Separation and purification of peptides by high-speed counter-current chromatography

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Abstract

Preparative separations of peptides have been accomplished using high-speed counter-current chromatography. This has been made possible by the use of a particular solvent system that does not exhibit solvent carryover at high speed flow and centrifugation conditions. The solvent system composed of tert.-butyl methyl ether, n-butanol, acetonitrile and aqueous trifluoroacetic acid which can be adjusted in volume ratios and percent acid is employed in two instruments for counter-current chromatography. The cross-axis coil planet centrifuge was used for separation of various dipeptides. Superior resolution was obtained with the multi-layer coil planet centrifuge in the separation of six dipeptides and in the preparative purification of two synthetic peptides.

1. Introduction

Counter-current chromatography (CCC) [1] is a method of separation whereby substances are separated by partitioning between two liquid phases. In the coil planet centrifuge (CPC) this process takes place in open coiled tubing. Since there is no solid support, certain complications such as sample loss due to adsorption or deactivation on a solid phase are avoided. High-speed CCC, carried out in the multi-layer CPC, provides high partition efficiency and has been widely used for the separation of natural products and synthetic organic compounds [2]. Applying high-speed CCC to polar compounds,

however, has been limited mainly due to less stationary phase retention of viscous polar solvent systems leading to less resolution. Consequently, peptide separations have been performed mostly on the horizontal flow-through CPC [3] which produces more reliable retention of the stationary phase but yields less efficient separations.

Recently this difficulty was overcome by the use of less viscous polar solvent systems composed of tert.-butyl methyl ether, n-butanol, acetonitrile and aqueous trifluoroacetic acid (TFA) at various volume ratios. The use of acetonitrile and aqueous TFA with another organic solvent has been introduced in a previous experiment [4]. The present paper describes the preparative separation by high-speed CCC of various peptides with the above two-phase sol-

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vent systems using two types of coil planet centrifuges.

2. Experimental

2.1. CCC instruments

The following two different types of coil planet centrifuges were employed: a prototype of the cross-axis coil planet centrifuge (cross-axis CPC) and a commercial model of the type-J multi-layer coil planet centrifuge (multi-layer CPC). The

design of these instruments have been described previously [2,5].

The cross-axis CPC (Fig. 1A) used in the present study, recently fabricated at the National Institutes of Health, is especially configured for the use of the viscous aqueous polymer phase systems for separating proteins [5]. This instrument is distinct from the other CPCs in that the rotating tubing coil revolves perpendicularly to the centrifuge axis (Fig. 1B), whereas the tubing coil in the other CPCs rotate parallel to the centrifuge axis. There are two multi-layer coils that are interconnected and opposite each other.
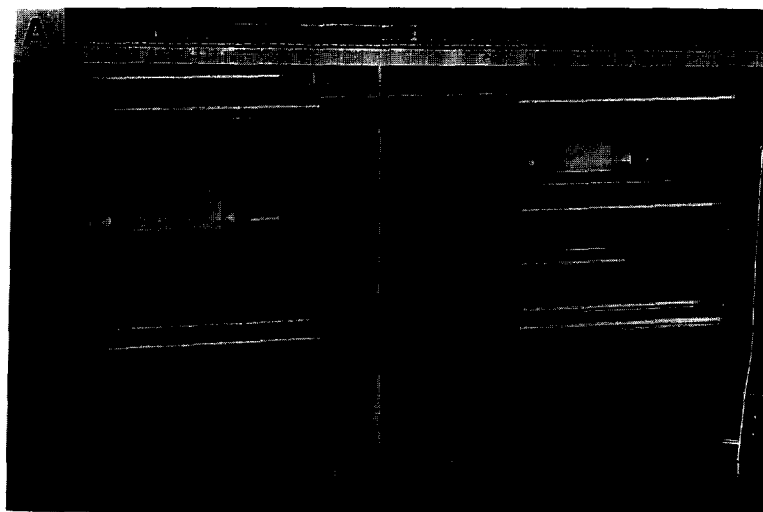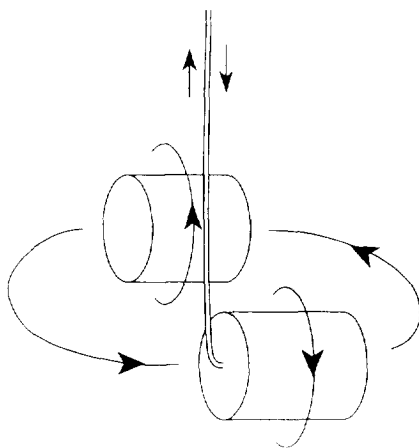

Fig. 1. The cross-axis coil planet centrifuge: photograph (A) and diagram of flow tubing, column-coil and motion (B).

These rotate on an axis perpendicular to the central centrifuge axis. In one revolution around the central axis, these rotate about their own axis one time. The coiled tubing column can be mounted either in the center or off-center on the frame. In the central position, the column is subjected to a strong radial centrifugal force across the diameter of the column which provides stable retention of the stationary liquid phase in the column. In the off-center position, the column is subjected to an additional force field around the rotating holder axis to produce very high mixing of the two phases in the column. Each column is a left-handed coil of 2.6 mm I.D. polytetrafluoroethylene (PTFE) tubing (Zeus Industrial Products, Raritan, NJ, USA) prepared by winding the tubing in multiple layers. The two columns are connected with a flow tube (0.85 mm I.D. PTFE) with a total volume of 570 ml.

The multi-layer CPC (Fig. 2A) is a commercial

B

Fig. 2. The commercial model of multi-layer CPC; photograph (A) and diagram of flow tubing, column and motion (B).

model (Ito Multilayer Separator/Extractor, P.C. Inc., Potomac, MD, USA). There is one multi-layer coil with an opposite counterweight, both at a distance of 10 cm from the central axis of the centrifuge. The planetary gear mounted on the holder axis is engaged to an identical sun gear rigidly mounted around the centrifuge axis. The gear arrangement produces a synchronous planetary motion of the holder: one rotation about its own axis during one revolution around the central axis of the centrifuge both in the same direction (Fig. 2B). The multi-layer coil is of 160 m × 1.6 mm I.D. PTFE tubing in 16 coiled layers on the holder hub for a total volume capacity of 330 ml.

Both planet centrifuges were equipped with a controller (Bodine Electric, Chicago, IL, USA) which regulates the revolution speed up to 1000 rpm.

2.2. Reagents

tert.-Butyl methyl ether, acetonitrile, *n*-butanol, methanol and TFA were glass-distilled chromatographic grade (Burdick & Jackson Lab., Muskegon, WI, USA). Water is distilled and passed through a Nanopure cartridge system (Barnstead, Boston, MA, USA).

Peptides were synthesized by automated solid-phase methods, coupling the fluorenylmethyloxycarbonyl-protected amino acids to the amino acyl hydroxybenzylalcohol resin [4,6]. The peptides were cleaved from the resin, and the protecting groups removed, by reaction with TFA. The peptides were extracted in a suitable solvent and lyophilized. The peptides were analyzed by HPLC and the amino acid composition determined.

2.3. Preparation of solvent systems and sample solutions

The suitable solvent system was selected by means of partition coefficient measurement. The present studies employed two-phase solvent systems composed of *tert.*-butyl methyl ether, *n*-butanol, acetonitrile and aqueous TFA where the partition coefficient values (K) were adjusted
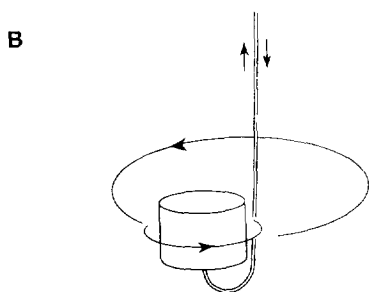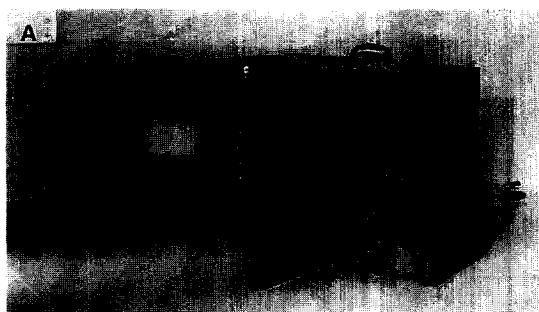
to 0.5–1 for each sample mixture by changing the solvent volume ratios and/or the TFA concentration. The solvent system selected for the CCC separation was thoroughly equilibrated in a separatory funnel at room temperature and the phases separated.

The sample solution was prepared by dissolving a sample mixture in 4–20 ml (equal volumes of each phase) of the solvent system used for separation.

2.4. Separation procedure

The CCC separations were performed according to the standard technique as follows. The column was first completely filled with the organic stationary phase. Then the sample solution was injected into the coil and the aqueous mobile phase was pumped into the column at 2–5 ml/min while the apparatus was rotated at 700–800 rpm. The effluent from the outlet of the column was continuously monitored with a UV monitor (Uvicord S, LKB Instruments, Bromma/Stockholm, Sweden) and collected in 3–5-ml fractions in a fraction collector (Ultrorac, LKB Instruments). After the desired peaks were eluted, the apparatus was stopped and, by connecting the column inlet to a pressurized nitrogen line, the column contents were emptied into a graduated cylinder to measure the volume of the stationary phase retained in the column.

2.5. Analysis of CCC fractions

Contents of the peaks were analyzed by HPLC as previously described [6] on a S-5 C_{18} column (15 cm × 0.4 cm, 200 pores of 5-μm spherical silica; YMC, Wilmington, NC, USA) in 0.1% aq. phosphoric acid and gradients of acetonitrile at 0.8 ml/min in Waters analytical equipment (Waters, Milford, MA, USA) consisting of a U6K injector, Model 510 dual pumps. Model 481 UV detector and an SE120 chart recorder. The fractions determined to be pure were lyophilized.

3. Results and discussion

3.1. The cross-axis CPC experiments

Fig. 3 contains the chromatograms of three dipeptides separated in the cross-axis CPC in two column positions: the central position (A) and off-center position (B). Both separations were performed with a binary two-phase solvent system composed of *n*-butanol–water and eluting the aqueous phase at 5 ml/min under 700 rpm revolution. The sample solution contained 200
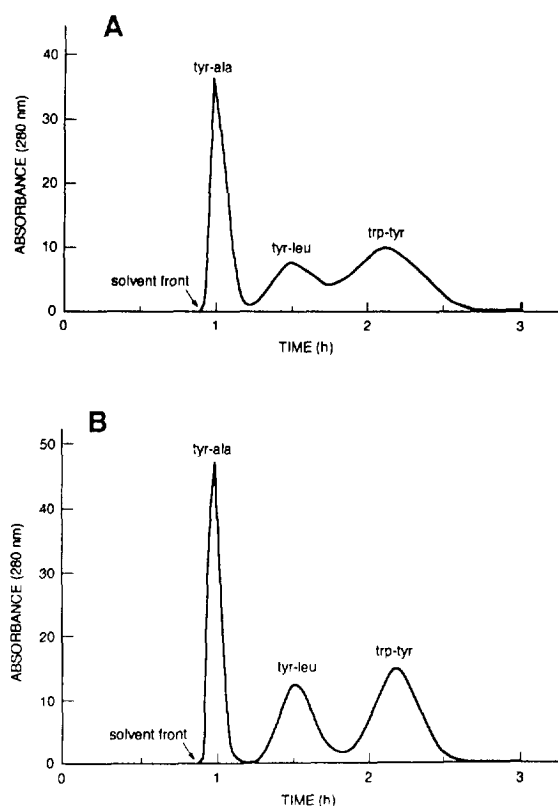

Fig. 3. Separation of 100–200 mg of three dipeptides in the cross-axis CPC in two column positions. (A) Central column position; (B) off-center column position. Experimental conditions are described in the text. Absorbance at 280 nm was determined manually of the fractions and total units are plotted per hour. The lower phase of *n*-butanol–water was used as the mobile phase at a flow-rate of 5 ml/min with a centrifugation at 700 rpm. Retention of stationary phase was 49.6% (A) and 60% (B).

mg each of Tyr–Ala, Tyr–Leu and 100 mg Trp–Tyr dissolved in 40 ml solvent (20 ml each phase). Presence of carried-over mobile phase droplets in the stationary phase produced too much noise in the on-line monitoring of the elution. Therefore, these chromatograms were drawn from the data obtained by manual absorbance measurements of the collected fractions. The results clearly indicated that the off-center position produces much higher peak resolution.

In Fig. 4 are shown similar chromatograms obtained from the separation of a set of 5
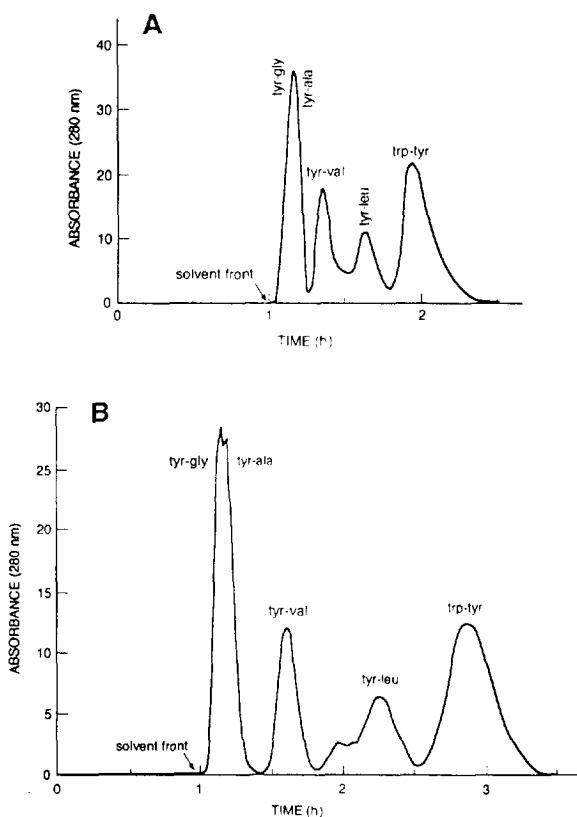
dipeptides (100 mg each) using the off-center column position. A two-phase solvent system composed of tert.-butyl methyl ether–acetonitrile–aq. TFA (2:2:3, v/v) was used by modifying the TFA concentration at 0.5% (v/v) (A) and 5% (v/v) (B). The separations were performed under the same experimental conditions used in the previous experiment. Higher TFA concentration increased the K value, hence the longer retention time of the analytes, resulting in better peak resolution. However, the first peaks (Tyr–Gly and Tyr–Ala) are only partially resolved.

The above results indicate that the present system is useful for separating several hundred milligrams of peptides with a fairly broad range of hydrophobicity from Tyr–Gly to Trp–Tyr.

3.2. Type-J multi-layer CPC experiments

The chromatograms of 50 mg each of 4 dipeptides (Fig. 5) run in the multi-layer CPC using the solvent system composed of tert.-butyl methyl ether–acetonitrile–5% aq. TFA (2:2:3,

Fig. 4. Chromatogram of five dipeptides by the cross-axis CPC in off-center column position using the ternary solvent system composed of tert.-butyl methyl ether–acetonitrile–aq. TFA (2:2:3, v/v) with varying TFA concentrations. (A): 0.5% TFA; (B): 5% TFA. The lower aqueous phase was used as the mobile phase at a flow-rate of 5 ml/min at 700 rpm. Retention of stationary phase in this experiment was 60%.
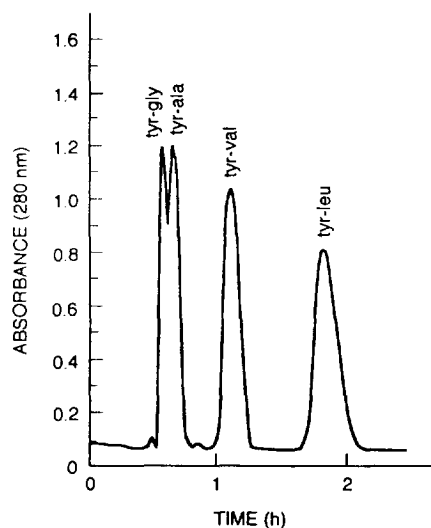

Fig. 5. CCC separation of four dipeptides in the multi-layer CPC. Sample size of each peptide was 50 mg. tert.-Butyl methyl ether–acetonitrile–5% aq. TFA (2:2:3, v/v) was the solvent system with the lower aqueous phase used as the mobile at a flow-rate of 3 ml/min at 800 rpm and retention of stationary phase was 68.5%.

v/v) are shown. The separation was performed at a flow-rate of 3 ml/min at 800 rpm. Because the present system provided a stable retention of the stationary phase, the on-line monitoring produced a noise-free elution curve.

The result shows that the resolution between the Tyr–Gly and Tyr–Ala peaks is much improved and the Tyr–Leu peak is eluted in about 2 h suggesting that the peak resolution may be further improved by increasing the polarity of the solvent system.

Fig. 6A is a chromatogram of 6 dipeptides (50 mg each) including peptides of similar composition but different sequence in the multi-layer CPC. The separations were performed with a more polar solvent system composed of tert.-butyl methyl ether–acetonitrile–1% aq. TFA (2:2:3, v/v) under otherwise identical experimental conditions as described in Fig. 5. The increased polarity of the solvent system further improved the peak resolution between the Tyr–Gly and Tyr–Ala but Tyr–Val and Val–Tyr are only partially resolved. Finally in Fig. 6B is illustrated a separation performed with a polar quaternary solvent system composed of tert.-butyl methyl ether–n-butanol–acetonitrile–1% aq. TFA (2:2:1:5, v/v). In this solvent system, all components are completely resolved and eluted in about 6 h. These results indicate that the multi-layer CPC has a high partition efficiency when used with polar solvent systems. The foregoing experiments showed that this instrument has higher resolution than the cross-axis CPC.

The present system was applied for purification of synthetic peptides using the above quaternary solvent system with various volume ratios adjusted to meet the polarity of the products. In Fig. 7 is shown a chromatogram of 50 mg of a synthetic undecapeptide using the solvent system tert.-butyl methyl ether–n-butanol–acetonitrile–1% aq. TFA (2:1:1:4, v/v). The major peak eluted in slightly over 1 h contained the purified peptide as shown in the analysis in Fig. 8. The yield after lyophilization was 16 mg.
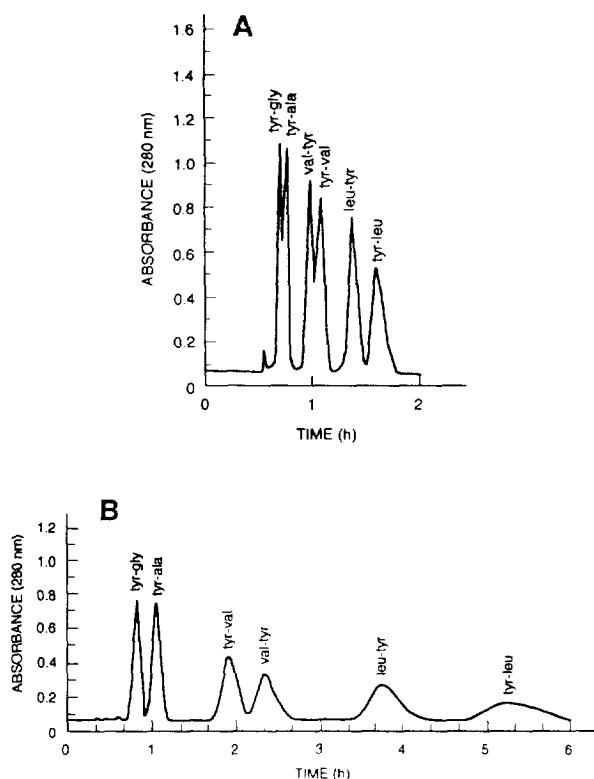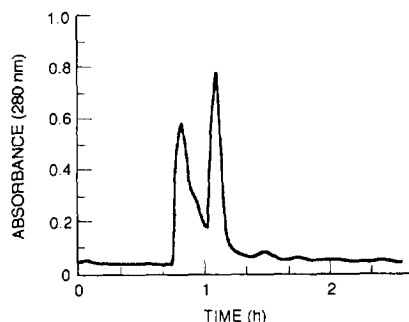

Fig. 6. Separation of 50 mg each of six dipeptides in the multi-layer CPC in two polar solvent systems: (A) tert.-butyl methyl ether–acetonitrile–1% aq. TFA (2:2:3) and (B) tert.-butyl methyl ether–n-butanol–acetonitrile–1% aq. TFA (2:2:1:5) under the same conditions as described in Fig. 5. Retention of stationary phase was 76.5% (A) and 73.4% (B).

Fig. 7. CCC purification of 50 mg synthetic peptide (Cys.Asp.His.2Ile.Lys.2Arg.Thr.Trp.Tyr) in the multi-layer CPC using tert.-butyl methyl ether–n-butanol–acetonitrile–1% aq. TFA (2:1:1:4) with the lower phase mobile at a flow-rate of 2 ml/min and 800 rpm. Retention of stationary phase was 67.4%.
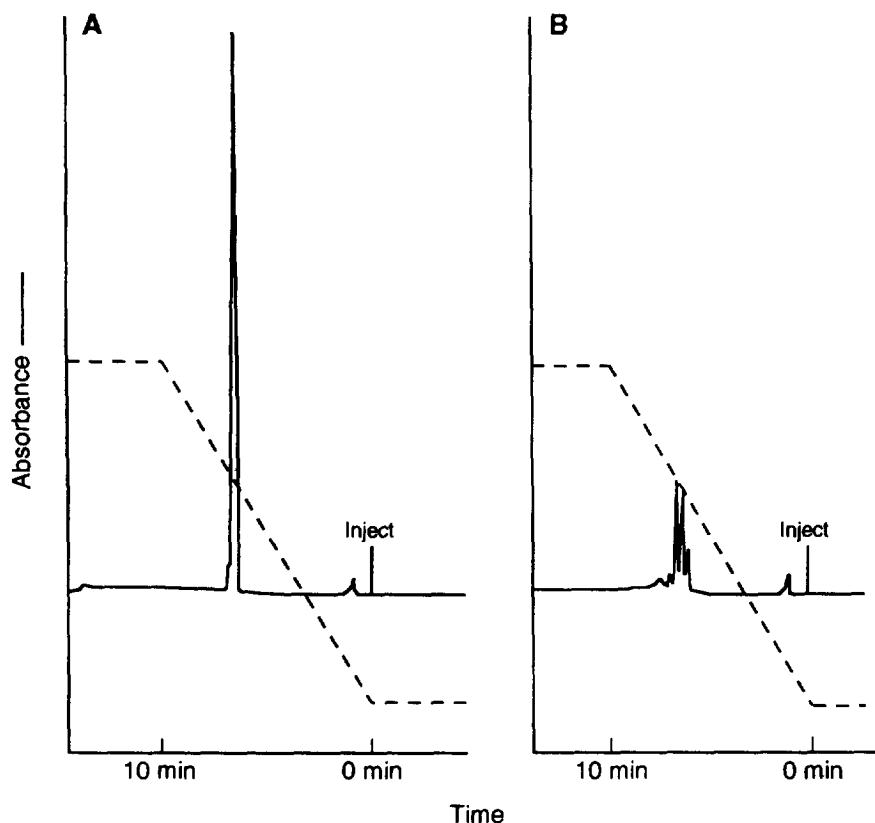
Fig. 8. HPLC analyses of peak fractions of the CCC of the peptide of Fig. 7. (A) Fraction 72, 5-μl injection, of the peak eluting at 1 h; (B) 5 μl of fraction 58 which is the peak after the solvent front (peak before 1 h). The HPLC column used is S-5 ODS 200 Å, 15 × 0.49 cm (YMC) with the solvent system A = 0.1% aq. phosphoric acid and B = 10% A in acetonitrile at a flow of 0.8 ml/min and a gradient of 5 to 40% B in 10 min. The detection is at 215 nm with 0.5 AUFS.

In Fig. 9 is shown a chromatogram of 50 mg of a synthetic 25-mer peptide using the same solvent components. Because of the higher charge of the peptide, the polarity of the solvent system was increased by modifying the volume ratios of the solvent system to 4:3:2:9. The peak at 2.5 h contained the separated peptide as shown in Fig. 10. The experiment using the solvent ratio of 2:1:1:4 eluted the major product near the solvent front while the solvent ratio of 2:2:1:5 retained the product over 4 h with an excessively long baseline (CCC chromatograms not shown). Analysis by HPLC (Fig. 10) of the 2:1:1:4 separation shows the material near the solvent front and the later-eluting purified peptide.
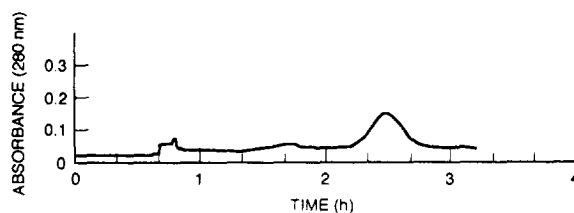

Fig. 9. (A) CCC purification of 50 mg of a 25-mer synthetic peptide [2Ala.Asp.8Glu.Phe.2Gly.Ile.Lys.2Leu.2Asn.Arg. Ser.Val.2Trp]under the same conditions as in Fig. 7 except that the volume ratios of the solvent system are 4:3:2:9. Retention of the stationary phase was 70.0%.
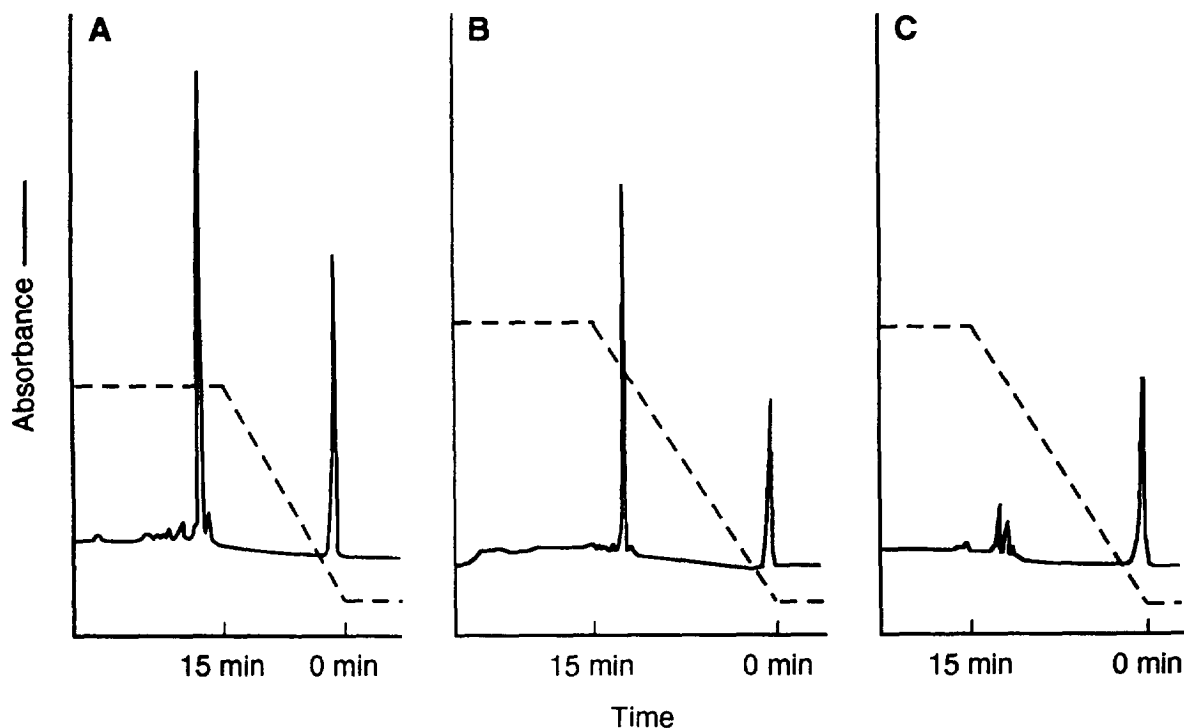
Fig. 10. HPLC analyses of CCC fractions. (A) Analysis of 50 μl of fraction 160, the peak at approximately 2.5 h. HPLC conditions are the same as Fig. 8. (B. C) Analyses of the CCC of the same peptide with a different solvent system as described in the text. The peptide was eluted closer to the solvent front and the HPLC analysis of the pure peptide peak is fraction 70 (B) and the material closer to the solvent front is the last chromatogram, fraction 54. The HPLC conditions are the same as in Fig. 8 except that the gradient is from 5 to 50% B in 15 min and sample injected was 25 μl.

4. Conclusions

The overall results of the present studies indicate potential utility of the two types of CPCs for the preparative purification of peptides.

(1) The cross-axis CPC has a large-scale preparative capability with a fair efficiency using the off-center column position.

(2) The type-J multi-layer CPC produces higher efficiencies for separations of up to 300 mg of peptides.

(3) The *tert.*-butyl methyl ether, *n*-butanol, acetonitrile and aq. TFA components can be adjusted for the optimal partition coefficient for most peptides. This solvent system makes it possible to use the multi-layer CPC at high-speed

conditions for good efficiency in preparative peptide separation.

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