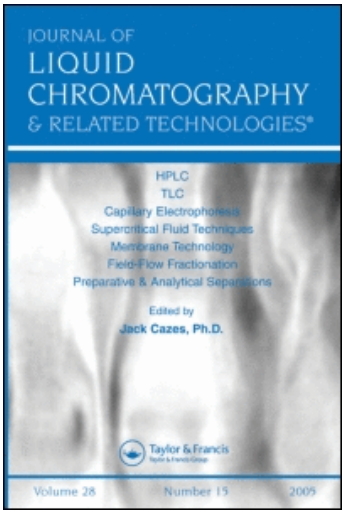

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Dual Countercurrent Chromatography

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DUAL COUNTERCURRENT CHROMATOGRAPHY

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ABSTRACT

A modern method of performing conventional countercurrent distribution called Dual Countercurrent Chromatography is described. The system consists of a multilayer coiled column integrated with two inlet and two outlet lines for solvent phases and a sample feed line. Subjecting the system to a particular combination of centrifugal and planetary motions produces a unique hydrodynamic effect which allows two immiscible liquids to flow countercurrently through the coiled column. The sample solution is fed at the middle portion of the column and eluted simultaneously through the column in the opposite direction by two liquids. This distinct feature of maintaining a constant fresh two phases within the coiled column permits a rich domain of application, such as continuous sample process and continuous extraction.

The present paper describes the application of this dual countercurrent system to the separation of natural products and synthetic intermediates. Preliminary results indicate that this new method has remarkably improved the efficiency of conventional

countercurrent distribution. It is conceivable that dual countercurrent chromatography will become an essential tool for research in natural products, organic synthesis and biotechnology.

INTRODUCTION

The separation of multicomponent mixtures by differential partition between two immiscible solvents has long been used in liquid-liquid extractions. This technique was automated effectively with a multistage countercurrent distributor in the 1950's (1). Its high resolving power was remarkably demonstrated in the fractionation of a commercial preparation of insulin into two subfractions which differ only by an amide group in a molecular weight of 6000 (2). However, the inconvenience of the system and the time involved in using it limit its future applications.

In recent years, significant improvements have been made in the countercurrent chromatography field (3-5). A number of convenient and effective procedures such as droplet CCC (6), centrifugal droplet CCC (7,8), rotation locular CCC (7), flow-through coil planet centrifugal CCC (4) and high speed multilayer coil centrifugal CCC (9) have been developed and proven advantageous for the isolation and purification of a diverse array of natural products. One common feature of these procedures is that a mobile phase is partitioning through a finite stationary phase which is retained in the system either by gravitational or centrifugal force.

Separation based on such continuous liquid-liquid partitioning proves to be ideal in fractionation of crude natural products

for bioactivity because all the complications arising from solid support such as degradation, deactivation, and contamination are eliminated. However, the advantage and versatility of the countercurrent distribution method cannot be fully effected with the ordinary mode of one way elution. A recently developed high speed countercurrent chromatographic method (11,12) utilizes a particular combination of coil orientation and planetary motion to produce a unique hydrodynamic effect which permits a true countercurrent flow of the two solvent phases through the coiled column (10,13). Basically, the system consists of a multilayer coiled column integrated with two solvent inlets, two solvent outlets, and one sample feed line. The two solvent phases can be simultaneously eluted through the column in the opposite directions while the sample solution is continuously fed at the middle portion of the column. This dual countercurrent system provides a rich domain of applications such as continuous extraction, enrichment, and stripping of solutes or particles.

The present paper describes the application of this dual countercurrent system to the separation of complex mixtures of steroids and synthetic intermediates. Preliminary results indicate that this new method has remarkably improved both the separation time and efficiency of the conventional countercurrent distribution method. It is conceivable that dual countercurrent chromatography will become an essential tool for research in fractionation of natural products, organic synthetic intermediates, and biotechnology products.

PRINCIPLE

The principle of the dual countercurrent system is illustrated in foam countercurrent chromatography (10). Basically, a cylindrical multilayer coiled column holder is equipped with a planetary gear which is coupled with an identical stationary sun gear placed around the central axis of the centrifuge. This gear arrangement produces a particular type of synchronous planetary motion. This synchronous rotation of the holder unwinds the twist of the flow tubes caused by revolution, thus eliminating the need for the rotary seals. When the multilayer coiled column is subjected to the synchronous planetary motion, the two solvent phases in the coiled tube distribute in such a way that one phase entirely occupies the head side and the other phase, the tail side of the coil. As shown in Figure 1 the white phase if introduced into the black phase would move toward the head and the black phase, if introduced into the white phase, would move toward the tail. Consequently, the system permits simultaneous introduction of the two phases through the respective ends of the coil to produce true countercurrent flow. This requires an additional flow channel at each end of the coil to collect the effluent and also a sample feed line at the middle portion of the coil, a total of five channels.

EXPERIMENTAL

Reagents

Organic solvents used for preparation of the two phase solvent systems, including n-hexane, ethyl acetate, methanol, etha-

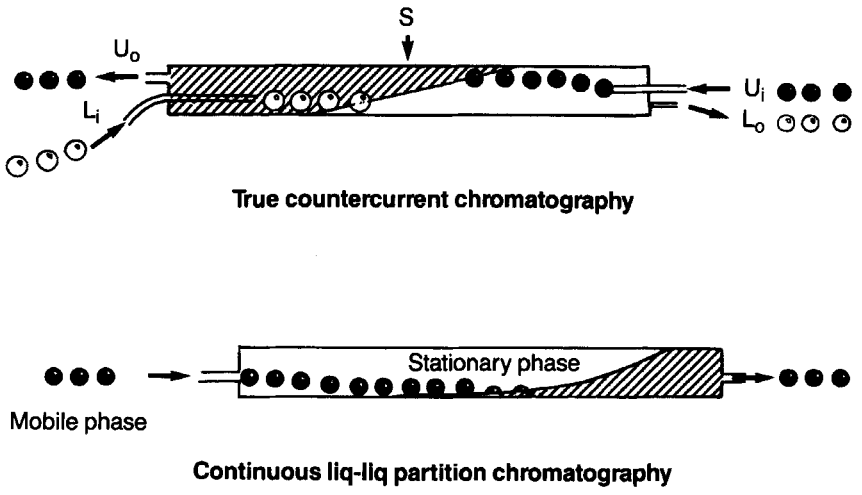


FIGURE 1. Flow Diagram of Dual Countercurrent Chromatography.

nol, were glass distilled chromatographic grade purchased from Burdick and Jackson Laboratories, Inc., Muskegon, MI. Experiments were performed with either (A) a two phase solvent system composed of n-hexane, ethyl acetate, methanol and water with a volume ratio of 3:7:5:5 or (B) a 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.

Apparatus

The dual countercurrent experiments were performed with a table top model high speed planet centrifuge equipped with a multilayer coiled column (for detail see reference 10).

The multilayer coiled column was prepared from 1.6 mm i.d. PTFE tube (Zeus Industrial Products, Raritan, NJ) by winding it coaxially onto the holder with a total volume capacity of 400 mL. In order to facilitate the countercurrent process, the head terminal was located at the outer layer and the tail terminal at the inner layer of the coiled column. Each terminal is equipped with a 3-way adaptor which connects inlet and outlet flow tubes to the coiled column whereas at the middle portion of the column a sample feed tube opens through another 3-way adaptor. At each terminal the feed line is passed through the adaptor to extend into the separation column for about 50 cm or one complete helical turn. This prevents the introduced phases from flowing back toward the immediate outlet opening at each terminal. The liquid collection lines were made of 0.85 mm i.d. PTFE tubes and other three feed lines, 0.55 mm i.d. tubes. As mentioned earlier, these 5 flow tubes from the separation column are free from twisting.

The rotational speed of the apparatus is continuously adjustable up to 1000 rpm but the rate was limited to 450 rpm throughout the present study. Two Model 6000A HPLC pumps (Waters Assoc. Inc., Milford, MA) were used to pump the liquid phases. The flow rate through the liquid collection line (upper phase) was regulated by the use of a needle valve.

Method

In a typical operation mode the multilayer coiled column is first filled with one solvent phase, then both solvent phases are

pumped into the system simultaneously from opposite end of the column while the apparatus is run at 450 rpm. The flow rates of two phase solvents are adjusted initially to facilitate the equilibrium. Once the two solvent phases reach almost equal volume inside the column, the flow rates are readjusted back to equal. Sample solution is introduced to the middle of the coiled column through a sample feed line. The effluents from the respective collection lines are separately fractionated into a series of test tubes with a Buchler LC 200 fraction collector. The fractions are monitored with thin layer chromatography and with suitable solvent systems.

RESULTS AND DISCUSSION

The capability and versatility of dual countercurrent chromatography are evidenced in the separation of an indole mixture, a synthetic intermediate and a mixture of steroids. Because of the preparative nature of these separations, fractions eluted from both mobile phases were analyzed by thin layer chromatography with appropriate solvent systems.

Separation of Indole Mixture

An indole mixture consisting of indole-3-acetic acid, indole and biphenyl was used for our initial feasibility study. AS shown in Figure 2, the three component mixture was completely resolved with a pentane, ethanol and water (5:4:1) two phase system. The fractions eluted from upper phase contained biphenyl and the fractions eluted from lower phase contained indole-3-acetic acid (F5-F8) and indole (F10-F13). This preliminary

Sample:

1. Biphenyl: 50 mg
2. Indole: 30 mg
3. Indole-3-acetic acid: 25 mg

Forward rotation: 450 rpm
Solvent: Pentane: Ethanol: H_2O (5:4:1)
Flow rate: 1.5 mL/min (both sides)
Fractions: tube/4 min
Total column capacity: 410 mL
Phase distribution: Upper 125 mL
Lower: 285 mL

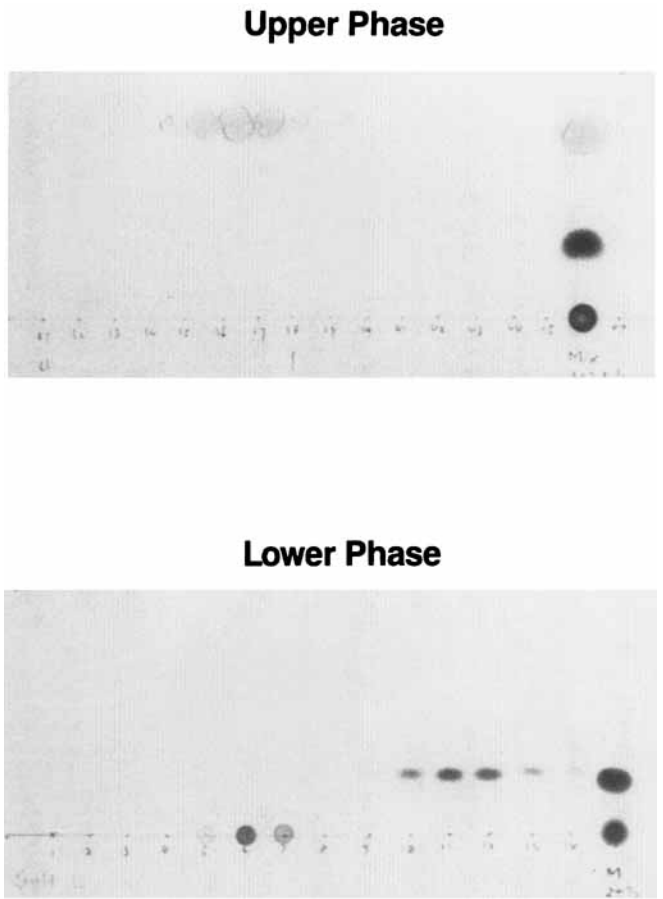


FIGURE 2. DCC Separation of Indole Mixture.

result clearly demonstrates the feasibility of dual countercurrent chromatography in preparative separation of multicomponent mixture.

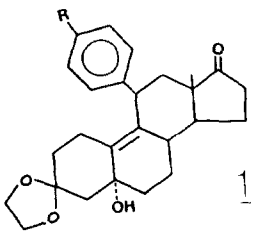
Separation of Synthetic Intermediates

A crude chemical reaction product usually consists of desired product, undesired products, unreacted starting material and unconsumed reagents. Isolation of desired product from such complex mixture presents a constant challenge to synthetic chemists. As shown in Figure 3, a multicomponent reaction mixture (250 mg) was separated by dual countercurrent chromatography employing a two phase system composed of hexane, ethanol and water (6:5:4). The desired product 1 (110 mg) was obtained in reasonable purity from the lower phase (F21-F24), while the excess reagents were eluted simultaneously from upper phase (F14-F25). The entire separation took less than 2 h to complete. This example demonstrates that dual countercurrent chromatography is applicable to preparative purification of synthetic intermediates.

Separation of Steroid Mixture I

The feasibility of dual countercurrent chromatography is evidenced in the previous relatively simple separations. Its resolving power and capability can be exemplified with more complex mixtures. A steroid mixture I, as shown in Figure 4, consisting of five steroid components bearing various functional groups was subjected to dual countercurrent chromatography. The

Sample: Steroid Intermediates 250 mg
Forward Rotation: 450 rpm
Flow Rates: 2.0 mL/min, 4 min/tube
Solvent System: Hexane:Ethanol:H_2O (6:5:4)



1

Upper phase

Lower phase

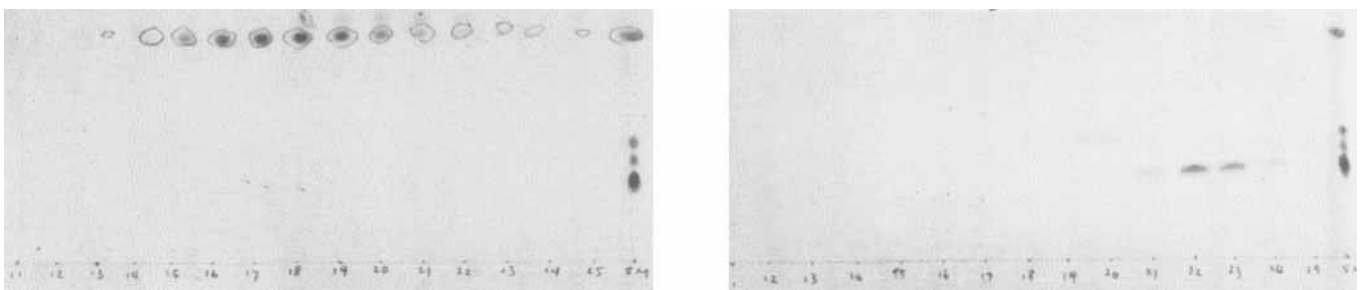
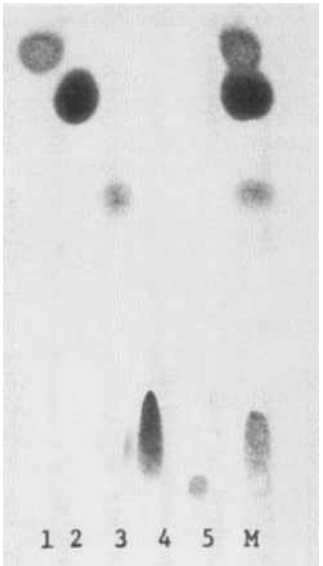
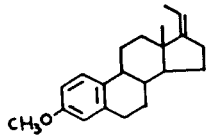


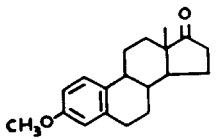
FIGURE 3. DCC Separation of Synthetic Intermediate.



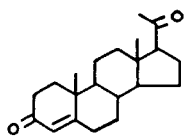
1. **Estrone-3-methyl ether derivative**



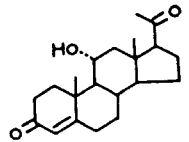
2. **Estrone-3-methyl ether**



3. **Progesterone**



4. **11 α-OH-Progesterone**



5. **Prednisone (Δ^1-cortison)**

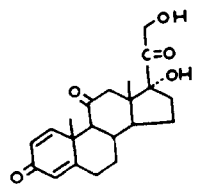


FIGURE 4. Steroid Mixture I (5 Components).

relative R_f value of each individual steroid reflects the overall polarity of the molecule which is attributed to the functional groups attached. A two phase solvent system composed of hexane, ethyl acetate, methanol and water (6:5:5:5) was used.

As shown in Figure 5, the polar components (4 & 5) were eluted by the lower mobile phase in a sequence similar to reverse phase chromatography; the most polar component 5 (F16-F18) eluted first, followed by less polar component 4 (F25-F31). The non-polar components (1, 2 and 3) were eluted simultaneously from the upper mobile phase in a sequence similar to normal phase chromatography; the least polar component 1 (F29-F32) eluted first followed by component 2 (F30-F34) then component 3 (F36-F42). There were some overlapping fractions of component 1 and component 2, but the remaining components were well resolved. This example demonstrates the capability of dual countercurrent chromatography to resolve a steroid mixture in a true countercurrent fashion.

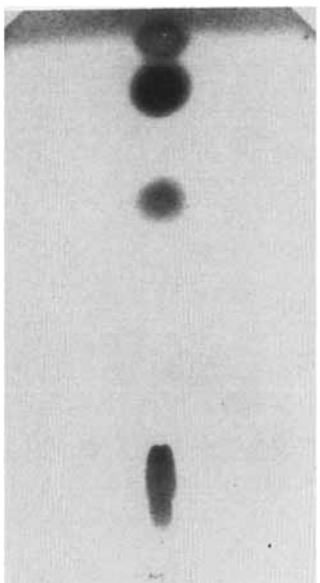
Separation of Steroid Mixture II

A steroid mixture II consisting of nine components (Figure 6) was employed to evaluate the resolving capability of dual countercurrent chromatography. As shown in Figure 6 these nine steroid components bearing identical tetracyclic ring skeletons are substituted with a variety of functional groups and side chains. Because of the structural similarities, these complex mixtures would be a difficult sample for any means of chromatographic separation.

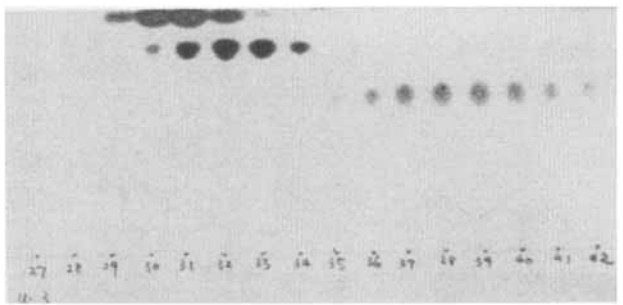
Sample:

1. 25 mg
2. 25 mg
3. 30 mg
4. 25 mg
5. 15 mg

Solvent System:
Hexane: EtOAc: MeOH: H_2O (6:5:5:5)
Flow rates: 1.8 mL/min, 4 min/tube
Forward rotation: 450 rpm
Phase distribution: Upper 265 mL
Lower: 155 mL



Upper Phase



Lower Phase

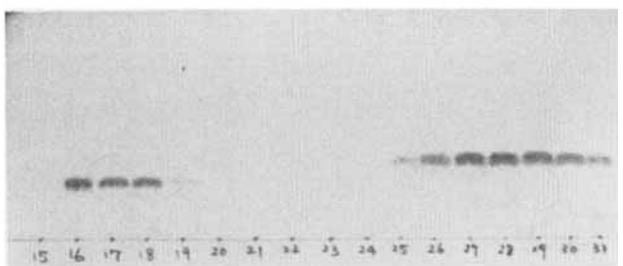
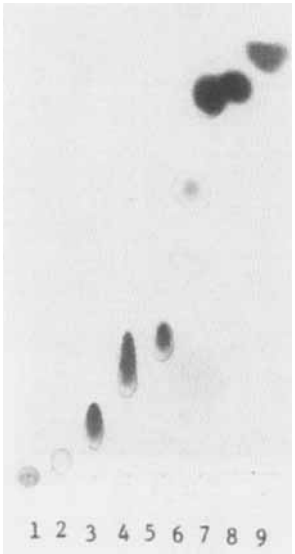
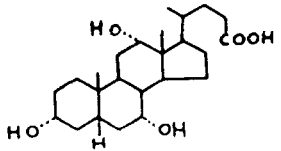


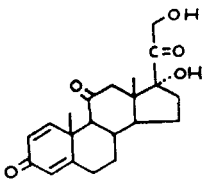
FIGURE 5. DCC Separation of Steroid Mixture I.



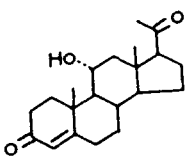
1 Cholic acid



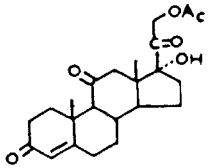
2 Prednisone (Δ^1-cortison)



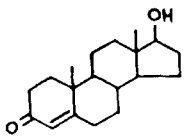
3 11 α-OH-Progesterone



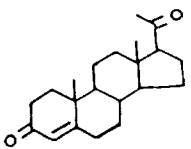
4 Cortisone Acetate



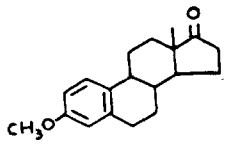
5 Testosterone



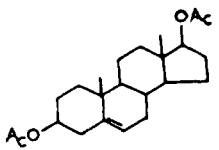
6 Progesterone



7 Estrone-3-methyl ether



8 Δ^5-Androsten-3, 17-diacetate



9 Estrone-3-methyl ether derivative

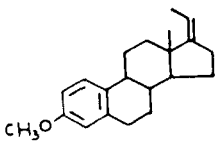


FIGURE 6. Steroid Mixture II (9 Components).

Sample: 1 ~ 9 (25 mg each)
Solvent System: Hexane: EtOAc: MeOH: H_2O (6:5:5:5)
Flow rates: 1.8 mL/min, 4 min/tube
Forward rotation: 450 rpm
Phase distribution: Upper 260 mL
Lower: 160 mL

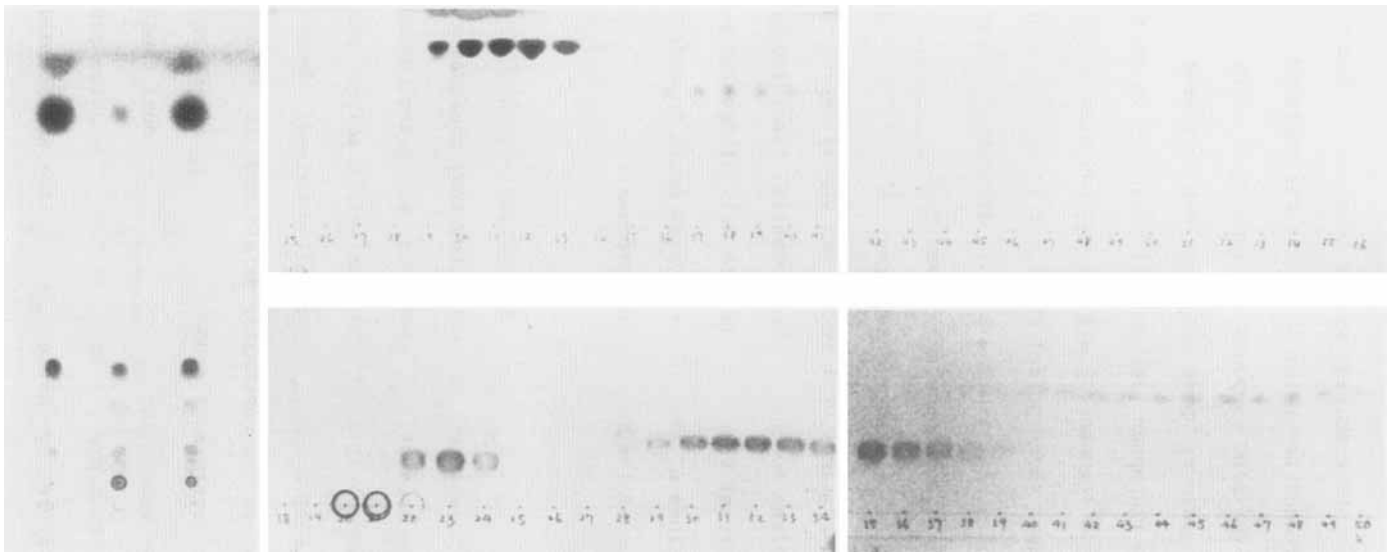


FIGURE 7. DCC Separation of Steroid Mixture II.

A two phase solvent system composed of hexane, ethyl acetate, methanol and water (6:5:5:5) was employed. As shown in Figure 7 the nine component mixture of steroids was resolved simultaneously by upper and lower mobile phases. The steroids eluted in the upper (less polar) phase are in normal phase chromatographic sequence; 9 → 8 → 7 → 6 and steroid components eluted in the lower (more polar) phase are in reverse phase chromatographic sequence; 1 → 2 → 3 → 4. Although there was only partial separation of components 7, 8 and 9, the remaining six components were completely resolved as shown. Steroid 5, which has a partition coefficient of 1 in the two phases employed, was located in the column contents. The potential resolving power of dual countercurrent chromatography is well illustated by this example. Modifications of the solvent system could be made to enhance resolution of the less polar components.

CONCLUSION

Dual countercurrent chromatography provides a novel method to perform conventional countercurrent distribution in an efficient manner. It allows two immiscible solvent phases to countercross within a multilayer coiled column. Because two fresh mobile phases are constantly maintained, it is capable of continuous fractionation of crude samples with a wide range of polarities. The resolution and versatility of dual countercurrent chromatography are evidenced in the preparative separation of indole mixture, synthetic intermediates and steroid mixtures. It

is conceivable that dual countercurrent chromatography will become an important preparative chromatographic method for research in natural products, synthesis and biotechnology.

REFERENCES

1. Craig, L. C., Hausmann, W., Ahrens, P. and Harfenist, E. J., Analyt. Chem., 23, 1326, 1951.

2. Ham's, J. I., Sanger, F. and Naughton, M. A., Arch. Biochem. Biophys., 65, 427, 1956.

3. Ito, Y. and Conway, W. D., Anal. Chem., 56, 534A, 1984.

4. Ito, Y., Advances in Chromatography, Giddings, J. C., Grusha, E., Cazes, J. and Brown, P. R., eds., Dekker, New York, 1977, Vol. 24, p. 181.

5. Conway, W. D. and Ito, Y., LC Magazine, 2, 5, May, 1984.

6. Hostettmann, K., J. Liq. Chromatogr., 7, 231, 1984.

7. Snyder, J. K., J. Liq. Chromatogr, 7, 243, 1984.

8. Murayama, W., J. Chromatogr., 239, 643, 1982.

9. Ito, Y. and Lee, Y. W., J. Liq. Chromatogr., 391, 290, 1987.

10. Ito, Y., J. Liq. Chromatogr., 8 (12), 2131, 1985.

11. Ito, Y., J. Chromatogr., 214, 122 (1981).

12. Ito, Y., Sandlin, J. and Bowers, W. G., J. Chromatogr., 244, 247, 1982.

13. Ito, Y., J. Chromatogr., 301, 377, 1984.