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Amino acid analysis by high-performance liquid chromatography after derivatization with diethyl ethoxymethylenemalonate

Manuel Alaiz, José L. Navarro, Julio Girón and Eduardo Vioque*

Instituto de la Grasa y sus Derivados (CSIC), Apartado 1078, E-41012 Seville (Spain)

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ABSTRACT

Amino acids were determined by precolumn derivatization with diethyl ethoxymethylenemalonate and reversed-phase high-performance liquid chromatography (HPLC) with spectrophotometric detection at 280 nm. The reaction time was 50 min and the derivatives were stable at room temperature. Chromatographic resolution of a mixture of the derivatives of seventeen amino acids, including proline and cystine, was achieved within 35 min using a binary gradient system. The detection limit was 3 pmol. Amino acid analyses of acid hydrolysates of two proteins gave results equivalent to those obtained by conventional ion-exchange-based amino acid analysis. The simplicity of the procedure allows its use on any multi-purpose HPLC system.

INTRODUCTION

In an effort to increase instrument productivity and sensitivity with respect to amino acid analyses, several alternatives to traditional ion-exchange chromatography and postcolumn derivatization with ninhydrin have been explored [1]. For the most part, the alternative approaches rely on precolumn derivatization with hydrophobic reagents, which in turn allows rapid and efficient resolution of the derivatives by reversed-phase high-performance liquid chromatography (HPLC). Moreover, the nature of these compounds is such that the absorbance or fluorescence signals of the compounds often extend the sensitivity to the picomole or femtomole range.

The most common reagents used for amino acid derivatization in this context are *o*-phthalaldehyde [2–6], 5-dimethylaminonaphthalene-1-sulphonyl chloride [7–10], 4-dimethylaminoazobenzene-4′-sulphonyl chloride [11,12], phenyl isothiocyanate [13–15], 9-fluorenylmethyl chloroformate [16,17], 1-fluoro-2,4-dinitrobenzene [18], 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide [19] and 4-N,N-dimethylaminoazobenze-4′-isothiocyanate [20]. Each of these reagents provides the general advantages associated with precolumn derivatization.

A new reagent which gives amino acid derivatives detectable in the ultraviolet region is diethyl ethoxymethylenemalonate [21]. The derivatives are easily obtained and are very stable. In this paper, we report the HPLC determination of the derivatives of all the commonly occurring L-amino acids in proteins (except those which are destroyed by acid hydrolysis). The run, using a binary elution programme, is complete in 35 min.

EXPERIMENTAL

Reagents and samples

Diethyl ethoxymethylenemalonate was obtained from Fluka (Buchs, Switzerland). Sodium acetate, sodium hydroxide, boric acid and hydrochloric acid were purchased from Panreac (Barcelona, Spain). Acetonitrile of HPLC grade was from Romil Chemicals (Loughborough, UK). Sodium azide was obtained from Merck (Darmstadt, Germany) and L-amino acids from Serva (Heidelberg, Germany). Insulin and lysozyme were purchased from

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Boehringer (Mannheim, Germany). Deionized, distilled water was used for the preparation of buffers. The buffers used for the HPLC analysis were filtered through a 0.22-μm filter (Millipore, Bedford, MA, USA). All the HPLC solvents were degassed with helium.

Instruments

The HPLC system (Waters) consisted of a Model 600E multi-solvent delivery system, a Wisp Model 712 automatic injector, a Model 484 UV–VIS detector and an APC IV NEC personal computer. Data adquisition and processing were effected with Maxima 820 3.3 version software (Waters). Separations were attained using a 300 × 3.9 mm I.D. reversed-phase column (Nova-Pack C_{18}, 4 μm; Waters). The column was maintained at 18°C by a temperature controller (Julabo F 10).

Hydrolysis

Mixtures of amino acids, or a protein, with D,L-α-aminobutyric acid as internal standard were dissolved in 6.0 *M* hydrochloric acid. The solutions were gassed with nitrogen and sealed in hydrolysis tubes under nitrogen, then incubated in an oven at 110°C for 24 h.

Derivatization

Formation of N-[2,2-bis(ethoxycarbonyl)vinyl] derivatives of mixtures of amino acids, or protein hydrolysates, was achieved as follows: to a dried sample of standard amino acid mixture, or a protein hydrolysate (2–200 μg), in 1 *M* sodium borate buffer (pH 9.0) (1 ml) containing 0.02% of sodium azide was added 0.8 μl of diethyl ethoxymethylenemalonate. The reaction was carried out at 50°C for 50 min with vigorous shaking. The resulting mixture was cooled to room temperature and 15 μl were injected into the chromatograph.

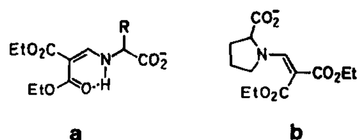

Fig. 1. Derivatives obtained in the reaction of diethyl ethoxymethylenemalonate with amino acids. (a) R = characteristic lateral chain of each amino acid, except proline; (b) derivative of proline. Et = Ethyl.

Chromatography

Resolution of the amino acid derivatives was routinely accomplished using a binary gradient system. The solvents used were (A) 25 m*M* sodium acetate containing 0.02% of sodium azide (pH 6.0) and (B) acetonitrile. Solvent was delivered to the column at a flow-rate of 0.9 ml/min as follows: Time 0.0–3.0 min, linear gradient from A–B (91:9) to A–B (86:14); 3.0–13.0 min, elution with A–B (86:14);

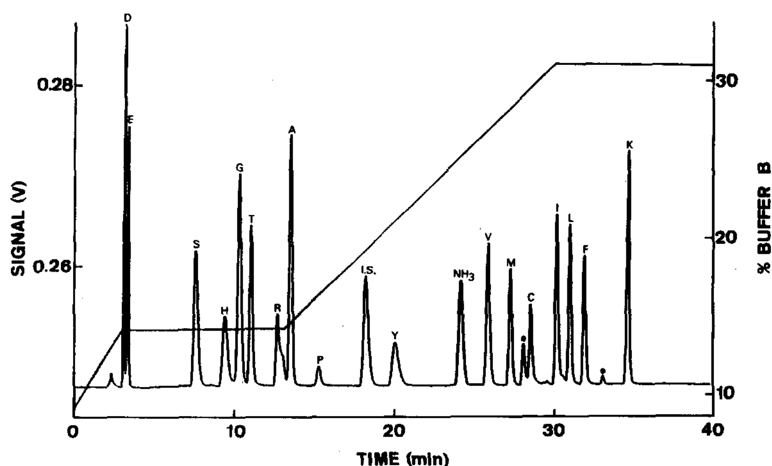

Fig. 2. Elution pattern of a standard amino acid mixture chromatographed as described in the text. The amount of each standard N-[2,2-bis(ethoxycarbonyl)vinyl]amino acid present in the mixture ranged from 50 to 200 pmol, except proline (300 pmol). Peaks are labelled with single-letter notations for amino acids. I.S. = D,L-α-aminobutyric acid (internal standard); ● = unidentified peaks.

13.0–30.0 min, linear gradient from A–B (86:14) to A–B (69:31); 30.0–35.0 min, elution with A–B (69:31).

RESULTS AND DISCUSSION

Fig. 1 shows the structures of the derivatives obtained in the reaction of L-amino acids with diethyl ethoxymethylenemalonate.

Reversed-phase HPLC resolution of the N-[2,2-bis(ethoxycarbonyl)vinyl] derivatives of seventeen commonly occurring L-amino acids is shown in Fig. 2. The 2,2-bis(ethoxycarbonyl)vinyl group provided not only the neccesary interactions with the apolar stationary phase but also a high absorptivity for easy spectrophotometric detection at 280 nm [21]. Another characteristic of this derivatization that should be noted is the absence of the reagent peak in the chromatogram because the diethyl ethoxymethylenemalonate has an absorption maximum at 240 nm. The derivatization blank did not give any peak. The limit of detection was 3 pmol with a signal-to-noise ratio of 5. The identity of each peak was established either by adding a threefold molar excess of the amino acid in question, or by omitting the amino acid from the standard mixture. Lysine and cystine were separated as disubstituted derivatives. Analysis of individual amino acids standards allowed the identification of the unknown peak with a retention time of 28.10 min produced in cystine hydrolysis. Direct derivatization of cystine standard without hydrolysis did not afford this peak. Although the separation between aspartic and glutamic acids seems to be too close, the retention times of the peaks show good repeatability and allow reliable integration of the areas.

The derivatization of free amino acids with diethyl ethoxymethylenemalonate is easy and quantitative, and the N-[2,2-bis(ethoxycarbonyl)vinyl] derivatives are stable for several weeks at room temperature [21]. Response factors for all derivatives were calculated from calibration runs employing 10–2000 pmol of amino acid, using D,L-α-aminobutyric acid as an internal standard. The response was found to be linear in all instances (correlation coefficient $\geqslant$ 0.996). Fig. 3 shows a typical calibration graph for the range 10–1000 pmol for glycine. The derivatives of aspartic acid, glycine, isoleucine and

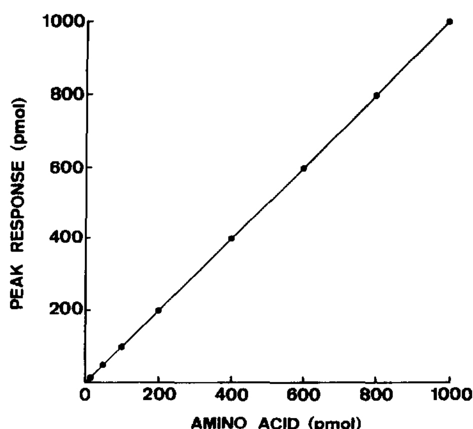

Fig. 3. Plot of peak response (area/area$_{I.S.}$ × amount of I.S.) of N-[2,2-bis(ethoxycarbonyl)vinyl] derivative for glycine. Each point represents the mean of five different analyses.

TABLE I

RETENTION TIMES AND RESPONSE FACTORS FOR N-[2,2-BIS(ETHOXYCARBONYL)VINYL] DERIVATIVES OF AMINO ACIDS

Amino acid	Retention time (min)[a]	Response factor
Aspartic acid	3.10	1.00
Glutamic acid	3.31	1.04
Serine	7.48	0.96
Histidine	9.35	0.92
Glycine	10.25	1.00
Threonine	11.02	1.02
Arginine	12.66	0.98
Alanine	13.45	1.03
Proline	15.25	0.04[b]
D,L-α-Amino butyric acid	18.15	1.00[c]
Tyrosine	20.05	0.91
Ammonia	24.21	–
Valine	25.84	1.04
Methionine	27.22	0.92
Cystine	28.52	1.08
Isoleucine	30.19	1.00
Leucine	31.02	1.00
Phenylalanine	31.95	0.95
Lysine	34.73	1.86

[a] Values represent averages determined from twenty standard chromatograms with a standard deviation of 0.1 min for each compound.

[b] The lowest response factor of the compounds evaluated; this is due to its different structure (Fig. 1).

[c] 1 pmol of D,L-α-aminobutyric acid (internal standard) represents 1380 μV s.

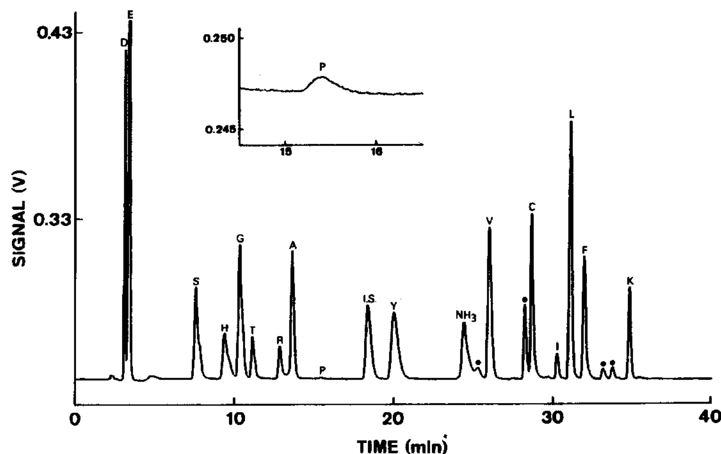

Fig. 4. Elution pattern of N-[2,2-bis(ethoxycarbonyl)vinyl] derivatives in an insulin hydrolysate. The sample contained 9488 pmol of amino acids and 617 pmol of internal standard. Peaks are designated as in Fig. 2.

leucine essentially follow the same line. The slope, 1.00, represents the response factor.

Table I summarizes the retention times and response factors for the N-[2,2-bis(ethoxycarbonyl) vinyl] derivatives evaluated.

After the verification of the method using several mixtures of amino acid standards with different compositions, its applicability to the determination of amino acids in a protein hydrolysate was assessed by the determination of the amino acid compositions of insulin and lysozyme (Figs. 4 and 5). The results agreed with those obtained with a conventional amino acid analyser based on ion-exchange chromatography and were equivalent to the reported values (Table II).

Several widely used methods for amino acid analysis have lower detection limits [13–17] and some of them shorter derivatization [2–10, 13–18] or analysis times [2–17] than the method presented here. However, many of them have disadvantages such as the use of unstable derivatives [2–10, 13–15], the presence of reagent or reagent-derived peaks in the

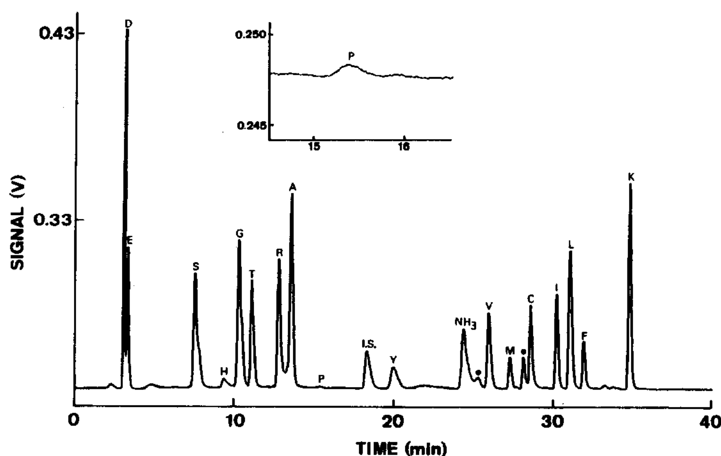

Fig. 5. Elution pattern of N-[2,2-bis(ethoxycarbonyl)vinyl] derivatives in a lysozyme hydrolysate. The sample contained 9188 pmol of amino acids and 308 pmol of internal standard. Peaks are designated as in Fig. 2.

TABLE II

AMINO ACID COMPOSITIONS OF PROTEINS DETERMINED BY THE DESCRIBED HPLC METHOD AND BY CONVENTIONAL ION-EXCHANGE CHROMATOGRAPHY (IEC)

Amino acid	Insulin[a,b]			Lysozyme[a,c]		
	Present	HPLC	IEC[d]	Present	HPLC	IEC[e]
Aspartic acid	3	3.0	2.9	21	20.0	22.2
Glutamic acid	7	6.8	6.8	5	5.1	5.2
Serine	3	3.1	2.8	10	9.9	8.1
Histidine	2	2.1	1.8	1	1.1	0.8
Glycine	4	4.0	4.0	12	11.6	12.5
Threonine	1	1.0	2.8	7	6.9	6.2
Arginine	1	0.9	1.1	11	9.9	11.6
Alanine	3	3.1	1.1	12	12.4	12.0
Proline	1	1.0	1.0	2	1.9	2.4
Tyrosine	4	4.0	3.7	3	3.2	3.5
Valine	5	4.3	3.0	6	5.6	5.3
Methionine	0	0.0	0.0	2	2.4	1.8
Cystine (half)	6	6.4	4.9	8	8.1	6.6
Isoleucine	1	0.6	1.2	6	5.7	5.0
Leucine	6	6.1	5.9	8	9.8	8.3
Phenylalanine	3	3.2	2.8	3	3.0	2.8
Lysine	1	1.0	1.0	6	6.1	5.3

[a] For hydrolysis and derivatization, see Experimental.
[b] Residues per 51 total residues.
[c] Residues per 123 total residues.
[d] Ion-exchange on a Durrum 500 column provided by J. Johansen, Carlsberg Bio Tech, Carlsberg, Sweden.
[e] See ref. 22.

chromatograms [7–20] or extra steps (*e.g.*, extraction, concentration and evaporation) in sample processing after the derivatization reaction [7–20].

The present derivatization reaction is clean (no side-reaction products have been observed) and the shift in the absorbance maximum from 240 to 280 nm on derivatization provides a novel means for eliminating the interference from excess of reagent. These aspects, in addition to the stability and the good chromatographic behaviour of the N-[2,2-bis (ethoxycarbonyl)vinyl] derivatives in reversed-phase HPLC, make possible an easy and reproducible derivatization, injection and elution process.

In conclusion, the derivatization of amino acids with diethyl ethoxymethylenemalonate and their subsequent reversed-phase HPLC is recommended as an alternative to the dedicated amino acid analyser when subpicomole sensitivity is not required.

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