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Preparative isolation and purification of notopterol and isoimperatorin from *Notopterygium forbessi* boiss (Chinese traditional medicinal herb) by high-speed counter-current chromatography

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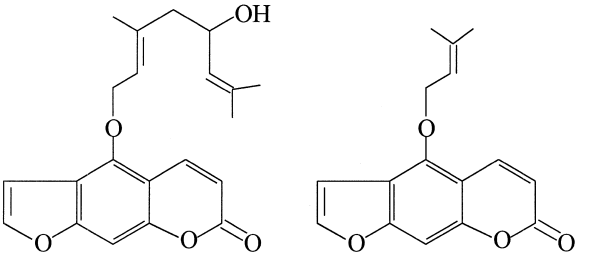
Abstract

Preparative high-speed counter-current chromatography was successfully used for isolation and purification of coumarins from Chinese traditional medicinal herb *Notopterygium forbessi* Boiss (Qianghuo in Chinese) using stepwise elution with a pair of two-phase solvent systems composed of light petroleum–ethyl acetate–methanol–water at volume ratios of 5:5:4.8:5 and 5:5:5:4. Four major components including notopterol and isoimperatorin were isolated, each at over 98% purity.

Keywords: Counter-current chromatography; *Notopterygium forbessi*; Pharmaceutical analysis; Notopterol; Isoimperatorin; Coumarins

1. Introduction

Notopterygium forbessi Boiss (Qianghuo in Chinese) is a traditional Chinese medicinal herb which has been used as a diaphoretic, an antifebrile and an anodyne [1–3]. The major active constituents of this herb are coumarins, including notopterol and isoimperatorin, whose chemical structures are given in Fig. 1. Notopterol and isoimperatorin are used as standards in the quality control of Qianghuo products.



1: notopterol 2: isoimperatorin.

Fig. 1. Chemical structures of coumarins from *N. forbessi* Boiss.

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High-speed counter-current chromatography (HSCCC), being a support-free liquid–liquid partition chromatography, eliminates irreversible adsorption of sample onto the solid support [4], and has been used for preparative separation of various natural products such as coumarins [5–7].

The present paper describes HSCCC separation of notopterol, isoimperatorin and two unknown compounds from a crude ethyl acetate extract of *N. forbessi* Boiss. Analytical HSCCC is first used for selecting a pair of two-phase solvent systems and their combined use in stepwise elution led to the successful preparative separation of notopterol and isoimperatorin.

2. Experimental

2.1. Apparatus

The analytical HSCCC instrument employed in the present study is a Model GS-20 analytical high-speed counter-current chromatograph designed and constructed at Beijing Institute of New Technology Application, Beijing, China. The apparatus holds a pair of column holders symmetrically on the rotary frame at a distance of 5 cm from the central axis of the centrifuge. The multilayer coil separation column was prepared by winding a 50 m, 0.85 mm I.D. PTFE (polytetrafluoroethylene) tube directly onto the holder hub forming multiple coiled layers with a total capacity of 30 ml. The β values varied from 0.4 at the internal terminal to 0.7 at the external terminal ($\beta = r/R$ where r is a distance from the coil to the holder shaft, and R, the revolution radius or a distance between the holder axis and central axis of the centrifuge). While the revolution speed of the apparatus could be regulated with a speed controller in the range between 0 to 2000 rpm, an optimum speed of 1500 rpm was used in the present studies. Preparative HSCCC was performed using a Model GS10A2 multilayer coil planet centrifuge (Beijing Institute of New Technology Application) equipped with a PTFE multilayer coil with 1.6 mm I.D. and 110 m in length with a total capacity of 230 ml. The β values of the preparative column ranged from 0.5 to 0.8. Revolution speed can be regulated between 0 and 1000 rpm where the optimum speed of 800 rpm was used in the present studies.

The solvent was pumped into the column with a Model NS-1007 constant-flow pump (Beijing Institute of New Technology Application). A Model 8823A-UV Monitor (Beijing Institute of New Technology Application) was used to continuously monitor the effluent at 254 nm. A manual sample injection valve with a 1.0 ml loop (for the analytical HSCCC) or a 20 μl loop (for the preparative HSCCC) (Tianjin High-New Science and Technology Company, Tianjin, China) was used to introduce the sample into the column. A portable recorder (Yokogawa Model 3057, Sichuan Instrument Factory, Chongqin, China) was used to draw the chromatogram.

The high-performance liquid chromatography (HPLC) equipment used was a Shimadzu LC-10AVP system including two LC-10ATVP solvent delivery units, a SPD-M10AVP UV–VIS photodiode array detector, a Model 7726 injection valve with a 20&#61549 (1 μl loop), an SCL-10AVP system controller, a CTO-10ASVP column oven, a DGU-12A degasser, and a Class-VP-LC workstation (Shimadzu, Kyoto, Japan).

2.2. Reagents

All organic solvents used for HSCCC were of analytical grade and purchased from Beijing Chemical Factory, Beijing, China. Methanol and acetonitrile used for HPLC analysis were of chromatographic grade and purchased from Tianjin Huaxi Special Reagent Factory, Tianjin, China. The crude ethyl acetate extract of *N. forbessi* was provided by Professor Youfu Sun of the Institute of Chinese Materia Medica, China Academy of Traditional Chinese Medicine.

2.3. Preparation of two-phase solvent systems and sample solutions

Following three two-phase solvent systems were prepared:

Solvent system 1: light-petroleum (b.p. 30–60°C)–EtOAc–MeOH–water (1:1:1:1; v/v)

Solvent system 2: light-petroleum (b.p. 30–60°C)–EtOAc–MeOH–water (5:5:4.8:5; v/v)

Solvent system 3: light-petroleum (b.p. 30–60°C)–EtOAc–MeOH–water (5:5:5:4, v/v).

Each solvent system was thoroughly equilibrated in a separatory funnel at room temperature and the two phases were separated shortly before use.

The sample solutions were prepared by dissolving the crude extract in the organic phase at suitable concentrations according to the analytical or preparative purpose.

2.4. Separation procedure

Analytical HSCCC was performed with a Model GS 20 HSCCC instruments using solvent systems 1–3 as follows: The multilayer coiled column was first entirely filled with the upper organic stationary phase. Then, the lower aqueous mobile phase was pumped into the head end of the column at a flow-rate of 1.0 ml/min, while the apparatus was rotated at 1500 rpm. After hydrodynamic equilibrium was reached, as indicated by a clear mobile phase eluting at the tail outlet, the sample solution (10 mg dissolved in 1 ml of the upper organic phase) was injected through the sample port. The effluent from the tail end of the column was continuously monitored with a UV detector at 254 nm. The retention of the stationary phase relative to the total column capacity was computed from the volume of the

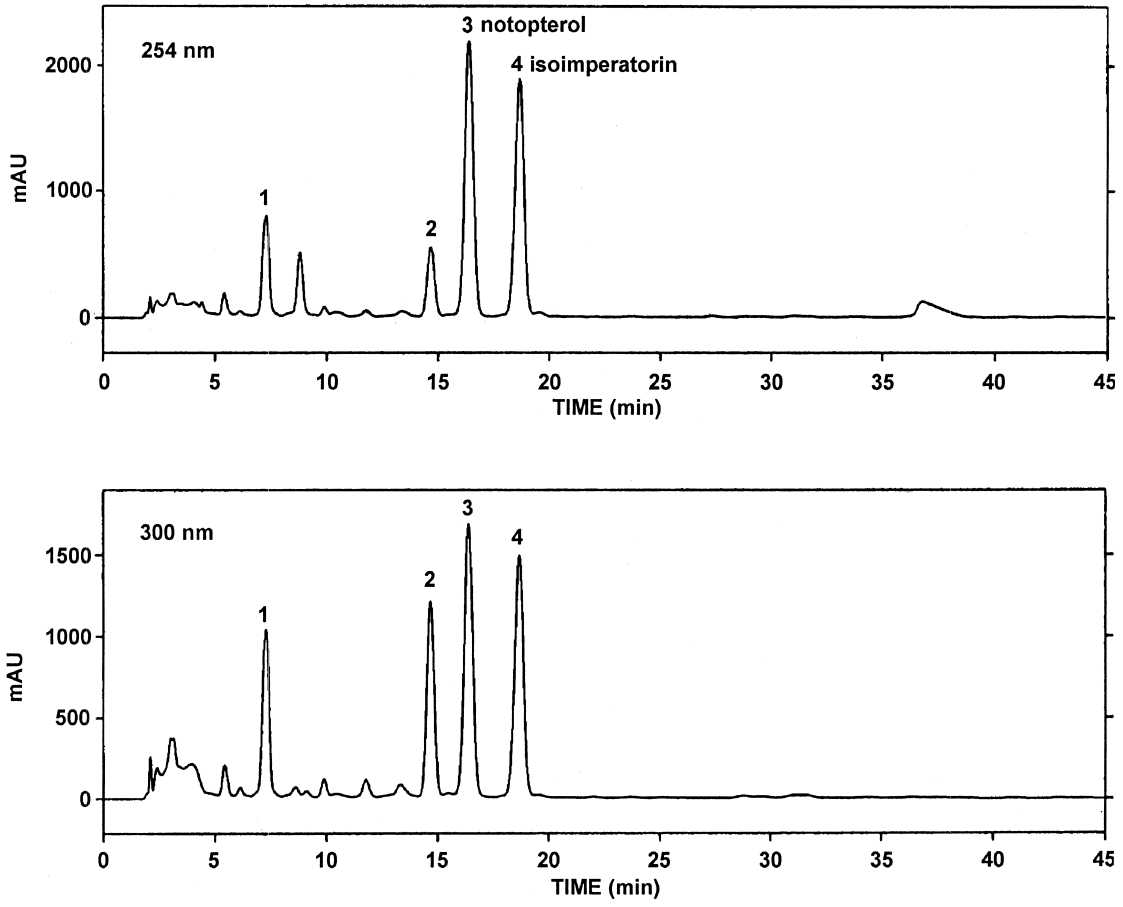

Fig. 2. The result of HPLC analyses of the crude ethyl acetate extract from *N. forbessi* Boiss. Column: Shim-pack CLC-ODS column (150×4.6 mm I.D.); column temperature: 40°C; mobile phase: Methanol–acetonitrile–water (30:30:40, v/v); Flow-rate: 1.0 ml/min. 1: Unknown 1; 2: notopterol; 3: unknown 2; 4: isoimperatorin.

stationary phase collected from the column after the separation was completed.

Preparative HSCCC was performed with a Model GS10A2 HSCCC instruments using solvent systems 2 and 3 as follows: The multilayer coiled column was first entirely filled with the upper organic phase of solvent system 2 as a stationary phase. Then, the lower aqueous phase was pumped into the head end of the column at a flow-rate of 2.0 ml/min, while the apparatus was rotated at a 800 rpm. After hydrodynamic equilibrium was reached, as indicated by a clear mobile phase eluting at the tail outlet, the sample solution was injected through the injection valve. The effluent from the outlet of the column was continuously monitored with a UV detector at 254 nm. Peak fractions were manually collected into test tubes according to the chromatogram. After five hours, the mobile phase was changed to the lower aqueous phase of solvent system 3, and elution, detection and fraction collection were resumed.

2.5. Analyses and identification of CCC peak fractions

The crude ethyl acetate extract of *N. forbessi* Boiss and each peak fraction obtained by HSCCC were analyzed by HPLC. The analyses were performed with a Shim-pack CLC-ODS column (150 mm×4.6 mm I.D.) at a column temperature of 40°C. The mobile phase composed of MeOH–MeCN–water (30:30:40, v/v) was isocratically eluted at a

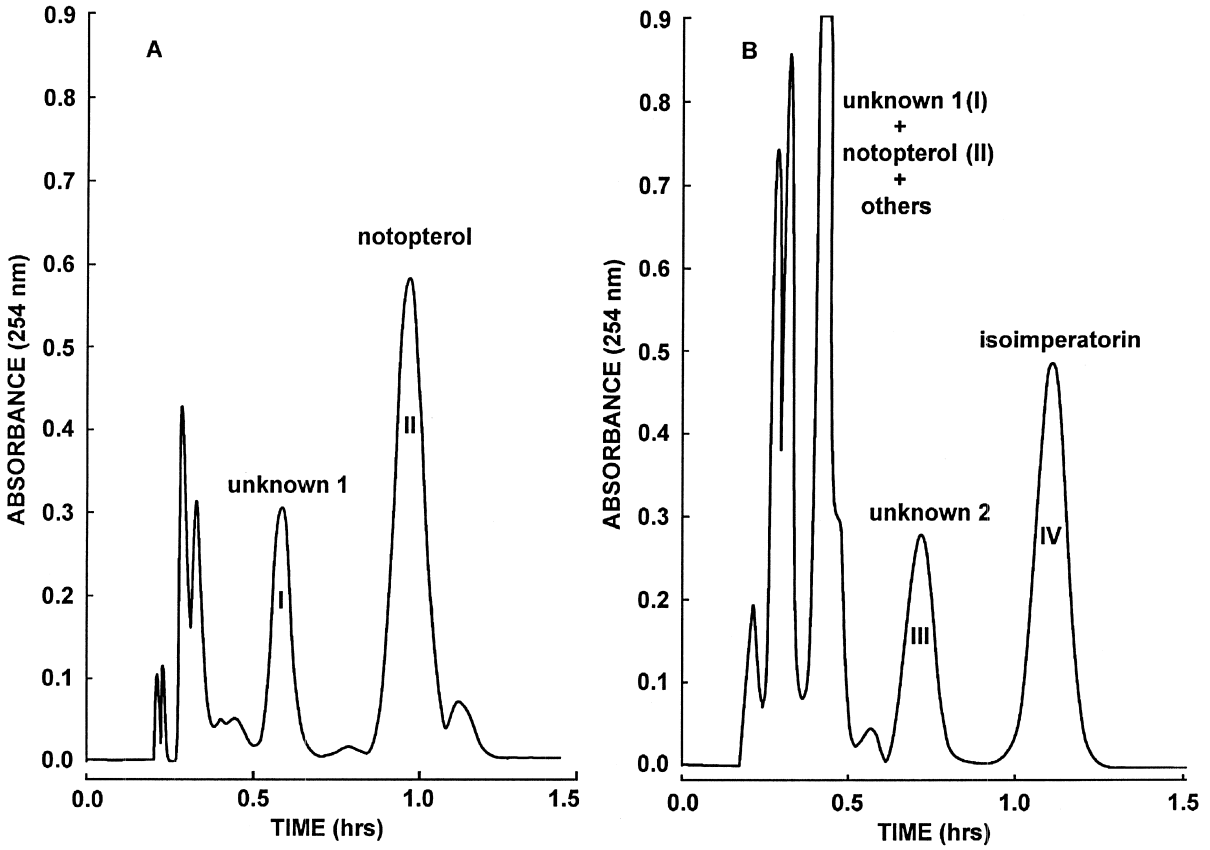

Fig. 3. Chromatograms of crude ethyl acetate extract from *N. forbessi* Boiss by analytical HSCCC. Solvent systems: A: Solvent system 2 (light petroleum–ethyl acetate–methanol–water, 5:5:4.8:5, v/v), B: Solvent system 3 (light petroleum–ethyl acetate–methanol–water, 5:5:5:4, v/v); Stationary phase: Upper organic phase; mobile phase: Lower aqueous phase; flow-rate: 1.0 ml/min; revolution speed: 1500 rpm; sample size: 6–10 mg dissolved in 1 ml organic phase; retention of the stationary phase: About 55%.

flow-rate of 1.0 ml/min and the effluent monitored by a photodiode array detector.

Identification of HSCCC peak fractions was carried out by MS (Finnigan MAT711), ^{1}H-NMR and ^{13}C-NMR spectra (Varian Mercury 300). The UV spectra were taken from the HPLC three-dimensional spectrum of absorbance versus time and wavelength.

3. Results and discussion

The crude ethyl acetate extract of *N. forbessi* Boiss was first analyzed by HPLC. As indicated in Fig. 2, it contained four major compounds including notopterol and isoimperatorin.

In order to achieve an efficient resolution of target compounds, three different solvent systems were examined using analytical HSCCC. The results indicated that the solvent system 1 could only partially separate notopterol. Solvent system 2 could resolve notopterol and unknown 1 well, while isoimperatorin and unknown 2 were retained in the column (Fig. 3A). Solvent system 3, on the other hand, could separate isoimperatorin and unknown 2 well, but failed to resolve notopterol and unknown 1 (Fig. 3B). Consequently, the combined use of solvent systems 2 and 3 in stepwise elution resulted in a successful separation of notopterol, isoimperatorin, and unknown compounds in a single run. Fig. 4 shows the result obtained from 200 mg of the crude ethyl acetate extract of *N. forbessi* Boiss by preparative HSCCC. After five hours of elution using solvent system 2, the mobile phase was switched to the lower phase of solvent system 3 to elute the retained two peaks. This separation yielded four pure peak fractions each at over 98% purity as determined by HPLC. The results of HPLC analysis and the UV spectra characteristic of each fraction were given in Fig. 5A–D.

Here it is worthwhile to discuss the advantage of the stepwise elution applied in the present studies. After some desired peaks eluted, other peaks with much greater partition coefficient will be retained in the column for a long period of time. In this case one

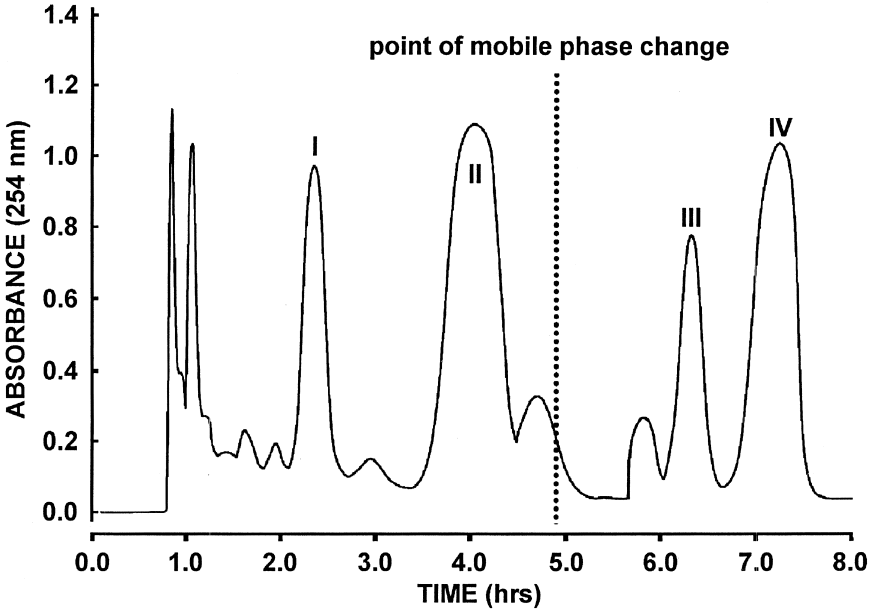

Fig. 4. Chromatogram of crude ethyl acetate extract of *N. forbessi* Boiss by preparative HSCCC using stepwise elution with solvent systems 2 and 3, i.e., light petroleum–ethyl acetate–methanol–water (5:5:4.8:5, v/v) and (5:5:5:4, v/v), respectively; Stationary phase: Upper organic phase of system 2; mobile phase: 590 ml of lower phase of solvent system 2 and 360 ml of lower phase of solvent system 3; flow-rate: 2.0 ml/min; revolution speed: 800 rpm; sample size: 200 mg dissolved in 5 ml organic phase of system 2; retention of the stationary phase: 57%. I: Unknown 1; II: Notopterol; III: unknown 2; IV: Isoimperatorin.

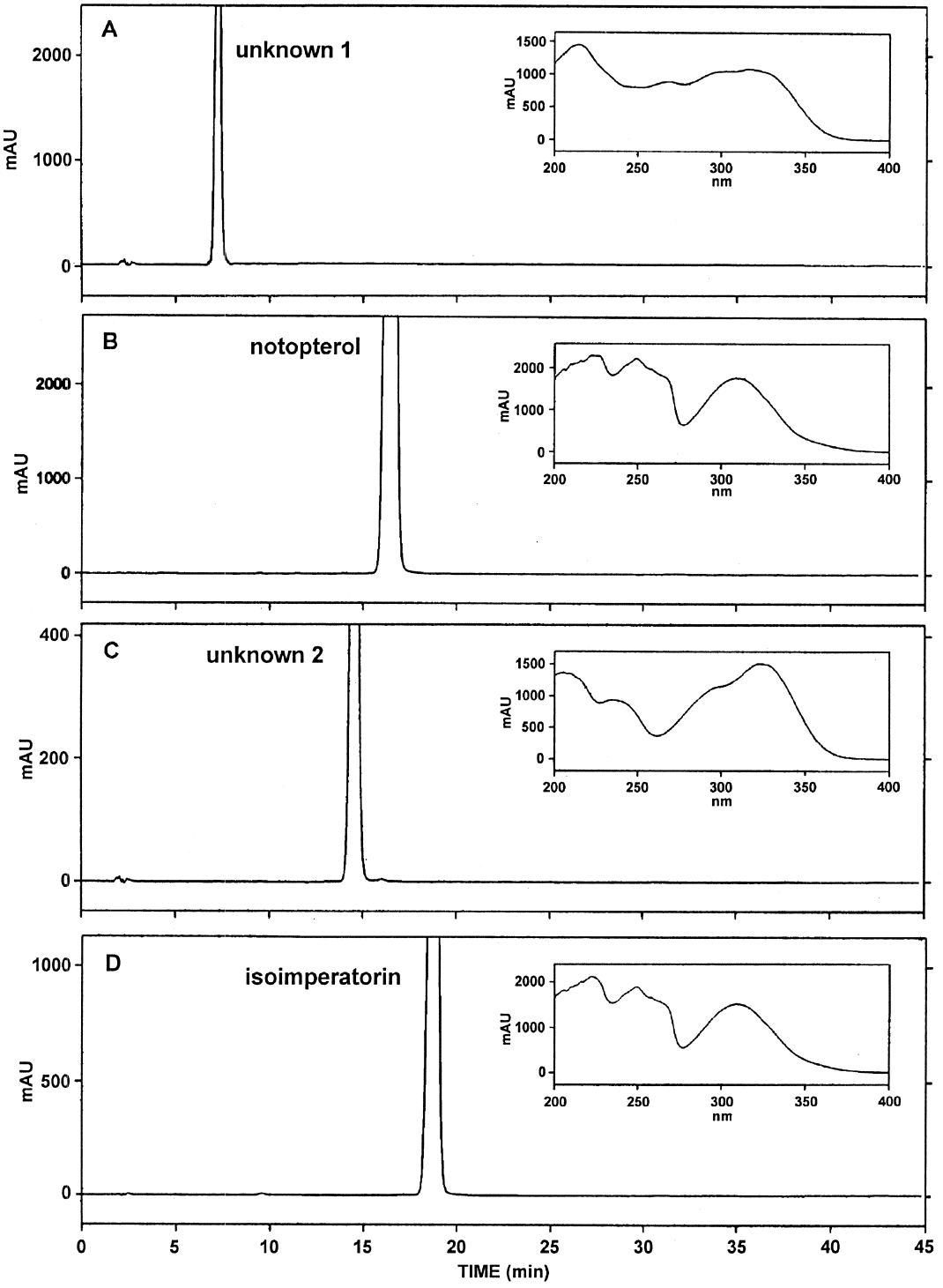

Fig. 5. The result of HPLC analyses and UV spectrum of the purified CCC peak fractions. (A): Peak I; (B): Peak II; (C): Peak III and (D): Peak IV of the preparative HSCCC separation shown in Fig. 3. Column: Shim-pack CLC-ODS column (150 mm×4.6 mm I.D.); column temperature: 40°C; mobile phase: Methanol–acetonitrile–water (30:30:40, v/v); flow-rate: 1.0 ml/min.

can change the chromatographic condition to facilitate elution of the retained peaks without loss of peak resolution. This can be done by the following two different ways: The first method is a dual elution method in which the column is eluted in the opposite direction with the other phase that was originally used as the stationary phase. Although this method is simple, the target peaks still present near the original inlet of the column due to its large partition coefficient (K) may be immediately eluted without further separation from the surrounding impurities. The second method is the stepwide elution applied to the present method. This method requires a careful choice of the second mobile phase in such a way that the volume of the stationary phase is not significantly altered after the two phases reestablish the hydrodynamic equilibrium in the column. However, the method allows a choice of the second mobile phase with suitable K values for the target compounds so that the peak resolution is further increased. The structural identification of HSCCC peak fractions was carried out by MS, ^{1}H-NMR and ^{13}C-NMR spectra as follows: Notopterol (HSCCC peak II in Fig. 4): Electron impact ionization (EI) MS m/z: 354[M^{+}], 202(100); ^{1}H-NMR (300 MHz, $C^{2}HCl_{3}$): 8.13(^{1}H, d, J=9.9 Hz, C4–H), 7.58(^{1}H, d, J=2.1 Hz, C10–H), 7.12(^{1}H, s, C8–H), 6.94(^{1}H, d, J=1.5 Hz, C9–H), 6.25(^{1}H, d, J=9.6 Hz, C3–H), 5.60(^{1}H, m, C2′–H), 5.16(^{1}H, d, J=8.4 Hz, C6′–H), 4.95(^{2}H, d, J=6.6 Hz, C1′–H), 4.50(^{1}H, m, C5′–H), 2.25(^{2}H, m, C4′–2H), 1.75(^{3}H, s, C8′–Me), 1.70(^{3}H, s, C10′ or C9′–Me), 1.67 (^{3}H, s, C9′ or C10′–Me), ^{13}C-NMR (300 Mhz, $C^{2}HCl_{3}$), 17.0 (q, C10′), 18.2 (q, C9′), 25.7 (q, C8′), 47.6 (t, C4′), 66.3 (d, C5′), 69.4 (t, C1′), 94.2 (d, C8), 105.0 (d, C9), 107.0 (s, C4a), 112.5 (d, C3), 113.9 (s, C6), 122.0 (d, C2′), 127.2 (d, C6′), 135.9 (s, C7′), 139.5 (d, C4), 140.5 (s, C3′), 144.9 (d, C10), 148.7 (s, C8a), 152.5 (s, C5), 158.1 (s, C7), 161.3 (s, C2).

Isoimperatorin (HSCCC peak IV in Fig. 4): EI–MS m/z: 270 [M^{+}], 202(70), 69 (100); ^{1}H-NMR (300 MHz, $C^{2}HCl_{3}$), 8.13(^{1}H, d, J=9.9 Hz, C4–H), 7.58(^{1}H, d, J=1.5 Hz, C10–H), 7.13(^{1}H, s, C8–H), 6.95(^{1}H, m, C9–H), 6.26 (^{1}H, d, J=9.9 Hz, C3–H), 5.53 (^{1}H, m, C2′–H), 4.95 (^{2}H, d, J=6.9 Hz, C1′–H), 1.79 (^{3}H, s, C5′–Me), 1.68 (^{3}H, s, C4′–Me); ^{13}C-NMR (300 MHz, $C^{2}HCl_{3}$), 18.2 (q, C5′), 25.8 (q, C4′), 69.4 (t, C1′), 94.1 (d, C8), 105.0 (d, C9), 107.4 (s, C4a), 112.4 (d, C3), 114.0 (s, C6), 119.0 (d, C2′), 139.6 (d, C4), 139.8 (s, C3′), 144.8 (d, C10), 148.9 (s, C8a), 152.6 (s, C5), 158.0 (s, C7), 161.3 (s, C2).

The results of our studies clearly demonstrate that a combined use of two solvent systems in stepwide elution by HSCCC provides highly efficient preparative separation of notopterol and isoimperatorin from a crude extract of *N. forbessi* Boiss.

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