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Note

Gas–liquid chromatography of the N-isobutyloxycarbonyl methyl esters of non-protein amino acids

MASAMI MAKITA, SHIGEO YAMAMOTO, KEIKO SAKAI and MASAMI SHIRAISHI

Faculty of Pharmaceutical Sciences, Okayama University, Tsushima, Okayama 700 (Japan)

The use of gas–liquid chromatography (GLC) for the analysis of amino acids is attractive because of the speed, sensitivity and resolving power of the method. However, the low volatility of the amino acids has prevented their direct analysis by
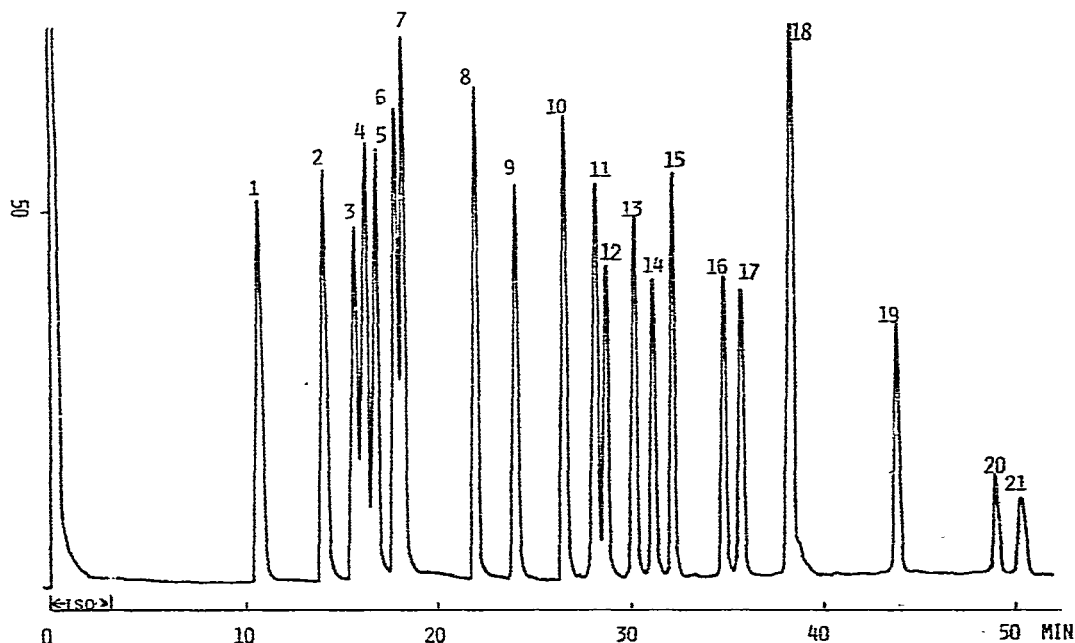

Fig. 1. Chromatogram of the N-isoBOC methyl esters of selected non-protein amino acids. Column: 0.5% FFAP on silanized Gas-Chrom P (100–120 mesh), 2 m × 3 mm I.D., glass. Conditions: isothermal at 80° for 3 min, then programmed at a rate of 4°/min to a final temperature of 275°; attenuation, 32 × 10². N₂ flow-rate, 40 ml/min. Each peak represents 2 μg of amino acid. Peaks: 1 = sarcosine; 2 = α-amino-*n*-butyric acid; 3 = alloisoleucine; 4 = norvaline; 5 = β-aminoisobutyric acid; 6 = β-alanine; 7 = norleucine; 8 = γ-aminobutyric acid; 9 = S-methylcysteine; 01 = ε-aminocaproic acid; 11 = ethionine; 12 = homoserine; 13 = α-aminoadipic acid; 14 = δ-amino-levulinic acid; 15 = kainic acid (internal standard); 16 = S-carboxymethylcysteine; 17 = homo-cysteine; 18 = 2,4-diaminobutyric acid; 19 = methionine sulphone; 20 = lanthionine; 21 = δ-hydroxylysine.

GLC; therefore, suitable volatile derivatives of the amino acids must be prepared.

GLC investigations of non-protein amino acids so far described have generally made use of the N-trifluoroacetyl (N-TFA) methyl esters[1], N-TFA n-butyl esters[2,3] or the trimethylsilyl derivatives[4,5]. Recently, Makita et al.[6,7] demonstrated that the twenty protein amino acids can be quantitatively analyzed as their N-isobutoxycarbonyl (N-isoBOC) methyl esters, which are conveniently prepared by a simple procedure involving isobutyloxycarbonylation with isobutyl chloroformate in aqueous media, followed by esterification with diazomethane*. We now report the logical extention of this work to the analysis of a variety of non-protein amino acids.

EXPERIMENTAL

Reagents

Sarcosine, α-amino-n-butyric acid, norvaline, β-aminoisobutyric acid, β-alanine, norleucine, γ-aminobutyric acid, ethionine, δ-aminolevulinic acid·HCl, α-aminoadipic acid, homocysteine, δ-hydroxylysine·HCl, taurine, cysteic acid, anthranilic acid, m-aminobenzoic acid, p-aminobenzoic acid, 3-hydroxyanthranilic acid, 5-hydroxytryptophan and kainic acid were obtained from Nakarai Chemicals (Kyoto,
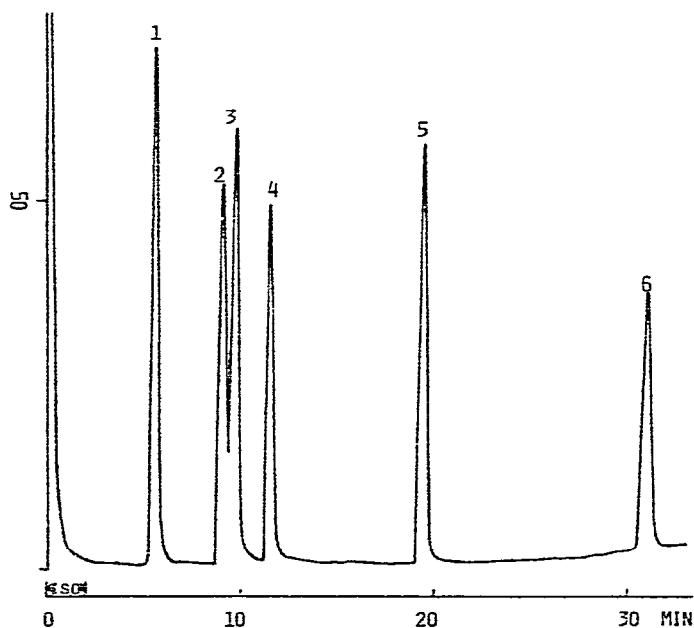

Fig. 2. Chromatogram of the N-isoBOC methyl esters of selected non-protein amino acids. Column: 1% OV-1 on silanized Gas-Chrom P (100–120 mesh), 1 m × 3 mm I.D., glass. Conditions: isothermal at 130° for 2 min, then programmed at a rate of 4°/min to a final temperature of 260°; attenuation, 32 × 10²; N₂ flow-rate, 40 ml/min. Each peak represents 2 μg of amino acid. Peaks: 1 = anthranilic acid; 2 = m-aminobenzoic acid; 3 = p-aminobenzoic acid; 4 = kainic acid (internal standard); 5 = 3-hydroxyanthranilic acid; 6 = 5-hydroxytryptophan.

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* For arginine, an additional reaction step (arginase treatment) is required.

TABLE I

RELATIVE WEIGHT RESPONSE (RWR) OF N-isoBOC METHYL ESTERS OF SELECTED NON-PROTEIN AMINO ACIDS

$$RWR = \frac{\text{(peak height/weight) for the amino acid}}{\text{(peak height/weight) for kainic acid}} ; 1, 2 \text{ and } 3 \text{ are independent determinations.}$$

Amino acid	RWR				Relative standard deviation (%)
	1	2	3	Average	
Sarcosine	0.966	0.979	0.949	0.965	1.56
α-Amino-n-butyric acid	1.093	1.088	1.119	1.100	1.51
Alloisoleucine	0.983	0.951	0.963	0.966	1.67
Norvaline	1.137	1.138	1.109	1.128	1.46
β-Aminoisobutyric acid	1.009	1.012	1.022	1.014	0.67
β-Alanine	1.102	1.100	1.091	1.098	0.53
Norleucine	1.288	1.303	1.294	1.295	0.58
γ-Aminobutyric acid	1.226	1.198	1.222	1.215	1.25
S-Methylcysteine	0.963	0.934	0.945	0.947	1.54
ε-Aminocaproic acid	1.192	1.182	1.139	1.187	0.43
Ethionine	0.963	0.992	0.978	0.978	1.48
Homoserine	0.770	0.761	0.757	0.763	0.87
α-Aminoadipic acid	0.897	0.871	0.870	0.879	1.74
δ-Aminolevulinic acid	0.712	0.688	0.701	0.700	1.72
S-Carboxymethylcysteine	0.737	0.742	0.731	0.737	0.74
Homocysteine	0.705	0.689	0.713	0.702	1.74
2,4-Diaminobutyric acid	1.259	1.231	1.204	1.231	2.23
Methionine sulphone	0.615	0.607	0.603	0.608	1.01
Lanthionine	0.264	0.257	0.258	0.260	1.46
δ-Hydroxylysine	0.215	0.210	0.199	0.208	3.93
Anthranilic acid	1.446	1.422	1.430	1.433	0.85
m-Aminobenzoic acid	1.152	1.142	1.124	1.139	1.25
p-Aminobenzoic acid	1.250	1.236	1.264	1.250	1.12
3-Hydroxyanthranilic acid	1.149	1.126	1.158	1.144	1.44
5-Hydroxytryptophan	0.736	0.756	0.766	0.753	2.03
Kainic acid	1.000	1.000	1.000	1.000	

Japan); ε-aminocaproic acid, homoserine and citrulline from Kyowa Hakko Kogyo (Tokyo, Japan); S-methylcysteine, S-carboxymethylcysteine, 2,4-diaminobutyric acid·$(HCl)_2$, methionine sulphone and lanthionine from Sigma (St. Louis, Mo., U.S.A.); alloisoleucine from Tokyo Chemical Industry (Tokyo, Japan). Isobutyl chloroformate stabilized with $CaCO_3$ was obtained from Tokyo Chemical Industry and used without further purification. N-Methyl-N-nitroso-p-toluenesulphonamide for use in the generation of diazomethane was obtained from Wako (Osaka, Japan). Diethyl ether was purified as described previously[7], and stored over $FeSO_4$.

Derivatization

The derivatization procedure described by Makita et al.[7] was used for the conversion of non-protein amino acids into their N-isoBOC methyl esters.

Gas chromatography

A Shimadzu 5A gas chromatograph, equipped with a dual-column oven bath

TABLE II

CALIBRATION LINEARITY OF SELECTED NON-PROTEIN AMINO ACIDS AS N-isoBOC METHYL ESTERS

$$\langle RWR \rangle \ (\%) = \frac{RWR \ \text{value at each level}}{RWR \ \text{value at the highest level (at } 100 \, \mu g)} \times 100; \ RWR \ \text{values were determined using kainic}$$

acid (50 μg) as internal standard. Each $\langle RWR \rangle$ value represents an average of three independent determinations. RSD = Relative standard deviation.

Amino acid	Derivatized amount (μg)							
	10		20		50		100	
	$\langle RWR \rangle$ (%)	RSD (%)	$\langle RWR \rangle$ (%)	RSD (%)	$\langle RWR \rangle$ (%)	RSD (%)	$\langle RWR \rangle$ (%)	RSD (%)
Sarcosine	9.2	2.74	20.3	2.74	51.7	1.55	100	2.25
α-Amino-n-butyric acid	9.4	1.06	19.8	1.62	50.9	1.56	100	0.99
Alloisoleucine	9.6	2.17	20.2	1.03	51.2	1.67	100	1.39
Norvaline	10.1	1.51	20.8	0.55	52.4	1.49	100	0.49
β-Aminoisobutyric acid	9.8	3.58	20.9	1.99	51.9	0.70	100	0.27
β-Alanine	10.0	1.54	19.7	0.78	48.9	0.54	100	0.92
Norleucine	9.5	2.19	19.7	0.78	48.6	0.63	100	0.38
γ-Aminobutyric acid	9.8	1.02	19.3	1.08	49.8	1.18	100	1.17
S-Methylcysteine	10.0	1.53	19.8	1.01	48.2	1.57	100	0.67
ε-Aminocaproic acid	9.7	1.58	20.3	0.99	51.1	0.41	100	1.14
Ethionine	10.2	0.98	19.8	1.55	50.3	1.49	100	1.12
Homoserine	9.8	3.28	19.8	1.82	48.9	0.85	100	0.66
α-Aminoadipic acid	10.1	1.71	19.5	1.65	48.6	1.78	100	1.01
δ-Aminolevulinic acid	10.0	2.52	19.7	1.55	48.5	1.65	100	0.69
S-Carboxymethylcysteine	10.0	2.08	19.9	2.30	49.8	0.80	100	1.25
Homocysteine	10.0	2.00	19.9	1.54	49.5	1.74	100	1.15
2,4-Diaminobutyric acid	10.0	3.06	20.1	1.25	48.8	2.25	100	1.25
Methionine sulphone	10.2	1.70	20.2	0.76	50.0	1.03	100	0.95
Lanthionine	9.2	4.52	20.0	2.93	49.4	0.84	100	2.71
δ-Hydroxylysine	9.4	9.28	20.6	4.23	48.1	3.93	100	3.21
Anthranilic acid	9.8	3.12	19.8	0.78	49.4	0.84	100	0.76
m-Aminobenzoic acid	9.2	1.25	18.7	1.24	48.6	1.26	100	0.66
p-Aminobenzoic acid	9.6	3.19	19.9	1.76	49.7	1.11	100	0.44
3-Hydroxyanthranilic acid	9.5	2.79	19.5	1.07	50.1	1.44	100	0.70
5-Hyroxytryptophan	9.4	2.21	19.5	1.85	48.8	2.08	100	0.60
Theory	10.0		20.0		50.0		100	

with dual differential hydrogen flame detectors, on-column injection ports and a linear temperature programmer (Shimadzu TP-5), was employed for the analyses. Column packings were prepared from commercial liquid phases and silanized Gas-Chrom P by the "solution-coating" technique[8]. Columns (1 % OV-1 and 0.5 % FFAP) were conditioned with a nitrogen flow-rate of *ca.* 20 ml/min at 275° for 22 h.

RESULTS AND DISCUSSION

In order to evaluate the gas chromatographic properties of N-isoBOC methyl esters of non-protein amino acids, each non-protein amino acid was derivatized and chromatographed. Three amino acids, taurine, citrulline and cysteic acid, gave no

detectable peaks, but all of the remaining 26 amino acids which were tested gave single clean peaks. Figs. 1 and 2 illustrate the chromatograms of mixtures of the derivatives of these non-protein amino acids.

The N-isoBOC methyl esters of these non-protein amino acids were very stable under normal laboratory conditions. Recovery, as determined by peak height, was essentially quantitative for all of the derivatives which had been allowed to stand in solution in ethyl acetate for a week at room temperature. The relative weight responses (RWR) were measured and are given in Table I. The precision of the data is good as evidenced by the relative standard deviations, which in most cases are *ca.* $\leqslant 2\%$.

In experiments to test calibration linearity, three replicate samples at each level in the range of 10–100 µg were derivatized and analyzed. As shown in Table II, the calibration linearity of each amino acid in the range studied and its reproducibility were found to be satisfactory.

These experiments have conclusively demonstrated that a wide range of non-protein amino acids, as well as protein amino acids, can be successfully analyzed by GLC as their N-isoBOC methyl esters. It is anticipated that this simple and convenient method will find extensive applications in biochemical and medical researches.

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