ELSEVIER

Journal of Chromatography A, 808 (1998) 95–104

JOURNAL OF CHROMATOGRAPHY A

Toroidal coil counter-current chromatography Achievement of high resolution by optimizing flow-rate, rotation speed, sample volume and tube length

Kazuhiro Matsuda[a], Sachie Matsuda[b], Yoichiro Ito[a,*]

[a]*Laboratory of Biophysical Chemistry, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, MD 20892, USA*

[b]*Pulmonary Critical Care Medicine Branch, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, MD 20892, USA*

Received 16 October 1997; received in revised form 9 February 1998; accepted 9 February 1998

Abstract

This paper deals with optimization of a new seal-free compact toroidal coil centrifuge to achieve high resolution in analytical counter-current chromatography (CCC). Toroidal coil CCC (hydrostatic motion) has advantages compared with high-speed CCC (efficiently mixing solution with planetary motion) in the separation of protein or easily emulsified samples. A toroidal coil separation column of 0.4 mm I.D. PTFE tubing was accommodated around the periphery of the cylindrical centrifuge bowl. Using a two-phase solvent system composed of chloroform–acetic acid–0.1 *M* hydrochloric acid (2:2:1, v/v/v) and a set of dinitrophenyl-amino acids as test samples, a series of experiments was performed with parameters such as the column length, sample volume, flow-rate, elution mode of the mobile phase and rotation speed. The highest efficiency, over 10 000 theoretical plates, was achieved with a 100 m long coiled tube and an 11 ml total capacity at a flow-rate of 0.01 ml/min at 800 rpm.

Keywords: Counter-current chromatography; Optimization; Toroidal coil centrifuge; Amino acids

1. Introduction

Counter-current chromatography (CCC) is a liquid–liquid partition chromatography technique that avoids the use of solid supports. Therefore, it eliminates complications sometimes encountered with other chromatographic systems, such as sample loss and deactivation, tailing of solute peaks and contamination [1,2]. The method was first introduced as helix CCC using a long, narrow-bore helical tube as a separation column that was accommodated around the periphery of the centrifuge bowl [1]. In this device the solvent was introduced through a rotating syringe and the effluent was collected through a rotating seal. Using an 0.2 mm I.D. helical column with a toroidal coil arrangement, partition efficiencies of several thousand theoretical plates were achieved. Later, this centrifuge system was improved by eliminating the use of rotary seal which often became the cause of leakage, clogging and contamination [3]. And a seal-free flow-through centrifuge device was applied to CCC with a toroidal coil separation column [4,5].

Further improvement of the toroidal coil cen-

*Corresponding author.

0021-9673/98/$19.00
PII: S0021-9673(98)00114-9

trifuge in our laboratory has lagged behind because of intensive efforts focused on the development of high-speed CCC which gives efficient preparative separations for many substances in a short elution time [6–8]. However, the toroidal coil centrifuge system has an important advantage over the high-speed CCC system in that the method provides stable retention of the stationary phase for analytical-scale separations. This is especially important in the micro-scale separations of biologically active compounds such as proteins, polysaccharides, phospholipids and glycolipids, since these compounds tend to produce emulsification in the high-speed CCC system, often resulting in a detrimental loss of the stationary phase. Toroidal coil CCC is based on the hydrostatic equilibrium system in which the separation of the two phases is enhanced by increasing the centrifugal force field while the extent of two-phase mixing is governed by the flow-rate of the mobile phase. Therefore, regardless of the interfacial tension of the two phase solvents, one can attain a satisfactory level of the retention of the stationary phase by adjusting the rotation speed and the flow-rate of the mobile phase. For example, even a particular type of the solvent system containing a surfactant can be retained by applying a suitable combination of a high rotation speed and a slow flow-rate.

In the present work we evaluate the performance of a newly designed compact model of the toroidal coil centrifuge for establishing an efficient analytical CCC system. A series of experiments was conducted by changing various parameters such as flow-rate, rotation speed, column length, sample volume and elution mode of the mobile phase using a solvent system composed of chloroform–acetic acid–0.1 *M* HCl (2:2:1, v/v/v) and a set of dinitrophenyl (DNP)-amino acids as test samples in a preliminary experiment to compare its resolution with earlier data on the high-speed CCC.

2. Experimental

2.1. Apparatus

The present studies employ a commercial model of the toroidal coil centrifuge purchased from Pharma-Tech Research Corp., Baltimore, MD, USA. The apparatus is a compact table top unit measuring 30 cm×30 cm×40 cm in dimensions. It is equipped with a flow-through device without the use of rotary seals according to the principle described earlier [3]. The rotation speed is continuously adjustable up to 3000 rpm with a speed regulator equipped with a digital display. The toroidal coil separation column was prepared by winding a 15–100 m×0.4 mm I.D. PTFE (polytetrafluoroethylene) tubing (Zeus Industrial Products, Raritan, NJ, USA) onto a nylon pipe of 1.5 mm O.D. making right-handed coil. Then the coiled tube was affixed against the inner wall of the cylindrical centrifuge bowl (12 cm in diameter and 5 cm in height) forming doughnut-shaped configuration (toroidal coil). A long toroidal coil consisted of two to three coiled layers. The standard toroidal coil measures about 6 m in length (made from 60 m long PTFE tubing) consisting of 12 000 helical turns with a total capacity of about 8 ml. The longest toroidal coil (10 m in length made up from 100 m long PTFE tubing) consisted of three layers of the coil with a total capacity of approximately 11 ml.

The inlet and outlet flow lines were made from 0.35 mm I.D., thick wall PTFE tubing to withstand its constant flexing motion. A chromatographic metering pump (Model series 200 lc pump, Perkin-Elmer, Norwalk, CT, USA) was used to pump the mobile phase, and a fraction collector (Ultrorac, LKB Instruments, Stockholm, Sweden) was used to collect the eluate into test tubes.

2.2. Reagents

Chloroform and methanol both of glass-distilled chromatographic grade, were purchased from Burdick-Jackson Labs. (Muskegon, MI, USA) and a reagent grade of glacial acetic acid and hydrochloric acid from Fisher Scientific (Fairlawn, NJ, USA). Various *N*-2,4-dinitrophenyl (DNP)-amino acids including DNP-L-leucine (DNP-Leu), DNP-L-valine (DNP-Val), DNP-L-alanine (DNP-Ala), di-DNP-L-cystine [di-DNP-L-$(Cys)_2$], DNP-L-glutamic acid (DNP-Glu), DNP-L-aspartic acid (DNP-Asp) and

DNP-L-ornithine (DNP-Orn) were all obtained from Sigma (St. Louis, MO, USA).

2.3. Preparation of two-phase solvent system and sample solution

A solvent system composed of chloroform–acetic acid–0.1 *M* hydrochloric acid (2:2:1, v/v/v) was thoroughly equilibrated in a separatory funnel at room temperature. The sample solution was prepared by dissolving a set of seven DNP-amino acids, DNP-Leu, DNP-Val, DNP-Ala, diDNP-$(Cys)_2$, DNP-Glu, DNP-Asp and DNP-Orn each 100 mg except for diDNP-$(Cys)_2$ (50 mg) in 10 ml of the upper phase and 0.05 ml (3.25 mg) was applied to the column in each run unless otherwise indicated.

2.4. CCC procedure

In each separation the toroidal coil was first entirely filled with the stationary phase (either the upper or the lower phase) followed by injection of the sample solution. Then the mobile phase was pumped into the column while the column was rotated at a desired rate. The effluent from the outlet of the column was collected in test tubes at a rate of 0.1 ml/tube.

After the desired peaks eluted, the centrifuge run was terminated and the column contents were fractionated into test tubes at 0.2 ml/tube by eluting the column with the solvent initially used as the stationary phase.

2.5. Analysis of CCC fractions

To each fraction 2 ml of methanol was added and the absorbance measured at 430 nm using a spectrophotometer (Model PM6, Zeiss, Hanover, MD, USA). The elution curve was manually drawn by plotting the absorbance values against the fraction number. From the chromatogram, the partition efficiency of each separation was measured and expressed in terms of theoretical plate number (TP) and peak resolution (R_s) using the conventional equations as follows:

$$TP = (4R/W)^2 \quad (1)$$

$$R_s = 2(R_1 - R_2)/(W_1 + W_2) \quad (2)$$

where R indicates the retention time (volume) and W the peak width.

3. Results and discussion

The performance of the new toroidal coil centrifuge was evaluated by the separation of a set of DNP-amino acids on a two-phase solvent system composed of chloroform–acetic acid–water (2:2:1, v/v/v). The use of DNP-amino acids as the standard samples with the above solvent system provides a broad range of partition coefficient values and their bright yellow color facilitates observation of the separation patterns during centrifugation under stroboscopic illumination as well as in the collected fractions. These samples have been intensively used for assessing the performance of CCC instruments in the past by us, and the results can be readily compared with earlier data.

A series of experiments was performed to study the effects of various parameters such as flow-rate, rotation speed, column length, sample volume and elution mode of the mobile phase.

3.1. Effects of flow-rates

The first series of studies demonstrated the effects of the flow-rate on the partition efficiency. Using the standard toroidal coil with a 8 ml capacity, the flow-rate was changed from 0.5 to 0.01 ml/min under a fixed rotation speed of 1000 rpm and a sample volume of 0.05 ml (Fig. 1). The lower phase was used as the mobile phase and eluted through the column in such a way that the flow direction is opposite to that of the rotation (parallel direction to the Coriolis force) (see Section 3.3). In Table 1, the resolution is indicated in terms of TP and peak resolution (R_s). As the flow-rate is decreased, both the TP and R_s were improved, and at a flow-rate of 0.01/min, the partition efficiency reached 5800 TP and peak resolution between DNP-Ala and DNP-$(Cys)_2$ and between DNP-$(Cys)_2$ and DNP-Glu were 4.0 and 5.8, respectively, while it required 16 h.

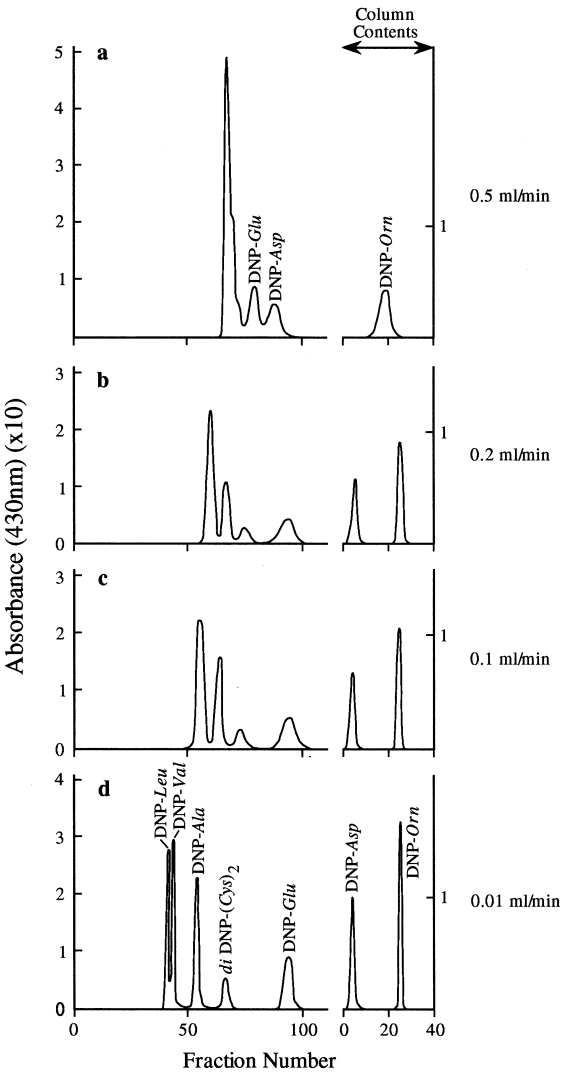

Fig. 1. Effects of flow-rates. The flow-rate was changed as 0.5 (a), 0.2 (b), 0.1 (c) and 0.01 ml/min (d) under a fixed rotation speed at 1000 rpm and a sample volume of 0.05 ml. The lower phase was injected as the mobile phase in the ''reversed mode'' (Coriolis parallel). After the separation was completed, the centrifuge was stopped and the column contents were eluted by pumping the upper phase at 1 ml/min and collected at 0.2 ml/tube. The column length was 60 m.

At a low flow-rate the solvent front emerged earlier because of improved retention of the stationary phase whereas at a high flow-rate of 0.5 ml/min the solvent front appeared much later indicating poor retention of the stationary phase. The 15 m tubes showed same tendency (Fig. 5B).

3.2. Effects of rotation speed

The effects of the rotation speed on the partition efficiency were investigated using the standard toroidal coil. Fig. 2 shows the results of changing the rotation speed and flow-rate. At a low rotation speed or a high flow-rate, sufficient retention could not be obtained. The results indicate the minimum requirement of revolution speed for each flow-rate in the present toroidal coil, i.e., 600 rpm for 0.1 ml/min, 800 rpm for 0.2 ml/min and 1000 rpm for 0.5 ml/min.

At 600 rpm a flow of 0.01 ml/min produced sufficient retention and excellent peak resolution almost equivalent to those obtained from 800 rpm at a flow-rate of 0.01 ml/min (data not shown).

3.3. Effects of Coriolis force

Previously, we reported that the direction of the mobile phase flow through the toroidal coil affected the resolution of protein peaks in the aqueous–aqueous polymer phase system composed of polyethylene glycol 1000 and dibasic potassium phosphate [9]. It was speculated that this effect may be caused by the Coriolis force acting on the flowing mobile phase in the rotating column. In the present study, this Coriolis effect was examined by introducing the mobile phase into the toroidal coil in two opposite directions under otherwise identical experimental conditions. If the flow is introduced along the direction of revolution, it is called ''normal mode'', and if the flow is against the direction of revolution, it is called ''reversed mode''. In the previous studies, when the lower phase was mobile, elution in the normal mode is defined ''Coriolis crossing'' and the elution against the revolution (reversed mode), ''Coriolis parallel''. This relationship is reversed if the mobile phase is the upper phase, i.e., the elution along the rotation (normal mode) is called ''Coriolis parallel'' and the elution against the rotation (reversed mode), ''Coriolis crossing''. The relationship between the elution mode and Coriolis force is summarized in Table 2. From the results of the previous studies with a polymer phase

Table 1
Effects of flow-rate on partition efficiency of DNP-amino acid separation. Resolution indicated in terms of theoretical plate number (TP) and resolution (R_s)

Flow-rate (ml/min)	TP			R_s	
	DNP-Ala	Di-DNP-$(Cys)_2$	DNP-Glu	DNP-Ala Di-DNP-$(Cys)_2$	Di-DNP-$(Cys)_2$ DNP-Glu
0.01	5800	5170	3930	4.0	5.7
0.1	3240	1557	1310	1.5	2.5
0.2	1718	1406	1168	1.2	2.0
0.5	–	–	1764	–	–

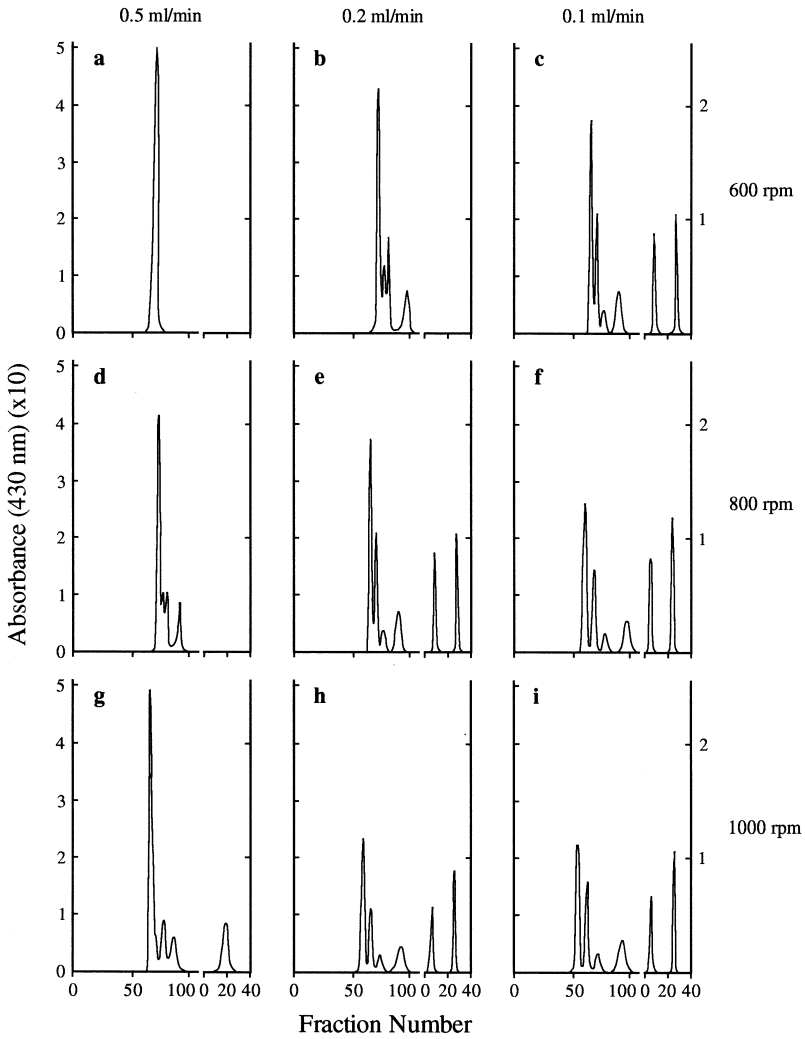

Fig. 2. Effects of rotation speed and flow-rate. Both flow-rate and rotation speed were changed under a fixed sample volume of 0.05 ml. The lower phase was injected as the mobile phase in the ''reversed mode'' (Coriolis parallel). The column contents were eluted with the upper phase at 1 ml/min and collected at 0.2 ml/tube. Flow-rate: 0.5 ml/min (a, d, g), 0.2 ml/min (b, e, h), and 0.1 ml/min (c, f, i). Rotation speed: 600 rpm (a, b, c), 800 rpm (d, e, f) and 1000 rpm (g, h, i). The column length was 60 m.

Table 2
Relationship between the elution mode and Coriolis force

	Normal mode	Reversed mode
Upper phase mobile	Parallel	Crossing
Lower phase mobile	Crossing	Parallel

In this study we used a right-handed coil and the direction of rotation was counter-clockwise.

system [9,10], it is assumed that if the Coriolis force acts on the mobile phase flow at an angle (Coriolis crossing), the mobile phase can form a continuous stream along the tube wall resulting in better retention of the stationary phase but less efficient separation due to the lack of droplet formation.

The effect of the Coriolis force was observed in retention of the lower phase at the low revolution speed (Fig. 3). At the revolution speeds of 1000 rpm, 800 rpm and 600 rpm, both the retention of the stationary phase and the peak resolution were found to be much better in the Coriolis crossing mode at a flow-rate of 0.1 ml/min. However, at higher revolution speeds or lower flow-rates in which sufficient retention was observed, there was no significant difference between the chromatograms obtained from these two elution modes.

3.4. Effects of sample volume

To study the effects of the sample volume on the partition efficiency, 2.5 mg of the DNP-amino acid samples was dissolved in various volumes of the upper phase, 0.01 ml, 0.05, 0.1, 0.2 and 0.5 ml. At the revolution speed of 1000 rpm, each sample solution was loaded into the column (Fig. 4). As the sample volume is increased, the resolution became worse however, this effect was much less produced than the result brought about by a change in flow-rates.

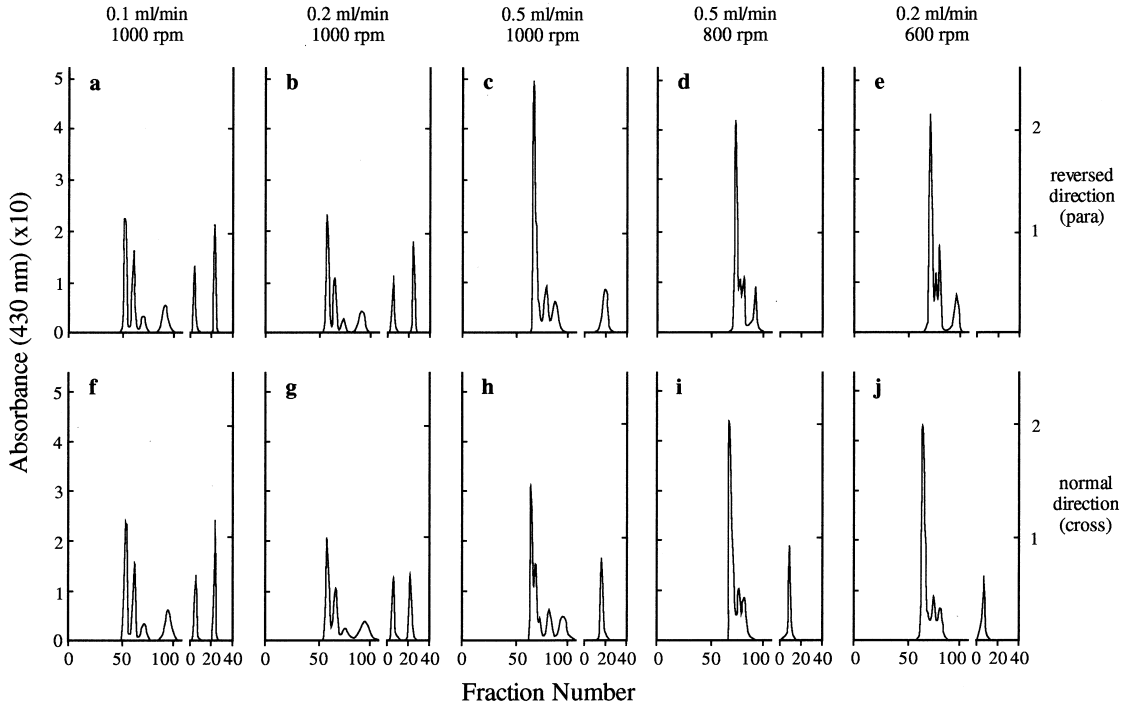

Fig. 3. Effects of Coriolis force. The lower mobile phase was eluted in the ''reversed mode '' (Coriolis parallel) (a, b, c, d and e) or ''normal mode'' (Coriolis crossing) (f, g, h, i and j) under various rotation speeds and flow-rates (as indicated in the Figure). The column length was 60 m.

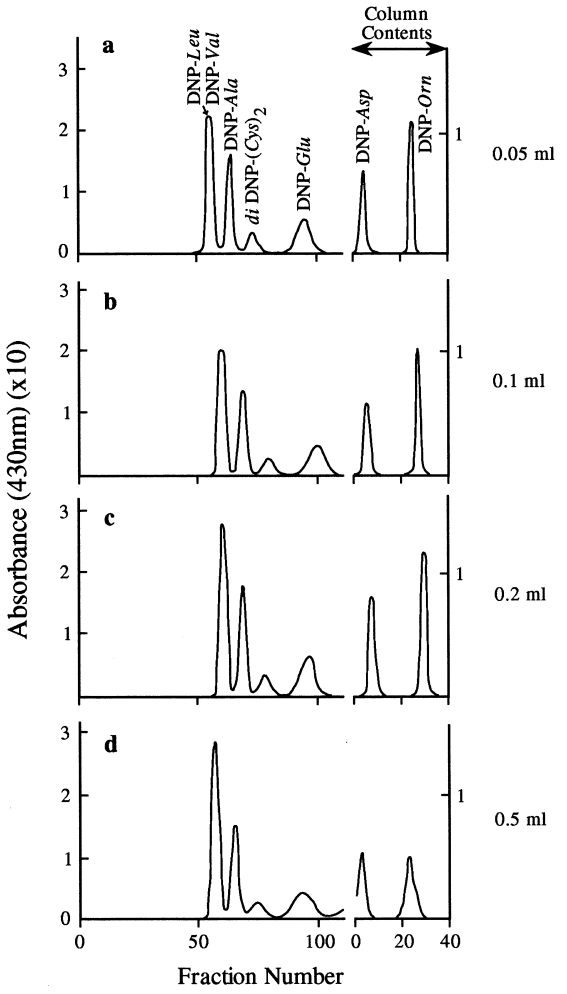

Fig. 4. Effects of sample volume. Sample volume was 0.05 (a), 0.1 (b), 0.2 (c) and 0.5 ml (d). The lower phase was eluted in the ''reversed mode'' (Coriolis parallel). The column contents was eluted with the upper phase at 1 ml/min and collected at 0.2 ml/tube. The column length was 60 m.

3.5. Effects of rotation speed

The results presented in Fig. 2 suggests that further increasing the rotation speed might improve the separation. However, the application of a high rotation speed such as 1200 rpm to the standard toroidal column would increase the column pressure and ruptures the connections over 500 p.s.i. (1 p.s.i. = 6894.76 Pa). Therefore, we made a shorter column of 15 m in length to observe the effect of higher rotation speeds.

Fig. 5A shows the effects of rotation speed at a flow-rate of 0.05 ml/min and the sample volume of 0.05 ml observed in the short column. These results indicate no significant effect observed by a higher revolution speed. Fig. 5B shows the effect of flow-rate in the short column. The results are consistent with those observed with the standard column (60 ml capacity) indicating that an increase in the tube length will proportionally increase the partition efficiency.

3.6. Effect of tube length

In order to examine the effect of column length, we prepared a long toroidal coil measuring 100 m in length (5/3 times that of the standard column). At the rotation speed of 800 rpm the lower phase was eluted through the column in the Coriolis parallel mode. Fig. 6 shows four chromatograms all obtained at a flow-rate of 0.01 ml/min and at 800 rpm. The two chromatograms on the left were obtained from the standard column (60 m in length) and those on the right from the long column (100 m in length) at 800 rpm. Comparison between the chromatograms obtained with the two different column capacity reveals that increasing the length of the column improves the sharpness of the peaks and nearly proportionally increases the partition efficiency as indicated in Tables 3 and 4. The chromatograms obtained with the 100-ml capacity column exhibit partition efficiencies exceeding 10 000 theoretical plates.

4. Conclusion

A new compact model of the toroidal coil centrifuge enables an efficient analytical separation achieving over 10 000 theoretical plates. The retention of the stationary phase can be improved simply by increasing the revolution speed and/or decreasing the flow-rate of the mobile phase. The overall results of the present studies indicate that the partition efficiency will be further improved by the use of a longer and/or narrower toroidal coil.

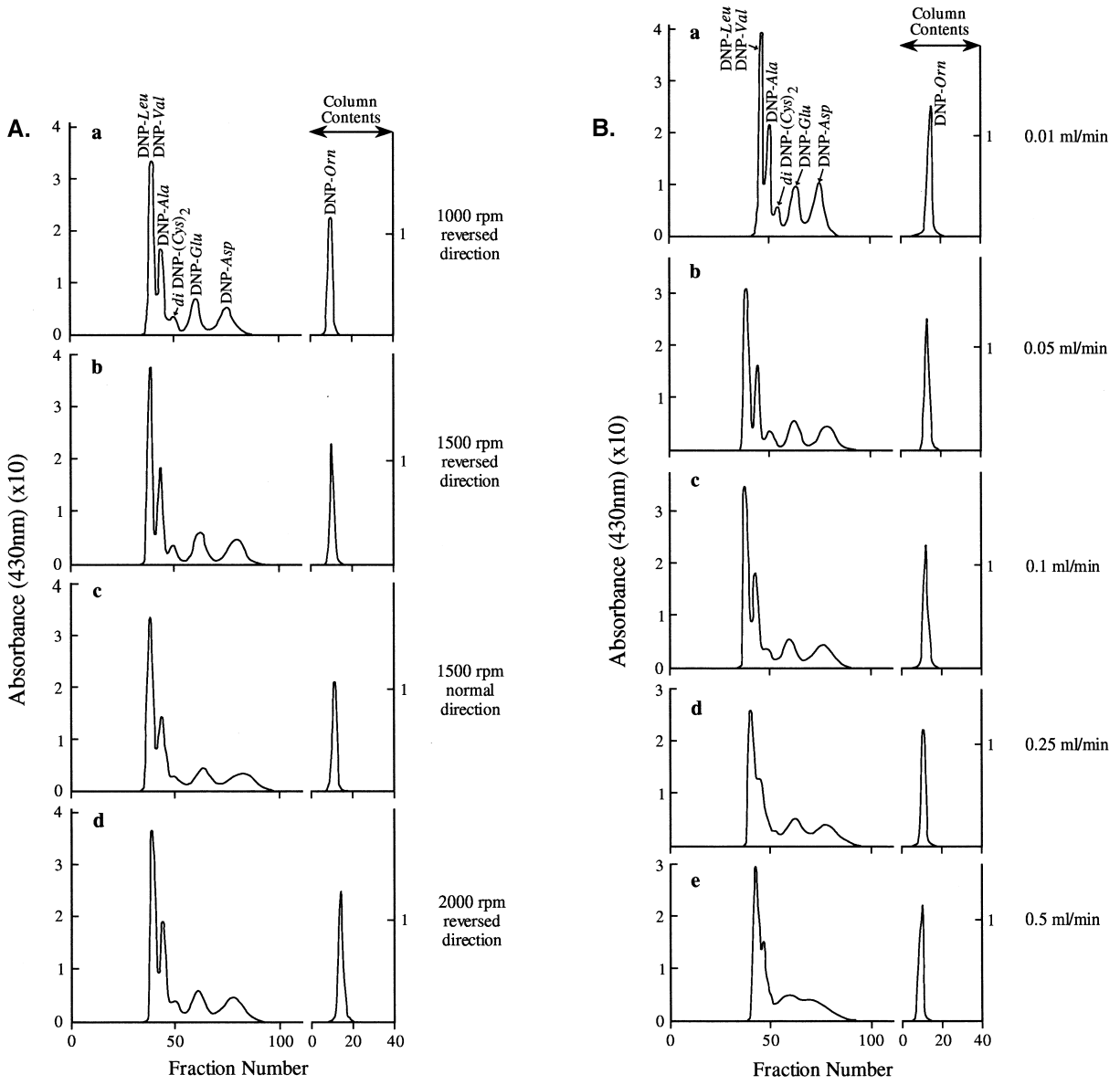

Fig. 5. (A) Effects of rotation speed and Coriolis force in a short column (15 m). The rotation speed was varied as indicated in the Figure. The lower phase was eluted in the ''reversed mode'' (Coriolis parallel) (a, b, d) or ''normal mode'' (Coriolis crossing) (c). The column contents were eluted with the upper phase at 1 ml/min and collected at 0.2 ml/tube. (B) Effects of flow-rate in a short column (15 m). Flow-rates were 0.01 (a), 0.05 (b), 0.1 (c), 0.25 (d) and 0.5 ml/min (e) at 2000 rpm with a sample volume of 0.5 ml. The lower phase was eluted in the ''reversed mode'' (Coriolis parallel). The column contents were eluted with the upper phase at 1 ml/min and collected at 0.2 ml/tube.

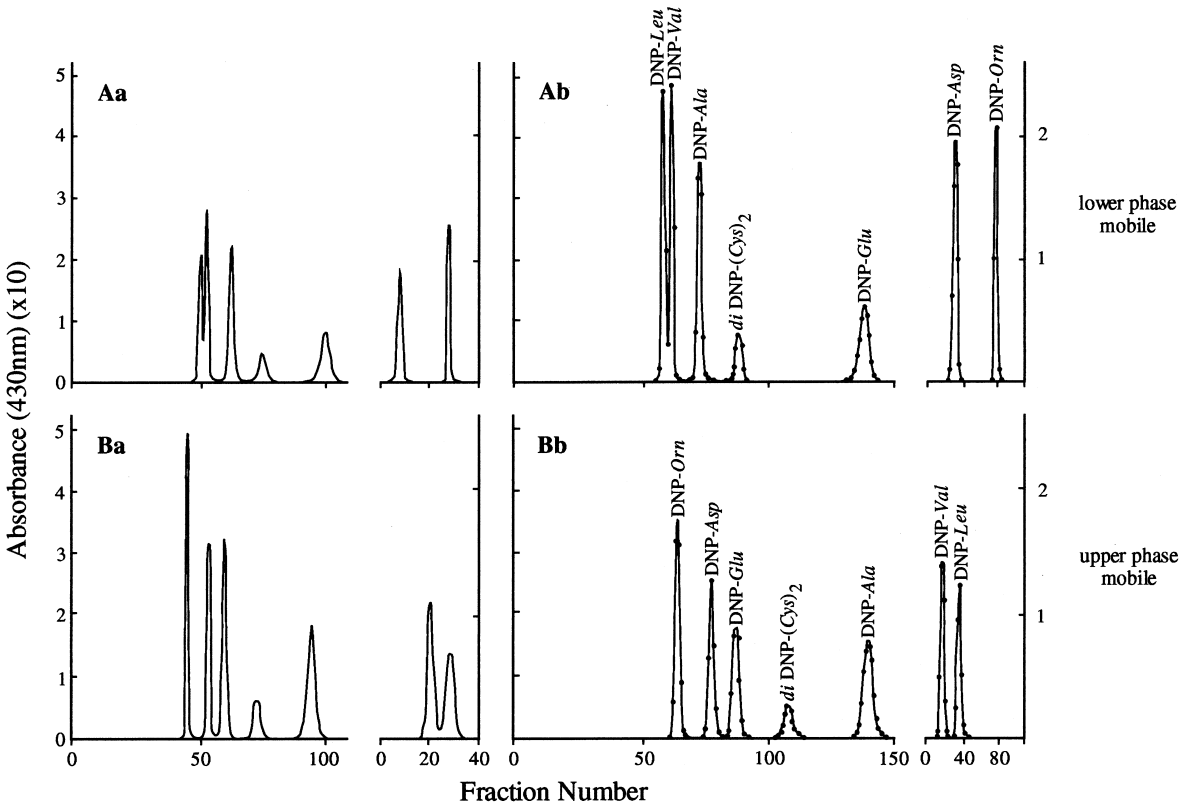

Fig. 6. Effects of column length. A sample volume of 0.05 ml was eluted at 0.01 ml/min at 800 rpm using the toroidal coils of 60 m (A) and 120 m (B). DNP-Amino acids were separated with lower phase (A a, B b) or upper phase (A b, B b) mobile. The column contents were eluted with the upper phase at 0.1 ml/min and collected at 0.2 ml/tube.

Table 3
Effects of column length on partition coefficient. Lower mobile phase (reversed direction)

Column length (m)	TP			R_s	
	DNP-Ala	Di-DNP-$(Cys)_2$	DNP-Glu	DNP-Ala / Di-DNP-$(Cys)_2$	Di-DNP-$(Cys)_2$ / DNP-Glu
60	5800	5170	3930	4.0	5.7
100	18 000	14 000	8460	5.0	9.8

Table 4
Effects of column length on partition coefficient. Upper mobile phase (reversed direction)

Column length (m)	TP					R_s			
	DNP-Orn	DNP-Asp	DNP-Glu	Di-DNP$(Cys)_2$	DNP-Ala	DNP-Orn / DNP-Asp	DNP-Asp / DNP-Glu	DNP-Glu / Di-DNP-$(Cys)_2$	Di-DNP-$(Cys)_2$ / DNP-Ala
60	10 000	7464	7345	5329	4011	4.2	2.3	3.8	4.4
100	10 650	10 816	10 000	7464	8649	4.9	2.9	4.8	5.7

We believe that the present system will be extremely useful for the analytical separation of various biologically active compounds which tend to produce emulsification in the high-speed CCC system.

Acknowledgements

The authors are indebted to Dr. Henry M. Fales for editing the manuscript.

References

[1] Y. Ito, R.L. Bowman, Science 167 (1970) 281–283.
[2] Y. Ito, in N.B. Mandava and Y. Ito (Editors), Countercurrent Chromatography – Theory and Practice, Marcel Dekker, New York, 1988, pp. 79–442.
[3] Y. Ito, J. Suaudeau, R.L. Bowman, Science 189 (1975) 999–1000.
[4] Y. Ito, R.L. Bowman, Anal. Biochem. 85 (1978) 614–617.
[5] S.D. Flanagan, G. Johansson, B. Yost, Y. Ito, I.A. Sutherland, J. Liq. Chromatogr. 7 (1984) 385–402.
[6] Y. Ito, J. Sandlin, W.G. Bowers, J. Chromatogr. 244 (1982) 247–258.
[7] Y. Ito, CRC Crit. Rev. Anal. Chem. 17 (1986) 65–143.
[8] Y. Ito, in Y. Ito and W.D. Conway (Editors), High-Speed Countercurrent Chromatography, Wiley-Interscience, New York, 1996, pp. 3–43.
[9] Y. Ito, Y. Ma, J. Liq. Chromatogr. 21 (1998) 1–17.
[10] Y. Ito, K. Matsuda, Y. Ma, L. Qi, J. Chromatogr. A 802 (1998) 277–283.