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Short communication

pH-Zone-refining counter-current chromatography of lappaconitine from *Aconitum sinomontanum* Nakai I. Separation of prepurified extract

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Abstract

pH-Zone-refining counter-current chromatography was applied to the separation of diterpenoid alkaloids from a crude sample from a crude prepurified sample containing lappaconitine at about 90% purity using a multilayer coil planet centrifuge. The experiment was performed with a two-phase solvent system composed of methyl *tert*.-butyl ether–tetrahydrofuran–distilled water (2:2:3, v/v) where triethylamine (10 m*M*) was added to the upper organic stationary phase as a retainer and hydrochloric acid (10 m*M*) to the aqueous mobile phase as an eluter. The separation of 10.5 g of the sample yielded 9.0 g of lappaconitine at a high purity of over 99% as determined by HPLC.

Keywords: Counter-current chromatography; *Aconitum sinomontanum*; Preparative chromatography; Pharmaceutical analysis; Lappaconitine; Alkaloids; Terpenoids; Ranaconitine

1. Introduction

pH-Zone-refining counter-current chromatography (CCC) [1–18] is a recently developed preparative separation method which provides many important advantages over the conventional CCC method including an over 10-fold increase in sample loading capacity, high purity and high concentration of fractions, concentration of minor impurities, etc. The method uses a retainer base (or acid) in the stationary phase to retain the analytes in the column and an eluter acid (or base) to elute the analytes according to their pK_a values and hydrophobicities. It produces a succession of highly concentrated rectangular peaks with minimum overlap. This method has been successfully applied to the separation of a variety of natural and synthetic compounds including both acidic [1,3–8] and basic [9] amino acid derivatives, many hydroxyxanthene dyes [1,10–12]; alkaloids [3,13,14]; coumarin [15]; chiral compounds [16]; geometrical [17] and structural [18] isomers, etc.

Aconitum sinomontanum Nakai is a medicinal herb found only in the central and western parts of China. The major active constituents of this herb are diterpenoid alkaloids, including lappaconitine, ranaconitine, *N*-deacetyllappaconitine and *N*-

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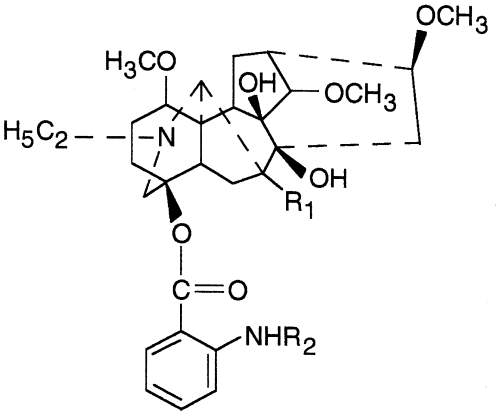
deacetylranaconitine, whose chemical structures are given in Fig. 1 [19,20]. Although most of the diterpenoid alkaloids from the family of *Aconitum* are very toxic, lappaconitine – the major component from *A. sinomontanum* Nakai – was found to be neither toxic nor addictive, and therefore used as a pain reliever mainly for cancer patients [21–24]. The separation and purification of lappaconitine from a crude ethanol extract from *A. sinomontanum* Nakai was usually performed by several steps using conventional silica gel column chromatography and recrystallization. The conventional method can only yield lappaconitine with a purity no more than 95% as determined by high-performance liquid chromatography (HPLC). On the other hand, the high purity standard lappaconitine is still short for the quality control of the related products and further clinical research.

In the present paper, pH-zone-refining CCC is applied to the multigram preparative separation of lappaconitine from a prepurified sample from *A. sinomontanum* Nakai.



	R_1	R_2
lappaconitine:	H	Ac
ranaconitine:	OH	Ac
N-deacetyllappaconitine:	H	H
N-deacetylranaconitine:	OH	H

Fig. 1. Chemical structures of diterpenoid alkaloids from *A. sinomontanum* Nakai.

2. Experimental

2.1. Apparatus

The CCC apparatus used in the present study is a multilayer coil planet centrifuge for performing high-speed CCC (P.C. Inc., Potomac, MD, USA). It holds a column holder and a counterweight in the symmetrical positions at a distance of 10 cm from the central axis of the centrifuge. The separation column was prepared by winding a single piece of Tefzel tubing (Zeus Industrial Products, Raritan, NJ, USA) of 1.6 mm I.D. (SW 14), 160 m in length around the column holder hub making 12 layers between a pair of flanges spaced 2 in. apart (1 in.=2.54 cm). The total capacity of the column is about 320 ml. The β values varied from 0.5 at the internal terminal to 0.75 at the external terminal. A pair of flow tubes from the column is first led through the hollow column holder shaft downward, and then makes an arc to enter the side hole of the central pipe, finally exiting the centrifuge at the top plate where they are tightly supported by a pair of clamps. The revolution speed of the apparatus was regulated with a speed controller (Bodine Electric, North Chicago, IL, USA) where an optimum speed of 860 rpm was used in the present studies.

The HPLC equipment used was a Shimadzu LC-6A system including an LC-6A solvent delivery unit, an SPD-6AV UV–Vis spectrophotometric detector, a Model 7726 injection valve with a 20-μl loop, and a CR501 Chromatopac integrator (Shimadzu, Kyoto, Japan).

2.2. Reagents

Methyl *tert*.-butyl ether (MtBE) (HPLC grade), tetrahydrofuran (THF) (HPLC grade), water (HPLC grade), hydrochloric acid (reagent grade), and triethylamine (TEA) (HPLC grade) were purchased from Fisher Scientific (Fair Lawn, NJ, USA).

The prepurified alkaloid sample (ca. 90% lappaconitine) from the *A. sinomontanum* Nakai was

provided by Lanzhou Pharmaceutical Factory (Lanzhou, Gansu Province, China).

2.3. Preparation of two-phase solvent system and sample solution

The two-phase solvent system utilized in the present study was composed of MtBE–THF–distilled water (2:2:3, v/v). The solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature, and the two phases were separated shortly before use. Then, TEA (retainer) was added to the upper organic stationary phase to obtain a final concentration of 10 m*M* and hydrochloric acid (eluter) was added to the lower aqueous mobile phase to obtain a final concentration of 10 m*M*.

The sample solution was prepared dissolving 2.0 to 10.5 g of sample in 20 to 100 ml of the solvent mixture consisting of about equal volumes of each phase.

2.4. Separation procedure

The column was first entirely filled with the organic stationary phase containing TEA at 10 m*M*. This was followed by sample injection through the sample port. Then the aqueous phase containing hydrochloric acid (eluter acid) at 10 m*M* was pumped into the inlet of the column at a flow-rate of 3.0 ml/min in the head-to-tail elution mode, while the apparatus was rotated at 860 rpm. The effluent from the outlet of the column was continuously monitored with a UV monitor (Uvicord S, LKB Instruments, Bromma/Stockholm, Sweden) at 206 nm and collected into test tubes at 2-min intervals (6.0 ml/tube) using a fraction collector (Ultrorac, LKB Instruments). After the desired peaks were eluted, the apparatus was stopped and the column contents were collected into a graduated cylinder by connecting the inlet of the column to a nitrogent line at 100 p.s.i. (1 p.s.i.=6894.76 Pa). 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.

2.5. Analyses and identification of CCC fractions

The pH value of each fraction was manually determined with a portable pH meter (Accumet Portable Laboratory, Fisher Scientific, Pittsburgh, PA, USA).

Each alkaloid-containing fraction was analyzed by HPLC with a Kaseisorb LC ODS-300-5 column (250×4.6 mm I.D.) at 254 nm with methanol–water–chloroform–TEA (60:40:2:0.1, v/v) as mobile phase.

Identification of the major components was carried out by electrospray ionization mass spectrometry (MS) using a Finnigan TSQ-700 mass spectrometer (Finnigan, San Jose, CA, USA).

3. Results and discussion

Successful operation of pH-zone-refining CCC of basic compounds requires suitable partition coefficient (K) values in both acidic ($K_{acid}<<1$) and basic ($K_{base}>>1$) conditions as well as good solubility of the sample in the solvent system. We first evaluated a binary two-phase solvent system composed of MtBE–water which has been used for many kinds of compounds [3,4,9]. Although this solvent system produced suitable K values, the maximum sample size applicable to pH-zone-refining CCC was limited to 2 g due to poor solubility of the alkaloids in this solvent system. The solubility of alkaloids was substantially improved by adding THF to the above solvent system, and the K values were optimized by selecting MtBE–THF–water (2:2:3, v/v). Adding the retainer base (triethylamine) to the solvent system gave a small K_{base} of 0.2 while adding eluter acid (HCl) produced a large K_{acid} of over 5. Thanks to the high solubility of the alkaloid mixture in this solvent system, up to 10.5 g of alkaloid sample was separated at high purity.

Fig. 2A–C show three typical pH-zone-refining counter-current chromatograms of alkaloids from *A. sinomontanum* Nakai obtained from the separations of 2.0, 6.5 and 10.5 g of sample as indicated. The target compound, lappaconitine, formed a rectangular peak whereas impurities or minor alkaloid components were highly concentrated at its front and rear boundaries.

Increasing the sample size from 2.0 up to 10.5 g resulted in broadening of the rectangular peak without loss of peak resolution. The pH-zone-refining

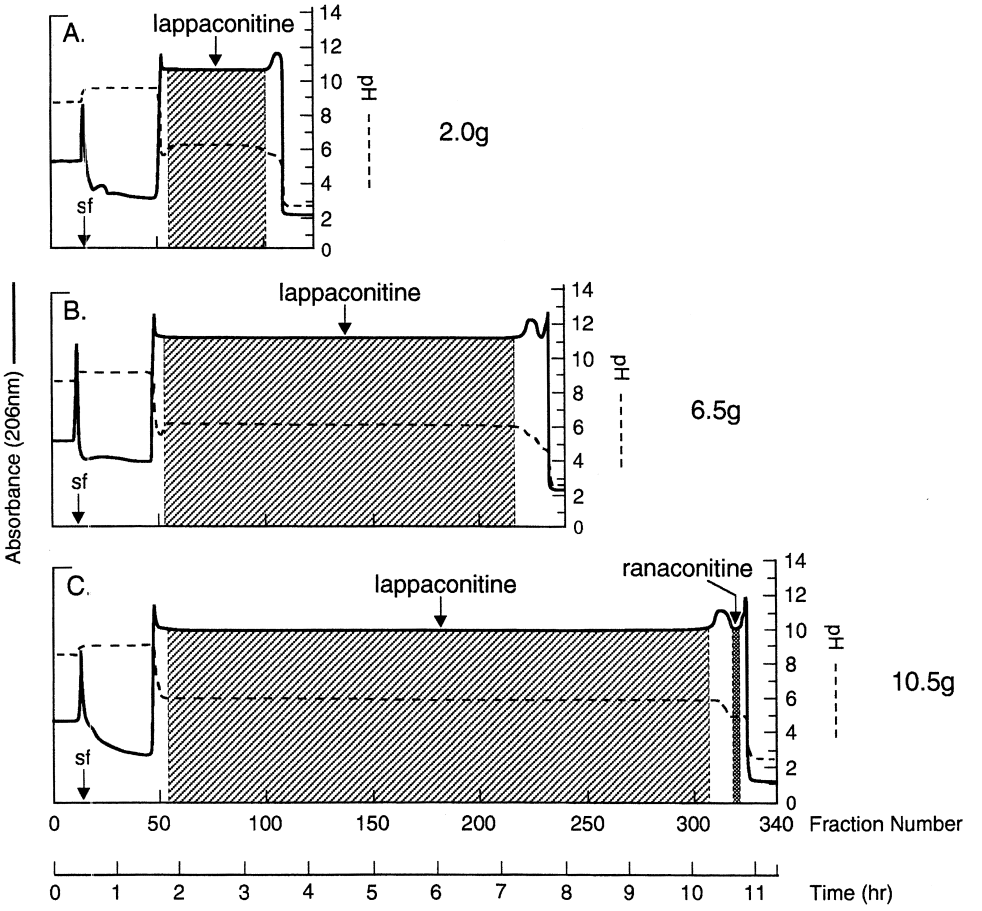


Fig. 2. Separation of lappaconitine from a prepurified extract of *A. sinomontanum* Nakai by pH-zone-refining CCC. Solvent system: MtBE–THF–water (2:2:3, v/v), 10 m*M* TEA in the upper organic stationary phase and 10 m*M* HCl in the lower phase; sample size: 2.0 g (A), 6.5 g (B) and 10.5 g (C); flow-rate: 3 ml/min; detection: 206 nm; revolution speed: 860 rpm; retention of stationary phase: 75.8% (A), 75% (B) and 75.6% (C);.

CCC separations yielded 1.75 g (A), 5.6 g (B) and 9.0 g (C) of pure lappaconitine ($[M+H]^+$: 585.35) with over 99% purity as determined by HPLC (Fig. 3).

In addition, the chromatogram of 10.5 g sample size (Fig. 2C) shows a narrow plateau of another alkaloid, which resulted from the increased sample size, and identified as ranaconitine ($[M+H]^+$: 601.50) by MS.

The present study has been conducted to obtain multigram quantities of pure lappaconitine standard from the prepurified alkaloid fraction from *A. sinomontanum* Nakai through a conventional multi-step process in Lanzhou Pharmaceutical Factory in China. Currently the continued study is underway in our laboratory using pH-zone-refining CCC to recover pure lappaconitine and other alkaloids from the depleted residues of crude sample produced by the above factory.

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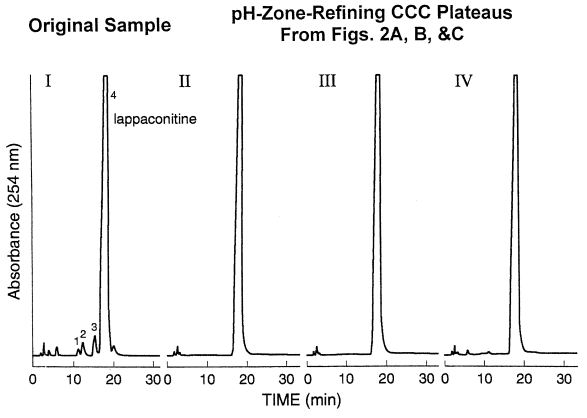

Fig. 3. HPLC analyses of original sample and three pH plateaus fractions from pH-zone-refining CCC separations with different sample size. Column: Kaseisorb LC ODS-300-5 column (250×4.6 mm I.D.); mobile phase: methanol–water–chloroform–TEA (60:40:2:0.1, v/v); UV detection wavelength: 254 nm; flow-rate: 1.0 ml/min. *N*-deacetylranaconitine (1), *N*-deacetyllappaconitine (2), ranaconitine (3) and lappaconitine (4). Compound identities confirmed by MS.

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