin was still present in ethyl acetate layer. The distribution of the active compounds as described above seems to represent their amphiphatic property (2).

Stepwise HPLC chromatograms during the optimized partial purification Based on the above finding, ethyl acetate was excluded from fractionating organic solvents used in the purification procedure for a maximal recovery of the active compounds in peony (5). In this study, diethyl ether and *n*-butanol were selected as the fractionating organic solvents for partial purification before gradient preparative HPLC. The partition with diethyl ether was used to wash out non-polar substances in the herbal extract such as pigments, and that with *n*-butanol was used to remove soluble sugars and water-soluble starch (12).

The analytical HPLC chromatograms of individual fractions during the partial purification, comprising diethyl ether washing and *n*-butanol partition, and acetone dissolution, are shown in Fig. 1. Albiflorin and paeoniflorin were eluted within 20 min with the retention time of 13.28 and 16.30 min, respectively. The clarity of peak pattern was improved according to the progress of partial purification from the herbal extract to *n*-butanol fraction and then to acetone fraction.

Purification of the active compounds using gradient preparative HPLC The active compounds in acetone fraction from previous partial purification were separated by gradient preparative HPLC at various flow rates and injection volumes. As shown in Fig. 2, the peak resolution was obtained at the flow rate and injection volume of 3.0 mL/min and 100 μL, respectively. In this case, the retention time of paeoniflorin and albiflorin were present within 25 min. To purify the active compounds in peony, however, the first gradient preparative HPLC was performed at the flow rate of 3.0 mL/min and injection volume of 300 μL to speed up the process. The eluates corresponding to the retention time of the standard

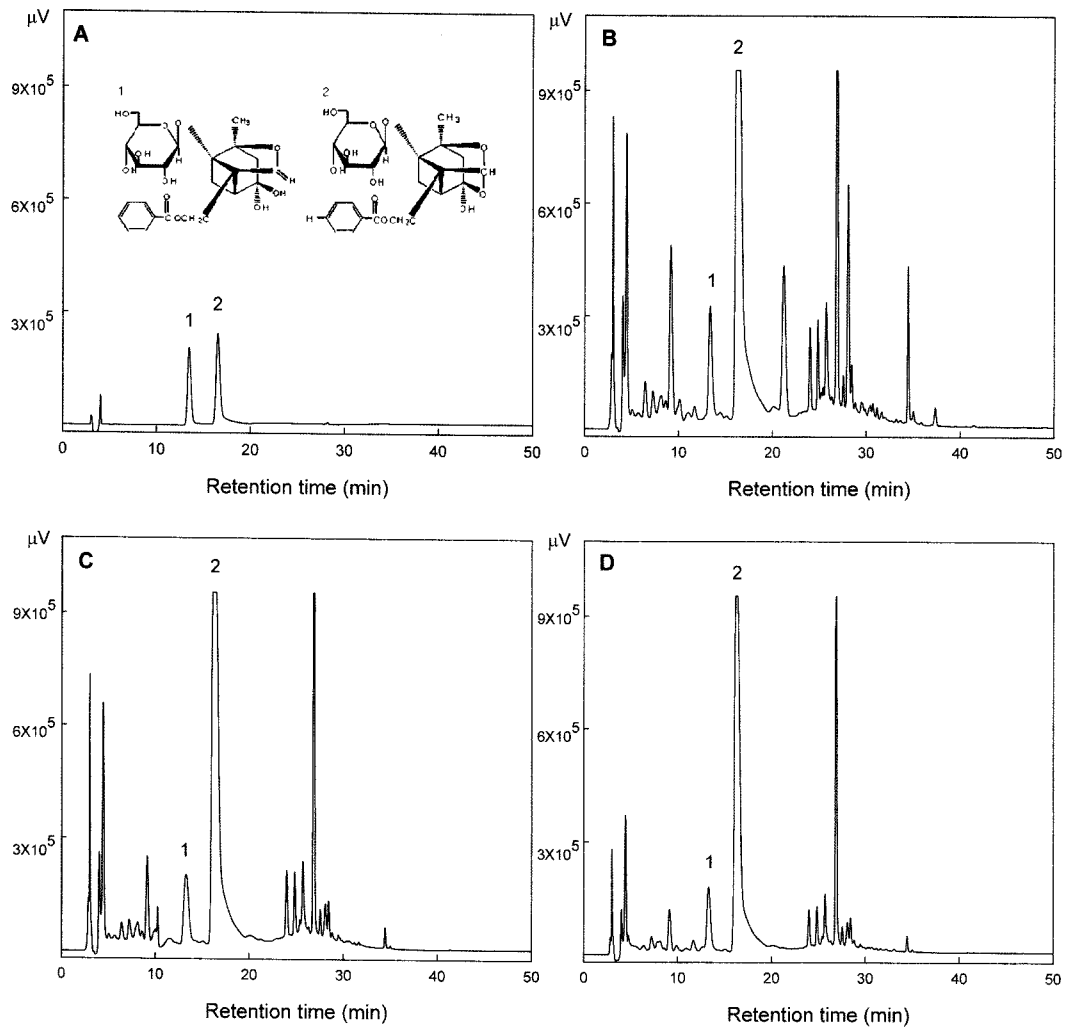

Fig. 1. Analytical HPLC chromatograms on the fractions during the partial purification. A, standard samples of paeoniflorin and albiflorin; B, herbal extract from peony; C, *n*-butanol fraction; D, acetone fraction. 1, albiflorin; 2, paeoniflorin. The inset figures in panel A were adopted from the report of Ikeda *et al.* (2).

paeonoflorin and albiflorin were individually combined, followed by a rechromatography at the above best conditions to improve peak resolution. When the rechromatographically purified paeoniflorin and albiflorin were compared with the standard ones using analytical HPLC and TLC, retention time and R_f values were correctly matched (data not shown).

Changes of yield and purity during the purification procedure During the purification procedure, the changes of yield for the active compounds in peony were determined (Fig. 3). The contents of paeoniflorin, albiflorin, and the sum of them in *n*-butanol fraction amounted to 97.2, 87.2, and 95.4% of those found in the herbal extract, respectively. The contents of paeoniflorin, and the sum of paeoniflorin and albiflorin after gradient preparative HPLC were still high, corresponding to 75.0 and 68.7% of those found in the herbal extract. On the other hand, the content of albiflorin in this fraction decreased by 61.2%, compared to that in the herbal extracts.

The purity of total paeoniflorin and albiflorin increased from 6.8% of the herbal extract to 96.0% of gradient preparative HPLC fraction. On the other hand, the purity of individual paeoniflorin and albiflorin in gradient preparative HPLC fraction were found as 96.2 and 93.8%, respectively. Until now, the active compounds in peony have been purified by a chromatographic procedure exploiting HPD 100 macroporous resin having adsorption and desorption property (16), by a consecutive procedure including partition, silica gel column chromatography, medium-pressure RP chromatography, and HPLC (17), and by a high speed counter-current chromatography employing two phase solvent system composed of *n*-butanol-ethyl acetate-water (1:4:5, v/v/v) (9). According to these studies, the purity of paeoniflorin in the range of 30.0-98.2% was found.

In this work, a novel purification procedure for the active compounds in peony was developed. During the partial purification comprising diethyl ether washing and *n*-butanol partition, and acetone dissolution, impurities present in peony were considerably removed. Through the following gradient preparative HPLC, paeoniflorin, albiflorin, and the sum of them were separated with the purity more than 93.8% without a considerable loss in

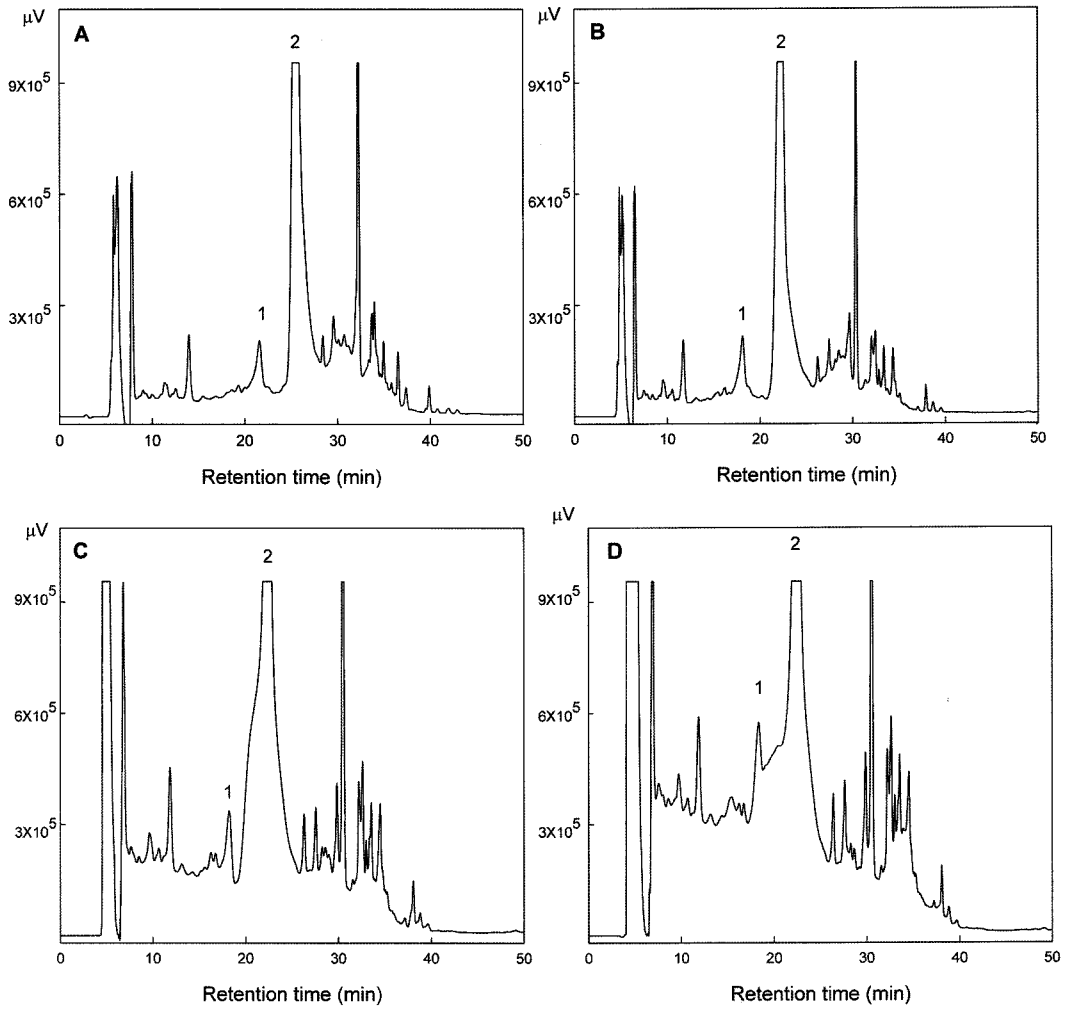

Fig. 2. Gradient preparative HPLC profiles for albiflorin (1) and paeoniflorin (2) in acetone fraction at various flow rates and injection volumes. The flow rates (injection volumes) of the panels A, B, C, and D were 2.5 (100), 3.0 (100), 3.0 (300), and 3.0 mL/min (500 μL), respectively.

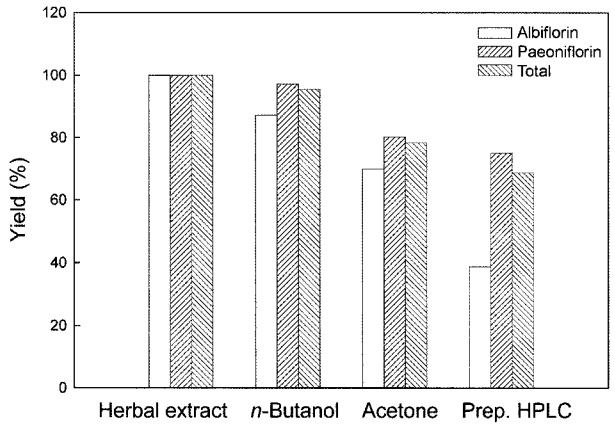

Fig. 3. Changes of yield for the active compounds in peony during the purification procedure.

their yield. From these results, gradient preparative HPLC following partial purification, as described above, seems to be an efficient purification protocol for the active compounds in peony.

Acknowledgments

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