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Novel design for centrifugal counter-current chromatography: VI. Ellipsoid column

Dongyu Gu^{1,3}, Yi Yang^{1,2}, Xuelei Xin², Haji Akber Aisa², and Yoichiro Ito^{1,*}

¹Bioseparation Technology Laboratory, Biochemistry and Biophysics Center, National Heart, Lung, and Blood Institute, National Institutes of Health, 10 Center Drive, Building 10, Room 8N230, Bethesda, MD 20892, USA

²Key Laboratory of Xinjiang Indigenous Medicinal Plants Resource Utilization, Xinjiang Technical Institute of Physics and Chemistry, Chinese Academy of Sciences, Urumqi 830011, China

³School of Marine Science and Environment Engineering, Dalian Ocean University, Dalian 116023, China

Abstract

A novel ellipsoid column was designed for centrifugal counter-current chromatography. Performance of the ellipsoid column with a capacity of 3.4 mL was examined with three different solvent systems composed of 1-butanol-acetic acid-water (4:1:5, v/v) (BAW), hexane-ethyl acetate-methanol-0.1 M HCl (1:1:1:1, v/v) (HEMH), and 12.5% (w/w) PEG1000 and 12.5% (w/w) dibasic potassium phosphate in water (PEG-DPP) each with suitable test samples. In dipeptide separation with BAW system, both stationary phase retention (Sf) and peak resolution (Rs) of the ellipsoid column were much higher at 0° column angle (column axis parallel to the centrifugal force) than at 90° column angle (column axis perpendicular to the centrifugal force), where elution with the lower phase at a low flow rate produced the best separation yielding Rs at 2.02 with 27.8% Sf at a flow rate of 0.07 ml/min. In the DNP-amino acid separation with HEMW system, the best results were obtained at a flow rate of 0.05 ml/min with 31.6% Sf yielding high Rs values at 2.16 between DNP-DL-glu and DNP-β-ala peaks and 1.81 between DNP-β-ala and DNP-L-ala peaks. In protein separation with PEG-DPP system, lysozyme and myoglobin were resolved at Rs of 1.08 at a flow rate of 0.03 ml/min with 38.9% Sf. Most of those Rs values exceed those obtained from the figure-8 column under similar experimental conditions previously reported.

Keywords

ellipsoid column; hydrostatic counter-current chromatographic system; centrifugal counter-current chromatography; retention of the stationary phase; peak resolution

*Corresponding author: Yoichiro Ito, Bioseparation Technology Laboratory, Biochemistry and Biophysics Center, National Heart, Lung, and Blood Institute, National Institutes of Health, 10 Center Drive, Bldg.10, Room 8N230, Bethesda, MD 20892-1762, USA, Tel.301-496-1210; FAX:301-402-0013; itoy2@mail.nih.gov.

INTRODUCTION

High-speed countercurrent chromatography (HSCCC) has been widely used for the separation and purification of natural and synthetic products^[1–4]. This chromatographic technology is particularly suitable for the preparative-scale separation of natural products.^[5–8] However, this hydrodynamic counter-current chromatography (CCC) system can not be efficiently applied to analytical separations due to a strong cohesive force between liquid and the tube wall in small diameter tubing which results in a serious loss of stationary phase from the column. This problem can be solved by the hydrostatic CCC system, such as centrifugal CCC, which provides a stable centrifugal force field to facilitate counter-current movement of the two phases through a narrow-bore coiled column. Consequently, this hydrostatic CCC system can produce highly efficient analytical separations as reported earlier^[9–11].

In our previous studies, a series of novel column designs has been introduced to further improve the performance of centrifugal counter-current chromatography, including triangular coil^[12], zigzag^[13, 14], saw tooth^[15] and figure-8 columns^[16]. Recently, we found that the highest resolution (R_s) was obtained when the figure-8 column was mounted parallel to the radially acting centrifugal force field. The performance of the system was examined in various test samples including amino acids, dipeptides and proteins using moderately hydrophobic solvent system, high polarity solvent system and aqueous two-phase system^[17, 18]. This high partition efficiency of this system may be explained by hydrodynamic motion and interaction of the two phases in the column at various angles. When the column angle is set at 0° against the radially acting centrifugal force field, the second loop of figure-8 provides two separate partition segments for droplet flow to improve R_s as shown in Fig. 1A^[17]. Based on this finding, the ellipsoid column is designed in the present study, which can provide a much longer partition segment than that in the figure-8 column (Fig. 1B). The performance of this novel centrifugal CCC system was examined by the separations of dipeptides, DNP-amino acids and proteins each with a suitable two-phase solvent system using a rotary-seal-free continuous-flow centrifuge system.

EXPERIMENTAL

Apparatus

The present study uses a rotary-seal-free centrifuge fabricated by Pharma-Tech Research Corporation, Baltimore, Maryland, USA. It holds an aluminum rotary plate measuring about 34 cm in diameter to support an ellipsoid separation column. Each column unit is made by hooking a 0.46 mm ID FEP (Fluorinated ethylene propylene) (Zeus Industrial Products, Orangeburg, SC, USA) tubing onto a pair of screws upstanding from the rotary plate making plural layers of ellipsoid loops (Fig. 2). Multiple column units are serially connected with transfer tubing to form an ellipsoid separation column. The total capacity is 3.4 mL. Each terminal of the column is connected to a PTFE flow tube (0.46 mm I.D., Zues Industrial Products) with a set of tubing connectors (Upchurch Scientific, Palm Spring, CA, USA) as shown in Fig. 2. A pair of flow tubes is put together and passed through the center of the central shaft downward and the hollow horizontal shaft of a miter gear, then led upward into

the vertical hollow tube support, and finally exits the centrifuge from the center of the upper plate where they are tightly held with a pair of clamps^[19].

A metering pump (Shimadzu LC-10ADVP, Columbia, MD, USA) was used for pumping the solvents. In order to improve the tracing of protein separation, hollow fiber membranes (20 cm, Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA) inserted between the outlet of the column and the inlet of the detector were eluted with water at a flow rate of 0.01 ml/min. The effluent was continuously monitored with a UV detector (Model Uvicord SII, LKB Instruments, Stockholm, Sweden) and the elution curve was traced using a strip-chart recorder (Pharmacia, Stockholm, Sweden).

Reagents

1-Butanol, hexane, ethyl acetate and methanol were purchased from Fisher Scientific, Fair Lawn, NJ, USA and other solvents such as acetic acid and hydrochloric acid, from Mallinckrodt Chemicals, Phillipsburg, NJ, USA. PEG (polyethylene glycol) 1000, dibasic potassium phosphate, lysozyme (chicken egg), myoglobin (horse skeletal muscle), tryptophyl-tyrosine (Trp-Tyr), valyl-tyrosine (Val-Tyr), N-2, 4-dinitrophenyl-L-alanine (DNP-ala), N-2, 4-dinitrophenyl-β-alanine (DNP-β-ala), N-2, 4-dinitrophenyl-D, L-glutamic acid (DNP-glu) were all obtained from Sigma Chemicals, St. Louis, MO, USA.

Partition coefficient measurement^[20]

The partition coefficients (K_U) of each sample in the two-phase solvent system were determined using the conventional test tube method with a UV spectrophotometer (Genesis 10 UV, Thermo Spectronic, Rochester, NY, USA) at 280 nm. The absorbance of the upper phase was recorded as A_U and that of the lower phase as A_L . Then the K_U value was calculated according to the following equation: $K_U = A_U/A_L$.

Two-phase solvent systems and sample solutions

In the present study, three typical two-phase solvent systems each with different polarity including hexane-ethyl acetate-methanol-0.1 M HCl (1:1:1:1, v/v) (HEMH), 1-butanol-acetic acid-water (4:1:5, v/v) (BAW), and 12.5% (w/w) PEG1000 and 12.5% (w/w) dibasic potassium phosphate in water (PEG-DPP) were used to separate a set of test samples, DNP-amino acids, dipeptides and proteins, respectively. Each solvent mixture was thoroughly equilibrated in a separatory funnel by vigorous shaking and degassing several times, and the two phases separated shortly before use.

The sample solution for HEMH was prepared by dissolving 5.7 mg of DNP-ala, 7.1 mg of DNP-β-ala and 5.4 mg of DNP-glu in 10 ml of the upper phase of HEMH, and 50 μl was charged in each run. The sample solution for BAW was prepared by dissolving 25 mg of Trp-Tyr and 100 mg of Val-Tyr in 20 ml of the upper phase of BAW, and 50 μl was charged in each run. For protein separation lysozyme and myoglobin each 100 mg were dissolved in 20 ml of the upper phase of PEG-DPP used for separation, and after eliminating insoluble particles by filtration 100 μl of this stock solution was charged into the sample loop for each separation.

Separation procedure

In each separation, the separation column was entirely filled with the stationary phase, either upper or lower phase, followed by sample injection, and the column was rotated at 1000 rpm while the mobile phase was pumped into the separation column at a given flow rate. The effluent from the outlet of the column was continuously monitored with a Uvicord IIS at 280 nm and the elution curve was traced using a strip-chart recorder. In order to improve the tracing of the elution curve for organic-aqueous solvent systems, ethanol was mixed to the effluent at a volume ratio of 1:3 at the inlet of the detector using a tee connector and a fine mixing tube (PTFE 0.4 mm ID $\times$ ca 1 m). In the protein separation using an aqueous-aqueous polymer phase system (PEG-DPP), a hollow fiber membrane dialyzer (20 cm, spectrum laboratories, Inc., Rancho Dominguez, CA) was inserted between the column outlet and the eluted with water at a flow rate of 0.01 ml/min to dilute the effluent to improve the recording of the chromatogram. After the desired peaks were eluted, the run was stopped and the column contents were collected into a graduated cylinder by pressured air to determine the volume of the stationary phase retained in the column. The retention of the stationary phase was computed by dividing the volume of the retained stationary phase with the total column volume.

Evaluation of partition efficiency

The partition efficiency of separation column in each run was evaluated by computing theoretical plate number (N) for each peak and peak resolution (R_s) between the peaks using the following conventional equations:

$$N=(4t/W)^2 \quad (1)$$

$$R_s=2(t_2 - t_1)/(W_1+W_2) \quad (2)$$

where t and W indicate the retention time and the peak width in Eq. 1 and those for the specified peaks in Eq. 2, respectively.

RESULTS AND DISCUSSION

Performance of ellipsoid column parallel and perpendicular to the radially acting centrifugal force field in dipeptide separation

Because the performance of figure-8 column was very different at the 0° and 90° angle against the radially acting centrifugal force field in our previous studies^[17], two column models were investigated in this study where the ellipsoid column was mounted parallel and perpendicular to the radially acting centrifugal force field as shown in Fig. 3.

The first series of experiments was performed on separation of dipeptides (Trp-Tyr and Val-Tyr) with a high polarity solvent system (BAW) using the ellipsoid column under a revolution speed of 1000 rpm. Fig. 4 schematically illustrates the relationship between flow rate, retention of stationary phase and peak resolution in the dipeptide separation with 0° and 90° column angles. Four different conditions were examined as follow: 0° column angle with the lower mobile phase (Condition A); 0° column angle with the upper mobile phase

(Condition B); 90° column angle with the lower mobile phase (Condition C); 90° column angle with the upper mobile phase (Condition D).

Fig. 4A clearly indicates that the retention of stationary phase (Sf) was remarkably higher at 0° column angle than at 90° column angle. And Sf was linearly decreased with the increased flow rate. Sf with the lower mobile phase was lower than that with the upper mobile phase at 0° column angle (Table 1). The results of Rs were shown in Fig. 4B. Rs was much higher at 0° column angle than at 90° column angle. And with the decreased flow rate, Rs was improved in all groups (Table 1). The column pressure was higher at 0° column angle than at 90° column angle. When the lower phase was mobile, the values of these two factors were the best. And these results can explain why Rs was the best under condition A (Table 1). Fig. 5 shows the chromatogram of dipeptide separation with BAW at a flow rate of 0.07 mL/min under a revolution speed of 1000 rpm by centrifugal CCC in the ellipsoid column, yielding Rs at 2.02.

Comparison of the performance of dipeptide separation between ellipsoid and figure-8 column

The performance of figure-8^[17] and ellipsoid columns was compared under the identical experimental conditions, including column capacity, column length, column diameter, aluminum rotary plate, etc. When the flow rates were 0.03–0.04 mL/min, Rs of the Figure-8 column was better than that of the ellipsoid column because of higher Sf. When the flow rate was increased to over 0.05, however, Rs of ellipsoid column became higher than that of the Figure-8 column. When the flow rate was 0.05–0.07 mL/min, Rs of ellipsoid and Figure-8 column was 2.21–2.02 and 1.93–1.48, respectively (Table 1)^[17]. Under the flow rates from 0.03–0.07 mL/min, higher column pressure of the ellipsoid column suggests that the new design can produce more efficient mixing of two phases in much larger partition segments (Fig. 1) accompanied with slight loss of the stationary phase. Since the lower flow rates (0.03–0.04 mL/min) provide the longer time for mass transfer of solute in two-phase solvent system, Rs of the Figure-8 column was higher due to higher retention of the stationary phase (Sf). However, under the higher flow rates of 0.05–0.07 mL/min time required for mass transfer was limited and, therefore, Rs of the ellipsoid column becomes higher than that of the Figure-8 column because of its longer partition segment.

Separation of DNP-amino acids with HEMW

DNP-amino acids (DNP-ala, DNP-β-ala and DNP-glu) were successfully separated at a flow rate of 0.05 mL/min under a revolution speed of 1000 rpm by centrifugal CCC using ellipsoid column (Fig. 6). Rs was 2.16 between DNP-DL-glu and DNP-β-ala, and 1.81 between DNP-β-ala and DNP-L-ala with the retention of stationary phase at 31.6 % using the lower mobile phase. But Rs of the Figure-8 column was 1.77 between DNP-DL-glu and DNP-β-ala, and 1.52 between DNP-β-ala and DNP-L-ala with the retention of stationary phase at 27.3 % under the same separation conditions^[17].

Separation of proteins with PEG-DPP

In our previous study, the centrifugal CCC with a Figure-8 column was successfully applied for proteins separation. Therefore, this new ellipsoid column was also tested for protein

separation. Fig. 7 shows the separation of lysozyme and myoglobin using the PEG-DPP solvent system with various flow rates (0.03–0.07 ml/min) by ellipsoid centrifugal counter-current chromatography. The revolution speed was 1000 rpm. The results indicated that both R_s and S_f increased with decreased flow rate. When the low flow rate of 0.03 ml/min was used, R_s was 1.08 with S_f of over 38.9 % (Fig. 8). Compared with the Figure-8 column, the performance of centrifugal CCC was slightly improved by the ellipsoid column^[18]. The above result suggests that higher efficiency of the protein separation can be obtained with a lower flow rate. The lower flow rate can provide enough time to produce a prolonged droplet flow of the mobile phase, thus increasing interface area to enhance the mass transfer process. This may explain high performance of the ellipsoid column obtained by a lower flow rate of the mobile phase.^[17]

CONCLUSIONS

Using the novel ellipsoid column, satisfactory peak resolution and stationary phase retention were obtained for DNP-amino acid and dipeptide separations with organic-aqueous solvent systems and also for protein separation with an aqueous-aqueous polymer phase system. Overall results of our present studies indicated that the performance of centrifugal counter-current chromatography was further improved by the ellipsoid column.

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Figure 1. Hydrodynamic motion and interaction of the two phases in the figure-8 and ellipsoid column. The column was parallel to the acting centrifugal force.

Figure 2.
Photography of the ellipsoid column for centrifugal counter-current.

Figure 3.
The ellipsoid column mounted model. A) column was parallel to the centrifugal force; B) column was perpendicular to the centrifugal force.

Figure 4.

Comparison of performance of dipeptide separation with 0° and 90° angles at various flow rates in centrifugal counter-current chromatography with ellipsoid column. Sample: Trp-Tyr, Val-Tyr; sample size: 40 μ L; solvent system: BAW; revolution speed: 1000 rpm; column capacity: 3.4 mL. (A) Retention of stationary phase; (B) Resolution. Condition A: the angle of column against the centrifugal force was 0° and lower phase was mobile phase; Condition B: the angle of column against the centrifugal force was 0° and upper phase was mobile phase; Condition C: the angle of column against the centrifugal force was 90° and lower phase was mobile phase; Condition D: the angle of column against the centrifugal force was 90° and upper phase was mobile phase.

Figure 5.

Chromatograms of dipeptide separation by centrifugal counter-current chromatography using ellipsoid column. Rotational speed: 1000 rpm, Mobile phase: lower phase, Solvent system: BAW, Flow rate: 0.07 mL/min.

Figure 6.

Chromatograms of DNP-amino acid separation by centrifugal counter-current chromatography using ellipsoid column. Rotational speed: 1000 rpm, Mobile phase: lower phase, Solvent system: HEMW, Flow rate: 0.05 mL/min.

Figure 7.

The performance of the protein separation at the various flow rate by centrifugal counter-current chromatography using ellipsoid column. Samples: lysozyme, myoglobin; solvent system: PEG-DPP; rotational speed: 1000 rpm; capacity: 3.4 mL.

Figure 8.

Chromatogram of protein separation by centrifugal counter-current chromatography using ellipsoid column. Samples: lysozyme, myoglobin; solvent system: PEG-DPP; flow rate: 0.03 mL/min; rotational speed: 1000 rpm; capacity: 3.4 mL; detection wavelength: 280 nm; retention of stationary phase: 38.9%.

Table 1

Two-phase solvent systems and K values of the test samples

Two-phase solvent system	Abbreviations	Test samples	K value
n-hexane-ethyl acetate-methanol-0.1 M HCl (1:1:1:1, v/v)	HEMH	DNP-L-ala	2.36
		DNP- β -ala	1.18
		DNP-L-glu	0.44
1-butanol-acetic acid-water (4:1:5, v/v)	BAW	Trp-Tyr	1.69
		Val-Tyr	0.53
12.5% (w/w) PEG 1000 and 12.5% (w/w) dibasic potassium phosphate in water	PEG-DPP	Lysozyme	1.69
		myoglobin	0.51

Table 2

Comparison of the performance of the ellipsoid column amounted at 90° and 0° in dipeptide separation with the various flow rates by centrifugal counter-current chromatography using ellipsoid column.

Mobile phase	Flow rate (ml/min)	0°				90°			
		Sf (%)	Rs	Pressure (Psi)	Sf (%)	Rs	Pressure (Psi)	Sf (%)	Rs
Lower phase	0.07	27.8	2.02	214	16.7	1.08	89		
	0.06	31.6	2.18	212	19.1	1.18	87		
	0.05	33.9	2.21	211	20.7	1.44	85		
	0.04	36.2	2.28	207	23.5	1.52	81		
	0.03	38.1	2.35	203	25.1	1.56	76		
Upper phase	0.07	37.5	0.97	240	16.9	0.52	96		
	0.06	39.1	0.98	233	17.6	0.54	92		
	0.05	40.5	1.08	226	18.9	0.58	89		
	0.04	42.1	1.21	222	20.6	0.73	85		
	0.03	44.4	1.36	218	23.6	0.81	81		

Note: rotational speed: 1000 rpm. Solvent system: BAW.

Table 3

comparison of the performance of dipeptides separation between ellipsoid and figure-8 column at the 0° angle by centrifugal counter-current chromatography

Flow rate (mL/min)	Figure-8 column				Ellipsoid column			
	Sf (%)	Rs	Pressure (Psi)		Sf (%)	Rs	Pressure (Psi)	
0.07	35.3	1.48	205		27.8	2.02	214	
0.06	36.4	1.67	202		31.6	2.18	212	
0.05	37.8	1.93	201		33.9	2.21	211	
0.04	39.1	2.34	198		36.2	2.28	207	
0.03	39.8	3.28	193		38.1	2.35	203	

Note: samples: Val-Tyr and Trp-Tyr, solvent system: BAW, mobile phase: lower phase, rotational speed: 1000 rpm.