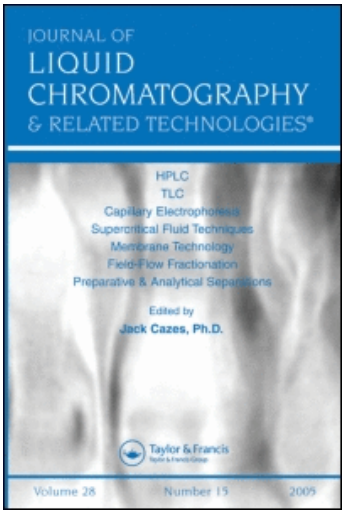

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M. Knight[a]; A. M. Kask[a]; C. A. Tamminga[a]
[a] Experimental Therapeutics Branch, National Institute of Neurological and Communicative Disorders and Stroke, Bethesda, Maryland

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PURIFICATION OF SOLID-PHASE SYNTHESIZED PEPTIDES ON THE COIL PLANET CENTRIFUGE

M. Knight, A. M. Kask and C. A. Tamminga
Experimental Therapeutics Branch
National Institute of Neurological and
Communicative Disorders and Stroke
Building 10, Room 5C106
National Institutes of Health
Bethesda, Maryland 20205

ABSTRACT

The analytical flow-through coil planet centrifuge, an instrument for countercurrent chromatography, performs the preparative purification of synthetic peptides. Various two-phase solvent systems have been tried with either phase mobile to purify many synthesized peptides. A series of N-terminal fragment peptides of cholecystokinin octapeptide (CCK 26-33) were synthesized by solid-phase techniques and purified on the coil planet centrifuge. The peptides were sulfated and chromatographed again. For hydrophobic peptides, purification is effected in solvent systems with a mobile aqueous phase. The n-butanol, acetic acid and water system (4:1:5 by volume) with the lower phase mobile was utilized. For sulfated peptides, the neutral system, 0.2 M ammonium acetate and n-butanol was generally applied.

INTRODUCTION

The horizontal flow-through coil planet centrifuge (2) has been used extensively for the preparative purification of peptides prepared for neuropharmacological experiments. Countercurrent chromatography (CCC) has been a useful method for separating truncated or deletion sequences and possible side products bearing

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protecting groups or products due to the oxidation of Trp and Met residues. Opioid peptides, cholecystokinin fragments and analogues have been synthesized by solid-phase techniques and purified in various two-phase solvent systems. The elution of the peptides was modified by changes in the composition of the solvent system to afford better separations. The equipment and methodology used in these experiments including the development of the solvent systems will be described. With this experience hopefully the behavior of peptides in the chromatography will become more predictable. One solvent system useful for purifying any synthetic product or adequate as an initial and routine purification step is a goal of our developmental work.

EXPERIMENTAL

Synthesis Of Peptides

Peptides were synthesized by standard solid-phase procedures whereby N-t-butyloxycarbonyl-L-amino acids including β-Benzyl-L-Asp and 2Br carbobenzoxy-L-Tyr (Bachem, Torrance, CA; Peninsula, Belmont, CA; and Chemalog, So. Plainfield, NJ) were coupled in the presence of dicyclohexylcarbodiimide to an amino acyl resin (copolymer of polystyrene and divinylbenzene (BioRad, Richmond, CA) (3). The amino-protecting group was removed by 25% trifluoroacetic acid in methylene chloride and neutralized by 10% triethylamine in methylene chloride and the next amino acid was coupled in sequence. After deprotection, removal from the solid support was effected by reaction in anhydrous hydrogen fluoride in the presence of ethyl methyl sulfide and anisole. The syntheses were conducted automatically in the Model 990 B synthesizer (Beckman, Palo Alto, CA). After synthesis the peptides were submitted to countercurrent chromatography as described below. After purification the peptides were analyzed for purity by TLC, HPLC and amino acid analysis of a mercaptoethanesulfonic acid hydrolysis at 110°C, for 22 hr. Peptides of the cholecystokinin fragment series were sulfated by reaction with the pyridine · sulfur trioxide complex. After removal of solvent, the reaction was treated with 1 M $NaHCO_3$ and

lyophilized and purified by countercurrent chromatography. The products were analyzed for homogeneity and for the presence of the sulfate group by spectroscopic techniques.

Apparatus

The countercurrent chromatography was carried out in the analytical flow-through coil planet centrifuge. Two instruments were available for our use. The Kontes prototype model (Vineland, NJ) mounted with coiled columns of either 1.5 mm i.d. fluorinated ethylene propylene (FEP) coiled tubing with a total volume of 150 ml or 2.6 mm i.d. FEP tubing with a volume of 260 ml. The other instrument was built by the Laboratory of Technical Development (National Heart, Lung, and Blood Institute, Bethesda, MD) and mounted with PTFE tubing with a volume of 260 ml. The coil is mounted on the gear side. Further details of the design of the instrument are described elsewhere (4). The Kontes prototype instrument with accessory equipment is shown (Figure 1). The coil is driven by an EC motomatic drive (Model E-652-M) (Electro-Craft Corp.). The solvent pump is a Milton Roy sapphire piston minipump, 16/160 ml/hr capacity, (Riviera Beach, FL). The sample loading device, an MTS 4-way slide valve, 0.8 mm bore (Pierce, Rockland, IL) is connected between the pump and coil. The rest of the components are Instrumentation Specialties Co. I.S.C.O. (Lincoln, NE) equipment; Model 328 fraction collector, Type 6 optical unit and Model UA-5 absorbance monitor and recorder.

Methods

Crude peptide in amounts ranging from 200 to 600 mg which were typical synthetic yields but usually not more than 300 mg were chromatographed. The coil was filled with stationary phase, either upper or lower phase of an equilibrated two-phase solvent system. All solvent systems included n-butanol as the upper phase. Solvents were reagent grade from Baker (Phillipsburg, NJ) or Fisher Scientific Co. (Fair Lawn, NJ). Water was deionized, Hydro Service and Supplies (Durham, NC) and distilled or charcoal filtered. The

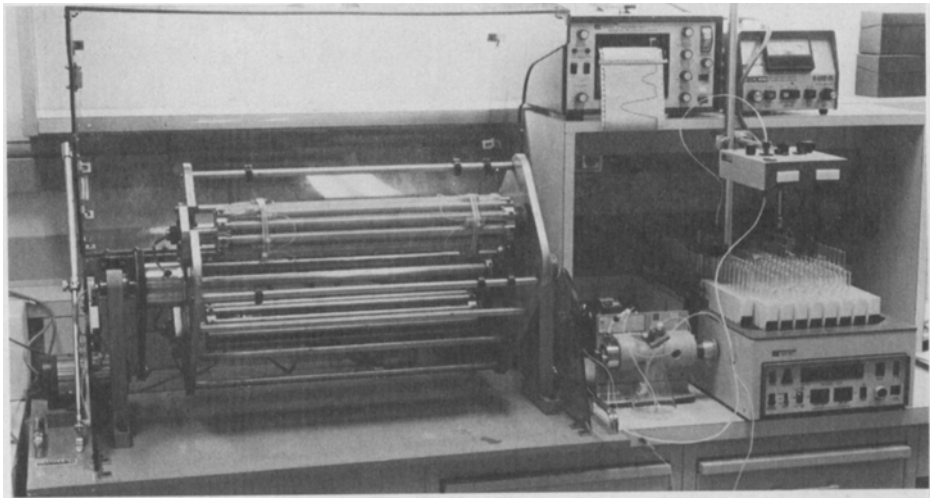

FIGURE 1

The analytical flow-through coil planet centrifuge, Kontes prototype instrument (serial no. 2) and accessory equipment described in the text. On the gear side (upper coil) is mounted the coil for preparative chromatography. On the pulley side (lower coil) is the 0.55 mm i.d. coil for analytical scale chromatography.

sample was dissolved in 3 to 5 ml of the solvent system and charged via the 4-way slide valve. The mobile phase was pumped at a flow rate of 24 ml/hr while the coil was rotated at 400 rpm in the counterclockwise direction. The effluent was monitored at 280 nm through a 40 microliter flow cell in the optical unit. The flow was down through the flow cell when the upper phase was used as the mobile phase and upward for an aqueous mobile phase. Fractions of 6 ml were collected. Samples were usually loaded late in the day and left to proceed overnight. The chromatography of an unknown sample was allowed to proceed for 3 to 4 column volumes. If necessary, the column contents were pumped out while fractions were collected and the coil rotated at 20 rpm in the clockwise direction as recommended by Y. Ito (4). Fractions containing peptide were pooled and concentrated by rotary evaporation, lyophilized and analyzed by TLC, HPLC

and amino acid analysis. The coil was washed with acetone and water between runs.

RESULTS

The peptides purified to date by the coil planet centrifuge method are listed in Tables 1 and 2 with the solvent systems utilized and resulting partition coefficients. The solvent systems are described in Table 1. Most of the peptides are cholecystokinin octapeptide (CCK 26-33) related peptides which were synthesized and purified in the conditions listed in Table 2. The peptides were sulfated and repurified in the systems shown in the table. Other peptides prepared in our laboratory are presented in Table 1.

The volume of elution of the substance permits the calculation of the partition coefficient (K) of the sample. The partition coefficient defined as (solute concentration in the mobile phase)/(solute concentration in the stationary phase) is computed as the gas chromatographic K:

V_m = excluded volume = solvent front tube x volume of fractions

V_c = total capacity of coil (260 ml or 150 ml)

V_s = $V_c - V_m$; retained stationary phase volume

$$K\,(\text{Peak fraction} \times \text{fraction volume} - V_m) = V_s$$

For a sample eluting at peak tube 85 and solvent front at tube 26 in the 260 ml coil and fractions of 6 ml:

$$V_m = 26 \times 6 \text{ ml} = 156 \text{ ml}$$
$$V_s = 260 \text{ ml} - 156 \text{ ml} = 104 \text{ ml}$$
$$V_{85} = 510 \text{ ml} - 156 \text{ ml} = 354 \text{ ml}$$
$$K_{85}\,(354 \text{ ml}) = 104 \text{ ml}$$
$$K_{85} = .29$$

TABLE 1

Synthesized Peptides

Name and Sequence	Solvent System	Mobile Phase	K
CCK Asp Tyr (SO_3H) Met Gly Trp Met Asp Phe NH_2	NH_4OAc*	Upper	.27
($D\text{-}Ala^4$) CCK8 Asp Tyr (SO_3H) Met D-Ala Trp Met Asp Phe NH_2	EBHAW*	Upper	1.22
(Lys^{31}) CCK 26-31 Ac Asp Tyr (SO_3H) Met Gly Trp Lys	BAW*	Upper	.91
(Tfa Lys^{31}) CCK 26-31 Ac Asp Tyr Met Gly Trp Tfa Lys	NH_4OAc	Upper	1.22
βLPH 61-69 Tyr Gly Gly Phe Met Thr Ser Glu Lys	.1%TFA*	Lower	1.26
βLPH 66-77 Thr Ser Glu Lys Ser Gln Thr Pro Leu Val Thr Leu	.1%TFA	Upper	.19
(Tyr^{11}) Head activator peptide PCA Pro Pro Gly Gly Ser Lys Val Ile Leu Tyr	BAW	Lower	1.51

*NH_4OAc = 0.2 to 0.5M ammonium acetate, n-butanol (1:1 by volume)
EBHAW = ethanol, n-butanol, hexane, acetic acid, water (1:3:2:1:5)
BAW = n-butanol, acetic acid, water (4:1:5)
.1% TFA = n-butanol, 0.1% trifluoroacetic acid (1:1)

The purification of two peptides are shown in Figures 2 and 3. The chromatography of Ac Asp Tyr Met Gly Trp NH_2 (unsulfated Ac CCK 26-30 amide) in the BAW system is presented in Figure 2. The peptide which had an R_f of .58 in silica gel TLC with the BAW system (4:1:1) was purified in the BAW system with the lower phase mobile. The results are shown in Figure 2. The main peak of material emerged at fraction 149, K of .14 and contained 102 mg of pure peptide from a 0.5 m mole synthesis.

The isolation of a sulfated peptide Ac Asp Tyr (SO_3H) Met Gly Trp Met NH_2 (Ac CCK 26-31 NH_2) from the sulfation reaction and side

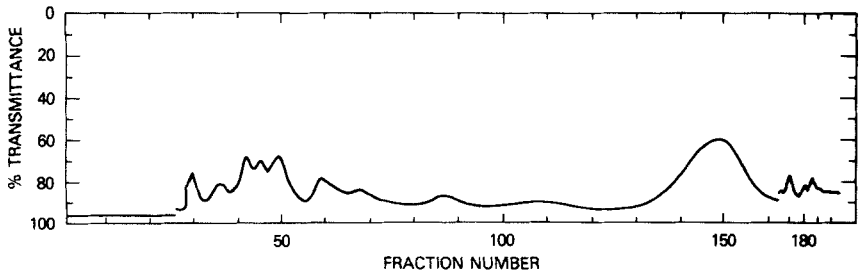

FIGURE 2

Purification of Ac Asp Tyr Met Gly Trp NH_2 in the BAW system with the lower phase as mobile phase. The 260 ml coil planet centrifuge with LKB (Bromma, Sweden) Uvicord S monitor and 6 channel recorder was used for this separation. The solvent front emerged at tube 26. Peptide, 102 mg, was collected from tubes 130 to 161 and contents of coil were pumped out beginning at fraction 163.

TABLE 2

Cholecystokinin Fragment Peptides

Sequence	Solvent System	Mobile Phase	K
Asp Tyr Met Gly Trp Met Asp NH_2	BAW	Lower	2.40
Asp Tyr (SO_3H) Met Gly Trp Met Asp NH_2	BAW	Upper	.27
Ac Asp Tyr Met Gly Trp Met Asp NH_2	BAW	Lower	.03
Ac Asp Tyr (SO_3H) Met Gly Trp Met Asp NH_2	NH_4OAc	Upper	.03
Ac Asp Tyr Met Gly Trp Met Asp	BAW	Lower	.49
Ac Asp Tyr (SO_3H) Met Gly Trp Met Asp	BAW	Upper	.88
Ac Asp Tyr Met Gly Trp Met NH_2	NH_4OAc	Upper	8.80
Ac Asp Tyr (SO_3H) Met Gly Trp Met NH_2	NH_4OAc	Upper	.90
Ac Asp Tyr Met Gly Trp Met	BAW	Lower	.17
		Upper	5.08
Ac Asp Tyr (SO_3H) Met Gly Trp Met	NH_4OAc	Upper	.03
Ac Asp Tyr Met Gly Trp NH_2	BAW	Lower	.14
Ac Asp Tyr (SO_3H) Met Gly Trp NH_2	NH_4OAc	Upper	.16
Ac Asp Tyr Met Gly	BAW	Upper	.48
Ac Asp Tyr (SO_3H) Met Gly	BAW	Upper	.12

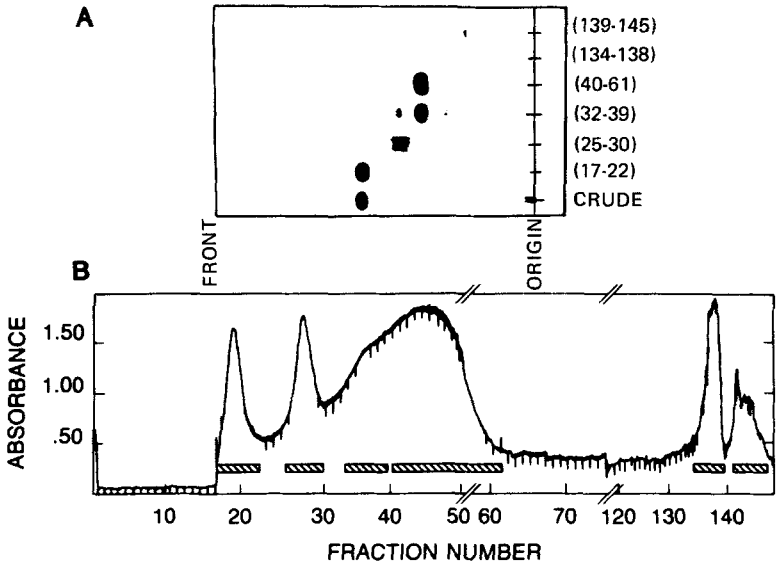

FIGURE 3

Panel A is the TLC analysis of the CCC fractionation (panel B) of the sulfation reaction of Ac Asp Tyr (SO_3H) Met Gly Trp Met NH_2. The pooled fractions indicated by diagonal lines were concentrated and lyophilized and 5 µg analyzed by TLC on silica gel in BAW (4:1:1) and developed by the Ehrlich spray (3). Unsulfated peptide, 206 mg, had been reacted with 660 mg of pyridine·sulfur trioxide complex and later treated with 15 ml of 1 M $NaHCO_3$ and neutralized with HCL. The dried mixture was chromatographed in the Kontes instrument in the 0.4 M NH_4OAc system. The solvent front emerged at fraction 17 and at fraction 118, the contents were pumped out. The first peak, 17-22, contained 102 mg of unsulfated peptide, fractions 25-30 contained 10 mg, 32-39 had 24 mg of the sulfated peptide and 40-61, 42 mg of pure sulfated peptide. Fractions 134-138 and 139-145 contained large amounts of salt.

products is shown in Figure 3. The TLC analysis of the products is included in the figure.

DISCUSSION

The partition coefficients indicate the relative elution volume and is determined by the hydrophobicity of the peptides. The majority of peptides in this group have many aromatic amino acids in their sequence and thus are highly hydrophobic. The R_f of these peptides in silica gel TLC are 0.5 or higher in the BAW system. Most of the peptides when chromatographed on the coil planet centrifuge eluted close to the solvent front in the BAW solvent system with the upper phase mobile. Better separation of impurities was achieved when the lower phase was used as the mobile phase. The peptide emerged at a later time and was distributed in a wider peak. If in TLC the R_f was high, it was better to elute in the lower K mode and if the peptide was hydrophilic, as indicated by a low R_f on TLC and there were more hydrophobic contaminants, it was useful to elute with the upper phase mobile. However if the peptide was not eluted after three column volumes then pumping out contents of the coil was warranted. A satisfactory fractionation was achieved in many cases.

Ac Asp Tyr Met Gly Trp Met as seen in Table 2 was an example of the direct determination of K's in both phases used as the mobile phase. In the BAW system with lower phase mobile, the K was 0.17 with the peak at fraction 128; and with the upper phase used as the mobile phase, the K was 5.08 (peak at tube 27). The experimental results agree with the theoretical expectation of the K's being the inverse of each other.

A means of decreasing the elution of peptides in the upper phase was to adjust the composition of the solvent system such that the sample was less soluble in the upper phase. This could be done by substituting ethyl acetate for n-butanol in the BAW system or using the system ethanol, n-butanol, hexane, acetic acid and water (EBHAW, 1:3:2:1:5). The peptide D-Ala^4 CCK 26-33 was eluted with

the solvent front in the system ethyl acetate, n-butanol, acetic acid and water (1:3:1:5) and in EBHAW it was eluted later with a K of 1.22.

The peptides were sulfated and repurified in either the BAW or NH_4OAc system with the upper phase mobile. The sulfation reaction mixture contained large quantities of sodium bicarbonate, NaCl and pyridine · sulfur trioxide decomposition products which remained in the lower phase as the peptides were extracted during the chromatography. The NH_4OAc system was preferred if adequate separation was achieved because the tyrosine sulfate ester function remained stable in the neutral pH of this system. The sulfation reaction mixture of Ac Asp Tyr (SO_3H) Met Gly Trp Met NH_2 was chromatographed in 0.4 M NH_4OAc and after two peaks (one of which was unsulfated peptide) a wide peak emerged (Figure 3). The earlier fractions in this peak were heterogeneous so the fractions were pooled separately from the rest of the peak. In the figure the elution of the chromatography and the TLC analysis of the peak contents are presented. The order of elution correlated with the R_f of the pooled fraction. The sulfated peptide was the third peak, fractions 40-61 (containing 42 mg) and on TLC had an R_f of .35. Thus the fractionating potential of CCC can be assessed by TLC. In order to separate the impurities better, product from a resynthesis plus the pool of fractions 32-39 from the synthesis in Figure 3 were combined and chromatographed in 0.2 M NH_4OAc. The sulfated peptide was eluted as a symmetrical peak at fraction 77 (not shown). The product was very pure by TLC. The lower ionic strength of the NH_4OAc decreased partitioning in the upper phase and thus resulted in better fractionation in this case. The peptide Ac Asp Tyr Met Gly Trp Tfa Lys was also better fractionated with 0.2 M than with 0.4 M NH_4OAc. In some cases the sulfated peptide had a low K and separation from the salt required another step, usually HPLC. This was the case for Ac CCK 26-32 amide, CCK 26-31, Ac CCK 26-29 and Ac CCK 26-30.

Generally the solvent systems selected for CCC application have been those described for countercurrent distribution (5).

These solvents have negligible absorbance in the ultraviolet range so the effluent can be monitored. The system 0.1% trifluoroacetic acid, n-butanol (1:1) has been used for β Lipotropin 61-69 (6) and for des enkephalin γ endorphin (β LPH 66-77) (7). Pyridine acetate (0.1% acetic acid, n-butanol and pyridine 11:5:3) which absorbs in the UV has been utilized for relatively hydrophilic peptides with upper phase mobile, but a dual flow cell would be necessary if monitoring is desired. All the solvent systems used in this work are volatile hence desalting steps are unnecessary. The examples where these other solvent systems were used are listed in Table 2.

CONCLUSION

From this experience we have found countercurrent chromatography on the coil planet centrifuge a convenient alternative to CCD because the apparatus is small in comparison to the glass manifold and resolution is better. As in CCD total recovery of material is always possible. If a run is to be repeated in another system, the sample is easily recovered. Furthermore, the capacity of the coil is enough for the yields of average solid-phase syntheses. The time required for purification may be longer than that for HPLC, but the sample can be loaded and left unattended usually overnight thus requiring less personal involvement than HPLC. The coil planet centrifuge has established itself well as a "work horse" instrument in our peptide chemistry laboratory.

ACKNOWLEDGEMENTS

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1. Abbreviations used: CCC = countercurrent chromatography; CCD = countercurrent distribution; TLC = thin layer chromatog-

raphy; HPLC = high performance liquid chromatography; K = partition coefficient; R_f = mobility relative to front; UV = ultraviolet; Tfa = trifluoroacetyl; NH_4OAc = ammonium acetate; B = n-butanol; H = hexane; A = acetic acid; W = water; E = ethanol; CCK = cholecystokinin; TFA = trifluoroacetic acid; Ac = acetyl; LPH = lipotropin.

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