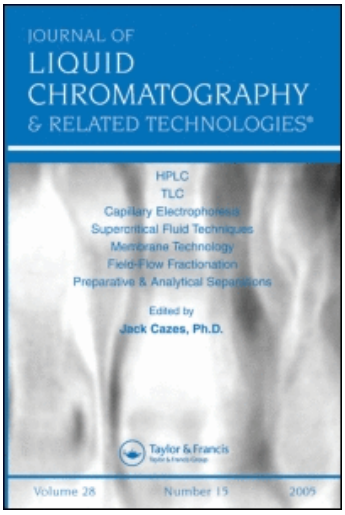

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SEPARATION AND PURIFICATION OF STRYCHNINE FROM CRUDE EXTRACT OF *STRYCHNOS NUX-VOMICA L.* BY HIGH-SPEED COUNTERCURRENT CHROMATOGRAPHY

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ABSTRACT

High-speed countercurrent chromatography (CCC) was applied to the separation of strychnine and brucine from crude extract of *Strychnos nux-vomica L.* using a two-phase solvent system composed of chloroform/0.07 M sodium phosphate, 0.04 M citric buffer (pH 5.08) (1:1, v/v). Fractionated components were identified with authentic pure compounds on TLC and also analyzed by HPLC, IR and FTMS. The results showed that from 40 g of the seed powder, 48.2 mg of strychnine was purified at 99.9 % purity (83.3% recovery) while 18.1 mg of brucine fraction was obtained after rechromatographed by preparative TLC improving the purity to 91.2%.

INTRODUCTION

Being a liquid-liquid partition chromatographic system free of solid support matrix in the separation column, high-speed countercurrent chromatography (HSCCC) is ideal for separation of active principles from traditional Chinese medicinal herbs and other natural products.[1] In the past, HSCCC has been successfully applied to various components,[2-4] such as alkaloids,[5,6] hydroxyanthraquinones,[7] and flavonoids.[8,9]

Strychnine and brucine (Fig. 1), isolated from *Strychnos nux-vomica L.*, possess strong biological activity on central nervous system. In order to isolate these compounds from strychnos extract, partition silica gel column chromatography has been used in China, but it is time-consuming and laborious. The present paper describes isolation of strychnine and brucine from a crude alkaloid extract of *S. nux-vomica L.* by HSCCC.

EXPERIMENTAL

Reagents

Chloroform, sulfuric acid, citric acid, and sodium phosphate are all of analytical grade and were obtained from Shanghai Chemical Reagents Company, Shanghai, China. Distilled water and the seed powder of *Strychnos nux-vomica L.* were purchased from Shanghai Herb Medicine Company, Shanghai, China.

Preparation of Sample Solution

A 40 g amount of seed power of *S. nux-vomica L.* was soaked in mixture of 120 mL of chloroform and 20 mL of 34% aqueous ammonia solution for 24 hours. After removing insoluble residues by filtration, the extract of alkaloids was extracted three times with 0.5 M sulfuric acid (40 mL, 20 mL, 20 mL).

The extracts were combined and basified to raise the pH to 9.0, and the alkaloids were again extracted three times with chloroform (40 mL, 20 mL, 20 mL). Finally, the extracts were combined and diluted to 100 mL with chloroform.

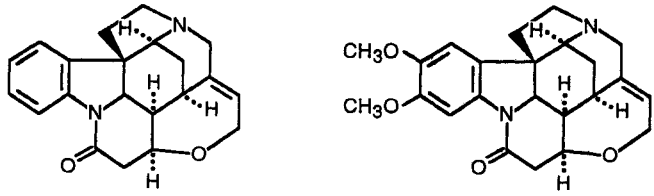

Figure 1. Chemical structures of strychnine and brucine.

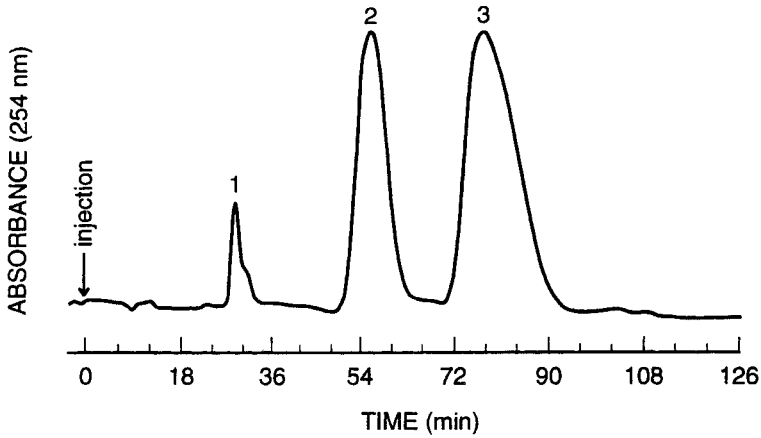

Figure 2. HSCCC separation of a crude extract of strychnos powder.

High-Speed Countercurrent Chromatography (HSCCC)

The multilayer coil planet centrifuge used in the present study was obtained from the Beijing Institute of New Technology Application, Beijing, China. The apparatus holds a multilayer coil separation column and the counterweight symmetrically on the rotary frame at a distance of 8 cm from the central axis of the centrifuge. A 130 m long, 1.5 mm ID PTFE (polytetrafluoroethylene) tube was directly wound around the holder hub forming multiple coiled layers with a total capacity of 230 mL. The revolution seed of the apparatus is continuously adjustable from 0 to 1000 rpm with a speed controller while an optimum speed of 800 rpm was used in this study. A

Milton Roy metering pump (model 196-31, LDC/Milton Roy, Florida, USA) was used to deliver the solvent and a UV detector (Model 57WA, Factory of Shanghai Optical Instruments, Shanghai, China) with a strip chart recorder (Model XWX 1042, Shanghai Dahua Instrument Factory, Shanghai, China) to monitor the effluent at 254 nm.

A two-phase solvent system composed of chloroform/0.07M sodium phosphate buffer solution (pH 5.08) (1:1, v/v) was selected in the present study. The solvent mixture was thoroughly equilibrated in a separatory funnel by repeating vigorous shaking; the two phases were separated shortly before use. In each separation, the column was first entirely filled with the aqueous stationary phase, followed by injection of the sample solution. Then the organic mobile phase was pumped into the column at a flow rate of 2 mL/min while the apparatus was rotated at 800 rpm. The effluent from the outlet of the column was continuously monitored with a UV monitor at 254 nm and collected with a fraction collector at a 2 min interval per tube.

Thin Layer Chromatography (TLC)

Both crude sample solution and HSCCC fractions were subjected to TLC analysis for tentative identification. The TLC plates were prepared with SiO_2-gel and 0.4% CMC, and activated at 105°C for one hour. TLC was developed with toluene-acetone-ammonia solution-ethanol (4:5:0.6:0.4) and stained with $KBiI_4$ reagent for detection.

High-Performance Liquid Chromatography (HPLC)

A Shimadzu HPLC set (Shimadzu, Kyoto, Japan) consisting of a constant-flow pump (Model: LC-6A) and a variable-wavelength UV detector (Model: SPD-6A) was used. The separation was performed on C_{18}-ODS column (150 mm X 4.5 mm ID) (Shimadzu) with methanol-0.05 M acetic acid-sodium acetate buffer as a mobile phase at a flow-rate of 1.0 mL/min and 226 nm for detection.

RESULTS AND DISCUSSION

Figure 2 shows a chromatogram of the crude extract of *Strychnos nux-vomica L.* obtained by HSCCC. The separation was performed with a two-phase solvent system composed of chloroform/0.07M sodium phosphate, 0.04M citric acid buffer (pH 5.08) (1:1, v/v) by elution with the lower nonaqueous

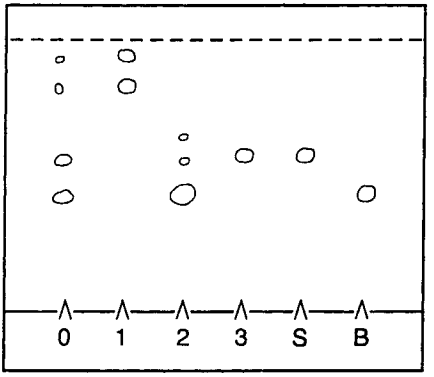

Figure 3. TLC analysis of strychnos crude extract and HSCCC fractions. Developed with toluene-acetone-ammonia solution-ethanol (4:5:0.6:0.5) and stained with $KBiI_4$ reagent for detection. "O" represents crude extract; "1", "2" and "3", peaks 1, 2 and 3 of HSCCC shown in Fig. 2; and "S" and "B", strychnine and brucine references, respectively.

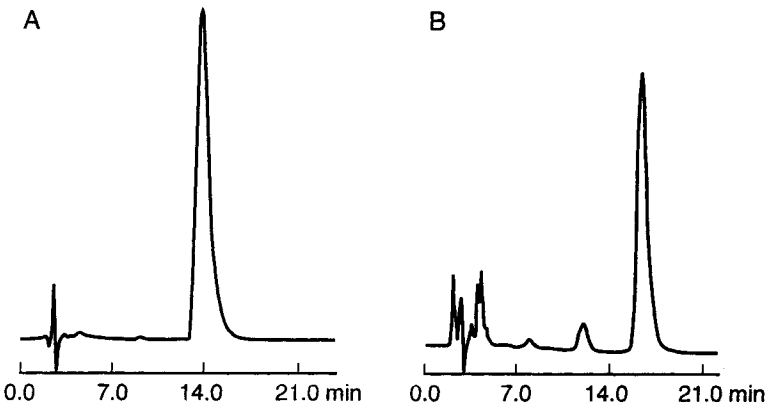

Figure 4. HPLC analysis of CCC fractions. (A) peak 3: strychnine and (B) peak 2: brucine. Experimental conditions: Shimadzu HPLC system, including an LC-6A constant-flow pump, 150 mm x 4.5 mm Zorbax-C_{18} (5 μm), an SPD variable wavelength detector and a CR-3A recorder. Mobile phase: methanol-0.5 M acetic acid/sodium acetate buffer (pH 4.6) (50:42, v/v); flow rate: 1 mL/min, temperature: 400C; sample size: 20 μL.

phase at a flow rate of 2.0 mL/min. Two major peaks were well resolved in 90 min. TLC analysis of each peak fraction is shown in Fig. 3. Fractions corresponding to peaks 2 and 3 were identified as brucine and strychnine by their standards, respectively. Fractions from peaks were analyzed by Nicolet FTMS and IR. The mass spectrum showed important fragment at m/z 335, and the IR spectrum showed strong absorption in 2860 cm^{-1} (=CH_2), 1670 cm^{-1} (-C=O), 1610 cm^{-1} (=C-C=), 1470 cm^{-1} indicating that strychnine is present in peak 3. Two impurities found in the TLC chromatogram of peak 2 fraction could be separated from brucine using preparative TLC.

In order to estimate purity and recovery rate of the analytes, the crude extract and the fractions corresponding to peaks 2 and 3 were analyzed by HPLC (Fig. 4). The results indicated that over 99% pure strychnine was obtained at 83.2% recovery. Brucine was further purified by preparative TLC to improve the purity to 91.2% with a low recovery rate.

It was found that pH of the solvent system has a profound effect on peak resolution between strychnine and brucine. The above separation was achieved at pH 5.08 where the distribution ratios for strychnine and brucine were 0.59 and 0.34, respectively. When the pH was adjusted to 5.00 for reducing the separation time, distribution ratios for strychnine and brucine became 0.62 and 0.38, respectively, without affecting the resolution between the two peaks. At pH 4.91, the concentration of free strychnine is nearly equal to the concentration of ionized strychnine in the solution, with distribution ratio of near 1 while, at pH 4.74, brucine has the same distribution ratio of 1. At pH values between 4.60 and 5.00, both the peak resolution and separation time were satisfactory. However, at the pH values below 4.60, the separation time became much longer associated with excessive peak broadening. On the other hand, upon raising the pH above 5.08, the peak resolution rapidly decreased.

Since the crude sample was a free base, after many successive injections (30 times), the pH value of the stationary phase rose and the resolution was slightly reduced, but it was still over 1.5, which meets the demand for preparative separation. The column can be rejuvenated by replenishing a fresh stationary phase, if necessary. Ionic strength of solvent system also affects the resolution. When the buffer concentration was 10 times that of the applied concentration (0.07 M), the peak resolution between strychnine and brucine became less than 1.0. Therefore, the lowest buffer concentration is desirable as far as the solvent system remains stable. Increasing the concentration of the sample may result in emulsification, since both strychnine and brucine are surface active. In the present separation, the crude extract contained 6.8 mg/mL of these alkaloids.

We were able to separate and purify strychnine from the crude alkaloid extract of *Strychnos nux-vomica L.* using HSCCC in a one step operation. The strychnine fraction was 99.9% pure, at a high recovery rate of 83.3 %, which can be used as a reference standard.

The overall results of the present studies suggest that HSCCC is an excellent technique for purification of natural products.

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