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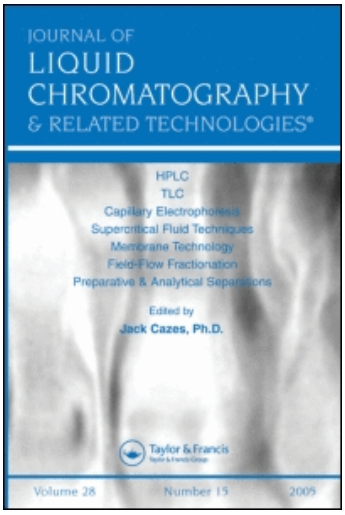

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USE OF THE MULTI-COIL COUNTERCURRENT CHROMATOGRAPH

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ABSTRACT

The multi-coil countercurrent chromatograph which is an Ito coil planet centrifuge equipped with 8 multi-layer coils in series is useful for preparative chromatography. A table top unit was tested with three solvent systems suitable for peptide separation. Preparative separations of 2 cholecystokinin peptides were performed in the instrument resulting in their complete purification in one step.

INTRODUCTION

A new design of the Ito coil planet centrifuge has been made for countercurrent chromatography with increased capacity. The multi-coil countercurrent chromatograph or serial multi-layer coil planet centrifuge is equipped with 8 column-coils, 4 more coils than in a previously described instrument <2,3>. Here we describe its features and operation.

The coil planet centrifuge assembly is encased in a steel covered box lined with anodized aluminum. The sides of the instrument are insulated. A top lid that opens includes a front window made of Lexan and the rim opening is lined with a

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1/2-in silicone rubber "P" gasket. The dimensions are 13-in wide, 19.5-in deep and 14-in high and at the rear left side is a control box extending out 9-in. On the control box are digital displays of rpm and temperature. When turned on by a key, the coil assembly rotates at a low rate of 200 rpm which can be set higher up to 1000 rpm by a dial. The 1/4 hp motor is variable speed with feedback control. A second dial is for temperature control. Inside the rear is a metal coil attacheable to a coolant or water. Forced air heating is provided via a copper coil and plates. Thus a heating-cooling sink mechanism exists for temperature control from room temperature to 50°C. When the lid is raised centrifugation ceases which serves as a safety feature. With the top open, there is full access to the frame and coils which is good for observation or making adjustments. The door is secured closed with a positive locking latch.

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The rotary frame holds 2 coil holders placed opposite each other at a distance of 8.75-cm from the central axis of rotation. Each holder shaft has a planetary gear on the left side engaged to the identical stationary sun gear on the central axis to produce the desired planetary motion of the holder about its own axis at the same angular velocity and direction. The column holder is removable by loosening a pair of screws on each bearing block. There are 4 identical multi-layer coils symmetrically around each holder shaft. The radius of each coil is 3.75-cm, thus B = 0.43 within the range of suitable functioning (B = ratio of radius of coil to radius of revolution). Each coil is made of 1.6 mm i.d. polytetrafluoroethylene (PTFE) thick walled tubing (0.064-0.072-in i.d., 0.020-0.028-in wall, Zeus Industrial Products, Inc. Raritan, NJ) wound in layers between the flanges; the total length giving a volume of 50 ml/coil. The ends of the

coils are connected by pieces of 0.85 mm i.d. tubing with commercial connectors. These are anchored on a rod between the coils. Each coil of tubing is secured by a vinyl heat shrunk covering. The coils are connected in series with the outer end to the inner entry of the next coil. The flow tubing enters the unit through a left side opening through which it is clamped. It passes through the central axis stationary pipe up through a side hole in the coupling pipe through a center opening of the holder shaft where it connects to the coiling. From the right end of the coil, the tubing passes back across on a supporting bar to the left side of the next coil and so on to the fourth coil, then through the center hole of the coupling pipe to a holder on the other side and around those coils up to the right side opening of the central pipe and out of the opening on the right side of the instrument through a deldrin tube clamp. The tubing where contacting metal moving parts is covered with tygon tubing filled with silicone grease. Because of the orientation of the tubing there occurs no twisting upon centrifugation, serving for continuous elution without a rotary seal.

The instrument has a significant amount of weight which is advantageous to prevent movement during operation and the covering is heavier than that of other instruments, thus adding to the safety factor in its operation. The coil assembly has to be well balanced for smooth operation without shaking. The parts key to the planetary motion have to be very tight and not move relative to each other. These are particularly each coil rod relative to the column holder and to the flanges. Figure 1 is a photograph of the multi-coil countercurrent chromatograph.

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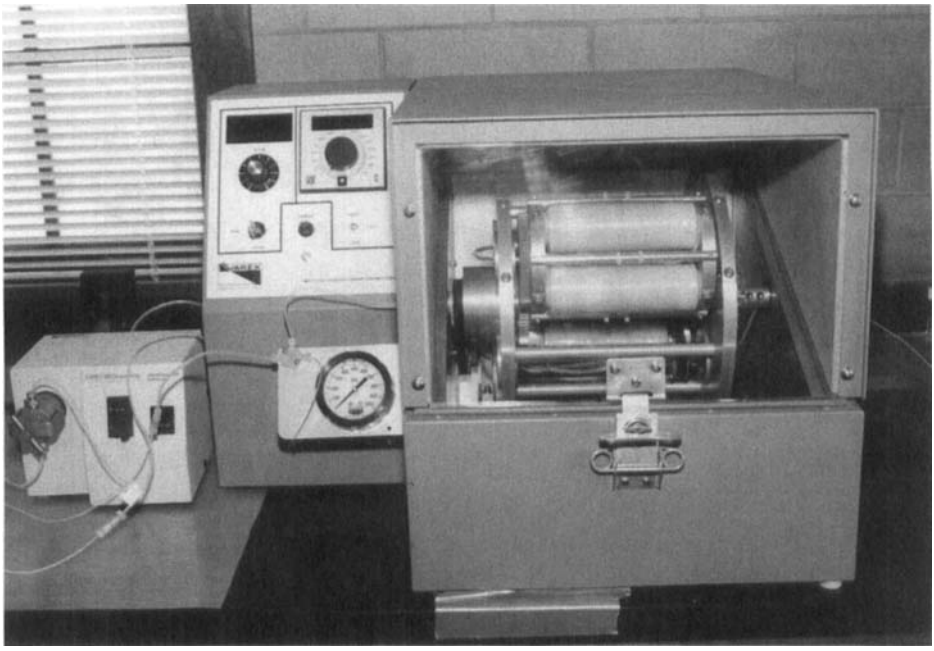

FIGURE 1

Photograph of the multi-coil countercurrent chromatograph. Details of the table-top instrument are described in the text.

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METHODS

Solvents used were from Fisher Scientific (Springfield, NJ). Water was passed through a Nanopure cartridge system (Barnstead, Boston MA). Unsulfated cholecystokinin peptides were synthesized by solid-phase methods <4>. Acetyl CCK 26-29 amide (Ac-Asp-Tyr-Met-Gly-NH_2) was synthesized manually with a shaker (St. Johns Assoc. Beltsville, MD) and Acetyl CCK 26-30 amide

(Ac-Asp-Tyr-Met-Gly-Trp-NH_2) was made using a Biosearch 9500 AT peptide synthesizer (Novato, CA) by coupling Boc-protected amino acid derivatives to p-methyl benzhydrylamine resin (U.S. Biochemical Corp. Cleveland OH). Peptides were removed from the resin and side chain protecting groups simultaneously deprotected by reaction with hydrogen fluoride. The resulting products were submitted to countercurrent chromatography for preparative purification. Subsequently the peptides were sulfated for use in biochemical experiments.

The table-top instrument was fabricated at Varex Corporation (Burtonsville, MD). This is similar to the instrument described previously <2,3> but with an additional set of 4 coils counterbalancing the other set of coils. Two-phase solvent systems were mixed in a large separatory funnel, equilibrated and separated at room temperature. The coil was filled with one phase which served as the stationary phase and the other was pumped through under the operating conditions as the mobile phase. The solvent was delivered by a LDC/Milton Roy Mini Pump (range 1-5 ml/min) connected to a Tefzel tee to which a pressure gauge, 0-1000 psi, was also connected. The gauge and the tee connecting the flow tubing were mounted on a bracket attached to the side of the multi-coil countercurrent chromatograph (Fig.1).

Initial studies were made to determine the volume of the instrument and the stationary phase retention of particular solvent systems under similar conditions of operation. The functioning of the chromatograph pertains to its ability to retain the stationary phase <5>. This is determined by measuring the stationary phase volume. After the coil is filled with the stationary phase, the rotation is set at 500 rpm, then the mobile phase is pumped through at 1 ml/min. In time the solvent

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front is eluted. This volume, V_m, is the excluded volume after the establishment of the equilibrium. V_m subtracted from the coil total volume, V_c, gives the stationary phase volume, V_s. The phase retention is the ratio of the stationary phase volume to the total volume. The total volume of this instrument with 8 coils is 385 ml.

For the preparative chromatography runs, a sample of 200 to 300 mg was dissolved in 5 to 10 ml of both phases and loaded after filling of the coil with the stationary phase. Then chromatography with the mobile phse was started. Fractions of 10 ml were collected in a LKB Ultro Rac fraction collector (LKB/Pharmacia Gaithersburg MD). Presence of peptide was determined manually by absorbance measurement at 280 nm. The purity of peak fractions and the final recovered peptide was determined by HPLC as described previously <6> using a S-5 ODS column (0.4 or 0.6 X 15-cm, 200 A pore, ODS bonded spherical 5 um silica, YMC, Inc. Morris Plains NJ) in 0.1 % aqueous phosphoric acid and acetonitrile gradients at 0.8 ml/min in Waters analytical HPLC equipment consisting of a U6K injector, 2 Model 510 pumps, Model 680 gradient controller, Model 481 variable-wavelength UV detector and an SE120 recorder (Waters/Millipore, Milford MA).

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RESULTS

Phase retention with three solvent systems was determined by filling the coils with one phase and chromatographing the other phase and some time after elution of the solvent front, the contents are removed and the two phases are measured. The ratio or percent of retained stationary phase in the coil to the total coil volume is calculated and given in Table 1. The experiments were run at 500 rpm with a flow rate of 1 ml/min. During

TABLE 1

Stationary Phase Retention
as Per Cent Total Volume (V_m/V_c)

Solvent Composition	Mobile Phase	
	Upper	Lower
n-butanol/acetic acid/water (4:1:5 by volume)	30%	40%
1% trifluoroacetic acid/n-butanol (1:1)	43%	39%
chloroform/acetic acid/water (2:2:1)	28%	37%

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the introduction of the mobile phase the back pressure increased incrementally up to a level maintained constant after emergence of the solvent front of the mobile phase. This is due to the attainment of equilibrium and ensuing balance of the liquid mass. We decreased the rpm if the back pressure exceeded 350 psi. However, during recent sample runs the back pressure has not been exceeding 213 psi. We are currently observing the performance at 500 to 600 rpm.

In the table are the results of determining the stationary phase retention in various solvent systems under conditions of either phase mobile. In these experiments the retained stationary phase varied between 105 and 150 ml.

The centrifugal rate was lowered to 450 rpm in the case of the first two solvent systems and to 384 rpm with the chloroform system. The back pressure increased significantly because of the the greater density difference between the phases <7>. The

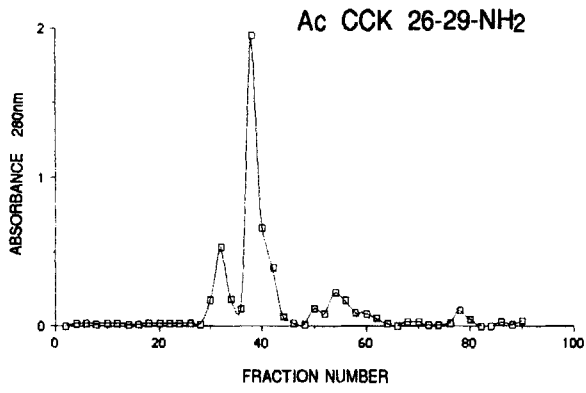

FIGURE 2

Preparative chromatography of 300 mg Ac-Asp-Tyr-Met-Gly-NH_2 in the multi-coil countercurrent chromatograph in n-butanol/acetic acid/water (4:1:5) with the upper phase used as the mobile phase. The flow was 1 ml/min and centrifugal rate was 600 rpm. The solvent front came out at fraction no. 27. Fractions of 10 ml were collected.

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mean phase retention is 36%. In most experiments done since and with earlier experiments performed at higher rpm with other tubing, the phase retention averaged 40% which is suitable for chromatography. This result means that compared to the performance of the older horizontal flow-through instrument <8>, the phase retention is similar but the flow rate able to be used is over 2 times faster which constitutes enhanced performance.

Recent synthetic products of two CCK fragment peptides were chromatographed in the new instrument. In Fig. 2 is shown the result of the separation of 300 mg AcCCK(26-29)amide. The major peak of fractions 37-40 contained 278 mg pure peptide as determined by analytical HPLC, shown in Fig. 3. The early peak at the solvent front was yellow and had little mass and in fractions (49-62) there were 26 mg side product. The K is 1.05, close to

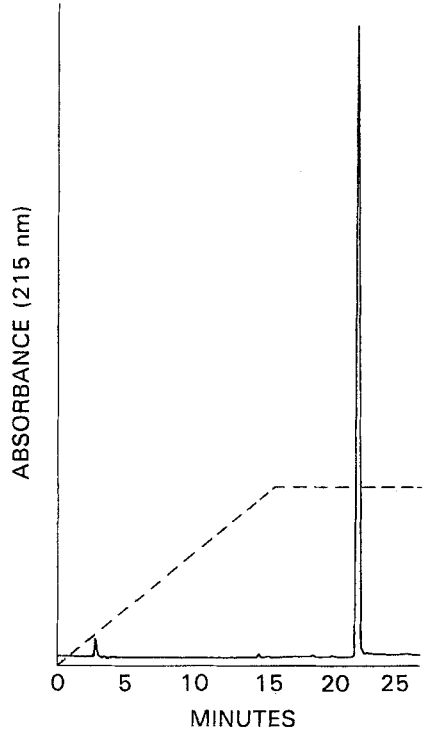

FIGURE 3

Analytical HPLC of the contents of the major peak of the chromatography of AcCCK(26-29)NH_2 (Fig. 2). Approximately 15 ug sample chromatographed on a 5 um spherical 200 A ODS, 0.4 X 15 cm, YMC, Inc. column in 0.1 % aq. phosphoric acid and a gradient of acetonitrile, 5 to 30 % in 15 min at a flow of 0.8 ml/min. Detection is at 280 nm with 0.5 absorbance units full scale.

the previously published value of 0.95 (K = conc. in mobile phase/conc. in stationary phase). The resolution calculated from the peak shape was 1444 which is suitably high for preparative chromatography. The material emerged in 6 hr compared to over 9 hr in the old instrument <9> using the same solvent and mobile phase. Another run was made of 274 mg peptide with similar results (not shown).

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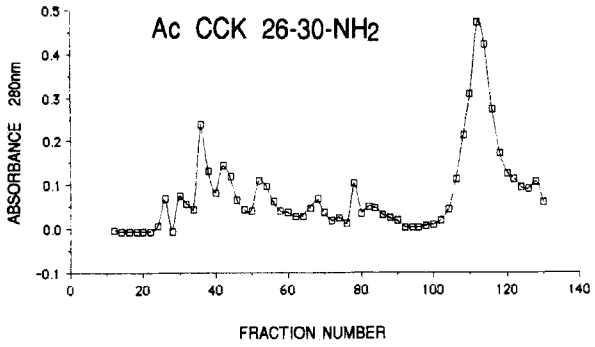

FIGURE 4

Countercurrent chromatography of Ac-Asp-Tyr-Met-Gly-Trp-NH_2 in the BAW system with the lower phase mobile at 500 rpm with a flow of 1 ml/min. Sample load was 200 mg and pure peptide recovered totaled 56 mg.

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The other peptide, AcCCK(26-30)amide, 200 mg, was run in the BAW system (n-butanol/acetic acid/water, 4:1:5) with the lower phase mobile. The result is shown in Fig. 4. Fractions 107-117 contained the pure peptide. N calculated from the run is 1600 and the K is 0.157 close to the reported value of 0.14. Analytical HPLC of the crude and purified peptide after recovery and lyophilization indicated purification (Fig. 5). The mass was 56 mg. A preparative HPLC of the rest of the synthetic product gave a similar mass recovery of purified peptide. Therefore what peptide there was in the sample was isolated. This peptide was also chromatographed in the 4-coil multi-coil countercurrent chromatograph with similar results <3>.

Experiments of operating the instrument at higher flow rates and rpm are necessary to assess the possibility of even higher resolution. We are currently chromatogaphing other peptides of

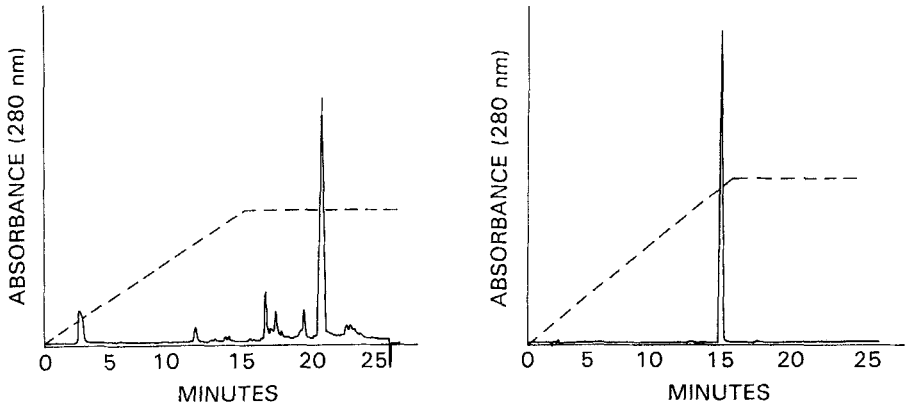

FIGURE 5

Analytical HPLC of unpurified and purified AcCCK(26-30)NH_2 (Fig. 4). Left is approximately 20 ug sample at 280 nm, 1 absorbance unit full scale, with a gradient of acetonitrile from 10 to 30 % in 15 min. Right is purified peptide in a gradient of 5 to 30 % in 15 min.

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different composition to determine their purification with respect to resolution and recovery as we have done with the older instrument. In some cases we can compare results with preparative reverse-phase chromatography <10-12>. Both peptides described here have given N values higher than 500 which is good for preparative chromatography. These results indicate superior performance of the present instrument which also can be used for proteins <2> as well as small MW compounds, important for biotechnology and natural product isolations, respectively. All solvent systems can be used, and with the added feature of temperature control (not used here), the multi-coil countercurrent chromatograph has the capability of universal application.

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1. Abbreviations used: HPLC = high performance liquid chromatography; CCK = cholecystokinin; Boc = butyloxycarbonyl; ODS = octadecylsilyl; rpm = revolutions per minute.

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