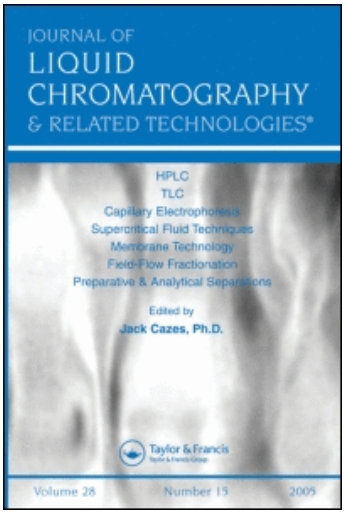

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Analytical High Speed Countercurrent Chromatography

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ANALYTICAL HIGH SPEED COUNTERCURRENT CHROMATOGRAPHY

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ABSTRACT

The resolution of countercurrent distribution has long been demonstrated. However, the long elution time limits its analytical applications. The development of a high speed planet centrifuge has markedly improved the efficiency of the system; thus the countercurrent chromatography can be completed within several hours instead of days. Further reduction in separation time is still needed for analytical use, however.

The most effective approach to improve the efficiency is to use a smaller diameter tubing for the multilayer coiled column, because it provides for better phase mixing. Under standard operation conditions, the increased phase interaction has resulted in steady carry-over of the stationary phase. This was solved with a high revolutional speed which provides the necessary centrifugal force for sustaining the stationary phase.

Excellent phase retention and resolution have been achieved with a 0.85 mm i.d. column and a 2000 rpm revolutional speed. The analytical capability of the new system is evidenced by the

separation of indole plant hormones, plant alkaloids and herbicides. The current resolution and speed of CCC together with its other advantages such as mild conditions, high recovery and ready semipreparative scale-up suggest the CCC can be complementary to HPLC as an analytical technique.

INTRODUCTION

Countercurrent chromatography employing an Ito's planet centrifugal system is a novel method for purification of a wide class of compounds (1-4) which eliminates complications arising from conventional solid support liquid chromatography. The high resolving power of the system has been demonstrated in the fractionation of actinomycin C complex into its components (5). However, the method required long elution times and the resolution depends largely on individual's experience in selecting the proper solvent system and the correct mode of elution. For analytical applications, the overall resolution of the system must be further improved and standardized.

Recently, the development of a high speed planet centrifugal system has markedly reduced separation time to several hours instead of days (6-8). Attempts to improve the resolution by employing a 0.85 mm i.d., multilayer coiled column with a 20 cm revolutional radius, however, resulted in steady carry-over of the stationary phase, possibly due to excessive mixing of the two phases.

In order to sustain the stationary phase in a smaller diameter column, while the mobile phase is passing through it, it is necessary to apply a higher revolutional speed to provide the

needed centrifugal force. Excellent phase retention and resolution have been achieved in our recent study employing a multilayer coiled column of 0.85 mm i.d. and a revolutional speed of 2000 rpm (8). In this paper, the analytical capability of the system is further evidenced by separations of plant indole hormones, plant alkaloids and herbicides. Comparison with reverse phase HPLC revealed that the high speed analytical countercurrent system is capable of separation similar to HPLC. It is conceivable that analytical high speed countercurrent chromatography will become an essential tool complementary to HPLC for analytical applications.

EXPERIMENTAL

Apparatus

The apparatus employed is a newly developed analytical high speed planet centrifuge with a 5 cm revolutional radius and a 0.85 mm diameter multilayer coiled column. The motor directly drives the rotary frame around the central axis of the centrifuge. The rotary frame consists of a pair of aluminum plates rigidly bridged by links and holds a column holder (top) and the counterweight holder (bottom) in the symmetrical positions at 5 cm from the central axis of the centrifuge. The holder shaft is equipped with a plastic planetary gear which is coupled to an identical stationary sun gear rigidly mounted on the central axis of the centrifuge. This gear coupling produces a desired synchronous planetary motion of the column holder. The holder

revolves around the central axis of the centrifuge and simultaneously rotates about its own axis at the same angular velocity. As described elsewhere (7), this particular type of planetary motion permits the flow tubes to rotate around the central axis of the centrifuge without twisting, thus facilitating continuous elution of the mobile phase through the rotating column. The revolutional speed of the centrifuge is continuously adjustable up to 2000 rpm with a speed control unit. Both column holder and counterweight holder can be removed from the rotary frame simply by loosening a pair of screws in each bearing block, hence facilitating the column preparation and determination of the counterweight mass required for balancing the centrifuge system.

The multilayer coiled column was prepared by winding a long piece of PTFE tubing (0.85 mm i.d.) onto the holder with a 5 cm hub diameter, making multiple coiled layers. The β value varied from 0.5 at the internal terminal to 0.8 at the external terminal. The total column capacity of the multilayer coil measured approximately 38 mL.

The coiled column was equipped with a pair of flow tubes measuring 0.5 mm i.d. and 0.5 mm wall thickness (Pierce Chemical Co., Rockford, IL). The junction between the flow tube and the column terminal was made with a short piece of PTFE tubing of 1.7 mm i.d. as an adaptor. Both inlet and outlet flow tubes from each column were first led through the center hole of the holder shaft, and then by making a loop passed through the side hole of the short coupling pipe to enter the opening of the central sta-

tionary pipe. At the exit from the centrifuge (left) the flow tubes were clamped between silicone rubber sheets by a tube support mounted on the centrifuge wall.

The HPLC system consisted of a Model 6000A pump (Waters Assoc.), and a Model 46K injector (Waters Assoc.), and a Model 440 UV detector (Waters Assoc.). HPLC separations were performed on a 4.6 mm x 25 cm Zorbax-ODS column (DuPont Inc.).

Reagents

Organic solvents used for preparation of the two phase solvent systems, including n-hexane, ethyl acetate, methanol, ethanol, are glass distilled chromatographic grade purchased from Burdick and Jackson Laboratories, Inc., Muskegon, MI. Experiments were performed with either (A) the two phase solvent system composed of n-hexane, ethyl acetate, methanol and water with a volume ratio of 8:2:5:5 or 3:7:5:5 or (B) the two phase system composed of n-hexane, ethanol and water with a volume ratio of 6:5:5. The two phase solvent system was prepared by thoroughly equilibrating the solvent mixture in a separatory funnel at room temperature followed by filtration through a 5 μm filter and degassing.

Methods

The analytical countercurrent chromatography was performed with an analytical high speed planet centrifuge system equipped with a multilayer coil column of 0.85 mm i.d. and at a revolutional speed of 2000 rpm.

In each separation the column was first filled with the stationary phase (upper phase) followed by injection of the sample solution through the head inlet. The mobile phase (lower phase) was then pumped into the column head inlet while the apparatus was run at a revolutional speed of 2000 rpm. The effluent from the tail outlet of the column was continuously monitored with a UV detector at 280 nm and fractionated into test tubes with an LKB fraction collector. Each fraction was mixed with 2.5 mL of methanol and the absorbance was measured at 280 nm with a Zeiss spectrophotometer.

RESULTS AND DISCUSSION

The analytical separation of S-triazine herbicides, plant indole hormones and plant alkaloids with the new analytical countercurrent system has been compared with corresponding HPLC analyses with a reverse phase system (Zorbax-ODS, 4.5 mm x 15 cm, 15% H_2O in MeOH).

Chlorinated S-Triazine Herbicides

Triazine herbicides have been widely used for control of broadleaf weeds in cropland (9,10). These compounds exhibit persistence in the soil and contamination due to spraying and run-off. Methods for the analysis of these compounds and their metabolites are important from both environmental and agricultural points of view. Analytical methods presently approved for analyzing these chlorinate S-triazine herbicides is gas

liquid chromatography (GLC) (11-13), although HPLC appears to be equally suitable for the analysis. Previous attempts with countercurrent chromatography showed excellent resolution, however, the separation took three hours to complete (14). In the present study, a two phase solvent system composed of hexane-ethyl-acetate-methanol-water (8:2:5:5) was employed. The upper phase was used as the mobile phase at a flow rate of 1 mL/min. As shown in Figure 1, the four components of s-triazines were well resolved and the entire separation took less than 1 h. Thus the new CCC system is about three times as efficient as the previous CCC system. This represents the overall results of employing a smaller diameter column combined with 2000 rpm revolutional speed. Figure 2 shows the corresponding HPLC analysis with a reverse phase system (Zorbax ODS column 4.5 mm x 25 cm; 15% H_2O in MeOH) which gave comparable separation within 20 min.

Plant Alkaloids

(+)-Vincamine 1 is the major alkaloid of Vinca minor (15). Its principal activity is in cerebral vasodilation. Clinical studies have demonstrated that IV administration of vincamine can reduce the arterial blood pressure and increase cerebral blood flow and oxygen consumption (16). Besides vincamine, a number of analogs are present in the Vinca minor. The isolation of vincamine has been complicated by the presence of vincine 2, an 11-methoxy analog, and other isomers (17). Because of the structural similarity, vincine has always cochromatographed with vincamine under various preparative chromatographic conditions. As

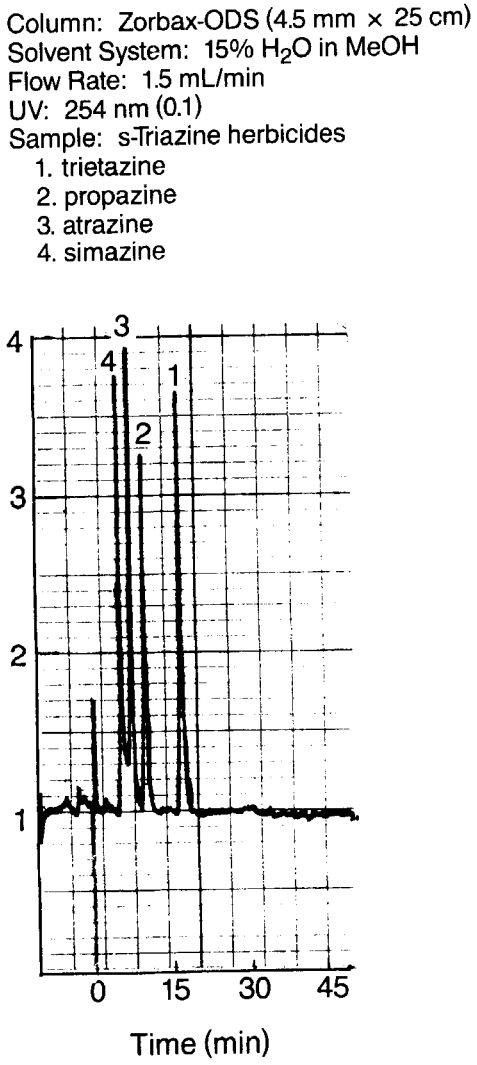

FIGURE 1. Separation of the Chlorinated S-Triazine by Analytical High Speed Countercurrent Chromatography. Absorbance Data From Each One Minute Fraction were Plotted and Connected by a Smooth Curve.

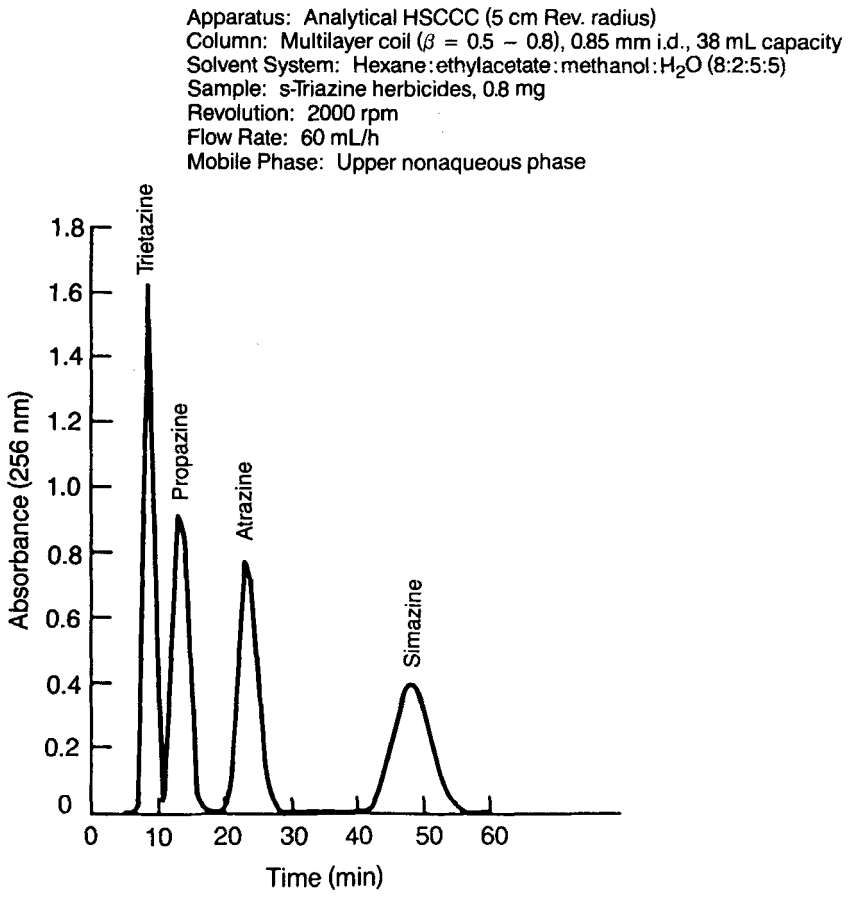

FIGURE 2. Separation of the Chlorinated S-Triazine by HPLC.

shown in Figure 3, vincamine and vincine were well resolved with an analytical CCC employing hexane-ethanol-water (6:5:5) as the two phase solvent system. The lower phase was employed as the mobile phase at a flow rate of 0.8 mL/min. The corresponding HPLC analysis with a reverse phase column and a buffered solvent

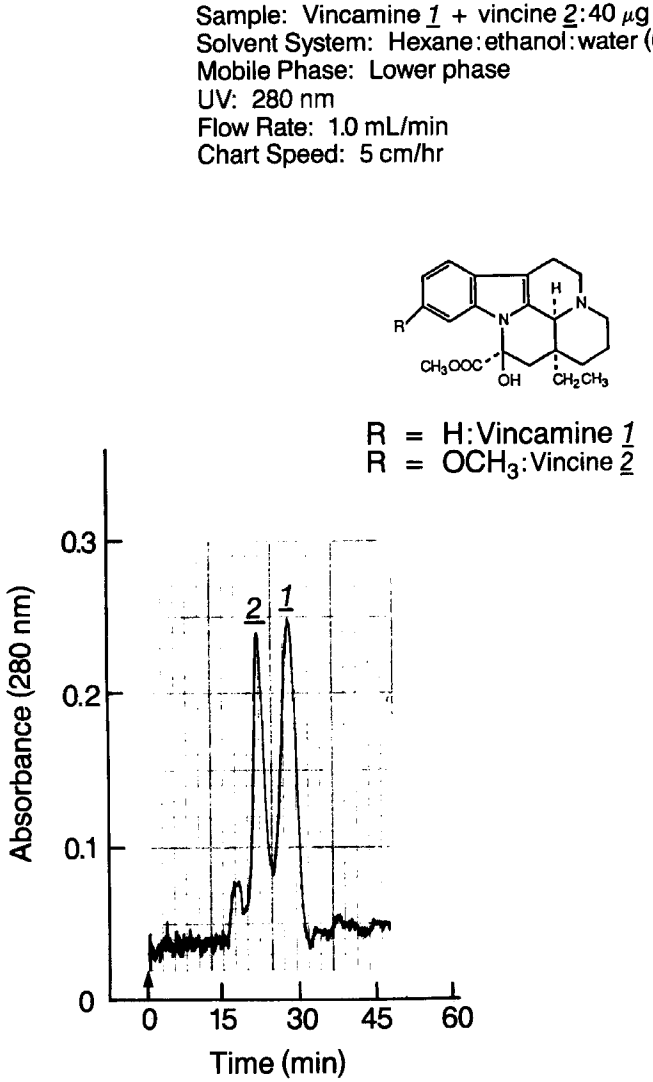

FIGURE 3. Separation of Vincamine and Vincine by Analytical High Speed Countercurrent Chromatography. Absorbance Recorded Continuously at 280 nm on a Choeffel SF770 UV Detector.

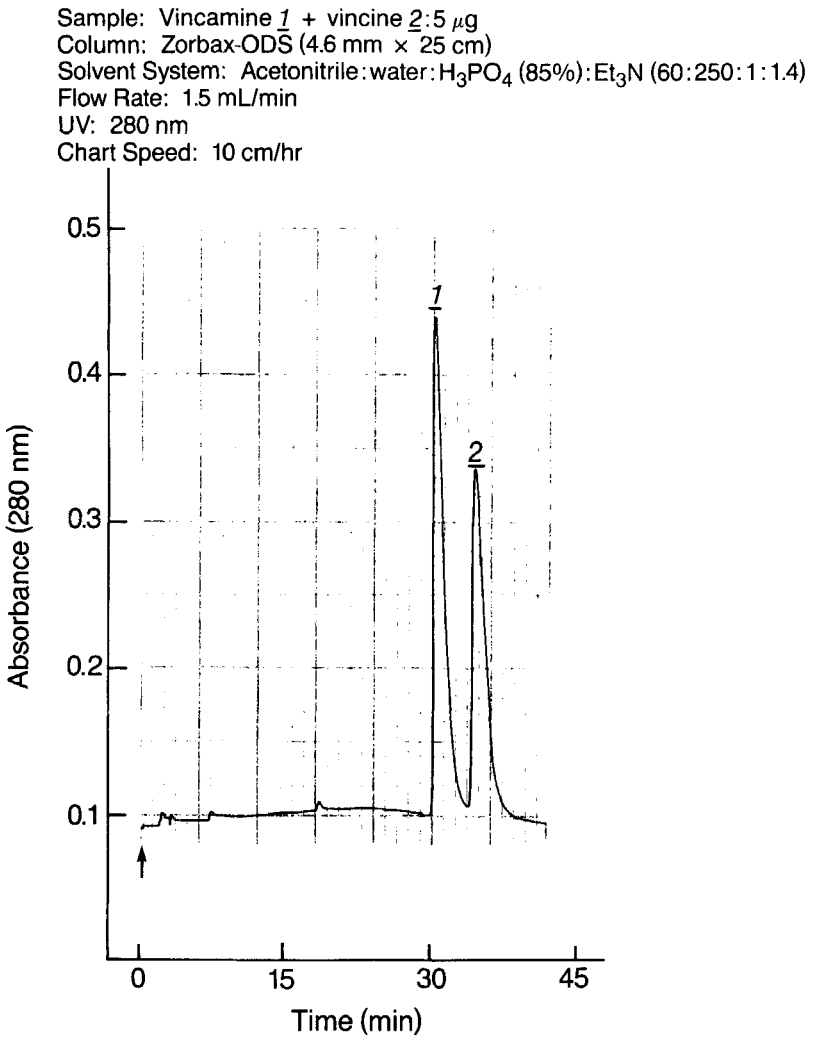

FIGURE 4. Separation of Vincamine and Vincine by HPLC.

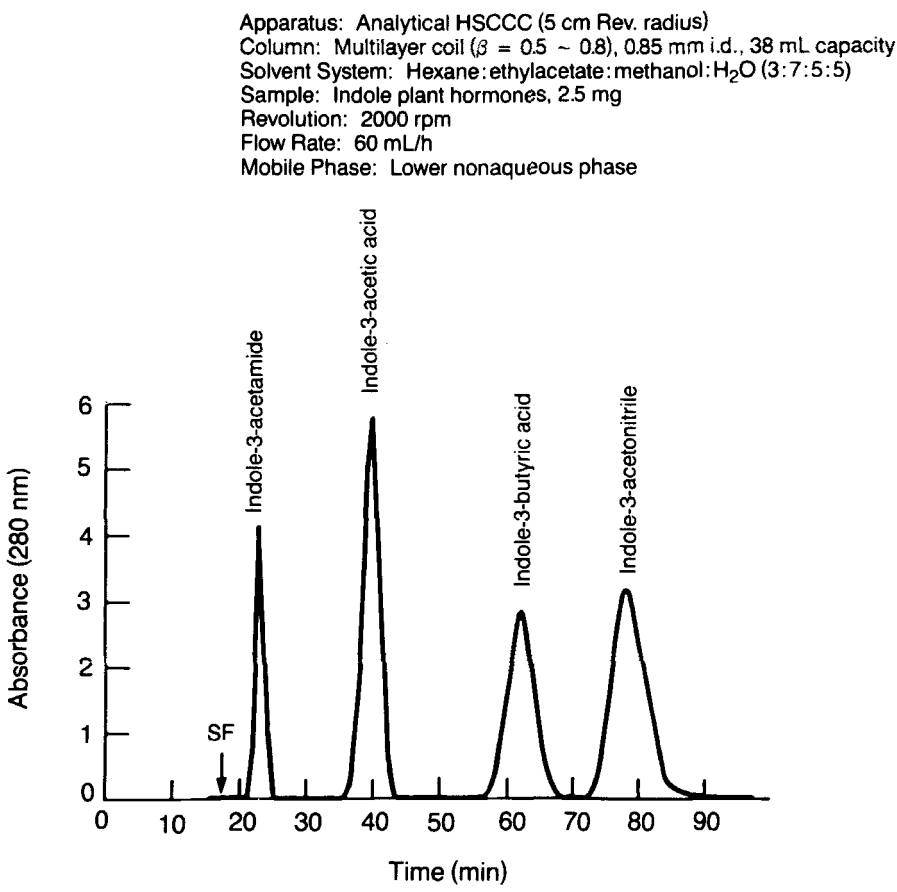

FIGURE 5. Separation of Indole Plant Hormones by Analytical High Speed Countercurrent Chromatography. Absorbance Data From Each One Minute Fraction were Plotted and Connected by a Smooth Curve.

system is shown in Figure 4. A minor impurity shown in analytical CCC was not observed in the HPLC the chromatogram. The analytical high speed countercurrent chromatography provides an excellent means not only to determine the purity of vincamine but also to isolate vincine for pharmacological evaluations.

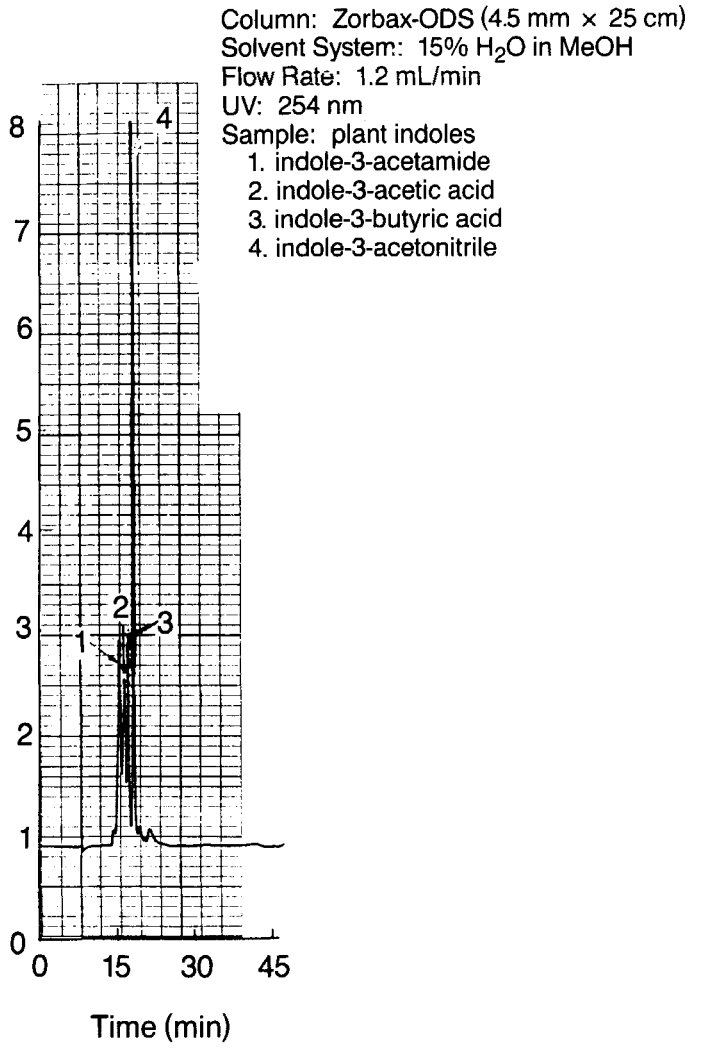

FIGURE 6. Separation of Indole Plant Hormones by HPLC.

Indole Plant Hormones

The separation of indole plant hormones is shown in Figure 5. A two phase solvent system composed of hexane-ethyl-acetate-methanol-water (3:7:5:5) was employed. The lower phase was used as the mobile phase at a flow rate of 1 mL/min. The four indole components were completely resolved within 90 min. The retention of the stationary phase was 55% of the total volume capacity. As shown in Figure 6, the corresponding HPLC analysis showed incomplete separation under the identical experimental conditions used in the first example. It is possible that the resolution can be improved under optimum HPLC conditions. This example clearly demonstrates the potential of the present CCC technique in performing analytical scale separations.

Conclusion

Analytical high speed countercurrent chromatography provides a complementary method to HPLC for the analysis and isolation of a wide range of complex natural products. The major advantages are (1) high resolution; (2) avoidance of contamination or deactivation from solid adsorbance; (3) total recovery of bioactivity and sample; (4) inexpensive operation; (5) analytical and semi-preparative applications.

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