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Preparative separation of lappaconitine, ranaconitine, *N*-deacetyllappaconitine and *N*-deacetylranaconitine from crude alkaloids of sample *Aconitum sinomontanum* Nakai by high-speed counter-current chromatography

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Abstract

Analytical high-speed counter-current chromatography (HSCCC) was used for the systematic selection and optimization of the two-phase solvent system to separate alkaloids from *Aconitum sinomontanum* Nakai. The optimum solvent systems $CHCl_3$–MeOH–0.3 M/0.2 *M* HCl (4:1.5:2, v/v) thus obtained led to the successful separation of lappaconitine, ranaconitine, *N*-deacetyllappaconitine and *N*-deacetylranaconitine from 60 to 500 mg of crude alkaloid sample by preparative HSCCC separation.

Keywords: *Aconitum sinomontanum*; Counter-current chromatography; Preparative chromatography; Alkaloids; Lappaconitine; Ranaconitine; Deacetyllappaconitine; Deacetylranaconitine

1. Introduction

Aconitum sinomontanum Nakai (Gaowutou in Chinese) contains several diterpenoid alkaloids, including lappaconitine, ranaconitine, *N*-deacetyllappaconitine and *N*-deacetylranaconitine [1,2]. The major alkaloid, lappaconitine, was found to be neither toxic nor addictive, and has been used as a pain reliever mainly for cancer patients [3–6]. The conventional separation methods only yielded lappaconitine with a purity no more than 95% as determined by HPLC. As previously reported, this prepurified sample has been purified by pH-zone-refining counter-current chromatography (CCC) [7]. However, the remaining residues still contain four useful compounds including lappaconitine, ranaconitine, *N*-deacetyllappaconitine and *N*-deacetylranaconitine, which are difficult to separate by conventional means.

High-speed counter-current chromatography (HSCCC), a support-free liquid–liquid partition chromatographic technique, eliminates irreversible adsorption of the sample onto the solid support [8], and has been widely used for the preparative separation of natural products [9,10]. The selection of the solvent system is the first and most important step in performing HSCCC. Although a systematic search for the two-phase solvents has been introduced for

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CCC [11,12], the method is focused on hydrophobicity of the solvent system. In order to separate charged analytes such as alkaloids, however, an additional adjustment is required with respect to pH and ionic strength of the solvent system [13]. Because of its speedy separation and minimum solvent consumption, analytical HSCCC offers a very efficient means of testing the solvent system for providing a proper range of partition coefficients of analytes.

In the present paper, analytical HSCCC is used for the systematic selection and optimization of the two-phase solvent system for separation of alkaloids from a crude sample from *A. sinomontanum* Nakai. Using the optimized solvent systems, the preparative HSCCC separations of the alkaloids are performed from the crude sample from *A. sinomontanum* Nakai.

2. Experimental

2.1. Apparatus

The analytical HSCCC instrument (I) employed is a type-J coil planet centrifuge. It 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 coiled separation column was prepared by winding a 50 m×0.85 mm I.D. PTFE (polytetrafluoroethylene) tubing directly onto the holder hub forming multiple coiled layers with a total capacity of 30 ml. The β-value varied from 0.4 at the internal terminal to 0.7 at the external terminal ($\beta = r/R$ where r is the distance from the coil to the holder shaft, and R, the revolution radius or the distance between the holder axis and central axis of the centrifuge). The revolution speed of the apparatus could be regulated with a controller in the range between 0 and 2000 rpm; an optimum speed of 1500 rpm was used in the present studies.

The analytical HSCCC instrument (II) employed is a triplet type-J coil planet centrifuge fabricated in the NIH machine shop. It holds three columns symmetrically around the rotary frame at a distance of 7.5 cm from the central axis of the centrifuge. Each column consists of 0.85 mm I.D. Tefzel tubing (Zeus Industrial Products, Raritan, NJ, USA) which is coaxially wound around the holder hub forming 11 coiled layers. These three multilayer coils are connected in series with 0.4 mm I.D. PTFE tubing (Zeus Industrial Products) to make up a total capacity of 175 ml. The β-value varied from 0.5 at the internal terminal to 0.75 at the external terminal. The revolution speed of the apparatus could be regulated with a controller in the range between 0 and 1500 rpm; an optimum speed of 900 rpm was used in the present studies.

The preparative HSCCC 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) 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″ apart. 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 high-performance liquid chromatography (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

Chloroform, methanol and water were of HPLC grade. Hydrochloric acid and triethylamine (TEA) were of reagent grade. All were purchased from Fisher Scientific (Fair Lawn, NJ, USA).

The crude alkaloid sample 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 solutions

The HSCCC separation study used a series of two-phase solvent systems composed of chloroform, methanol and dilute hydrochloric acid. Each solvent system was prepared by mixing the solvents at the specified volume ratios and thoroughly equilibrating in a separatory funnel at room temperature. The two phases were separated shortly before use.

The sample solutions were prepared by dissolving 2.5 mg of sample in 1.0 ml of the mobile phase for the selection of solvent system; 60 mg of sample in 3 ml of the mobile phase for the semi-preparative separation by analytical HSCCC; and 300–500 mg of sample in 5–10 ml of mobile phase for preparative HSCCC separation.

2.4. Separation procedure

The HSCCC separation was performed as follows: the column was first entirely filled with the upper aqueous stationary phase. This was followed by sample injection through the sample port. The mobile phase was pumped into the inlet of the column at a flow-rate of 1.0 ml/min (analytical) and 3.0 ml/min (preparative) in the head-to-tail elution mode, while the apparatus was rotated at 1500 rpm (analytical HSCCC), 900 rpm (analytical triplet coil planet centrifuge) and 800 rpm (preparative). The effluent from the outlet of the column was continuously monitored with a UV monitor (Uvicord S, LKB Instruments, Bromma/Stockholm, Sweden) at 254 or 275 nm and collected in test tubes at 2-min intervals 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 nitrogen line at 100 p.s.i. (1 p.s.i.=6894.76 Pa). The percent 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

Each alkaloid-containing fraction was analyzed by HPLC with a phenomenex Luna 5μ C_{18}(2) column (250×4.6 mm I.D., 5 μm) at a wavelength of 300 nm and with methanol–water–chloroform–TEA (70:30:2:0.1, 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

The crude alkaloid sample from *A. sinomontanum* Nakai was first analyzed by HPLC and the results are shown in Fig. 1. The target alkaloids are eluted at retention times between 20 and 40 min as labeled (peaks 1–6).

Studies on the selection of two-phase solvent system for HSCCC were carried out using the

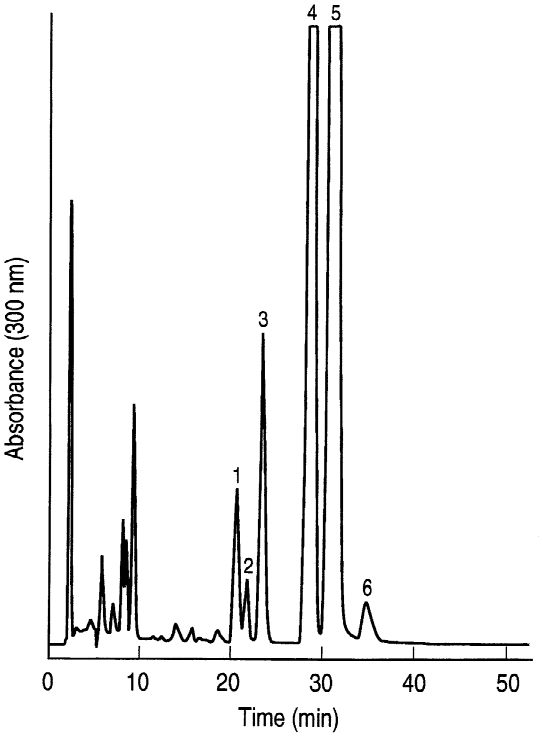

Fig. 1. HPLC analyses of the crude soybean extract. Column: phenomenex Luna 5μ C_{18}(2) (250×4.6 mm I.D., 5 μm); mobile phase: methanol–water–chloroform–TEA (70:30:2:0.1, v/v); flow-rate: 1.0 ml/min; UV wavelength: 300 nm. 1, *N*-Deacetylranaconitine; 2, unknown; 3, *N*-deacetyllappaconitine; 4, ranaconitine; 5, lappaconitine; 6, unknown.

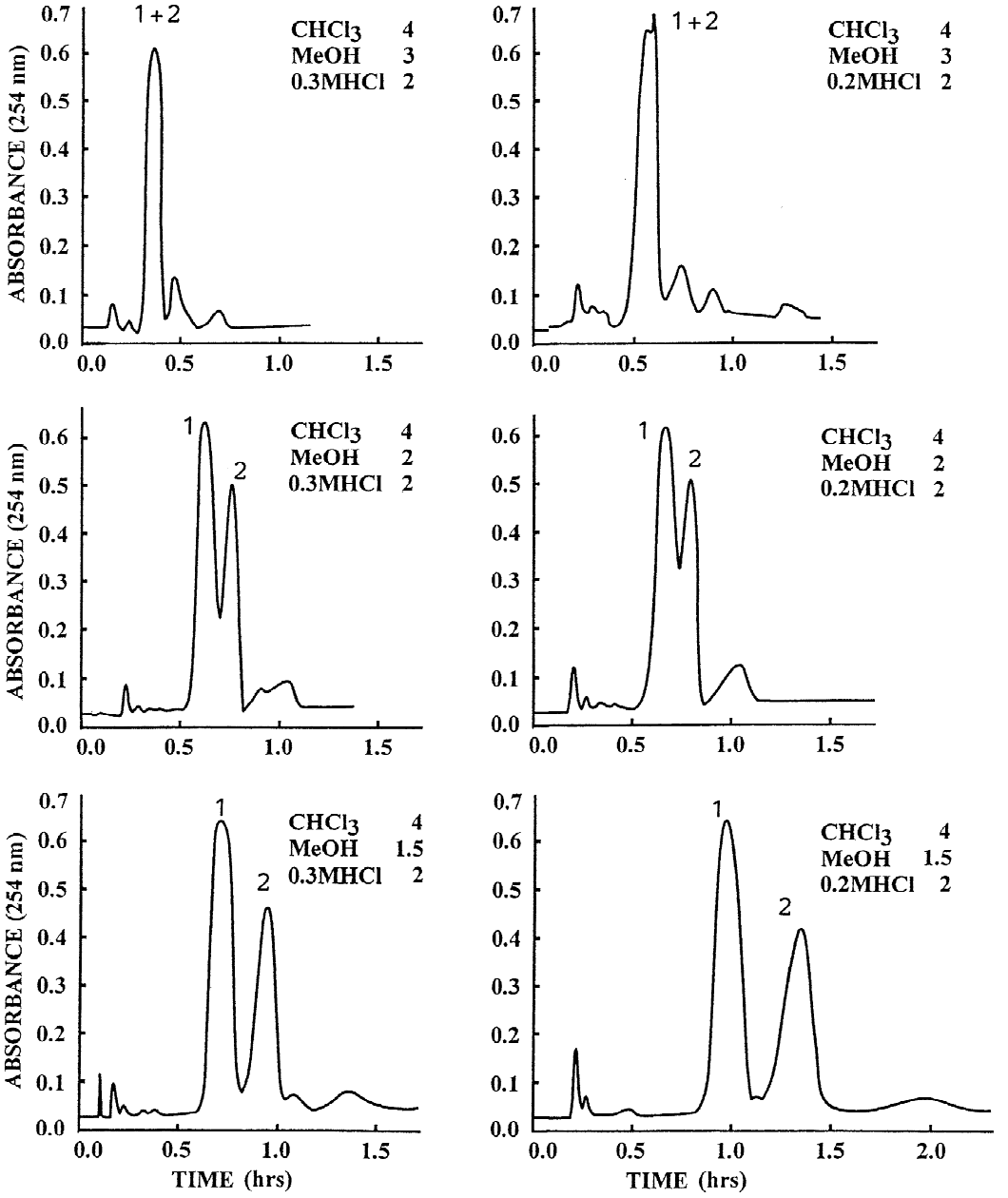

Fig. 2. Chromatograms of the crude alkaloids from *A. sinomontanum* Nakai by analytical HSCCC (I). Six chromatograms are arranged in such a way that the effects of methanol and HCl concentrations on the separation of alkaloids are each clearly visualized. Solvent system: shown above each chromatogram; mobile phase: lower organic phase; flow-rate: 1.0 ml/min; revolution: 1500 rpm; retention of the stationary phase was as follows: $CHCl_3$–MeOH–0.2/0.3 *M* HCl (4:3:2), 77%; $CHCl_3$–MeOH–0.2/0.3 *M* HCl (4:2:2), 80%; $CHCl_3$–MeOH–0.2/0.3 *M* HCl (4:1.5:2), 77%; sample size: 2.5 mg in 1 ml mobile phase. 1, lappaconitine; 2, ranaconitine.

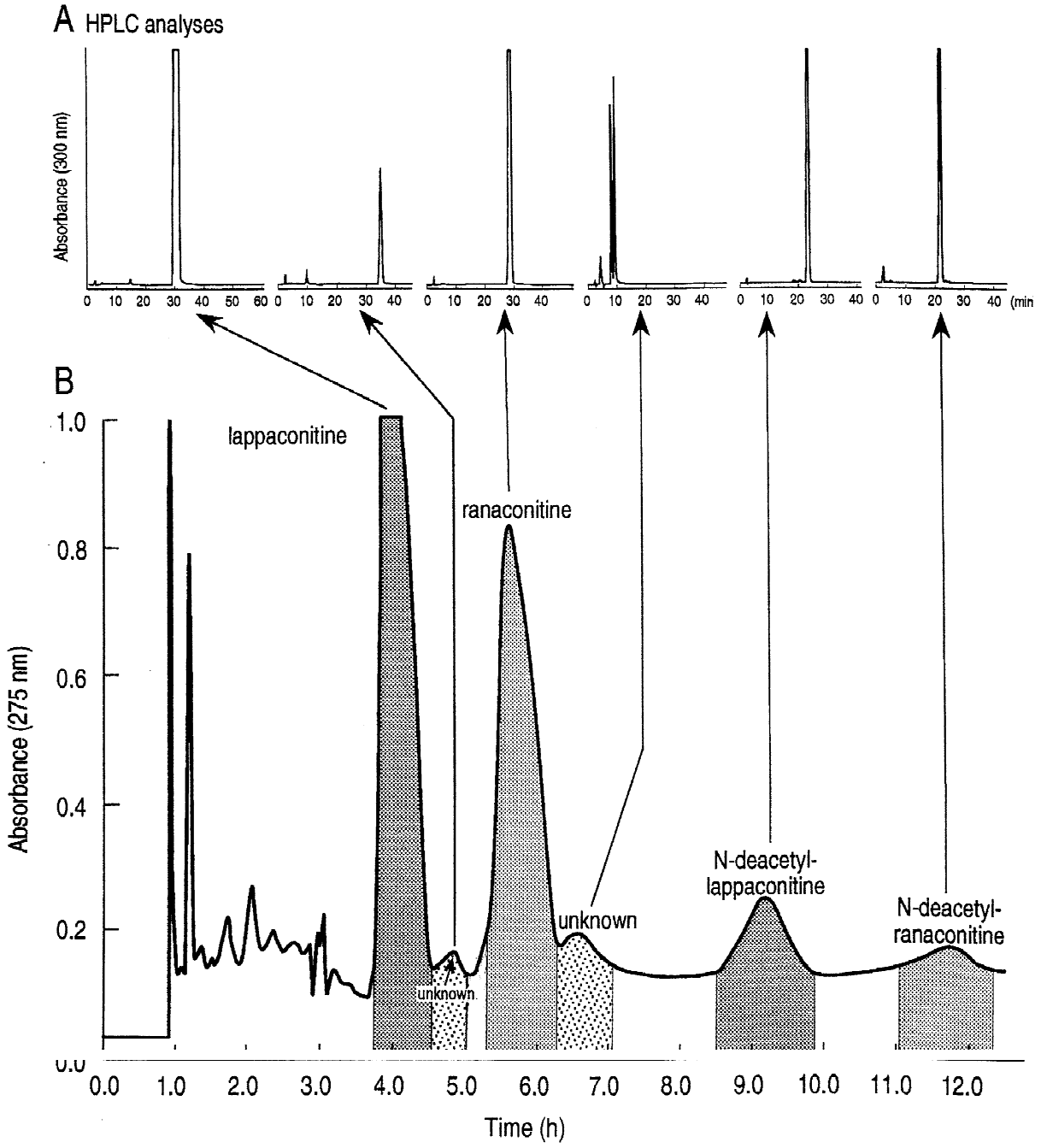

Fig. 3. Chromatogram of the crude alkaloids from *A. sinomontanum* Nakai by analytical HSCCC (II) and the results of HPLC analyses of each peak fraction. Solvent system: $CHCl_3$–MeOH–0.3 *M* HCl (4:1.5:2, v/v); mobile phase: lower organic phase; flow-rate: 1.0 ml/min; sample size: 60 mg dissolved in 3 ml of solvent consisting of about equal volumes of each phase; revolution speed: 900 rpm. Retention of the stationary phase: 75%. HPLC conditions same as in Fig. 1.

solvent system composed of chloroform, methanol and dilute HCl by systematically modifying the relative volumes of methanol (4:3:2–4:1.5:2) and the concentration of HCl (0.3–0.2 *M*). Fig. 2 shows the results of separations of the crude alkaloids from *A. sinomontanum* Nakai by analytical HSCCC with a column capacity of 30 ml, and using a set of the above mentioned solvent systems. In this figure, six chromatograms are arranged in such a way that the effects of methanol and HCl concentrations on the separation of alkaloids are each clearly visualized. As seen from the top to the bottom rows, decreasing the relative volumes of methanol in the solvent system from 4:3:2: to 4:1.5:2 increases the retention time and resolution of alkaloids. A similar effect is also produced by decreasing the concentration of HCl from 0.3 to 0.2 *M* in the solvent system (from left to right in each row). Among those, both of the solvent systems composed of $CHCl_3$–MeOH–0.3 *M* HCl (4:1.5:2, v/v) and $CHCl_3$–MeOH–0.2 *M* HCl (4:1.5:2, v/v) produced baseline separation of two major alkaloids.

In order to separate the four alkaloids from the crude sample from *A. sinomontanum* Nakai by analytical and preparative HSCCC, the solvent system composed of $CHCl_3$–MeOH–0.3 *M* HCl (4:1.5:2, v/v) was chosen first. Fig. 3 shows the separation of 60 mg of the crude alkaloids sample from *A. sinomontanum* Nakai by analytical HSCCC with a column capacity of 175 ml. The results of HSCCC and HPLC indicate that four alkaloids are completely separated from each other and also from unknown impurities, and with 99% purity as determined by HPLC. They are identified by MS as lappaconitine ($[M+H]^+$: 585.35), ranaconitine ($[M+H]^+$: 601.50), *N*-deacetyllappaconitine ($[M+H]^+$: 543.31), and *N*-deacetylranaconitine ($[M+H]^+$: 559.46).

Fig. 4 shows the separation of 300 mg of the crude alkaloid sample from *A. sinomontanum* Nakai by preparative HSCCC. The result indicates that four alkaloids are completely separated from each other and each with 98.5% purity as determined by HPLC.

Fig. 5 shows the separation of 500 mg of the crude alkaloids sample from *A. sinomontanum* Nakai by preparative HSCCC using the solvent system composed of $CHCl_3$–MeOH–0.2 *M* HCl (4:1.5:2, v/v). The separation is still good, but it took a little longer and the *N*-deacetylranaconitine peak was retained in the column over 10 h.

The results of our studies clearly demonstrate the advantage of HSCCC in both analytical and prepara-

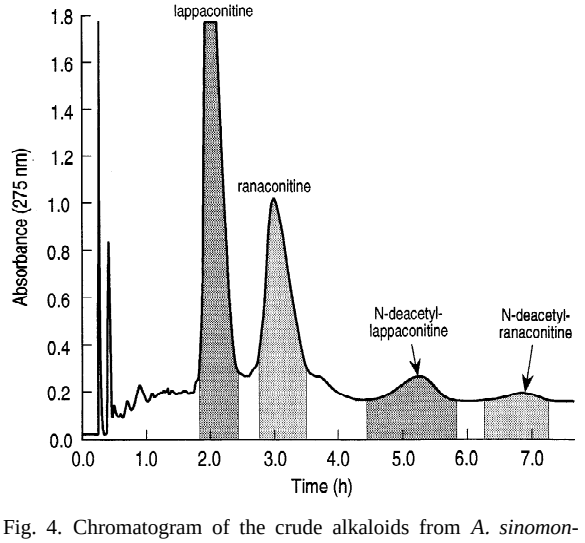

Fig. 4. Chromatogram of the crude alkaloids from *A. sinomontanum* Nakai by preparative HSCCC. Solvent system: $CHCl_3$–MeOH–0.3 *M* HCl (4:1.5:2, v/v); mobile phase: lower organic phase; flow-rate: 3.0 ml/min; sample size: 300 mg dissolved in 6 ml of solvent consisting of about equal volumes of each phase; revolution speed: 800 rpm; retention of the stationary phase: 83%.

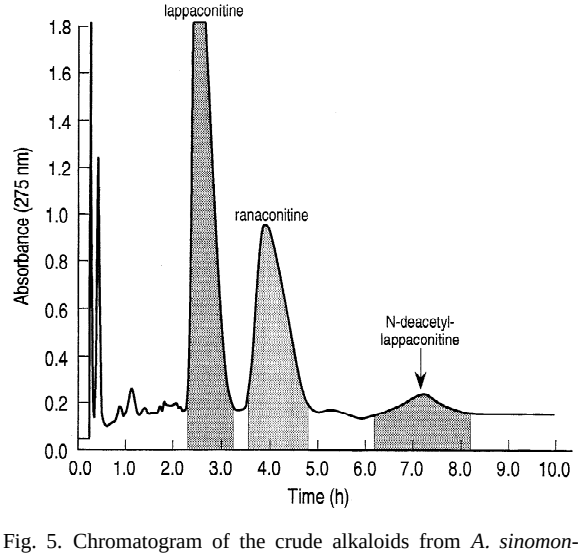

Fig. 5. Chromatogram of the crude alkaloids from *A. sinomontanum* Nakai by preparative HSCCC. Solvent system: $CHCl_3$–MeOH–0.2 *M* HCl (4:1.5:2, v/v); mobile phase: lower organic phase; flow-rate: 3.0 ml/min; sample size: 500 mg dissolved in 10 ml of solvent consisting of about equal volumes of each phase; revolution speed: 800 rpm; retention of the stationary phase: 83%.

tive separations of alkaloids from a crude alkaloid sample of *A. sinomontanum* Nakai. In particular, analytical HSCCC with its speedy separation and minimum solvent consumption offers a very efficient means to carry out optimization of solvent systems for separation and purification of natural products by preparative HSCCC.

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