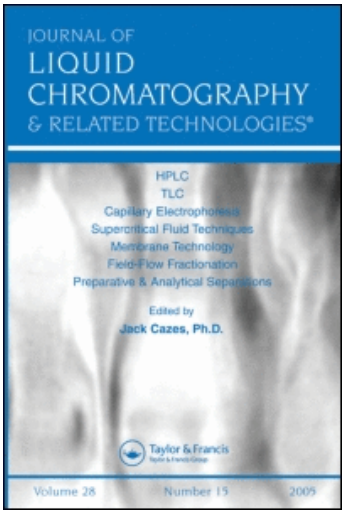

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PREPARATIVE PURIFICATION OF PEPTIDES BY COUNTERCURRENT CHROMATOGRAPHY ON THE ITO COIL PLANET CENTRIFUGES

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ABSTRACT

For the purification of up to 1000 mg of synthetic peptides by countercurrent chromatography, the coil planet centrifuges have proven useful in the research laboratory. Besides the earlier described horizontal flow-through coil planet centrifuge which chromatographs substances in all solvent systems at room temperature, the multi-layer coil planet centrifuge affords more rapid chromatography. However, separations using n-butanol, especially suited for peptides, have to be conducted at elevated temperatures. A new machine that has characteristics of both of the foregoing instruments is the compact horizontal flow-through coil planet centrifuge which promises rapid chromatography with full retention of the stationary phase.

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INTRODUCTION

The research of Yoichiro Ito over the last 15 yrs in countercurrent chromatography, elucidating the behavior of two-phase solvent systems has led to the design of instruments capable of chromatography. These instruments are basically continuous open tubing arranged in coils which serve as the separation units in which equilibration occurs. In a rotating coil filled with two phases, one phase migrates to one end, the head, whereas the other phase goes to the other end, the tail. Therefore, pumping a liquid phase in the direction opposite to which it would migrate causes the other phase to be retained and hydrodynamic equilibrium results. The earlier non-rotating coil arrangement producing hydrostatic equilibration was the droplet countercurrent coil (2). Subsequently, the observation was made that by centrifuging a coil rotating on its own axis, produced complete or hydrodynamic mixing of the solvents. Thus the efficiency of separating substances according to their partitioning in the liquid system without a solid support was greatly increased. Planetary motion applied to a horizontal or eccentric orientation of a series of coils arranged in columns parallel to the axis of rotation comprised the horizontal flow-through coil planet centrifuge (HFTCPC). This was the first coil planet centrifuge extensively used in research laboratories for preparative-scale chromatography. To date, the HFTCPC has been used to purify over 100 synthetic peptides in amounts ranging from 20 to 1000 mg. A description including the methodology of its use is the subject of an earlier report (3). The ensuing instruments which have been developed are herein compared to the HFTCPC instrument in utility.

Winding the tubing in layers such as in a spool forms a compact multi-layer coil which is more mechanically stable to fast rotation than the wider column-coils of the HFTCPC. Thus a modular table-top instrument housing a 3 in wide coil holder is more compact than the 2 ft wide column-coil arrangement. In this type of coaxial coil when rotated at a high, critical rate, a unilateral hydrodynamic equilibrium occurs where the phases separate to the opposite ends. Thus in normal elution, the column is filled with head phase and the other phase is passed from head to tail. Under rapid centrifugation and flow rates, over 80% of the stationary phase is retained. When the coil is filled with the tail phase and the head phase is pumped from tail to head, retention of the stationary phase and very high resolution result. The coil equipped with 1.6 mm i.d. PTFE tubing has a total volume of 285 ml and the 2.6 mm i.d. tubing has an even higher volume, approximately 400 ml. Therefore the amount of sample soluble in approximately 1/10 these volumes is the limiting amount that can be loaded which is similar for the HFTCPC. Since this chromatographic system is soley liquid in open tubing, total recovery of sample is always possible. The resolution for compounds is very high when operated at a partition coefficient of 1. Previous separations of peptides such as cholecystokinin-related peptides and bombesin have been described (4,5). For the common solvent systems containing chloroform and other hydrophobic solvents used for relatively non-polar compounds, the separations can be run rapidly at room temperature. However, it was found that volatile n-butanol solvent systems, the very ones used primarily for peptides, were not retained well in the multi-layer coil. Raising the temperature

to 45°C lowered the viscosity making possible the retention of the stationary phase and hence successful chromatography (6).

Recently, the design of the HFTCPC has been re-evaluated and modifications have been made incorporating features of the above machines. The chief advantage of the earlier instrument is the complete versatility in solvent systems capable of being used at room temperature. A major advantage of the MLCPC is the high speed of the chromatography, a characteristic that overcomes the remaining disadvantage of this method relative to HPLC. A hybrid instrument called the compact HFTCPC has been constructed. The tubing is wrapped around columns of about 1/2 the width of the original machine. They are wrapped in dual layers to maintain a high volume. A faster rotation is possible and with moderate heating the flow can be increased with viscous solvent systems. Examples of chromatography of various peptides on the multi-layer and the compact instrument will be described. From the experience to date, the operating conditions of all the machines are summarized.

MATERIALS AND METHODS

Solvents and chemicals were reagent or HPLC grade. Peptides were synthesized by solid-phase techniques (7). Bovine insulin was purchased from Sigma.

Horizontal Flow-through Coil Planet Centrifuge. Two instruments described previously have been utilized for the experiments summarized here. Both instruments have 2.6 mm i.d. PTFE tubing with a total volume of 260 ml. The column coils are 24 in wide. One instrument was made by Laboratory of Technical Development, National Heart, Lung, and Blood Institute, (National Institutes

of Health, Bethesda, MD) and the other is a prototype built by Kontes Scientific Glassware/Instruments (Vineland, NJ) (3).

High Speed Multi-layer Coil Planet Centrifuge. The instrument used in these experiments also built by the Laboratory of Technical Development has been described previously (4,5,6). MLCPC instruments are commercially available from P. C. Inc. (Potomac, MD). A table top model (17.5" x 18" x 18.5") is equipped with heating pads and a temperature controller (Boonton, NJ) (Fig. 1). The 3 in wide coil consists of 1.6 mm i.d. PTFE tubing with a total volume of 285 ml.

Compact Horizontal Flow-through Coil Planet Centrifuge. The compact apparatus has 8 columns of double layered coils of 1.6 mm i.d. tubing mounted on a holder with a 10 cm radius of revolution (Fig. 2). The total capacity is 100 ml. The centrifuge is contained in housing similar to that of the MLCPC. If desired the coil can be interchanged in the holder with a multi-layer coil; thus the instrument can have dual capability. This prototype has been constructed by the Laboratory of Technical Development, National Heart, Lung, and Blood Institute.

Accessory equipment for these instruments consists of LC flow cells, detecting at 280 nm, recorders and fraction collectors holding 200 tubes. Milton-Roy Duplex mini-pumps (Sunnyvale, CA) are suitable for high speed operation. Peptides are loaded in small volumes from 5 to 10 ml of 1:1 mixture of each phase and the instruments are rotated at the RPM indicated in the Table and mobile phase pumped at flow rates specified for each instrument. Fractions of 6 ml are collected; those containing peptide are pooled, lyophilized and analyzed by HPLC and TLC for homogeneity and for amino acid composition as previously described (3).

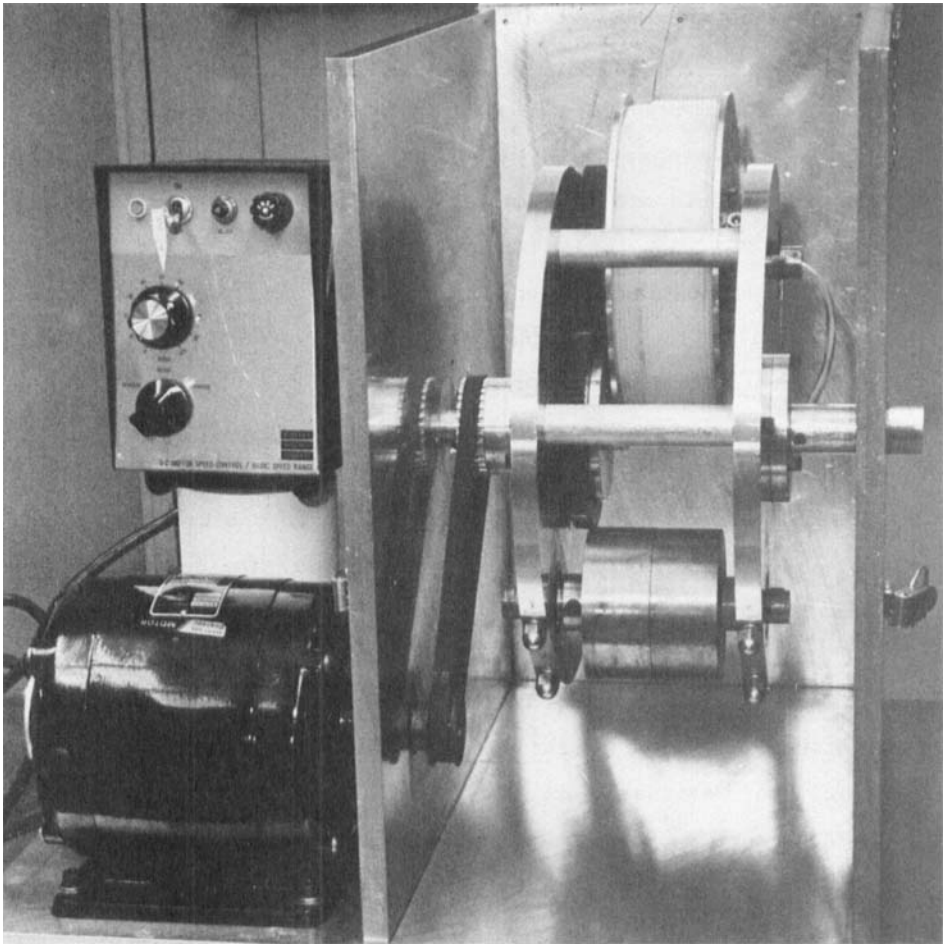

FIGURE 1

Multi-Layer Coil Planet Centrifuge with the coil consisting of PTFE tubing (upper side) mounted 10 cm from central axis of centrifuge. Counter weight on lower side. A gear mounted on the coil holder shaft is coupled to the central axis to produce the planetary motion. Not shown are electric heating pads inside the walls of the container and a temperature control unit outside. The motor and controller are shown. The inlet and outlet tubing is introduced from the left (clamped down on the outside wall) through the central axis and into the center of the coil (upper right).

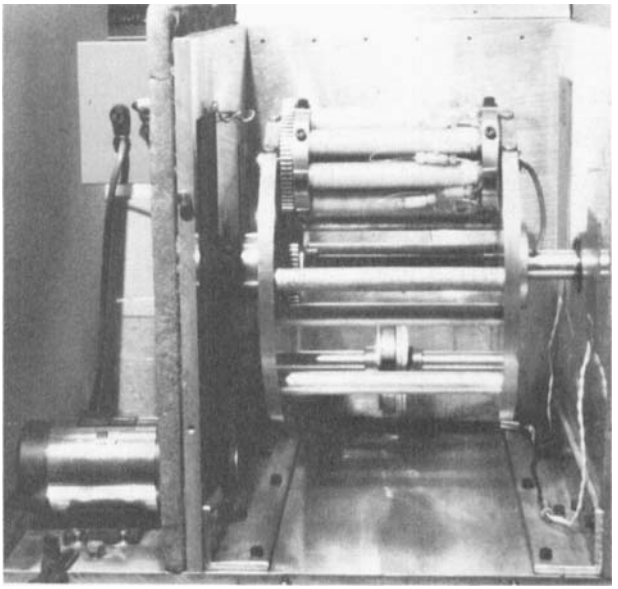

FIGURE 2

Compact horizontal flow-through (or dual-layer) coil planet centrifuge. The column holder (upper side) supports 8 rods wrapped with 2 layers of tubing. Length of the coils are 8 in. Other aspects of the construction are similar to the MLCPC. The heating pads are shown as well as the temperature controller (upper left). Urethane insulation is on outside of housing.

RESULTS

The purification of bombesin has been previously accomplished in the MLCPC using 1% dichloroacetic acid in n-butanol (5). However, a totally volatile solvent system was tried here in which bombesin was found to have a partition coefficient of 1.2 at 50°C. Synthetic bombesin, 300 mg, was chromatographed in 0.5% trifluoroacetic acid/n-butanol (1:1) with the lower phase mobile at a flow of 120 ml/hr. The chromatography appeared similar to that reported previously; the peptide eluting in fractions 51-56. With this solvent a final extraction step was avoided.

A cholecystokinin analog, Ac-Asp-Tyr-Met-Ala-Trp-Met-Asp-Phe-NH_2, was prepared by solid-phase and purified on the MLCPC (Figure 3). The conditions used were a stationary phase of the lower phase of 0.2 M ammonium acetate, pH 9.0/ n-butanol and the upper phase pumped from the tail to the head end of the coil at a rate of 150 ml/hr. The results of chromatographing 100 mg of synthetic product gave 24 mg of purified peptide and 22 mg of side product which eluted at the solvent front. Analytical HPLC indicated that purification to homogeneity was achieved in this step.

Samples of 50 and 100 mg of insulin were chromatographed at 50°C on the compact HFTCPC in 2% dichloroacetic acid/sec-butanol (1:1) with the lower phase used as the mobile phase. The flow was 50 ml/hr and rotational speed 600 RPM. The chromatogram of 100 mg is presented (Fig. 4). The resolution is equivalent to that achieved in an earlier instrument (8). If 2.6 mm i.d. tubing were used, the equivalent flow would be 150 ml/hr. Thus a high flow comparable to that in the MLCPC is possible.

DISCUSSION

The present experience with the three types of instruments has indicated particular operating conditions for peptides for each type of machine (Table). These are presented as guidelines for developing methods for polar compounds such as peptides. The last two solvent systems listed under the HFTCPC were used in purifying hydrophobic peptides and are relatively non-polar solvent systems. Apparently by decreasing the flow and centrifugation rate, it is possible to perform separations at room temperature in the multi-layer and compact HFTCPC with the polar solvents and either phase mobile (9). Operation at 45°C is

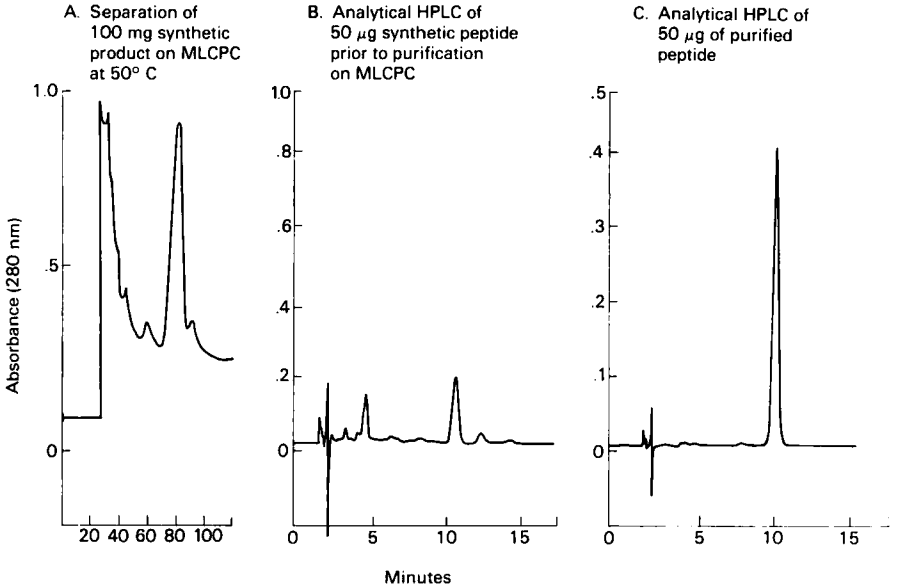

FIGURE 3

A. Purification of [N-Ac, Ala^{29}]cholecystokinin 26-33 on the MLCPC in the n-butanol/0.2 M ammonium acetate, pH 9.0, solvent system. The stationary phase used was the lower phase and the mobile upper phase was pumped from the external tail-end of the coil to the internal head-end at 150 ml/hr. The rotational speed was 800 rpm. Fractions of 6 ml or 2 min/tube were collected. The solvent front emerged at tube 14, and the purified peptide in fractions 37-44, the contents of the peak at 80 min. The yield of peptide was 24 mg.

B. HPLC of the starting material on a μ-Bondapak C_{18} column (0.4 x 30 cm, Waters Assoc. system, Milford, MA) eluted isocratically in 0.1% phosphoric acid and 33% acetonitrile at a flow of 2 ml/min.

C. Analytical chromatography of the purified peptide in the same conditions.

TABLE

PEPTIDE SEPARATION

HORIZONTAL FLOW-THROUGH COIL PLANET CENTRIFUGE

Conditions:
1. Either phase mobile
2. Room temperature operation
3. Flow, 24 ml/hr, 400 RPM

Solvent System	Volume Ratio	
0.5 to 2% dichloroacetic acid/sec.-butanol	1:1	(11)
0.2 to 0.4 M NH_4OAc/n-butanol (pH 6 to 8.5)	1:1	(3,4)
n-butanol/acetic acid/water	4:1:5	(3,12,13)
Ethanol/n-butanol/hexane/acetic acid/water	1:3:2:1:5	(3)
0.1% trifluoroacetic acid/n-butanol	1:1	(3,14)
Dichloromethane/acetic acid/water	2:1:2	(15)
Chloroform/benzene/methanol/water	15:15:23:7	(16)

MULTI-LAYER COIL PLANET CENTRIFUGE

Conditions: (for polar solvents)
1. Lower phase mobile
2. 40-50°C
3. Flow, 60-150 ml/hr, 800 RPM

Solvent System	Volume Ratio	
0.2 M NH_4OAc/n-butanol (Upper ph. mobile possible)	1:1	(4)
0.5 to 1% trifluoroacetic acid/n-butanol	1:1	(15)
0.3 to 3% dichloroacetic acid/n-butanol	1:1	(5)
n-butanol/acetic acid/water	9:1:10	(15)

COMPACT HORIZONTAL FLOW-THROUGH (OR DUAL-LAYER)

COIL PLANET CENTRIFUGE

Conditions:
1. Same conditions as HFTCPC
2. At 40 to 50°C - flow increased to 100 ml/hr
3. Centrifugation increased to 800 RPM

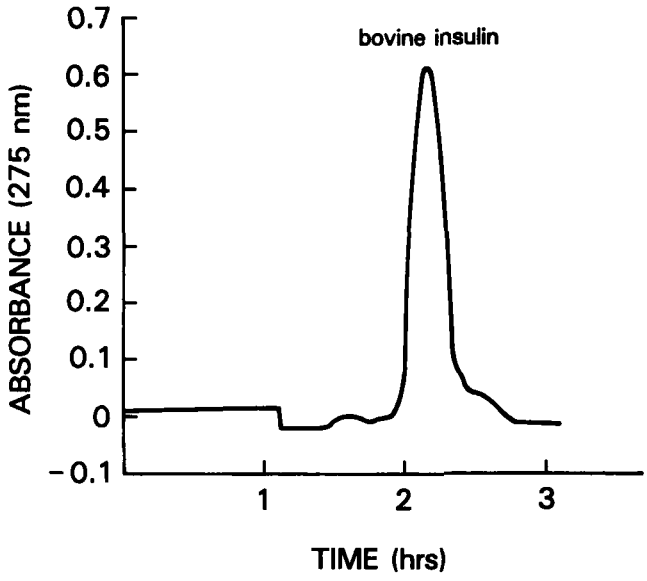

FIGURE 4

Countercurrent chromatogram of 100 mg bovine insulin. Absorbance at 275 nm of the run is shown. Fractions of 6 ml were collected. The chromatography was conducted at 50°C at a flow of 50 ml/hr and at 600 RPM. The lower phase of 2% dichloroacetic acid/n-butanol was pumped from head to tail. The solvent front emerged at 1 hr.

recommended for rapid chromatography using n-butanol or sec.-butanol. The prediction of elution is possible by a simple partition coefficient determination which can help in the selection of the solvent system and the best conditions for resolution using either the upper or the lower phase as the mobile phase (3).

The multi-layer CPC and the compact HFTCPC offer the advantage of high speed chromatography. It is important for future machines being made that the temperature be well controlled at the minimun necessary for good stationary-phase retention. The current maximum capacity of these instruments afford-

ing good resolution is 1000 mg. Development in instrument design is underway to enable separation with high efficiency of multiple grams of material (10).

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1. Abbreviations used: HPLC = high performance liquid chromatography; LC = liquid chromatography; MLCPC = multi-layer coil planet centrifuge; HFTCPC = horizontal flow-through coil planet centrifuge; CPC = coil planet centrifuge; TLC = thin layer chromatography; PTFE = polytetrafluoroethylene.
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