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Note

Preparative purification of the peptide des-enkephalin γ-endorphin

Comparison of high-performance liquid chromatography and counter-current chromatography

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Considerable importance is attached to the development of procedures for the purification of synthesized peptides where good chromatographic fractionation can be achieved. Partition chromatography in volatile solvent systems has for some time been a useful procedure for large amounts of material. Amounts of peptide in the range 100–500 mg were easily separated in the counter-current distribution machine of the Craig–Post design. This large apparatus, however, is no longer commonly available. Methods utilizing more modern instruments which are relatively compact and accessible were evaluated for their applicability to purifying large amounts of synthesized peptides. These methods provide potentially easy and routine initial fractionation steps. One procedure studied in this experiment is counter-current chromatography performed in the horizontal flow-through coil planet centrifuge[1,2]. This liquid–liquid partition system allows excellent recovery of material and is versatile in that either phase is used as the mobile phase. The other procedure evaluated was high-performance liquid chromatography (HPLC) using a semi-preparative C_{18} column, 30 cm × 7.8 mm I.D. μBondapak (Waters Assoc., Milford, MA, U.S.A.). In this case the resolution was expected to be better. Both these procedures were tried in the purification of the dodecapeptide β-LPH (66–77), or des-enkephalin γ-endorphin, a β-endorphin fragment studied for its possible neuroleptic-like behavioral effects[3]. The results were compared on the basis of purity and recovery.

EXPERIMENTAL

All chemicals were analytical grade reagents. *n*-Butanol was from Burdick &

Jackson (Muskegon, MI, U.S.A.). Acetonitrile and methanol were HPLC grade from Waters Assoc. Water used in HPLC was deionized and glass distilled.

The peptide des-enkephalin γ-endorphin (Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu) was synthesized by the automated solid-phase procedure on a Beckman 990B instrument[4]. The N-α-Boc amino acids in 2.5 molar excess were coupled in the desired sequence to 0.92 g Leu substituted chloromethyl 1% divinylbenzene cross-linked polystyrene resin (0.4 mmol) using dicyclohexylcarbodi-imide as the coupling agent. A 4-molar excess of Boc-Gln was coupled as the *p*-nitrophenyl ester in dimethylformamide. The side-chain-protected N-α-Boc amino acids were benzyl Thr, benzyl Ser, ε-2Cl carbobenzoxy Lys, γ-benzyl Glu and Gln *p*-nitrophenyl-ester (purchased from BaChem or Chemalog). Each coupling step was repeated with half the amount of coupling amino acid. The peptide resin was cleaved and deprotected with 10 ml of anhydrous hydrogen fluoride and 1 ml of anisole for 45 min at 0°C.

Counter-current chromatography was carried out in the horizontal flow-through coil planet centrifuge with 2.6 mm I.D. PTFE tubing coiled in 1000 turns with a capacity of 260 ml. The column was charged with 230 mg of peptide in 5 ml of each phase of the solvent system, 0.1% trifluoroacetic acid–*n*-butanol (1:1, v/v). The sample had a K value of 0.19 in this system. The coil was filled with lower phase and the upper phase served as the mobile phase. The upper phase was pumped at 24 ml/h and the column-coil was rotated at 400 rpm. Fractions of 6 ml or 15 min were collected. The solvent front emerged at tube 28 (168 ml). The mobile phase was pumped for three times the column volume, then the column contents were pumped out emerging between fractions 130 and 170. The fractions were assayed by Folin-Lowry determination[5], and tubes containing peptide were pooled, evaporated in a rotary evaporator and lyophilized.

For preparative HPLC a 50–100 mg sample was dissolved in 0.02 M acetic acid (0.1%) and passed through a Millipore filter and injected with the 6UK manual injector in a volume of 2 ml onto the semi-preparative C_{18} μBondapak column, pumped at a flow-rate of 3 ml/min with 0.1% acetic acid. After 5 min, a linear gradient of methanol was pumped from 0 to 60% methanol in 60 min. Two 6000A Waters pumps were used with a 720 systems controller. The column was run through the chromatography conditions prior to loading the sample. Absorbance at 210 nm at the full scale of 2 was monitored. Fractions of 1 min were collected and assayed by the Folin-Lowry method, and tubes containing peptide were pooled and concentrated as described above.

Solutions of 10 mg/ml were made of the different chromatographic products and these were analyzed by TLC, analytical HPLC, and amino acid analysis of hydrolysates. Analytical HPLC was done on the 30 cm × 4 mm I.D. C_{18} μBondapak column in 0.1% phosphoric acid and 17% acetonitrile at a flow-rate of 3 ml/min. Absorbance at 210 nm at 0.04 absorbance units was recorded.

RESULTS AND DISCUSSION

The yield of synthesized product after lyophilization was 382 mg of off-white powder. On cellulose TLC in the system *n*-butanol–acetic acid–water (4:1:1, v/v/v), there were two spots (not discrete but smeared) with R_F values of 0.41 and 0.13.

The results of an HPLC experiment are shown in Fig. 1. Absorbance at 210 nm was monitored. The peaks, determined by the Folin-Lowry method, were well separated. The yield of material from a chromatography of 50 mg was 2 mg in the peak emerging at 37 min and 9 mg in the peak emerging at 48 min. In a chromatography of 100 mg, 3 and 29 mg were obtained, respectively. Amino acid analysis showed the expected integral molar ratios of the amino acids in the material of the second peak.

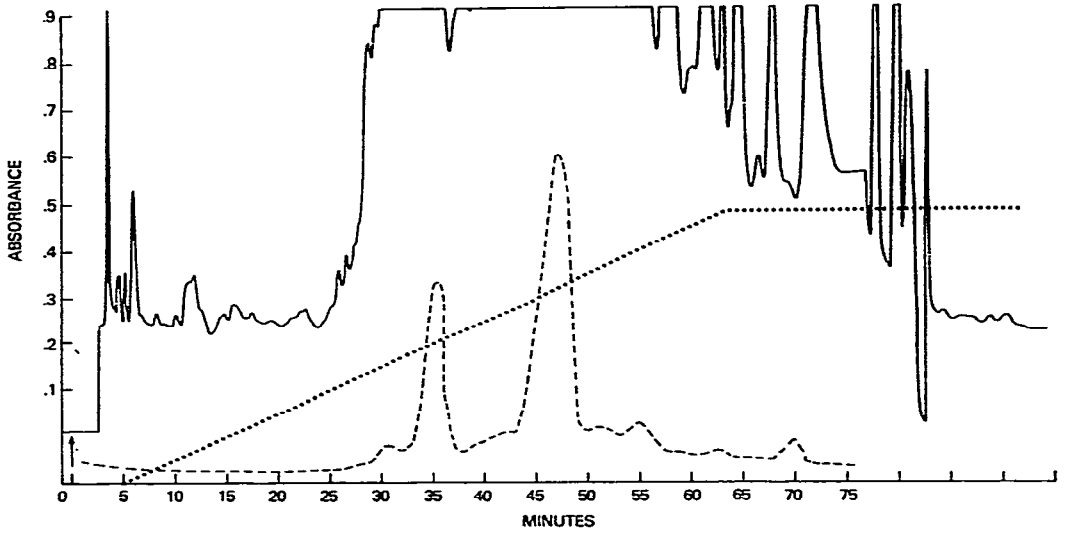

Fig. 1. Preparative HPLC of 50 mg des-enkephalin γ-endorphin. The procedure is described in the text. The absorbance recording of the Model 440 variable-wavelength detector (Waters Assoc.) at 210 nm with the full scale of 2 units is the solid line. The dashed line is absorbance at 700 nm. Samples of 20 μl were taken from 3-ml fractions and analyzed by the Folin-Lowry method. The dotted line indicates percentage of methanol. The column was pumped at 3 ml/min in 0.1 % acetic acid, and 5 min after injection of sample a gradient of methanol at 1 %/min was started.

Since the yield was so low the other procedure was tried. A sample of 230 mg was run in the horizontal flow-through coil planet centrifuge. As seen in Fig. 2, only a single peak was found and it was not symmetrical, indicating lack of resolution. The yield of the major fraction of the peak was 148 mg and of the following material, 33 mg. Much more material was recovered in this procedure. Fig. 3 contains the traces of the analytical HPLC of each of the fractions from both procedures. In the upper panel (A) the major peak of the reversed-phase chromatography was pure. The major peak of the counter-current chromatogram contained impurities but was highly purified (C). This partially purified product was passed through the reversed-phase column, affording complete purification but low yield (23 %).

Des-enkephalin γ-endorphin, a neutral, non-aromatic, moderate-sized peptide would be expected to be resolved only with difficulty from impurities of similar structure, such as deletion or truncated peptides or diastereoisomers. The resolution of the semi-preparative reversed-phase column in purifying this peptide was excellent. The impurities were separated well from the major product by 10 min. This separation was not complete in the counter-current chromatography. Nevertheless, the coil

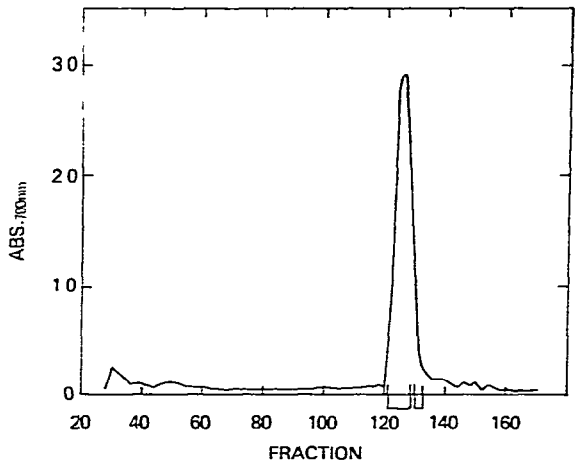

Fig. 2. Analysis of horizontal flow-through planet centrifuge fractions. The absorbance of 100-μl samples analyzed by the Folin-Lowry method is shown. Fractions 121–128 were pooled as the major product and fractions 129–132 as the minor product. The solvent front emerged at tube 28, the lower phase at tube 121 and column contents between tubes 130 and 170. The conditions of the chromatography are described in the text.

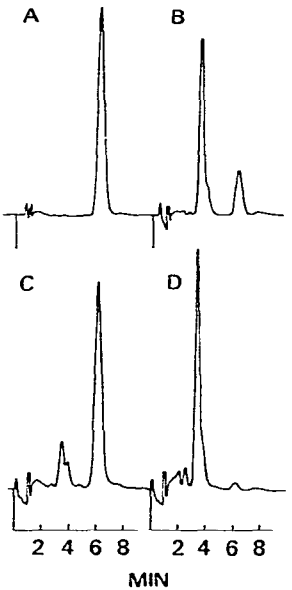

Fig. 3. Analytical HPLC of 5-μg samples of each chromatographic product. Recording at 210 nm absorbance (0.04 units full scale) is shown. The products of the HPLC preparative fractionation are shown in the upper panel. A is the large peak emerging at 47 min and B is the smaller peak at 35 min (Fig. 1). In the lower panel C is the major component of the counter-current chromatography fraction (121–128) and D is the remainder of the material (129–132) (Fig. 2).

could have been run longer or recycled to achieve a better separation. Compared with the major product, the impurities were less hydrophobic eluting earlier in the reversed-phase and later in the counter-current chromatography.

A serious problem with the reversed-phase chromatography, however, was the recovery. Pure peptide attained by this method (29% yield) was one half that of the counter-current procedure (65%). The counter-current fraction appeared to be highly purified (at least 85%) in the analytical HPLC. With other peptides the horizontal flow-through coil planet centrifuge has provided chromatographically pure products[6,7]. Conditions will have to be developed to improve the recovery of peptide from the reversed-phase column. Other volatile solvents or flow-rates or gradients could be tried. The column packing material may be affecting the results as well. The flow-rate utilized was not very high and back pressure was *ca.* 3500 p.s.i. Furthermore, in one example (D-Ala^2 enkephalin Arg^6) which has a relatively early retention time, the recovery from HPLC after two chromatographic runs on a column was quite high (50%). Thus the recovery may depend on the structure of the peptide. It is still preferable to perform a chromatography that provides quantitative recovery and then resort to HPLC for further purification. If high recoveries were consistently possible with HPLC then this method could be used exclusively.

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