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Isolation and purification of honokiol and magnolol from cortex *Magnoliae officinalis* by high-speed counter-current chromatography

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Abstract

High-speed counter-current chromatography was used to isolate and purify honokiol and magnolol from cortex Magnoliae Officinalis (*Magnolia officinalis Rehd. et* Wils.), a plant used in the traditional Chinese medicine. A crude sample, 150 mg, was successfully separated with a two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water (1:0.4:1:0.4, v/v), and the fractions were analyzed by high-performance liquid chromatography. The separation produced 80 and 45 mg of honokiol and magnolol with purities of 99.2 and 98.2%, respectively, in 2.5 h.

Keywords: Counter-current chromatography; *Magnoliae officinalis*; Preparative chromatography; Pharmaceutical analysis; Honokiol; Magnolol

1. Introduction

Magnolia officinalis Rehd. et Wils. (Houpu in Chinese) is one of the most popular traditional Chinese medicines. This herb has been used to treat a wide variety of clinical diseases such as wind-stroke, cold damage, headache, cold and heat, fright qi, blood impediment and dead muscle [1]. The major active constituents of Houpu are considered to be honokiol and magnolol (Fig. 1) [2]. Honokiol had strong antioxidative activity in biological systems [3] and protected rat liver from peroxidative injury [4]. In fact, the antioxidative activity of honokiol was found to be 1000-fold greater than that of Vitamin E [4]. Honokiol inhibited platelet aggregation [5] and ameliorated cerebral infarction from ischemia-reperfusion injury in rats [6,7]. In cell culture, honokiol inhibited cancer cell growth and induced apoptosis in cancer cell lines including human squamous lung cancer CH27 [8], human fibrosarcoma HT-1080 [9], human lymphoid leukemia Molt 4B cells [10] and human leukemic HL-60 cells [11]. Recently, honokiol was found to have potent antiangiogenesis activity in vitro and is highly effective against angiosarcoma in nude mice [12]. Honokiol is a γ-aminobutyric acid A (GABA-A) receptor agnoist, producing strong anxiolytic activity [13–18] and increasing hippocampal acetylcholine release in freely-moving rats [19]. In cortical neurons cultured in serum-free medium supplemented with B27, honokiol promoted neurite outgrowth [20]. In addition, the survival and growth of neurons were significantly enhanced by adding honokiol to the primary cultures in serum-free medium supplemented with N2 [20]. Its neurotrophic activity was comparable to 40 ng/ml of bFGF at a concentration of 10 μM [20].

Because of the importance of its biological properties, a large quantity of pure materials is urgently needed for further studies. However, the preparative separation and purification of honokiol from other constituents of the plant by classical methods are tedious, requiring multiple chromatographic steps on silica gel, polyamide column, etc. [21]. High-speed counter-current chromatography (HSCCC) is a unique liquid–liquid partition chromatography technique that uses no solid support matrix. HSCCC eliminates the irreversible adsorptive loss of samples onto the solid support matrix used in the conventional chromatographic column. This method has been successfully applied to the analysis and separation of several natural products [22–28]. No report has been seen on the use of HSCCC for the isolation

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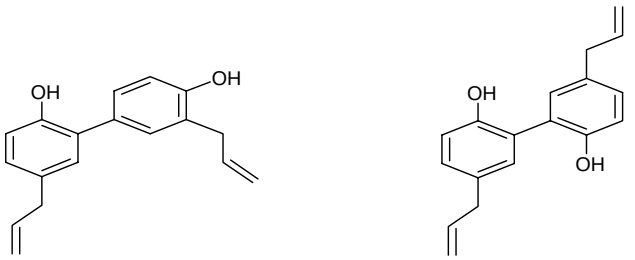

Honokiol Magnolol

Fig. 1. Chemical structures of honokiol and magnolol.

and purification of honokiol and magnolol from plants. We herein report an efficient new method for the preparative isolation and purification of honokiol by HSCCC.

2. Experimental

2.1. Apparatus

The preparative HSCCC instrument employed in the present study is a Model GS10A-2 miltilayer coil planet centrifuge (Beijing Institute of New Technology Application, Beijing, China) equipped with a polytetrafluoroethylene multilayer coil of 110 m × 1.6 mm i.d., with a total capacity of 230 ml. The β value of the preparative column varied from 0.5 at internal to 0.8 at the external ($\beta = r/R$, where r is the rotation radius or the distance from the coil to the holder shaft, and R is the revolution radius or the distances between the holder axis and central axis of the centrifuge). The rotation speed is adjustable from 0 to 1000 rpm, and 800 rpm was used in the present studies. The system was also equipped with one NS-1007 constant flow pump, a Model 8823A-UV monitor operating at 254 nm, a Yakogawa 3057 recorder and a manual injection valve with a 2 or 10 ml sample loop.

The HPLC system used throughout this study consisted of a Waters 660 pump, a Waters 660 controller (Waters, Milford, MA, USA), a sample injector (Rheodyne, Cotati, CA, USA) with a 10 μl loop, and a Waters 996 photodiode array detector. Evaluation and quantification were made on a Millenium 32 workstation (Waters).

2.2. Reagents

All organic solvents used for HSCCC were of analytical grade and purchased from Tianjin Guangcheng Chemical Factory, Tianjin, China. Acetonitrile used for HPLC analysis was of chromatographic grade and purchased from Tianjin Siyou Special Reagent Factory, Tianjin, China.

2.3. Measurement of partition coefficient

Approximately 1 mg of the test sample was weighed in a 10 ml test tube to which 2 ml of each phase of the equilibrated two-phase solvent system was added. The tube was caped and shaken vigorously for 1 min to equilibrate the sample thoroughly with the two phases. Equal volumes of each phase were then analyzed by HPLC to obtain the partition coefficients (K). The K value was expressed as the peak area of compound in the upper phase divided by the peak area of compound in the lower phase.

2.4. Preparation of the two-phase solvent system

The two-phase solvent system utilized in the present study was composed of *n*-hexane–ethyl acetate–methanol–water (1:0.4:1:0.4, v/v). After thoroughly equilibrating the mixtures in a separation funnel at room temperature, the two phases were separated shortly before use where the aqueous phase was used as the stationary phase and the organic phase as the mobile phase.

2.5. Sample and the preparation of sample solution

The raw material, an ethanol extract of cortex Magnoliae Officinalis, was purchased from Qingdao Sun-Star, China. The contents of honokiol and magnolol are approximately 90%. A solution of the sample was prepared by dissolving the raw material in the mobile phase of the solvent system used for separation.

2.6. Separation procedure

First, the multilayer coiled column was entirely filled with the upper organic phase as the stationary phase. Then, the lower aqueous phase was pumped into the head end of the column at a suitable flow-rate of 2.0 ml/min for Model GS10A-2 while the apparatus was rotated at an optimum speed of 800 rpm. After hydrodynamic equilibrium was reached as indicated by a clear mobile phase eluting from the tail outlet, the solution of the sample was injected through the injection valve. The effluent from the tail end of the column was continuously monitored by UV detection at 254 nm, and the peak fractions were collected according to the chromatogram. The retention of the stationary phase relative to the total column capacity was computed from the volume of the stationary phase collected from the column after the separation was completed.

Table 1
Partition coefficients and separation factors (a) of these compounds

Hexane–ethyl acetate–methanol–water	Honokiol, K_1	Separation factors (a)	Magnolol, K_2
1:1:1:1	6.96	2.18	15.20
1:0.8:1:0.8	2.92	2.20	6.43
1:0.4:1:0.4	0.38	2.39	0.91
1:0.4:1.2:0.4	0.21	2.86	0.60
1:0.2:1:0.2	0.06	3.50	0.21
1:0.6:1:0.6	1.03	2.51	2.59
1:0.4:1.1:0.4	0.26	3.12	0.81

2.7. HPLC analyses and identification of HSCCC peak fractions

The crude ethanol extract and each purified fraction from the preparative HSCCC separation were analyzed by HPLC with a Shim-pack VP-ODS column (250 mm × 4.6 mm, i.d.) at 288 nm and at a column temperature of 30 °C. The mobile phase, acetonitrile–0.1% phosphoric acid (55:45, v/v), was eluted at a flow rate of 1.0 ml/min. The effluent was monitored by a photodiode array detector [29].

3. Results and discussion

3.1. Selection of the two-phase solvent system

A successful separation of the target compounds using HSCCC requires a careful search for a suitable two-phase solvent system to provide an ideal range of partition coefficients for the applied material. Generally, the two-phase solvent system needs to satisfy the following requirements [30–32]: (1) for ensuring a satisfactory retention of the stationary phase, the settling time of the solvent system should be considerably shorter than 30 s; (2) for an efficient separation, the partition coefficient (k) value of the target compounds should be close to 1, and the separation factor between two components ($\alpha = k_2/k_1, k_2 > k_1$) should be greater than 1.5. In general, smaller K values may result in a loss of peak resolution, while larger K values tend to produce excessive sample band broadening. In this case, the K value was determined after partitioning the sample between the two solvent phases and aliquots of the upper and lower layers were analyzed by HPLC. From these two chromatograms, the K value of each component is determined by computing the ratio of the peak heights (or areas)

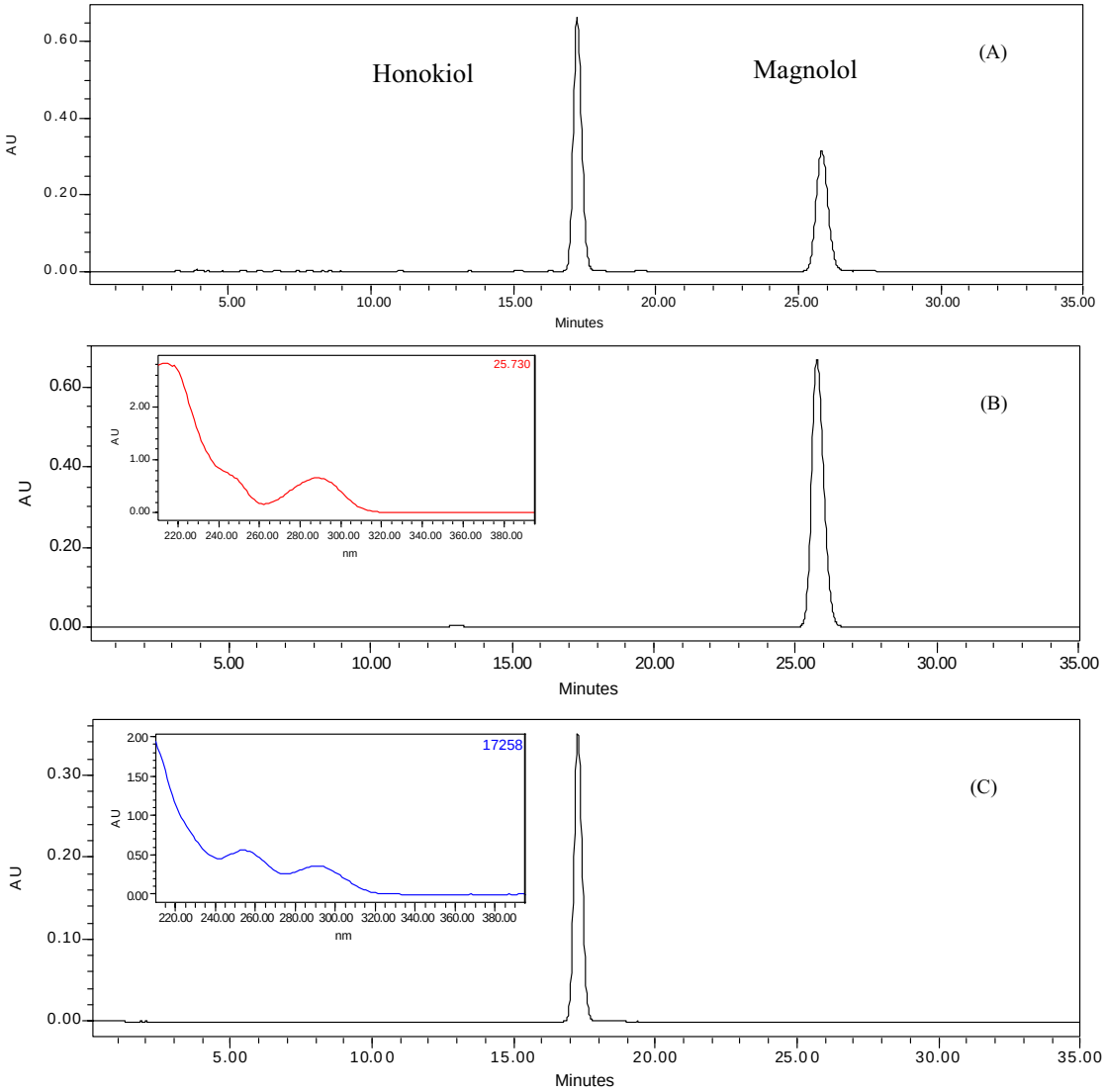

Fig. 2. (A) HPLC chromatogram of the crude ethanol extract; (B) HPLC analyses and UV spectrum of the purified magnolol; (C) HPLC analyses and UV spectrum of the purified honokiol. Conditions—a Shim-pack VP-ODS column (250 mm × 4.6 mm i.d.); column temperature: 30 °C; mobile phase: acetonitrile–0.1% phosphoric acid (55:45, v/v); flow rate: 1.0 ml/min; detection: 288 nm.

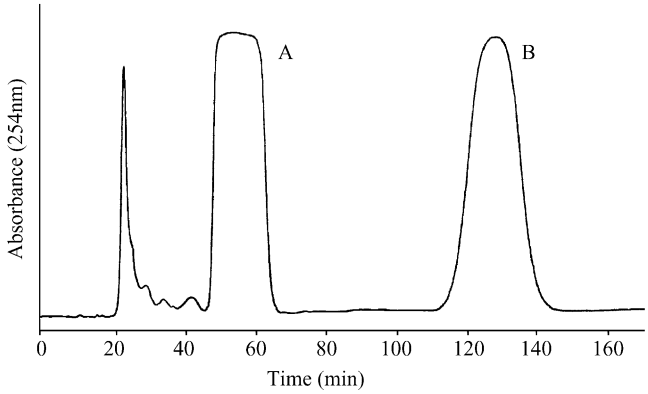

Fig. 3. Chromatogram of the crude extract by preparative HSCCC. Conditions—column: multilayer coil of 1.6 mm i.d. PTFE tube with a total capacity of 230 ml; revolution speed: 800 rpm; solvent system: *n*-hexane–ethyl acetate–methanol–water (1:0.4:1:0.4, v/v); stationary phase: upper organic phase; mobile phase: lower aqueous phase; flow-rate: 2.0 ml/min; detection: 254 nm; sample size: 150 mg; injection volume: 10 ml; retention of the stationary phase: 80.0%; A: honokiol; B: magnolol.

between the corresponding peaks. Fig. 2A shows the resulting chromatogram of the crude extract that contained honokiol and magnolol.

We have selected a two-phase solvent system composed of *n*-hexane, ethyl acetate, methanol and water because it provided a broad range of hydrophobicity by modifying the volume ratio of the four solvents. Table 1 shows that the *n*-hexane–ethyl acetate–methanol–water ratios ranging from 1:0.8:1:0.8, 1:0.6:1:0.6, 1:0.4:1.2:0.4, 1:0.4:1.1:0.4 and 1:0.4:1:0.4 can be used to separate the samples. After trying all the above solvent systems, the ratio of 1:0.4:1:0.4 (v/v) was found to be the best to effect the separation. Fig. 3 showed the separation of HSCCC using this solvent system.

3.2. Separation of honokiol and magnolol by HSCCC

A crude ethanol extract of the raw herb, 150 mg, was separated using the above solvent system. The retention of the stationary phase was 80.0%. The total separation time was 2.5 h with a total elution volume of 300 ml. The HSCCC fractions were analyzed by HPLC, and their absorbance was measured at 254 nm to draw the elution curve (Fig. 3). As expected, the HPLC analysis of each fraction revealed that the components eluted in the order of peaks A (honokiol) and B (magnolol). The structures of honokiol and magnolol were carried out by MS, ^{1}H NMR and ^{13}C NMR spectra as follows: honokiol (peaks A in Fig. 3): electron impact ionization (EI) MS *m*/*z*: 266 (M^{+}, 100), 237, 224, 210, 197, 184, 165, 152, 133, 115. ^{1}H NMR (300 MHz, $C^{2}HCl_3$) δ ppm: 3.33 (2H, d, aryl-CH_2–CH=), 3.45 (2H, d, aryl-CH_2–CH=), 5.04–5.25(4H, m, 2 × –CH=CH_2), 5.92–6.08 (2H, m, 2 × –CH=CH_2), 6.90–7.26 (6H, m, ArH). ^{13}C NMR (300 MHz, $C^{2}HCl_3$) δ ppm: 126.3 (C-1), 130.2 (C-2), 129.6 (C-3), 153.9 (C-4), 116.6 (C-5), 128.6 (C-6), 35.2 (C-7), 135.9 (C-8), 115.6 (C-9), 127.6 (C-1′), 150.7 (C-2′), 116.9 (C-3′), 128.8 (C-4′), 132.2 (C-5′), 131.1 (C-6′), 39.4 (C-7′), 137.8 (C-8′), 115.5 (C-9′). Magnolol (peaks B in Fig. 3): electron impact ionization (EI) MS *m*/*z*: 266 (M^{+},100), 247, 237, 225, 207, 197, 184, 165, 115, 41. ^{1}H NMR (300 MHz, $C^{2}HCl_3$) δ ppm: 3.35 (2H, d, aryl-CH_2–CH=), 3.37 (2H, d, aryl-CH_2–CH=), 5.04–5.12 (4H, m, 2 × –CH=CH_2), 5.89–6.02 (2H, m, 2 × –C–CH=C), 6.91–7.24 (6H, m, ArH). ^{13}C NMR (300 MHz, $C^{2}HCl_3$) δ ppm: 123.9 (C-1, 1′), 150.9 (C-2, 2′), 116.6 (C-3, 3′), 129.9 (C-4, 4′), 133.2 (C-5, 5′), 131.2 (C-6, 6′), 39.3 (C-7, 7′), 137.5 (C-8, 8′), 115.8 (C-9, 9′). Based on the HPLC analysis and the elution curve, all collected fractions were combined into two pooled fractions. Fig. 2B and C shows the HPLC analysis of these combined fractions. In the HPLC analysis of the original sample, Fig. 2A, peaks A and B constituted about 53 and 34% of the total peak area at 288 nm, respectively. After only one-step of operation by HSCCC, the purity of the components was increased to 99.2% (Fig. 2B), and 98.2% (Fig. 2C), respectively. These results demonstrate the high resolving power of HSCCC.

4. Conclusion

Using HSCCC, we were able to purify honokiol and magnolol efficiently and quickly. From a 150 mg mixture, we obtained 45 mg of magnolol with a purity of 98.2% and 80 mg of honokiol with a purity of 99.2% in 2.5 h. Our studies demonstrate that HSCCC is a powerful new technique for the purification of honokiol and magnolol.

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