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CAPILLARY GAS CHROMATOGRAPHIC ANALYSIS OF AMINO ACIDS IN BLOOD AND PROTEIN HYDROLYSATES AS *tert.*-BUTYLDIMETHYLSILYL DERIVATIVES

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SUMMARY

A method is given for a one-step derivatization and gas chromatography of amino acids in blood and protein hydrolysates. Blood samples are partially purified by solvent extraction. Protein hydrolysates are neutralized with a triethylamine solution. Then *tert.*-butyldimethylsilyl derivatives of the amino acids are prepared in a one-step procedure and separated on a 30-m fused-silica SE-30 capillary column. Except for tryptophan and cystine, amino acids are eluted within 30 min. Amino acids are derivatized more rapidly than their corresponding trimethylsilyl derivatives and do not degrade on the long fused-silica columns

INTRODUCTION

Gas chromatography (GC) of amino acids has generally been performed using two types of derivatives, acylated amino acid esters and trimethylsilyl (TMS) derivatives [1]. During the two-step preparation of acyl amino acid esters, partial destruction of tryptophan occurs as well as the oxidation of asparagine and glutamine to aspartic acid and glutamic acid, respectively. Peptides present in the preparation will also hydrolyze to the respective amino acids. Trimethylsilyl derivatives have advantages over acyl ester derivatives in that derivatization is a non-oxidative, single-step procedure and will not hydrolyze peptides. However, this derivative results in multiple peaks for some amino acids and some deriva-

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tives with higher boiling points tend to decompose on long fused-silica capillary columns [2].

Corey and Venkateswarlu [3] developed a silylation reagent, N-methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA), to protect hydroxyl groups during organic synthesis. Compounds derivatized with this reagent were considerably more stable to solvolysis than the corresponding TMS derivatives [4]. The use of this reagent for the analysis of the amino acid composition of protein hydrolysates has just recently been reported [5,6]. We have found, however, that different conditions may be required for the derivatization and separation of amino acids in blood because of a number of non-protein amino acids present. These conditions are provided in the present study as well as conditions for the derivatization and separation of amino acids in protein hydrolysates. The analysis of blood urea by this method is also presented.

EXPERIMENTAL

Apparatus

GC analyses were performed with a Varian Model 3700 gas chromatograph on a 30 m × 0.25 mm fused-silica SE-30 capillary column (film thickness, 0.25 mm; J. & W. Scientific, Rancho Cordova, CA, U.S.A.). A 30 m × 0.25 mm fused-silica SE-54 capillary column (film thickness, 0.25 mm; J. & W. Scientific) was also investigated as a potential column for separating amino acid derivatives. Injector and detector temperatures were both 300°C. The oven temperature was programmed to rise from 155 to 255°C at a rate of 3°C/min and then immediately increased to 300°C. A split injection mode (1:50) maintained the helium carrier gas flow-rate at approximately 0.75 ml/min through the column. The make-up gas (helium) flow-rate through the detector was 30 ml/min. Air and hydrogen gas flow-rates to the detector were 300 and 30 ml/min, respectively.

Compound retention times and peak areas were determined by a Hewlett-Packard Series 3353 laboratory automation system (Avondale, PA, U.S.A.). Relative molar responses were calculated as the ratio of amino acid peak area to internal standard peak area at equivalent molar concentrations of amino acid and internal standard. Results were graphically plotted by a Fisher Recordall Series 5000 recorder (Fisher Scientific, Edmonton, Canada).

Chemicals

L-Amino acid standard solution for protein hydrolysates (Cat. No. AA-S-18), asparagine, glutamine, 3-methylhistidine, hydroxyproline, tryptophan, cycloleucine, urea, type I collagen and casein were purchased from Sigma (St. Louis, MO, U.S.A.). Acetone, ethanol, methylene chloride, triethylamine, hexane, pyridine and N,N-dimethylformamide (DMF) were purchased from Fisher Scientific and were either HPLC grade or redistilled before use. MTBSTFA and *tert.*-butyldimethylchlorosilane (TBDMCS) were purchased from either Pierce (Rockford, IL, U.S.A.) or Sigma.

Standards

The amino acid standard solution for protein hydrolysates was diluted to a 100 nmol/ml working solution. Stock solutions (10 m*M*) were prepared for 3-methylhistidine, hydroxyproline and cycloleucine and stored at −20°C until diluted to a working standard. Stock solutions of asparagine, glutamine and tryptophan were prepared daily. A single working solution (100 nmol/ml) was prepared from the stock solutions of amino acids except for cycloleucine. A separate working solution was prepared for cycloleucine which served as an internal standard.

Solvent extraction of blood

A 0.5-ml volume of blood or standard was added to 0.5 ml of cycloleucine internal standard. Ice-cold acetone (4 ml) was added to deproteinize the sample and the supernatant was separated by centrifugation at 4°C (10 min at 1800 *g*). The supernatant was acidified with one drop of concentrated hydrochloric acid and then extracted three times with chloroform. The aqueous layer was transferred to a 13×100 mm screw-capped culture tube and evaporated just to dryness at 65°C under a stream of nitrogen. The residue was derivatized as described below.

Protein hydrolysis

A 50-mg amount of protein was weighed into a 13×100 mm screw-capped culture tube. A 5-ml volume of redistilled 6 *M* hydrochloric acid was added, the head space purged with nitrogen gas and the culture tube tightly capped. The sample was hydrolyzed in a forced-draft oven at 110°C for 24 h. At the end of hydrolysis, 0.5 ml of 10 m*M* (stock solution) cycloleucine (internal standard) were added to the tube. The sample was transferred to a 125-ml round-bottom flask and the hydrochloric acid was removed under reduced pressure at 50°C with a rotary evaporator. Distilled water (10 ml) was added to the dry residue. A 0.2-ml aliquot was transferred to a 13×100 mm screw-capped culture tube and an additional 2 ml of 20% triethylamine in 20% aqueous acetone were added. The solution was extracted three times with chloroform (centrifugation facilitates the separation of layers). The aqueous layer was evaporated to dryness under a stream of nitrogen at 65°C and the residue derivatized as described below.

Derivatization procedure

Residual moisture in the amino acid residues from the blood clean-up procedure or protein hydrolysis was removed with 0.5 ml of methylene chloride. *tert.*-Butyldimethylsilyl (TBDMS) derivatives were prepared by adding 50 µl each of MTBSTFA and DMF to the culture tube, purging the head space with nitrogen gas, tightly capping the tube and then heating the tube on a sand bath at 50°C for 1 h. A 1-µl aliquot was injected into the gas chromatograph. In order to determine the proper conditions for derivatization, hexane, methylene chloride, acetonitrile and pyridine were investigated as solvents in place of DMF and 1% and 10% TBDMCS in the MTBSTFA as catalysts. Derivatization temperatures ranging from room temperature to 150°C were also investigated.

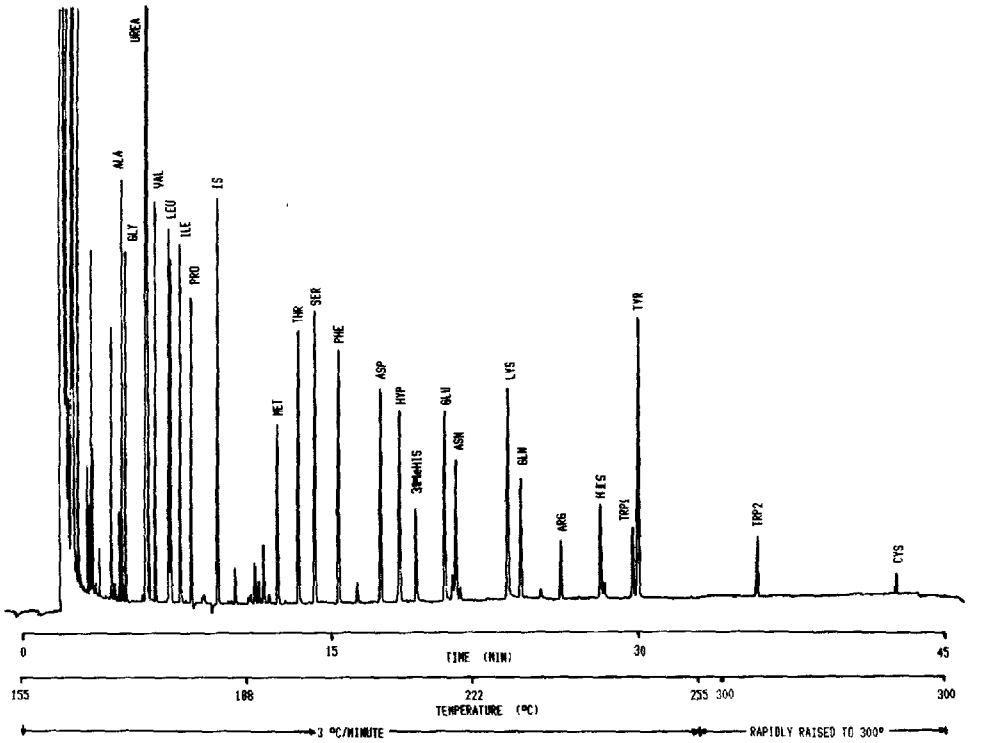

Fig. 1. Gas chromatographic separation of *tert*-butyldimethylsilyl derivatives of amino acid standards on a 30-m fused-silica SE-30 capillary column. Standard concentration: 100 μM each. IS = cycloleucine, internal standard. TRP1 and TRP2 are two peaks of tryptophan.

RESULTS AND DISCUSSION

Chromatography

Chromatograms of TBDMS amino acid derivatives in a standard mixture and in bovine whole blood analyzed using a 30-m fused-silica column are shown in Figs. 1 and 2, respectively. A slow temperature gradient was necessary in order to separate ornithine from asparagine and the first peak of tryptophan (tryptophan 1) from tyrosine. Following the elution of tyrosine at 255 °C, the temperature was immediately increased to 300 °C to allow for the faster elution of the second peak of tryptophan (tryptophan 2) and cystine. The TBDMS derivatives had later eluting times than the corresponding TMS derivatives [2] and contrary to observations with TMS derivatives, the TBDMS derivatives of the later-eluting amino acids, histidine, tyrosine, tryptophan and cystine, did not degrade on longer fused-silica columns. An SE-54 fused-silica column was also investigated, this column being slightly more polar than the SE-30 column. The use of even higher polarity columns was limited due to the lower temperature maxima of these columns. The SE-54 column adequately separated tryptophan 1 from tyrosine, but 3-methylhistidine, glutamate, ornithine and asparagine were not adequately separated. Therefore, there appeared to be no additional advantage to using the SE-54 column over the SE-30 column. Biermann et al. [5] used a 60-m capillary column

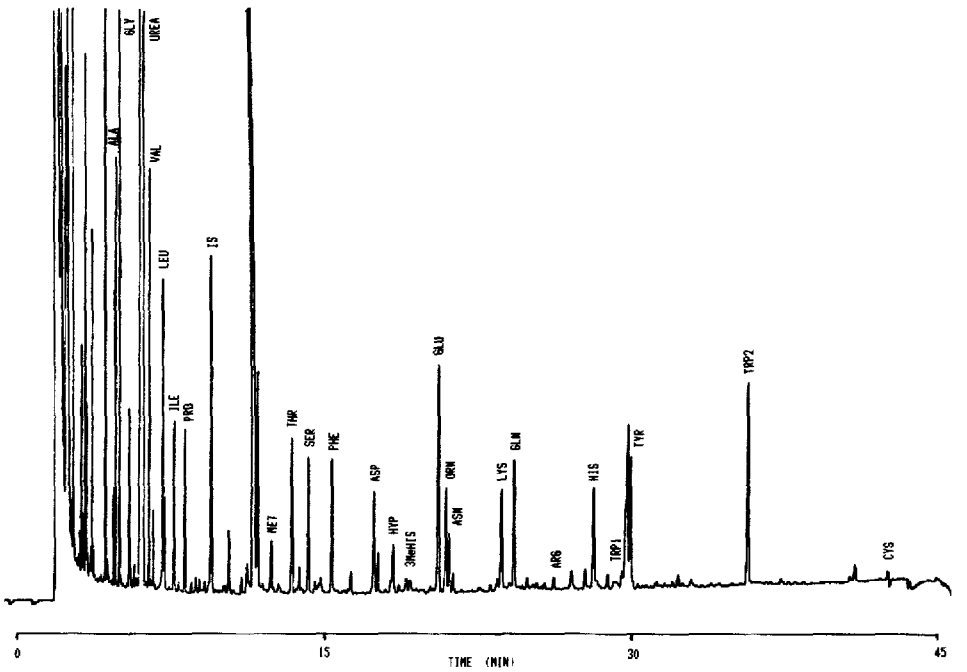

Fig. 2. Chromatogram of amino acids in bovine whole blood. IS = cycloleucine, internal standard (100 μM).

(SPB-5; Supelco, Bellefonte, PA, U.S.A.) of similar polarity to the SE-54 phase and found adequate separation of most amino acids except for glutamic acid and arginine. Under their derivatization conditions, they found two peaks for arginine. Mawhinney et al. [6] observed adequate separations for eighteen amino acids found in protein hydrolysates using a shorter (12 m) SE-30 (Varian, Park Ridge, IL, U.S.A.) and a 25-m bonded OV-17 (Quadrex, New Haven, CT, U.S.A.) fused-silica capillary column. They also observed two peaks for arginine and two peaks each for lysine, tryptophan and cystine for the SE-30 and OV-17 phase columns, respectively.

Optimizing derivatization conditions

Derivatization of most amino acids was essentially complete within 1 h at room temperature. Hydroxyproline, however, required a longer derivatization time and the rate increased with temperature. Glutamine, asparagine and tryptophan showed decreased responses with both time and temperature. The condition selected as optimal was 1 h at 50 °C since most amino acids were derivatized under these conditions with the least destruction to asparagine, glutamine and tryptophan. The detector response was linear in the concentration range 10–500 μM (Table I) for most amino acids. The detector response to arginine, histidine and cystine declined rapidly in the range of 10–50 μM. Tryptophan response remained low up to 100 μM but increased rapidly from 100 to 500 μM (Fig. 3). Our most consistent responses were obtained with injections immediately after derivati-

TABLE I

LINEAR REGRESSION COEFFICIENTS AND *y*-INTERCEPTS OF MOLAR RESPONSES RELATIVE TO CYCLOLEUCINE VERSUS CONCENTRATION OF TBDMS AMINO ACIDS

Regressions were calculated over a concentration range of 10–500 μM amino acid except where the range is specified in parentheses. Relative molar responses were calculated relative to cycloleucine internal standard (100 μM).

Amino acid	Regression coefficient	*y*-Intercept	
Alanine	0.88	– 5.09	
Glycine	0.92	0.26	
Urea	1.02	–22.78	(100–5000)
Valine	0.74	– 2.88	
Leucine	0.68	– 3.30	
Isoleucine	0.67	– 0.75	
Proline	0.77	5.81	
Methionine	0.84	7.18	
Serine	0.60	– 1.61	
Threonine	0.58	– 1.03	
Phenylalanine	0.58	0.74	
Aspartate	0.76	– 1.71	
Hydroxyproline	0.64	8.20	
3-Methylhistidine	1.58	6.92	
Glutamate	0.73	7.44	
Asparagine	0.85	4.81	
Lysine	0.65	– 2.39	
Glutamine	1.09	– 3.18	
Arginine	2.68	13.70	(50–500)
Histidine	0.99	22.19	(50–500)
Tyrosine	0.51	2.60	
Cystine	3.97	29.82	(50–500)

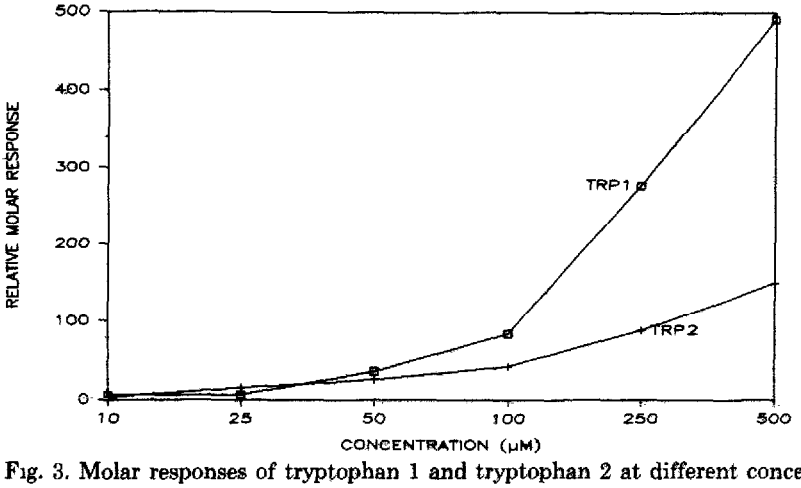

Fig. 3. Molar responses of tryptophan 1 and tryptophan 2 at different concentrations relative to cycloleucine (100 μM).

TABLE II

BOVINE WHOLE BLOOD AMINO ACID CONCENTRATIONS AND THE PERCENTAGE RECOVERY OF ADDED AMINO ACID STANDARDS

Amino acid	Initial concentration (mean ± S.D.) (μM)	Recovery (mean ± S.D.) (%)				
		Concentration of amino acid added (nmol/ml of blood)				
		10	25	50	100	250
Alanine	200 ± 11	147 ± 43	107 ± 17	94 ± 15	95 ± 4	93 ± 7
Glycine	231 ± 18	258 ± 80	98 ± 29	105 ± 26	102 ± 5	92 ± 11
Urea*	2888 ± 147	319 ± 28	237 ± 12	153 ± 5	111 ± 30	115 ± 23
Valine	274 ± 11	123 ± 47	105 ± 22	95 ± 13	95 ± 4	92 ± 10
Leucine	138 ± 11	103 ± 84	103 ± 23	99 ± 19	99 ± 6	93 ± 13
Isoleucine	103 ± 5	99 ± 43	106 ± 13	96 ± 10	95 ± 7	96 ± 11
Proline	71 ± 2	100 ± 41	94 ± 20	93 ± 12	97 ± 8	97 ± 12
Methionine	40 ± 1	110 ± 20	98 ± 10	108 ± 15	105 ± 16	103 ± 10
Serine	79 ± 8	103 ± 36	102 ± 10	98 ± 7	80 ± 1	78 ± 5
Threonine	81 ± 4	109 ± 42	96 ± 9	90 ± 7	95 ± 1	89 ± 5
Phenylalanine	73 ± 2	116 ± 22	96 ± 9	99 ± 2	91 ± 7	91 ± 8
Aspartate	34 ± 2	106 ± 46	106 ± 7	104 ± 15	108 ± 4	101 ± 6
Hydroxyproline	28 ± 1	82 ± 14	100 ± 15	104 ± 17	96 ± 12	95 ± 9
3-Methylhistidine	46 ± 5	102 ± 86	91 ± 5	89 ± 3	89 ± 3	57 ± 4
Glutamate	107 ± 8	129 ± 42	104 ± 19	116 ± 21	96 ± 2	96 ± 2
Asparagine	42 ± 4	74 ± 13	83 ± 9	92 ± 3	90 ± 2	80 ± 3
Lysine	74 ± 4	112 ± 6	55 ± 3	65 ± 6	36 ± 5	30 ± 4
Tyrosine	269 ± 3	99 ± 21	91 ± 3	93 ± 5	96 ± 5	87 ± 4

*Urea recoveries were determined over a concentration range of 100–2500 nmol/ml of blood.

zation (particularly for arginine, histidine, glutamine and tryptophan) and most derivatized amino acids were stable for at least seven days at room temperature in tightly closed, nitrogen-purged culture tubes.

Hexane and methylene chloride resulted in multiple-peak formation for all amino acids. Acetonitrile, which was the preferred solvent for the TMS amino acid derivative [7,8] and for the MTBSTFA derivatizing procedure of Biermann et al. [5], required longer derivatization time and higher temperatures for derivative formation of serine, threonine, ornithine, hydroxyproline and cycloleucine (150 °C for at least 8 h). Glutamine, asparagine, arginine and tryptophan decomposed under these conditions. Pyridine as a solvent increased the molar responses of glycine, 3-methylhistidine, asparagine, glutamine, lysine, histidine and tryptophan 1 and decreased the molar responses of proline, serine, threonine, hydroxyproline, arginine, tryptophan 2 and cystine relative to cycloleucine. Pyridine could therefore be used as a solvent for analyses which had particular emphasis on the former amino acids. The addition of 1 or 10% TBDMCSA to the reaction mixture did not improve the rate or extent of derivative formation.

Analysis of amino acids in blood

Since a variety of compounds in blood will silylate, it was necessary to perform a partial purification prior to derivatization. The recovery of amino acid and urea standards added to whole blood determined using the chloroform extraction method was greater than 90% for most amino acids over a wide range of added

TABLE III

AMINO ACID COMPOSITION OF COLLAGEN AND CASEIN HYDROLYSATES

Amino acid	Composition (mean ± S.D.) (molar % of total concentration)	
	Collagen*	Casein**
Alanine	12.2± 0.59 (10.1, 13.0)	4.8±0.08 (3.7)
Glycine	32.4±10.0 (34.4, 33.2)	3.6±0.10 (2.8)
Valine	2.4± 0.03 (3.1, 1.4)	7.5±0.04 (8.2)
Leucine	2.7±0.07 (3.0, 2.0)	9.6±0.62 (9.3)
Isoleucine	1.3±0.01 (1.8, 0.6)	5.5±0.03 (6.2)
Proline	12.5±1.18 (11.7, 11.9)	11.4±0.01 (11.4)
Methionine	0.3±0.002 (0.3, 0.8)	1.4±0.15 (2.5)
Serine	2.9±0.03 (2.9, 2.9)	5.7±0.05 (6.9)
Threonine	1.5±0.04 (2.0, 1.9)	4.0±0.04 (4.5)
Phenylalanine	1.4±0.01 (1.4, 1.4)	3.8±0.04 (3.9)
Aspartate	5.5±0.46 (5.2, 4.2)	7.7±0.05 (6.4)
Hydroxyproline	9.9±0.02 (9.5, 10.3)	
Glutamate	8.1±0.73 (6.5, 7.8)	19.9±0.22 (18.7)
Lysine	0.9±0.08 (1.7, 3.0)	4.8±0.27 (6.8)
Arginine	4.3±1.67 (5.0, 5.1)	2.7±0.32 (2.7)
Histidine	0.9±0.02 (0.7, 0.2)	2.9±0.14 (2.3)
Tyrosine	0.5±0.004 (0.3, 0.2)	3.9±0.01 (3.7)

*Values in parentheses were calculated from data previously reported [9] for the two-chain sources of type I collagen.

**Values in parentheses were calculated from data previously reported [10] for dried casein.

amino acid and urea standards (Table II). Arginine, histidine, tryptophan, glutamine and cystine had inconsistent detector responses and consequently the recoveries of these amino acids were variable and ranged from 5 to 45%.

Protein hydrolysates

The hydrolysis was a standard procedure, but the additional steps of triethylamine neutralization and chloroform extraction (removes triethylamine hydrochloride) have been included because the amino acids did not derivatize as well as hydrochlorides. The amino acid compositions of collagen and casein hydrolysates are shown in Table III. These data were generally more consistent (lower S.D.) than were the amino acid analyses on blood. The amino acid compositions of these two proteins, as reported elsewhere [9,10], are also given in Table III. Our compositional patterns compared well with these data.

In summary, the TBDMS amino acid derivative appears to be a suitable derivative for the capillary GC analysis of amino acids in blood and protein hydrolysates. It offers the additional convenience of derivatizing urea; thus, one may avoid having to conduct a separate assay for this compound when experimental or clinical situations require its content.

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