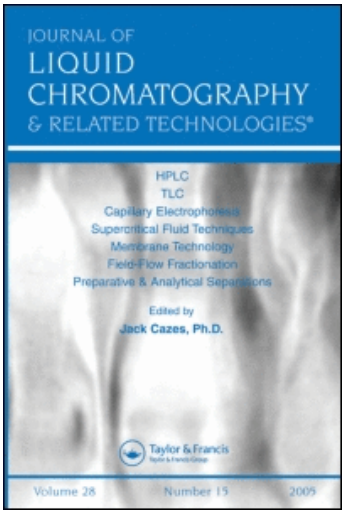

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SYNTHETIC PEPTIDE PURIFICATION IN THE MULTI-COIL COUNTERCURRENT CHROMATOGRAPH

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ABSTRACT

The multi-coil countercurrent chromatograph (MC-CCC) is used for preparative separations of various synthetic peptides. For peptides that are water insoluble and that have a high partitioning in the upper phase of n-butanol, acetic acid and water, the use of the more hydrophobic solvent system comprised of chloroform, acetic acid and water provides purification. An alternative two-phase solvent system made up of equal volumes of three components such as ethyl acetate, acetonitrile and an aqueous acid or salt solution is proposed for peptide separation as well.

INTRODUCTION

In recent years the Ito coil planet centrifuges, used for liquid-liquid partition chromatography, have been modified in design to in-

crease capacity while maintaining resolution. The horizontal flow-through coil planet centrifuge or eccentric multi-layer coil planet centrifuge with eight multi-layer coils in series is a compact apparatus with a capacity of 385 ml which is enough to load up to 500 mg of sample. Peptide separations in the n-butanol/acetic acid/water (BAW, 4:1:5 by volume) system have been reported previously for this instrument (2,3). This solvent system has been useful in all the earlier instruments except for the single multi-layer coil planet centrifuge in which high speed countercurrent chromatography (HSCCC) is conducted (4). The BAW solvent system can be used in the newer MC-CCC instrument because the centrifugal rate used is lower than that for HSCCC. Peptides, that are more hydrophobic than those able to be chromatographed in the BAW system, can be separated in the MC-CCC using CHL/HOAc/H_2O (2:2:1) a strongly polar hydrophobic solvent system (5).

It continues to be important to find more 2-phase solvent systems whose components can be adjusted to provide a partition coefficient for maximum separation of a particular sample. A new solvent system has been devised containing acetonitrile as a modifier between diethyl ether, ethyl acetate or methyl t-butyl ether and aqueous dilute acid or salt solutions. There is a suitably high density difference between the

phases such that this type of solvent system can even be used in HSCCC (6). Therefore, we have applied this solvent system in the present MC-CCC instrument for a 21-mer peptide separation.

METHODS

Chemicals were reagent grade from Aldrich (Milwaukee,WI) and solvents were reagent or HPLC grade from Fisher Scientific (Springfield, NJ). Water was purified through a Nanopure cartidge system (Barnstead, Boston, MA). Peptides were synthesized either manually or in a Biosearch 9600 AT peptide synthesizer using protocols for coupling fluorenylmethyloxycarbonyl (Fmoc) protected amino acids to the Fmoc amino acyl hydroxybenzylalchohol (Wang) resin. The peptides were removed from the resin and deprotected by trifluoroactic acid treatment in the presence of scavengers. The peptides were extracted in water or formic acid and lyophilized. The peptides were submitted to CCC by dissolving in equal volumes of each phase of a solvent system and injecting into the coil filled with stationary phase. The apparatus is centrifuged at 450 rpm and mobile phase is pumped at 1 to 3 ml/min. The instrument used is the table-top multi-coil countercurrent chromatograph (Peptide Technologies Corporation, Washington, DC) described previously (2). This coil planet centrifuge is equipped with eight 50-ml multi-layer coils connected in series. Four

coils on a column holder are counterbalanced with 4 coils on the other column holder. Solvent is pumped with a LDC Milton Roy mini-pump (Riviera Beach, FL). Fractions of 6 to 15 ml of the mobile phase are collected in a LKB Ultrorac fraction collector (LKB-Pharmacia, Gaithersburg, MD) and absorbance read manually in a Hewlett-Packard diode array spectrophotometer (San Francisco, CA).

Contents of the peaks are analyzed by HPLC as previously reported (7) on a S-5 ODS column (0.4 or 0.6 x 1.5 cm, 200Å pores spherical 5 μm silica, YMC, Inc., Morris Plains, NJ) in 0.1% aq. phosphoric acid and gradients of acetonitrile at 0.8 ml/min in Waters analytical equipment (Millipore Corporation, Milford, MA) comprised of a U6K injector, Model 510 dual pumps, Model 481 or 484 variable UV detector, and the Maxima 820 data system with a NEC 286 computer and printer. The peak fractions found to contain pure peptide are pooled, evaporated in a rotary evaporator, dissolved in a small amount of water or glacial acetic acid and lyophilized to a powder. The peptide is analyzed by HPLC or TLC and amino acid analysis.

RESULTS

Previous work has shown that most peptides can be purified in the BAW system. As an example, a positively charged, aromatic

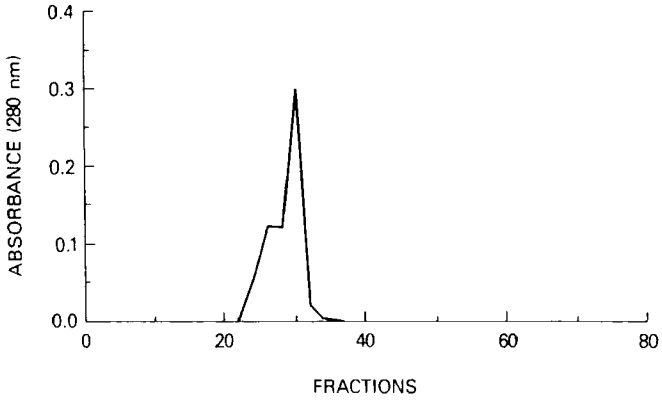

FIGURE 1

Countercurrent chromatography of 60 mg HLDIIW in the BAW system with upper phase elution. The planet centrifuge was rotated at 450 rpm and flow was 60 ml/hr. Fractions of approximately 15 ml were collected. The solvent front was at fraction 22. Absorbance at 280 nm was read as described in the text.

peptide had a high partition coefficient or K (conc. in upper phase to conc. in lower phase) of 1.7 in the BAW solvent system. HLDIIW corresponding to the carboxyl tail of endothelin was purified in the MC-CCC. An amount of 60 mg was chromatographed with the upper phase mobile. The hexapeptide emerged after the solvent front and from the main peak eluting after a shoulder, 28 mg of pure peptide was recovered (Fig. 1). Analyses by HPLC show the composition of the early material and the major peak. The impurities eluting first are more

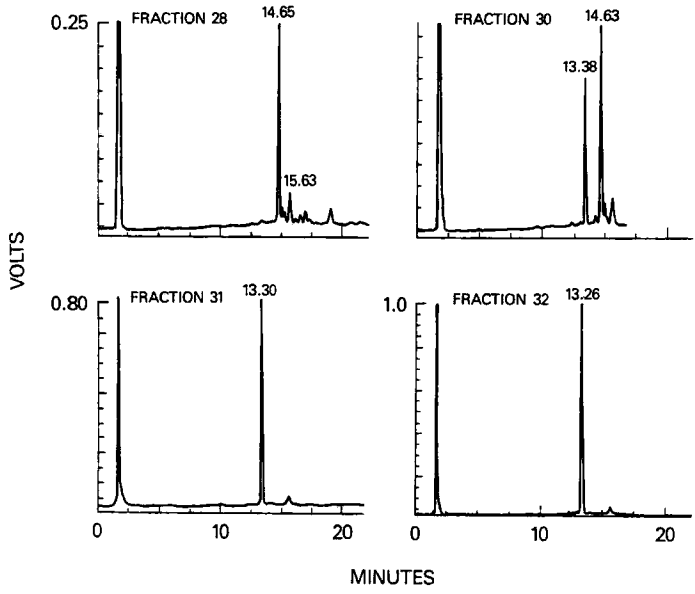

FIGURE 2

HPLC analysis of 10-15 µl of chromatography fractions of the experiment of Fig. 1. The absorbance at 215 nm is measured in volts. HPLC conditions are described in the "Methods". A gradient of 5% to 30% acetonitrile in 10 min was run. The void volume absorbance occuring at 1.67 min is due to the presence of acetic acid in the injected sample. The peak at 13.3 min is the peptide which is pure in fractions 31 and 32.

hydrophobic and elute later in reverse phase chromatography (Fig. 2). Fractions 31-33 were taken as purified peptide.

Two highly similar 26-mers with the same composition but different sequences were not water soluble and not easily analyzed by HPLC. Their composition was C.17L.Nle.3R.3S.W. The peptides were

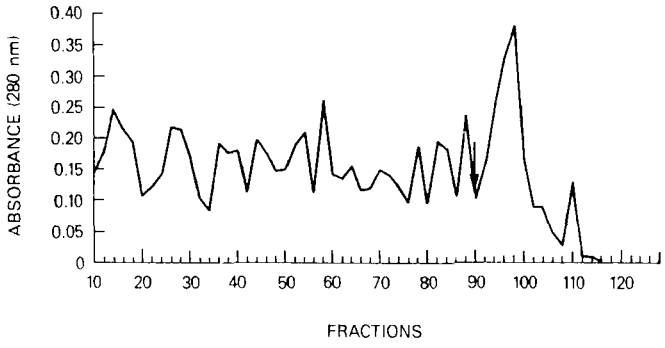

FIGURE 3

Separation of 222 mg of a 26-mer in the CHL/HOAc/Water system with the upper aqueous phase used as the mobile phase. The aborbance at 280 nm was determined of the 2 to 3 ml fractions. Solvent front is at fraction 90. The presence of chloroform in the lower phase fractions prior to the solvent front causes high absorbance readings. Peptide is present after fraction 91. Fractions (91-94) contained 42 mg; (95-99), 60 mg; (100-106), 22 mg and 70 mg of side product were present in the coil contents, after fraction 120. Total recovery of material was 194 mg.

chromatographed in the CHL/HOAc/water system using the aqueous upper phase as the mobile phase. The peptides eluted after the solvent front and fractions were taken and analyzed by HPLC. Much of the mass in both runs was recovered. The purest fraction pool of the peptide of Fig. 3 was 95-99 containing 60 mg. The material from CCC fractions 67-71 to 80-93 of the other peptide (Fig. 4) looked similar by TLC. The recovery of total peptide material from crude was high, 87%

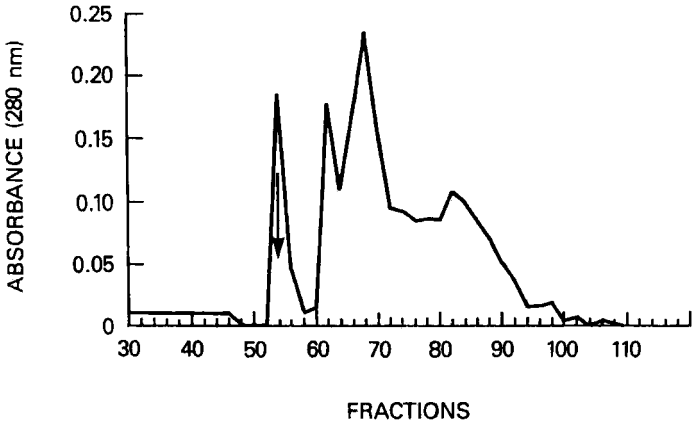

FIGURE 4

Chromatography of 276 mg of another 26-mer of a different sequence in the same solvent system and conditions except that the fractions collected were 12 to 15 ml. The solvent front was at 53. Fractions (53-66) contained 31 mg, (67-71) 62 mg, (73-79) 34 mg, and (80-93) 41 mg, respectively.

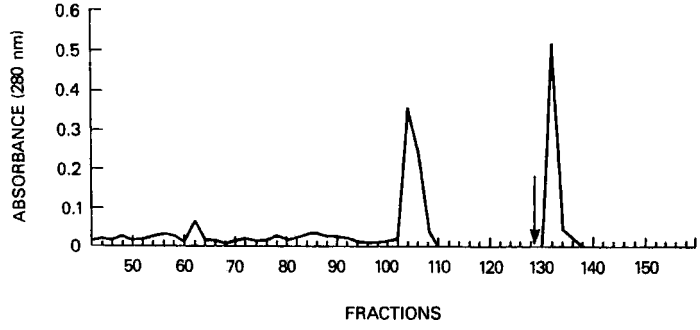

FIGURE 5

Chromatography of 200 mg of a 21-mer (D.E.2F.K.L.N.-2Q.3R.S.4T.2V.2W) in ethyl acetate, acetonitrile and 1% aq. Tfa with the upper phase mobile. At fraction number 129 the rotation was stopped and contents pumped out. Fractions of 3 ml were taken.

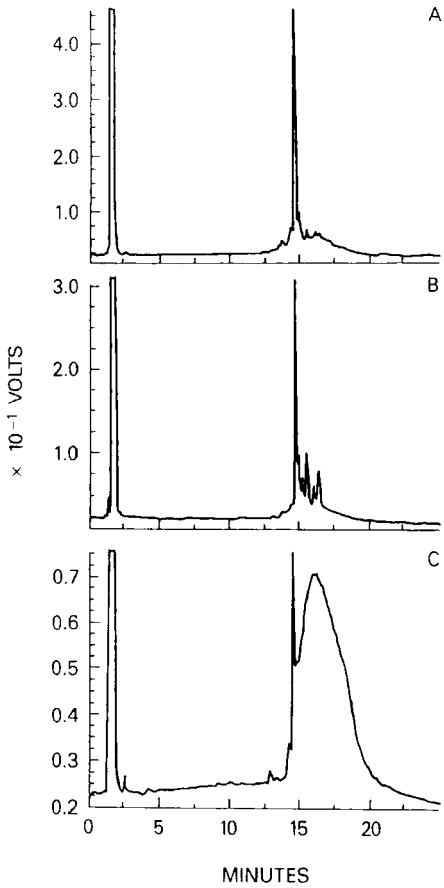

FIGURE 6

Analysis by HPLC of 10-15 μg samples of pooled fractions from the CCC of Fig. 5 in conditions as described in the "Methods". The gradient of acetonitrile used was 5% to 40% in 15 min. Upper panel A is analysis of purest fraction (105-107) containing 60 mg, B is analysis of early part of CCC peak (103-104) which contains 35 mg and C is material left in coil which has a mass of 59 mg, fraction (131-132).

and 61%, respectively. These peptides could not be chromatographed by reverse phase chromatography.

Finally, a new 2-phase solvent system has been proposed as suitable for peptide fractionation. Equivolumes of ethyl acetate, acetonitrile and aq. salt or diute acid form a stable 2-phase system. A 21-mer peptide, 200 mg was chromatographed with the upper phase mobile of ethyl acetate, acetonitrile and 1% trifluoroacetic acid in the MC-CCC at a constant temperature of 22°C (Fig. 5). The recovery of material was 77%. The HPLC analysis of pooled fractions taken (103-104, Fig. 6B) and (105-107, Fig.6A) showed improved purification in the later fraction. A large amount of impurities was eluted when the contents were pumped out (fractions 131-132, Fig. 6C). This material had been retained in the coil. Further studies of this solvent system are underway. For example, a radioactive peptide was eluted in methyl t-butyl ether, acetonitrile and 1% Tfa by HSCCC performed in the faster single multi-layer CPC instrument (not shown). Hopefully, useful partition coefficients can be found with aq. Tfa, HCl, HOAc and ammonium acetate at various pH.

In summary, an increasing range of solvent systems are being utilized for preparative peptide separation in countercurrent chromatography. Hydrophobic peptides that are not water soluble or easily recovered from HPLC can be usefully purified by these methods.

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