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Flat-Twisted Tubing: Novel Column Design for Spiral High-Speed Counter-Current Chromatography

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Abstract

The original spiral tube assembly for high-speed counter-current chromatography (HSCCC) is further improved by a new tube configuration called “flat-twisted tubing” which was made by extruding the tube (1.6 mm I.D.) through a narrow slot followed by twisting along its axis forming about 1 cm twisted screw pitch. This modification interrupts the laminar flow of the mobile phase through the tube and continuously mixes the two phases through the column. The performance of this spiral tube assembly was tested by three types of two-phase solvent systems with different polarity each with a set of suitable test samples such as DNP-amino acids, dipeptides and proteins at the optimal elution modes. In general all these test samples yielded higher resolution with the lower mobile phase than the upper mobile phase. In the most hydrophobic two-phase solvent system composed of hexane-ethyl acetate-metanol-0.1M hydrochloric acid (1:1:1:1, v/v), DNP-amino acids were separated with *Rs*-a (peak resolution based on the same column capacity adjusted for comparison) at 4.40 and 73 % of stationary phase retention at a flow rate of 0.5 ml/min with the lower mobile phase. In the polar solvent system composed of 1-butanol-acetic acid-water (4: 1: 5, v/v), dipeptide samples were resolved with *Rs*-a at 4.06, compared to 2.79 with the cross-pressed tube assembly at 45 % stationary phase retention, each at a flow rate of 1 ml/min. Finally in the aqueous-aqueous polymer phase systems composed of polyethylene glycol 1000 – dibasic potassium phosphate each 12.5% (w/w) in water, protein samples were resolved with *Rs*-a at 2.53 compared to 1.10 with the cross-pressed tube assembly at 52 % of stationary phase retention, each at a flow rate of 1 ml/min. These results indicate that the present system substantially improves the partition efficiency with a satisfactory level of stationary phase retention by the lower mobile phase.

Keywords

spiral tube assembly; flat-twisted tubing; high-speed counter-current chromatography; retention of the stationary phase; resolution; separation of DNP-amino acid; dipeptide and protein

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1. Introduction

High-speed counter-current chromatography (HSCCC) has been widely used for the separation and purification of natural products [1–3]. When it was used to separation of polar compounds, such as peptides and proteins, however, it often produced low resolution due to insufficient retention of the station phase.

In order to cope with this problem, the spiral tube assembly was developed, which can provide a centrifugal force gradient along the radius of the spiral channel to enhance the retention of the stationary phase [4] and it has been successfully applied to separation of dipeptides with polar two-phase solvent systems. However, the system gave low efficiency in protein separation with an aqueous-aqueous polymer phase system apparently due to a lack of mixing of the viscous phases within the column. Then the system was improved by changing the shape of the tubing, by compressing the tubing perpendicularly at 1 cm intervals. This “cross-pressed” spiral tube assembly improved separation of proteins and peptides by inducing intermittent change of flow pattern which interrupts laminar flow of the mobile phase [4,5]. In the past, Degenhardt et al. reported similar effects produced by a conventional multilayer coil separation column made of twisted rectangular tubing for separation of natural products with an organic-aqueous two-phase solvent system [6].

The present study describes a new column design composed of “flat-twisted tubing” that further improves spiral tube support assembly and provides efficient separation of various test samples using the lower phase as a mobile phase.

2. Experimental

2.1 Apparatus

The apparatus of hydrodynamic equilibrium system used in the present study is a type-J coil planet centrifuge manufactured by P.C. Inc., Potomac, MD, USA. It holds a separation column on one side and a counterweight on the other side of the rotor symmetrically at 10 cm from the central axis of the apparatus. The original spiral tube assembly for HSCCC was designed in our laboratory and fabricated in the National Institutes of Health Machine Instrumentation Facility. The spiral tube assembly used in the present study was made from a plastic spiral tube support (CC Biotech LLC, Rockville, MD, USA) with channels, 5 cm deep and 3 mm in width forming four interwoven spirals interconnected with 4 radial grooves. Both ends of each radial groove were rounded to prevent kinking of the tubing. The column was made by inserting a single piece of fluorinated plastic tubing (Zeus Industrial Products, Orangeburg, SC, USA) (approximately 1.6 mm × 35 m) which was first extruded through a narrow slit, to form a flattened tube (approximately 4.0 mm × 38 m), followed by twisting along its axis to form ca 1 cm spiral pitch, into the grooves making a number of spiral layers. The present design of the spiral tube assembly requires placing a set of four transfer tubes in radial grooves between the neighboring spiral tube layers, thus limiting the number of spiral layers accommodated in the spiral tube support. In order to increase the column capacity, the tubing in the four radial grooves is pressed down with a specially made disk with 2 or 4 extrusions that fit into the radial grooves. The present studies were performed with a spiral tube assembly with 7 spiral layers and the total column capacity of 60 ml.

2.2 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, tryptophyl-tyrosine (Trp-Tyr), valyl-tyrosine (Val-Tyr) and N-2, 4-dinitrophenyl-L-alanine (DNP-ala), N-2,4-dinitrophenyl-β-alanine (DNP-β-ala), N-2,4-dinitrophenyl-DL-

glutamic acid (DNP-glu), lysozyme (chicken egg), myoglobin (horse skeletal muscle) were obtained from Sigma Chemicals, St. Louis, MO, USA.

2.3 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 such as DNP-amino acids, dipeptides and proteins, respectively, Table 1. 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 5.7mg 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 0.4 ml 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 0.4 ml 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 1 ml of this stock solution was charged into the sample loop for each separation.

2.4. 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 800 rpm while the mobile phase was pumped into the coiled column at a given flow rate. The effluent from the outlet of the coiled column was continuously monitored with a Uvicord IIS (LKB, Stockholm, Sweden) at 280 nm and the elution curve was traced using a strip-chart recorder (Pharmacia, Stockholm, Sweden). In order to improve the tracing, a suitable solvent such as ethanol or water was mixed with the effluent at the inlet of the detector using a tee connector and a narrow-bore mixing tube (PTFE 0.4 mm ID $\times$ ca 1 m). The flow rate is 30 % that of the mobile phase. After the desired peaks were eluted, the run was stopped and the column contents were discharged 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.

According to the results of the previous studies [5,7], two elution modes, L-I-T (lower mobile phase pumped into the internal tail terminal of the spiral) and U-O-H (upper mobile phase pumped into the external head terminal of the spiral) were examined for separation of dipeptides; two elution modes, L-I-H (lower mobile phase pumped into internal head terminal of the spiral) and U-I-T (upper mobile phase pumped into internal tail of the spiral) were examined for DNP-amino acids; and L-I-T (lower mobile phase pumped into the internal tail terminal of the spiral) and U-O-H (upper mobile phase pumped into the external head terminal of the spiral) were examined for separation of proteins.

2.5. Evaluation of Partition Efficiency

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

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

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

Where R and W indicate the retention time and the baseline peak width in Eq. 1 and for the specified peaks in Eq. 2, respectively.

In order to make a fair comparison between the present results and the previous data obtained from the column with a larger capacity, the peak resolution (R_s) obtained from the present flat-twisted spiral tube assembly was adjusted using the following equation:

$$R_{s-a} = R_s(V_2/V_1)^{1/2} = R_s(100/60)^{1/2} = 1.29R_s \quad (3)$$

where R_{s-a} is the adjusted peak resolution and V indicates the column volume being specified by the subscript 1 for the present column and 2 for a column of 100 ml volume.

3. Results and discussion

High-speed counter-current chromatography has been successfully applied to purification of natural products using organic/aqueous solvent systems. However, when the method was applied to the separation of polar analytes such as proteins using polymer phase systems, it failed to retain a satisfactory amount of the stationary phase in the column resulting in low peak resolution. As mentioned earlier, in order to improve the retention of the stationary phase the spiral tube support has been developed, which accommodates a long piece of PTFE tube into the spiral grooves to form multiple spiral layers without junctions [4]. This system worked well for separation of peptides, but it showed low peak resolution for protein separation as expected from the previous studies on the spiral disk assembly [8]. The efficiency of protein separation in the spiral tube support, however, has been substantially improved by pressing the PTFE tubing at regular intervals to prevent laminar flow which tends to produce longitudinal broadening of peak [5]. Degenhardt et al used twisted custom-made rectangular tubing which yielded 15–30% higher efficiency in separation of natural products than that of the conventional standard multilayer coil [6]. We have produced the flat-twisted tube by modifying the plain tubing for spiral tube assembly to further improve the peak resolution, especially for protein separation. In order to demonstrate the versatility of this flat-twisted spiral tube assembly three types of typical two-phase solvent systems were each tested with a set of suitable test samples as summarized in Table 1.

Experiments were performed on separation of DNP-amino acids with moderately hydrophobic solvent system composed of HEMH. Fig. 1 illustrates the variations of peak resolution, retention of stationary phase and column pressure with the increased flow rate in the DNP-amino acid separation with spiral tube assembly using the flat-twisted tube. The best resolution ($R_s = 3.41$) with 73% of stationary phase retention was obtained with the lower mobile phase at a flow rate of 0.5 ml/min (Fig. 2A). When the lower phase was the mobile phase, both peak resolution and retention of stationary phase decreased with the increased flow rate while column pressure increased. Similar results were obtained for the upper mobile phase (Fig. 1A and Fig. 1B). The peak resolution and retention of stationary phase for the lower mobile phase was much better than for the upper mobile phase, while the column pressure was higher for the lower mobile phase (Fig. 1C).

A second series of experiments was performed on separation of dipeptides with the polar BAW solvent system, Fig. 3. When the lower phase was the mobile phase, the best peak resolution ($R_s = 3.15$) with 45% of stationary phase retention was obtained at a flow rate of 1 ml/min (Fig. 2B) while a flow rate at 0.5 ml/min resulted in considerable peak broadening with the

lower phase mobile. With the upper phase mobile, peak resolution was decreased with the increased flow rate (Fig. 3A). Retention of the stationary phase decreased sharply with increasing flow rate for either phase, while the upper mobile phase always gave much higher phase retention (Fig. 3B). The column pressure on the other hand increased with increasing of flow rate, especially for the upper mobile phase, apparently due to its higher viscosity (Fig. 3C). At a high flow rate of 3 ml/min, lower mobile phase gives poor peak resolution due to the low retention of stationary phase (14 %). Similar results were also obtained when the upper phase was the mobile phase. Peak resolution obtained from the lower mobile phase was substantially better than that with the upper mobile phase. But the degree of retention of stationary phase was just opposite. The retention of stationary phase was higher with the upper phase mobile probably due to its high affinity to the tube wall.

The third series of experiments was performed on protein separation with PEG-DPP aqueous polymer phase solvent system (Fig. 4) and exhibited a trend similar to the dipeptide separation. When the lower phosphate-rich phase was the mobile phase, the best peak resolution ($R_s = 1.92$), with 52% of stationary phase retention, was obtained at the 1 ml/min flow rate (Fig. 2C), after which resolution decreased with increasing flow rate of either mobile phase (Fig. 4A). The retention of stationary phase also decreased sharply with increasing of flow rate of either mobile phase (Fig. 4B), while the column pressure increased (Fig. 4C). Peak resolution with the lower mobile phase was always better than with the upper mobile phase. But, the retention of stationary phase was better with the upper phase mobile, while column pressure was higher with the lower mobile phase.

In both protein and dipeptide separation using flat-twisted tube, the retention of stationary phase with the upper viscous mobile phase was much higher than with the lower mobile phase, whereas the results of peak resolution was just opposite. This may be explained as follows: when the lower phase was mobile, due to a lack of tube wall affinity, it tends to form a droplet flow providing a large interface area for mass transfer (Fig. 5A). In contrast, when the upper phase was mobile, it smoothly flows along the wall surface of the tubing, due to its high affinity, providing high retention of the stationary phase while giving a limited interface area for mass transfer. This hypothesis is diagrammatically illustrated in Fig. 5.

Compared with the spiral tube assembly made of the cross pressed tubing, the peak resolution was further improved by the flat-twisted tube (table 2). The CCC separation is a complexity course of interaction of K , α and S_f [9]. S_f and N are two significant contributors to R_s . We calculated the resolution according to the eq. 3 to compare the present and previous data, each obtained from column of different capacity. The peak resolution obtained from the flat-twisted tube was much better than that from the cross-pressed tube. The resolution of proteins obtained from the flat-twisted tube was over two times than that of the cross-pressed tube. Most of N is remarkably much greater than that from the cross-pressed tube. But the retention of stationary phase using cross-pressed tube was better except for DNP-amino acid separation with a moderately hydrophobic system. Higher flow rate led to the lower resolution because of the loss of the stationary phase retention. The present system yields high partition efficiency with the lower aqueous mobile phase using the flat-twisted tube because of the high retention of stationary phase and higher N . However, when upper phase was mobile, the resolution obtained from the cross-pressed tube was better than that from the flat-twisted tube in the dipeptide separation, while in the separation of the DNP-amino acid and protein separation, the results were mixed as shown in Table 2 [4,5].

4. Conclusions

Flat-twisted tube can be successfully applied to the separation of DNP-amino acids, dipeptides and proteins with excellent peak resolution by spiral tube HSCCC using a variety of two-phase

solvent systems including a moderately hydrophobic solvent system, a polar butanol solvent system and an aqueous-aqueous polymer phase system. Although the peak resolution using the upper mobile phase was less efficient, the peak resolution with the lower mobile phase was remarkably improved and exceeds that obtained by the conventional cross-pressed spiral tube assembly. The overall results of the present studies indicated that the flat-twisted tube assembly yields high partition efficiency using the lower mobile phase, especially for protein separation with the polymer phase system.

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Figure 1.

Comparison on performance of various flow rates in DNP-amino acid separation of spiral tube assembly using the flat-twisted tube. A) variation of resolution; B) variation of stationary phase retention; C) variation of column pressure. Test sample: DNP-L-glu, DNP- β -ala and DNP-L-ala, revolution: 800 rpm. L-I-H: lower mobile phase pumped into internal head terminal of the spiral and U-I-T: upper mobile phase pumped into internal tail of the spiral. Solvent system: HEMH. Detection: 280 nm.

Figure 2.

Separation of dipeptides, DNP-amino acids and proteins by the flat-twisted tube assembly. Apparatus: Type-J coil planet centrifuge with 10 cm revolution radius; separation column: flat-twisted spiral tube assembly: 7 spiral layers of ca 40 m long, 1.6 mm I.D. FEP tube with 1 cm screw pitch and 60 ml capacity; Detection: 280 nm. Revolution: 800 rpm. A) sample: DNP-L-glu, DNP- β -ala and DNP-L-ala, elution mode: L-I-H, solvent system: HEMH, flow rate: 0.5 ml/min. B) sample: Val-Tyr and Trp-Tyr, elution mode: L-I-T, solvent system: BAW, flow rate: 1 ml/min. C) sample: Lysozyme and myoglobin, elution mode: L-I-T, solvent system: PEG-DPP, flow rate: 1 ml/min.

Figure 3.

Comparison of performance of various flow rates in dipeptide separation of spiral tube assembly using the flat-twisted tube. A) variation of resolution; B) variation of stationary phase retention; C) variation of column pressure. Test sample: Val-Tyr and Trp-Tyr, revolution: 800 rpm. L-I-T: lower mobile phase pumped into the internal tail terminal of the spiral and U-O-H: upper mobile phase pumped into the external head terminal of the spiral. Solvent system: BAW. Detection: 280 nm.

Figure 4.

Comparison of performance of various flow rates in protein separation of spiral tube assembly using the flat-twisted tube. A) variation of resolution; B) variation of stationary phase retention; C) variation of column pressure. Test sample: Lysozyme and myoglobin, revolution: 800 rpm. L-I-T: lower mobile phase pumped into the internal tail terminal of the spiral and U-O-H: upper mobile phase pumped into the external head terminal of the spiral. Solvent system: PEG-DPP. Detection: 280 nm.

Figure 5.

Diagrammatic illustration of hydrodynamic behavior of the two phases flowing through a narrow bore tubing. A: upper organic phase mobile, B: lower aqueous phase mobile.

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 resolution and retention of stationary phase between the flat-twisted tube and cross-pressed tube for spiral tube assembly separation ^{a)}

sample	Solvent ^{b)}	mobile	Flow rate (ml min ⁻¹)	Sf (%) ^{c)}	Sf (%) ^{c)}	Rs/a/f	Rs/C	N-a ^{e)} /F	N/C
ALAN β-ALAN GLU	HEMH	Lower phase (LH)	0.5	73	---	4.40/3.12	---	802/656/463	---
			1	69	---	3.53/3.01	---	398/698/451	---
			2	67	60	3.50/2.66	3.18/2.38	639/500/410	757/614/423
		Upper phase (UH)	3	60	57	3.44/2.50	2.78/2.23	1352/760/326	816/480/400
			0.5	41	---	3.84/2.48	---	293/488/528	---
			1	38	---	2.91/2.08	---	228/512/528	---
trp-tyr val-tyr	BAW	Lower phase (LH)	2	33	83	2.86/1.84	3.48/1.93	338/726/490	253/294/324
			3	23	76	2.29/1.29	2.28/1.18	710/582/383	204/171/138
		Upper phase (UH)	0.5	57	---	3.92	---	145/250	---
			1	45	82.5	4.06	2.79	248/289	92/157
			2	27	65.3	3.57	3.38	161/579	359/291
			3	14	57.5	---	2.94	---	300/267
Lysozyme myoglobin	PEG-DPP	Lower phase (LH)	0.5	83	---	2.99	---	173/161	---
			1	80	69.1	2.86	3.60	238/221	262/282
			2	52	64.8	2.79	4.74	394/190	424/784
		Upper phase (UH)	3	43	37.3	2.24	3.25	209/1178	344/705
			0.5	73	75.7	2.26	1.53	45/83	24/39
			1	51.67	66.3	2.53	1.10	96/126	31/31
Lysozyme myoglobin	PEG-DPP	Lower phase (LH)	2	30	54.0	1.66	1.39	115/67	55/53
			0.5	81.67	72.0	1.09	1.44	82/30	46/57
			1	70	63.5	1.70	1.40	85/79	52/73
		Upper phase (UH)	2	41.67	49.4	1.08	1.74	41/146	72/137
			0.5	81.67	72.0	1.09	1.44	82/30	46/57
			1	70	63.5	1.70	1.40	85/79	52/73

a) Notes:

The data of flat-twisted tube (capacity: 60 ml) were calculated according to eq. (3) to compare with the data of the cross-pressed tube whose capacity is 100 ml.

- b) BMW: n-Butanol/Acetic acid/Water (4:1:5, v/v); HEMH: Hexane/EtOAc/MeOH/0.1M HCl (1:1:1:1, v/v); PEG-DPP: 12.5% (w/w) PEG 1000 and 12.5% (w/w) dibasic potassium phosphate in water
- c) F: flat-twisted tube
- d) C: cross-pressed tube
- e) $N_a = N L_2 / L_1 = N (100/60) = 1.32 N$
 where N_a is the adjusted theory plate number and L indicates the column length being specified by the subscript 1 for the present column of approximately 38 m length and 2 for a column of approximately 50 m length.