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SENSITIVE METHOD OF DETECTION, QUANTITATION AND PURIFICATION OF PEPTIDES USING PRE-COLUMN DERIVATIZATION WITH PHENYL ISOTHIOCYANATE

SIMON LEMAIRE*, MICHEL DUMONT and SERGE NOLET*

Department of Pharmacology, Faculty of Health Sciences, University of Ottawa, 451 Smyth Road, Ottawa, Ontario K1H 8M5 (Canada)

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SUMMARY

A sensitive method for the detection, quantitation and purification of peptides is described. The method is based on pre-column derivatization of peptides with phenyl isothiocyanate to form phenylthiocarbamoyl derivatives (PTC peptides). The derivatized peptides are analysed by reversed-phase high-performance liquid chromatography on a Zorbax ODS column (5 μm) and detected at 269 nm with a sensitivity limit of 1–5 pmol. The technique was utilized for the separation of a mixture of closely related synthetic peptides. The eluted PTC peptides were collected with an average recovery yield of 75% as determined by amino acid analysis. This method of separation of PTC peptides was also combined with the determination of the complete structure of recovered PTC-dynorphin A-(1–13) using the solid-phase sequenator (Sequemat). The advantages of the derivatization method are the rapidity and completeness of the reaction, the stability of the product, the sensitivity and specificity of the detection of derivatized peptides and the compatibility of the technique with subsequent analytical procedures. A particular application of this method was exemplified by the dosage of enkephalins secreted from perfused bovine adrenal glands.

INTRODUCTION

The purification and characterization of natural peptides present difficulties associated with the determination of the purity and amount of the product obtained at the last step of purification. Recent advances have been made in the application of high-performance liquid chromatography (HPLC) with microbore columns for the final step of purification of peptides [1, 2]. However, the eluted material is most commonly detected by UV absorbance at low wavelengths (210–220 nm), which provides high sensitivity but low selectivity, or at 230 nm

* Present address: Clinical Research Institute of Montreal, Montreal, Canada.

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for the detection of the amide bond, which limits the sensitivity [3, 4]. At low wavelengths, salts and impurities are detected as well as peptides. Other methods based on pre- or post-column derivatization of free amino groups with *o*-phthalaldehyde (OPA) [5], dansyl chloride [6] and fluorescamine [7] have also been used for the detection of peptides at picomole levels. The reaction products with these latter techniques provide high detection sensitivity and some selectivity for peptide material, but they cannot be submitted to subsequent Edman degradation [8].

Recently, Chang [9] has introduced a derivatization technique for the isolation and characterization of peptides at picomole levels. This technique is based on the pre-column formation of dimethylaminoazobenzenethiocarbamoyl (DABTC) peptides. Such a procedure, when combined with HPLC, allows the separation and detection of peptides with high sensitivity. The products are compatible with subsequent methods of analysis of the amino acid composition and structures. However, the derivatization reaction with dimethylaminobenzene isothiocyanate (DABITC) presents the following difficulties: (1) the reaction is relatively slow and incomplete; (2) several products can be obtained from a single peptide, depending on the completeness of the reaction; (3) the products are highly hydrophobic and sometimes difficult to separate from each other. Herein, we present an analytical method that is based on the formation of phenylthiocarbamoyl (PTC) peptides followed by their separation by HPLC. The method was applied in combination with amino acid analysis and sequencing, and for the identification and quantitation of secreted peptides from perfused bovine adrenal medulla.

EXPERIMENTAL

Materials

Pyridine, dimethyl-N-allylamine (DMAA), triethylamine (TEA), phenyl isothiocyanate (PITC), trifluoroacetic acid (TFA), 1-chloro butane, dimethylformamide (DMF), hydrochloric acid and OPA were purchased from Pierce (Rockford, IL, U.S.A.). N-Dimethylaminopropyl-N-ethylcarbodiimide (EDC), aminopolystyrene resin, N-methylmorpholine and N-propylamine were products of Sequemat (Boston, NJ, U.S.A.). All these solvents and reagents were sequenal grade. Acetonitrile and methanol (HPLC grade) were obtained from Fisher (Montreal, Canada). Polypropylene microcentrifuge tubes (1.5 ml) were products of Walter Sarstedt (Princeton, NJ, U.S.A.). They were extensively washed with 2 *M* hydrochloric acid, rinsed with bidistilled water and air-dried. The microcentrifuge (Model 5414, Eppendorf) and the heating block (Model 5320, Eppendorf) were purchased from Mandel Scientific (Montreal, Canada). Argon (ultrapure) was obtained from Liquid Carbonic (Montreal, Canada). A vortex evaporator (Buchler) and an HPLC system (Waters) including two M6000 A pumps, one UV detector (Model 410), an automatic injector (WISP 710), a fluorimeter (Model 420), a system controller (Model 720) and a data module (Model 730), were also used. Dynorphin A (Dyn A), Dyn A-(1–13), Dyn A-

(1–12), Dyn A-(1–9), Leu-enkephalin (Leu-Enk) and Met-Enk were synthesized in our laboratory as previously described [10].

Derivatization of peptides with PITC

The synthetic peptides (1–500 pmol each) were first added to a polypropylene centrifuge tube in 0.1% TFA. They were lyophilized, dissolved in 20 μl of ethanol–triethylamine–water (2 : 1 : 2, v/v) and dried under vacuum. The derivatization reaction was started by addition of 20 μl of PITC–ethanol–triethylamine–water (1:7:1:1, v/v). The tube was filled with a stream of argon and the reaction was allowed to proceed at room temperature (22°C) for 20 min. The reaction medium was dried under high vacuum after addition of 1 ml of methanol. The drying step was repeated twice in order to remove excess reagents and by-products. Impurities could also be removed by washing the product (dissolved in 100 μl of water) with two 100-μl portions of 1-chlorobutane. The reaction product was either kept dry in the freezer (−20°C) or submitted to HPLC analysis. It was stable for weeks when kept under these conditions.

High-performance liquid chromatography

Separation of PTC peptides (1–500 pmol samples) was achieved by HPLC on a Zorbax ODS column (25 cm×0.3 cm I.D., 5 μm, Dupont; Maynard Scientific, St. Laurent, Canada) with two different elution systems. In system 1, PTC peptides were eluted at 1.5 ml/min with a stepwise gradient of acetonitrile between solution A (20% acetonitrile in 0.08% TFA) and solution B (80% acetonitrile in 0.08% TFA). The chromatography was started with 25% solution B increasing to 40% solution B at 5 min. System 2 was operated at 1 ml/min under different conditions using 6% acetonitrile in 0.06 *M* sodium acetate buffer (pH 6.5) as solution A and 60% acetonitrile as solution B. The chromatography was started with 100% solution A and PTC peptides were eluted with linear gradients between solutions A and B to reach 12, 50, 92 and 100% solution B at 12, 30, 45 and 48 min, respectively. In all chromatograms, PTC peptides were detected at 269 nm [11].

Amino acid analysis

Samples of PTC peptides or underivatized peptides (100 pmol) were poured into the bottom of a glass tube (50 mm×6 mm) in 50 μl of methanol. The solvent was removed under vacuum. After drying, the vacuum was released and 100 μl of constant boiling 6 *M* hydrochloric acid containing 0.1% mercaptoethanol were added. The tube was sealed under high vacuum and the samples were hydrolysed at 108°C for 24 h. After hydrolysis, the residual hydrochloric acid was removed under vacuum. Blanks were analysed by incubating the acid solution in the absence of peptide. The hydrolysates were dissolved in 100 μl of $Na_2B_4O_2 \cdot 10H_2O$ (0.1 *M*, pH 9.5) and filtered through MS1 Cameo filters (0.45 μm, MS1 Micron Separations, Honcoye Falls, NY, U.S.A.). The amino acid analysis of the sample was performed by HPLC on a C_{18}/Resolve column (10 cm×0.25 cm I.D., 5 μm, Waters). The injection was effected in three steps, thus allowing the pre-column derivatization of amino acids with *o*-OPA in presence of mercaptoethanol. A time

of 1 min each was allowed for the first two injections with a flow-rate of 0 ml/min. The first injection contained 10 μl of a solution of *o*-OPA (50 mg in a mixture of 1.25 ml of methanol and 11.2 ml of 0.1 *M* $Na_2B_4O_2 \cdot 10H_2O$, pH 9.5). The second injection was made with 10 μl of a solution of mercaptoethanol (10 μl mercaptoethanol in 6.5 ml of methanol). The third injection contained the sample in 10 μl of 0.1 *M* $Na_2B_4O_2 \cdot 10H_2O$ (pH 9.5). The reaction was started at the beginning of the third injection when the sample and reagents were passing through an enlarged coil (10 cm×5 mm I.D.) localized at the outlet of the automatic injector. Derivatized (OPA) amino acids were eluted with a system gradient between solution A (2% methanol and 2% tetrahydrofuran in 0.05 *M* sodium acetate and phosphate buffer, pH 7.5) and solution B (65% methanol). Initially, the flow-rate was 0 ml/min. It was set at 0.1 ml/min at 2 min and thereafter increased linearly to reach 1 ml/min at 2.5 min. It was then maintained at 1 ml/min for the rest of the analysis. The column was pre-equilibrated with 100% solution A and, starting at 2.5 min, linear gradients were effected between solutions A and B to reach 40, 60, 80 and 100% B at 30, 35, 55 and 56 min, respectively. The column was then washed with 100% B for 5 min and returned to the initial conditions at 65 min. Derivatized amino acids were detected on the HPLC fluorometer at the emission wavelength of 425 nm and the excitation wavelength of 338 nm.

Solid-phase Edman degradation

PTC peptides were attached to aminopolystyrene via activation of the C-terminal carboxyl group by a water-soluble carbodiimide (EDC) procedure according to L'Italien and Strickler [12]. The coupling yield (20–75%) was generally improved by 5–15% when PTC peptides were used instead of the underivatized compounds. Briefly, the coupling procedure was as follows. The peptide material (0.5 nmol in 0.1% TFA) was added to a polypropylene centrifuge tube and lyophilized. The resin (15 mg) was preincubated with 1 ml of a solution of pyridine–water (8.1 : 85) in hydrochloric acid (pH 5.0) for 1 h at room temperature, and washed with three 1.5-ml portions of water and two 1.5-ml portions of DMF. The peptide sample was then added. The coupling reaction was carried out for 1 h at 37°C after the addition of 200 μl of DMF, 50 μl of pyridine–water (8.1 : 85, pH 5.0) and 4 mg of EDC (in 100 μl of DMF–water, 80 : 20). After the reaction, the resin was washed with two 1-ml portions of a mixture of the sequencer coupling buffer (pyridine–14% N-methylmorpholine, 3 : 2; pH 8.5) and DMF (1 : 3). The free amino groups were blocked by the addition of 100 μl of PITC (12% in acetonitrile) and a coupling period of 1 h at 37°C. The resin was washed with four 1-ml portions of methanol and two 1-ml portions of diethyl ether, and air-dried. The peptides were sequenced by solid-phase Edman degradation using an unmodified 60-min sequenator program on a Sequemat Mini-15 solid-phase sequencer equipped with a P-6 autoconvertor [12]. Each cycle from the sequenator (after conversion) was dried twice under the evapo-mix (Buchler). The samples were then dissolved in 50 μl of 50% acetonitrile and applied to a Microsorb C_{18} column (100 mm×4.2 mm I.D., 3 μm; Rainin, Woburn, MA, U.S.A.). PTH-amino acids were eluted at room temperature with the following solutions: (A) 20% acetonitrile in 0.1% TFA (pH 2.2); (B) 60% acetonitrile in 0.1% TFA. A linear

gradient between solutions A and B was effected, starting with 100% of solution A at time 0 to reach 45% of solution B at 25 min. The derivatized amino acids were detected at 269 nm [11].

Secretory product analysis

The HPLC analysis of PTC peptides was used for the identification and quantitation of Leu-Enk and Met-Enk released from perfused bovine adrenal glands. Bovine adrenal glands, freshly obtained from a local slaughterhouse, were perfused at 10 ml/min in a retrograde manner with Krebs buffer at 37°C. After a preequilibration period of 60 min, carbamylcholine (500 μM) was added to the perfusion buffer during 1.5 min. The perfusate was collected 5 min before stimulation with carbamylcholine (control) and 5 min starting from the beginning of the stimulation. The samples of perfusate were passed through a Sep-Pak C_{18} cartridge (Waters) and peptides were eluted with 6 ml of acetonitrile (70% in 0.1% TFA). The eluted fraction was evaporated under vacuum and submitted to the derivatization procedure combined to HPLC on Zorbax ODS (system 2, see above). Comparative radioimmunoassay (RIA) measurements of Leu-Enk were achieved as previously described [13].

RESULTS

HPLC of PTC peptides

PTC peptides were highly retained on reversed-phase HPLC when analysed under the same conditions as the underivatized compounds. However, using a Zorbax ODS column with system gradient 1 (Experimental, Fig. 1), an excellent separation of closely related peptides was obtained. The detection limit was ca. 1–5 pmol under these conditions (data not shown). The side-products of the reaction eluted after the PTC peptides (after 32 min, Fig. 1). In general, it was useful to analyse a blank (Fig. 1, bottom) in order to determine the level of background activity. Peaks corresponding to PTC-Dyn A-(1–11) and Dyn A-(1–13), were collected and submitted to amino acid analysis as described in Experimental. An average recovery yield of 75% was obtained for these two peptides, starting from 100 pmol of material. The amino acid compositions were as follows. For Dyn A-(1–11): Gly, 2.12; Arg, 2.97; Tyr, 0.46; Phe, 0.88; Ile, 1.0; Leu, 1.07; Lys, 0.83. For Dyn A-(1–13): Gly, 2.0; Arg, 2.99; Tyr, 0.95; Phe, 0.76; Ile, 1.21; Leu, 1.73; Lys, 1.99. The amino acid composition corresponded to that expected for both peptides with the exception of Pro, which does not react with the reagent used (OPA) for amino acid analysis.

Micro sequence analysis of PTC-dynorphin A-(1–13)

The amino acid sequence of PTC peptides can be determined by Edman degradation using a peptide or protein sequencer, provided that their N-terminal function is not blocked by any other group than the PTC moiety. As an example, PTC Dyn A-(1–13) (500 pmol) was submitted to HPLC on a Zorbax ODS column under the conditions described in Fig. 1. The fractions corresponding to the PTC peptide were collected, lyophilized and sequenced using a solid-phase au-

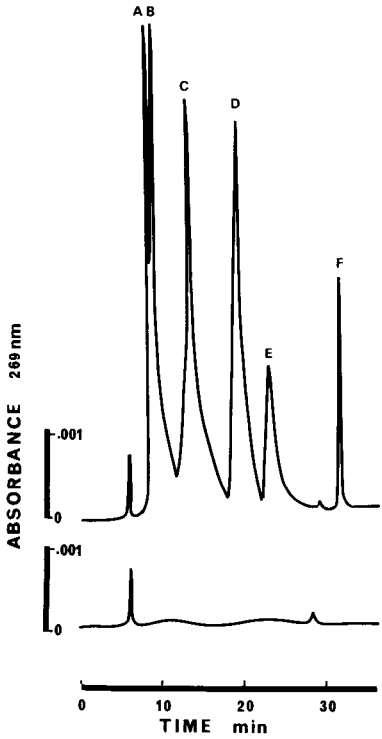

Fig. 1. Separation of a mixture of PTC-Dyn A-(1-9) (A), PTC-Leu-Enk (B), PTC-Dyn A-(1-11) (C), PTC-Dyn A-(1-12) (D), PTC-Dyn A (E) and PTC-Dyn A-(1-13) (F) by reversed-phase HPLC on a Zorbax ODS column. Synthetic peptides (100 pmol each) were derivatized with PITC and submitted to HPLC as described in Experimental, with gradient system 1 (Experimental).

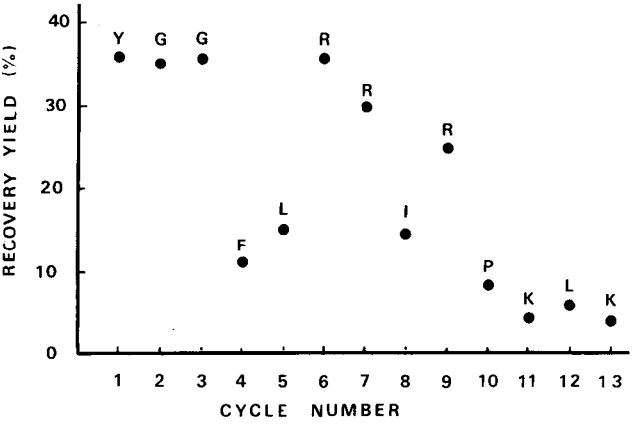

Fig. 2. Solid-phase Edman degradation of PTC-Dyn A-(1-13) (0.5 nmol) collected from HPLC on Zorbax ODS column (elution system 1; data not shown). The eluted material was coupled with 15 mg of aminopolystyrene resin and the degradation was performed on an automatic sequenator (Sequemat) as described in Experimental. The results are expressed as the amount (nmol) of amino acids detected in relation to the degradation cycle. The sequence obtained (YGGFLRRIRPKLK) corresponds to that expected.

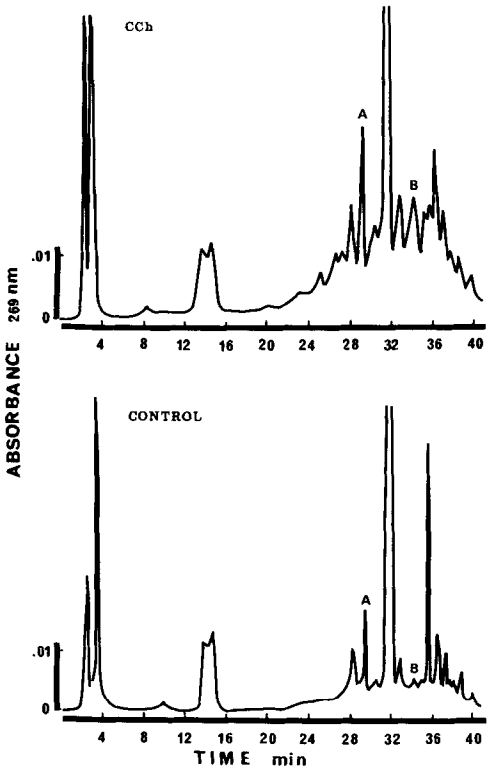

Fig. 3. HPLC analysis of perfusates from bovine adrenal medulla before (control) or after stimulation with carbamylcholine (CCh; 500 μM). The gland was perfused with Krebs buffer (pH 7.4) at 10 ml/min for 0.5 h. The perfusate was then collected for 5 min prior to and for 5 min following the beginning of the stimulation. It was passed through Sep-Pak cartridges (C_{18}; Waters), and the peptide material was eluted with 6 ml of 70% acetonitrile in 0.1% TFA (pH 2.1). It was evaporated, lyophilized, derivatized with PITC and analysed by HPLC on a Zorbax ODS column using the elution system 2 (Experimental). A and B indicate the retention times of synthetic PTC-Met-Enk and PTC-Leu-Enk, respectively.

tomatic sequenator (Sequemat, Fig. 2). The first cycle gave a recovery yield of 36% (Tyr: 0.18 nmol). The recovery of Arg at cycle 9 was 24%, and the loss of peptide from cycle to cycle was relatively low. The HPLC profiles for the identification of PTH-amino acids were all clean and unambiguous. The whole structure of Dyn A-(1–13) was thus obtained and corresponded to that expected.

Analysis of secreted peptides

The adrenal medulla is known to contain a large variety of neuropeptides, including Enk, Enk-containing peptides, dynorphin, substance P, neurotensin and vasoactive intestinal peptide [13]. Opioid peptides have already been shown to be released from perfused adrenal glands, using a radioreceptor assay as the detection procedure [14]. Herein, we used retrogradely perfused bovine adrenal medulla and the perfusate was analysed before (Fig. 3, lower part) and after stimulation with carbamylcholine (500 μM; Fig. 3, upper part). The control chro-

matogram indicated that peptides are released under basal conditions at a rate of 312 pmol per 5 min of perfusion for Met-Enk and 31 pmol per 5 min for Leu-Enk. Perfusion of the gland with carbamylcholine for 1.5 min induced a 2-fold increase in the secretion of Met-Enk (from 312 to 625 pmol per 5 min) and 10.5-fold increase in the release of Leu-Enk (from 31 to 325 pmol per 5 min). The release of Leu-Enk in response to carbamylcholine as measured by the derivatization procedure coupled to HPLC (325 pmol per 5 min) was of the same order of magnitude as that measured by RIA (500 pmol per 5 min).

DISCUSSION

The advent of HPLC has greatly improved the capacity of separation of peptide mixtures. For peptide detection, HPLC has been combined with various analytical methods, including UV absorption at low wavelengths (210–220 nm), post-column derivatization with fluorescamine or ninhydrin and, recently, electrochemical detection [15]. All these methods present great improvements over those used a decade ago. However, the UV absorption is not selective to peptide material, and the derivatization and oxidation techniques give products that are not suitable for subsequent structural analysis. Recently, Chang [9] has developed a detection method (formation of DABTC derivatives) that can be combined with amino acid analysis and Edman degradation. However, the derivatization of the side-chain of Lys with DABITC is rather slow and often incomplete. Conversely, PITC reacts quite rapidly and completely with peptides and gives a single PTC peptide product (starting with one peptide). PTC peptides can be separated quite nicely by HPLC and they are detected with great sensitivity, thus providing another separation means for peptide material (Fig. 1).

Derivatization with PITC has also been successfully applied for the analysis of amino acid composition of peptides and proteins [16]. PTC-amino acids are stable and can be separated by HPLC. In the present study, it was found that PTC peptides are also stable under slightly acidic chromatographic conditions (0.08% TFA, pH 2.2) that do not seem to interfere with subsequent amino acid analysis or Edman degradation. The initial recovery yield (average of 75%) of PTC peptides obtained after HPLC was relatively low. This can be explained by the possible oxidation of the thiocarbamoyl bond. The peptides were derivatized under argon but oxidation may occur in the steps between derivatization and HPLC. Full automatization of the analytical procedure (automatic derivatization coupled on-line to HPLC) may help to increase its efficiency.

Our method can be used for the final step of purification of naturally occurring peptides as well as for the identification and quantitation of peptides secreted from gland cells (Fig. 3). HPLC on Zorbax ODS provides a complete separation of closely related peptides, and the eluted material can be recovered and submitted to further analytical determinations. Moreover, the UV detection of PTC peptides is so sensitive that only a small percentage of a peptide sample (1 pmol) need be derivatized and chromatographed in order to provide useful information

on the purity and amount of the peptide material before it is submitted to analysis that may require more material.

The analysis of Leu-Enk secreted from the adrenal gland by derivatization with PITC gave poorer results than those obtained by RIA (325 pmol compared with 500 pmol secreted per 5 min after stimulation with carbamylcholine). This difference may be explained by the low degree of cross-reactivity of our antibody against Met-Enk (4%) or other closely related peptides (Met-Enk-Arg^6, Phe^7, Met-Enk-Arg^6, Gly^7, Leu^8 and other naturally occurring Enk-containing peptides). The proposed procedure allows the separation of closely related peptides, and the integration of their respective UV absorbances, although less sensitive than RIA, is more specific and more likely to correspond to the concentration of the peptide studied. The level of sensitivity (1–5 pmol) is ca. three to six times less than that obtained by RIA (15–30 fmol). However, PTC peptides may be detected with more sensitivity if they are separated on microbore HPLC columns [1,2,17] or if they are eluted with systems containing ion-pairing agents in order to increase peak sharpness.

In conclusion, the present data indicate that pre-column derivatization of peptide hormones with PITC may be used as an excellent means to verify the purity and amount of natural products before conducting further structural analysis or as a putative means to produce peptide map digests [5, 18]. The derivatization is complete within a few minutes, and the product is stable and provides UV signals that can be detected with great sensitivity. Further improvements of the methods of separation of PTC peptides may still be possible, and these may lead to other methods for the measurement of naturally occurring peptides in biological tissues or secreted from glands.

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