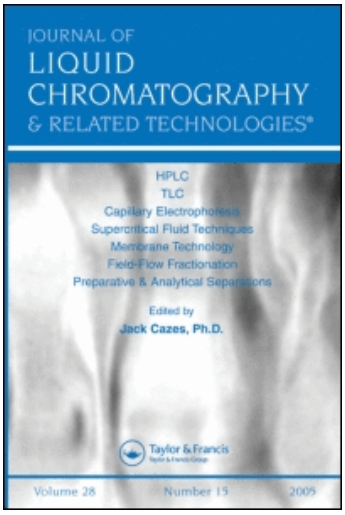

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RAPID PURIFICATION OF SYNTHETIC BOMBESIN BY COUNTERCURRENT CHROMATOGRAPHY ON THE MULTI-LAYER COIL PLANET CENTRIFUGE

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ABSTRACT

A temperature-controlled multi-layer coil planet centrifuge rapidly yields highly efficient preparative separations of polar compounds. Capability of the method was demonstrated on a one-step purification of crude synthetic bombesin with a two-phase solvent system composed of n-butanol/dichloroacetic acid/water (100:1:100). Under an elevated temperature at 45°C to 50°C, the bombesin peak was eluted within two hours. Reversed phase HPLC analysis of the bombesin fractions showed over 98% purity. The method may be applicable to many other peptides and polar compounds.

INTRODUCTION

The last few years have witnessed a rapid development of new methodology for countercurrent chromatography (CCC). This method is

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capable of separating large amounts of substances according to their partitioning between the stationary and the mobile phases of a two-phase solvent system (1-3). This process, which utilizes an apparatus consisting of a long continuous coil, has led to the design of the horizontal flow-through coil planet centrifuge (4). More recently the instrumentation has been modified to a more compact apparatus, the multi-layer coil planet centrifuge, which contains features that allow more rapid rotation and flow rates (5).

The present advance in CCC technology has come about from the discovery of the unilateral hydrodynamic equilibrium behavior of two-phase solvent systems within a rotating coaxial or multi-layer coil. During rapid rotation at a critical rate, the two phases therein separate to the opposite ends. In general, the upper or less dense phase (head phase) is distributed to the head side and the lower phase (tail phase) goes to the tail end of the coil. Thus countercurrent chromatography can be performed at a high centrifugation rate in a normal elution mode where the column is filled with the head phase and the other phase is pumped from head to tail at a rapid flow rate. A great deal of the stationary phase is thus retained (80%). Also the reversed elution mode is possible whereby the coil is filled with the tail phase and the head phase is pumped from tail to head. The rapid centrifugation rate which enhances the acceleration field produces high retention of the stationary phase. The multi-layer coil planet centrifuge possesses great resolving power and high sample capacity. Recently it has been observed that the hydrodynamic distribution of highly viscous solvent phases in the coiled column results in less retention of the stationary phase. It was later found that performing the separations under elevated temperature prevented loss of the stationary phase at high flow rates (6). This

modification allowed the separation of peptides efficiently at elevated temperatures (7) in n-butanol solvent systems. On the multi-layer instrument separations have been achieved in 30 min, as compared to 7 hr on the flow-through coil planet centrifuge, an earlier CCC instrument (7). With the high-speed CCC possible with the multi-layer coil planet centrifuge, the last "disadvantage" of CCC is surmounted and rapid and efficient preparative separations are now possible.

The multi-layer coil planet centrifuge was evaluated using an n-butanol system for the purification in one step of solid-phase synthesized bombesin. This peptide, isolated from frog skin, has various physiological effects and is found in the mammalian central nervous system and small cell lung carcinoma (8). Synthetic bombesin that appeared heterogeneous by HPLC was obtained in the synthesis. The peptide was chromatographed on the instrument in a solvent composition that gave a partition coefficient of 1. The recovered peptide was analyzed for purity.

MATERIALS AND METHODS

All reagents were analytical grade and solvents were analytical or HPLC grade. Trifluoroacetic acid was from Halocarbon (Hackensack, NJ). The amino acid derivatives were from Bachem or Peninsula Laboratories (Belmont, CA) and p-methyl benzhydrylamine resin was from U. S. Biochemical Corporation (Cleveland, OH). A standard of bombesin was purchased from Bachem (Torrance, CA).

Synthesis

Bombesin (PCA-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2) was synthesized in a Beckman Model 990B synthesizer (Palo Alto, CA) starting with 1 g of Boc Met p-methyl benzhydrylamine resin with a

substitution of Boc Met of 0.5 mmol/g (9,10). The synthesis proceeded with 3 X molar excess of Boc amino acid and the coupling agent, dicyclohexylcarbodiimide (DCC). The schedule of synthesis involved washing with methylene chloride, deprotection with 25% trifluoroacetic acid and 1 mg/ml indole, washing, neutralization with 10% triethylamine in methylene chloride, washing, addition of Boc amino acid then DCC, coupling for 2 hr, then washing with methylene chloride and repetition of the cycle through the amino-terminal residue which was coupled twice. Pyroglutamic acid and the Boc amino acids, im Tosyl His, Xanthydryl Gln and Xanthydryl Asn were coupled with DCC in dimethylformamide. The peptide-resin was cleaved by treatment with anhydrous hydrogen fluoride at 0°C for 45 min in the presence of 1 ml anisole and 200 µl ethyl methyl sulfide. The resin was washed with ethyl acetate, dried and extracted with glacial acetic acid. The yield of crude product was 260 mg.

Chromatography on the Multi-layer Coil Planet Centrifuge

The partition coefficient of the peptide was determined in a number of solvent systems (Table 1). The chromatography runs were performed in a table top model of the multi-layer coil planet centrifuge equipped with a temperature control system (Laboratory of Technical Development, NHLBI, Bethesda, MD) (Fig. 1). Aliquots of 100 to 120 mg synthetic peptide were chromatographed to determine best conditions. However, the coil could have been loaded with all of the sample. The apparatus had a medium-sized coil of 1.6 mm i.d. polytetrafluoroethylene tubing with a total capacity of 280 ml. The column was mounted on a holder 4-in away from the central axis (β = 0.5-0.8, ratio of radius of the column coil to radius of centrifuge). The two-phase solvent system was equilibrated in a separatory funnel at 45°C and the coil was filled with the upper phase. The lower phase used as the mobile phase was introduced through the inner head end of the coil from the reservoir kept at 45°C in a water bath.

TABLE 1

Partition Coefficients of Bombesin in N-butanol Solvent Systems at 45°C

Solvent Composition	Volume Ratio	Partition Coefficient*
n-butanol/dichloroacetic acid/water	1:0:1	0.27
	1:.01:1	1.00
	1:.02:1	1.59
	1:.04:1	2.06
	1:.06:1	2.78
n-butanol/acetic acid/ water	4:1:5	0.19

* Partition coefficient is defined here as solute concentration in the nonaqueous phase divided by that in the aqueous phase.

The solvent was pumped at a rate of 150 ml/hr by a Milton Roy mini-pump (Sunnyvale, CA). The planet centrifuge was rotated at 800 rpm under a controlled temperature of 45-50°C. The effluent passed through an LKB Uvicord S monitor (Gaithersburg, MD) where the absorbance at 275 nm with 0.5 units full-scale was detected. Fractions of 5 ml or 2 min were collected. After the chromatography the absorbance of the tubes were measured manually and the tubes containing the major peak fractions were pooled and extracted 3 times with equal volumes of anhydrous ethyl ether and the resulting solution was lyophilized.

Analysis

The purified peptide was analyzed for homogeneity by high performance liquid chromatography (HPLC). Samples were chromatographed on a μBondapak reversed phase column (0.4 x 30 cm, Millipore Water's

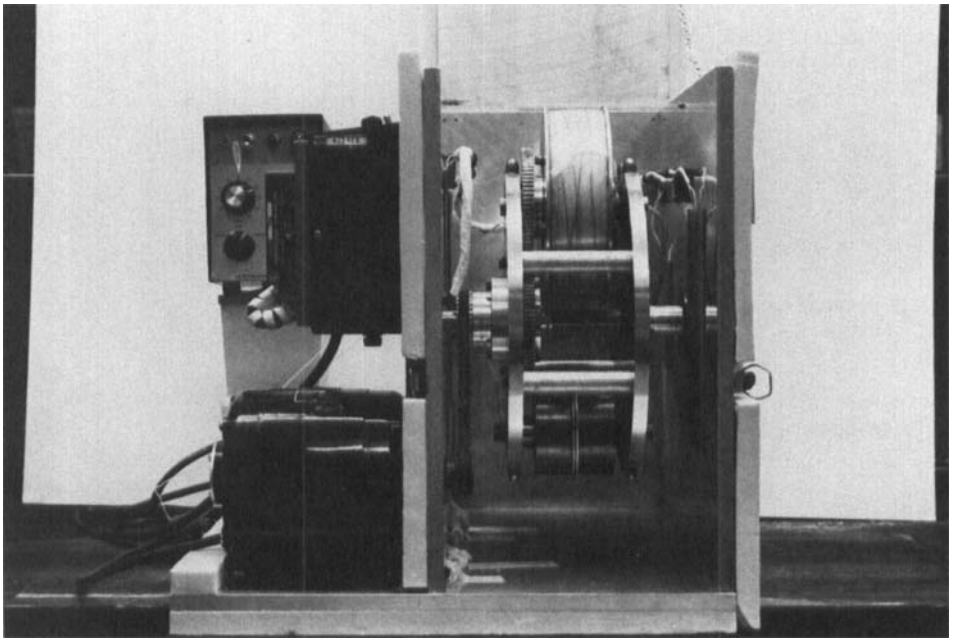

Figure 1

High-Speed CCC apparatus (17.5" x 18" x 18.5") equipped with a temperature control system. The rotary frame of the coil planet centrifuge holds a multilayer coiled column (upper side) and a counterweight (lower side) at a distance of 10 cm from the central axis of the centrifuge. An aluminum gear mounted on the column holder shaft is coupled to an identical stationary gear mounted on the central pipe to produce the desired planetary motion of the column holder. Several electric heating pads are pasted onto the inner wall while the whole unit is insulated with urethane on the outside. The column temperature is regulated up to 60°C with a temperature control unit.

Chromatography Division, Milford, MA) in 0.1% phosphoric acid with a gradient of acetonitrile from 10% to 30% in 15 min at a flow rate of 2 ml/min. Amino acid analysis was performed on a 6 N hydrochloric acid hydrolysate.

RESULTS

Since the peptide was found to have a partition coefficient of 1 in n-butanol/1% dichloroacetic acid (Table 1), this solvent system was used for the chromatography. The aqueous phase solvent front emerged at the 11th fraction. During the run the retention of stationary phase was 80%

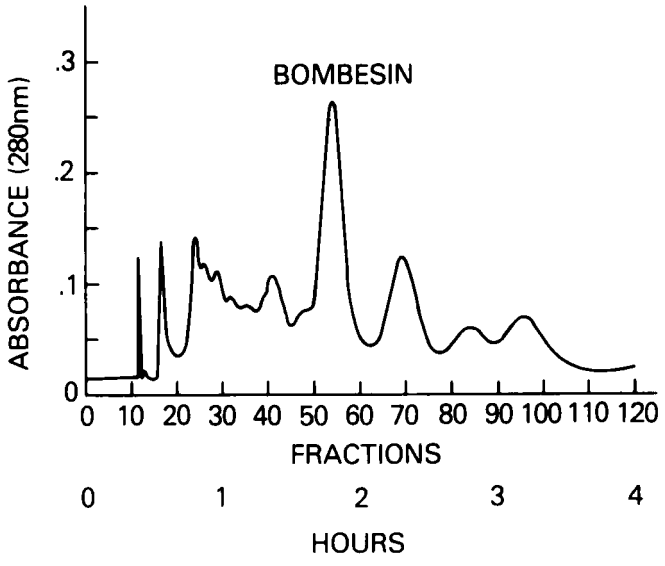

Figure 2

Countercurrent chromatogram of 120 mg synthetic bombesin. Absorbance at 280 nm of the 6 ml fractions is shown.

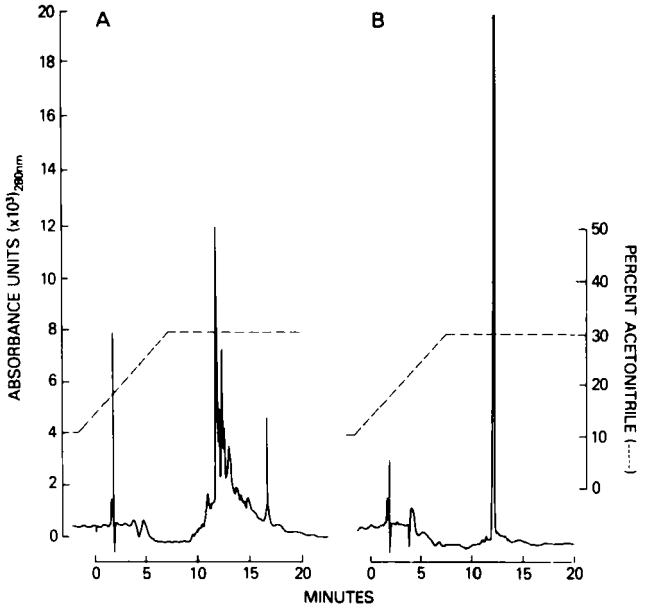

Figure 3

(A) Reversed phase HPLC analysis of 7.5 µg of the crude synthetic product and (B) analysis of 10 µl of fraction 55 of the countercurrent chromatography. Absorbance tracing at 280 nm is shown. The dotted line is the percent of acetonitrile.

and no carry-over of upper phase occurred. The recorder tracing of the chromatography of 120 mg indicated that the major material eluted between fractions 53-58 (Fig. 2). The absorbance of the fractions were read manually to confirm the location of the material in case the speed of the elution may have caused artifacts. The absorbance readings of the peak did coincide. The K calculated from the elution volume of the peptide was 1 as predicted from the previous partition coefficient determination. The pH of the pooled fraction containing the aqueous phase of the solvent system was 2.7 and was raised to 6.5 after 3 extractions with ethyl ether.

Yield of lyophilized powder was 98 mg or 81.6% recovery by weight. In analytical HPLC the peptide appeared homogeneous and over 98% pure (Fig. 3). Amino acid analysis indicated the presence of the amino acid residues in the following molar ratios: His, 0.90; Arg, 0.99; Asp, 1.07; Glu, 2.80; Gly, 1.83; Ala, 1.02; Val, 1.00; Met, 0.83; Leu, 2.03.

DISCUSSION

The past advances in peptide synthesis made possible by the solid-phase chemistry, has resulted in the demand for reliable purification methods suitable for the effective fractionation of preparative amounts of synthetic peptides. At present limited amounts of peptides are purified by tedious procedures of multiple injections on HPLC columns (11). The rigid qualities of the silica spheres with chemically bonded phases allow good resolution. However, the scaling up of column chromatography to preparative amounts has remained a challenge. The excellent resolution is sacrificed by the difficulty of maintaining high flow rates and high pressure. For preparative separations, on HPLC theoretical plates of 100 are operationally attained. Column methodologies are taxed with loss of sample and limited reusability of the supports which are a significant cost factor. Thus a reconsideration of countercurrent chromatography as performed in the multi-layer coil planet centrifuge is encouraged.

The dichloroacetic acid system used in the experiment proved to be a versatile system because a desirable partition coefficient of 1 could be attained by adjusting the amount of acid. Despite the fact that dichloroacetic acid is not volatile it was removed by extraction. However, care should be taken to avoid ether with significant amounts of

peroxides that may oxidize the peptide. The synthesis resulted in 50% yield of crude peptide. Nevertheless, the product appeared to be composed primarily of the expected peptide as determined by HPLC which is a good synthetic result considering that no monitoring or re-coupling was done during the automatic synthesis except for the final amino acid. Since the purpose of the experiment was to purify a non-homogeneous product, highly involved analytic and synthetic maneuvers were not performed during the synthesis. The purification was easily accomplished as the results presented here indicated. A large amount of the peptide was purified in less than 2 hr of chromatography. From the shape of the chromatographic peak the theoretical plates were calculated to be over 500, which is better than that achieved with 1 inch HPLC columns. These results along with those of other peptides (7) hopefully demonstrate the promise of reliable preparative chromatography that is possible with a simple instrument that requires only solvents and no solid supports. The choices of using any solvent system (12) and of either upper phase or lower phase for elution allow the development of conditions for total purification of peptides and other compounds in one rapid step.

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