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SEMI-PREPARATIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC PURIFICATION OF A 28-AMINO ACID SYNTHETIC PARATHYROID HORMONE ANTAGONIST

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SUMMARY

Structure–activity studies of parathyroid hormone (PTH) over the last decade have led to the design and synthesis of a peptide hormone inhibitor of PTH action *in vivo*, [Tyr-34]bPTH-(7–34)amide*. To evaluate the biological properties of this 28-amino acid hormone analogue, sufficient amounts of the peptide needed to be prepared in a high state of purity to permit continuous infusion in groups of animals for periods of several hours. For this purpose, gel chromatography followed by semi-preparative, reversed-phase high-performance liquid chromatography (HPLC) using a lyophilizable solvent gradient system of acetonitrile in 0.1 % trifluoroacetic acid was utilized. In a parallel purification scheme, gel filtration followed by ion-exchange chromatography was employed. Comparison of yields and product purity were determined by a battery of chemical analytical techniques. Semi-preparative HPLC generated a highly purified product; substantial heterogeneity was detected in the ion-exchange-purified material. The HPLC-purified product retains the biological activities of binding to PTH receptors *in vitro* and inhibiting PTH action *in vivo*.

INTRODUCTION

Parathyroid hormone (PTH), an 84-amino acid single-chain polypeptide, serves a critical physiological role in mineral ion homeostasis. The structural determinants necessary for full biological activity lie within the amino-terminal one-third (residues 1–34) of the hormone molecule[1]. The amino acid sequence is Ala - Val - Ser - Glu - Ile - Gln - Phe - Met - His - Asn - Leu - Gly - Lys - His - Leu - Ser - Ser - Met - Glu - Arg - Val - Glu - Trp - Leu - Arg - Lys - Lys - Leu - Gln - Asp - Val - His - Asn - Phe. Multiple *in vivo* and in vitro assays for PTH-stimulated effects on adenosine-3′,5′-cyclic phosphoric acid (cyclic AMP) levels and calcium and phosphate fluxes have demonstrated identical potency between the native hormone and an amino-terminal 1–34 synthetic fragments[2–5].

Further structure–activity studies with synthetic PTH analogues have de-

* bPTH = Bovine parathyroid hormone.

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lineated within the hormone molecule separable domains for receptor binding and activation[2,6]. An effective inhibitor of PTH action would compete with the native hormone for receptor occupancy, but would have no agonistic properties. The 3–34 region of the molecule contains such an inhibitory core. Multiple *in vitro* bioassays have demonstrated that a 3–34 synthetic peptide analogue, [Nle-8, Nle-18, Tyr-34]bPTH-(3–34)amide, possesses avidity for the PTH receptor comparable to that of the native hormone: the inhibitory constant (K_i) of the 3–34 analogue is approximately equal to the affinity constant (K_m) of PTH. Further, the analogue is devoid of PTH-like agonist activity *in vitro*[7].

However, when tested *in vivo*, the 3–34 analogue demonstrated weak but definite PTH-like agonist properties in the dog[8] and the rat[9], although its potency was less than 1% that of PTH on a molar basis. These results suggested that further modifications or truncation of the N-terminus might eliminate the final traces of agonist activity from the 3–34 sequence without concomitant loss of avidity for PTH receptors.

The sequence 7–34 can still bind to PTH receptors and inhibit completely the specific binding of a fully biologically active radioligand analogue of PTH[6]. The receptor affinity of the 7–34 sequence is diminished by only 1/10–1/100th that of the 3–34 sequence. In addition, in all assay systems tested, the substitution of tyrosine amide at the carboxy terminus (position 34) for phenylalanine carboxylic acid increased the potency of analogues. Using these established directions, we selected for synthesis and biological evaluation as an *in vivo* inhibitor the analogue [Tyr-34]bPTH-(7–34)amide.

A several milligram amount of highly purified peptide was necessary for the extensive *in vivo* evaluation that was planned. The analogue was prepared by the solid-phase method of Merrifield[10]. To maximize yields and product purity, we employed semi-preparative, reversed-phase high-performance liquid chromatography (HPLC)[11–13] with lyophilizable solvents consisting of a gradient of acetonitrile in 0.1% trifluoroacetic acid–water[14].

Previously, synthetic PTH analogues had been purified by gel filtration followed by ion-exchange chromatography. To compare the yield and purity of product obtained, material from the same synthesis of [Tyr-34]bPTH-(7–34)amide was prepared by both purification schemes.

MATERIALS AND METHODS

Synthesis of [Tyr-34]bPTH-(7-34)amide

A fragment of bovine parathyroid hormone, [Tyr-34]bPTH-(7–34)amide, was synthesized on a Beckman Model 990 automated synthesizer using a modification[15] of the Merrifield solid-phase method.

Benzhydrylamine hydrochloride resin (polystyrene–1% divinylbenzene, 0.54 m*M* amine per gram of resin; Beckman) was used as the solid support to effect the carboxyamide ($CONH_2$) COOH-terminal modification. The Boc-L-amino acids were obtained from Bachem Fine Chemicals. Each amino acid-incorporating step was monitored for completeness of reaction by the fluorescamine test[16]. The details of the synthetic procedure have been reported previously[17].

The peptide was cleaved from the resin, and side-function protecting groups

were removed simultaneously by treatment with anydrous hydrogen fluoride for 1 h at 0°C in the presence of anisole.

Gel chromatography

All crude peptide product obtained from hydrogen fluoride cleavage underwent preliminary gel chromatographic purification prior to either semi-preparative HPLC or ion-exchange chromatography. Peptide was applied to a polyacrylamide Bio-Gel P-6 (Bio-Rad) column (100 × 2.0 cm) and eluted with 1.0 *M* acetic acid.

Analytical and semi-preparative HPLC

Reversed-phase HPLC was performed with a Waters liquid chromatograph using both semi-preparative (30 × 7.8 mm I.D.) and analytical (30 × 3.9 mm I.D.) μBondapak C_{18} columns.

Acetonitrile (HPLC grade/MCB reagents) was used to make the solvent gradient. Solvent 1 was 20% acetonitrile and 80% water with 0.1% trifluoroacetic acid throughout; solvent 2 was 70% acetonitrile and 30% water with 0.1% trifluoroacetic acid throughout[14]. Flow-rates of 4.0 and 2.0 ml/min were used for the semi-preparative and analytical modes, respectively. The column effluent was monitored at both 220 and 254 nm.

Ion-exchange chromatography

An alternative method of purification involved the application of the partially purified product obtained after the Bio-Gel P-6 chromatography to an ion-exchange column of carboxymethylcellulose (CMC) (30 × 12 mm I.D., CM-52; Whatman), using an ammonium acetate buffer gradient system[15,18]. The column effluent was monitored at 206 and 280 nm.

Amino acid analysis

The amino acid composition was determined after acid hydrolysis in 5.7 *M* hydrochloric acid at 110°C in an evacuated desiccator for 24 h in the presence of 1:2000 (v/v) mercaptoethanol; amino acid analyses were conducted using a Beckman Model 121M analyzer.

Sequence analysis

Automated Edman sequence analysis was performed using a Beckman 890C sequencer. The single-coupling, double-cleavage method of Edman and Begg[19] and other previously described methods[20] were used. Edman degradations were carried out manually, as previously reported[21]. Phenylthiohydantoin derivatives of amino acids were identified by thin-layer chromatography (TLC) and reversed-phase HPLC using a 0.02–0.1 *M* lithium acetate gradient[22].

Chemical analytical methods

TLC and thin-layer electrophoresis (TLE) were performed on the peptide at various stages of purification using cellulose-coated plates (100-μm layers)[18]. The TLC solvent system was *n*-butanol–pyridine–acetic acid–water (15:10:3:12) and the TLE solvent system was pyridine–acetic acid–water (30:1:270), pH 6.5 (600 V, 11 mA, 1.5 h). The plates from both systems were stained with ninhydrin.

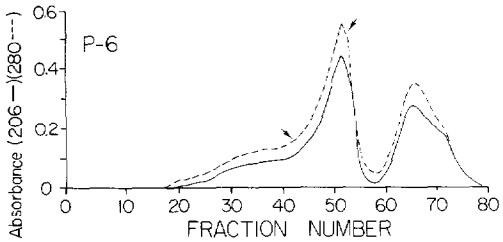

Fig. 1. Elution profile obtained by gel chromatography of crude synthetic [Tyr-34]bPTH-(7–34)amide on a Bio-Gel P-6 column. The fractions between the arrows were pooled and lyophilized for further purification.

Polyacrylamide gel isoelectric focusing (IEF) was performed using slab gels [gel concentration $T = 5\%$, cross-linkage $C = 3\%$, pH = 3.5–10.5, ampholine concentration = 2.4% (v/v); LKB]. Bands were detected by 30-min treatment of the gel with a solution of 150 ml of methanol, 350 ml of water, 17.2 g of sulfosalicylic acid and 57.5 g of trichloroacetic acid. The precipitated peptide bands appear white and can be readily made visible against a black background[23].

RESULTS AND DISCUSSION

The first step in the purification scheme was the application of 250 mg of crude peptide (Fig. 2A) to a P-6 column for preliminary purification (Fig. 1). The major peaks, collected by pooling the appropriate fractions, weighed 86.5 mg, corresponding to a yield of 34.6%. At this stage of partial purification, the material was heterogeneous by TLC, TLE, IEF and analytical HPLC, and hence was not evaluated biologically. Further purification was achieved by either semi-preparative HPLC or ion-exchange chromatography.

The chromatograms from semi-preparative HPLC were identical at 220 and 254 nm, and showed multiple peaks (Fig. 2B). Only one peak had the correct amino acid composition and sequence analysis. TLC, TLE, IEF and analytical HPLC (Fig. 3) demonstrated a high degree of purity.

The yield of material from this peak was 12.4% by weight of the crude peptide starting material. The CMC ion-exchange chromatogram of the P-6 purified material

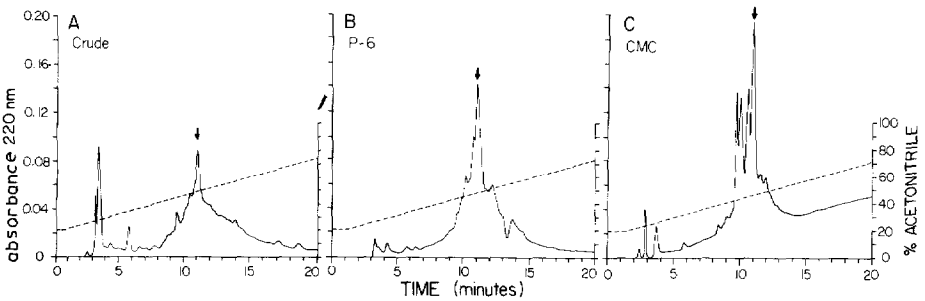

Fig. 2. Analytical and semi-preparative HPLC of [Tyr-34]bPTH-(7–34)amide. A, Analytical HPLC profile of crude peptide without previous purification; B, semi-preparative HPLC profile of peptide after gel chromatography on a Bio-Gel P-6 column; C, analytical HPLC profile after purification by P-6 and CMC ion-exchange chromatography.

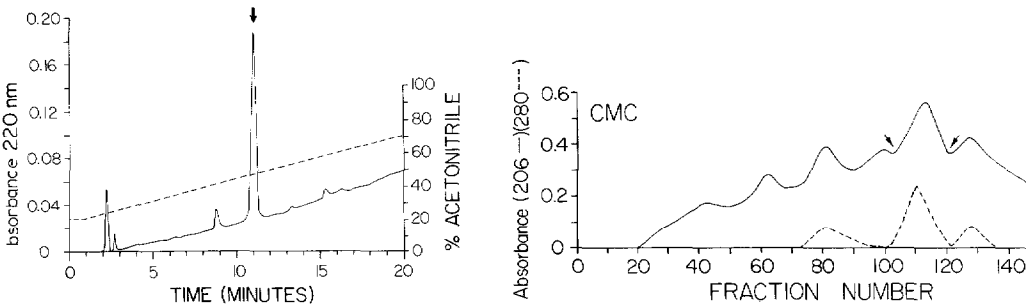

Fig. 3. Analytical HPLC of [Tyr-34]bPTH-(7–34)amide, purified by semi-preparative HPLC.

Fig. 4. Elution profile by ion-exchange chromatography of [Tyr-34]bPTH-(7–34)amide using a CMC column. Three peaks absorb at both 206 and 280 nm. The second peak, enclosed by arrows, contains the desired peptide.

showed only three peaks which absorbed at both 280 and 206 nm (Fig. 4). As the aromatic moiety of tyrosine at position 34 absorbs at 280 nm, the desired material was in one of these three fractions. Only the second peak had the correct amino acid composition.

The material from this fraction accounted for 26.6% of the original P-6 purified material applied to the CMC column. However, by TLC, TLE and IEF, the CMC-purified material was heterogeneous. When analyzed by HPLC (Fig. 2C), eight peaks were obtained.

The amino acid composition of the semi-preparative HPLC-purified material was Asp(3) 3.1, Ser(2) 1.8, Glu(3) 3.2, Gly(1) 1.0, Val(2) 2, Met(2) 1.6, Leu(4) 4.0, Tyr(1) 1.1, Phe(1) 1.0, His(3) 3.1, Lys(3) 3.1 and Arg(2) 2.0; and for the CMC-purified material Asp(3) 3.0, Ser(2) 1.9, Glu(3) 3.0, Gly(1) 1.1, Val(2) 1.9, Met(2) 1.8, Leu(4) 4.1, Tyr(1) 1.1, Phe(1) 1.1, His(3) 3.1, Lys(3) 3.1 and Arg(2) 1.9.

Automated Edman sequence analysis, through ten cycles, confirmed the presence of the desired sequence for peptides obtained from both purification routes. Furthermore, contamination by deletion-containing error peptides was quantitated as "preview", indicated by the presence of more than one phenylthiohydantoin amino acid derivative per cycle; the minor phenylthiohydantoin amino acid is present one cycle prior to expected occurrence and hence is termed "preview". Preview was determined at a sensitivity of less than 1%[24]. No preview was detected in the HPLC-purified material, indicating complete absence of deletion-containing error peptides among the first ten residues. In contrast, preview analysis of the CMC-purified material showed 3% Met/Phe at cycle 1, 5.1% Asn/His at cycle 3, 4.3% Leu/Asn at cycle 4, 8.23% Gly/Leu at cycle 5 and 7.3% Lys/Gly at cycle 6, indicating a purity of no greater than 92–93% for this peptide.

The product from each purification scheme was assayed *in vivo* for antagonist activity. The [Tyr-34]bPTH-(7–34)amide obtained by semi-preparative HPLC inhibited the action of native parathyroid hormone[26]. The ion-exchance purified peptide lacked demonstrable antgonistic properties. Because of its impurity, the CMC-purified material may have contained either insufficient amounts of the desired material, or contaminating side-products possessing agonist-like activity which prevented demonstration of antagonist properties for the heterogeneous product. The side-products present in the CMC-purified material were analyzed for the presence of methionine sulfoxide. Less than 1% of methionine was present in oxidized form as determined by amino acid analysis after enzymic hydrolysis[25]. Oxidized PTH is devoid of bioactivity and does not bind to PTH receptors[25]. Hence, oxidation of the ana-

logue during purification does not account for the loss of antagonistic properties observed. This result is not surprising, as PTH-(1–34) can be purified by CMC or HPLC with full retention of bioactivity[25] (and unpublished data).

In summary, semi-preparative HPLC was an effective method for purifying a synthetic peptide inhibitor of PTH, [Tyr-34]BPTH-(7–34)amide. With this technique, several milligrams of highly purified material were obtained.

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