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Separation and Purification Technology xxx (2007) xxx–xxx
Separation and Purification Technology
www.elsevier.com/locate/seppur

Separation and purification of DL-tetrahydropalmatine from *Corydalis yanhusuo* by high-speed counter-current chromatography

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Received 24 January 2007; received in revised form 12 May 2007; accepted 15 May 2007

Abstract

High-speed counter-current chromatography (HSCCC) was applied to preparative separation and purification of DL-tetrahydropalmatine from *Corydalis yanhusuo* by a one-step separation. The two-phase system consisted of petroleum ether–ethyl acetate–methanol–water (15:30:21:20, v/v) was employed. An orthogonal design was used for optimizing the revolution speed of the separation column, flow rate of the mobile phase and separation temperature, which was 850 rpm, 1.2 ml/min and 20 °C, respectively. HPLC analysis of the fractions collected by preparative HSCCC of 200 mg of crude extracts showed that the purity of DL-tetrahydropalmatine (8.6 mg) was 96.4%. The chemical identity of the component was confirmed by ^{1}H NMR and EI-MS.
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Keywords: Counter-current chromatography; DL-Tetrahydropalmatine; *Corydalis yanhusuo*; Orthogonal design

1. Introduction

Corydalis yanhusuo W.T. Wang, a perennial herb belonging to the Papaveraceae family, is well known as a traditional Chinese herbal medicine. *C. yanhusuo* grows wild in Siberia and Northern China and is cultivated principally in Zhejiang, China. The dried and pulverized tubers of *C. yanhusuo* is also known as Rhizoma Corydalis or Yanhusuo [1]. It has been used to promote blood circulation, reinforce vital energy and alleviate pain such as spastic, abdominal, and menstrual pain [2,3]. Its major active components are alkaloids and among them are tertiary alkaloids with very similar structures and basicity, and of which DL-tetrahydropalmatine (DL-THP, Fig. 1) is the most important [4]. Thus separation and purification of DL-THP are very desirable.

Due to the complexity of this herb composition, and the extremely low DL-THP content, tedious procedures involving several liquid–liquid extractions are generally performed in the traditional methods for the extraction and purification of DL-THP [2]. High-speed counter-current chromatography (HSCCC), being as a support free liquid–liquid partition chromatography, eliminates irreversible adsorption of sample onto the solid support. This method has been successfully applied to the separation and purification of various natural products [5–10]. However few studies have been published on the use of HSCCC for separation and purification of DL-THP from *C. yanhusuo*. This paper describes HSCCC separation and purification of DL-THP from *C. yanhusuo* by a one-step separation. Furthermore, an orthogonal design was used for optimizing the important separation parameters, such as, revolution speed of the separation column, flow rate of the mobile phase and separation temperature.

2. Experimental

2.1. Instrumentation

The preparative HSCCC instrument (Model TBE-300, Shanghai Tauto Biological Company, China) was equipped with three preparative coils connected in series (diameter of polytetrafluoroethylene (PTFE) tube, 2.6 mm; total volume, 119 ml) and a 10 ml sample loop. The revolution speed of the instrument could be regulated with a speed controller in the range between 0 and 999 rpm. Constant temperature circulator (HX-1050, Beijing Boyikang Experimental Apparatus Company, China) was used to control the temperature. The solvent was pumped into

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doi:10.1016/j.seppur.2007.05.011

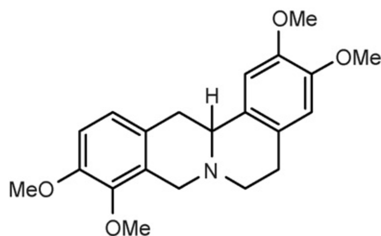

Fig. 1. Chemical structure of DL-tetrahydropalmatine ($C_{12}H_{25}NO_4$).

the column with the AKTA purifier pump P-900 (Amesham, USA) at a flow rate of up to 10 ml/min and pressure up to 25 MPa. The continuous monitoring of the effluent was achieved with a monitor UV-900, a multi-wavelength UV–vis monitor for simultaneous monitoring of up to three wavelengths in the range 190–700 nm (Amersham, USA).

An Agilent/HP 1100 series HPLC-DAD system consisting of a vacuum degasser, quandary pump, thermostated column compartment and diode array detection (DAD) (Agilent, Palo Alto, CA, USA) was used for acquiring chromatogram. Pure water was treated with SAGA-30D Super Pure Water System (Saga Electronic Technology Co. Ltd., Shanghai, China). Disintegrator (Shanghai Experimental Instrument Company, Shanghai, China) and vacuum desiccator (ZK 82J electrothermal vacuum desiccator, Shanghai Experimental Instrument Company, China) were also used in experiment.

2.2. Materials

C. yanhusuo (Zhejiang, China) was purchased from Shanghai Kangqiao Medical Factory, authenticated by Shanghai Chinese Traditional Medicine Research Institute and fitted for Chinese Pharmacopoeia. All solvents used for the preparation of crude extracts and HSCCC separation were of analytical grade (Chinese Medication Group Shanghai Chemical Reagent Company, Shanghai, China). Methanol (Merck) and pure water used for HPLC were of chromatographic grade. DL-THP standard sample was purchased from National Institute for the Control of Pharmaceutical and Biological Products, the Ministry of Health, Beijing, China.

2.3. Preparation of crude samples

Different concentrations of ethanol were tested during the preparation of crude samples (30%, 50%, 70%, 90%). After analysis of HPLC, the highest DL-THP content appeared in the extracts of 70% ethanol. The 10.0 g *C. yanhusuo* was ground to powder (60 mesh) by a disintegrator, 40 ml concentrated ammonia water and 60 ml 70% ethanol was added into the flask, heated to reflux for 1.5 h. Then the extraction solution was filtered and concentrated to dryness by rotary evaporator under reduced pressure and further dried under vacuum, the yield of extraction was 7.63%. The residue was stored in a desiccator.

2.4. HSCCC separation procedure

A mixture of petroleum ether–ethyl acetate–methanol–water (15:30:21:20, v/v) was prepared in a separatory funnel and set at room temperature until there were two clearly separated phases. The upper phase and lower phase should be degassed using an ultrasonic bath for 15 min. The multilayer coiled column was first filled with the upper phase at a flow rate of 10 ml/min. Then the lower phase was pumped into the column at a flow rate of 1.2 ml/min. In the meantime, the HSCCC apparatus was rotated at 850 rpm, and the system was kept at 20 °C. After the upper phase emerged and the volume unchanged, the liquid-liquid equilibrium was established in the column. A sample solution containing 200 mg *C. yanhusuo* extracts in 10 ml of the lower phase of the two-phase solvent system was injected through the injection valve. The effluent was continuously monitored with UV detector at 280 nm and peak fractions were collected according to the chromatogram. After using thin layer chromatography (TLC) as a primary comparison with the standard sample of DL-THP, HPLC analysis was carried out.

2.5. HPLC analysis, mass spectrometry, and ^{1}H NMR identification of the fractions

HPLC analysis was performed with an Agilent/HP 1100 series (Agilent, USA), which consisted of a vacuum degasser, quandary pump, thermostated column compartment, diode array detection (DAD) (Agilent, Palo Alto, CA, USA) and an injection valve with a 20 μl loop. The crude extracts of *C. yanhusuo* and each purified peak fraction from the preparative HSCCC separation were analyzed by HPLC (YMC C_{18} column, 5.0 μm, 4.6 mm × 150 mm), eluted with methanol–0.1% phosphoric acid (diethylamine added, pH 6.0) (65:35, v/v) at a flow rate of 1.0 ml/min with the temperature being 30 °C. The purified fraction of DL-THP obtained from the preparative HSCCC separation was analyzed by electron impact mass spectroscopy (EI-MS) (GC-TOFMS, Micromass, UK) and ^{1}H NMR (Bruker Advance 500 MHz spectrometer referenced to tetramethylsilane), respectively.

3. Results and discussion

3.1. HSCCC separation of DL-THP from the crude extracts

3.1.1. Selection of two-phase solvent system

The selection of appropriate two-phase solvent system for the target compound is the most important step in HSCCC where searching for a suitable two-phase solvent system may be estimated to be 90% of the entire work in HSCCC. Ideally, the mobile phase passes through the system, while more than 50% of the stationary phase is retained. This can be tested by determination of the separation time of the two phases, which should not exceed 20 s after gently inverting for several times. Furthermore, a suitable partition coefficient (K), which is the ratio of solute distributed between the mutually equilibrated two solvent phases. The suitable K value for HSCCC are in the range of

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Table 1
The K and retention of the stationary phase values of DL-THP in solvent systems

Solvent system (v/v)	K value	Retention of the stationary phase (%)
Petroleum ether–ethyl acetate–methanol–water		
1:3:2:2	0.48	50.21
11:29:21:19	0.39	48.96
15:30:21:20	0.88	55.21
16:31:19:21	1.59	49.37

0.5–1.0, higher retention of the stationary phase normally results in better peak resolution [11].

In the experiment, preliminary HSCCC studies were carried out with eight different two-phase solvent systems, including chloroform–methanol–0.2 mol L^{-1} hydrochloric acid (8:2:3, 8:4:3, 10:4:4, 10:6:4, v/v) and petroleum ether–ethyl acetate–methanol–water (1:3:2:2, 11:29:21:19, 15:30:21:20, 16:31:19:21, v/v). The two-phase solvent systems of chloroform–methanol–0.2 mol L^{-1} hydrochloric acid with different ratios did not give effective separation and led to serious emulsification, so this solvent system was not chosen for further research. The solvent system of petroleum ether–ethyl acetate–methanol–water would be evaluated. The K value was expressed as the peak area of target compound in the upper phase versus the lower phase. The retention of the stationary phase relative to the total column volume was determined by collecting the stationary phase during the separation. The measured K value and ratio of the stationary phase retention of two-phase system was shown in Table 1. After the comparison, we found petroleum ether–ethyl acetate–methanol–water (15:30:21:20) gave the best separation result.

3.1.2. Optimization of the separation condition

The revolution speed of the separation column, flow rate of the mobile phase and separation temperature have different effect on the separation result. The flow rate of the mobile phase determines the separation time, the amount of stationary phase retained in the column, and the peak resolution. Use of a lower speed will reduce the volume of the stationary phase retained in the column, which may lead to lower peak resolution. On the other hand, higher speed may produce excessive sample band broadening by violent pulsation of the column because of the elevated pressure [10]. An orthogonal design L_9 (3^4) was used to determine the optimum combination. The factors and levels were listed in Table 2. The retention value of the stationary phase was shown in Table 3. It can be seen that these three factors do affect the retention of the stationary phase in the order of speed of the separation column (B), flow rate of the mobile phase (A), and separation temperature (C). The optimum combination of these three factors should be $A_2B_2C_1$. Under the conditions of 1.2 ml/min of flow rate of the mobile phase, 850 rpm of revolution speed of the separation column, and 20 °C of the separation temperature, petroleum ether–ethyl acetate–methanol–water (15:30:21:20) gave the best separation. The retention of the stationary phase was 55.21%. The preparative HSCCC separation chromatogram was shown in Fig. 2, 8.6 mg of DL-THP was obtained from the 200 mg crude extracts.

Table 2
L_9 (3^3) orthogonal test design

Test. no.	Flow rate of the mobile phase, A (ml/min)		Revolution speed of the separation column, B (rpm)		Separation temperature, C (°C)	
1	A_1	1.0	B_1	750	C_1	20
2	A_1	1.0	B_2	850	C_2	25
3	A_1	1.0	B_3	950	C_3	30
4	A_2	1.2	B_1	750	C_2	25
5	A_2	1.2	B_2	850	C_3	30
6	A_2	1.2	B_3	950	C_1	20
7	A_3	1.5	B_1	750	C_3	30
8	A_3	1.5	B_2	850	C_1	20
9	A_3	1.5	B_3	950	C_2	25

Table 3
The results of orthogonal design L_9 (3^4)

No.	A (ml/min)	B (rpm)	C (°C)	Retention of the stationary phase (%)
1	A_1	B_1	C_1	47.79
2	A_1	B_2	C_2	53.31
3	A_1	B_3	C_3	48.46
4	A_2	B_1	C_2	48.49
5	A_2	B_2	C_3	54.12
6	A_2	B_3	C_1	50.86
7	A_3	B_1	C_3	46.01
8	A_3	B_2	C_1	52.85
9	A_3	B_3	C_2	47.97
k_1	49.85	47.43	50.50	
k_2	51.16	53.43	49.92	
k_3	48.94	49.10	49.53	
R	2.22	6.00	0.97	

$k_i^A = \sum$(retention of the stationary phase at A_i)/3; $R_i^A = \max\{k_i^A\} - \min\{k_i^A\}$.

3.2. HPLC analysis

The crude samples and peak fractions collected from HSCCC were analyzed by HPLC under the analytical conditions described in Section 2.5. The results were shown in Fig. 3. The purified sample of DL-THP calculation was made by comparison of the peak area with the standard. The results indicted that peak 5 corresponded to DL-THP, and its purity was 96.4%.

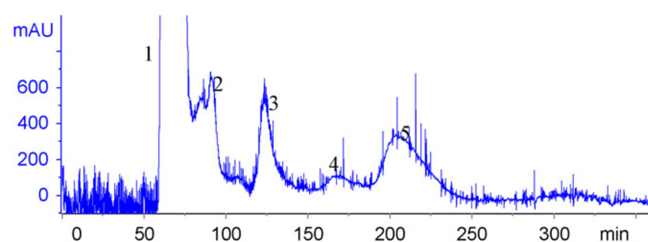

Fig. 2. Chromatogram of crude extracts of *Corydalis yanhusuo* by HSCCC. Peak 5, DL-THP. Experimental condition: two-phase solvent system, petroleum ether–ethyl acetate–methanol–water (15:30:21:20); stationary phase, upper phase; mobile phase, lower phase; flow-rate, 1.2 ml/min; rotary speed, 850 rpm, separation temperature, 20 °C; sample size, 200 mg of crude extracts dissolved in 10 ml of the lower phase; retention of the stationary phase, 55.21%. Detection at 280 nm (the peak 5 purity was 96.4% estimated by HPLC).

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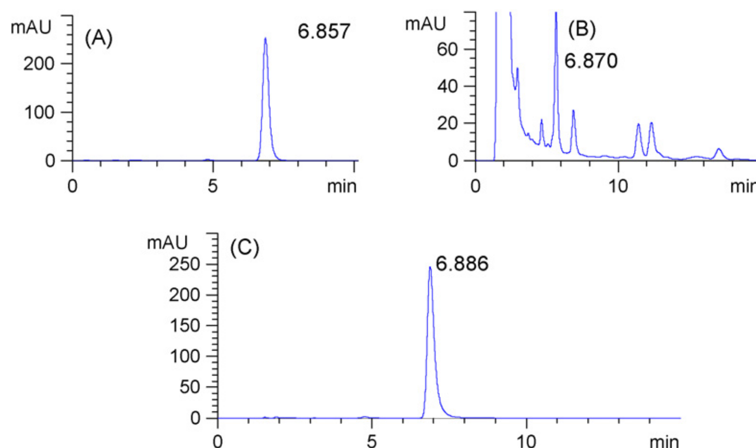

Fig. 3. HPLC chromatogram of the standard sample of DL-THP (A), crude extracts of *C. yanhusuo* (B), peak 5 from preparative HSCCC (C). Experimental condition: YMC C_{18} column (5.0 μm, 4.6 mm × 150 mm); mobile phase, methanol–0.1% phosphoric acid (diethylamine added, pH 6.0) (65:35, v/v); flow rate, 1.0 ml/min; temperature, 30 °C; detection at 280 nm.

3.3. Structural identification

The identity of peak 5 in HSCCC was determined by electron impact mass spectroscopy (EI-MS) and ^{1}H NMR. EI-MS: *m*/*z*, 355 (100%), 324 (20%), 164 (64%), 149 (56%), 121 (9%). The molecular ion at *m*/*z* 355 corresponds to the formula $C_{21}H_{25}NO_4$ of DL-THP, other ionic peaks are the fragments of the molecule. ^{1}H NMR ($CDCl_3$) δ (ppm): 2.66 (m, 2H), 2.85 (t, 1H, J = 1.7 Hz), 3.12 (m, 1H), 3.20 (t, 1H, J = 6.0 Hz), 3.27 (m, 1H), 3.55 (t, 2H, J = 7.7 Hz), 3.88 (m, 12H), 4.26 (d, 1H, J = 15.8 Hz), 6.62 (s, 1H), 6.73 (s, 1H), 6.80 (d, 1H, J = 8.4 Hz), 6.89 (d, 1H, J = 8.4 Hz). Compared with the data given in reference [12], these data indicate the compound in peak 5 is DL-THP.

4. Conclusion

The overall results indicate that HSCCC was successfully used for separation and purification of DL-THP from *C. yanhusuo*, which is a bioactive substance in *C. yanhusuo*. About 8.6 mg of DL-THP was obtained from the 200 mg crude extracts with purity over 96%. HSCCC has been demonstrated to be a powerful tool in separation and purification of bioactive components from the natural medicines.

Acknowledgements

Financial supports from Shanghai Commission of Science and Technology and National Engineering Research Center for Traditional Chinese Medicine are gratefully acknowledged.

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