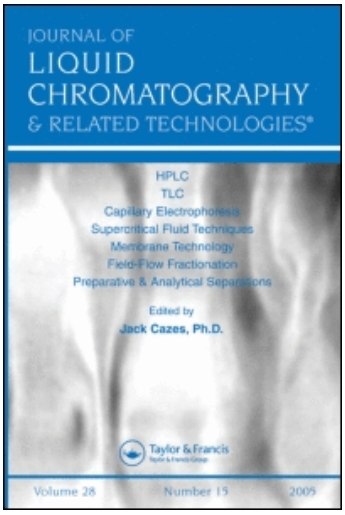

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SEPARATION OF ALKALOIDS EXTRACTED FROM *STEPHANIA TETRANDRA S. MOORE* BY ANALYTICAL HIGH-SPEED COUNTER-CURRENT CHROMATOGRAPHY

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ABSTRACT

High-Speed countercurrent chromatography is a recently developed separation method which has been remarkably improved in both partition efficiency and separation time. In the present study, this advanced countercurrent chromatographic method was applied to separation of sample mixture containing tetrandrine, fangchinoline, and cyclanoline originally extracted from

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Stephania tetrandra S. Moore. Separations were performed with a two-phase solvent system composed of n-hexane/ethyl acetate/methanol/water in two different elution modes. Sample mixture containing 3 mg of alkaloids was efficiently separated in 100 min. The peak fraction of each component was analyzed with a mass spectrometer for structure identification.

INTRODUCTION

Since the last decade, countercurrent chromatography has been remarkably improved in both partition efficiency and separation time. Recently, high-speed countercurrent chromatography has been developed by Ito and his coworkers based on the discovery of a unique hydrodynamic phenomenon in a rotating coil (1,2). It is characterized by high partition efficiency and large retention capacity of the stationary phase at high flow rates of the mobile phase. Several types of coil planet centrifuges have been constructed for performing high-speed countercurrent chromatography (1). The authors have successfully used one of these instruments for separating various kinds of compounds extracted from the medicinal herbs (3,4).

The present paper describes the separation of alkaloids from a methanol extract of medicinal herbs by analytical high-speed countercurrent chromatography. A 3 mg-quantity of the sample mixture containing tetrandrine, fangchinoline and cyclanoline at a ratio of 10:5:2 in weight was separated in 100 min. The peak fractions, determined by UV absorbance measurement, were analyzed by a Finnigan MAT mass spectrometer for identification of each compound.

MATERIALS AND METHODS

Apparatus

The apparatus used in this experiment was a Pharma-Tech model CCC-2000 Analytical Countercurrent Chromatograph (Pharma-Tech Research Corporation, Baltimore, MD). It is a coil planet centrifuge which provides a synchronous planetary motion of the column holder, i.e., rotation of the column holder about its own

axis and revolution around the central axis of the centrifuge at the same angular velocity and in the same direction. This mode of planetary motion of the holder prevents twisting of the flow tubes and permits continuous elution of the mobile phase through the rotating column without the use of conventional rotary seal device (1).

This apparatus is equipped with a column holder at a 2.5-inch revolution radius. A multilayer coil prepared from about a 70 m length of heavy wall #20 (0.85 mm I.D.) PTFE (polytetrafluoroethylene) tubing (Zeus Industrial Products, Raritan, NJ) is coaxially mounted on the holder. The β values (ratio of the radius of rotation to the radius of revolution) ranged from 0.5 at the internal terminal to 0.75 at the external terminal. The total capacity of the column is 43 ml which includes 3 ml in the flow tubes. The maximum revolutional speed of this centrifuge is 2000 rpm. This commercial countercurrent chromatographic model is equipped with an LDC/Milton Roy metering pump, a speed controller (Bodine Electric Co.) with digital rpm display, and pressure gauge, etc.

Sample and Two-Phase Solvent System.

Dried roots of Stephania tetrandra S. Moore (Menispermaceas) or Fenfangji in China is one of the famous Chinese traditional drugs used for rheumatism and arthritis. The content of total alkaloid or the active compounds in this natural product is 2.3%. By means of conventional selective solvent extraction and neutral Al_2O_3 column chromatography, three major alkaloids were identified as tetrandrine (1%), fangchinoline (0.5%), and cyclanoline (0.2%): Tetrandrine and fangchinoline were inseparable by the conventional method resulting in a mixed product, while cyclanoline was well separated from other two compounds. As illustrated in Fig. 1, tetrandrine and fangchinoline are both bisbenzylisoquinoline alkaloid, whereas cyclanoline is a water-soluble quaternary protoberberine-type alkaloid.

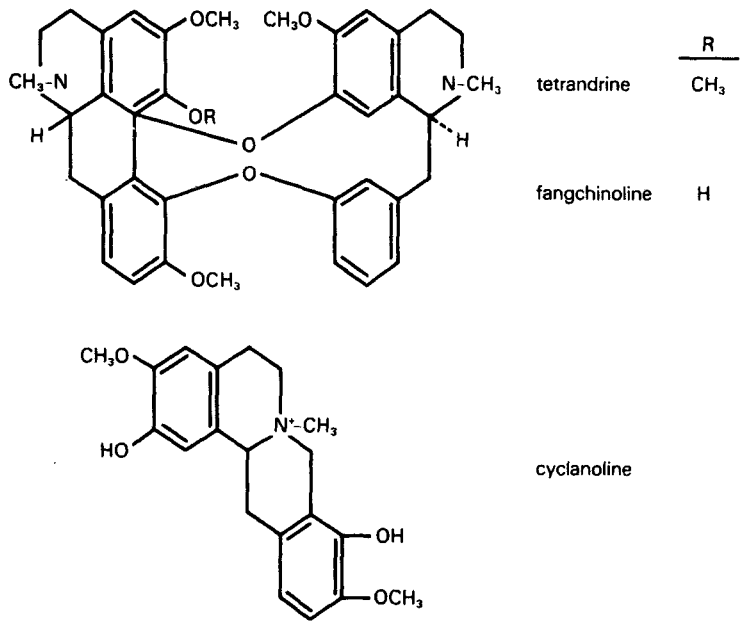

FIGURE 1. Chemical structures of tetrandrine, fangcholine, and cyclanoline.

The sample solution was prepared as follows: The above mixture of tetrandrine and fangchinoline was added to the purified cyclanoline to obtain the ratio of the three compounds at 10:5:2 in weight to simulate their composition in the natural drug. A 3 mg quantity of this sample mixture was dissolved in 0.5 ml of upper stationary phase of the following two-phase solvent system and charged for each separation.

Separations were performed with two solvent systems composed of n-hexane/ethyl acetate/methanol/water at different volume ratios of 1:1:1:1 and 3:7:5:5. Each solvent mixture is thoroughly equilibrated in a separatory funnel at room temperature, and two phases separated shortly before use.

Experimental Procedure.

In the first experiment, a two-phase solvent system composed of n-hexane/ethyl acetate/methanol/water at a 3:7:5:5 volume ratio was used. The coiled column was first entirely filled with the upper stationary phase with the Milton Roy metering pump, followed by injection of 0.5 ml of the above sample solution through the sample port. Then the apparatus was rotated at the optimum revolutional speed of 1800 rpm while the lower mobile phase was pumped into the head end of the column at a flow rate of 60 ml/h. Effluent from the tail of the column was continuously monitored with an LKB Uvicord S at 278 nm and fractionated into test tubes with an LKB fraction collector. After two peaks were collected (70 min), the column was eluted with the original stationary phase (upper phase) in the reversed direction to collect the third peak still remaining in the column. In order to avoid the excess flow, due to the column pressure in this reversed elution mode, a narrow capillary tube was connected to the outlet of the UV monitor to maintain the original flow rate of 1 ml/min (1). Aliquot of each fraction was diluted with methanol and absorbance was determined at 280 nm with a Zeiss spectrophotometer (model PM6) In this experiment, the retention of the stationary phase was 50%, and the maximum pressure at the outlet of the pump measured 70 psi.

In the second experiment, a two-phase solvent system composed of n-hexane/ethyl acetate/methanol/water at a 1:1:1:1 volume ratio was used. Separation was similarly performed as the first experiment except that all three peaks were collected with the lower mobile phase in the normal elution mode. In this case, the retention of the stationary phase was improved to 72.5% while the maximum pressure at the outlet of the pump was increased to 110 psi.

RESULTS

Fig. 2 shows the chromatogram obtained from the first experiment. In the normal elution mode, peak 1 and peak 2 were

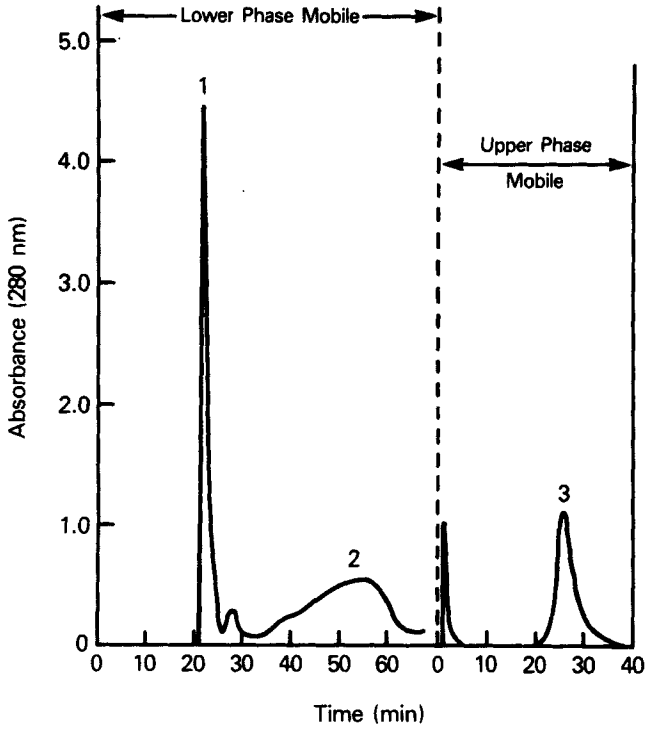

FIGURE 2. Chromatogram of the sample mixture (tetrandrine:fangchinoline:cyclanoline = 10:5:2). Solvent System: n-hexane/ethyl acetate/methanol/water (3:7:5:5).

completely resolved and collected in 70 min. This was followed by the reversed elution mode without interrupting the centrifuge run to collect the third peak in additional 30 min. The results clearly show that this analytical high-speed countercurrent chromatography enables highly efficient separations of several milligrams of samples in a short period of time. In addition, a very small amount of impurity present between peaks 1 and 2 was also well resolved in this chromatogram.

The chromatogram obtained by the second experiment is shown in Fig. 3. Due to the modified volume ratio of the solvent

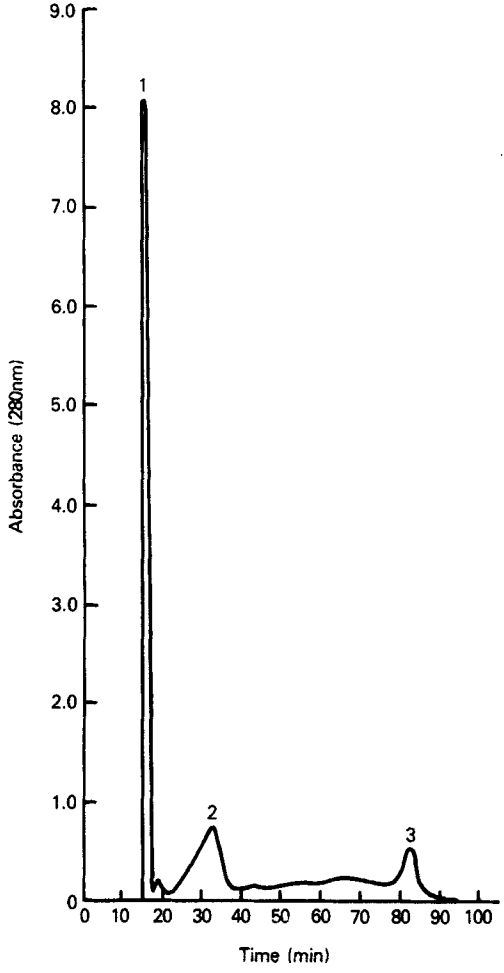

FIGURE 3. Chromatogram of the sample mixture (tetrandrine: fangchinoline:cyclanoline = 10:5:2). Solvent System: n-hexane/ethyl acetate/methanol/water (1:1:1:1).

system, all peaks were eluted earlier at high concentrations compared to the previous experiment. The retention of the stationary phase was also substantially increased probably due to increased interfacial tension of the two phases. Because the partition coefficient K(L/U) (solute concentration in the lower phase divided by that in the upper phase) of the purified cyclanoline in the n-hexane/ethyl acetate/methanol/water (1:1:1:1) system measured as high as 120, cyclanoline was eluted as the first peak immediately after the solvent front as shown in Fig. 3.

The partition coefficient of each compound can be estimated from the chromatogram by using the following equation:

$$K(C_m/C_s) = (C - R_f)/(R - R_f)$$

where $K(C_m/C_s)$ is the partition coefficient obtained by solute concentration in the mobile phase divided by that in the stationary phase; C, the total column capacity; R_f, the retention volume of the solvent front; and R, the retention volume of the solute peak. From the chromatogram shown in Fig. 3, the partition coefficients K(L/U), of fangchinoline and tetrandrine were computed as 1.37 and 0.38, respectively.

In order to identify the compounds in peak 2 and peak 3 in Fig. 3, a Finnigan MAT mass spectrometer was used to analyze the peak fractions. The mass spectrum obtained from the fraction of peak 2 is shown in Fig. 4, which determines the molecular weight of this material as 609, indicating that fangchinoline is the major compound in peak 2. The molecular weight of the compound from the fraction of peak 3 was similarly determined as 623 according to the mass spectrum shown in Fig. 5, which indicates that tetrandrine is the major component in peak 3 (Fig. 3).

DISCUSSION

High-speed countercurrent chromatography is a recently developed separation technique characterized by high partition efficiency and short separation time. These advantages are clearly demonstrated by a commercial model used in the present

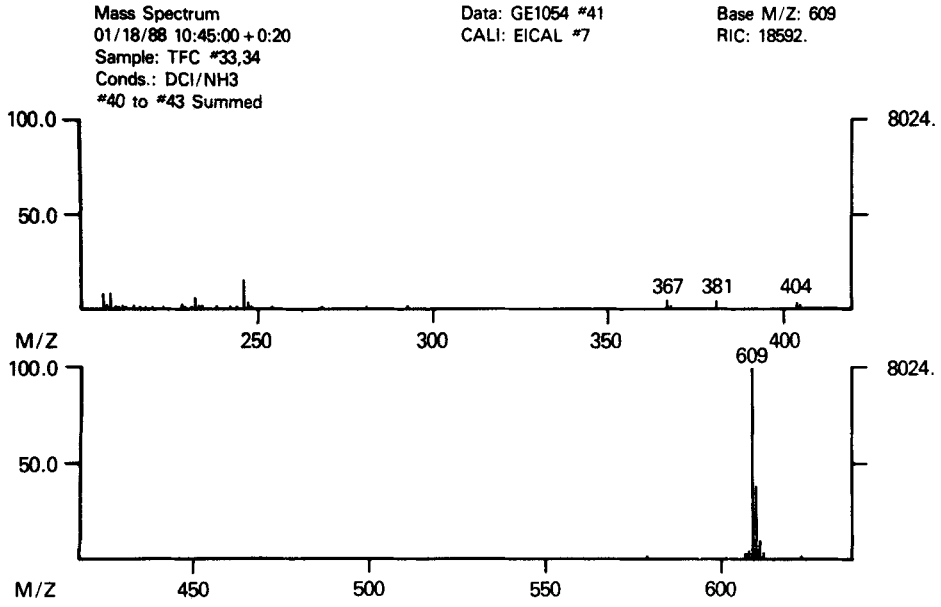

FIGURE 4. The mass spectrum of the fraction from peak 2 in Fig. 3.

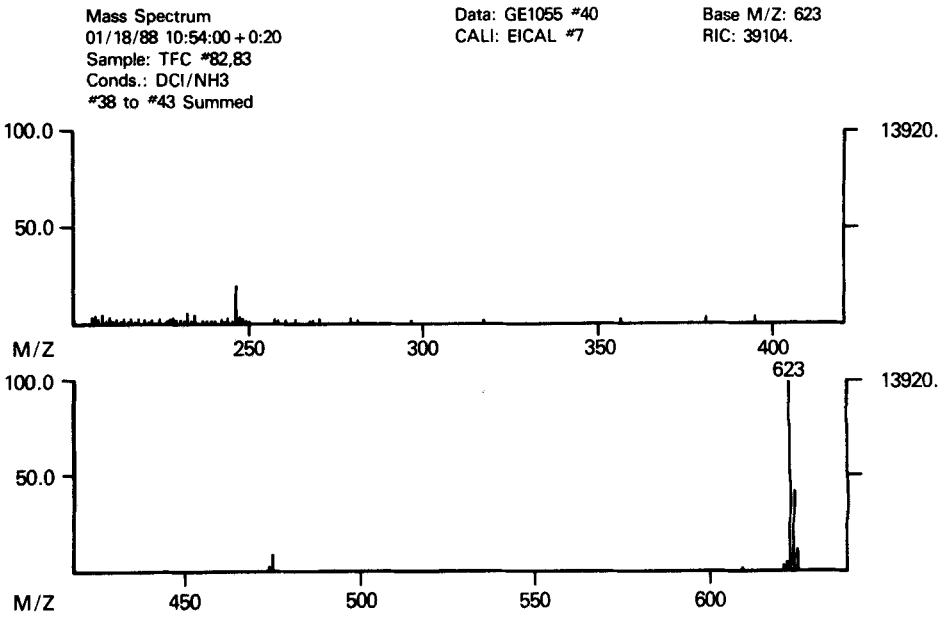

FIGURE 5. The mass spectrum of the fraction from peak 3 in Fig. 3.

experiments. Several milligrams of the sample mixture is separated completely in 1 to 2 hr; hence such separation can be repeated several times a day. For each separation, requirement of the solvent is no more than 100 ml for each phase.

As shown in the first experiment, either phase of the two-phase solvent system can be used as the mobile phase. This provides a particular advantage for separation and purification of natural products, because a crude biological extract, which usually contains multiple components with a wide spectrum in polarity, can be efficiently processed in a short period of time without excessive dilution. The second experiment demonstrates an alternative approach where the solvent composition is adjusted to modify the partition coefficients of the compounds to shorten the separation time without the use of the reversed elution mode.

Mass spectrometric analysis has proved that all three compounds in the sample mixture were satisfactorily purified. Here, it should be emphasized that fangchinoline and tetrandrine were separated easily in the present method while they are almost inseparable with the conventional chemical methods and/or column chromatography.

Because of high efficiency, speedy separation and versatility, we believe that the present method can be widely used in separation and purification of a variety of natural products in the future.

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