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HORIZONTAL FLOW-THROUGH COIL PLANET CENTRIFUGE: SOME PRACTICAL APPLICATIONS OF COUNTERCURRENT CHROMATOGRAPHY

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SUMMARY

A horizontal flow-through coil planet centrifuge was constructed that holds a pair of coiled separation columns made of PTFE tubing of different inner diameters. With this scheme, samples from a few to hundreds of milligrams can be separated in each operation. The experimental methods are discussed from three aspects: the selection of the two-phase solvent systems, the determination of the optimal operational conditions and the general separation procedures. The capabilities of this apparatus were demonstrated with separations of several biological samples such as antibiotics, agricultural chemicals and medicinal herbs.

INTRODUCTION

Countercurrent chromatography (CCC)[1,2] is an efficient method of partition chromatography that totally eliminates the use of solid supports. It retains the high sample recovery, high purity of fractions and excellent reproducibility inherent in countercurrent distribution methods (CCD), and yet yields a high partition efficiency by continuous elution as in liquid chromatography (LC).

The coil planet centrifuge (CPC) is a newly developed apparatus for performing CCC[3]. Its continuous flow-through system permits the influx and efflux of mobile phase through the rotating column without the use of rotating seals. The advantages of this scheme are that it is leak free, there is no mechanical sample damage or no heat disruption, contamination problems are eliminated and there is a wide choice of solvent systems. In the past, several types of horizontal flow-through CPCs have been developed for performing both micro-scale and large-scale separations with a high partition efficiency[4–8].

This paper introduces one type of horizontal flow-through CPC constructed in our laboratory. It is suitable for separating sample mixtures from a few to hundreds of milligrams in each operation, which is usually completed within several hours. The solvent requirement is only a few hundred millilitres. This CCC apparatus can easily be fabricated and can be used for a wide range of separations and extractions.

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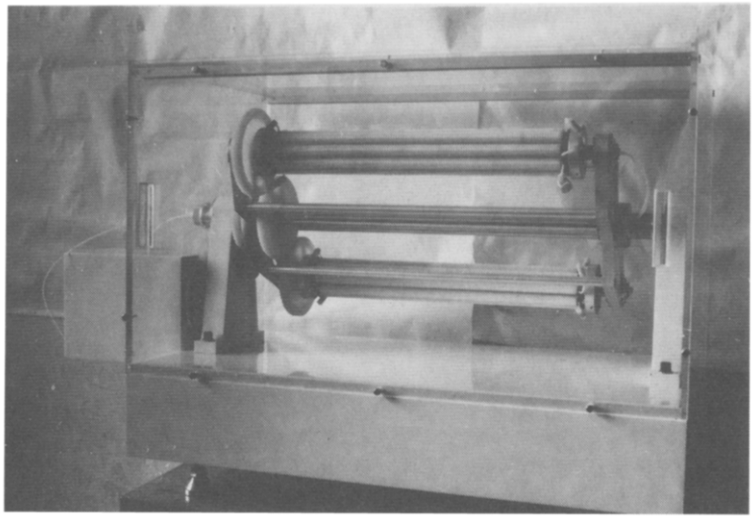

Fig. 1. Horizontal flow-through coil planet centrifuge.

EXPERIMENTAL

Apparatus

Fig. 1 shows the prototype CPC. The design of the apparatus is based on the original horizontal flow-through system[4,5] with minor modifications. The rotary frame holds a pair of column holders symmetrically at a distance 14 cm from the central axis of the centrifuge. The column unit was prepared by winding a piece of PTFE tubing on to a metal pipe of 1.25 cm O.D. Two different sizes of tubing were used, 2.5 mm I.D. with a 0.5 mm wall thickness for large-scale separations and 0.8–1.0 mm I.D. with a 0.3–0.5 mm wall thickness for small-scale separations. Eight column units were arranged symmetrically around each holder and interconnected in series with suitable PTFE tubing connectors. A counterweight was added on the lighter side in order to obtain the necessary balance between the two different columns. The rotational radius for each column unit was 3.2 cm, giving a β value of 0.23. Either column can be used for separations by arranging the pair of flow lines through the opening of the central stationary pipe. As described elsewhere[4], the present design of the flow-through CPC requires no rotating seals.

The revolutional speed can be continuously adjusted from 0 to 500 rpm with a control unit that provides high stability and constantly displays the speed to three digits during operation. Except for the main centrifuge and its control unit, the whole test system utilizes conventional liquid chromatographic equipment, including an elution pump, sample injector, UV detector and fraction collector, as illustrated in Fig. 2.

Method

In order to use this apparatus most efficiently, three problems must be solved: selection of the two-phase solvent system, determination of the optimal operational conditions and establishment of operating procedures for successful separation.

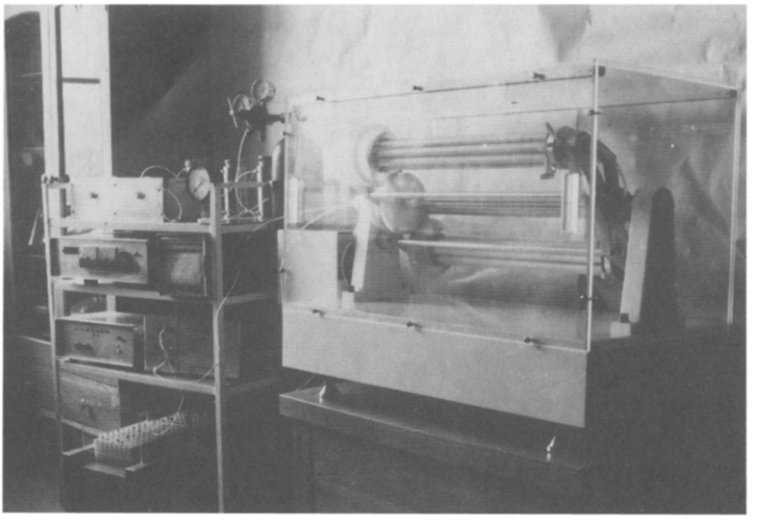

Fig. 2. Overall test system.

In order to select a suitable solvent system, we can consider successful experiences with CCD and droplet countercurrent chromatography (DCCC)[9,10]. In addition, the solvent system can be pre-determined according to the solubility and partition coefficient of the sample components. By means of a simple test-tube experiment[11] or using other chromatographic analysis such as paper, thin-layer (TLC) and high-performance liquid chromatography (HPLC)[12], further refinements can be made. As the revolutional speed can be adjusted and the column can be changed easily, the apparatus is appropriate for commonly used organic–aqueous solvent systems, especially for systems with low to medium interfacial tensions.

Although this apparatus is easy to operate, there are two parameters, the revolutional speed (rpm) and the flow-rate of the mobile phase (ml/h), that must be optimized after the solvent system has been selected. It is convenient to use the phase distribution diagram which describes the steady-state relative volume occupied by the two phases as a function of revolutional speed under given conditions[13]. On the other hand, it is possible to determine a suitable revolutional speed and flow-rate by measuring the linearly increasing relationship between the pumping pressure and elution volume or the elution time during which the flow-rate is constant. When the mobile phase is pumped into the moving column containing the stationary phase, the pressure, P, at the outlet of the pump necessary to maintain a constant flow-rate is

$$P = n(d_h - d_l)g_a hC$$

where n is the number of helical turns in which the distribution of two phases has reached dynamic equilibrium, d_h and d_l are the densities of the heavier and lighter phases, respectively, g_a is the centrifugal acceleration, h is the helix diameter and C is the pressure constant of a given elution system. Except for n, all these factors are constants, so that the pressure P increases in proportion to n. Obviously, to maintain a steady equilibrium between the two phases in the coil, the revolutional speed should

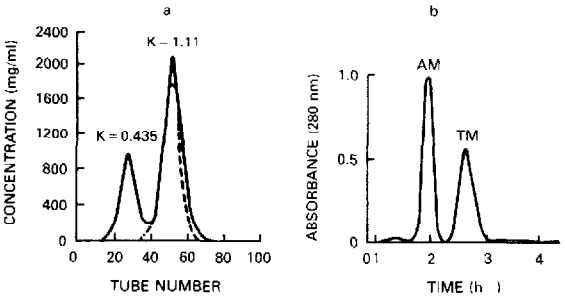

Fig. 3. Separation of terramycin and aureomycin by (a) CCD and (b) CCC. (a) No. of tubes, 100; solvent system, *n*-butanol–0.01 *N* HCl (1:1); mobile phase, aqueous phase. (b) Sample, 0.5 ml containing aureomycin (10 mg/ml) and terramycin (10 mg/ml); revolutional speed, 300 rpm; flow-rate, 60 ml/h; detection, 280 nm.

be limited according to the number of helical turns so that the pumping pressure does not exceed the critical range.

The normal operation procedure for CPC has been reported previously[14]. One phase of the solvent system, *e.g.*, the stationary phase, is pumped into the column until the entire column space is filled and all air has been excluded. With the centrifuge running at the desired speed, the other phase, *e.g.*, the mobile phase, is then pumped through the system. The sample can be dissolved in either phase and introduced at the sample port with an injector. Theoretically, the sample can be injected at the front of the mobile phase, but we often impose a short, suitable delay to ensure perfect separation and symmetrical elution of the component with a large partition coefficient in the mobile phase, which is detected as the first peak near the solvent front.

In the other operating mode, the eluate containing the sample peaks may be selectively recycled through the column to obtain better resolution. In general, a crude sample or a large amount of sample can be separated by the large-scale column and the fraction obtained can then be separated into multiple peaks by the small-scale column.

APPLICATIONS

Antibiotics

Fig. 3a shows the separation of terramycin and aureomycin obtained by

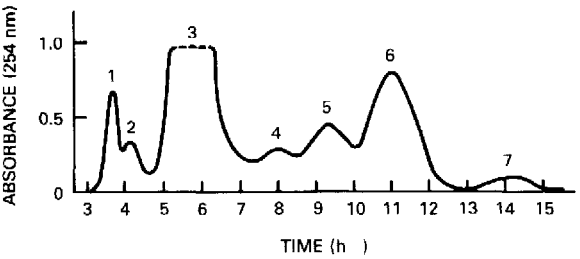

Fig. 4. Countercurrent chromatogram of tetracycline, obtained by the present CPC method. Solvent system, nitromethane–chloroform–pyridine–0.1 *M* ethylenediaminetetraacetic acid (pH 7) (20:10:3:33); mobile phase, lower non-aqueous phase; column, 1.5 mm I.D.; sample, 0.5 ml tetracycline at a concentration of 5 mg/ml; revolutional speed, 260 rpm; flow-rate, 30 ml/h; detection 254 nm.

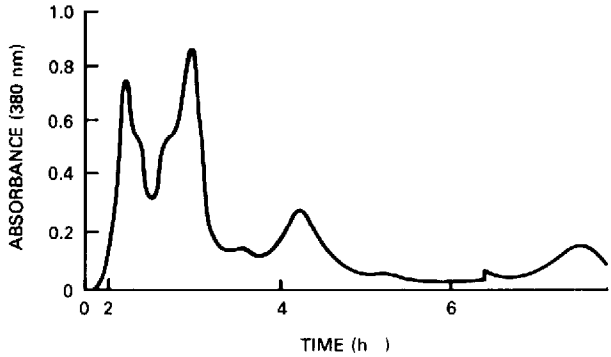

Fig. 5. Countercurrent chromatogram of trichomycin, obtained by the present CPC method. Solvent system, chloroform–methanol–borate buffer (4:4:3); mobile phase, upper aqueous phase; sample, trichomycin, 0.3 ml at 10 mg/ml; revolutional speed, 420 rpm; flow-rate, 60 ml/h; detection, 381 nm.

CCD[15] with the two-phase solvent system *n*-butanol–0.01 *N* hydrochloric acid (1:1). Using the same solvent system, we separated the same mixture using the CPC system. Fig. 3b shows the result of the CCC separation, which was completed in 3 h. With the present method, two peaks were separated more completely and efficiently.

Fig. 4 shows the countercurrent chromatogram of tetracycline obtained by the CPC method. Seven components were collected, of which four, the tetracycline peak (3), its dehydrated peak (1), its epimer peak (6) and its dehydrated epimer peak (5), have been identified previously. However, the other peaks, 2, 4 and 7, are impurities of an unknown nature. The substance in peak 7, which was present in the smallest amount, was particularly difficult to detect and/or separate by means of paper or thin-layer chromatography.

The apparatus is suitable for the separation of polyene antibiotics and we have used it to study the components of trichomycin, globoroseamycin (a Chinese-made polyene antibiotic) and nystatinum.

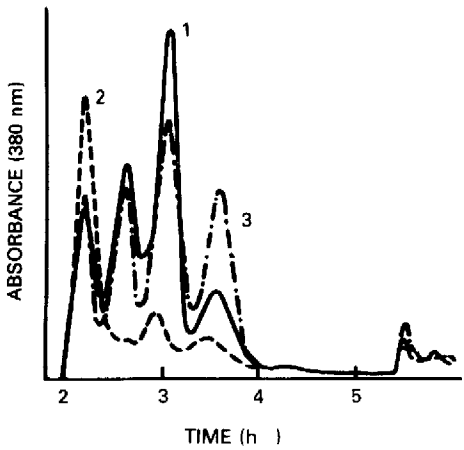

Fig. 6. CCC separation of globoroseamycin fractions from silica gel column. For experimental conditions, see Fig. 5. (1) Mixture of 414a and 414b, 0.5 ml at 10 mg/ml; (2) 414a, 0.4 ml at 1 g/ml; (3) 414b, 0.6 ml at 10 mg/ml.

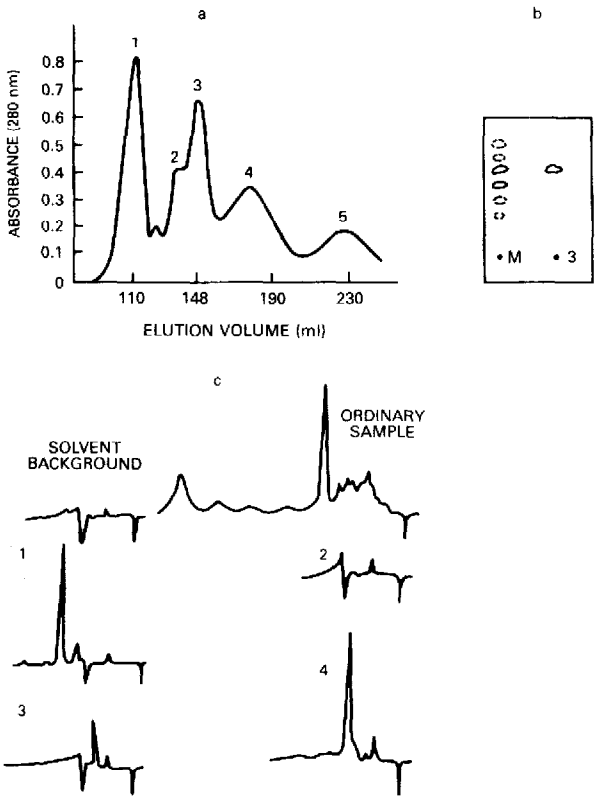

Fig. 7. Chromatograms of nystatin obtained by CCC, TLC and HPLC. (a) CCC separation of nystatin. Solvent system, chloroform–methanol–borate buffer (2:4:3); mobile phase, lower non-aqueous phase; sample, nystatin, 0.6 ml at 10 mg/ml; revolutional speed, 360 rpm; flow-rate, 30 ml/h; detection, 280 nm. (b) TLC analysis of peak 3 fraction obtained by CCC. (c) HPLC (LC-30) analysis of peaks 1–4 obtained by CCC. Solvent system, methanol water (7:3); pressure, 120 kg/cm^2; flow-rate, 1 ml/min; Column, ODS C_{18} (Shimadzu, Kyoto, Japan), 25 cm × 3.4 mm I.D., column efficiency, 40,000 theoretical plates per metre; detectionn, 380 nm.

The CCC separation of trichomycin is shown in Fig. 5. This result is identical with that previously obtained by a different type of CPC[16].

By means of silica gel column liquid chromatography, globoroseamycin could be separated into two fractions, named 414a and 414b. With the present CPC method under the same experimental conditions as applied to the separation of trichomycin above, we achieved more efficient separations of these two fractions. Fig. 6 shows chromatograms of three different samples, *viz.*, (1) the mixture of 414a and 414b, 10 mg/ml, 0.5 ml, (2) 414a, 1 g/ml, 0.4 ml and (3) 414b, 10 mg/ml, 0.6 ml. Each fraction was separated into more than seven components. We extracted hundreds of milligrams of the main effective component, opeak 1, for further analysis and examination.

The result of the separation of nystatin by CCC is shown in Fig. 7a. Components 1, 2, 3 and 4 were collected and tested for their purities by means of TLC and HPLC. Fig. 7b shows the TLC analysis of component 3. Fig. 7c shows the

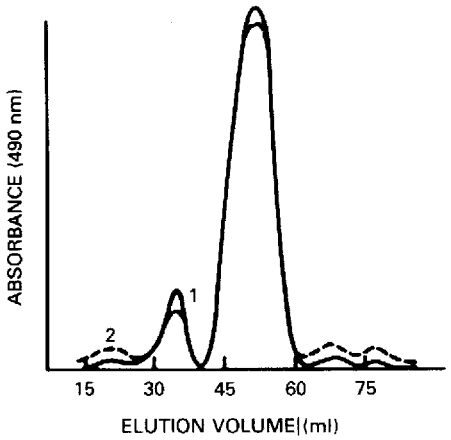

Fig. 8. Countercurrent chromatogram of Chinese-made erythromycin, obtained by the present CPC method. Solvent system, methyl isobutyl ketone–acetone–0.2 *M* phosphate–citrate buffer (pH 6.5) (20:1:21); mobile phase, lower aqueous phase; sample, erythromycin, 889 units/mg, 0.6 ml at 10 mg/ml (1) and 740 unit/mg, 0.6 ml at 10 mg/ml (2); revolutional speed, 360 rpm; flow-rate, 45 ml/h; detection, 490 nm (treated with 27 *N* H_2SO_4).

chromatograms of components 1–4 obtained by HPLC (LC-30). These results clearly indicate that the fractions yielded by the CPC method were pure.

Using the CPC apparatus, we made comparisons between antibiotic samples of the same kind but with different efficiency values. As an example, Fig. 8 shows the chromatogram of Chinese-made erythromycin, which shows two main components, A and C. The lower the efficiency value, the less of the main components is present and the more of the impurities.

Kangdisu is a Chinese-made polypeptide antibiotic but although its curative effect is similar to that of the international standard colistin E, no instrumental

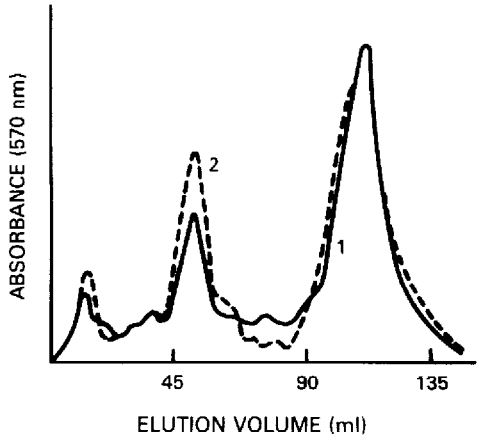

Fig. 9. CCC analysis of kangdisu and colistin E. Solvent system, *n*-butanol–2% dichloroacetic acid (5% NaCl solution) (6:7); mobile phase, lower aqueous phase; sample, (1) kangdisu, 0.6 ml at 10 mg/ml, (2) colistin E, 0.6 ml at 10 mg/ml; revolutional speed, 380 rpm; flow-rate, 30 ml/h; detection, 580 nm.

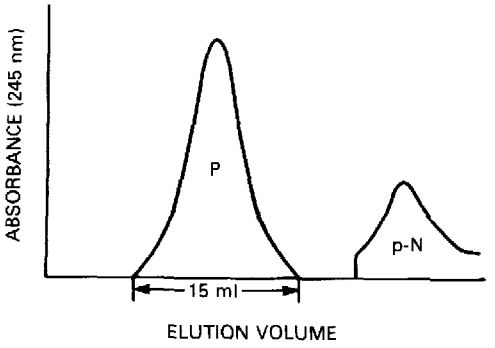

Fig. 10. Countercurrent chromatogram of parathion-ethyl, obtained by the present CPC method. Solvent system, carbon tetrachloride–water (1:1); Column, 2.6 mm I.D., two column units with 400 helical turns; sample, parathion-ethyl (purity 99%), 10 mg + *p*-nitrophenol (purity 99%), 1 mg; revolutional speed, 300 rpm; flow-rate, 45 ml/h; detection, 254 nm.

analysis has been carried. We compared the chromatograms of kangdisu and colistin E obtained by the CPC method and the results are shown in Fig. 9. Using the large-scale column, samples of the order of 100 mg could be separated. Three biologically active components, E_1, E_2 and E_3, were extracted. By further analysing the fractions obtained we found that kangdisu and colistin were identical.

Agricultural chemicals

Parathion-ethyl is a widely used agricultural chemical, which often contains *p*-nitrophenol as an impurity. Fig. 10 shows the CCC separation of the mixture. Parathion-ethyl was collected in 15 ml of mobile phase at a high recovery of over 99%. The impurity, *p*-nitrophenol, remained in the column and could be pumped out after the pure agricultural chemical had been extracted. In this way the pure chemical is efficiently extracted and the purity of the products is easily determined.

Medicinal herbs

Rutinum is a common herb used in Chinese medicine. It can be extracted from

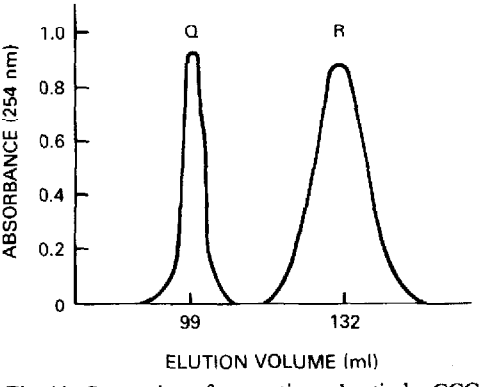

Fig. 11. Separation of quercetin and rutin by CCC. Solvent system, ethanol–0.07 *M* NaOH–ethyl acetate (1:2:2); mobile phase, upper non-aqueous phase; sample, mixture of rutin standard (2 mg/ml) and quercetin standard (6 mg/ml); revolutional speed, 460 rpm; flow-rate, 60 ml/h; detection, 254 nm.

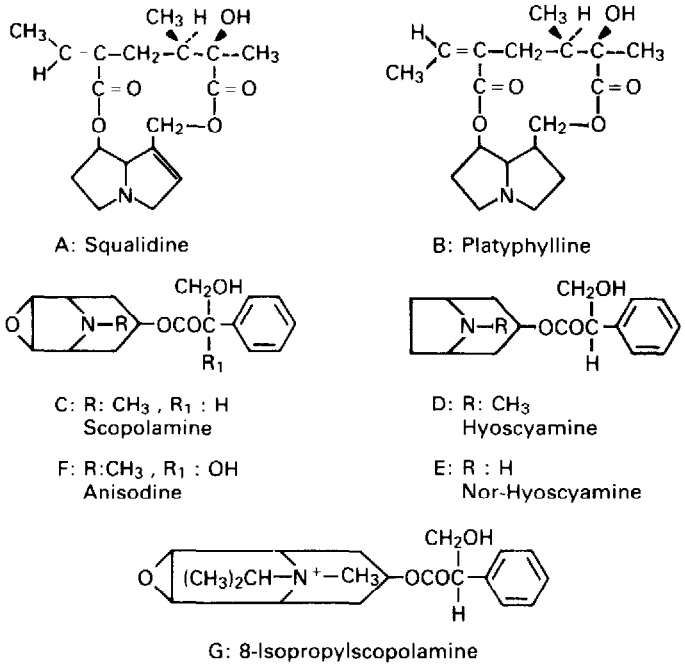

Fig. 12. Structural formulae of seven similar compounds extracted from medicinal herbs.

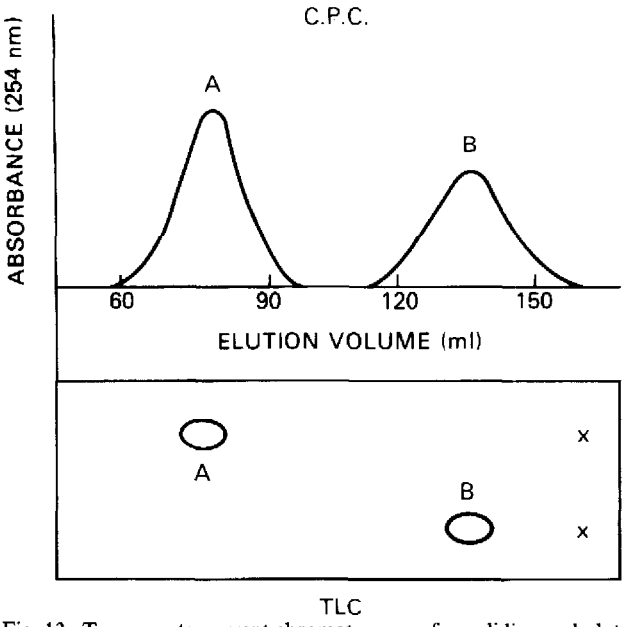

Fig. 13. Top: countercurrent chromatogram of squalidine and platyphylline obtained by the present CPC method. Solvent system, chloroform–0.2 *M* phosphate buffer (pH 6.2) (1:1); mobile phase, lower non-aqueous phase; sample, squalidine, 5 mg, and platyphylline, 5 mg, dissolved in 0.2 ml of chloroform; column, large-scale preparative column with 4 units; revolutional speed, 320 rpm; flow-rate, 60 ml/h, detection; 280 nm. Bottom: TLC analysis of the above fractions obtained with the CPC method.

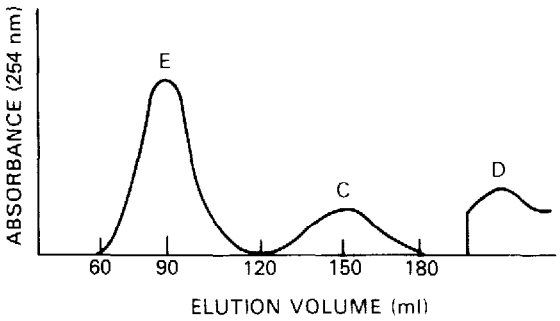

Fig. 14. Countercurrent chromatogram of scopolamine, hyoscyamine and nor-hyoscyamine with the present CPC method. Solvent system, chloroform–0.07 *M* phosphate buffer (pH 6.5) (1:1); mobile phase, lower non-aqueous phase; sample, mixture of the above three compounds, 5 mg each, dissolved in 0.2 ml of chloroform; other experimental conditions as in Fig. 13.

Flos sophorae, but quercetin ($C_{15}H_{10}O_7$) always accompanies with the main component rutin ($C_{27}H_{30}O_{16}$). We successfully separated these two substances, as shown in Fig. 11. The two peaks are completely resolved with excellent reproducibility.

The other application was to extract the principle alkaloids from a crude extract of *Herba scandentis*, which had the appearance of a thick brown oil. Using the large-scale column, we separated 500 mg of this sample in each operation. It is an advantage of this apparatus that there is no need for the crude samples to be cleaned carefully before application.

In order to test the capability of the CPC, we separated a group of similar substances prepared from medicinal herbs, including (A) squalidine, (B) platyphylline, (C) scopolamine, (D) hyoscyamine, (E) nor-hyoscyamine, (F) anisodine and (G) 8-isopropylscopolamine. Their structural formulae are shown in Fig. 12.

Figure 13a shows chromatogram of a mixture of A and B obtained by the CPC method. The two substances were completely separated and each fraction was subsequently analysed by TLC with a satisfactory result, as shown in Fig. 13b.

Fig. 14 shows the separation of a mixture of C, D and E, and Fig. 15 shows the separation of a mixture of C, F and G.

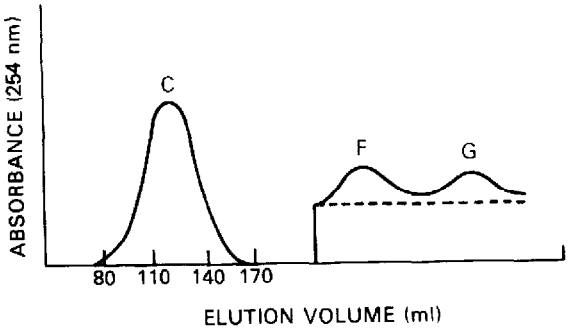

Fig. 15. Countercurrent chromatogram of scopolamine, anisodine and 8-isopropylscopolamine with the present CPC method. Solvent system, chloroform–0.07 *M* phosphate buffer (pH 6.3) (1:1); mobile phase, lower non-aqueous phase; sample, mixture of the above three compounds, 5 mg each, dissolved in 0.2 ml of chloroform; other experimental conditions as in Fig. 13.

CONCLUSION

The overall results clearly demonstrate the versatility of the horizontal flow-through CPC in the separation and purification of natural products. The method is capable of yielding highly pure fractions without adsorptive losses in the solid support matrix and therefore it is a reliable tool for the detection and identification of minor components and impurities present in crude samples. The CPC gives efficient separations on a preparative-scale at relatively high flow-rates so that purification of samples of the order of hundreds of milligrams are usually completed in several hours of elution. We believe that the present method will be widely used in various research laboratories for the separation and purification of natural products and synthetic drugs.

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REFERENCES

1 N. B. Mandava, Y. Ito and W. D. Conway, *Amer. Lab.*, Oct. (1982) 62.
2 N. B. Mandava, Y. Ito and W. D. Conway, *Amer. Lab.*, Nov. (1982) 48.
3 Y. Ito, *J. Biochem. Biophys. Methods*, 5 (1981) 105.
4 Y. Ito and R. L. Bowman, *Anal. Biochem.*, 82 (1977) 63.
5 Y. Ito and R. L. Bowman, *J. Chromatogr.*, 147 (1978) 221.
6 Y. Ito, *J. Chromatogr.*, 188 (1980) 33.
7 Y. Ito, *J. Chromatogr.*, 188 (1980) 43.
8 Y. Ito and G. J. Putterman, *J. Chromatogr.*, 193 (1980) 37.
9 T. Tanimura, J. J. Pisano, Y. Ito and R. L. Bowman, *Science*, 169 (1970) 54.
10 K. Hostettmann, *Planta Med.*, 39 (1980) 1.
11 W. D. Conway and Y. Ito, *J. Liq. Chromatogr.*, 7 (1984) 275.
12 W. D. Conway and Y. Ito, *J. Liq. Chromatogr.*, 7 (1984) 290.
13 Y. Ito and R. L. Bowman, *J. Chromatogr. Sci.*, 11 (1973) 284.
14 I. A. Sutherland and J. E. E. Sharpe, *J. Chromatogr.*, 122 (1976) 333.
15 R. J. Hickey and W. F. Phillips, *Anal. Chem.*, 26 (1954) 1640.
16 J. W. Lightbown, P. Newland, I. A. Sutherland and J. W. A. Dymond, *Proc. Anal. Div. Chem. Soc.* 14 (1977) 34.