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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PHENYLTHIOCARBAMYL DERIVATIVES OF AMINO ACIDS AND SIDE-CHAIN DERIVATIZED AMINO ACIDS

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SUMMARY

Amino acid analysis by precolumn derivatization with phenyl isothiocyanate and subsequent characterization of the phenylthiocarbamyl derivatives by high-performance liquid chromatography on an inexpensive Supersphere C_8 column is described. The phenylthiocarbamyl derivatives of common amino acids from protein hydrolysates are separated within 15 min. Suggestions for optimization of the system with respect to the separation of closely eluting derivatives are given. The characterization of side-chain-derivatized, *e.g.*, phosphorylated and sulphated amino acids and of a series of biologically active amino acid analogues is described.

INTRODUCTION

Amino acid analysis by precolumn derivatization with phenyl isothiocyanate (PITC) and subsequent reversed-phase high-performance liquid chromatography (HPLC) was introduced in a classical paper by Heinrikson and Meredith[1]. The paper was dedicated to the memory of William Stein and Stanford Moore whose ion-exchange chromatographic method using post column derivatization with ninhydrin remains the most commonly used method for amino acid analysis almost 30 years after its introduction[2]. Precolumn derivatization with other reagents, *e.g.*, *o*-phthalaldehyde, dansyl chloride or 4′-dimethylaminoazobenzene-4-sulphonyl chloride, had previously been suggested but had not generally been accepted[3–7]. The uniform and quantitative reaction of all commonly occurring amino and imino acids with PITC, the stability of the resulting phenylthiocarbamyl (PTC) derivatives and their relative ease of separation by HPLC indicated that this method may gain wide acceptance[1].

In their original paper on the use of PITC for precolumn derivatization, Heinrikson and Meredith showed that both C_{18} and C_8 columns could separate the PTC derivatives of the common proteinous amino acids using acetonitrile, methanol or a mixture of both as solvent "B"[1]. It is therefore not surprising that several subsequent papers have described comparable reversed-phase HPLC protocols for separation of these PTC derivatives[8–10], Table I. It has been substantiated that amino acid analysis

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TABLE I

HPLC SYSTEMS FOR SEPARATION OF PTC DERIVATIVES OF AMINO ACIDS

Column	*Solvent A*	*Solvent B*	*Temperature (°C)*	*Flow-rate (ml/min)*	*Gradient type**	*Chromatography time (min)***	*Ref.*
25 cm × 0.46 cm C_{18} (IBM) 5 μm	0.05 *M* Ammonium acetate pH 6.8 (with phosphoric acid)	0.1 *M* Ammonium acetate pH 6.8 in water–acetonitrile (50:50)	52	?	2 linear increments	34	1
25 cm × 0.46 cm C_{18} (IBM) 5 μm	0.05 *M* Ammonium acetate pH 6.8 (with phosphoric acid)	0.1 *M* Ammonium acetate pH 6.8 in water–methanol (20:80)	52	?	3 linear increments	35	1
25 cm × 0.46 cm C_{18} (IBM) 5 μm	0.05 *M* Ammonium acetate pH 6.8 (with phosphoric acid)	0.1 *M* Ammonium acetate pH 6.8 in water–Methanol–acetonitrile (46:10:44)	52	?	2 linear increments	35	1
25 cm × 0.46 cm C_8 (Altex ultrasphere) 5 μm	0.05 *M* Ammonium acetate pH 6.8 (with phosphoric acid)	0.1 *M* Ammonium acetate pH 6.8 in water–acetonitrile (50:50)	52	?	1 linear increment	32	1
15 cm × 0.39 cm Pico-Tag™ (Waters)	0.14 *M* Sodium acetate + 0.5% TEA pH 6.35 with acetic acid	Acetonitrile–water (60:40)	?	1.0	Convex curve	11	8
15 cm × 0.46 cm Spherisorb C_{18} (Custom LC) 3 μm	0.03 *M* Sodium acetate + 0.05% TEA + 6% acetonitrile pH 6.4	Acetonitrile–water (60:40)	47	0.8	2 linear increments	21	9
25 cm × 0.46 cm LiChrosphere C_{18} (Merck)	0.01 *M* Potassium phosphate pH 6.5	Acetonitrile–"A" (70:30)	50	0.8	2 linear increments	39	10
25 cm × 0.4 cm Supersphere C_8 (Merck)	0.1 *M* Ammonium acetate + 0.05% phosphoric acid pH 5.4 (or pH down to 5.1)	Acetonitrile–"A" (50:50) (or the same with pH up to 5.8 in "A")	50	1.0	Isocratic + 1 linear increment	14 (less with a 12.5-cm column)	Present study

* The timing of the individual gradients may vary depending on the particular column (see individual references for details).

** Time from injection to elution of the last PTC derivative.

of peptides with PITC precolumn derivatization after hydrolysis is a reliable method comparable to the traditional procedure[1,8-10].

In the present paper we present a separation system for PTC amino acids and PTC derivatives of assorted side-chain-derivatized amino acids using a Merck Supersphere C_8 column, chosen initially because it had proven to be satisfactory for separation of phenylthiohydantoin derivatives of amino acids even in an isocratic system[11,12].

MATERIALS AND METHODS

Reagents and solvents

Water was doubly distilled and stored in glass bottles. Ethanol, Art. 11727 (Merck, Darmstadt, F.R.G.), was used directly or after distillation from ninhydrin. Triethylamine (TEA) was Sequanal grade (Pierce, Rockford, IL, U.S.A.) and was further purified by distillation from ninhydrin. PITC was Sequanal grade (Pierce) and was stored in a 1-ml glass container at −20°C. Ammonium acetate (Merck, Art. 1116) was stored at 4°C. Phosphoric acid and glacial acetic acid were both analytical grade from Merck. Acetonitrile was chromatographic grade (Merck, Art. 30). A standard mixture of amino acids was obtained from LKB Biochrom (Cambridge, U.K.). Individual standard amino acids were taken from the Pierce kit (Cat. No. 20065). With the exception of β-hydroxyleucine (U.S. Biochemicals, Cleveland, OH, U.S.A.) all other amino acids were obtained from Sigma (St. Louis, MO, U.S.A.).

Coupling of amino acids with PITC

Amino acids, 0.1 mol, were dissolved in 1 ml of 50% methanol in water (a couple of droplets of TEA were added to bring certain amino acids into solution) and 25 nmol of each were transferred to reaction vials, 50 mm × 6 mm borosilicate tubes (Kimbel, IL, U.S.A.) and dried under vacuum in a small vial which holds twelve tubes and can be sealed with a simple PTFE valve (Waters, Milford, MA, U.S.A.). Pretreatment and coupling was performed according to Bidlingmeyer *et al.*[8] using water–ethanol–TEA (2:2:1) and ethanol–water–TEA–PITC (7:1:1:1) respectively.

Liquid chromatography of PTC amino acids

The separation system is presented in Table I in addition to other published systems. A Hewlett-Packard 1090A chromatograph was equipped with a filter-photodetector using the filter for 254-nm light. The columns were Merck Supersphere C_8 with 5-μm particles packed in either 25 cm × 0.4 cm or 12.5 cm × 0.4 cm cartridges (Hibar LiChroCART®, 250-4 and 125-4, LiChrospher 60 CH-8 SUPER; Cat. Nos. 16010 and 16052 respectively). Solvent "A" was 0.10 *M* ammonium acetate to which 0.05% phosphoric acid was added and the pH brought to the desired value, usually 5.40, with glacial acetic acid. Solvent B was a 1:1 mixture of acetonitrile and solvent A, or a similar mixture where the pH was higher, *e.g.*, 5.8. Solvent C was 70% acetonitrile in water. The column temperature was 50°C and a flowrate of 1 ml/min giving a back pressure of 175–220 bar for the 25-cm column was employed. The gradient elution consisted of an initial period of 2–5 min of isocratic elution with between 13 and 17% "B" (6.5 and 8.5% acetonitrile), depending on the specific

column and column length, followed by a linear increase in "B" to 60% over 10–15 min, again depending on the specific column. The chromatography was completed by a wash with 100% C for 2–3 min, followed by a re-equilibration period of 3–5 min giving a total time between injections of 19–27 min. While the system was in use the pumps were never stopped; the flow-rate remained at 0.1 ml/min in between periods of use in order to prevent precipitation of salts in the system. When the chromatograph was to be used for other purposes, the column was washed extensively in water and then in a methanol–water mixture (70:30), in which the column was stored.

RESULTS AND DISCUSSION

As shown in Table I the different chromatographic protocols published for the separation of PTC amino acids use rather similar solvents and columns, *i.e.*, state-of-the-art C_{18} or C_8 columns (although the material in the so-called "application specified Pico-Tag^{TM}" column from Waters is not specified). The chromatograms are also rather similar, including those presented in the present paper using a lower pH in the elution buffer than in other systems (Table I and Fig. 1). However, the elution times are quite different. In chromatography, speed is often considered a virtue. In

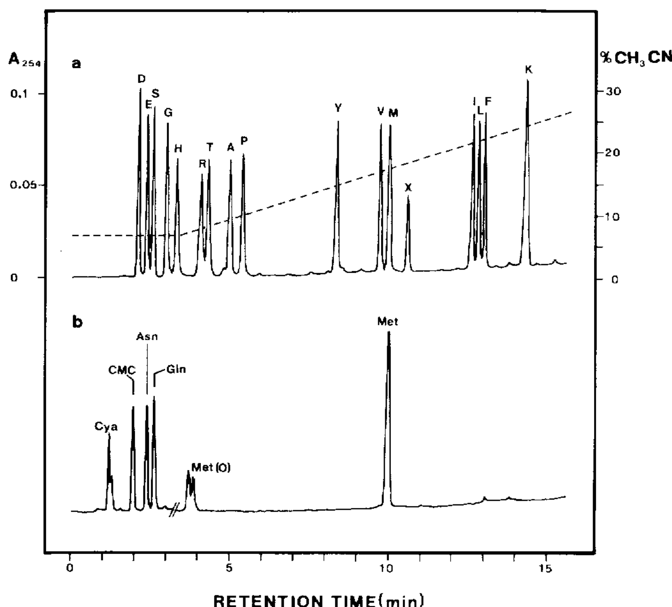

Fig. 1. Elution profile of phenylthiocarbamyl derivatives of standard amino acids occurring in protein hydrolysates (a) and of assorted amino acids which can be obtained by oxidation or derivatization of proteins or by other methods of hydrolysis (b); 500 pmol standard. Column: Merck Supersphere C_8, 25 × 0.4 cm. Solvents: A = 0.1 *M* ammonium acetate + 0.05% phosphoric acid brought to pH 5.1 with glacial acetic acid; B = 50% A pH 5.8 and 50% acetonitrile. For further details see the text. Amino acids: D = Asp; E = Glu; S = Ser; G = Gly; H = His; R = arg; T = Thr; A = Ala; P = Pro; Y = Tyr; V = Val; M = Met; I = Ile; L = Leu; F = Phe; K = Lys: X is possibly the PTC derivative of Cys, however this was not confirmed; Cya = cysteic acid; CMC = carboxymethylcysteine; Met(O) = methionine sulphoxide. (b) comprises two independent experiments.

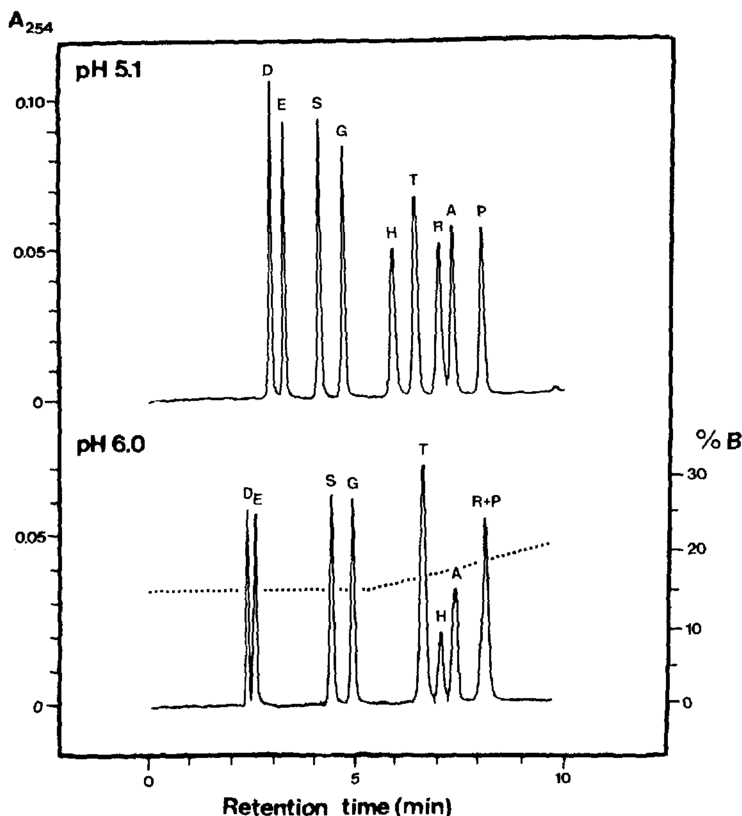

Fig. 2. The effect of pH on the elution profile of the early eluting PTC amino acids. A Merck Supersphere column (25 cm × 0.4 cm) was eluted with pH 5.1 or 6.0 in both solvent A and B. Solvent B was a mixture of solvent A, acetonitrile, and methanol (50:30:20).

certain systems the analysis can be completed after 10–15 min[8] (Fig. 1). Whether one chooses to use a more expensive column, where the manufacturer guarantees its application for the specific purpose of separating PTC amino acids[8], or, *e.g.*, a column known to exhibit good overall performance, like the one in the present study, depends on the resources and time available. The time to adjust a new column was usually a few hours; we used seven different Merck Supersphere columns.

The groups of amino acids that have shown to be most difficult to separate are described below; the term "amino acids" is used for brevity although it is the PTC derivatives that are meant. We found that changes in pH were particularly important in order to obtain the optimum separation of all amino acids. At high pH, amino acids Asp and Glu were eluted both close to each other and to the injection artefact (which does not appear to be a problem in most other systems). A better separation both from each other and from the start of the chromatogram can be obtained by decreasing the pH, Fig. 2. Through changes in the pH and depending on the column, Arg can be caused to elute in any position from before Thr to all the way down to the Pro peak, Fig. 2. The elution position of His will change more or

less in parallel with that of Arg. At low pH, Val and Met have a tendency to be eluted close together. We have often chosen the compromise of pH 5.8 in the buffer which is mixed with acetonitrile to form "B" and to adjust the pH to, *e.g.*, 5.1 with glacial acetic acid in solvent "A". In one case where the pH compromise did not give an acceptable separation, this was obtained by adding 20% methanol to solvent B.

In the characterization of protein hydrolysates, the identification and quantification of derivatives of Cys is often important. Cys itself is probably eluted just after Met (as x in Fig. 1a). Cysteic acid and carboxymethylcysteine are both eluted before Asp, the cysteic acid often too close or in the injection artefact (Fig. 1b). Oxidation products of Met can occur in protein hydrolysates. The methionine sulphoxide is eluted as two peaks just before Thr. Possibly two isomeric forms are generated during the oxidation process and separated on the column (Fig. 1b). Asn and Gln, which are converted into Asp and Glu by acid hydrolysis, can be released from peptides during sequence determination using carboxypeptidase digestion. These two amino acids are eluted around Ser and Gly, usually in the order Asn/Ser/Gln/Gly. On most long columns, a good separation of these four amino acids can be obtained; in Fig. 1 no attempt was made to optimize this separation.

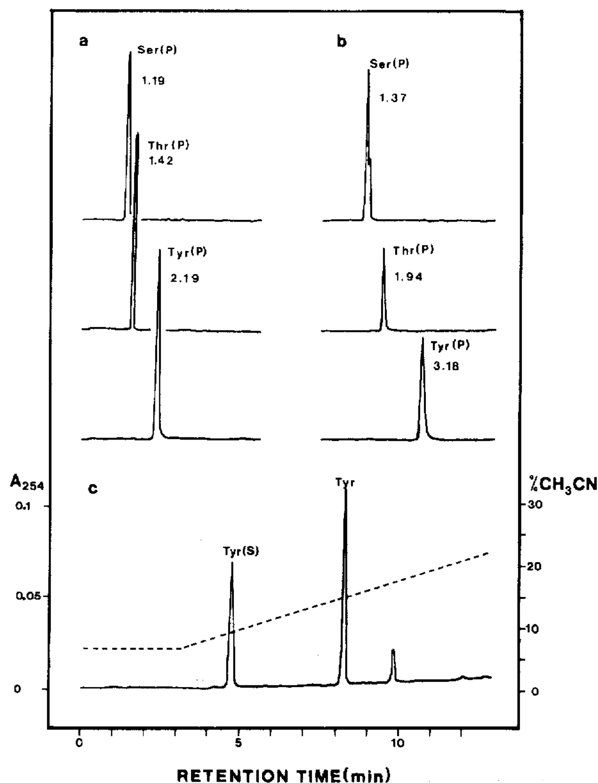

Fig. 3. Elution profile of PTC derivatives of phosphorylated and sulphated amino acids. (a) O-phospho-L-serine, Ser(P), O-phospho-L-threonine, Thr(P), and O-phospho-DL-tyrosine, Tyr(P), with the column and elution system in Fig. 1; 500 pmol of each amino acid were injected. (b) As (a) but using an isocratic elution with 9% B (4.5% acetonitrile). (c) PTC derivative of O-sulphated L-tyrosine together with normal Tyr in the system used in Fig. 1.

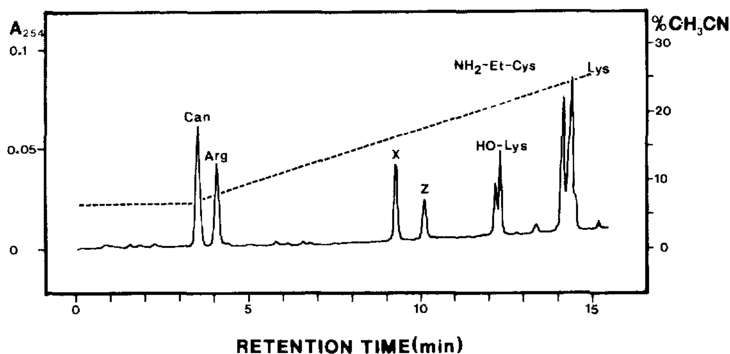

Fig. 4. Elution profile of PTC derivatives of basic amino acid analogues. Approximately 500 pmol of each derivative were injected. The column and elution system were as in Fig. 1. Can = L-Canavanine; HO-Lys = δ-hydroxylysine (mixture of D/L and D/L-*allo*); NH_2-Et-Cys = S-2-aminoethyl-L-cysteine; X and Z = contaminants of Can and Arg respectively.

Phosphorylation of Ser, Thr, and Tyr and sulphation of Tyr are post-translational modifications important for cell function. Systems to identify phosphorylated amino acids by precolumn derivatization with 4'-dimethylaminoazobenzene-4-sulphonyl chloride or *o*-phthalaldehyde have been reported[13,14]. The phosphorylated and the sulphated amino acids can also be identified and quantitated by the present pre-column derivatization method using PITC. The phospho-Ser and phospho-Thr are separated from each other and from the normal acidic amino acids, whereas phospho-Tyr is eluted very close to Asp, as shown in Fig. 3a. In order to get a better separation for collection of peaks for quantitation in labelling experiments with ^{32}P, the initial isocratic elution can be done with, *e.g.*, 4.5% acetonitrile (Fig. 3b) or even less. The time required for chromatography of the phosphorylated amino acids is still as short as, or less than that for the *o*-phthalaldehyde derivatives[14]. Cold standard amino acids are added to the sample for calibration during the collection of radioactive amino acids. The elution profile of sulphated Tyr and normal Tyr is shown in Fig. 3c. Sulpho-Tyr is eluted close to Ala, but this is not important since sulphorylated Tyr is generally identified and quantitated in ^{35}S labelling experiments after enzymatic or basic hydrolysis.

Certain amino acid analogues can be used to study co- and post-translational modifications of proteins[15]. In experiments with such analogues it is important to determine their purity and to identify and quantitate them in fluids when they are added, usually in millimolar concentrations. The chromatography of some of these analogues will now be described.

Basic amino acid analogues

Canavanine (Arg with the CH_2 in the δ position replaced by O), S-2-aminoethylcysteine (Lys with the CH_2 in the γ position replaced by S and δ-hydroxylysine are analogues which have been used to stop cellular processing at basic residues[15,16]. S-2-Aminoethylcysteine is also the identification product for cysteines after alkylation with, *e.g.*, β-bromoethyamine[17]. The basic amino acid analogues are all eluted earlier than their normal, more apolar counterparts, Fig. 4. The system is capable of differentiating between the two stereoisomeric forms of hydroxylysine.

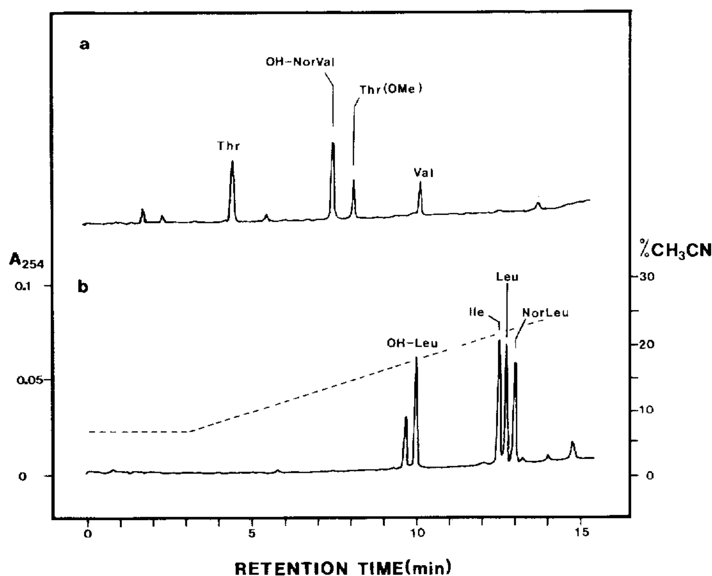

Fig. 5. Elution profile of PTC derivatives of analogues of aliphatic amino acids and related amino acids. Approximately 500 pmol of each amino acid derivative were injected. The column and the elution system were as in Fig. 1. OH-NorVal = DL-β-Hydroxynorvaline (DL-3-hydroxy-2-aminopentanoic acid); Thr(OMe) = O-methyl-L-threonine; OH-Leu = β-hydroxy-DL-leucine; NorLeu = L-norleucine.

Analogues for aliphatic amino acids

Norleucine, which has often been used as an internal standard in, *e.g.*, sequence determination with carboxypeptidases, is well separated from its isomeric counterparts (Fig. 5b). On most columns, norleucine is also separated from Phe. The polar Leu analogue, β-hydroxyleucine, in accordance with theory, is eluted earlier than Leu and again the system differentiates between the two stereoisomeric forms (Fig. 5b). β-Hydroxynorvaline is actually not a valine analogue; in cells it functions as a Thr analogue which can inhibit glycosylation of proteins in Asn residues when it is built into the recognition sequence, Asn-X-Thr[18]. In Fig. 5a the elution of the isomeric O-methylated Thr analogue is shown.

Analogues for aromatic amino acids

Trp, which is normally destroyed during acid hydrolysis, is well separated in the PTC system between Phe and Lys. The elution profile of a mixture of different Trp analogues is shown in Fig. 6a. The system is capable of separating three analogues fluorinated in position 4, 5 and 6 respectively. The separation of the mono- and di-iodinated Tyr derivatives shown in Fig. 6b is useful in the characterization of iodinated tracers of peptides used in radioimmunoassays and receptor-binding studies. After the tracer has been cleaved to separate the possible iodinated tyrosyl residues, the fragments are hydrolysed by enzymatic digestion. It is then determined whether the radioactivity is found in mono- or di-iodinated Tyr, in the same way as described above for the ^{35}S- and ^{32}P-labelled peptides. The fluoro-substituted Phe

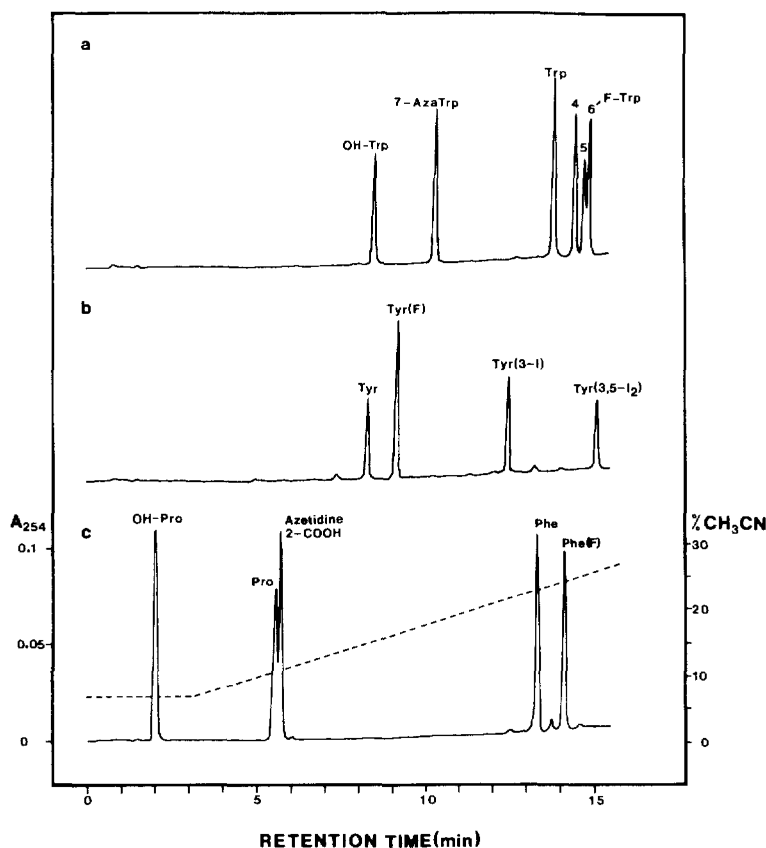

Fig. 6. Elution profile of PTC derivatives of analogues of aromatic amino acids and analogues of proline. Approximately 500 pmol of each amino acid derivative were injected. The column and elution system were as in Fig. 1. OH-Trp = 5-Hydroxy-DL-tryptophan; 7-AzaTrp = DL-7-azatryptophan; 4/5/6-F-Trp = 4-fluoro, 5-fluoro and 6-fluoro-DL-tryptophan respectively; Tyr(F)-3-fluoro-DL-tyrosine; Tyr(3-I) = 3-iodo-L-tyrosine; Tyr(3,5-I_2) = 3,5-diiodo-L-tyrosine; Phe(F) = 4-fluoro-DL-phenylalanine; OH-Pro = L-hydroxyproline; azetidine 2-COOH = L-azetidine-2-carboxylic acid.

shown in Fig. 6c can be used as an internal standard in enzymatic sequence determinations and also functions as a Phe analogue in cellular experiments.

Proline analogues

In the presented system, hydroxyproline is eluted together with Asp; as regulatory peptides usually do not contain hydroxyproline, no attempt was made to separate the two coeluting amino acids. The four-carbon-ring analogue of proline, azetidine-2-carboxlic acid, is used in cellular experiments[15] and in this laboratory has also been used as an internal standard in enzymatic sequence determination of peptides. It is eluted at a good position in the chromatogram which is free from interfering peaks when biological samples are analysed. Usually the analogue is also totally separated from proline, however we present data for different analogues ob-

tained on one column and with one solvent system, with no special optimization applied for any particular amino acid analogue.

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