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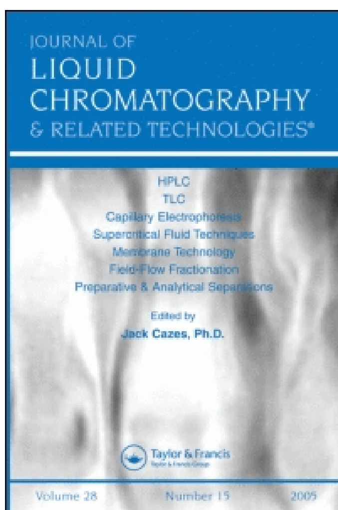

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
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Chromatography of AC-ASP-TYR-MET-GLY-TRP-MET-ASP-NH_2 on the Horizontal flow-Through Coil Planet Centrifuge and the High-Speed Multi-Layer Coil Planet Centrifuge

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To cite this Article Knight, M. , Ito, Y. , Kask, A. M. and Chase, T. N.(1984) 'Chromatography of AC-ASP-TYR-MET-GLY-TRP-MET-ASP-NH_2 on the Horizontal flow-Through Coil Planet Centrifuge and the High-Speed Multi-Layer Coil Planet Centrifuge', Journal of Liquid Chromatography & Related Technologies, 7: 13, 2525 — 2533

To link to this Article: DOI: 10.1080/01483918408067022

URL: http://dx.doi.org/10.1080/01483918408067022

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JOURNAL OF LIQUID CHROMATOGRAPHY, 7(13), 2525-2533 (1984)

CHROMATOGRAPHY OF AC-ASP-TYR-MET-GLY-TRP-MET-ASP-NH_2 ON THE HORIZONTAL FLOW-THROUGH COIL PLANET CENTRIFUGE AND THE HIGH-SPEED MULTI-LAYER COIL PLANET CENTRIFUGE

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ABSTRACT

The peptide Ac-Asp-Tyr-Met-Gly-Trp-Met-Asp-NH_2 was purified by countercurrent chromatography in the horizontal flow-through coil planet centrifuge. The solvent system used was ammonium acetate, pH 8.5 and n-butanol (1:1 by volume). The high pH served to maintain the peptide in solution. When the upper phase was utilized as the mobile phase better separation of the peptide from impurities resulted. The peptide was also chromatographed in a new apparatus, the high-speed multi-layer coil planet centrifuge. With the lower phase mobile and at a higher temperature, the peptide was fractionated very rapidly in 30 min compared to 7 hr on the other instrument.

INTRODUCTION

Ac-Asp-Tyr-Met-Gly-Trp-Met-Asp-NH_2 is an N- and C-terminal protected cholecystokinin fragment that is important in the study of the mechanism of action of cholecystokinin (CCK) because the sulfated peptide is a CCK antagonist in the pancreas and interacts

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0148-3919/84/0713-2525$3.50/0

with CCK receptors in pancreas and brain (2). We previously reported the synthesis and purification of this peptide on the horizontal flow-through coil planet centrifuge (3,4), but have recently modified the purification procedure. The solvent system used previously, an n-butanol, acetic acid system, caused a large loss of peptide which came out of solution during the run. Since the peptide proved to be highly soluble in dilute basic solutions we changed to an n-butanol, ammonium acetate system and modified the pH. In addition, purification of the synthetic peptide at high speeds was tried on the multi-layer coil planet centrifuge (MLCPC), a new apparatus for countercurrent chromatography (CCC) which is designed for rapid fractionation of substances in two phase solvent systems with a large retention of stationary phase at higher rotation rates and flow rates. Details of the design of the MLCPC apparatus are described elsewhere (5,6).

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METHODS

Solvents used in synthesis and purification were reagent grade. Water was deionized and charcoal filtered (Hydro Service and Supplies, Durham, NC) and solvents for chromatography were HPLC grade. The peptide was synthesized by solid-phase procedures (2) on a Beckman Model 990B synthesizer (Beckman, Palo Alto, CA). The synthesis was started with 1 mm Boc β-Benzyl-L-Aspartyl benzhydrylamine resin to which 2.5 mm Boc amino acids were coupled for 2 hr with equimolar dicyclohexylcarbodiimide in the desired

sequence. After each coupling the peptide was deprotected with 25% trifluoroacetic acid in methylene chloride and neutralized with 10% triethylamine in methylene chloride. After coupling with the N-terminal aspartate residue the peptide was acetylated with acetyl-imidazole. The peptide was deblocked and cleaved from the resin by liquid hydrogen fluoride. A Kontes prototype flow-through coil planet centrifuge (serial no. 2) (Vineland, NJ) equipped with 2.6 mm i.d. PTFE tubing for a total volume of 260 ml was used for counter-current purification (7,4). The solvent system of n-butanol and 0.2 M ammonium acetate, pH 8.5 (adjusted with NH_4OH) (1:1) was used with either the upper or lower phase mobile. The column-coil was rotated at 400 rpm. The effuent was monitored at 280 nm. Fractions of 15 min or 6 ml were collected as previously reported (4).

The peptide was also chromatographed on a table top model of a MLCPC (Laboratory of Technical Development, NHLBI, Bethesda, MD) equipped with a controlled heating system that maintained the coil at 45°C during the run. For the n-butanol solvent systems carryover of stationary phase occurred during the chromatography at room temperature. This was observed to be related to the longer separation time of the phases. The increase in temperature shortened the separation time by decreasing the viscosity of the solvent system (8). Another modification of the instrument was the substitution of a metal gear in place of the plastic gear which became deformed during the high temperature conditions. The multi-layer coil is comprised of 1.6 mm i.d. PTFE tubing with a total capacity of 285 ml coiled concentrically around a 3-in wide spool-

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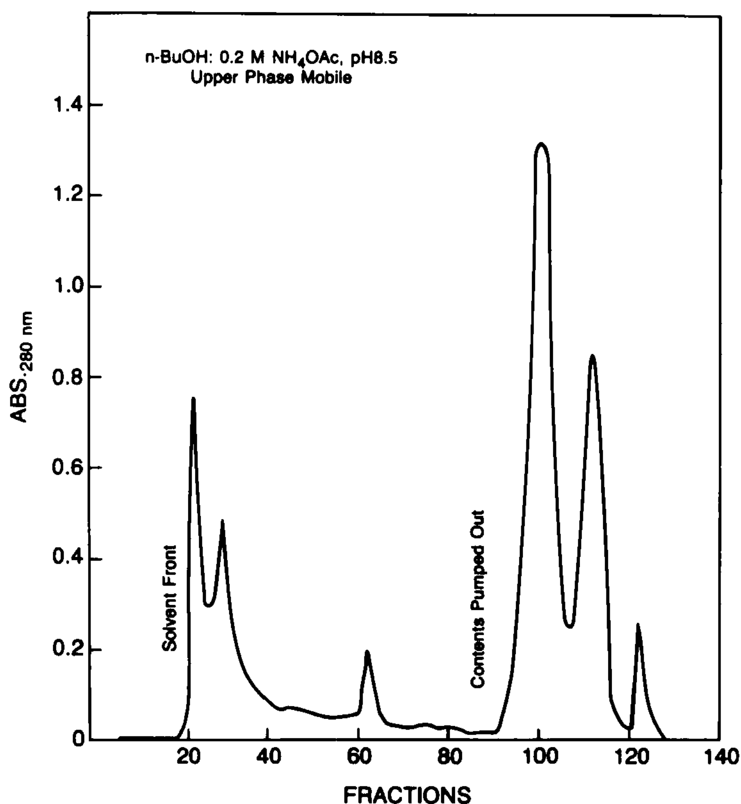

FIGURE 1

Chromatography of 300 mg peptide on the HFCPC in 0.2 M ammonium acetate pH 8.5 and n-butanol, upper phase mobile. Solvent front emerged at fraction 20. Rotation was stopped at fraction 86. Fraction (94-106) contained purified peptide. Absorbance of the fractions diluted 1/200 is shown.

shaped column holder which was mounted 4-in away from the central axis of revolution. The solvent system was equilibrated at 50°C in a water bath and during the separation both the coiled column and the mobil phase reservoir were maintained at the same temperature. These conditions produced an extremely high retention of the stationary phase at 82% of the total column capacity. Apparently the

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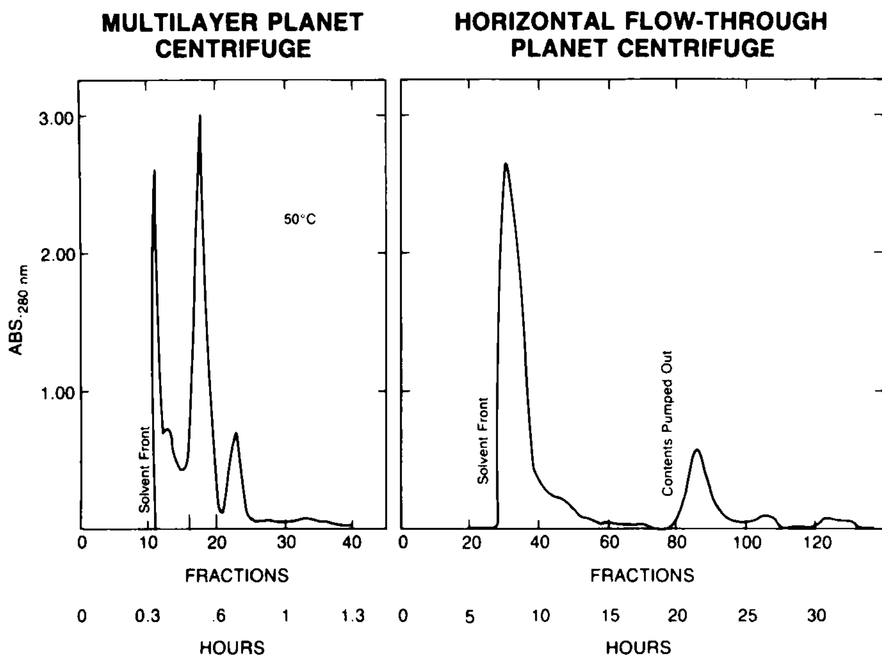

FIGURE 2

Comparison of the chromatography of synthetic peptide on the MLCPC (left) and HFCPC (right). The absorbance of the fractions is shown. The fractions are indicated by tube number and time. Lower phase of ammonium acetate system was mobile in both experiments. Pure peptide was contained in fractions (16-20) and (33-39) respectively.

hydrodynamic behavior of this volatile solvent system in the high-speed MLCPC is different from other more hydrophobic solvent systems which commonly contain chloroform. The basis for these modifications of the operating conditions of high-speed CCC are described in detail by Ito and Conway (8). The flow rate was 150 ml/hr and rotational speed was 800 rpm. The effluent was monitored by U.V. absorbance and fractions of about 6 ml or 2 min were collected. The purified peptide was sulfated by pyridine-

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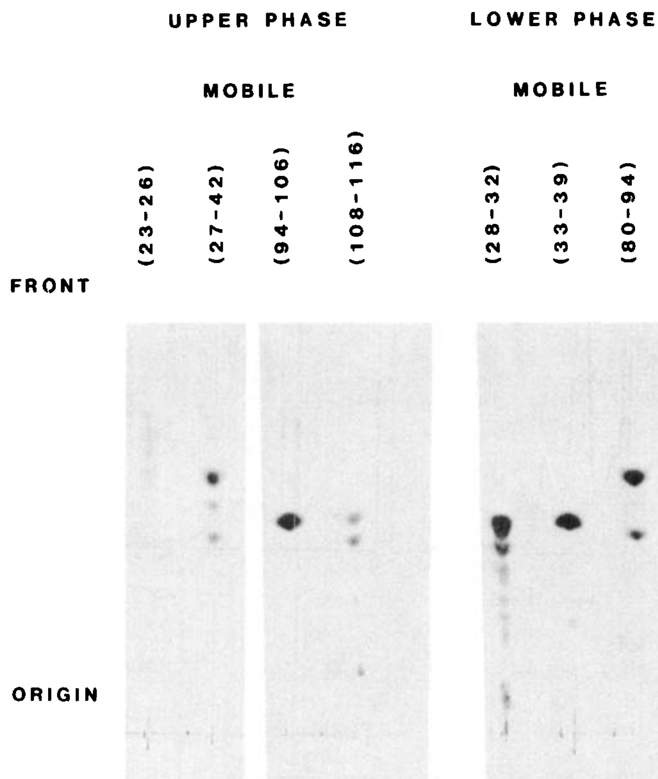

FIGURE 3

TLC (Silica gel, BAW system 4:1:1 by volume) of the content of the fractions from HFCPC run in both conditions of eluting with upper and lower phases of the ammonium acetate system, pH 8.5. Compounds were revealed by the Ehrlich spray.

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sulfur trioxide and repurified according to previously described procedures (2).

RESULTS

From a 1 mmole synthesis about 600 mg of product resulted. A sample of 300 mg was chromatographed on the HFCPC in ammonium

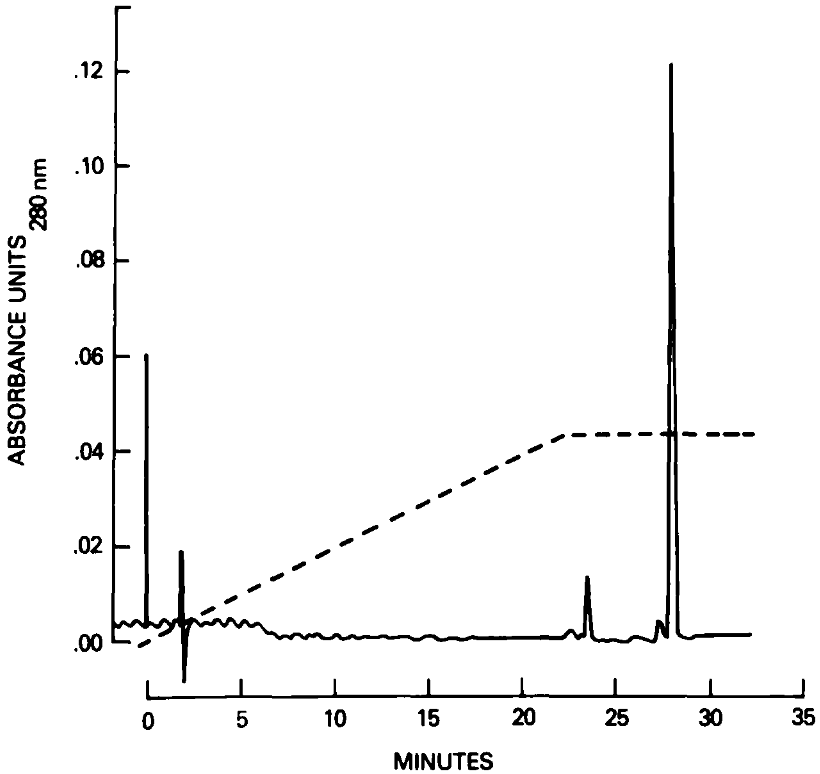

FIGURE 4

HPLC of MLCPC-purified peptide. A sample of 10 µg was chromatographed in 0.1% phosphoric acid on a C_{18} µ Bondapak column (Waters 0.4 x 30 cm) and a linear gradient of acetonitrile (dotted line) from 7% to 30% in 25 min at a flow of 2 ml/min. Absorbance at 280 nm is shown. Full scale is 0.02 absorbance units.

acetate, pH 8.5 and n-butanol with the upper phase mobile (Fig. 1). The rotation was ceased at fraction 86 and contents were pumped out. The yield of purified peptide in fraction (94-106) was 140 mg. The rest of the sample was run with lower phase mobile (Fig. 2, right panel) but these conditions resulted in less resolution of the material. Fraction (33-39) contained 170 mg of peptide which was

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relatively pure by TLC (Fig. 3), R_f = 0.5. Fraction (28-32), material which eluted ahead with the solvent front, contained the same peptide plus impurities. The yield of purified peptide from the solid-phase synthesis was 32%. Amino acid analysis gave the molar ratios: Asp, 2.09; Gly, 1.08; Met, 1.86; Tyr, 0.97.

A small sample of the synthetic product, 15 mg, was chromatographed in the MLCPC in the conditions described in the Methods with lower phase mobile (Fig. 2). The contents of fraction (16-20) were 10 mg and were highly purified (90%) as determined by HPLC (Fig. 4). The solvent front emerged at tube 11 and the peak tube of the major material was fraction 18.

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CONCLUSIONS

The modification in the pH of the aqueous phase of n-butanol and ammonium acetate increased the solubility of the negatively charged peptide. The peptide salt was maintained in solution under these conditions. In an earlier experiment when 365 mg was chromatographed in the BAW system with the lower phase mobile 60 mg or 16.4% was recovered (3). In these conditions the recovery was much better, 46.6%. In the HFCPC elutions with the upper phase resulted in better chromatography which took 24 hr. With the lower phase mobile the peptide emerged in 5 hr but was less well fractionated.

Chromatography of the peptide on the MLCPC in the conditions of lower phase mobile showed fractionation in a much shorter time, 30 min. This is probably due to the greater retention of stationary phase in this instrument design. If more experiments show a

significant degree of purification in such fast conditions then this method which does not require a solid support would be very promising as a rapid purification process.

REFERENCES

1. Abbreviations used: BAW = n-butanol, acetic acid and water; Boc = t-butyloxycarbonyl; CCC = countercurrent chromatography; CCK = cholecystokinin; HFCPC = horizontal flow-through coil planet centrifuge; HPLC = high performance liquid chromatography; MLCPC = multi-layer coil planet centrifuge; TLC = thin layer chromatography; PTFE = polytetrafluoroethylene.

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