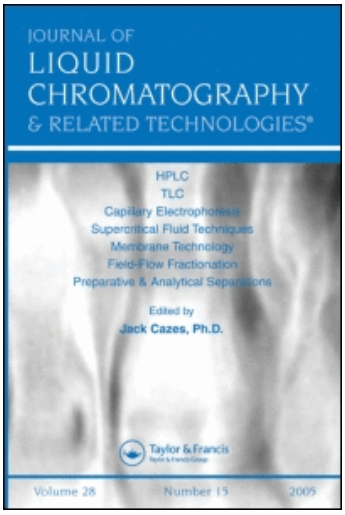

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Synergistic Use of Countercurrent Chromatography and High Performance Liquid Chromatography for the Purification of Synthetic Peptides

Gerald J. Putterman[a]; Mary Beth Spear[a]; Fulvio Perini[a]
[a] Frederick Cancer Research Facility, Biological Markers Program, National Cancer Institute, Frederick, Maryland

To cite this Article Putterman, Gerald J. , Spear, Mary Beth and Perini, Fulvio(1984) 'Synergistic Use of Countercurrent Chromatography and High Performance Liquid Chromatography for the Purification of Synthetic Peptides', Journal of Liquid Chromatography & Related Technologies, 7: 2, 341 — 350
To link to this Article: DOI: 10.1080/01483918408073971
URL: http://dx.doi.org/10.1080/01483918408073971

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(2), 341-350 (1984)

SYNERGISTIC USE OF COUNTERCURRENT CHROMATOGRAPHY AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY FOR THE PURIFICATION OF SYNTHETIC PEPTIDES

Gerald J. Putterman(1), Mary Beth Spear, and Fulvio Perini
Biological Markers Program, National Cancer Institute-
Frederick Cancer Research Facility
P.O. Box B, Frederick, Maryland 21701

ABSTRACT

Examples are given demonstrating how countercurrent chromatography (CCC) and high performance liquid chromatography (HPLC) can be used together to purify synthetic peptides. In one example, CCC provided a preliminary purification of Met-Arg-Asp-Val-Val-Leu-Phe-Glu-Lys by enabling separation of ultraviolet absorbing, ninhydrin-negative material from the desired peptide. Final purification was achieved with HPLC without risk of harming the HPLC column. In a second example Tyr-Ala-Ala-Nle-Ala-Ala was completely purified by CCC with the CCC separation rapidly and conveniently monitored by HPLC. CCC appears to be a very useful technique for the peptide chemist.

INTRODUCTION (2)

In previous reports we described the solid phase synthesis (3) and purification by countercurrent chromatography (CCC)

0148-3919/84/0702-0341$3.50/0

(3,4) of a peptide having the sequence: Tyr-Ala-Ala-Nle-Ala-Ala-Met-Arg-Asp-Val-Val-Leu-Phe-Glu-Lys. This purified peptide was subsequently coupled to bovine serum albumin and the conjugate injected into rabbits generated antibody to the peptide (5). In order to estimate which portion of the peptide contains antibody combining sites, the following peptides were prepared by solid phase synthesis: Tyr-Ala-Ala-Nle-Ala-Ala and Met-Arg-Asp-Val-Val-Leu-Phe-Glu-Lys. This report describes the purification of these synthetic peptides and illustrates how CCC can be used in conjunction with high performance liquid chromatography (HPLC) to facilitate the frequently arduous task of peptide purification.

EXPERIMENTAL

Reagents

Water used for CCC and HPLC was obtained by passing our standard laboratory grade water through a Critical Applications Adsorption Column (Hydro Services and Supplies, Inc., Durham, NC) which contains highly purified, activated charcoal and has a 0.2 μm polycarbonate filter (Nucleopore Corp, CA) attached to its outlet. Butanol and acetonitrile were obtained from Burdick and Jackson Laboratories (Muskegon, MI). Trifluoroacetic acid (TFA) Sequanal Grade was purchased from Pierce Chemical Co. (Rockford, IL). The enzymes D-amino acid oxidase and leucine aminopeptidase (LAP) were purchased from Sigma Chemical Co. (St. Louis, MO) and carboxypeptidase A (CPA) was obtained from Boehringer Mannheim (Indianapolis, IN). Other chemicals and solvents were reagent grade or better.

Peptides were prepared by a modification of the Merrifield solid-phase method (6) using a Vega (Vega Biochemicals, Tucson, AZ) Model 96 automated synthesizer. The benzyl group was used to protect Ser, Asp and Glu; Arg, Tyr and Lys were protected by p-toluenesulfonyl, o-bromobenzyloxycarbonyl and o-chlorobenzyl-oxycarbonyl groups, respectively. Carboxyl terminal residues were coupled to the resin as their cesium salts (7). The average load obtained was 0.4 mmoles amino acid/g resin. Peptides were custom-cleaved with HF by Peninsula Labs. (Belmont CA).

Apparatus

CCC was performed with the new horizontal flow-through coil planet centrifuge whose design and function has been described in

detail elsewhere (8). In brief, the rotary frame of the centrifuge contains a pair of column holders; each is subjected to a specific mode of synchronous planetary motion provided by a set of gears and pulleys. In this work a column mounted on the gear-side holder was used. This gear-side holder (coupled with gears) rotates about its own axis and simultaneously revolves around the central axis of the apparatus at the same angular velocity and in the same direction. The column is made of PTFE tubing (2.6 mm inner diameter) and has a total capacity of 260 ml.

HPLC was performed with an Altex Model 312 MP (Altex Instruments, Berkeley, CA) system which is a microprocessor controlled, gradient system described in detail elsewhere (9). Separations were performed with 0.39x30 cm μBondapak C_{18} columns (Waters Associates, Milford, MA). The particle size of the column packings was 10 microns. Runs were monitored at 206 nm with a Model 970A variable wavelength detector from Tracor Instruments (Austin, TX). To maintain a stable baseline a back pressure was placed on the outlet tubing as previously described (9).

Amino acid analyses were performed with a Durrum (Dionex Corp, Sunnyvale, CA) D-500 amino acid analyzer equipped with a fluorescence detector. Amino acids were made fluorescent by reaction with o-phthalaldehyde.

Procedures

For CCC of both synthetic peptides the solvent system butanol:acetic acid:water (4:1:5 by volume) was used with the aqueous, lower phase serving as the mobile phase. This two-phase solvent system was thoroughly equilibrated in a separatory funnel at room temperature and separated before use. The column was filled with stationary phase as previously described (4) and the samples (100 mg) dissolved in mobile phase (10 ml) were injected through the sample port into the column which was rotated at 400 or 500 rpm. Following sample addition, the mobile phase was pumped through the rotating column at the desired flow rate using a Milton Roy miniPump (American Scientific Products, Baltimore, MD). Column eluates were continuously monitored with an LKB (LKB Instruments, Gaithersburg, MD) Uvicord S detector at 280 nm.

For HPLC work a stock solution obtained by mixing 10 ml of TFA with 100 ml of purified water was used to prepare the chromatographic solvents. Solvent A was prepared by diluting 5

ml of the stock solution to 1 liter with water and solvent B was prepared by diluting 4.25 ml of the stock solution to 1 liter with acetonitrile.

CPA digestions were performed in bicarbonate solution and LAP digestions were performed in 0.1 M TRIS buffer, pH 8.6-.005 M $MgCl_2$. Oxidations with D-amino acid oxidase were performed using a modification of a published procedure (10).

RESULTS AND DISCUSSION

The profile obtained from CCC for the synthetic nonapeptide is shown in Figure 1. Thin-layer chromatography (TLC) of aliquots from the CCC fractions using the solvent system butanol: pyridine:acetic acid:water (90:60:18:72 by volume) indicated which tubes should be pooled and that fractions eluting prior to and later than the pooled fraction contained little ninhydrin-positive material, even though their ultraviolet absorption was higher. The stationary phase also contained ninhydrin-negative, ultraviolet absorbing material. HPLC of the pooled fraction (Figure 2) indicated that this fraction was still heterogeneous. However, comparison of Figure 2 with Figure 3, which is an HPLC run of the synthetic starting material, indicates that CCC has provided a most useful preliminary purification of the peptide. Final purification was achieved with HPLC by running isocratically at 15% B with a flow rate of 0.5 ml/min. The purified synthetic peptide was obtained in 50% yield and was subsequently shown to contain an antigenic determinant recognized by antibody raised to Tyr-Ala-Ala-Nle-Ala-Ala-Met-Arg-Asp-Val-Val-Leu-Phe-Glu-Lys (5).

CCC was used to completely purify Tyr-Ala-Ala-Nle-Ala-Ala. The chromatogram showed two large, partially separated peaks. Aliquots were removed from tubes comprising each peak but located far from the region of overlap, and these aliquots were evaporated to dryness. TLC using the solvent system butanol:acetic acid: water (4:1:5 by volume) indicated that each tube contained a single ninhydrin-positive peptide and that the peptide that eluted later in CCC also appeared to have a slightly larger R_f. Each fraction had the same amino acid composition, i.e. the expected composition. Aliquots of these purified peptides were

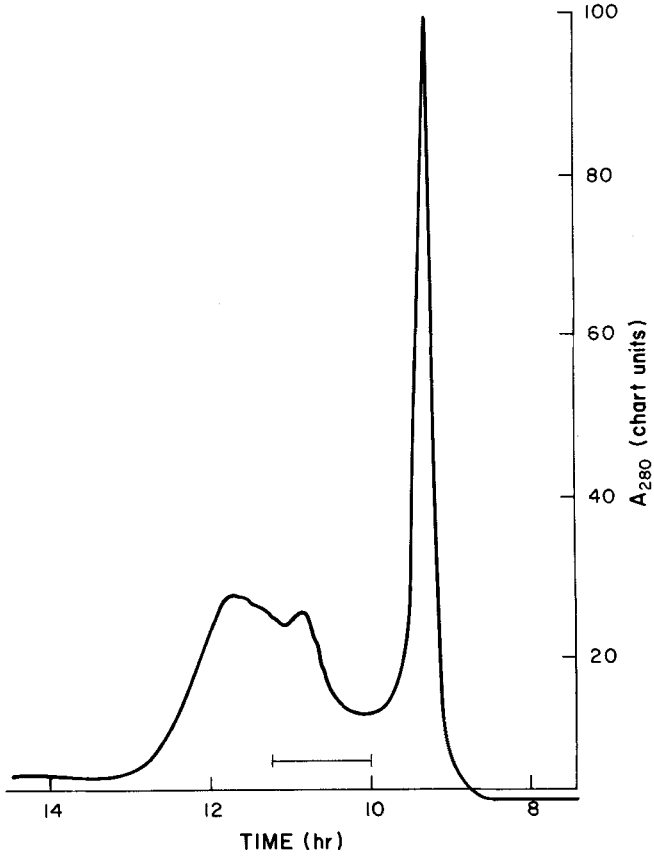

FIGURE 1: Countercurrent chromatogram of Met-Arg-Asp-Val-Val-Leu-Phe-Glu-Lys. revolution:500 rpm; flow rate: 15ml/hour; fraction volume: 3 ml. The region underlined in the figure was pooled and purified by HPLC.

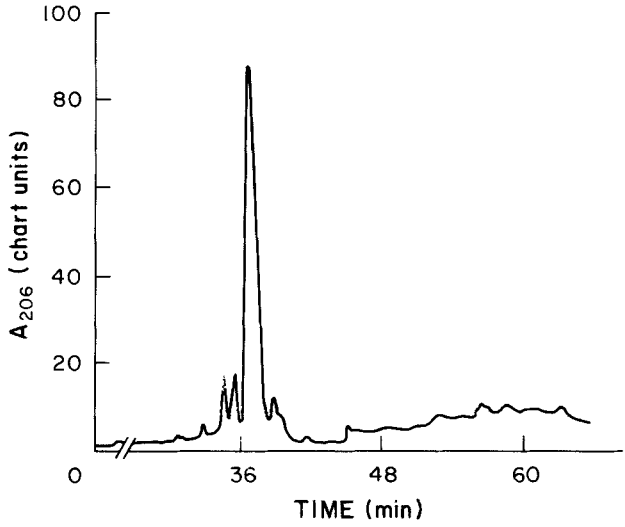

FIGURE 2: HPLC of an aliquot from the pooled fraction shown in Figure 1. The sample was run isocratically at 0% B for 5 min and then a linear gradient from 0% B to 60% B in 60 min was employed.

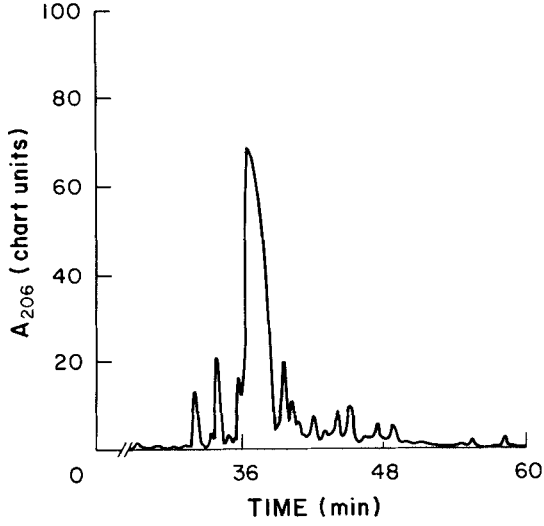

FIGURE 3: HPLC of Met-Arg-Asp-Val-Val-Leu-Phe-Glu-Lys (500 μg) before CCC. Conditions for HPLC are the same as in the legend to Figure 2.

then used to develop an HPLC technique for monitoring the CCC run. By running isocratically at 10% B at a flow rate of 1 ml/min, both peaks could be readily separated in 18 minutes. Aliquots from appropriate tubes comprising the CCC run were then analyzed by HPLC. The results (Figure 4) clearly indicate which tubes contained pure peptide and which tubes contained mixtures of the two peptides. The fraction which eluted later in CCC when the organic phase was used as the stationary phase, also appeared to be more hydrophobic on HPLC. Based on the data given in Figure 4, tubes 101-107 and 115-150 were pooled to give Fraction I and Fraction II respectively.

Physical and biochemical techniques were used to characterize the two fractions and determine which was the desired peptide. Mass spectra showed some differences, but it could not be concluded which was the desired peptide. Mass spectra did not show the presence of bromine suggesting that the heterogeneity was not due to incomplete deblocking of the o-bromobenzyloxycarbonyl group used to protect the tyrosyl residue during peptide synthesis. Exopeptidase digestions indicated differences between the two fractions. CPA digestion of Fraction I liberated both alanine and nor-leucine while CPA digestion of Fraction II only liberated alanine. LAP digestion of Fraction I liberated tyrosine, alanine and nor-leucine in a molar ratio of 1:4:1 while LAP digestion of Fraction II liberated tyrosine and alanine in equal molar ratios but nor-leucine was absent. Treating lyophilized acid hydrolysates of each peptide with D-amino acid oxidase gave tyrosine, alanine and nor-leucine in a molar ratio of 1:4:1 for Fraction I while a tyrosine to alanine molar ratio of 1:4 was obtained for Fraction II. Thus, the difference between Fraction I and Fraction II was that Fraction I contained L-Nle and Fraction II contained D-Nle. (A comparison of CPK space-filling models of the two hexapeptide isomers shows that with Fraction II, the butyl side-chain (of Nle) is quite exposed while with Fraction I the butyl side chain is better able to fold back on other groups in the same peptide. Thus, model work indicates that Fraction II would appear more hydrophobic on CCC and HPLC.) Since D-amino acid oxidase treatment of an acid hydrolysate of Tyr-Ala-Ala-Nle-Ala-Ala-Met- Arg-Asp-Val-Val-Leu-Phe-Glu-Lys, which had been prepared earlier using the same synthetic reactions, showed that the peptide contained no D-Nle, it must be concluded that Fractions I and II were obtained this time because our synthetic intermediate (t-butyloxycarbonyl-nor-leucine) was

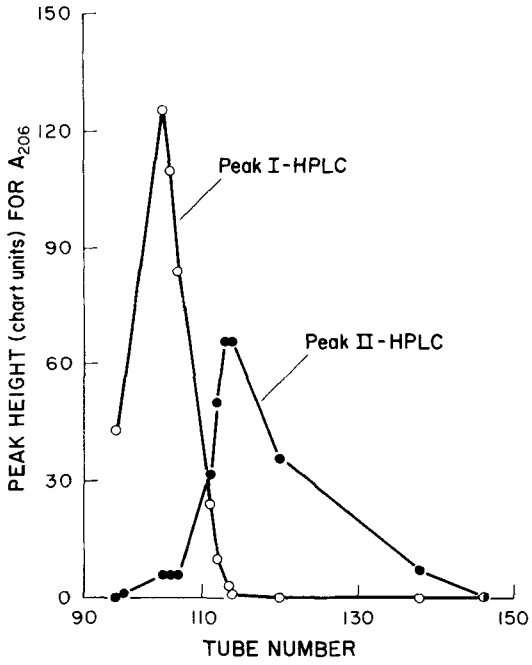

FIGURE 4: Countercurrent chromatogram of Tyr-Ala-Ala-Nle-Ala-Ala as monitored by HPLC. Conditions for CCC were: revolution: 400 rpm; flow rate: 10 ml/hour; fraction volume: 2 ml.

a racemic mixture. Fraction I contained no antigenic determinants recognized by antibody raised to this pentadecapeptide (5).

In a previous paper (3,4) it was noted that CCC appears to be ideally suited to the purification of synthetic peptides, since it gives peptide products free of salts, resin by-products, products of deblocking, and blocked peptides. This paper illustrates further that CCC can be used either for a preliminary purification with a final purification by HPLC or that CCC can be used for complete purification with the run conveniently monitored by HPLC. Thus far, only a few solvent systems have been used for CCC of peptides (3,4,11,12). Hopefully, other laboratories will employ CCC and provide additional solvent systems for the peptide chemist.

ACKNOWLEDGMENTS

The authors wish to thank C.V. Hixson for amino acid analyses, Jon Peace for mass spectra, Mark Handschumacher for help with the molecular models, and R. Ruddon for continued encouragement. This work was supported by the National Cancer Institute under Contract N01-CO-75380 with Litton Bionetics, Inc.

REFERENCES

1. Correspondence should be sent to Dr. G.J. Putterman, Dept. of Ophthalmology and Visual Sciences, Yale University School of Medicine, New Haven, CT; Ms. Spear's present address: ISI, Philadelphia, PA; Dr. Perini's present address: Dept. of Pharmacology, University of Michigan School of Medicine, Ann Arbor, MI.

2. Abbreviations: CCC = countercurrent chromatography; HPLC = high performance liquid chromatography; TFA = trifluoroacetic acid; LAP ◘ leucine aminopeptidase; CPA = carboxypeptidase A; TLC = thin-layer chromatography.

3. Putterman, G.J, Perini, F., White, E.L. and Ito, Y. Purification of Synthetic Peptides By Means of Countercurrent Chromatography With the Horizontal Flow-Through Coil Planet Centrifuge. Peptides: Structure and Biological Function, Proc. Sixth American Peptide Symposium, Gross, E. and Meienhofer, J., eds., Pierce Chemical Co., Rockford, IL, 1979, p.113.

4. Ito, Y. and Putterman, G.J. New Horizontal Flow-Through Coil Planet Centrifuge For Countercurrent Chromatography III. Separation and Purification of Dinitrophenyl Amino Acids and Peptides. J. Chromatogr. 193,37,1980.

5. Aldenderfer, P.H., Meade-Cobun, K.S. and Putterman, G.J. Unpublished results.

6. Merrifield, R.B. Solid Phase Peptide Synthesis I. The Synthesis of a Tetrapeptide. J. Amer. Chem. Soc., 85,2149,1963.

7. Gisin, B.F. The Preparation of Merrifield-Resins Through Total Esterification With Cesium Salts. Helvet. Chim. Acta 56,1476,1973.

8. Ito, Y. New Horizontal Flow-Through Coil Planet Centrifuge For Countercurrent Chromatography II. The Apparatus and Its Partition Capabilities. J. Chromatogr. 188,43,1980.

9. Putterman, G.J., Spear, M.B., Meade-Cobun, K.S., Widra, M. and Hixson, C.V. A Rapid Isolation of Human Chorionic Gonadotropin and Its Subunits By Reversed-Phase High Performance Liquid Chromatography. J. Liq. Chromatogr. 5,715,1982.

10. Wellner, D. and Lichtenberg, L.A., Assay of Amino Acid Oxidase, Methods in Enzymol. 17B, 593, 1971.

11. Knight, M. Ito, Y., and Chase, T.N. Preparative Purification of the Peptide Des-Enkephalin-γ-Endorphin. Comparison of High-Performance Liquid Chromatography and Countercurrent Chromatography. J. Chromatogr. 212,356,1981.

12. Knight, M., Tamminga, C.A., and Chase, T.N. Purification of Synthetic Peptides by Countercurrent Chromatography on the Coil Planet Centrifuge. Abstr. Pittsburgh Conf. Anal. Chem. and Applied Spectros. Atlantic City, 1982, p. 614.