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Purification of homoharringtonine and removal of residual solvents by spray drying

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Abstract−Homoharringtonine was purified from *Cephalotaxus koreana* by a combination of extraction, synthetic adsorbent treatment, low-pressure chromatography, and high performance liquid chromatography (HPLC). A crude extract was obtained by methanol extraction of biomass, followed by liquid-liquid extraction using chloroform. The waxy compounds were efficiently removed by adsorbent (active clay P-1) treatment. The extract was purified to greater than 52% with an 86.4% step yield by silica gel low-pressure chromatography. High performance liquid chromatography steps, which were composed of an HPLC step with silica column and an HPLC step with ODS column, were applied to give 98% purity with high yield. Amorphous homoharringtonine, with a fine particle size, was simply made by dissolving homoharringtonine in methylene chloride/methanol (98/2, v/v), followed by spray drying. Residual solvents, methylene chloride and methanol, could be reduced to 250 ppm and 1,160 ppm by spray drying and successive drying in a vacuum oven.

Key words: *Cephalotaxus koreana*, Homoharringtonine (HHT), Purification, Residual Solvents, Spray Drying

INTRODUCTION

Homoharringtonine (HHT), an alkaloid isolated from the genus *Cephalotaxus*, is an alkyl-substituted succinic acid ester of cephalotaxine [1,2]. HHT possesses antileukemic activity and is a potent myelosuppressive agent [3-6]. Several researchers have investigated the antineoplastic mechanism of HHT and related alkaloids; all have concluded that these drugs inhibit protein biosynthesis in the cell. HHT and congeners cause the breakdown of polyribosomes to monosomes, the release of completed globin chains, and delayed inhibition of the initiation of protein synthesis without affecting chain elongation. HHT has been tested clinically in advanced breast cancer, acute myelogenous leukemia, myelodysplastic syndrome (MDS), and MDS evolving to acute myeloid leukemia [7-9]. Although the chemical synthesis of cephalotaxine and its esters has been reported, extraction from plants is still the major source of HHT [10,11].

There are few reports of procedures for isolating and purifying HHT from plants that are directly applicable to commercial scale operations [12,13]. Existing purification methods using solvent extraction and chromatography procedures primarily aim to obtain crude HHT of low purity (<10%) and provide a mixture of HHT and related compounds, such as terpenoids, lipids, chlorophyll, and phenols. Consequently, high-purity HHT has not been obtained, even with multiple chromatographic columns and large solvent volumes; moreover, there is a heavy impurity load on the chromatography columns used. Since the existing purification methods are impractical, there remains a need for a method of isolating high-purity HHT in a simple, economical manner. In this paper, we report the development of an efficient purification process from *Cephalotaxus koreana.* A combination of adsorbent treatment and silica gel low-pressure chromatography method was applied to maximize purity with high yield before purification by high performance liquid chromatography (HPLC). We also studied the ways of preparing amorphous HHT and removing the residual solvents in HHT by spray drying and successive drying in a vacuum oven. These methods are suitable for mass production of HHT from *Cephalotaxus koreana* for clinical purposes.

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MATERIALS AND METHODS

1. Plant Material

The bark and needles were collected from Korean plum yew (*Cephalotaxus koreana*) growing on Mt. Kyeryong, South Korea, in November 2003. The bark and needle samples were freeze-dried and ground in a mortar and pestle. The dried powder (bark/needle= 1/4, w/w) was used for all the subsequent process development work.

2. Analysis of HHT

An HPLC system (Waters) was used for the analytical characterization of the intermediate and finished products. A C18 column (Shiseido, 4.6×250 mm, 5 μm) was eluted with a methanol/0.1 M ammonium formate gradient from 25/75 (v/v) to 45/55 (v/v) at a flow rate of 1.0 mL/min. The injection volume was 20 μL, and the effluent was monitored at 290 nm with a UV detector. The dried residue was redissolved in methanol and used for the quantitative analysis of HHT. Authentic HHT (purity: 98.6%) was purchased from Sigma-Aldrich and used as a standard.

3. Biomass Extraction and Liquid-Liquid Extraction of HHT from Biomass

The plant biomass was mixed with methanol and stirred at room temperature for 30 min. This mixture was filtered through filter paper in a Buchner funnel under vacuum. The biomass was added to methanol at a ratio of 1/8 (w/v). The extraction was repeated at least three times. Each methanol extract was collected, pooled, and concentrated at 40 °C under reduced pressure to decrease the volume of the methanol extract to 20% of the original. The concentrated methanol extract was added to chloroform at a volume ratio of 4 : 1 for liquid-liquid extraction, and this was extracted at room temperature

for 30 min. The extraction was repeated at least four times, and the crude extracts were pooled and dried at room temperature under reduced pressure.

4. Adsorbent Treatment of the Crude Extract

The dried crude extract from the liquid-liquid extraction was dissolved in methanol at a ratio of 20 of dried crude extract (v/w), and synthetic adsorbent (active clays P-1, Mizukalife Chemical Co., Japan) was added at various ratios of dried crude extract. The mixtures were stirred at room temperature for 30 min and filtered to obtain the filtration solution. The adsorbent cake thus obtained was washed several times with chloroform/ethanol (1/2, v/v) and the washings were combined with the filtration solution. The solution was concentrated at 40 °C under reduced pressure for silica gel low-pressure chromatography.

5. Silica Gel Low-Pressure Chromatography

The filtrate obtained in the adsorbent treatment step was applied to a 10×900 mm column packed with silica gel (Merck, Germany), which was equilibrated with 20% (v/v) methanol in methylene chloride. The column was eluted by isocratic means with the same solution. The fractions containing the HHT were collected and dried by rotary evaporation.

6. High Performance Liquid Chromatography (HPLC) for Purification

High performance liquid chromatography steps were composed of an HPLC with a 50×400 mm column packed with silica (Merck, Germany) and an HPLC with a 50×400 mm column packed with ODS (C18, Shiseido, Japan). In an HPLC employing a silica column, HHT-containing fractions obtained in the silica gel low-pressure chromatography, were injected onto the silica column and eluted with a mixture of methylene chloride and methanol. Eluates were analyzed by UV detector by determining absorbance at 290 nm and active fractions containing HHT were pooled and dried under a vacuum for subsequent use in ODS HPLC. At this time, methylene chloride and methanol were mixed at a volume ratio of 93 : 7. Samples were injected onto the HPLC at a flow rate of 3 cm/min at a concentration of 50 mg/mL. In an HPLC employing an ODS column, HHT-containing fractions obtained in the previous HPLC work were injected onto the ODS column and eluted with a mixture of methanol and 0.1 M ammonium formate. Eluates were analyzed by UV detector by determining absorbance at 290 nm, and active fractions containing HHT were pooled and dried under a vacuum to give the crystallized HHT. At this time, samples were injected onto the HPLC at a concentration of 50 mg/mL, and eluted with a mixture of methanol and 0.1 M ammonium formate mixed at a volume ratio of 75 : 25. Purified HHT was obtained by drying of pooled fractions.

7. Preparation of Amorphous HHT

After evaporation, the HHT was dissolved in methylene chloride/methanol (98/2, v/v) solution, and the solution was loaded onto a spray dryer (SD-1000, EYELA, Japan), which was heated to an inlet temperature 70 °C. Residual methylene chloride and methanol present in the amorphous HHT recovered from the spray dryer were removed until below 250 ppm and 1,160 ppm by vacuum drying respectively.

8. Analysis of Residual Solvents

A gas chromatography system (HP 5890, Hewlett-Packard, CA, USA) with DB-5 column (0.32 mm ID×30 m, 0.25 μm film) was used for quantitative analysis of residual solvents (methylene chloride and methanol). The conditions were as follows: FID with a temperature program of 40 °C to 100 °C (10 °C/min), hold for 2 min,

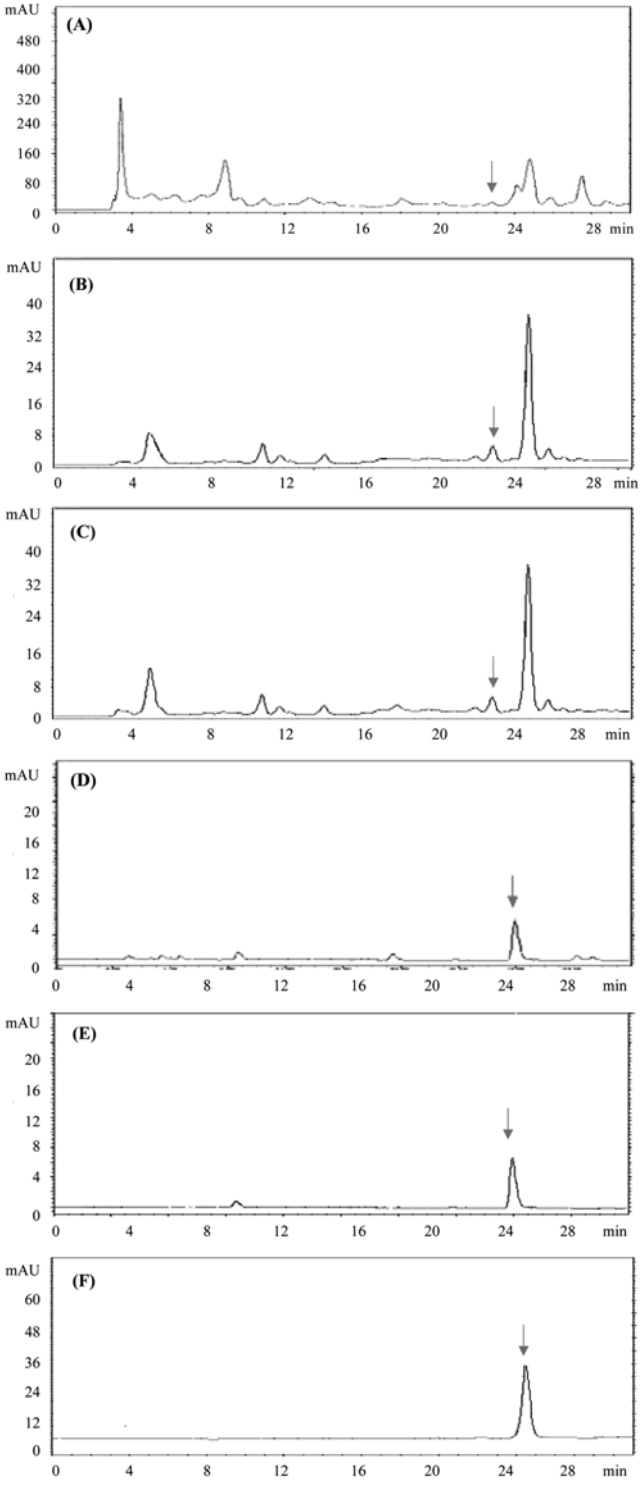

Fig. 1. Chromatogram of the separation/purification steps analyzed by using RP-HPLC: biomass extraction with methanol (A), liquid-liquid extraction with chloroform (B), adsorbent treatment with active clay (P-1) (C), low-pressure chromatography with silica gel (D), Silica-HPLC (E), and ODS-HPLC (F). The arrow indicates the position of homoharringtonine.

up to 300 ºC (30 ºC/min) fold for 15 min, flow rate 3 mL/min with helium.

RESULTS AND DISCUSSION

1. Development of Purification Process for HHT

Biomass extraction was carried out as a batch process by using methanol, which gave the best results in terms of HHT yield and efficient compared to alternative solvents or combinations of solvents (data not shown). The equilibrium between HHT in the biomass and methanol solution was reached within 20 min and the yield was 99% by three-time extraction (Fig. 1A). To eliminate polar impurities from biomass extract, liquid-liquid extraction was carried out with chloroform. At the step of liquid-liquid extraction, the HHT was recovered from the bottom phase (chloroform) and the polar impurities were efficiently removed from the top phase (methanol and water solution) (Fig. 1B). After further removal of impurities, which were a deeply colored, tar-like, insoluble material in chloroform, the crude extract was treated with active clay (P-1, Mizukalife Chemical Co., Tokyo, Japan). The optimal amount of active clay P-1 was 100% (w/w) of dried crude extract at room temperature for 30 min (Fig. 1C and Fig. 2). In this step, the purity seemed to show a small improvement but this treatment had a significant effect on convenience and feasibility of the following steps by removal of waxy compounds. Under isocratic conditions for silica gel low-pressure chromatography, the efficiency of purity and yield was compared for different solvent ratios. On elution with methylene chloride/methanol (80/20, v/v), the extract was purified to greater than 52% with an 86.4% step yield (Table 1 and Fig. 1D). The crude HHT from adsorbent treatment was pre-purified efficiently, and the purity increased from 10% to 52% with silica gel low-pressure chromatography under isocratic conditions. Compared with alternative processes [12,13], the use of active clay treatment and silica gel low-pressure chromatography in the pre-purification process allowed the rapid separation of HHT from interfering compounds. The purity (52%) of the crude HHT from silica gel low-pressure chromatography ensures that a minimum of material enters the HPLC purification process and costs are thereby minimized [14]. To obtain a higher purity of HHT, further purification was performed by HPLC composed of an HPLC with a silica column, and an HPLC with a hydrophobic resin column, e.g., ODS (octadecylsilylated, C18) column (Fig. 1E and F). A schematic diagram showing the major elements of the purification process and the results obtained for this purification of HHT from biomass is summarized in Table 1. The HPLC employing a silica column can obtain HHT of over 80% purity, but the impurities, which have to be removed, still remain in the solid. These impurities can be removed to over 98% purity of HHT by ODS-HPLC, which is performed by isocratic elution with methanol and 0.1 M ammonium formate mixture.

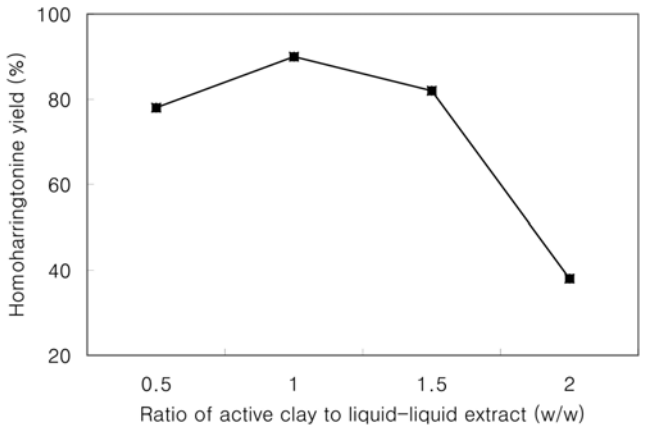

Fig. 2. Effect of the ratio of active clay (P-1) to liquid-liquid extract (w/w) on homoharringtonine yield.

Table 1. Summary of the purification of homoharringtonine from biomass (16 g)

	HHT (g)	Purity (%)	Step yield (%)	Yield (%)
Biomass	0.0550	-	100.0	100.0
Biomass extraction	0.0545	0.5	99.0	99.0
Liquid-liquid extraction	0.0540	8.0	99.0	98.0
Adsorbent treatment	0.0486	10.0	90.0	88.2
Low-pressure chromatography	0.0420	52.0	86.4	76.2
Silica-HPLC	0.0332	80.0	79.0	60.2
ODS-HPLC	0.0288	98.0	86.7	52.2

2. Removal of Residual Solvents in HHT

The morphology of HHT can be controlled by solvent treatment of HHT. Amorphous HHT, with a fine particle size, was simply made by dissolving HHT in methylene chloride/methanol (98/2, v/v), followed by spray drying. Residual solvents, methylene chloride and methanol, were easily removed to less than 250 ppm and 1,160 ppm by spray drying and successive drying in a vacuum oven on the temperature of 60 ºC for 72 h (Fig. 3). Actually, in the course of evaporation of solvents, a rigid layer seemed to be formed on the surface of dried HHT and the layer prevented further evaporation of residual solvents [15]. So, evaporating was not considered to be an acceptable method for removing these solvents for production of pharmaceutical materials. Finally, it was very hard to eliminate the residual solvents below the concentration limit for pharmaceutical drugs required by ICH (International Conference on Harmoni-

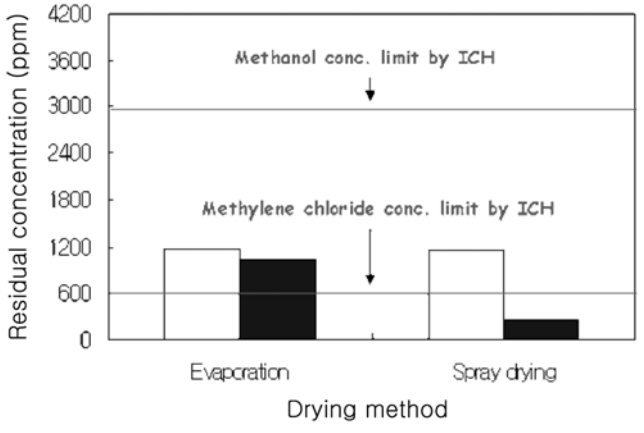

Fig. 3. The effect of drying method on residual solvents (□: methanol, ■: methylene chloride). The methanol and methylene chloride belong to class 2 in ICH guidance, and they should be limited in pharmaceutical products.

zation) guidance Q3C [16]. According to ICH guidance Q3C, methylene chloride and methanol are the residual solvents in the manufacturing process of pharmaceutical drugs; they belong to class 2, and their concentration limits showed 600 ppm for methylene chloride and 3,000 ppm for methanol, respectively. In case of evaporation, the content of methanol in HHT showed under the concentration limits of ICH guidance but the content of methylene chloride was much higher than the allowed maximum concentration limits (Fig. 3). In order to remove the residual solvents of amorphous HHT, HHT solved in methylene chloride/methanol (98/2, v/v) and put into spray drier at 70 °C with a peristaltic pump of a velocity 3 mL/min. Methylene chloride and methanol could be reduced by use of spray drier to 250 ppm and less than 1,160 ppm, respectively (Fig. 3). The high initial concentration (1,040 ppm) of methylene chloride could simply be reduced to 250 ppm by spray drying and successive drying in a vacuum oven.

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