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Rapid Isolation and Purification of Inotodiol and Trametenolic Acid from *Inonotus obliquus* by High-speed Counter-current Chromatography with Evaporative Light Scatting Detection

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ABSTRACT:

Introduction – In Eastern Europe, especially Russia, the fruiting body of *Inonotus obliquus* has been used as a folk medicine for cancer since the sixteenth or seventeenth century. Inotodiol and trametenolic acid are considered to be the main bioactive compounds of the fruiting body of the mushroom. These compounds show various biological activities, including anti-tumour, anti-viral, hypoglycaemic, anti-oxidant and cyto-protective. However, effective methods for isolating and purifying inotodiol and trametenolic acid from the fruiting body of *Inonotus obliquus* are not currently available.

Objective – To develop a suitable preparative method in order to isolate inotodiol and trametenolic acid from a complex *Inonotus obliquus* extract by preparative high-speed counter-current chromatography (HSCCC).

Methodology – Inotodiol and trametenolic acid were rapidly isolated and purified from the chloroform extract of *Inonotus obliquus* (Fr.) by HSCCC with evaporative light scatting detection (ELSD). The purity of the obtained target compounds was analysed by high-performance liquid chromatography (HPLC) with ELSD. The structures of the two compounds were identified by ^{1}H NMR and ^{13}C NMR.

Result – The target compounds were finally isolated and purified with a solvent system of hexane:ethyl acetate:methanol: water (1:0.4:1:0.4, v/v/v/v). In a single operation, 100 mg of the *I. obliquus* extracts yielded 13.0 mg of inotodiol and 7.0 mg of trametenolic acid. The entire separation and purification process took less than 5 h. The purities of obtained inotodiol and trametenolic acid were 97.51 and 94.04%, respectively.

Conclusion – HSCCC-ELSD was an efficient and rapid method for the separation and purification of inotodiol and trametenolic acid from *I. obliquus*.

Keywords: high-speed counter-current chromatography; evaporative light scattering detection; inotodiol; trametenolic acid; *Inonotus obliquus*

Introduction

Inonotus obliquus (Kabanoanatake in Japanese, Chaga in Russian, *Fuscoporia obliqua* in alternative taxonomy) is a white-rot fungus, belonging to the Hymenochaetaceae (Nakata *et al.*, 2007). In Eastern Europe, especially Russia, the fruiting body of this mushroom has been used as a folk medicine for cancer since the sixteenth or seventeenth century (Shin *et al.*, 2001a). *I. obliquus* has been documented to contain triterpenoid, steroids and polyphenolics. Some examples are inotodiol and trametenolic acid (Seikou *et al.*, 2009; Zheng *et al.*, 2010). These compounds show various biological activities, including anti-tumour, anti-viral, hypoglycaemic, anti-oxidant and cyto-protective (Kim *et al.*, 2006; Shin *et al.*, 2000; Taji *et al.*, 2008). For further investigation and potential clinical application of *I. obliquus* and its major constituents inotodiol and trametenolic acid, a large amount of the reference compounds in high purity is needed. Unfortunately, current preparative separation and purification methods for inotodiol and trametenolic acid from *I. obliquus* are tedious, time-consuming and susceptible to the highly adsorptive effect of the solid matrix (Shin *et al.*, 2000, 2001a, 2001b). Therefore, a more efficient separation method is needed.

High-speed counter-current chromatography (HSCCC) is a liquid–liquid partition chromatography technique that does not have a solid support matrix (Ito, 2005). This technique eliminates the irreversible adsorption of samples in the conventional solid-based stationary phase column chromatography (Sun *et al.*, 2006). As a method with unique advantages of high efficiency, high recovery

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and easy to scale-up, HSCCC has been widely used in preparative separation of natural products (Deng *et al.*, 2009; Ha *et al.*, 2007; OuYang *et al.*, 2007; Wang *et al.*, 2004). Recently, HSCCC has been widely applied as a convenient and efficient technique for the separation of natural compounds in medical plants and fungi. This method can save time, reduce complex procedures and produce high yield. As a new separation and purification tool, HSCCC has been scaled up to the industrial scale (Tian *et al.*, 2000). 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. The composition of a two-phase solvent system was selected according to the partition coefficient (*K*), detected by HPLC, of the target compounds of crude samples and the retention of the stationary phase, attained with a short settling time of the solvent system (<30 s; Oka *et al.*, 2002). The partition coefficient (*K*) value was expressed as the peak area of the target compound in the upper organic phase divided by that in the lower phase (Ito, 2005). In this work, several two-phase solvent systems were investigated.

Evaporative light scattering detection (ELSD) is a universal, nonspecific detection method that can provide a stable baseline even with gradient elution (Ju *et al.*, 2009). In this study, we explore an efficient and rapid method for the preparative isolation and purification of inotodiol and trametenolic acid from *I. obliquus* using HSCCC coupled with ELSD.

Experimental

Reagents and materials

All organic solvents used for HSCCC were of analytical grade and purchased from Shanghai Su ran Chemical Factory (Shanghai, China). Methanol used for HPLC analysis was of chromatographic grade and purchased from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China). Deionised water was used throughout the experiment.

The dried fruiting bodies of *I. obliquus* and standards of inotodiol and trametenolic acid were supplied by Key Laboratory for Biotechnology of Medicinal Plant of Jiangsu Province, Xuzhou Normal University, China.

Apparatus

The preparative HSCCC apparatus used in this study was a model HHS-400A multilayer coil planet centrifuge (Shanghai Tong Hong machine Co. Ltd, Shanghai, China) equipped with a polytetrafluoroethylene multilayer coil of 130 m×1.6 mm i.d. with a total capacity of 260 mL. The revolution radius or the distance between the holder axis and the central axis of the centrifuge (*R*) was 5 cm. The β-value of the preparative column varied from 0.33 at the internal to 0.86 at the external ($\beta = r/R$, where *r* is the distance from the coil to the holder shaft; Nakamura *et al.*, 2009). The rotational speed was adjustable from 200 to 850 rpm, and 800 rpm was used in this study. The system was also equipped with one NS-1007 constant flow pump, a model 8823B-UV monitor operating at 254 or 280 nm wavelength, a Yakogawa 3057 recorder, a manual injection valve with a 2 or 10 mL sample loop, and a fraction collector Beckman SC-100 (Iscor Inc., New York, USA).

A Beckman Technology HPLC system was used in this study which consists of a P3000 pump, a PL-ELS1000 detector (Polymer Laboratories Ltd Inc., Loughborough, UK) and a sample injector (model 7725; Hamilton Company, Reno, NV, USA) with a 20 µL loop. The column used in the experiment was an Alltima C_{18} column (250×4.6 mm, 5 µm). Evaluation and quantification were made using a CXTH-3000 (Beijing Chuangxin Tongheng Science And Technology Co. Ltd, Beijing, China). A Water Pro PS was used to produce deionised water (Labconco Co., Kansas, MO, USA).

^{1}H and ^{13}C NMR spectra were recorded on a Bruker AV 400 NMR spectrometer with tetramethylsilane as an internal standard. (Bruker AXS Inc., Madison, WI, USA).

Preparation of crude extract

The dried sclerotium of *I. obliquus* (50 g) was comminuted and extracted with 1 L of ethanol using a Soxhlet extractor for 20 min (Tian *et al.*, 2000). The insoluble materials were extracted three times. The extracts were combined, evaporated to dryness under reduced pressure at 40°C and redissolved in 500 mL of water. The aqueous solution was again extracted for three, each time with 500 mL petroleum ether. The aqueous phase was further extracted for three, each time with 500 mL chloroform. One gram of the chloroform extract was stored in a refrigerator for the subsequent HSCCC separation.

Preparation of the two-phase solvent system and sample solution

The two-phase solvent system utilised in the present study was composed of hexane:ethyl acetate:methanol:water (1:0.4:1:0.4, v/v/v/v). The mixtures were thoroughly mixed for about 2 min in a separation funnel and allowed to sit for more than 1 h at room temperature. The two phases were separated shortly before use. The upper organic phase was used as the stationary phase and the lower aqueous phase as the mobile phase. The sample solution was prepared by dissolving 100 mg of the chloroform extract of *I. obliquus* in 10 mL solvent comprising 5 mL of upper phase and 5 mL of lower phase (1:1, v/v).

HSCCC separation procedure

The multilayer coiled column was first filled with the upper organic phase as the stationary phase for 35 min with a flow-rate of 9.0 mL/min when the rotation was static. Then the apparatus was rotated at 800 rpm, while the lower phase (mobile phase) was pumped into the column at a flow-rate of 2.0 mL/min. After hydrodynamic equilibrium was reached in each column, as indicated by a clear mobile phase eluting from the tail outlet (Chen *et al.*, 2007), the sample (100 mg) was loaded into the injection valve after the system had reached hydrodynamic equilibrium. Each fraction was collected into 5 mL tubes when the solvent peak had been completely revealed. Analyses of fractions were performed by HPLC-ELSD.

HPLC analysis

Approximately 2 mg of the crude sample was weighed in a test tube. Two millilitres of each phase of the equilibrated two-phase solvent system was added into the same test tube. The chloroform extract from *I. obliquus* was analysed by HPLC-ELSD. The column temperature was set at 25°C. The mobile phase, which was composed of methanol and water (92:8, v/v) flowed at 1.0 mL/min. The effluent was continuously monitored by ELSD. The setting time, which was highly correlated with the retention of stationary phase, was expressed as the time to form a clear interface between the two phases when each phase (1:1, v/v) was mixed.

The fractions from the preparative HSCCC separation were analysed by HPLC-ELSD, and column temperature was 25°C. The mobile phase, composed of methanol and water (92:2, v/v), was eluted at a flow-rate of 1.0 mL/min.

Results and Discussion

HPLC-ELSD analysis

The selection of the HPLC conditions was guided by the requirement for obtaining chromatograms with better resolution

of adjacent peaks within a short time. The HPLC conditions developed in this study produced full peak-to-baseline resolution of the two target compounds, i.e. inotodiol and trametenolic acid. Because of the weak UV absorption of the target compounds, HPLC-ELSD was developed to detect these compounds in our separation procedure. The result showed that this unique hyphenated technology was a rapid and convenient way to detect the nonvolatile compounds. The operating conditions for ELSD such as the nebulising gas flow rate and the drift tube temperature were optimised to obtain the best *signal-to-noise level*. The nebuliser gas flow was set at 1.6 L/min, and the drift tube temperature was determined to be 90°C according to the data computed with the ELSD software. Figure 1 shows the HPLC-ELSD chromatograms of the crude sample extract.

Optimisation of HSCCC conditions

Appropriate two-phase solvent systems that provide values of the partition coefficient (K) in the range 0.5–2.0 for the target component are important for successful separation by HSCCC. Settling time of the solvent system was also considered in this work. Several two-phase solvents were tested and the K and α values of the target compounds were determined and are listed in Table 1. It can be seen that the K values of the ethyl acetate:methanol:water and chloroform:methanol:water systems were out of the range of 0.5–2. Hexane:ethyl acetate:methanol:water was found to be a suitable solvent system and crude extract could be resolved easily.

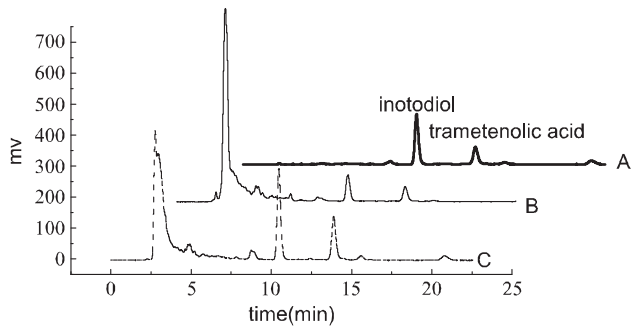

Figure 1. HPLC chromatograms of target compounds. Solvent system: hexane–ethyl acetate–methanol–water (1:0.4:1:0.4, v/v/v/v). (A) HPLC analysis of the lower aqueous phase of chloroform extract from *I. obliquus*; (B) HPLC analysis of the upper organic phase of chloroform extract from *I. obliquus*; (C) HPLC analysis of the crude sample. Experimental conditions: Alltima C_{18} column (250×4.6 mm, 5 μm); column temperature, 25°C; mobile phase, methanol and water (92:2, v/v); flow-rate, 1.0 mL/min detection, PL-ELS1000.

Several solvents of hexane:ethyl acetate:methanol:water with different volume ratios were tested. At first, hexane:ethyl acetate:methanol:water (1:0.6:1:0.6, v/v/v/v) was chosen. After HPLC analysis, the K value of the target component (0.12 for inotodiol and 0.18 for trametenolic acid) was not suitable for the HSCCC process (Table 1). A low K value means that the solubilities of the two target compounds in the upper phase, compared with the solubilities in the lower phase, were too low. In the solvent system of hexane:ethyl acetate:methanol:water, the ratio of ethyl acetate could adjust the solubilities of the two target compounds in both upper and lower phases. When the higher ratio of ethyl acetate was chosen, hexane:ethyl acetate:methanol:water (0.9:1:0.9:1), solubilities in upper phase tend to increase (Table 1). However, the settling time (>30 s) of the solvent system was not acceptable. Meanwhile decreasing ratio of ethyl acetate could also adjust the solubilities of the two target compounds and provide an acceptable settling time. As shown in Table 1, the solvent system composed of hexane:ethyl acetate:methanol:water (1:0.4:1:0.4, v/v/v/v) provided acceptable K values for inotodiol (0.60) and trametenolic acid (0.91). The inotodiol was primarily located between the retention time of 3.5 and 4 h, and trametenolic acid was primarily located between the retention time of 2 and 2.5 h. Figure 2 shows the HPLC-ELSD chromatograms of the purified inotodiol and trametenolic acid. The purities of inotodiol and trametenolic acid after HSCCC separation were 97.51 and 94.04%, and the recoveries were 85.5 and 85.7%, respectively.

The chromatograms of HPLC-ELSD were more useful in calculating the K value of solvent systems than the chromatograms of HPLC-UV because the ELSD detection method encountered less compound interference than UV detection.

Table 1. The partition coefficient (K) values and separation factors (α) of the targeted components under different solvent systems

Solvent system	K value		Separation factor (α)[a]
	Inotodiol (K_1)	Trametenolic acid (K_2)	
Hexane–ethyl acetate–methanol–water (0.9:1:0.9:1)	0.26	0.24	1.08
Hexane–ethyl acetate–methanol–water (1:1:1:1)	0.07	0.08	1.14
Hexane–ethyl acetate–methanol–water (1:0.8:1:0.8)	0.04	0.06	1.36
Hexane–ethyl acetate–methanol–water (1:0.6:1:0.6)	0.12	0.18	1.6
Hexane–ethyl acetate–methanol–water (1:0.8:1:0.4)	0.34	0.45	1.3
Hexane–ethyl acetate–methanol–water (1:0.6:1:0.4)	0.57	0.77	1.37
Hexane–ethyl acetate–methanol–water (1:0.4:1:0.4)	0.60	0.91	1.52
Hexane–ethyl acetate–methanol–water (1:0.4:1:0.6)	0.08	0.10	1.32
Hexane–ethyl acetate–methanol–water (1:0.3:1:0.4)	0.48	0.78	1.64
Hexane–ethyl acetate–methanol–water (1:0.4:1:0.3)	1.31	2.12	1.61
Chloroform– methanol–water(4:3:2)	234.56	95.14	2.46
Ethyl acetate–methanol–water(2:1:2)	0.1	0.3	3

[a]K_2/K_1 ($K_2 > K_1$). α-Value is expressed as separation factor of target component. A minimum α-value of 1.5 is required for baseline separation in HSCCC equipment.

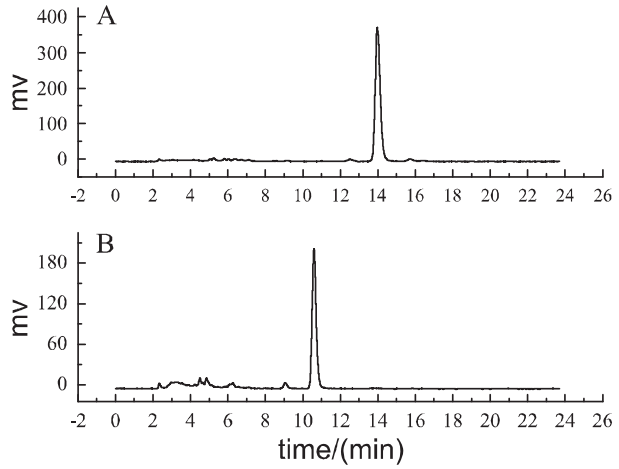

Figure 2. (A) HPLC analysis of trametenolic acid after HSCCC separation; (B) HPLC analysis of inotodiol after HSCCC separation. Experimental conditions: Alltima C_{18} column (250×4.6 mm, 5 μm); column temperature, 25°C; mobile phase, methanol and water (92:2, v/v); flow-rate, 1.0 mL/min detection, PL-ELS1000.

Structure identification

The chemical structure of the two target compounds purified by HSCCC was identified based on their ^{1}H NMR and ^{13}C NMR data. According to the reported data, the ^{1}H NMR and ^{13}C NMR data from our experiment were in agreement with the target compounds inotodiol and trametenolic acid (Shin *et al.*, 2001a, 2001b). The chemical structures of inotodiol and trametenolic acid were shown in Fig. 3.

^{1}H NMR ($CDCl_3$, 400 MHz) of inotodiol: δ0.75, 0.82, 0.88, 0.99, 1.01, 1.67, 1.77 (3H, s, H_3-18, 29, 30, 19, 28, 26, 27), 0.95 (3H, d, *J*=6.6 Hz, H_3-21), 3.24 (1H, dd, *J*=11.3, 4.6 Hz, H-5), 3.69 (1H, m, *J*=6.3, 3.3 Hz, H-22), 5.19 (1H, t, *J*=7.2 Hz, H-24).

^{13}C NMR ($CDCl_3$, 100 MHz) of inotodiol: δ12.32 (C-21), 15.13 (C-28), 15.39 (C-18), 17.69 (C-27), 17.95 (C-19), 18.85 (C-6), 20.71 (C-11), 24.01 (C-30), 25.70 (C-20), 26.22 (C-12), 26.95 (C-23), 27.54 (C-2), 27.67 (C-29), 28.81 (C-7), 30.66 (C-15, C-16), 35.29 (C-1), 36.74 (C-10), 38.60 (C-4), 41.39 (C-20), 44.56 (C-13), 46.97 (C-17), 49.11 (C-14), 50.09 (C-5), 73.08 (C-22), 78.68 (C-3), 121.07 (C-24), 133.90 (C-8), 134.28 (C-9), 134.87 (C-25).

^{1}H NMR (C_5D_5N, 400 MHz) of trametenolic acid: δ0.989 (3H, s, H_3-18), 0.955 (3H, s, H_3-30), 1.050 (3H, s, H_3-28), 1.141 (3H, s, H_3-19), 1.168 (3H, s, H_3-29), 1.596 (3H, s, H_3-25), 1.671 (3H, s, H_3-27), 3.420 (1H, dd, *J*=7.2 Hz, H-3), 4.430 (1H,d, *J*=4.8 Hz, OH), 5.302 (1H, t, *J*=7.2 Hz, H-24).

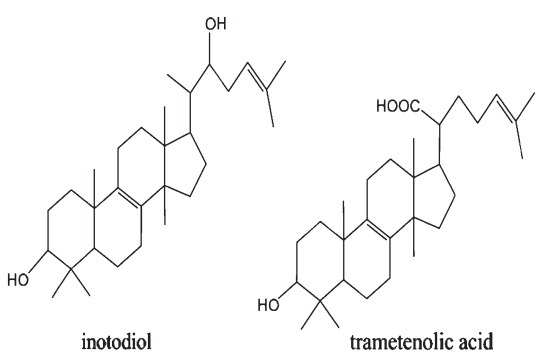

Figure 3. Chemical structure of inotodiol and trametenolic acid characterized by NMR.

^{13}C NMR (C_5D_5N, 100 MHz) of trametenolic acid: δ17.94 (C-29), 17.98 (C-18), 19.33 (C-27), 20.31 (C-6), 21.01 (C-19), 22.58 (C-11), 26.1 (C-30), 27.39 (C-26), 28.34 (C-23), 28.41 (C-22), 29.09 (C-16), 30.23 (C-7), 30.3 (C-28), 30.99 (C-2), 32.47 (C-12), 34.91 (C-15), 37.71 (C-1), 38.98 (C-10) 41.13 (C-4), 46.49 (C-13), 49.33 (C-20), 50.63 (C-17), 51.44 (C-14), 52.49 (C-5), 79.6 (C-3), 126.48 (C-24), 133.31 (C-25), 135.88 (C-9), 136.76 (C-8), 180.24 (C-21).

Conclusion

An efficient and rapid HSCCC-ELSD method was developed for the separation and purification of inotodiol and trametenolic acid from *I. obliquus*. Hexane:ethyl acetate:methanol:water (1:0.4:1:0.4, v/v/v/v) was selected as the two-phase solvent system. Triterpenoid compounds were obtained with high purity from the crude extract in one-step separation. In the crude extract, the purities of inotodiol and trametenolic acid were 15.28 and 9.33%, respectively. The purities of the prepared inotodiol and trametenolic acid were 97.51 and 94.04%, respectively, and the recoveries were 85.5 and 85.7%, respectively. HSCCC coupled with ELSD was an effective method to isolate components without UV absorptions.

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