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Vortex Counter-Current Chromatography: Performance of a New Preparative Column

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Abstract

A New preparative column for the vortex counter-current chromatograph was fabricated by making many (966) cylindrical separation units to a high-density polyethylene disk and threading them with 6-40 taps. The resulting column had a total capacity of 364 mL. The performance of this vortex column was examined with three different two-phase solvent systems each using a set of suitable test samples: hexane-ethyl acetate-methanol-0.1 M hydrochloric acid (1:1:1:1, v/v) for separation of DNP-amino acids; 1-butanol-acetic acid-water (4:1:5, v/v) for separation of dipeptides; and hexane-acetonitrile-water (20:15:2, v/v) for separation of Sudan dyes. Most of the separations show high partition efficiency of over a thousand theoretical plates, as expected based on the results previously obtained in preliminary separations with a small column. Overall, the results of the present study suggest that further improvement of the partition efficiency can be obtained by the modifying column configuration.

Keywords

Vortex counter-current chromatography; preparative separation; DNP-amino acids; dipeptides; Sudan dyes

Introduction

High-speed counter-current chromatography (HSCCC) has been widely used for the separation and purification of natural and synthetic products [1-5]. The separation is based on the Archimedean screw effect in the type-J synchronous planetary motion which efficiently mixes two solvent phases while retaining one of the phases as stationary phase in the multilayer coil [1]. While the method yields excellent partition efficiency, it also has some inherent problems derived from the Archimedean screw effect such as high column pressure and sample band broadening along the coiled column due to longitudinal phase mixing along the length of the coiled tube. In order to cope with these problems, we have

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developed a new CCC system called vortex CCC [6, 7]. It uses a type-I coil planet centrifuge [8] which produces circular motion of the two solvent phases in a cylindrical compartment. A series of cylindrical compartments are connected with narrow ducts in such a way that solvent enters through a side hole at one end of each compartment and exits through a hole in the center of the opposite end of the compartment [6,7]. This elution mode produces retention of the stationary phase in each compartment while the solute partitioning process takes place in two places: the rectangular vertical interface between the two phases; and the inner wall of the compartment where one of the phases (hydrophobic upper phase in the present study) forms a thin layer. In earlier work using a set of columns with different diameters, it was found that those of smallest diameter (3 mm ID) yielded the highest partition efficiency [6]. Furthermore, in a separate study, the partition efficiency was substantially improved by threading the inner wall with a 6-40 tap (UNF) and thus nearly doubling the area of the column wall [7]. The separation column that was tested in the previous studies consisted of 120 cylindrical compartments, each 3 mm ID and 5 cm long, with a total capacity of 42 mL. Despite a short column length of 6 m, the column produced a few hundred theoretical plates with height equivalent to one theoretical plate (HETP) of 1-2 cm [7]. By comparison, the conventional HSCCC multilayer coil of 1.6 mm ID and 180 m in length with a total capacity of 320 mL yielding 600 - 800 theoretical plates which corresponds to 15-20 cm for the HETP [9].

In the present study, the conclusions drawn from the previous work [6,7] were applied to fabricate a large preparative vortex column with 966 threaded cylindrical compartments of 3 mm ID, 48 m in length, and 364 mL total capacity. The performance of this new vortex column was tested using three different two-phase solvent systems broadly ranging in hydrophobicity, each with a suitable set of test samples.

Experimental

Apparatus

The type-I coil planet centrifuge used in the present studies was fabricated in the machine shop of the National Institutes of Health, Bethesda, MD, USA. The design of the apparatus has been reported in detail elsewhere [6]. Briefly, the apparatus holds a vortex column and the counterweight at 10 cm from the central axis of the apparatus (Fig. 1). It produces a type-I synchronous planetary motion in which the centrifugal force uniformly rotates around every point on the holder. The separation column was newly fabricated from a solid high-density polyethylene disk (16 cm in diameter and 5 cm in height) by making multiple holes on each side with a 3 mm diameter drill as shown in Fig. 2a. These holes are connected in series in such a way that the central hole (exit) (0.75 mm ID) of the first cylinder (left) is joined to the side hole (inlet) (1 mm ID) of the second cylinder and so on throughout the series. In order to improve the partition efficiency, each hole was threaded with a pair of 6-40 taps to form the right-handed threads in the odd numbered cylinders and the left-handed threads in the even numbered cylinders (Fig. 2b). This doubles the inner wall surface (effective mass transfer surface) while creating a weak Archimedean screw force effect [7]. If the central shaft in Fig. 1 is driven counterclockwise (viewed from the top of the centrifuge), the holes in the vortex column will gyrate in counterclockwise orbits. The force vectors within each hole will also rotate counterclockwise, placing the head end at the top in Fig. 2b, while the heads of the left-hand threaded holes will be at the bottom, making the flow from head to tail throughout the column illustrated in Fig. 2b. This head and tail pattern and the resulting flow designation will be reversed if the central shaft rotates clockwise. The present column contains 966 cylindrical partition compartments with a total length of ca. 48 m and a capacity of 364 mL.

The solvent was pumped through the column with a Waters model 510 HPLC pump (Boston, MA, USA) while the effluent was continuously monitored at 280 nm with an LKB Uvicord IIS (Bromma, Sweden) and a strip-chart recorder (Millipore, Boston, MA, USA). The effluent was collected into a graduated cylinder.

Reagents

Organic solvents including hexane, acetonitrile, ethyl acetate, methanol, and 1-butanol were of chromatographic grade and obtained through Fisher Scientific Co. (Fair Lawn, NJ, USA). Hydrochloric acid and acetic acid of analytical grade were purchased from Mallinckrodt Baker (Phillipsburg, NJ, USA). Test samples including Sudan I, Sudan III, Oil Red, DNP-amino acids, N-2,4-dinitrophenyl-L-lysine (DNP-lys), N-2,4-dinitrophenyl-L-aspartic acid (DNP-asp), N-2,4-dinitrophenyl-DL-glutamic acid (DNP-glu), N-2,4-dinitrophenyl- β -alanine (DNP- β -ala), N-2,4-dinitrophenyl-L-alanine (DNP-ala), N-2,4-dinitrophenyl-L-leucine (DNP-leu), L-tryptophyl-L-tryptophan (Trp-Trp), L-tryptophyl-L-tyrosine (Trp-Tyr), L-leucyl-L-tyrosine (Leu-Tyr), L-valyl-L-tyrosine (Val-Tyr), and L-tyrosyl-glycine (Tyr-Gly) were all purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Preparation of Two-Phase Solvent Systems and Sample Solutions

Three sets of two-phase solvent systems with a broad range in hydrophobicity were prepared according to the previous studies [6, 7]: Hexane/ethyl acetate/methanol/0.1 M hydrochloric acid (1:1:1:1, v/v) was used for separation of DNP-amino acids, 1-butanol/acetic acid/water (4:1:5, v/v) was used for separation of dipeptides, and hexane/acetonitrile/water (20:15:2, v/v) was used for separation of Sudan dyes (this solvent system was modified from that previously used in order to separate three components). Each solvent system was prepared by equilibrating the solvent mixture in a separatory funnel by vigorous shaking and degassing several times and the two phases were separated shortly before use. Sample solutions were each prepared by dissolving a suitable amount of sample mixture in the upper phase used for the CCC separation. In order to avoid the formation of a single phase in the sample compartment in the column, a small amount of lower phase was added until the sample solution became turbid which indicated formation of the two phases. The composition of the three solvent systems, the components of each test sample, and the partition coefficient value of each component are given in Table 1. The partition coefficients were determined as follows: a small amount of each sample component was added to a test tube containing 2 mL each of upper and lower phases of the equilibrated two-phase solvent system. After vigorous mixing with a vortex mixer to completely dissolve the sample, an equal volume (usually 200 μ l) of each phase was diluted with 2 mL of methanol to measure the absorbance at a suitable wavelength using a spectrophotometer. The partition coefficient (K) is expressed as the absorbance in the upper phase divided by that of the lower phase ($K_{up/lp}$) or in the lower phase divided by that of the upper phase ($K_{lp/up}$) (see Table 1).

VCCC Separation Procedure

In each separation the column was first entirely filled with the stationary phase, either upper or lower phase, followed by injection of sample solution through the sample port. Then, the column was rotated at a given speed while the mobile phase was eluted through the column at a given rate. The effluent eluted through the column was continuously monitored at 280 nm and the chromatogram was traced with the strip-chart recorder. The effluent was collected into a graduated cylinder to measure the volume of the stationary phase displaced by the mobile phase in order to compute the percentage retention of the stationary phase. The separated components were identified by comparing the order of their retention time to that of the partition coefficients.

Evaluation of Partition Efficiency and Stationary Phase Retention

Partition efficiency in each separation was estimated in terms of theoretical plate number (TP or N) from the chromatogram using the following conventional equation:

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

where V_R is retention volume of each peak and W the peak width. When the two peaks were only partially resolved, the front half width of the first peak (W_1') or the rear half width of the second peak (W_2') were used to approximate N value as

$$N'=\left[4V_R/2(W_1' \text{ or } W_2')\right]^2 \quad (2)$$

The percentage retention of the stationary phase (S_f) was calculated from the displaced stationary phase volume and the total column capacity.

Results and Discussion

Separation of Test Samples

Figs. 3a and 3b show the separation of a mixture of DNP-amino acids with a two-phase solvent system composed of hexane-ethyl acetate-methanol-0.1 M HCl (1:1:1:1, v/v) at a flow rate of 3 mL/min and revolution rate of 1000 rpm. The chromatogram in Fig. 3a shows separation of 6 DNP-amino acids. In the lower phase mobile (Fig. 3a) DNP-aspartic acid and DNP-glutamic acid were eluted as a single peak (2nd peak), but they were completely resolved with the upper phase mobile (Fig. 3b) where DNP-lysine was still retained in the column. As expected from the results obtained using a short column in a previous study [7], the current column produced high partition efficiency values of over one thousand theoretical plates (Table 2). This solvent system is used for separation of a wide variety of natural and synthetic products by modifying the volume ratio of the solvents. Either upper or lower phase can be efficiently used as the mobile phase depending on the partition coefficient values of the target compounds (Table 1).

Figs. 4a and 4b show the chromatograms obtained for separations of a dipeptide mixture. Five test components were well resolved with a polar two-phase solvent system composed of 1-butanol-acetic acid-water at a volume ratio of 4:1:5. Some disturbance of the base line was caused by a steady carryover of the stationary phase due to low interfacial tension and a small difference in density between the two phases. This particular solvent system is very useful for separation of peptides and other highly polar compounds despite low S_f (see Table 2), but the conventional multilayer coil in the type-J coil planet centrifuge also has low retention of the stationary phase.

Separation of hydrophobic compounds was examined with a two-phase solvent system composed of hexane-acetonitrile-water (20:15: 2, v/v). Figs. 5a and 5b illustrate chromatograms of the separation of a mixture of Sudan I, Sudan III, and Oil Red. With the upper phase mobile (Fig. 5b) the two closely related components in Oil Red eluted together near the solvent front with partial resolution while they are well resolved using the lower aqueous phase as a mobile phase (Fig. 5a) (These two components were not identified, since it is beyond the scope of the present study). These results clearly indicate that the method works efficiently when the sample has a relatively high K value without excessive sample band broadening, whereas samples with small K values may not be well resolved especially when the system has low retention of the stationary phase.

Table 2 lists the theoretical plate number of each sample and percentage retention of the stationary phase. Overall results of the present studies demonstrate that the vortex CCC may be efficiently used for separation of a variety of compounds with a broad range in hydrophobicity. Although the present work examined the performance of the new preparative vortex column with milligram quantities of test samples, its preparative capability has been successfully demonstrated by the separation of a gram quantity of synthetic dyes contaminanats[10].

Conclusions and Recommendations

In the past the capability of VCCC has been examined with a short column (6 m) for the separation of two dipeptides of Trp-Tyr and Val-Tyr with a polar two-phase solvent system composed of 1-butanol-acetic acid-water at volume ratios of 4:1:5. The separation was performed at 800 rpm eluting the upper phase at a flow rate of 1 mL/min. Figs. 6a and 6b shows a comparison of partition efficiency (a) and resolution (b) for the same peptide separation and solvent system using three different VCCC column designs (4 mm ID, 3 mm ID [6] and tapped 3 mm ID [7]) all with similar capacities and operating conditions as indicated in the figure caption. In these figures the theoretical plate numbers (TP) for two peaks together with percentage retention of stationary phase are given in the separation of dipeptides (trp-tyr and val-tyr) in a two-phase solvent system composed of 1-butanol-acetic acid-wate (4:1:5, v/v). The result clearly indicates that the 3 mm ID column shows substantially higher TP than the 4 mm ID column, and its efficiency is further improved by tapping the column wall. Fig. 7b similarly shows the peak resolution (Rs) obtained from the three columns. The tapped 3 mm ID column also yielded the highest Rs value. As mentioned earlier [7], the high performance of the tapped column is derived from the increased internal surface area where the hydrophobic phase forms a thin layer to provide an efficient mass transfer interface. On the basis of these findings, the future development of vortex CCC column may be considered in the following four directions: 1) these separations may be further improved by making a column from tapped 2 mm ID holes. 2) It is also planned to use laser sintering technology to make deep circular grooves in the inner wall of column segments instead of threading.

The circular grooves will prevent longitudinal mixing of the two phases to improve HETP, but it would be difficult tofabricate by standard machining procedures. 3) This system also has an advantage over the conventional type-J planetary centrifuge in that every point on the holder can provide the efficient column space. Therefore, the columnncapacity may be increased by mounting a larger column by eliminating the central shaft and supporting the column holder and counterweight between a pair of horizontal plates. 4) One important advantage of the present system over the conventional J-type HSCCC system is its low back pressure (stopping the revolution does not change the pressure at the outlet of the pump). Low column pressure in the present system will allow safe operation of a large preparative column. The conventional multilayer coil HSCCC or HPCCC produces a high column pressure at high revolution speed caused by an enhanced Archimedean screw effect. A hydrostatic CCC system called centrifugal partition chromatography uses a hydrostatic force to retain the stationary phase, and it again suffers from high column pressure at high rotation speeds. Consequently, elevation of column pressure in these CCC systems limits the application of high speed column rotation. Since vortex CCC can keep a low column pressure at a high revolution speed, future development may also be directed to application of much higher revolution speed which would allow application of higher flow rates of the mobile phase to shorten the separation time without losing the partition efficiency.

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Fig .1.
Diagram of type-I planetary centrifuge.

Threaded Cylindrical Column for Vortex CCC

Fig .2.

New vortex column

a. Photograph of the column with 966 holes of 3 mm ID threaded with 6-40 taps. b. .
Diagram of four units of the threaded column.

Fig. 3.

Separation of DNP-amino acids by vortex CCC

a. lower phase mobile; b. upper phase mobile. Experimental conditions: Apparatus: type-I planetary centrifuge with 10 cm revolution radius; column: vortex column with 966 tapped 3 mm ID cylindrical units connected in series with a total capacity of 364 mL; two-phase solvent system: hexane-ethyl acetate-methanol-0.1 M hydrochloric acid (1:1:1:1, v/v); mobile phase: lower phase (a) and upper phase (b); flow rate: 3 mL/min; detection: 280 nm; sample: DNP-Lys, DNP-asp, DNP-glu, DNP-β-ala, DNP-ala, and DNP-leu each 2 mg in 2 mL of upper phase; revolution: 1000 rpm; detection: 280 nm.

Fig. 4.

Separation of dipeptides by vortex CCC

a. lower phase mobile; b. upper phase mobile. Experimental conditions: solvent system: 1-butanol-acetic acid-water (4:1:5, v/v); mobile phase: lower phase (a) and upper phase (b); flow rate: 2 mL/min; sample: Tyr-Gly (8 mg), Val-Tyr (8 mg), Leu-Tyr (8 mg), Trp-Tyr (2 mg) and Trp-Trp (2 mg) in 4 mL of upper phase. Other conditions are described in the Fig. 4 caption.

Fig 5.

Separation of Sudan dyes by Vortex CCC

a. lower phase mobile; b. upper phase mobile. Experimental conditions: solvent system: hexabeacetonitrile-water (20:15:2, v/v); mobile phase: lower phase (a) and upper phase (b); flow rate: 3 mL/min; sample: Sudan I, Sudan III and Oil Red each 2 mg in 2 mL of upper phase. Other conditions are described in the Fig. 4 caption.

Fig. 6.

Comparison of partition efficiency between three different column designs of vortex CCC
 a. Comparison of theoretical plate number (TP) left bar indicates the first peak (val-tyr) (blue) and right bar the second peak(trp-tyr) (red); b. comparison in peak resolution between val-tyr and trp-tyr (Rs). Experimental conditions: Column: 4 mm ID column: 108 units, 5.4 m, and 44 mL capacity; 3 mm ID column (non-threaded): 120 units, 6.0 m, and 42 mL capacity; 3 mm ID threaded column: 120 units, 6 m, and 44 mL capacity; two-phase solvent system: 1-butanol-acetic acid-water (4:1:5, v/v), lower phase mobile: sample: trp-tyr (0.25 mg) and val-tyr (1 mg) in 1 mL of upper phase; flow rate: 1 mL/min; revolution: 800 rpm; detection: 280 nm.

Table 1

Partition coefficients of test samples in three different two-phase solvent systems

Solvent System	Test sample	($K_{up/lp}$)	($K_{lp/up}$)
HEMW [*]	DNP-lysine	0.005	1181
	DNP-aspartic acid	0.3	3.33
	DNP-glutamic acid	0.47	2.13
	DNP-beta-alanine	1.09	0.92
	DNP-alanine	1.97	0.51
	DNP-Leucine	7.8	0.13
BAW ^{**}	Gly-Tyr	0.31	3.23
	Ala-Tyr	0.53	1.89
	Leu-Tyr	0.85	1.18
	Trp-Tyr	1.69	0.59
	Trp-Trp	4.55	0.22
HAW ^{***}	Sudan I	0.85	1.18
	Sudan III	1.37	0.73
	Oil Red	2.25	0.44

^{*} HEMW: hexane-ethyl acetate-methanol-0.1M HCl (1:1:1:1, v/v)

^{**} BAW: 1-butanol-acetic acid-water (4:1:5, v/v)

^{***} HAW: hexane-acetonitrile-water (20:15:2, v/v)

Table 2

Partition efficiency of Vortex CCC for three different two-phase solvent systems

Solvent system	Elution mode	Theoretical plate number (TP)						Sf (%)
		DNP-asp	DNP-glu	DNP-β-ala	DNP-ala	DNP-leu		
HEMW*	upper phase mobile	1038	1058	1573	1900	8800		44
	lower phase mobile	-	-	1600	900	480		35
BAW**	upper phase mobile	Tyr-Gly	Ala-Tyr	Val-Tyr	Trp-Tyr	Trp-Trp		
		1942	1690	2085	11845	20000		23
HAW***	lower phase mobile	15550	4790	1936	1618	666		30
		Sudan I	Sudan III	Oil Red				
HAW***	upper phase mobile	2942	3872	3989, 4500				34
	lower phase mobile	3250	1620	669, 434				42

Sf: % retention of the stationary phase

* HEMW: hexane-ethyl acetate-methanol-0.1M HCl (1:1:1:1, v/v)

** BAW: 1-butanol-acetic acid-water (4:1:5, v/v)

*** HAW: hexane-acetonitrile-water (20:15:2, v/v)