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USE OF PICO-TAG METHODOLOGY IN THE CHEMICAL ANALYSIS OF PEPTIDES WITH CARBOXYL-TERMINAL AMIDES

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SUMMARY

A chemical method has been established for the detection of carboxyl-terminally amidated peptides in tissue extracts. Tissue was homogenized in an acidic medium designed to solubilize peptides while precipitating high-molecular-weight protein. The homogenate supernatant was in turn subjected to reversed-phase extraction with C_{18} Sep-Pak cartridges. The eluates were fractionated by reversed-phase high-performance liquid chromatography (RP-HPLC). Individual fractions were exhaustively digested with thermolysin, derivatized with phenylisothiocyanate (PITC), and then subjected to ethyl acetate extraction under basic conditions. The phenylthiocarbamyl (PTC)-amino acid amide derivatives were selectively taken up into the organic phase, while the other digestion products remained in the aqueous phase. The organic phase was analyzed by RP-HPLC on a Pico-Tag amino acid analysis column, monitoring eluates at 254 nm. PTC-amino acid amides were identified and quantitated by comparing their elution positions and peak areas, respectively, with those of standards. Their identities were confirmed by amino acid analysis, following hydrolysis with hydriodic acid. The technique was applied to extracts of bovine posterior pituitaries and a human medullary thyroid carcinoma. Vasopressin (-Leu-Gly-amide), oxytocin (-Gly-amide), Lys^1 γ_1-melanotropin (-Phe-amide), and various acetylated and non-acetylated forms of α-melanotropin (-Val-amide) were identified in the posterior pituitary extract. Various forms of calcitonin (-Val-Gly-Ala-Pro-amide) were detected in the tumour extract. For vasopressin and calcitonin the thermolytic digest resulted in di- and tetra-peptides, respectively, reflecting thermolytic cleavage at more favoured sites.

INTRODUCTION

Carboxyl-terminal amidation is a common post-transitional modification of neuropeptides[1]. Furthermore, it is, in most instances, an absolute requirement for the full expression of biological activity, and de-amidation is generally accompoanied by loss of biopotency. A thin-layer chromatographic (TLC) method for detecting C-terminally amidated peptides has been developed by Tatemoto and Mutt[2]. It is based upon the enzymatic release of C-terminal amino acid amides, followed by

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derivatization with dansyl chloride. The dansyl amino acid amides are selectively extracted from a basic aqueous solution into ethyl acetate. The dansyl derivatives of the free amino acids remain in the aqueous phase under these conditions. Analysis of the dansyl amino acid amides is then achieved by TLC. Through this novel approach several peptide hormones have been isolated from extracts of intestine. These include peptide PHI, peptide PYY, and gallanin[3–5]. In common with other gut hormones, these peptides have subsequently been discovered in the brain, where they probably serve as neurotransmitters or neuromodulators. Interest in α-amidation has intensified with the elucidation of the biosynthetic mechanism involved in carboxyl-terminal amidation.

All precursor proteins for amidated peptides contain the amino acid sequence X-Gly-basic-basic. The dibasic sequence (*e.g.*, -Lys-Arg-, -Arg-Arg-) is the biochemical signal for proteolytic cleavage of the precursor, while the glycine-extended product constitutes the penultimate intermediate in the generation of the carboxyl-terminal amide[1]. The α-amino nitrogen of the glycine residue becomes the amide with the concommitant release of glyoxylate[6]. The amidating enzyme of the pituitary has been localized in the secretory granule fraction and found to be dependent upon Cu^{2+}, ascorbate and molecular oxygen[7]. These findings add further evidence to the original hypothesis of Tatemoto and Mutt[2] that, if a naturally occurring peptide is carboxyl-terminally amidated, then a biological function for that peptide is strongly implied. Recently a reversed-phase high-performance liquid chromatography (RP-HPLC) method has been developed for the separation of dansylated amino acid amides[8].

In this paper we describe how we have adapted the Pico-Tag methodology[9,10] to the chemical detection of amidated amino acids. A similar protocol to that developed by Tatemoto and Mutt[2] is used. However, the Edman reagent, phenylisothiocyanate (PITC), is employed instead of dansyl chloride. The phenylthiocarbamyl (PTC) derivatives of the amino acid amides are separated by RP-HPLC. The method constitutes a useful means of screening tissue extracts for the presence of carboxyl-terminally amidated peptides.

MATERIALS AND METHODS

Reagents

Glycine amide, serine amide, proline amide, threonine amide, alanine amide, tyrosine amide, valine amide, methionine amide, isoleucine amide, leucine amide, tryptophan amide and phenylalanine amide were purchased from Sigma (St. Louis, MO, U.S.A.). Asparagine amide, glutamine amide, histidine amide and lysine amide were kindly donated by Viktor Mutt (Karolinska Institute, Stockholm, Sweden). Stock solutions of the amidated amino acids (100 μM) were made up in 0.1 M acetic acid. They were found to be stable during prolonged storage at −20°C. PITC and triethylamine were obtained from the Pierce (Rockford, IL, U.S.A.). Thermolysin, Type X, was obtained from Sigma. Hydriodic acid (47%) and ethyl acetate were obtained from Fisher Scientific (Montreal, Canada).

High-performance liquid chromatography

Chromatography was performed with a Waters Assoc. (Milford, MA, U.S.A.) HPLC system, consisting of two Model 510 pumps, a system interface module, a

WISP 710B automatic injector, a column temperature controller, and a Model 441 single-wavelength detector (set at 214 nm for HPLC of tissue extracts and at 254 nm for amino acid and amino acid amide analysis). This system was controlled by a Waters 840 Data and Chromatography Control Station.

Solvents and reagents for RP-HPLC of tissue extracts were prepared as described previously[12]. Chromatography of PITC derivatives of amino acid and amino acid amides was performed with Pico-Tag solvents A and B (either purchased from Waters or made up according to Water's instructions[9]). Solvent A consisted of 0.14 *M* sodium citrate containing 0.5 ml of triethylamine per litre. This solution was adjusted to pH 6.4 with glacial acetic acid. Solvent B consisted of acetonitrile–water (60:40, v/v).

Tissue extraction

A stock extract of twenty bovine posterior pituitaries (Pel-Freez Biologicals, Rogers, AR, U.S.A.) was prepared exactly as described previously[13]. A portion of a human medullary thyroid carcinoma (kindly provided by David Goltzman, Royal Victoria Hospital, Montreal, Canada) was defatted as described previously[14]. An amount of 5 g (wet weight) of tissue yielded 600 mg of dried powder. An amount of 50 mg was homogenized in 5 ml of an acidic medium, consisting of 1 *M* hydrochloric acid, containing 5% (v/v) formic acid, 1% (w/v) sodium chloride, and 1% (v/v) trifluoroacetic acid (TFA). The homogenate supernatant was subjected to batch extraction with a single octadecylsilyl silica (ODS-silica) cartridge (C_{18} Sep-Pak, Waters), as described previously[12].

RP-HPLC of tissue extracts

RP-HPLC was performed on the equivalent of one bovine posterior pituitary and the extract of 50 mg of medullary thyroid carcinoma powder. In each instance, the Sep-Pak eluate was reduced in volume by *ca.* 80% under a stream of nitrogen, diluted to 5 ml with 0.1% (v/v) TFA, and pumped directly onto a μBondapak C_{18} column (Waters), as described in detail elsewhere[12]. The column was eluted in each instance with a linear gradient of acetonitrile in water, containing 0.1% (v/v) TFA, at a flow-rate of 1.5 ml/min. The molecular weights of the two forms of calcitonin were estimated by using a Waters I-125 gel HPLC column, eluted with acetonitrile–water (40:60, v/v), containing 0.1% TFA, exactly as described previously[15].

Amino acid amide analysis by the Pico-Tag method

Derivatization with PITC. All peptide acid hydrolysates, amino acid amides, and thermolytic digests were derivatized with PITC according to the protocol developed by Bidlingmeyer *et al.*[10] and described in detail in the Pico-Tag amino acid analysis operator's manual[9]. Samples were incubated for 20 min with 20 μl PITC reagent, consisting of water–triethylamine–PITC–ethanol (10:10:10:70, v/v). Following evaporation of reagents, the residue was analyzed by RP-HPLC on a Pico-Tag amino acid analysis column (Waters). For enzyme digests, an ethyl acetate extraction was performed before RP-HPLC (see below).

Separation of PTC derivatives of amino acid amides. It was necessary to develop two systems to separate all the PTC-amino acid amides.

System 1 (isocratic) separates Asn-NH_2, Gly-HN_2, Gln-NH_2 and Ser-NH_2 (peaks 1–4 in Fig. 1): isocratic elution with 100% solvent A for 10 min, followed by purge (100% solvent B) and re-equilibration (100% solvent A).

System 2 (gradient) separates Thr-NH_2, Ala-NH_2, His-NH_2, Tyr-NH_2, Val-NH_2, Met-NH_2, Ile-NH_2, Leu-NH_2, Trp-NH_2, Phe-NH_2 and Lys-NH_2 (peaks 5–16 in Fig. 1): gradient elution with solvent A–solvent B, (85:15, v/v) to (54:46, v/v) over 15 min, using curve 7, then to 50:50 (v/v) over 5 min, using curve 6, followed by purge (100% solvent B) and re-equilibration [solvent A–solvent B (85:15, v/v)]. Curves 6 and 7 are gradients programmed by the Waters HPLC control station. Curve 6 is a linear gradient, while curve 7 is a slightly convex gradient. A flow-rate of 1 ml/min was used and the temperature was 38°C. The turn-around time for both systems was 30 min.

Peptide digestion, derivatization, and extraction. Portions of RP-HPLC fractions were evaporated in 50 × 6 mm Kimble borosilicate glass tubes (BDH Chemicals Canada) in a Speed-Vac Concentrator (Savant, Hicksville, NY, U.S.A.). All of the following operations were performed in the same tubes. Thermolytic digests were performed with 100 μg enzyme in 40 μl of 50 m*M* 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES) buffer, containing 10 m*M* calcium chloride (pH 7.5)[2], for 18 h at 37°C. Each digest was taken to dryness in the Speed-Vac and derivatized with PITC reagent, as outlined above. Excess reagent was removed in the Speed-Vac, and the residue was taken in 100 μl of 0.1 *M* sodium hydroxide (previously saturated with ethyl acetate). Then 100 μl of ethyl acetate was added to each tube and the two phases were vigorously mixed for 30 s in a Vortex mixer. Following low-speed centrifugation, 80 μl of the upper ethyl acetate layer was aspirated, using a pipetting gun, fitted with a plastic tip which had been extended by stretching over a Bunsen burner flame. The ethyl acetate phase was split into two 40-μl portions, and each was taken to dryness in WISP automatic injector inserts. One set of samples was analyzed by using system 1 while the other was analyzed by using system 3.

Confirmation of the identity of PTC-amino acid amide derivatives. Analysis of the thermolytic digests revealed the presence of several PTC-amino acid amides (two PTC-peptide derivatives were also observed). To confirm their identities, repeat derivatizations were performed on the peak tubes, and the entire analytical operation was repeated, with the exception that the PTC-derivatives were collected by hand into Kimble glass test tubes. The samples were taken to dryness in the Speed-Vac and then subjected to hydriodic acid hydrolysis, employing the Waters Pico-Tag work station (200 μl hydriodic acid for 1 h at 150°C). This apparatus permits acid hydrolysis to be performed without introducting acid into the sample tube. Instead, acid is placed at the bottom of a glass container into which the Kimble test tubes are introduced. The entire container is evacuated and incubated at elevated temperature to achieve hydrolysis. This is particularly useful for hydriodic acid hydrolysis, since samples can be hydrolyzed by the acid vapour but are essentially free of acid at the end of the incubation period. Thus, the slight amount of hydriodic acid which condenses inside the sample test tube can be removed under vacuum without having to deal with the breakdown of hydriodic acid and the contamination of the vacuum apparatus with iodine, which is a major problem of the method.

Amino acid analysis. The identity of amidated peptides was confirmed by amino acid analysis. This was achieved by hydrolysis with 6 *M* hydrochloric acid (containing phenol) in the Waters Pico-Tag work station, as outlined above for hydriodic acid

hydrolysis. Hydrolyses were performed at 110°C for 18 h. At the end of this period, one half of the sample was analyzed in a Beckman System 6300 high-performance analyzer (Palo Alto, CA, U.S.A.). The other half was analyzed with the Waters Pico-Tag amino acid analysis system according to the manufacturer's instructions[9,10]. Similarly, the products of hydriodic acid hydrolysis were identified by using both amino acid analysis systems.

RESULTS AND DISCUSSION

Pico-Tag amino acid amide analysis

Fig. 1 illustrates the resolution of the PTC-derivatives of the amino acid amides by the Pico-Tag HPLC column, eluted with Pico-Tag solvents A and B. Two runs are required to resolve all of them. This is not a too inconvenient, if the samples are introduced into the column with an automatic injector, as in this study. A single system for resolving all the PTC-amino acid amides would obviously be desirable, but this would require further methods development. The system described here does have the advantage of utilizing the Pico-Tag methodology, which is commercially available. For a variety of reasons, four amino acid amides have not been considered in the present method, namely, the amide forms of glutamic acid, aspartic acid, arginine and cysteine. The procedure of selective extraction of PTC-amino acid amides from enzymatic digests demands that the derivative have no charge at alkaline pH (see later). Glu-NH_2, Asp-NH_2 and Arg-NH_2 will all be highly charged under these conditions, even in the form of their PTC-derivatives. These three PTC-amino acid amides would be eluted early and could be incorporated into the isocratic system 1 (Fig. 1). Cys-NH_2 is not considered here, because it is unlikely to be liberated during

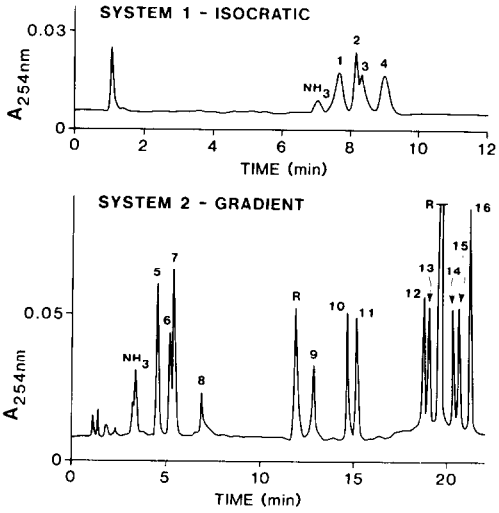

Fig. 1. Pico-Tag analysis of 250 pmoles of PTC-amino acid amides on a Waters Pico-Tag analysis column, eluted with Pico-Tag solvents A and B. See Materials and methods for details of systems 1 and 2. Peaks: 1 = Asn-NH_2, 2 = Gly-NH_2, 3 = Gln-NH_2, 4 = Ser-NH_2, 5 = Pro-NH_2, 6 = Thr-NH_2, 7 = Ala-NH_2, 8 = His-NH_2, 9 = Tyr-NH_2, 10 = Val-NH_2, 11 = Met-NH_2, 12 = Ile-NH_2, 13 = Leu-NH_2, 14 = Trp-NH_2, 15 = Phe-NH_2, 16 = Lys-NH_2. Peaks designated R are present in the reagent blank.

proteolytic digestion. Under most circumstances it will be connected through sulphur bridging with another cysteine residue (*i.e.* in the form of cystine).

Selective extraction of PTC-amino acid amides

The key to the method of Tatemoto and Mutt[2] is the selective extraction of derivatized amino acid amides from alkaline buffers into ethyl acetate. While that method is based on the dansyl reaction, our method is based on the PITC or Edman reaction[11]. PITC reacts with free amino groups to generate a PTC derivative. The separation of PTC derivatives of amino acids has recently been used as the basis for an amino acid analysis system[10,16,17]. This pre-column derivatization method compares favourably with conventional ion-exchange amino acid analysis employing the post-column ninhydrin reaction.

We found that PTC-amino acid amides were also efficiently extracted into ethyl acetate, but it is important to use a rather strongly alkaline phase to promote transfer of the PTC-derivatives into the ethyl acetate phase. The strongly basic conditions (*i.e.* 0.1 *M* sodium hydroxide) appear to be necessary to ensure complete suppression of the polarity of the PTC-amino acid amides and to facilitate their extraction by the organic phase. Incubation of PTC-amidated amino acids in 0.1 *M* sodium hydroxide for up to 1 h at room temperature revealed no apparent deamidation. Under basic conditions the PTC-derivatives of free amino acids are fully charged and tend not to be extracted by the ethyl acetate. In pilot experiments, free and amidated amino acids were derivatized and subjected to ethyl acetate extraction under basic conditions. In every instance except histidine, there was approximately 90% transfer of all the PTC-amino acid amides into the organic phase. The transfer of PTC-amino acids was slight, generally under 10%. The extent of carry-over was dependent upon the polarity of the amino acid. Thus, carry-over was nearly 0% for Pro but was greatest for the PTC-derivatives of Ile, Leu, Phe, Trp and Lys (*i.e.* di-PTC-Lys). In practical terms this extent of carry-over does not present a problem. The majority of products arising from proteolytic digestion of column fractions will result in production of rather large peptides and only small amounts free amino acids. Peptides are generally only poorly extracted into the ethyl acetate. A drawback of the present method is the poor extraction of PTC-His-amide (approximately 30–40%), due to its secondary p*K* value of 6. It is therefore quite highly charged under basic conditions and is not extracted efficiently. His-amide forms a didansyl derivative, and dansylated His-amide is efficiently extracted by ethyl acetate[2]. However, histidine is unlikely to be a carboxyl-terminal amide since the carboxyl-terminal amino acid of most amidated neuropeptides is usually hydrophobic in character. Extraction of PTC-Tyr-amide into ethyl acetate was in excess of 90%, despite its secondary p*K* of 10.

Liberation of carboxyl-terminal amino acid amides by proteolytic digestion

In their original method, Tatemoto and Mutt[2] used a number of proteolytic enzymes and buffers. They concluded that thermolysin was the most useful enzyme, because of its tendency to cleave on the amino-terminal side of hydrophobic residues (*e.g.*, Ile, Leu, Val). Since the majority of carboxyl-terminal amino acid amides found in naturally occurring neuropeptides are hydrophobic in nature, the choice of thermolysin seems ideal. We have used thermolysin in our standard procedure primarily for this reason. Other proteases can be substituted for thermolysin, but the method

demands the use of a vast excess of protease in order to maximize the chance of cleaving the terminal amino acid. In pilot experiments, we have found that most commercial enzymes give rise to a very troublesome UV background at the Pico-Tag analysis step. Fortunately, thermolysin gives the lowest UV background of all the enzymes that we investigated. In preliminary studies with synthetic peptides, we found that thermolysin successfully released carboxyl-terminal amino acid amides from α-melanotropin (-Val amide), vasopressin (-Gly amide), substance P (-Met amide) and cholecystokinin (-Phe amide). The method was then tested on extracts of two endocine tissues known to contain amidated peptides.

Analysis of tissue extracts for the presence of carboxyl-terminally amidated peptides

An extract of the equivalent of one bovine posterior pituitary was subjected to RP-HPLC (Fig. 2, upper panel). The reversed-phase extraction procedure gives rise to a peptide fraction, essentially free of high-molecular-weight protein. Thus all the UV-absorbing materials shown in Fig. 2 are derived from pro-opiomelanocortin[18] (POMC, from the intermediate lobe) or pro-oxyphysin or pro-pressophysin[19] (from the neural lobe). Fig. 3 shows the carboxyl-terminal sequences of four peptides wirth carboxyl-terminal amides which should be present in the posterior pituitary extract. Peaks eluted in the positions of PTC-Gly amide, PTC-Val amide and PTC-Phe amide were observed when column fractions were analyzed by the Pico-Tag

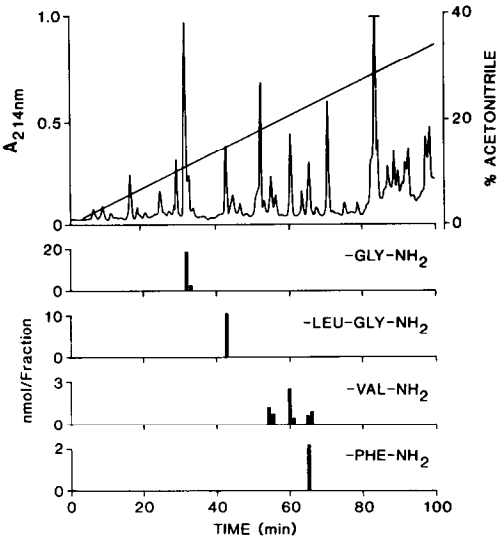

Fig. 2. Analysis of carboxyl-terminally amidated peptides found in an extract of one bovine posterior pituitary. The pituitary tissue was subjected to reversed-phase extraction with a C_{18} Sep-Pak cartridge[12]. This peptide-enriched fraction was then subjected to RP-HPLC on a μBondapak C_{18} column eluted with a linear gradient of 8% to 48% acetonitrile in water over 2 h; 1-min fractions were collected, and 400-μl aliquots from each fraction were dried and digested for 18 h with 100 μg thermolysin in 40 μl of HEPES buffer, containing 10 m*M* calcium chloride at pH 7.5. Digests were taken to dryness and derivatized with PITC according to the Pico-Tag method. Following evaporation of the reagents, the samples were taken up in 100 μl of 0.1 *M* sodium hydroxide and extracted with 100 μl of ethyl acetate. The upper layer (ethyl acetate) was aspirated, divided in two and evaporated. Each portion was subjected to Pico-Tag amino acid amide analysis, using systems 1 and 2 (see Fig. 1). See Materials and methods for full details.

method. Each was positively identified following hydrolysis with hydrodiodic acid (see Materials and methods for details). Amino acid analysis of the material in the peak tubes confirmed that the -Gly amide corresponded to arginine-vasopressin, the -Val amide peaks corresponded to various acetylated and non-acetylated forms of α-melanotropin (α-MSH) and the -Phe amide correspond to Lys^1 γ_1-melanotropin (γ_1-MSH). A fourth positive signal was obtained in the Pico-Tag analysis which did not correspond to any PTC-amino acid amide standard but fell in the valley between PTC-Met amide and PTC-Ile amide. Hydrolysis with hydrochloric acid yielded glycine, while hydrolysis with hydrodiodic acid yielded leucine and glycine. This peak therefore probably corresponded to oxytocin. This was confirmed by amino acid analysis of the peptide material in the peak tube. Thus, during thermolytic digestion of oxytocin, the -Pro-Leu- bond is preferred to the -Leu-Gly- bond (Fig. 3).

The yields of each of the amidated posterior pituitary peptides according to the chemical detection method (Fig. 2) compared favourably with those obtained by amino acid analysis of the purified peptides. The following yields, in nmoles per pituitary, were obtained (the values obtained from amino acid analysis are shown in parenthesis): vasopressin 21(25); oxytocin, 10.5(13); des-acetyl-α-MSH, 2(3); mono-acetyl-α-MSH, 3(4); di-acetyl-α-MSH, (1.5)2; γ_1-MSH, 2.5(3).

Our system was tested with an extract of another tissue known to contain a carboxyl-terminally amidated peptide. Fig. 4 shows the elution pattern resulting from RP-HPLC analysis of an extract of a human medullary thyroid carcinoma. A strong signal was obtained upon Pico-Tag amino acid amide analysis of column fractions. The UV peak did not correspond to any standard PTC-amino acid amide but fell in the valley between Tyr amide and Met amide. Back hydrolysis with hydrochloric acid yielded glycine, alanine and proline while back hydrolysis with hydroiodic acid yielded valine, in addition to these three amino acids. These four amino acids correspond to the carboxyl-terminal tetrapeptide sequence of calcitonin. Thus thermolysin, cleaves at the amino-terminal side of valine at position 29 (Fig. 3). Pico-Tag analysis of the products of thermolytic digests revealed the same peak in several column fractions. The amino acid composition of the two major peaks revealed that they were both apparently identical with that expected for human calcitonin. Gel HPLC of the major component yielded a molecular weight estimate compatible with that expected for calcitonin monomer (*i.e.* 3500 daltons). The second, more polar

VASOPRESSIN	-CYS-PRO-ARG↓-GLY-NH_2
OXYTOCIN	-CYS-PRO↓-LEU-GLY-NH_2
DES-, MONO- and DI-Ac-α-MSH	-GLY-LYS-PRO↓-VAL-NH_2
LYS^1-γ_1-MSH	-TRP-ASP-ARG↓-PHE-NH_2
CALCITONIN	↓-VAL-GLY-ALA-PRO-NH_2

Fig. 3. Carboxyl-terminally amidated peptides, found in the bovine posterior pituitary and in a human medullary thyroid carcinoma. The last four amino acid residues are shown and the site of cleavage by thermolysin.

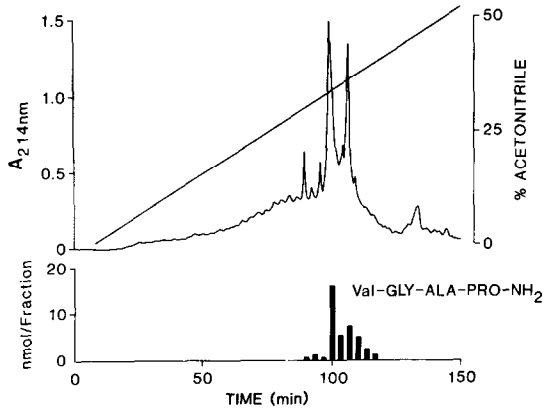

Fig. 4. Analysis of carboxyl-terminally amidated peptides, found in an extract of 50 mg of a human medullary thyroid carcinoma. The tissue extract was subjected to RP-HPLC on a μBondapak C_{18} column, eluted with a linear gradient of 1.6% to 61.6% acetonitrile in water over 3 h; 3-min fractions were collected, and 50 μl aliquots of each fraction were dried and treated as outlined in the legend to Fig. 2. See Materials and methods for full details.

component appeared to be much larger (*i.e.* 6000 daltons). This second component is probably a calcitonin dimer. It has previously been identified in medullary thyroid tumour extracts[20].

Our studies illustrate the use of a chemical method for detecting carboxyl-terminally amidated peptides, coupled with RP-HPLC, as a quantitative analytical tool. Inspection of the structures of known naturally occurring amidated peptides reveals that most of them have a hydrophobic amino acid residue at their carboxyl-terminus. Thermolysin is therefore the enzyme of choice for liberating the carboxyl-terminal amino acid amide prior to chemical analysis. The method should prove useful in the screening of tissue extracts for the presence of novel amidated peptides.

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