Preparative Separation of Two Alkaloids from Devil Pepper Radix (*Rauvolfia verticillata* [Lour.] Baill.) by pH-Zone-Refining Counter-Current Chromatography

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A method was developed for the preparative separation of two alkaloids from the crude extract of the radix of *Rauvolfia verticillata* (Lour.) Baill. in a single run. The two-phase solvent system composed of petroleum ether–ethyl acetate–methanol–water (5:5:2:8, v/v), where triethylamine (40 mmol/L) was added to the upper organic phase as the stationary phase and hydrochloric acid (10 mmol/L) was added to the lower aqueous phase as the mobile phase, was selected for this separation by pH-zone-refining counter-current chromatography (PZRCCC). For the preparative separation, the apparatus was rotated at a speed 850 rpm, while the mobile phase was pumped into the column at 2 mL/min. As a result, 112 mg of reserpine and 21 mg of yohimbine were obtained from 3 g of crude extract in a single run. The analysis of the isolated compounds was determined by high-performance liquid chromatography (HPLC) at 230 nm with purities of over 91.0%, and the chemical identification was carried out by the data of electrospray ionization–mass spectrometry (ESI–MS) and nuclear magnetic resonance (NMR) spectroscopy. The technique introduced in this paper is an efficient method for preparative separation of reserpine and yohimbine from devil pepper radix. It will be beneficial to utilize medicinal materials and also useful for the separation, purification, and pharmacological study of Chinese herbal ingredients.

Keywords: pH-zone-refining counter-current chromatography, *Rauvolfia verticillata* (Lour.) Baill., reserpine, yohimbine, alkaloids

Introduction

Devil pepper, the radix of *Rauvolfia verticillata* (Lour.) Baill., has been used for fever, sore throats, headache, and dizziness as a traditional Chinese medicine for a long time. It has been reported that devil pepper is antihypertensive, sedative, and removes blood stasis effect [1]. For now, hundreds of alkaloids were isolated from *R. verticillata* (Lour.) Baill., the major of which belong to indole and double hydrogen indole [2–4]. The major antihypertensive active constituents are reserpine and yohimbine; however, their content is low. In order to improve the preparation amount and meet the requirement of pharmacological effect research, establishing an efficient separation method for reserpine and yohimbine is necessary.

pH-zone-refining counter-current chromatography (PZRCCC) is a unique separation and isolation technique, which is developed from high-speed counter-current chromatography (HSCCC). There are two significant factors to realize the separation, a suitable solvent system composition for the target sample, target organic acid, and alkaloids in the solvent system for a better separation effect [5, 6]. The target compound realized separation is based on the difference of partition coefficient and hydrophobicity. Compared with HSCCC, chromatographic peak profile of PZRCCC is rectangular zone with steep border instead of Gaussian distribution. PZRCCC has many advantages including large sample loading capacity, high concentration, and high purity of targeted components [7–9]. Besides that, it detects samples by

their pH values, which is helpful for the compounds without ultraviolet absorbance [10]. Although PZRCCC has been employed for the separation of indole alkaloids from the leaves of *Rauvolfia* genus, a second purification step using medium pressure liquid chromatography (LC) to separate the yohimbine from reserpine was required [11]. Comparing with the above report, a PZRCCC method was established for preparative separation of alkaloids from devil pepper in one step, and reserpine and yohimbine with purity of 93.7% and 91.2%, respectively, were obtained. Their structures were shown in Figure 1.

Experimental

Reagents and materials. Methanol (MeOH), chloroform (CHCl₃), petroleum ether (Pet) (60–90 °C), ethyl acetate (EtAc), ethyl alcohol (EtOH), hydrochloric acid (HCl), and triethylamine (TEA) used in PZRCCC separation were of

Figure 1. Chemical structures of alkaloids from devil pepper radix

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analytical grade (Shanghai SSS Reagent Co., Ltd., Shanghai, China). Deuterated chloroform (CDCl_3) and tetramethylsilane (TMS) used in nuclear magnetic resonance (NMR) analysis were of analytical grade (Shanghai SSS Reagent Co., Ltd., Shanghai, China). Methanol and acetonitrile (CH_3CN) used for high-performance liquid chromatography (HPLC) analysis were of chromatographic grade (Tedia Company, Inc., Fairfield, USA). Reverse osmosis Milli-Q water (Millipore, Bedford, MA, USA) was used for all solutions and dilutions.

The root of *R. verticillata* (Lour.) Baill. was purchased from Chinese herbal medicine market (Bozhou, Anhui Province) and was identified by Professor Jia Li (Shandong University of TCM, Jinan, China).

Apparatus. The preparative HSCCC instrument employed in the present study was a Model TBE-300A commercial instrument (Shanghai Tauto Biotech Co., Ltd., Shanghai, China), with three preparative coils (2.6 mm inner diameter [i.d.] and 150 m in length with a total capacity of 300 mL) and a quantitative loop (the volume is 30 mL). The rotation of column is controlled by a speed controller in the range of 1 to 1000 rpm. The solvent was pumped into the column with a TBP1002 constant-flow pump (Beijing Bokang Experimental Equipment Co., Ltd., Beijing, China). The temperature of HSCCC instrument was kept at about 25 °C by DCW-0506 constant temperature circulator (Shanghai Baidian Instrument Co., Ltd., Shanghai, China). Continuous monitoring of the effluent was achieved with a Model 8823A-UV Monitor (Beijing Institute of New Technology Application, Beijing, China) at 254 nm and a PHS-3C pH meter (Wuhan Jisi Instruments Equipments Co., Ltd., Wuhan, China). A portable recorder (Yokogawa Model 3057, Sichuan Instrument Factory, Chongqing, China) was used to draw the chromatogram. The PZRCCC chromatogram was described by a model STR1001 recorder (Jiangsu Shun Tong Instrument Co., Ltd., Jiangsu, China). The crude sample and purified alkaloids were analyzed by the Waters Alliance Liquid Chromatography system where a 2695 pump, a 2998 Photodiode Array Detection (PAD) system, a 2695 System controller, a 2695 Multi-solvent Delivery System, and an Empower 3 workstation (Waters, Milford, USA) were used. The electrospray ionization–mass spectrometry (ESI–MS) analysis was carried out by Agilent 1100/mass selective detector (MSD) (Agilent Technologies, Santa Clara, USA). The NMR analysis was carried out by INOVA 600 MHz (Varian Medical systems, Palo Alto, USA).

Preparation of crude sample. The root of *R. verticillata* (Lour.) Baill. (1.0 kg) was pulverized and extracted three times with 90% ethanol for 2 h (ratio volume to weight, 5:1, v/w). After filtration, the extracts were combined and evaporated to dryness by rotary evaporation under reduced pressure. The residue was dissolved in 1 L water, acidized with HCl (2–3 mol/L) to pH 2.5, and then extracted with petroleum ether. The filtrate was basified with NH_4OH up to pH 9.5 and then extracted three times with CHCl_3 . The CHCl_3 extracts were combined and concentrated to dryness yielding 14.0 g crude alkaloids, which was stored in a refrigerator (4 °C) for subsequent PZRCCC separation.

Determination of the partition coefficient (K_D). The primary election of solvent system was based on the partition coefficient (K_D) of the target components abiding by the principle introduced by Ito [12]. The K_D values were determined using HPLC analysis as follows: (1) volumes (1 mL) of the upper and lower phases were placed into a 2 mL test tube; (2) a suitable amount of crude sample was also added into the test tube, as was 5 μL of HCl (K_{acid}) or TEA (K_{base}); (3) the solution was then shaken to distribute crude sample between the upper and lower phases; (4) the solution was allowed to stand for about 30 s before a biphasic solution was obtained; (5) volumes (200 μL) of the upper phase were placed into another test tube and dried with a stream of nitrogen; (6) the residues were diluted

Table 1. The K_D values of yohimbine and reserpine measured in different solvents

Solvent systems (v/v)	K_D values	
	Yohimbine	Reserpine
CHCl_3 –MeOH– H_2O (4:3:3)	k_{base}	14.4
	k_{acid}	0.01
Pet–EtAc–MeOH– H_2O (5:5:5:5)	k_{base}	4.6
	k_{acid}	0.57
Pet–EtAc–MeOH– H_2O (5:5:2:8)	k_{base}	7.6
	k_{acid}	0.22
		15.5
		0.03
		3.5
		0.87
		7.8
		0.26

to 200 μL with MeOH and analyzed by HPLC; (7) the lower phase was also prepared and analyzed as above; (8) the K_D value was defined as the peak area of target compound in the upper phase divided by that in the lower phase (Table 1) [13].

Preparation of biphasic solvent system and sample solution.

In this experiment, biphasic solvent system Pet–EtAc–MeOH– H_2O (5:5:2:8, v/v) was performed by adding each solvent to a separatory funnel and then by repeated vigorous shaking at room temperature. The two phases were separated shortly before use. The upper aqueous phase (the stationary phase) was alkalized with TEA, establishing a 40 mM solution, and the lower organic phase (the mobile phase) was acidified with HCl to the concentration of 10 mM. The two phases were degassed by sonication before use.

The sample solution was prepared by dissolving crude sample (2.0 g) in the mixture solution of 10 mL lower phase and 10 mL upper phase with TEA.

PZRCCC separation. The upper phase (stationary phase) was pumped into the multilayer-coiled column at a flow rate of 20 mL/min, and then, the sample solution was injected. The lower phase (mobile phase) was then pumped into the head end of the inlet column at a flow rate of 2.0 mL/min, while the apparatus was rotated at 800 rpm. The effluent of the column was continuously monitored with a UV detector at 254 nm and collected using manual collection at 3 min intervals (6 mL per tube). Simultaneously, the pH values of every collection were determined by a pH meter and the chromatogram was described by a portable recorder. The result showed that reserpine and yohimbine were eluted as irregular rectangle zones, where two absorbance plateaus were observed. As shown in Figure 2, the first absorption platform appeared on 150–180 min, and the second absorption platform appeared on 200–215 min; impurities were distributed in both sides of the rectangular peak. After the separation process was completed, the apparatus was shut down and column contents were forced out into a graduated cylinder with pressurized nitrogen. The retention rate of stationary phase was 46.7%, which was defined as the volume of stationary phase retained in column divided by the total volume of column. The purified fractions were freeze dried and weighted; 21 mg yohimbine and 112 mg reserpine were obtained. In the above condition, the target alkaloids were purified successfully and the separation time was significantly shortened.

Figure 2. PZRCCC chromatograms for the separation of the crude extract from devil pepper radix. Experimental conditions — solvent system: ether–ethyl acetate–methanol–water (5:5:2:8, v/v); 10 mM hydrochloric acid in aqueous stationary phase and 40 mM triethylamine in organic mobile phase; revolution speed: 850 rpm; flow rate: 2 mL/min; sample size: 3 g; UV detection wavelength: 254 nm

Analysis and identification of PZRCCC fractions. The total alkaloids and separated fraction by PZRCCC were analyzed by HPLC with a Hypersil ODS column (250 mm × 4.6 mm, 5 μm). The mobile phase was a linear gradient from methanol–0.1% trifluoroacetic acid (30:70, v/v) to methanol–0.1% trifluoroacetic acid (60:40, v/v) in 60 min. The flow rate was 1.0 mL/min, the injection volume was 20 μL, the column temperature was 25 °C, and the column effluent was monitored by a PDA at the wavelength of 230 nm.

ESI–MS data were measured on an Agilent 1100 LC/MSD mass spectrometer. The mass spectrometer was scanned between the range of 50 and 1200 m/z scan 1 s with an ionizing voltage of 70 eV. The temperature of dryer was 350 °C, the flow rate of dryer was 9.0 mL/min, and atomizing pressure was 2.38 MPa.

The ¹H-NMR and ¹³C-NMR experiments were performed on a VARIAN INOVA-600 (Varian Corporation, USA) NMR spectrometer using CDCl₃ as solvent and tetramethylsilane (TMS) as internal standard.

Results and Discussion. A successful separation by PZRCCC requires a suitable two-phase solvent system which provides ideal K_D values in both acidic ($K_{acid} \ll 1$) and basic ($K_{base} \gg 1$) conditions as well as good solubility of crude sample [13]. According to the literatures about separation of indole alkaloids, two-phase solvent system composed of CHCl₃–MeOH–H₂O (4:4:3, v/v) was first selected. As shown in Figure 2, although the two solvent systems provide suitable K_D values for the target compounds, the separation time was too long and target compounds were eluted with high polar impurities. Considering solubility and polarity, two-phase solvent systems Pet–EtAc–MeOH–H₂O (5:5:5:5, v/v) and Pet–EtAc–MeOH–H₂O (5:5:2:8, v/v) were selected. The K_D values were more suitable when Pet–EtAc–MeOH–H₂O (5:5:2:8, v/v) was used as two-phase solvent systems. In order to achieve ideal separation effect, the concentration of TEA was also tested. When the concentration of TEA was 10 mM and the HCl was 10 mM, the two target alkaloids have certain trend of separation. In order to improve the separation effect, concentration of TEA was increased to 20 mM; the degree of separation was better, but good separation effect of the target compounds could not be obtained. At last,

compounds 1 and 2 could be separated with purities of 93.7% and 91.2%, respectively, when two-phase solvent systems Pet–EtAc–MeOH–H₂O (5:5:2:8, v/v) with 40 mM TEA in aqueous stationary phase and 10 mM HCl in organic mobile phase was used as shown in Figure 3.

As shown in Figure 3, the mainly presented constituents (reserpine and yohimbine) of *R. verticillata* (Lour.) Baill. were obtained from 3 g of crude alkaloids in a single run with purities of all being over 91.0% using the selected condition.

Identification of the separated peaks. Identification of the alkaloids purified by PZRCCC was carried out by UV, ESI–MS, ¹H-NMR, and ¹³C-NMR.

Compound a (peak a in Figure 3): Positive ESI–MS, m/z 355.2 $[M + H]^+$; ¹H-NMR (600 MHz, CDCl₃) δ: 7.76 (brs, 1H), 7.49 (d, $J = 7.5$ Hz, 1H), 7.33 (d, $J = 7.9$ Hz, 1H), 7.18–7.09 (m, 2H), 4.25 (brs, 1H), 3.84 (s, 3H), 3.37 (dd, $J = 11.5, 1.9$ Hz, 1H), 3.13–3.09 (m, 1H), 3.06–2.94 (m, 3H), 2.77–2.72 (m, 1H), 2.66 (dt, $J = 11.2, 4.6$ Hz, 1H), 2.38 (dd, $J = 11.5, 2.0$ Hz, 1H), 2.28 (m, 1H), 2.09–1.98 (m, 3H), 1.60 (m, 3H), 1.46–1.44 (m, 1H), 1.40 (m, 1H). ¹³C-NMR δ: 175.5 (C), 135.8 (C), 134.2 (C), 127.2 (C), 121.3 (CH), 119.3 (CH), 118.0 (CH), 110.6 (CH), 108.1 (C), 66.8 (CH), 61.2 (CH₂), 59.7 (CH), 52.7 (CH₂), 52.2 (CH), 51.8 (CH₃), 40.6 (CH), 36.6 (CH), 34.2 (CH₂), 31.3 (CH₂), 23.2 (CH₂), 21.6 (CH₂). Compared with the data given in Ref. [14], compound a was identified as yohimbine.

Compound b (peak b in Figure 3): Positive ESI–MS, m/z 609.2 $[M + H]^+$; ¹H-NMR (600 MHz, CDCl₃) δ: 7.48 (brs, 1 H), 7.34 (d, $J = 8.79$ Hz, 1 H), 7.32 (s, 2H), 6.85 (d, $J = 2.05$ Hz, 1 H), 6.78 (dd, $J = 8.64, 2.20$ Hz, 1 H), 5.06 (ddd, $J = 11.64, 9.45, 4.98$ Hz, 1H), 4.48 (br. s., 1H), 3.92 (s, 9 H), 3.91 (m, 1H), 3.85 (s, 3H), 3.82 (m, 3H), 3.51 (s, 3H), 3.12–3.25 (m, 2H), 3.06 (dd, $J = 12.15, 2.78$ Hz, 1H), 2.96 (m, 1H), 2.70 (dd, $J = 11.13, 4.69$ Hz, 1H), 2.44–2.54 (m, 2H), 2.28–2.41 (m, 2H), 2.04–2.12 (m, 1H), 2.00 (ddd, $J = 12.67, 4.17, 0.73$ Hz, 1 H), 1.92 (d, $J = 11.72$ Hz, 1H), 1.80 (d, $J = 14.64$ Hz, 1H); ¹³C-NMR (126 MHz, CDCl₃): 172.79, 165.39, 156.28, 152.98, 142.28, 136.31, 130.36, 125.39, 122.20, 118.59, 109.09, 108.23, 106.78, 95.19, 77.98, 77.82, 60.93, 60.77, 56.27, 55.83, 53.72, 51.84, 51.77, 51.23, 49.06, 34.04, 32.31, 29.75, 24.35, 16.81.

Figure 3. HPLC chromatograms of the crude sample and the purified alkaloids from devil pepper radix. Experimental conditions — Hypersil ODS column (250 mm × 4.6 mm id, 5 μm); mobile phase: MeOH–H₂O (0.1% trifluoroacetic acid) 30:70, v/v; column temperature: 25 °C; flow rate: 1.0 mL/min; UV detection wavelength: 345 nm; injection volume: 10 μL

Compared with the data given in Ref. [15], compound b was identified as reserpine.

Conclusions

In this paper, two alkaloids were successfully separated from *R. verticillata* (Lour.) Baill. by pH-zone-refining counter-current chromatography. Reserpine (112 mg) with a purity of 93.7% and yohimbine (21 mg) with a purity of all 91.2% were successfully obtained in a single run within 4 h. The result showed that PZRCCC is an efficient and unique method for preparative separation and purification of alkaloids from TCM.

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