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DETERMINATION OF PHENYLTHIOCARBAMYL AMINO ACIDS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The quantitative high-performance liquid chromatographic determination of amino acids and precolumn derivatization with phenyl isothiocyanate has been investigated. Reproducible conditions for the detection and quantitation of the phenylthiocarbamyl derivatives of the major amino acids expected from protein hydrolysates are described. These derivatives could be completely separated with the aid of two types of octadecylsilyl reversed-phase columns, by elution with a binary gradient system consisting of a stepwise increase of acetonitrile up to a final concentration of 25% in 10 m*M* potassium phosphate (pH 6.5) within 40 min. The results obtained are comparable with those realized by conventional ion-exchange amino acid analysis. The sufficient reproducibility of peak areas and retention times calibrated daily with external amino acid standards allows the application of this method as a simple and low-priced way for the determination of amino acids in the range 50–500 pmole per residue.

INTRODUCTION

The routine determination of amino acids derived from protein hydrolysates or from biochemical manipulations of biological material is an important practice in the biological sciences. Stein and Moore[1] first developed a technique for the separation and quantitation of amino acids by use of ion-exchange chromatography, which became the method of choice until very recently. The post-column reaction of the isolated amino acids with ninhydrin, and its spectroscopic measurement, gave rise to automated amino acid analysers, thus allowing the routine detection of amino acids in the nanomole range[2,3]. Subsequently, with the aim of lowering the detection limit to the subnanomole range, some workers proposed the substitution of ninhydrin for *o*-phthalaldehyde as a fluorogenic post-column derivatization reagent, but this modification did not supersede the conventional technique[4–7].

The recent rapid development of high-performance liquid chromatography (HPLC) has opened new dimensions in biochemical analytics, promising higher sensitivities and shorter analysis times. A series of attempts has been made to search for

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a suitable post- or precolumn derivatization reagent of amino acids, so that the claims for the simplicity, completeness and sensitivity of HPLC analysis could be fulfilled. The use of *o*-phthalaldehyde as a pre-column derivatization reagent enables picomole amounts to be detected, but it suffers from the disadvantage of the impossibility of imino acid modification in a one-step reaction[8–10]. The insufficient reactivity of dansyl chloride and its coloured analogue dimethylaminoazobenzene-4-sulphonyl chloride renders the quantitation of the respective amino acid derivatives more difficult, and thus sets limits to the application of these reagents too[11–13].

Recent reports have highlighted the Edman reagent phenyl isothiocyanate (PITC) for precolumn derivatization, and the detection of phenylthiocarbamyl (PTC)amino acids[14,15]. This method permits the direct covering of all representative amino acids from protein hydrolysates, although the suspect reproducibility and durability of HPLC columns, possibly caused by the presence of excess PITC, must be born in mind.

The purpose of this work was to find favourable conditions for the derivatization of selected protein hydrolysates and the quantitative analysis of the respective PTC-amino acids by reversed-phase HPLC on octadecylsilyl columns. The results obtained, compared with those expected from the known amino acid sequences of the proteins investigated, indicate that the PITC method qualifies for large scale application in the routine amino acid analysis of peptides and proteins.

MATERIALS AND METHODS

Chemicals

Phenyl isothiocyanate (supplied in 1 ml vacuum-sealed ampoules), pyridine and triethylamine (sequanal grade), and a standard mixture of amino acids (2.5 μmol/ml in 0.1 *N* hydrochloric acid were purchased from Pierce (Rodgau, F.R.G.). Pyridine and triethylamine were successively distilled from ninhydrin and calcium hydride. Individual amino acids were products of Serva (Heidelberg, F.R.G.) and Sigma (Munich, F.R.G.). Acetonitrile was an HPLC-grade product of Baker (Gross-Gerau, F.R.G.). All other chemicals used were of analytical grade. Oxidized insulin B-chain was purchased from Serva. Aprotinin was a product of Boehringer (Mannheim, F.R.G.) and cytochrome *c* a product of Sigma.

Amino acid analysis

Compositional analyses of peptides and proteins were performed by ion-exchange chromatography on an automatic amino acid analyser, Biotronic LC 5000 (Biotronik, Puchheim, F.R.G.), according to Spackman *et al.*[3]. Samples were hydrolysed *in vacuo* for 18 h at 115°C in 6 *N* hydrochloric acid containing 0.1% phenol. Parts of each sample were coupled with PITC for analysis of the PTC-amino acids by reversed-phase HPLC.

Coupling procedure

Coupling of amino acids with PITC was done as described by Heinrikson and Meredith[14]. In order to achieve quantitative coupling of amino acids derived from acid hydrolysates the excess hydrochloric acid has to be completely removed. For this purpose the sample was evaporated to dryness, dissolved twice in water and then

twice in pyridine, being dried each time. Only after that was the respective coupling reaction started. The resulting PTC-amino acids were dissolved in 400 μl of 10 m*M* phosphate buffer (pH 6.5), and from this 20 μl of a 1:20 diluted aliquot was analysed by HPLC.

HPLC of PTC-amino acids

Analyses were performed using a LKB-HPLC system (LKB, Bromma, Sweden) consisting of one pump, a controller for gradient programming, an UV detector and a Shimadzu Chromatopac CR3A data processor. Elution gradients were formed by low pressure mixing, and the PTC-amino acids were detected by its UV absorption at 254 nm. Two reversed-phase C_{18} columns, Spherisorb ODS II (Bischoff, Leonberg, F.R.G.) and Lichrospher (Merck, Darmstadt, F.R.G.) (250 × 4.6 mm I.D., 5 μm particle size of the matrix) were tested; each column was repacked in an oven compartment, maintained at 40°C and 52°C, respectively, by an external water-bath. In all cases the elution solvent consisted of solvent A, containing 10 m*M* potassium phosphate in water, and solvent B, containing 70% acetonitrile in the same buffer (final concentration), both at pH 6.5. Gradient mixing was as follows: 0–15 min, 9% B; 15–35 min, 36% B; 35–38 min, 100% B; maintaining for 3 min at 100% B; 41–44 min 0% B; washing for 4 min.

RESULTS AND DISCUSSION

The use of PITC as a precolumn derivatization reagent for the detection of amino acids by HPLC will become an alternative to the use of *o*-phthalaldehyde. However, difficulties in quantitation of PTC-amino acids, the insufficient reproducibility of the resulting chromatograms, and the sensitivity of the HPLC columns are significant drawbacks of this theoretically elegant method of analysis.

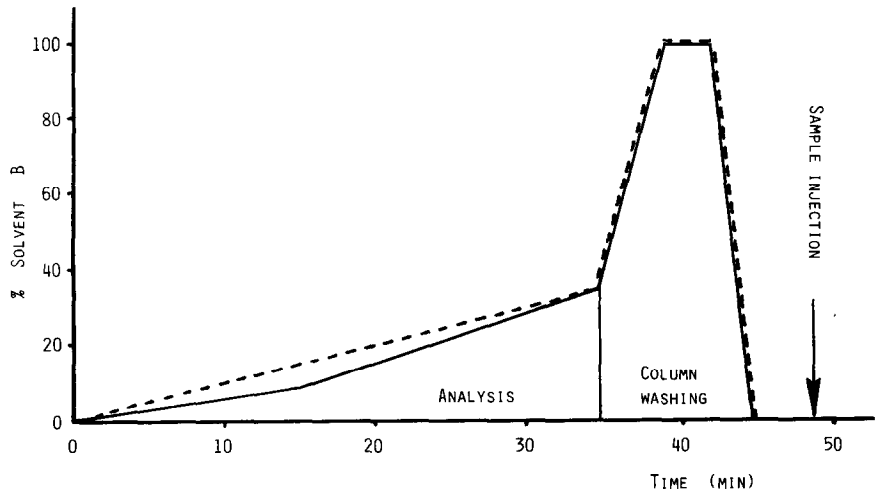

Fig. 1. Schematic representation of the gradient programming for the separation of PTC-amino acids by reversed-phase HPLC. Solvent A contained 10 m*M* potassium phosphate (pH 6.5), Solvent B contained 70% acetonitrile in 10 m*M* potassium phosphate (pH 6.5). Traces: (– – –) gradient using Spherisorb ODS II at 40°C; (———) gradient using Lichrospher RP-18 at 50°C.

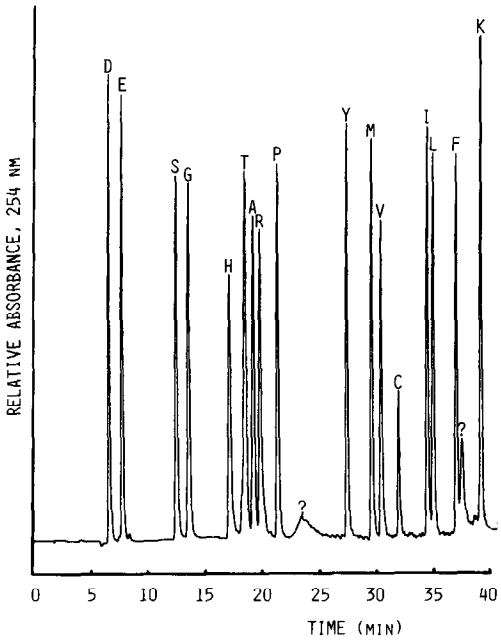

Fig. 2. Chromatogram of an amino acid standard containing 500 pmole each of seventeen PTC-amino acids. The separation was performed on a Lichrospher RP-18, column (5 μm) at 50°C. One-letter code: D = Asp; E = Glu; S = Ser; G = Gly; H = His; T = Thr; A = Ala; R = Arg; P = Pro; Y = Tyr; M = Met; V = Val; C = Cys; I = Ile; L = Leu; F = Phe; K = Lys.

The stability of silica gel matrices in HPLC columns is naturally limited by its solubility in water, especially in neutral aqueous media. We observed a further decrease of the durability of the columns when ammonium acetate was used as buffer substance in the solvents, as described by Heinrikson and Meredith[14]. The relatively high pressure of *ca.* 150 bar caused by ammonium acetate solutions, as well as the unfavourable high excess of PITC and other reaction products arising from the derivatization process, could be responsible for our finding that only four or five satisfactory analyses can be performed after new columns have been applied. When phosphate is substituted for ammonium acetate and a more effective washing step with up to 70% acetonitrile (final concentration) after each analysis run is employed, the column maintained its resolution capacity for at least four weeks of daily application at a pressure of 100 bar maximum. Furthermore, the separating power of the columns can be maintained by using minimal amounts of PITC during derivatization of amino acids and by the introduction of an additional column-rinsing with methanol once a week.

The coupling reaction with PITC is complete within 3–5 min and is simply performed, considering that thorough removal of the hydrochloric acid after the hydrolysis by repeated evaporation of the sample with the coupling buffer is absolutely necessary. In this way we were able to separate all important amino acids expected from protein hydrolysates within 40 min on both octadecylsilyl Lichrospher and Spherisorb columns. Elution of the PTC-amino acids on the Lichrospher column

was performed with a binary gradient of water–acetonitrile, containing 10 m*M* potassium phosphate (pH 6.5), by a two-step mixing of the solvents at a flow-rate of 0.8 ml/min and a pressure of 80–90 bar (Fig. 1).

The elution profile thus obtained from 500 pmole each of a PTC-amino acid standard, chromatographed at 50°C on the Lichrospher column (Fig. 2) is similar to those published by Heinrikson and Meredith[14]. As can be seen in the figure, the first four residues eluted Asp, Glu, Ser and Gly, are well separated, and the satisfactory resolution of the triplet Thr, Ala and Arg arises crucially, especially when a continuous linear gradient is applied. Thus, the increase in acetonitrile concentration to 90% in the first 15 min has been kept relatively flat, and the subsequent rise of the slope of the gradient allows the isolated desorption of the residual PTC-amino acids of the standard mixture within the remaining 25 min analysis time. The washing of the column by a further increase of the acetonitrile concentration to 70% (100% solvent B), which is held for 3 min, detaches most of the by-products from the coupling reaction. After a total of 48 min the column is ready for the next analysis. When a new Spherisorb ODS II column was used, a continuous linear gradient of water–acetonitrile (Fig. 1) was able to desorb all amino acids separately at 40°C at a flow-rate of 1 ml/min. With both HPLC columns used, the addition of methanol to solvent B, as suggested earlier[14], could be omitted without any detriment to the results.

The absorptivities of the PTC-amino acids and their corresponding retention times are listed in Table I. With the exception of Cys (its absorptivities are *ca.* 50% of the others) the integration values per picomole of residue are all of the same order.

TABLE I

ABSORPTIVITY (254 nm) AND RETENTION TIMES OF PTC-AMINO ACID DERIVATIVES ON REVERSED-PHASE HPLC

Amino acid	*Integration values per picomole*	*Time (min)*
$CysSO_3H$	1250*	5.7
Asp	1240	7.3
Glu	1234	8.4
Ser	1348	13.1
Gly	1447	14.1
His	1257	17.6
Thr	1512	19.0
Ala	1320	19.7
Arg	1415	20.3
Pro	1443	21.8
Tyr	1462	27.7
Val	1361	29.8
Met	1598	30.7
Cys	811	32.2
Ile	1428	34.6
Leu	1539	35.2
Phe	2114	37.1
Lys	2657	39.2

* Approximate; not determined.

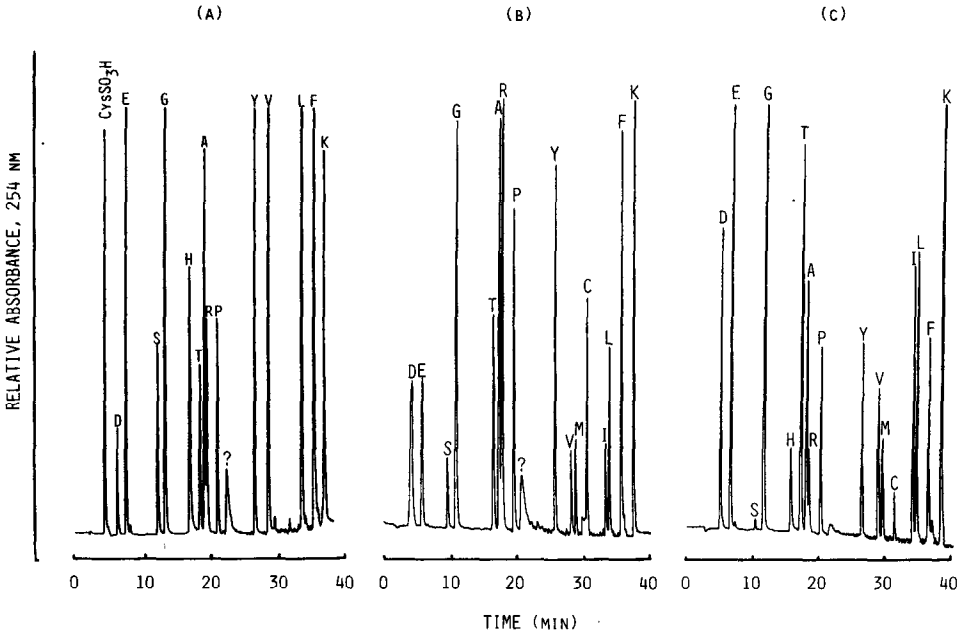

Fig. 3. Chromatograms of PTC-amino acids generated after hydrolysis and derivatization of oxidized insulin B-chain (A), aprotinin (B) and cytochrome *c* (C). The analyses were carried out at 50°C on the Lichrospher column with *ca.* 1 μg (300 pmole) of each peptide. For one-letter code see Fig. 2.

The slightly lower values of the PTC derivatives of the acidic amino acids may be due either to lower absorptivity or to a less than complete coupling reaction. Similar reasons could account for the abnormal behaviour of Cys, although its much lower absorptivity remains unclear.

The size of an unidentified peak emerging after proline (see Figs. 2 and 3) is dependent on the amount of PITC used for the coupling reaction in comparison with the amount of sample employed. Thus, if low concentrations of amino acids are expected, the use of only 0.5% PITC is recommended; this will also safeguard the durability of the HPLC columns (see above). The PTC derivative of hydroxyproline emerges between Glu and Ser, and PTC-homoserine appears before the respective histidine derivative. Cysteic acid occurs as the first peak before Asp, as can be inferred from the chromatogram of an protein hydrolysate of the oxidized insulin B-chain (Fig. 3a).

In Table II the amino acid analyses of selected proteins performed both by conventional ion-exchange chromatography and by reversed-phase HPLC are summarized. The quality of the results obtained is altogether comparable. The value for cysteic acid in insulin B-chain has been calculated on the molar absorptivity of PTC-Glu, giving rise to the expected two cysteines in this peptide.

Thus the present study supports the suggestion that amino acid analysis by reversed-phase HPLC will become an alternative to the more costly ion-exchange method. The disadvantage of precolumn derivatization with *o*-phthalaldehyde can be overcome by the use of PITC and the UV absorption of all the PTC derivatives

TABLE II

AMINO ACID ANALYSES OF OXIDIZED INSULIN B-CHAIN, APROTININ AND CYTOCHROME *c* BY REVERSED-PHASE HPLC AND BY ION-EXCHANGE CHROMATOGRAPHY

Values are given in mole residue per mole peptide.

Amino acid	*Oxidized insulin B-chain*		*Aprotinin*		*Cytochrome c*	
	Ion exchange	*HPLC**	*Ion exchange*	*HPLC*	*Ion exchange*	*HPLC*
$CysSO_3H$***	2.00	2.00 (2)**	–	–	–	–
Asx	1.03	0.71 (1)	5.13	4.31 (5)	8.38	6.88 (8)
Glx	3.39	2.36 (3)	3.20	3.19 (3)	12.02	12.01 (12)
Ser	0.95	1.09 (1)	1.16	1.09 (1)	0.57	– (0)
Gly	3.02	3.24 (3)	6.13	6.22 (6)	12.62	12.50 (12)
His	2.39	2.15 (2)	1.12	– (0)	5.18	3.21 (3)
Thr	0.95	0.81 (1)	2.93	3.14 (3)	9.51	9.82 (10)
Ala	2.02	2.31 (2)	6.12	6.62 (5)	6.69	6.42 (6)
Arg	0.95	1.10 (1)	5.10	6.66 (6)	1.99	2.09 (2)
Pro	1.01	1.14 (1)	3.33	4.34 (4)	3.12	4.39 (4)
Tyr	1.61	2.07 (2)	4.10	4.23 (4)	4.58	4.01 (4)
Val	2.95	3.33 (3)	0.82	1.11 (1)	2.75	3.66 (3)
Met	–	– (0)	1.18	1.06 (1)	1.02	2.13 (2)
Cys	–	– (0)	5.44	4.90 (6)	1.19	1.90 (2)
Ile	–	– (0)	0.74	1.25 (2)	4.86	6.19 (6)
Leu	3.94	4.32 (4)	2.11	2.22 (2)	6.10	6.42 (6)
Phe	2.79	2.67 (3)	4.20	3.42 (5)	4.19	3.49 (4)
Lys	0.99	0.97 (1)	4.25	4.22 (4)	18.32	17.80 (19)

* Values are means of three determinations.

** Expected values from amino acid sequences.

*** Values have been approximated based on similar absorptivities of the corresponding derivatives of Glu.

of amino acids can be recorded at 254 nm. This technique requires only moderately expensive apparatus and relatively short analysis times. Rapid progress in the production of more stable matrices for HPLC columns, promising still higher selectivities, indicates a good future for the routine application of HPLC to all aspects of amino acid analysis.

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