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Short communication

Determination of amino acids in human serum by capillary gas chromatography

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Abstract

A simple and rapid method for the determination of serum amino acids by gas chromatography (GC) has been developed. Following deproteinization of serum with perchloric acid, free amino acids in the supernatant were converted into their *N*(*O*,*S*)-isobutoxycarbonyl methyl ester derivatives and measured by GC with flame ionization detection using a DB-17 capillary column. All the derivatives of the 22 protein amino acids were completely resolved as single peaks within 9 min by GC The calibration curves were linear in the range 0.2–50 μg of each amino acid, and the correlation coefficients were above 0.998. By using this method, serum amino acids could be directly analysed without prior clean-up procedure such as ion-exchange column chromatography except for deproteinization of the samples, and without any interference from coexisting substances. Overall recoveries of amino acids added to serum samples were 88–108%. Analytical results for serum amino acids from normal subjects are presented.

Keywords: Amino acids

1. Introduction

Amino acids are fundamental units in the living organism and their extent in the body affects the healthy existence. Nutritional deficiencies of single amino acids or of total protein intake, and imbalances in the amino acids in dietary protein cause impairment of growth and other deleterious effects [1]. Further, several pathological conditions in man are known that occur because of a well-delineated abnormality in a single step of the metabolism of a specific amino acid [2]. Amino acid levels in blood are related to various factors such as diet, metabolism of each amino acid and protein metabolism. Therefore, the determination of blood amino acids is considered to be one of the most important indicators in the diagnosis and monitoring of the inherited disorders of amino acid metabolism and in evaluating the nutritional requirements of patients.

Several gas chromatographic methods for the determination of amino acids in blood samples have been developed on the basis of the preparation of *N*(*O*)-acyl alkyl esters [3–6], *N*(*O*,*S*)-alkyloxycarbonyl alkyl esters [7–9] and *N*(*O*)-*tert.*-butyldimethylsilyl derivatives [10]. However, some of these methods require anhydrous conditions, high temperatures and long reaction times for the derivatization, and most of them require laborious clean-up of the samples to remove the coexisting substances.

Recently, we have reported [11] improvements of the previous methods [7,8] in terms of speed and simplicity. The improved method is based on the

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preparation of *N(O,S)*-isobutoxycarbonyl (isoBOC) methyl ester derivatives of amino acids by using sonication technique and subsequent gas chromatography (GC) using a single capillary column. By using this method, we have demonstrated that amino acid compositions of proteins could be rapidly and simply determined without any influence from other coexisting substances. In this paper, we report the extension of these works to the determination of serum amino acids.

2. Experimental

2.1. Reagents

All of the standard amino acids were purchased from Ajinomoto (Tokyo, Japan) except for L-cysteine, L-ornithine, L-asparagine and L-glutamine, which were purchased from Nacalai Tesque (Kyoto, Japan). The standard stock solution containing the 22 protein amino acids (each 0.1 mg/ml) was prepared in 0.05 *M* hydrochloric acid and stored at 4°C. The working standard solution was made up freshly as required by dilution of the stock solution with 0.05 *M* hydrochloric acid. 4-Piperidinecarboxylic acid as an internal standard (I.S.) was purchased from Tokyo Kasei Kogyo (Tokyo, Japan) and was dissolved in 0.05 *M* hydrochloric acid at a concentration of 50 μg/ml. Dithioerythritol (DTE) was obtained from Nacalai Tesque and used as a 5 m*M* solution in distilled water. Arginase was purchased from Sigma (St. Louis, MO, USA). Arginase solution was prepared as follows: prior to use, 5 mg of arginase (40 IU/mg) were activated in 4.5 ml of 0.1 *M* ammonium acetate and 0.5 ml of 0.5 *M* manganese(II) sulphate at 37°C for 4 h. After the solution was centrifuged at 1600 *g* for 1 min, the supernatant was separated and this solution was stored at −20°C until used. This stored solution of activated arginase could be used without loss of activity for at least 15 days. Isobutyl chloroformate (isoBCF) was obtained from Tokyo Kasei Kogyo and used without further purification. Human control serum was obtained from Hyland Diagnostics (Round Lake, IL, USA). *N*-Methyl-*N*-nitroso-*p*-toluenesulphonamide and diethyleneglycol monomethyl ether for the generation of diazomethane [12] were obtained from Nacalai Tesuque. Peroxide-free diethyl ether was purchased from Dojindo Laboratories (Kumamoto, Japan). All other chemicals were analytical grade.

2.2. Preparation of samples

Venous blood samples from healthy volunteers were collected in vacutainer tube and centrifuged at 1600 *g* for 10 min. The serum layer was carefully collected and processed immediately. To 0.2 ml of serum sample was added 80 μl of 50 μg/ml 4-piperidinecarboxylic acid (I.S.) and 50 μl of 5 m*M* DTE, and then total volume was made up to 0.45 ml with distilled water. After adding 0.15 ml of 10% perchloric acid, the solution was vigorously mixed for 10 s with a vortex-type mixer and centrifuged at 2000 *g* for 1 min. The precipitate was re-extracted with 0.4 ml of 2.5% perchloric acid and centrifuged at 2000 *g* for 1 min. The supernatants were combined and neutralized with 30% sodium carbonate (ca. 0.1 ml). The neutralized supernatant was divided into two portions and each used for the analysis of ornithine plus 21 protein amino acids (except for arginine) and arginine, respectively.

For the analysis of ornithine plus 21 protein amino acids, a 0.6-ml portion of the neutralized supernatant was directly derivatized.

For the analysis of arginine, arginine was converted into ornithine by arginase treatment prior to derivatization. To a 0.3-ml portion of the neutralized supernatant was added 0.5 ml of 0.2 *M* sodium carbonate-sodium bicarbonate buffer (pH 9.5) and total volume was made up to 0.7 ml with distilled water, and then the activated arginase solution (50 μl) was added. The mixture was incubated at 37°C for 10 min and then used as the sample for derivatization.

2.3. Derivatization procedure

To the standard solution or the sample prepared by above method were added 40 μl of 50 μg/ml 4-piperidinecarboxylic acid (I.S.) if necessary and 0.25 ml of 10% sodium carbonate and the total volume was made up to 1 ml with distilled water. Then 20 μl of isoBCF was added immediately and the mixture was sonicated in a Model UT-104 Ultra sonic (39 kHz) cleaner (Sharp, Tokyo, Japan) for 30

s at room temperature after shaking for a few seconds by hand. The reaction mixture was extracted with 3 ml of peroxide-free diethyl ether in order to remove the excess of reagent, the ethereal extract being discarded. The aqueous layer was acidified to pH 1–2 with 2 *M* hydrochloric acid and saturated with sodium chloride, and then extracted twice with 3 ml of peroxide-free diethyl ether. The pooled ethereal extracts were methylated by bubbling diazomethane, generated according to the microscale procedure [12], through the solution until a yellow tinge became visible. This reaction should be performed in a well ventilated hood because diazomethane is explosive and toxic. After standing above 1 min at room temperature, the solvents were removed by evaporation to dryness at 60°C under a stream of dry air. The residue was dissolved in 0.05–0.1 ml of ethyl acetate and then 1 μl of this solution was injected into the gas chromatograph. The derivatization process is shown in Fig. 1.

2.4. Gas chromatography

GC analysis was carried out with a Hewlett-Packard 5890 Series II gas chromatograph equipped with a hydrogen flame ionization detector and split injection system. A fused-silica capillary column of cross-linked DB-17 (50% phenyl, 50% methylpolysiloxane, J & W, Folsom, CA, USA, 15 m×0.25 mm I.D., 0.25 μm film thickness) was used. The operation conditions were as follows: column temperature, programmed at 30 C°/min from 140–290°C; injection and detector temperatures, 300°C; helium carrier gas and make-up gas flow rates, 1 ml/min and 30 ml/min, respectively. Split ratio was 50:1.

2.5. Calibration curves and calculations

The peak heights of amino acids and the I.S. were measured and the peak-height ratios of amino acids against the I.S. were calculated to construct calibration curves. The contents of ornithine and 21 protein amino acids except for arginine in serum were calculated from the directly derivatized samples without arginase treatment. On the other hand, serum arginine content was calculated by subtracting the amount of ornithine obtained without arginase treatment from that obtained with arginase treatment.

3. Results and discussion

Protein amino acids except for arginine can be converted into their *N*(*O*,*S*)-isoBOC methyl ester derivatives as previously described [11]. This method is based on the *N*(*O*,*S*)-isobutoxycarbonylation of amino acids with isoBCF in aqueous alkaline medium and subsequent methylation with diazomethane. The *N*(*O*,*S*)-isobutoxycarbonylation was completed in 2.5% sodium carbonate solution with ≥10 μl of isoBCF within 15 s by sonication at room temperature. Sonication technique, which can easily mix aqueous solution and oily isoBCF reagent by its vibration effect, was effective for acceleration of the reaction. The resulting *N*(*O*,*S*)-isoBOC amino acids could be quantitatively and selectively extracted into diethyl ether, and the subsequent methylation of the ethereal extracts could be successfully carried out by bubbling diazomethane. The derivative preparation could be performed within 10 min. On the other hand, arginine requires conversion into ornithine with arginase, because the guanidino group on the side chain of arginine cannot be derivatized. Arginase treatment was carried out with 0.4 units of arginase in carbonate buffer (pH 9.5) at 37°C for 10 min. The conversion yield of arginine into ornithine was found to be above 93% in comparison with equivalent ornithine standard.

As shown in Fig. 2A, 22 amino acids could be completely resolved as single peaks within 9 min on a DB-17 capillary column. The calibration curves for

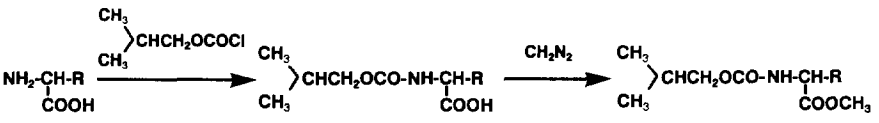

Fig. 1. Derivatization reaction of amino acids.

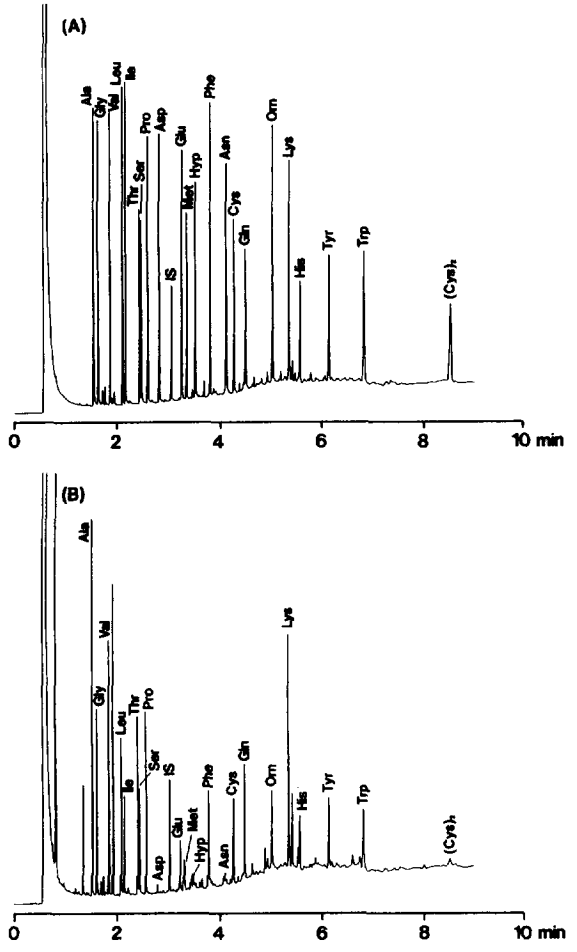

Fig. 2. Gas chromatograms obtained from standard and serum sample. (A) Standard solution containing 25 μg of asparagine, glutamine and cystine, and 5 μg of other amino acids, (B) serum (0.1 ml). GC conditions are given in Section 2. Peaks: Ala=alanine; Gly=glycine; Val=valine; Leu=leucine; Ile=isoleucine; Thr=threonine; Ser=serine; Pro=proline; Asp=aspartic acid; Glu=glutamic acid; Met=methionine; Hyp=hydroxyproline; Phe=phenylalanine; Asn=asparagine; Cys=cysteine; Gln=glutamine; Orn=ornithine; Lys=lysine; His=histidine; Tyr=tyrosine; Trp=tryptophan; $(Cys)_2$=cystine; IS=isonipecotic acid.

these amino acids were conducted using 4-piperidinecarboxylic acid, which showed similar behaviour to other amino acids during the derivatization and was well separated from other amino acids on a chromatogram as the I.S. A linear relationship was obtained with correlation coefficient being above 0.998 in the range 1–50 μg for asparagine, glutamine and cystine, 0.5–10 μg for arginine and 0.2–10 μg for other amino acids. The minimum detectable amounts of these amino acids to give a signal-to-noise ratio of 3 under our GC conditions were 0.2–4.0 ng injected.

The method developed was successfully applied to serum samples. Perchloric acid was used to remove serum proteins by precipitation. In order to prevent the oxidation of sulphur amino acids, DTE was added to serum sample prior to deproteinization. The protein-free supernatant could be directly derivatized without clean-up procedures such as ion-exchange column chromatography, solid phase extraction, and subsequent eluate evaporation. Fig. 2B shows a typical chromatogram obtained from 0.1 ml of serum sample. No peak was observed in the serum samples with the same retention time as that of isonipecotic acid (I.S.). Addition of the I.S. to serum at the first step in the overall procedure made the method reliable. All of the protein amino acids in serum could be analysed without any interference from coexisting substances. To confirm validity of this method, known amounts of amino acids were spiked to human control serum and their recoveries were calculated. As shown in Table 1, the overall recoveries of these amino acids were between 88–108%, and the relative standard deviations were 0.4–11.0% (n=4). The quantitation limits of amino acids in serum samples were ca. 1–5 μg/ml. The intra-assay C.V. values and inter-assay C.V. values for control serum throughout the overall procedure consisting of arginase treatment, derivatization and GC analysis were 1.8–13.5% (n=4) and 2.8–15.6% (n=4), respectively. Table 2 shows the results obtained from eight healthy adult volunteers, who were not restricted in food intake. These amino acid levels were within the range of the literature values obtained from normal subjects [4,7,8,13,14].

4. Conclusions

A convenient and reliable method for the determination of amino acids in serum samples has been established. The method is simple and rapid, and serum samples can be analysed without clean-up except for deproteinization and without any interference from other coexisting substances. We believe

Table 1
Recoveries of amino acids added to human control serum

Amino acids	Non-addition[a] (μg/ml)	Added (μg/ml)	Found[a] (μg/ml)	Recovery (%)	Added (μg/ml)	Found[a] (μg/ml)	Recovery (%)
Alanine	10.9±0.3	5	16.0±0.2	102	20	32.3±1.7	107
Glycine	5.6±0.1	5	10.5±0.2	98	20	26.3±1.5	104
Valine	7.0±0.2	5	12.1±0.3	102	20	26.2±0.7	96
Leucine	23.0±0.7	5	27.7±0.1	94	20	44.6±1.9	106
Isoleucine	2.5±0.2	5	7.2±0.3	94	20	22.6±0.9	101
Threonine	6.9±0.2	5	11.7±0.4	96	20	27.8±1.5	105
Serine	2.4±0.2	5	7.5±0.4	102	20	21.7±0.9	97
Proline	9.2±0.3	5	14.0±0.3	96	20	30.4±1.8	106
Aspartic acid	4.0±0.1	5	9.1±0.2	102	20	24.3±1.1	101
Glutamic acid	26.6±0.8	5	31.6±0.6	100	20	45.2±1.4	93
Methionine	4.1±0.1	5	8.5±0.3	88	20	21.7±0.4	88
Hydroxyproline	2.9±0.1	5	7.7±0.3	96	20	22.5±1.7	98
Phenylalanine	7.5±0.4	5	12.3±0.4	96	20	27.1±1.4	98
Asparagine	16.5±0.4	25	39.4±0.4	92	100	124.8±7.3	108
Cysteine	8.4±0.4	5	13.7±0.3	106	20	28.0±1.6	98
Glutamine	96.8±9.5	25	122.8±2.8	104	100	203.5±7.9	107
Ornithine	0.9±0.1	5	6.0±0.5	102	20	20.3±0.6	97
Lysine	17.2±0.6	5	22.3±0.4	102	20	36.5±1.8	97
Histidine	6.3±0.6	5	11.5±0.1	104	20	26.0±1.9	99
Tyrosine	14.3±1.0	5	19.7±0.6	108	20	34.5±1.3	101
Tryptophan	3.7±0.5	5	8.6±0.4	98	20	24.6±1.1	105
Cystine	ND[b]	25	26.3±2.9	105	100	106.3±5.5	106
Arginine	5.9±0.3	5	10.5±0.3	92	20	26.2±1.0	102

[a] Mean±SD (n=4).
[b] Not detectable.

Table 2
Serum amino acids in normal subjects

Amino acids	Content[a] (μg/ml serum)							
	1	2	3	4	5	6	7	8
Alanine	38.9±1.7	48.8±4.2	43.1±0.9	45.1±1.9	27.2±2.2	44.5±3.8	39.6±2.6	23.6±1.7
Glycine	25.2±0.8	21.4±1.5	22.5±0.4	17.2±0.4	19.0±1.3	17.5±0.8	20.9±1.2	11.6±0.8
Valine	34.8±1.0	37.2±0.7	30.1±0.5	32.7±1.1	27.1±2.1	21.7±1.3	23.0±1.4	27.6±1.8
Leucine	21.5±1.0	27.0±0.6	20.8±0.2	25.2±1.0	19.6±0.7	18.0±0.9	20.3±1.1	24.6±1.1
Isoleucine	12.0±0.1	12.6±0.3	11.5±0.4	12.8±0.5	8.0±0.3	8.0±0.7	7.5±0.6	6.0±0.4
Threonine	22.2±1.0	18.7±0.5	24.4±0.5	13.7±0.2	9.9±0.2	18.3±0.3	21.2±0.4	11.4±0.2
Serine	17.6±1.4	16.9±1.0	17.3±0.6	8.5±0.6	11.4±0.6	16.4±0.5	18.6±1.3	13.5±0.6
Proline	26.9±0.7	22.0±0.3	16.9±0.2	29.3±0.4	19.8±1.3	20.3±0.8	26.5±1.0	22.8±0.8
Aspartic acid	1.0±0.1	1.0±0.0	1.2±0.0	1.1±0.1	1.2±0.1	1.2±0.0	1.2±0.1	1.2±0.1
Glutamic acid	4.0±0.3	3.0±0.2	3.1±0.1	6.5±0.2	3.0±0.2	3.4±0.2	2.9±0.2	5.3±0.1
Methionine	7.0±0.5	8.7±0.5	6.0±0.5	4.8±0.1	6.3±0.4	5.3±0.3	4.1±0.2	4.1±0.2
Hydroxyproline	2.1±0.1	1.3±0.1	1.8±0.1	1.2±0.1	1.3±0.1	1.1±0.1	2.4±0.2	2.3±0.2
Phenylalanine	12.9±0.4	15.1±0.5	11.4±0.4	13.6±1.2	13.6±0.7	12.2±0.8	14.0±0.7	14.2±0.5
Asparagine	7.8±0.9	9.3±0.8	8.1±0.9	4.3±0.3	9.7±0.7	8.5±0.4	11.0±1.0	12.3±0.8
Cysteine	18.8±1.8	23.3±2.2	19.6±2.0	26.7±1.3	27.3±1.9	25.6±2.4	19.1±1.9	23.9±2.3
Glutamine	76.3±4.7	91.2±8.3	81.8±6.0	71.4±3.4	104.8±8.8	107.4±6.5	124.6±10.6	99.4±8.4
Ornithine	11.1±0.7	9.1±0.4	9.6±0.4	9.2±0.3	6.7±0.2	10.4±0.4	9.4±0.3	7.0±0.2
Lysine	33.5±2.3	34.2±1.3	30.5±1.7	39.6±1.8	21.4±1.0	29.9±0.7	32.2±0.9	30.5±0.1
Histidine	13.8±1.0	14.2±0.9	15.7±0.9	11.3±0.6	12.6±0.9	11.2±0.6	14.7±0.9	12.1±0.9
Tyrosine	11.1±0.8	12.1±0.6	13.9±1.2	17.0±1.4	12.9±1.2	9.7±0.9	11.5±0.9	11.5±0.9
Tryptophan	11.3±0.9	13.0±0.8	14.2±0.9	15.2±0.9	11.5±0.9	10.3±0.6	10.5±0.9	10.4±0.5
Cystine	12.6±0.4	21.0±0.8	19.1±1.1	23.4±0.9	12.7±0.3	16.6±0.7	12.4±0.2	13.0±0.3
Arginine	24.2±1.3	20.9±1.4	24.2±0.7	24.9±0.2	8.6±0.4	16.8±1.4	16.0±1.5	11.3±0.9

[a] Mean±S.D. (n=4).

that this method provides a useful tool in biochemical and clinical research.

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