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Determination of amino acids in biological fluids by capillary gas chromatography with nitrogen-phosphorus selective detection[1]

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Abstract

A selective and sensitive method for the determination of protein and non-protein amino acids in biological fluids by capillary gas chromatography (GC) has been developed. The amino acids in the samples were directly converted into their *N*(*O*,*S*)-isobutoxycarbonyl methyl ester derivatives and measured by GC with nitrogen-phosphorus selective detection (NPD) using a DB-17ht capillary column. Using this method, the derivatives of the 21 protein amino acids and the 25 non-protein amino acids provided excellent NPD responses and were quantitatively and reproducibly resolved within 28 min. The lower detection limits of these amino acids, at a signal-to-noise ratio of 3, were ca. 6–150 pg injected. The calibration curves for each amino acid in the range of 0.02–2 μg were linear and sufficiently reproducible for quantitative analysis. This method was successfully applied to small urine and serum samples without prior clean-up; there was no evidence of interference from coexisting substances. Overall recoveries of amino acids added to urine and serum samples were 83–112%. The intra-assay and inter-assay R.S.D. of amino acids in these samples were 0.3–8.9% ($n = 3$) and 1.9–15.8% ($n = 3$), respectively. © 1997 Elsevier Science B.V.

Keywords: Amino acids; Biological fluids; Isobutoxycarbonyl methyl ester derivatives; Gas chromatography; Nitrogen-phosphorus selective detection

1. Introduction

Amino acids are fundamental units in living organisms and play an important role in biological systems. 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, it is know that several diseases occur because of a well-delineated abnormality in the metabolism of a specific amino acid [2]. Amino acid levels in biological fluids such as urine and blood are related to various factors such as diet, metabolism of each amino acid and protein metabolism. Therefore, accurate quantita-

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tive analyses of amino acids in these samples are very important in diagnosing and monitoring inherited disorders of amino acid metabolism or in evaluating the nutritional requirements of patients. Sometimes, it is enough to analyse only one or small group of amino acids but, more often, a full amino acid profile is required.

Several gas chromatographic (GC) methods for the simultaneous determination of protein and non-protein amino acids in biological fluids have been developed on the basis of the preparation of *N*(*O*,*S*)-heptafluorobutyryl isobutyl esters [3–6], *N*-pentafluoropropionyl isopropyl esters [7], *N*(*O*,*S*)-pentafluorobenzoyl isobutyl esters [8], *N*(*O*,*S*)-isobutoxycarbonyl (isoBOC) methyl esters [9] or *N*(*O*)-*tert*-butyldimethylsilyl derivatives [10,11]. However, some of these methods require not only anhydrous conditions, high temperatures and long reaction times for the derivatization but also time-consuming GC separation. Furthermore, most require laborious clean-up of the samples to remove the coexisting substances.

Although a previous method [9] developed in these laboratories is relatively time-consuming and requires two packed columns on GC separation, the derivatives can be easily and quantitatively prepared in aqueous media without the use of heat and are stable to moisture. However, it has not been applied to the simultaneous determination of protein and non-protein amino acids in biological fluids. Recently, a simple and rapid method has been reported for the determination of protein amino acids based on the preparation of *N*(*O*,*S*)-isoBOC methyl ester derivatives of amino acids by a sonication technique and subsequent GC analysis with hydrogen flame ionization detection (FID) [12]; it was demonstrated that the amino acid composition of proteins [12] and the protein amino acid content of serum [13] could be easily determined. In the present paper, a more selective and sensitive method is described for the simultaneous determination of a variety of protein and non-protein amino acids by GC with nitrogen-phosphorus selective detection (NPD). By this method, the content of free amino acids in urine and serum samples was also studied.

2. Experimental

2.1. Reagents

The 46 amino acids shown in Table 1 were examined in this study. The standard stock solution (each 1 mg ml^{-1}) was prepared in 0.05 M HCl and stored at 4°C. The working standard solution containing 46 amino acids (each 2 μg ml^{-1}) was made up freshly as required by mixing the stock solutions and then dilution was 0.05 M HCl. 4-Piperidinecarboxylic acid as an internal standard (I.S.) was purchased from Tokyo Kasei Kogyo and was dissolved in 0.05 M HCl at a concentration of 2 μg ml^{-1}. Dithioerythritol (DTE) was obtained from Nacalai Tesque and used as a 0.5 mM solution in distilled water. Isobutyl chloroformate (isoBCF) was obtained from Tokyo Kasei Kogyo and used without further purification. *N*-Methyl-*N*-nitroso-*p*-toluenesulphonamide and diethyleneglycol monomethyl ether for the generation of diazomethane [14] were obtained from Nacalai Tesuque. Peroxide-free diethyl ether was purchased from Dojindo Laboratories (Kumamoto, Japan). Distilled water was used after fresh purification with a Model Milli-Q Jr. water purifier (Millipore, Bedford, MA, USA). All other chemicals were analytical grade.

2.2. Preparation of samples

Early morning urine sample from a healthy volunteer was collected and processed immediately or stored at −20°C until used. A urine sample (25 μl) was directly used for the analysis. A venous blood sample from a healthy volunteer was collected in a vacutainer tube and centrifuged at 1600*g* for 10 min. The serum layer was carefully collected and processed immediately. A serum sample (50 μl) was directly used for the analysis without deproteinization.

2.3. Derivatization procedure

An aliquot of the sample containing 0.02–2 μg of each amino acid and 0.1 ml of 2 μg ml^{-1} 4-piperidinecarboxylic acid (I.S.) were pipetted into a 10 ml Pyrex glass tube with a PTFE-lined

Table 1
Linear regression data and detection limits for protein and non-protein amino acids

Peak No.	Amino acid[a]	Abbreviation	Regression line[b]		Correlation coefficient[c] r	Detection limit (pg)
			Slope a	Intercept b		
1	α-Aminoisobutyric acid	α-AIBA	0.9981	0.9016	0.9967	9
2	Alanine	Ala	1.0901	0.9456	0.9944	10
3	Glycine	Gly	1.1637	1.0627	0.9952	9
4	α-Aminobutyric acid	α-ABA	1.0942	0.8731	0.9986	10
5	Valine	Val	1.0465	0.7292	0.9988	11
6	β-Alanine	β-Ala	1.2122	0.8740	0.9987	11
7	β-Aminobutyric acid	β-ABA	1.0833	0.8355	0.9990	11
8	β-Aminoisobutyric acid	β-AIBA	1.1227	0.7655	0.9993	11
9	Norvaline	NVal	1.0645	0.7950	0.9991	11
10	Leucine	Leu	1.0282	0.6375	0.9997	12
11	*allo*-Isoleucine	Alle	1.0163	0.6492	0.9989	12
12	Isoleucine	Ile	1.0185	0.6627	0.9997	12
13	Norleucine	NLeu	1.0670	0.6796	0.9999	12
14	γ-Aminobutyric acid	GABA	1.1886	0.8782	0.9991	11
15	Threonine	Thr	1.2551	0.7440	0.9995	28
16	Serine	Ser	1.4961	0.7727	0.9953	50
17	Proline	Pro	1.0113	0.8217	0.9996	9
18	Pipecolic acid	PCA	1.0078	0.7320	0.9998	11
19	Homoserine	HSer	1.2982	0.6959	0.9992	32
20	δ-Aminolevulinic acid	δ-ALA	1.1313	0.5799	0.9992	25
21	Aspartic acid	Asp	0.9660	0.6543	0.9997	14
22	Thioproline	TPro	1.2683	0.5937	0.9984	30
23	ε-Aminocaproic acid	ε-ACA	1.0060	0.5151	0.9994	16
24	Glutamic acid	Glu	1.0266	0.6256	0.9998	16
25	Methionine	Met	1.3815	0.2873	0.9971	135
26	Hydroxyproline	Hyp	1.1833	0.8384	0.9987	16
27	α-Aminoadipic acid	α-AAA	1.0597	0.5232	0.9992	24
28	Phenylalanine	Phe	0.9609	0.5718	0.9996	15
29	α-Aminopimelic acid	α-APA	1.0683	0.4584	0.9983	28
30	Asparagine	Asn	1.3192	0.3684	0.9961	56
31	*p*-Aminobenzoic acid	*p*-ABzA	1.1047	0.6917	0.9980	20
32	2,3-Diaminopropionic acid	DAPA	1.0236	1.0170	0.9984	9
33	Cysteine	Cys	1.1029	0.5802	0.9974	24
34	Glutamine	Gln	1.3119	0.1786	0.9968	150
35	2,4-Diaminobutyric acid	DABA	1.0891	1.0254	0.9991	10
36	Homocysteine	HCys	0.9898	0.1160	0.9931	60
37	Methionine sulphone	Met-S	1.0781	0.7250	0.9933	25
38	Ornithine	Orn	1.0267	1.1324	0.9982	6
39	Lysine	Lsy	1.0214	1.1195	0.9961	6
40	Histidine	His	1.0178	1.0684	0.9963	7
41	Tyrosine	Tyr	1.4371	0.6400	0.9964	64
42	δ-Hydroxylysine	δ-HLys	1.1698	0.6803	0.9934	34
43	Tryptophan	Trp	1.2711	0.8663	0.9951	24
44	Cystathionine	CTH	1.4594	0.7296	0.9936	30
45	Cystine	Cyt	1.2572	0.7120	0.9964	40
46	Homocystine	HCyt	1.2690	0.4084	0.9901	70

[a]1, 4, 6–9. 13, 14, 19, 22, 30, 33, 34, 36, 38, 46: Nacalai Tesque (Kyoto, Japan); 2, 3, 5, 10, 12, 15–17, 21, 24–26, 28, 39–41, 43, 45: Ajinomoto (Tokyo, Japan); 11, 18, 20, 27, 32, 35, 37, 42, 44: Sigma (St. Louis, MO, USA); 23, 29, 31: Tokyo Kasei Kogyo (Tokyo, Japan).

[b] $y = a \log x + b$; y, peak height ratio against the I.S.: x, amount of each amino acid (μg). Range: 0.04–2 μg for

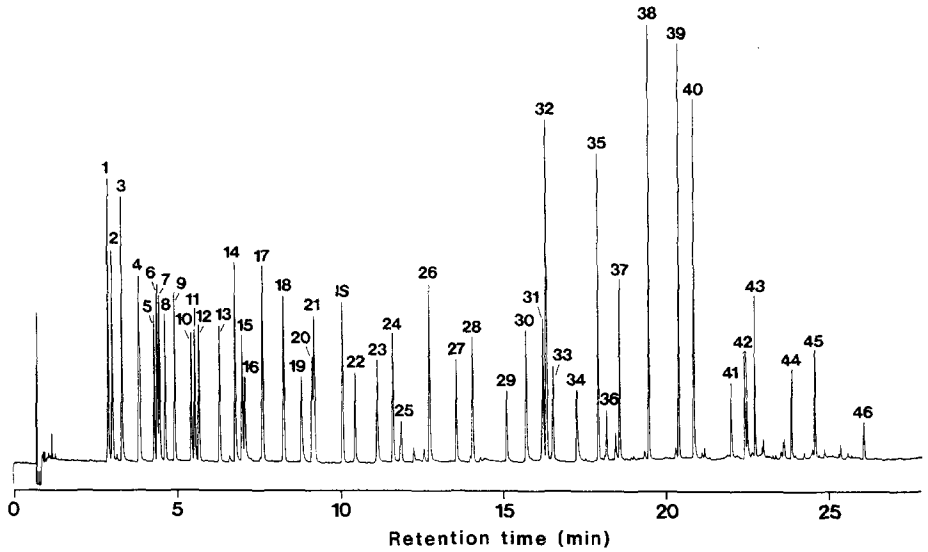

Fig. 1. Gas chromatogram obtained from the *N*(*O*,*S*)-isobutoxycarbonyl methyl ester derivatives of protein and non-protein amino acids (containing 0.4 μg of asparagine and glutamine and 0.2 μg of other amino acids) separated on a DB-17ht capillary column. GC conditions: see Experimental section. Peaks: 1, α-aminoisobutyric acid; 2, alanine; 3, glycine; 4, α-aminobutyric acid; 5, valine; 6, β-alanine; 7, β-aminobutyric acid; 8, β-aminoisobutyric acid; 9, norvaline; 10, leucine; 11, *allo*-isoleucine; 12, isoleucine; 13, norleucine; 14, γ-aminobutyric acid; 15, threonine; 16, serine; 17, proline; 18, pipecolic acid; 19, homoserine; 20, δ-aminolevulinic acid; 21, aspartic acid; 22, thioproline; 23, ε-aminocaproic acid; 24, glutamic acid; 25, methionine; 26, hydroxyproline; 27, α-aminoadipic acid; 28, phenylalanine; 29, α-aminopimelic acid; 30, asparagine; 31, *p*-aminobenzoic acid; 32, 2,3-diaminopropionic acid; 33, cysteine; 34, glutamine; 35, 2,4-diaminobutyric acid; 36, homocysteine; 37, methionine sulphone; 38, ornithine; 39, lysine; 40, histidine; 41, tyrosine; 42, σ-hydroxylysine; 43, tryptophan; 44, cystathionine; 45, cystine; 46, homocystine, I.S. = 4- piperidine-carboxylic acid.

screw-cap. To this solution were added 50 μl of 0.5 mM DTE and 0.25 ml of 10% Na_2CO_3, and the total volume was made up to 1 ml with distilled water. Then 50 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 HCl, saturated with NaCl 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 [14], through the solution until a yellow tinge became visible. This reaction had to be performed in a well ventilated hood because diazomethane is explosive and toxic. After standing for at least 1 min at room temperature, the solvents were removed by evaporation to dryness at 60°C. The residue was dissolved in 0.1 ml of ethyl acetate and then 1 μl of this solution was injected into the gas chromatograph by hot-needle injection technique (needle dwell time, 3 s).

2.4. Gas chromatography

GC analysis was carried out with a Hewlett-Packard 5890 Series II gas chromatograph equipped with an electronic pressure control (EPC) system, a split/splitless capillary inlet system, a nitrogen-phosphorus detector (NPD) and a hydrogen flame ionization detector (FID). A fused-silica capillary column of cross-linked DB-17ht (50% phenyl–50% methylpolysiloxane, J&W, Folsom, CA, USA: 20 m × 0.32 mm i.d., 0.15 μm film thickness) was used. The operating conditions

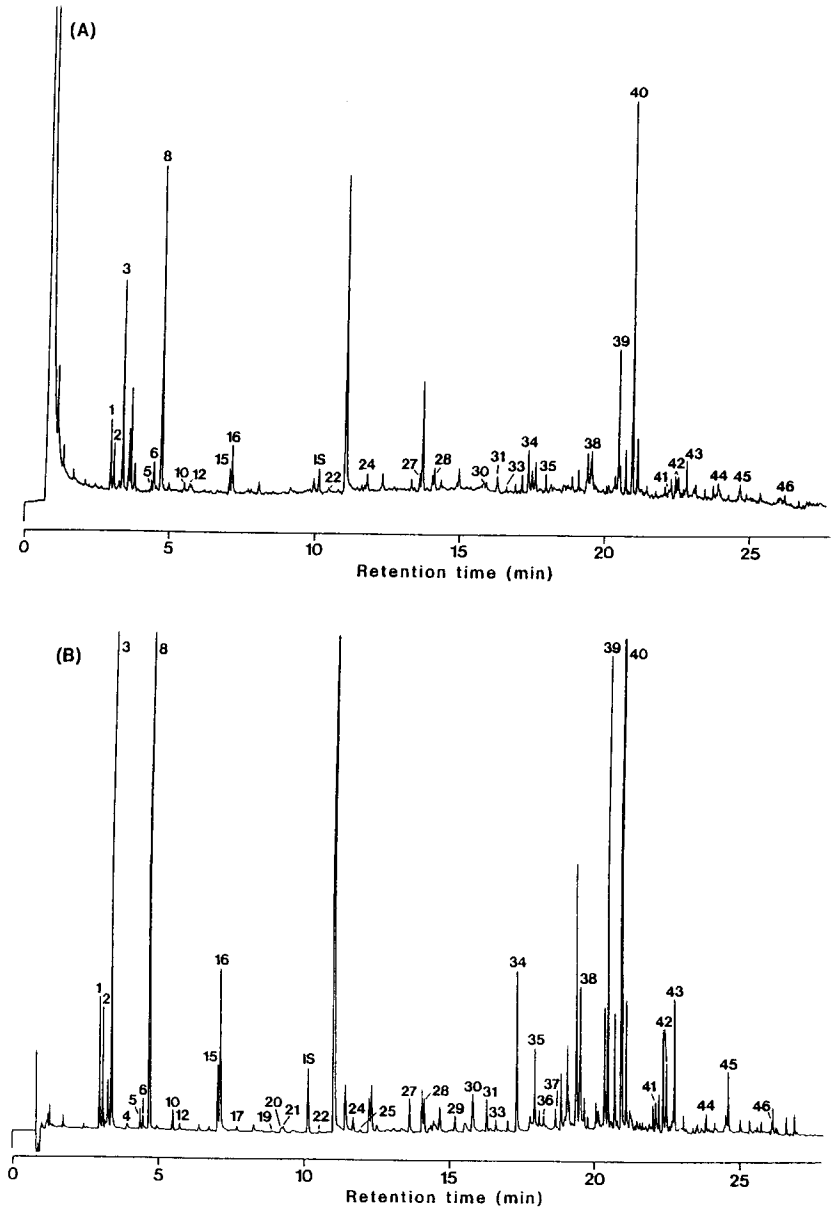

Fig. 2. Typical chromatograms obtained from 25 μl of urine sample by (A) FID-GC and (B) NPD-GC. GC conditions: see Experimental section. Peak No.: Fig. 1.

were: column temperature, programmed first at 4°C min^{-1} from 120 to 160°C, then at 6°C min^{-1} from 160 to 200°C and finally at 10°C min^{-1} from 200 to 310°C; inlet helium flow-rate was kept constant at 2 ml min^{-1} with EPC; split ratio, 10:1. Injection and detector temperatures and make-up gas flow-rate for the system were 320°C and 30 ml min^{-1}, respectively. The chromato-

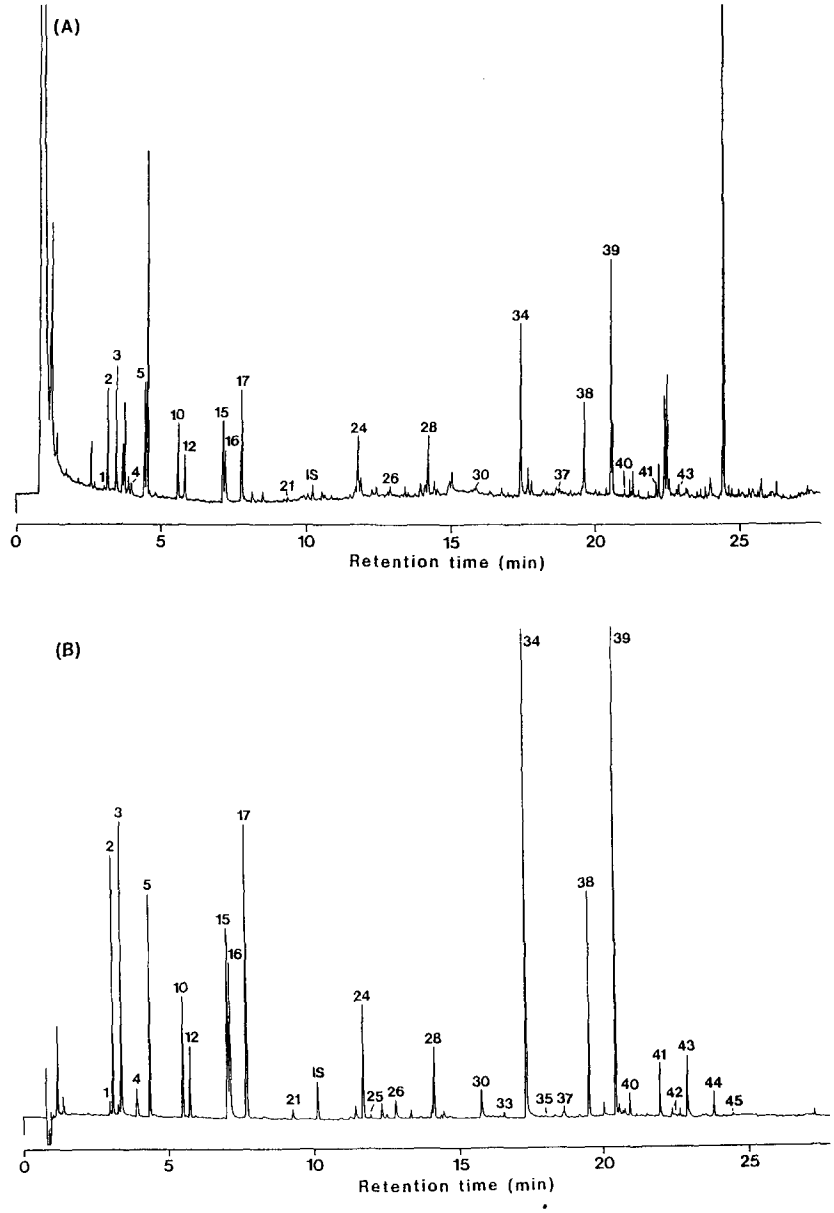

Fig. 3. Typical chromatograms obtained from 50 μl of serum sample by (A) FID-GC and (B) NPD-GC. GC conditions: see Experimental section. Peak No.: see Fig. 1.

graphic blank run (run made with no sample injected) data was subtracted from the sample run data to remove baseline drift (usually caused by-column bleed) using a single-column compensation function and then the baseline-corrected data were recorded on the chromatogram. 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 so that construct calibration curves could be constructed.

3. Results and discussion

Although NPD-GC is selective and sensitive for nitrogen-containing compounds, there have been few applications to the analysis of amino acids [3,15–18]. Therefore, a selective and sensitive method was investigated for the simultaneous determination of protein and non-protein amino acids by a combination of the convenient derivatization technique previously described [12] and the NPD-GC technique. The 21 protein amino acids except for arginine (the guanidino group on the side chain cannot be derivatized) and the 25 non-protein amino acids can be converted into their *N*(*O*,*S*)-isoBOC methyl ester derivatives by a previously reported method [12]. This method is based on the *N*(*O*,*S*)-isobutoxycarbonylation of amino acids with isoBCF in aqueous alkaline medium and subsequent methylation with diazomethane. In order to prevent the oxidation of sulphur amino acids during derivatization, DTE was added to the reaction mixture and peroxide-free diethyl ether was used as an extraction solvent as previously described [19]. The *N*(*O*,*S*)-isobutoxycarbonylation of amino acids was completed in 2.5% Na_2CO_3 solution with ≥ 10 μl of isoBCF within 15 s by sonication at room temperature. The sonication technique, which can easily mix the aqueous solution and the oily isoBCF reagent by its vibration effect, was effective for acceleration of the reaction. The resulting *N*(*O*,*S*)-isoBOC amino acids was quantitatively and selectively extracted into diethyl ether, and the subsequent methylation of the ethereal extracts was successfully carried out by bubbling diazomethane. The derivative preparation could be performed within 10 min. The derivatives were found to be very stable under normal laboratory conditions and no decomposition was observed during GC analysis.

The derivatives of protein amino acids could be completely resolved as single peaks within 9 min on a capillary column of cross-linked DB-17 (15 m × 0.25 mm i.d., 0.25 μm film thickness) as previously described [12] but the separation of the protein and non-protein amino acids examined in this study were incomplete on this column. Furthermore, high boiling-point derivatives were eluted slowly with a broader peak width and their sensitivities were reduced because of reduction in carrier gas flow-rate at higher temperature. In order to solve these problems, an attempt was made to introduce a EPC system and a thin-film-coated and high temperature (max. at 340°C) column DB-17ht (20 m × 0.32 mm id., 0.15 μm film thickness). Of several GC conditions tested for this column, the three-stage temperature programmes and the constant carrier gas flow-rate controlled with EPC were proved to give the most satisfactory separation of the 46 amino acids. For syringe manipulation, a hot needle injection technique was used to prevent the sample discrimination due to incomplete vaporization and flashback into other parts of the inlet. As shown in Fig. 1, the 21 protein amino acids and the 25 non-protein amino acids were well separated as single symmetrical peaks within 28 min on a DB-17ht capillary column under optimum GC conditions, except for δ-HLys which showed two peaks due to the *allo* form present in the standard. The derivatives of these amino acids provided excellent NPD responses and the minimum detectable amounts to give a signal-to-noise ratio of three under out GC conditions were 6–150 pg (injection amount) (Table 1). The NPD-GC system described here was over 10–50 times more sensitive than the FID-GC. In particular, the increase of sensitivity was remarkable for the nitrogen-rich amino acids such as DAPA, DABA, Orn, Lys and His. The calibration curves for amino acids were derived 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 was the I.S.. Various amounts of protein and non-protein amino acids (0.02–2 μg) were derivatized in the mixture and aliquots representing 0.2–20 ng were injected into the NPD-GC system. As mentioned previously [18], a significant curvature of the detector response was observed by NPD-GC, particularly at a low concentration of amino acid. Therefore, the calibration curves for each amino acid were constructed from both logarithmic plots of the peak height ratios and the amino acid amounts. A linear relationship was obtained with correlation coefficients being above 0.990, in the

Table 2
Recoveries of amino acids added to urine and serum

Amino acid	Urine				Serum			
	Added (μg ml^{-1})	Amount found (μg ml^{-1})[a]		Recovery (%)	Added (μg ml^{-1})	Amount found (μg ml^{-1})		Recovery (%)
		Non-addition	Addition			Non-addition	Addition	
α-AIBA	2	8.61 ± 0.16	10.59 ± 0.80	99.0	1	0.91 ± 0.08	1.89 ± 0.08	98.0
Ala	20	18.48 ± 1.41	38.12 ± 2.17	98.2	10	25.68 ± 0.45	36.07 ± 2.09	103.9
Gly	20	36.49 ± 0.43	57.59 ± 1.62	105.5	10	18.05 ± 0.35	28.23 ± 0.91	101.8
α-ABA	2	0.88 ± 0.07	2.96 ± 0.07	104.0	1	3.15 ± 0.13	4.10 ± 0.04	95.0
Val	2	3.69 ± 0.06	5.59 ± 0.22	95.0	10	29.33 ± 0.74	39.75 + 1.48	104.2
β-Ala	2	2.45 ± 0.09	4.41 ± 0.20	98.0	1	ND	1.04 ± 0.12	104.0
β-ABA	2	ND[b]	2.13 ± 0.02	106.5	1	ND	1.10 ± 0.09	110.0
β-AIBA	20	108.2 ± 2.3	127.8 ± 3.0	98.0	1	ND	0.99 ± 0.03	99.0
NVal	2	ND	2.15 ± 0.05	107.5	1	ND	1.01 ± 0.06	101.0
Leu	2	3.80 ± 0.07	5.80 ± 0.12	100.0	10	17.52 ± 0.52	26.60 ± 1.68	90.8
Alle	2	ND	2.03 ± 0.10	101.5	1	ND	1.12 ± 0.14	112.0
Ile	2	1.33 ± 0.02	3.41 ± 0.08	104.0	10	10.11 ± 0.20	19.87 ± 1.01	97.6
NLeu	2	ND	2.16 ± 0.12	108.0	1	ND	0.98 ± 0.05	98.0
GABA	2	ND	2.20 ± 0.07	110.0	1	ND	1.05 ± 0.05	105.0
Thr	20	11.13 ± 0.19	29.80 ± 0.72	93.4	10	19.85 ± 0.09	29.34 ± 0.74	94.9
Ser	20	19.77 ± 0.99	38.16 ± 1.96	92.0	10	14.03 ± 0.19	23.43 ± 0.76	94.0
Pro	2	0.79 ± 0.06	2.80 ± 0.07	100.5	10	27.65 ± 1.16	37.65 ± 0.71	100.0
PCA	2	ND	1.95 ± 0.06	97.5	1	ND	1.04 ± 0.04	104.0
HSer	2	0.88 ± 0.08	2.91 ± 0.08	101.5	1	ND	0.97 ± 0.11	97.0
δ-ALA	2	1.20 ± 0.07	3.35 + 0.23	107.5	1	ND	1.07 ± 0.03	107.0
Asp	2	0.80 ± 0.04	2.75 ± 0.16	97.5	1	1.10 ± 0.10	2.09 ± 0.08	99.0
TPro	2	1.12 ± 0.07	3.05 ± 0.26	96.5	1	ND	0.97 ± 0.12	97.0
ε-ACA	2	ND	1.88 ± 0.04	94.0	1	ND	1.07 ± 0.03	107.0
Glu	2	2.28 ± 0.08	4.35 ± 0.14	103.5	10	13.86 ± 0.52	23.51 ± 0.54	96.5
Met	2	1.93 ± 0.22	3.60 ± 0.14	83.5	1	1.00 ± 0.03	1.86 ± 0.04	86.0
Hyp	2	ND	1.83 ± 0.16	91.5	1	1.95 ± 0.07	2.95 ± 0.21	100.0
α-AAA	2	6.31 ± 0.02	8.25 ± 0.30	97.0	1	ND	1.04 ± 0.02	104.0
Phe	2	5.65 ± 0.06	7.75 ± 0.12	105.0	10	10.94 ± 0.39	21.06 ± 0.65	101.2
α-APA	2	3.60 ± 0.12	5.51 ± 0.30	95.5	1	ND	0.99 ± 0.02	99.0
Asn	4	16.40 ± 0.48	20.40 ± 0.22	100.0	2	7.52 ± 0.62	9.66 ± 0.38	107.0
p-ABzA	2	6.16 ± 0.08	8.28 ± 0.16	106.0	1	ND	1.09 ± 0.04	109.0
DAPA	2	ND	2.08 ± 0.18	104.0	1	ND	0.99 ± 0.08	99.0
Cys	2	8.41 ± 0.13	10.39 ± 0.46	99.0	1	0.94 ± 0.04	1.87 ± 0.24	93.0
Gln	40	59.54 ± 1.48	97.02 ± 6.58	93.7	20	100.2 ± 2.8	119.1 ± 3.2	94.5
DABA	2	6.57 ± 0.05	8.43 ± 0.53	93.0	1	0. 10 ± 0.01	1.07 ± 0.04	97.0
HCys	20	16.68 ± 0.08	34.87 ± 2.36	91.0	1	ND	0.97 ± 0.08	97.0
Met-S	2	1.68 ± 0.14	3.55 ± 0.26	93.5	1	0.51 ± 0.06	1.48 ± 0.22	97.0
Orn	2	6.60 ± 0.08	8.49 ± 0.13	94.5	10	8.97 ± 0.37	18.55 ± 1.23	95.8
Lsy	20	25.89 ± 0.54	45.17 ± 1.61	96.4	10	34.15 ± 1.37	44.64 ± 2.85	104.9
His	20	158.0 ± 7.0	178.2 ± 9.5	101.0	1	3.55 ± 0.29	4.49 ± 0.52	94.0
Tyr	20	10.33 ± 0.67	31.76 ± 0.76	107.2	1	8.49 ± 0.23	9.48 ± 0.63	99.0
δ-HLys	20	10.71 ± 0.05	28.73 ± 0.94	90.1	1	0.54 ± 0.05	1.46 ± 0.05	92.0
Trp	20	16.39 ± 0.22	36.01 ± 2.39	98.1	1	6.47 ± 0.30	7.38 ± 0.19	91.0
CTH	2	5.01 ± 0.06	7.04 ± 0.31	101.5	1	3.32 ± 0.10	4.23 ± 0.36	91.0
Cyt	20	10.33 ± 0.40	30.05 ± 1.24	98.6	1	0.51 ± 0.04	1.55 ± 0.10	104.0
HCyt	2	6.44 ± 0.04	8.27 ± 0.05	91.5	1	ND	1.08 ± 0.02	108.0

[a]Mean ± S.D. ($n = 3$).
[b]Not detectable.

ranges 0.04–2 μg for Asn and Gln and 0.02–1 μg for other amino acids (Table 1).

In order to demonstrate the applicability of the method to biological fluids, the contents of amino acids in human urine and serum samples were determined. The method developed was successfully applied to these samples without prior clean-up procedures such as deproteinization, ion-exchange column chromatography, solid phase extraction, and subsequent eluate evaporation. Figs. 2 and 3 show the typical chromatograms obtained from 25 μl of urine and 50 μl of serum by FID- and NPD-GC. In urine sample, hippuric acid was observed between TPro and Glu, but it did not overlap with ϵ-ACA. For FID-GC analysis, it was difficult to determine the amino acids in these samples because of the interfering peaks and low sensitivity. On the other hand, the amino acids in these samples could be analysed by NPD-GC without any such interference although some unknown peaks were observed on the chromatogram. To confirm validity of this method, known amounts of amino acids were added to human urine and serum and their recoveries were calculated. As shown in Table 2, the overall recoveries of these amino acids were 83.5–112.0% and the R.S.D. were 0.3–14.9% ($n = 3$). The quantitation limits of amino acids in urine and serum samples were ca. 0.1-0.4 μg ml^{-1}. The intra-assay and inter-assay R.S.D. for these samples throughout the overall procedure were 0.3–8.9% ($n = 3$) and 1.9–15.85% ($n = 3$), respectively.

In conclusion, the present method is simple, rapid and reproducible and the protein and non-protein amino acids can be simultaneously and quantitatively analysed within 28 min. Furthermore, it is selective and sensitive for amino acids; small urine and serum samples can be directly analysed without prior clean-up and any interference from other substances. It is believed that this method provides a useful tool in biochemical and clinical research.

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