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CHROMATOGRAPHY OF PEPTIDES ON A MULTI-COIL COUNTER-CURRENT CHROMATOGRAPH

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SUMMARY

A modified horizontal flow-through coil planet centrifuge has been constructed that has the potential for preparative chromatography. The multi-coil counter-current chromatograph, equipped with a set of four multi-layer coils, has have improved performance with polar solvents suitable for peptide elution. Separations of a group of dipeptides and purification of a cholecystokinin fragment and an undecapeptide were achieved with *n*-butanol–acetic acid–water at high flow-rates with good resolution.

INTRODUCTION

Coil planet centrifuges[1] are modern instruments for counter-current chromatography, a preparative method in use prior to the development of high-performance liquid chromatography (HPLC). Although peptides can be chromatographed by reversed-phase and ion-exchange chromatography, the option of the many solvent systems possible with counter-current chromatography makes this method attractive. With a suitable two-phase solvent composition devised for a particular separation, high selectivity is possible.

Over the years we have used a horizontal flow-through coil planet centrifuge for the preparative purification of synthetic peptides[1], but the flow-rates and centrifugal rates were slower than on later designed instruments for counter-current chromatography[2]. However, as this instrument was able to utilize the more polar solvent systems useful for peptides, this instrument has been continuously applied for this purpose and is the design that can be used with all solvent systems at room temperature[3]. Previously reported was an apparatus with a narrower frame holding a column-coil with more layers of tubing, thus maintaining the volume of the older instrument but able to be centrifuged faster[4]. When various types of coils were tested on the column holder, a multi-layer coil was found to have the highest resolution in chromatography. More recently, to increase the capacity further, an instrument fitted with four multi-layer coils connected in series by the flow tubing has been built and found to operate at increased centrifugal speed and flow-rate[5]. The separation of enzymes in a poly-

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ethylene glycol solvent system was achieved using this instrument. This instrument has been assessed for its capability to chromatograph peptides under high-performance conditions.

The serial multi-layer coil planet centrifuge, which we shorten to multi-coil counter-current chromatograph, is a modification of the horizontal flow-through coil planet centrifuge equipped with a set of four counterbalancing multi-layer column-coils of tubing around a column holder. These are parallel and displaced from the center axis of rotation of the centrifuge. The open tubing throughout the system connects the coils in series for increased volume in which the solvent equilibration and solute partitioning take place. Fig. 1 is a photograph of the prototype at the Laboratory of Technical Development, National Institutes of Health. The rotary frame carries a column holder on one side and a counterbalance on the other. Both are at a distance of 10 cm from the central axis. Engagement of the planetary gear mounted on the holder shaft with the identical stationary sun gear on the central axis of the apparatus produces the desired planetary motion of the holder, *i.e.*, revolution of the holder around the central axis of the apparatus and simultaneous rotation of the holder about its own axis at the same angular velocity in the same direction. The mixing induced by the motion maintains an equilibration of the two phases in the coils. The force holds a stationary phase of a two-phase liquid with the other phase being pumped through. Thus, if one phase is continuously pumped through, the excess leaves at the other end, allowing a continuous-flow elution mode.

As models for the performance of earlier prototype instruments, dipeptides have been chromatographed in flow-through coils, toroidal coils, non-synchronous instruments and the multi-layer coil planet centrifuge[6]. Dipeptides containing aromatic

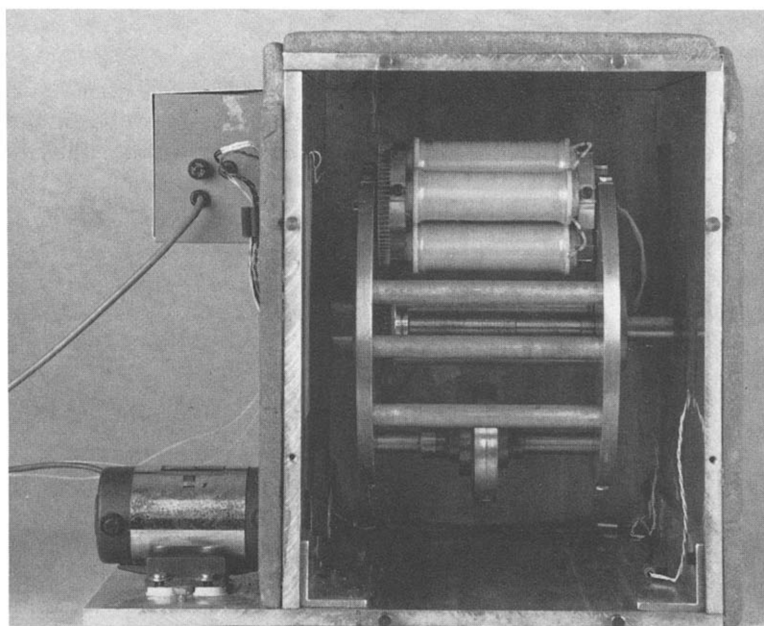

Fig. 1. Photograph of multi-coil counter-current chromatograph (from ref. 5).

amino acids for detection have been applied for the determination of the resolution and selectivity. Another peptide previously purified in the horizontal flow-through coil planet centrifuge is a cholecystokinin (CCK) fragment peptide CCK(26–30) amide[7], which when unsulfated is not soluble in water. This is an example of small, highly aromatic acidic peptides that contain substantial amounts of solid-phase synthesis side products, and the mixture is difficult to chromatograph on reversed-phase columns owing to solubility problems. Another peptide was chromatographed that had also been purified in the older instrument.

EXPERIMENTAL

Materials

The dipeptides were purchased from Sigma (St. Louis, MO, U.S.A.) and solvents from Burdick and Jackson (Muskegon, MI, U.S.A.). The peptides were synthesized manually in a table-top shaker (St. Johns Assoc., Beltsville, MD, U.S.A.) using methods described previously[8]. The identity and purity were established by amino acid analysis and HPLC.

Instrument

The apparatus has been described previously[5] (Fig. 1). The four coils around the holder are prepared from 1.6 mm I.D. polytetrafluoroethylene (PTFE) tubing (Zeus Industrial Products, Raritan, NJ, U.S.A.) wound on a spool 12.5 cm wide and 1.25 cm across in six layers. The entire coil and flanges are enclosed in a heat-shrunk poly(vinyl chloride) covering. The coils are equally spaced around the column holder shaft at a distance of 3.5 cm from the holder axis. Each coil has a volume of 50 ml, thus the total volume is 200 ml. The tubing is connected from the outside of the first column to the inside end of the next column and so on; the inlet and outlet tubing ends pass through the holder shaft and through the central stationary pipe. At the exit the flow tubes are clamped so they do not twist during the motion. The revolutional speed control unit regulates the speed up to 1200 rpm (Bodine Electric, Chicago, IL, U.S.A.). The unit is equipped with heating pads and a temperature-control unit (RFL Industries, Boonton, NJ, U.S.A.) that was not utilized in these experiments.

Procedure

The solvent system used for all the experiments was *n*-butanol–acetic acid–water (4:1:5, v/v/v), which was separated into two phases in a separatory funnel. The coil was filled with the upper phase, then the sample, dissolved in equal volumes of both phases, was loaded. The rotation was started and usually run at 800 rpm with the mobile phase or lower phase pumped through at 1 ml/min using a minipump (LDC/Milton Roy, Riviera Beach, FL, U.S.A.). The eluent was passed through an LKB Uvicord S flow cell detecting at 275 nm and recorded in an LKB six-channel chopper recorder (LKB, Gaithersburg, MD, U.S.A.) and collected in an LKB Ultrorac fraction collector in 3-ml fractions. For some experiments, aliquots of fractions were diluted with water or methanol and the absorbance was determined manually. After the run, the contents of the coil were flushed out with nitrogen and the volumes of the stationary and mobile phases were measured. The fractions from the undecapeptide chromatography were evaporated to dryness in a centrifugal evaporator (Savant Instruments, Farmingdale,

NY, U.S.A.). The fractions were analysed by HPLC as described previously[9] on a μBondapak C_{18} column (15 × 0.4 cm I.D.) (Waters/Millipore, Milford, MA, U.S.A.) in 0.1% aqueous phosphoric acid and acetonitrile gradients at 0.8 ml/min with detection at 280 nm using Waters/Millipore equipment consisting of a manual U6K injector, two Model 510 pumps with extended flow heads, a Model 680 gradient controller, a Model 681 variable-wavelength UV detector and an SE120 recorder.

RESULTS AND DISCUSSION

A sample load of 30 mg each of Tyr–Gly, Val–Tyr and Leu–Tyr and 10 mg of Tyr–Trp was dissolved in 6 ml of the solvent system and introduced into the coil (head end of first coil). The rotation was set at 850 rpm with the flow-rate at 1 ml/min in the head-to-tail direction and fractions of 3 ml were collected. The solvent front emerged at the 44th tube and all the components were eluted in 5 h. The run was stopped after 6 h. Samples of 0.1 ml were diluted with 1 ml of methanol and the absorbance was determined (Fig. 2). The retention of the stationary phase under these conditions was 33.8%. There was almost baseline separation of each dipeptide and the separation of Val-Tyr and Leu–Tyr showed good selectivity. The order of elution corresponds to increasing hydrophobicity.

CCK (26–30) amide, owing to the final extraction steps in the synthesis and the presence of Trp and Met, which can form many alkylation side-products, has a significant amount of extra mass. A load of 500 mg was chromatographed at 800 rpm and the chromatogram is shown in Fig. 3. Fractions 78–95 contained the purified peptide with a recovery of 67 mg. The large amount of impurities was easily removed with fractions 106–122 contaning a peptide side-product. The partition coefficient (*K*) calculated from the run as the concentration of the solute in the mobile phase divided by the concentration in the stationary phase[7,10], was 0.18. The retention of the stationary phase was 30.2% and the solvent front emerged at fraction 21. This was a high mass load for the 200-ml coil but examination of the mass recovered indicated that the peptide present was recovered. For a suitable solvent system for a compound, the capacity is limited by the solubility. The fractions with the pure peptide were flash

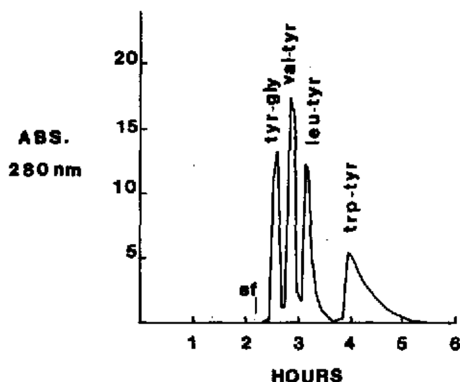

Fig. 2. Total absorbance units of the fractions from the chromatography of four dipeptides.

Ac-Asp-Tyr-Met-Gly-Trp-NH_2

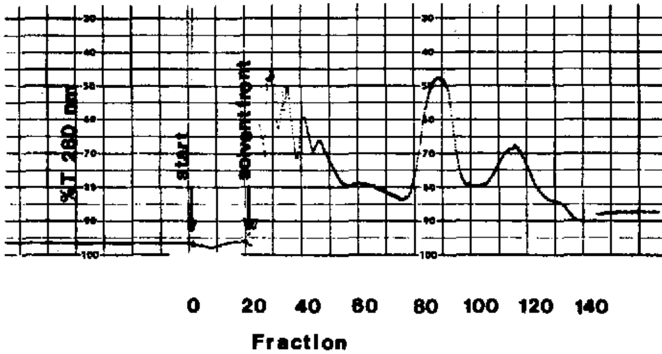

Fig. 3. Recording of chromatographic run of CCK (26–30) amide, 500 mg. Ac = Acetyl.

evaporated and lyophilized in a small volume of glacial acetic acid and the peptide was later sulfated.

The same conditions were used for the preparative purification of 225 mg of the undecapeptide acetyl–Glu–Glu–Trp–Asp–Pro–Ser–Asp–Gln–Glu–Pro–Cys–NH_2 at a centrifugal rate of 850 rpm (Fig. 4). The fractions across the peak were analysed by HPLC. The results are shown in Fig. 5. The peak tubes contained the most pure peptide. The fractions were combined in three pools with recoveries of (53–56) 27 mg, (57–59) (purest fraction) 54 mg and (60–67) 37 mg, with a total mass recovery of 52% in the peak. The K value for this compound was 1.6 in this solvent system and the stationary phase retention was 40%. The earlier and later eluting material was analysed by HPLC and appeared heterogeneous, as shown in Fig. 6, and the retention times corresponded to the order of elution from the counter-current coil.

These results indicate that the multi-coil counter-current chromatograph is useful for the preparative-scale chromatography of polar compounds such as peptides. The CCK fragment and the undecapeptide that had also been purified in the older horizontal flow-through instrument were chromatographed faster here. Specifically, the CCK peptide was eluted in 9 h compared with almost 40 h in the older instrument

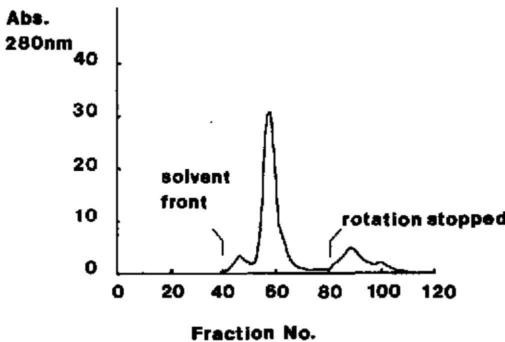

Fig. 4. Total absorbance of the fractions from the chromatography of the synthetic undecapeptide.

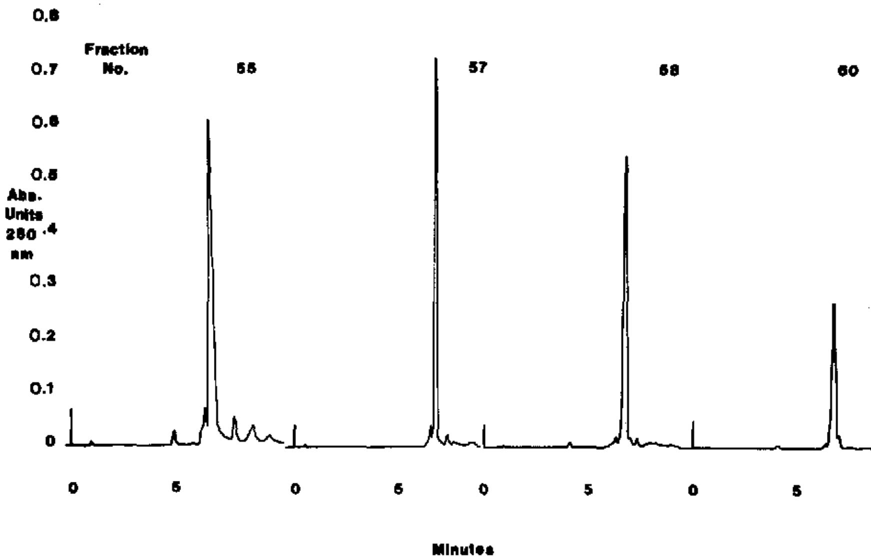

Fig. 5. Analytical HPLC of peak fractions on a μBondapak C_{18} column (15 × 0.4 cm I.D.) in 0.1% phosphoric acid with a gradient of 10–20% acetonitrile in 10 min at a flow-rate of 0.8 ml/min. Detection was at 280 nm (1 a.u.f.s.).

with the same solvent system and mobile phase[7]. The undecapeptide was eluted in 3 h whereas, using the upper mobile phase in the older instrument it was eluted in 13 h. For particular peptides that are not water soluble, this procedure can remove insoluble impurities easily without the requirement for elaborate procedures to clarify solutions

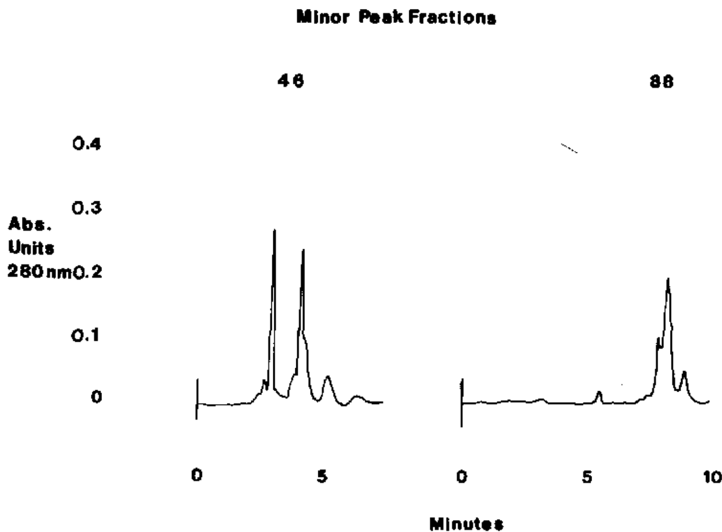

Fig. 6. Analytical HPLC of side-fractions of the undecapeptide separation under the same conditions as in Fig. 5.

for loading onto the chromatograph. We are currently assessing a new prototype (project with Varex, Burtonsville, MD, U.S.A.) with more coils for increased volume and sample capacity.

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