The presence of free D-serine, D-alanine and D-proline in human plasma

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Abstract. Twelve neutral free amino acids, i.e. serine, threonine, glutamine, asparagine, alanine, proline, methionine, tyrosine, valine, leucine, isoleucine and phenylalanine, were surveyed for the presence of D-enantiomers in plasma samples from patients with renal diseases and from normal subjects. D-serine, D-alanine and D-proline were found in the patient's plasma. The highest concentrations (D/L ratio) of D-serine, D-alanine and D-proline were 0.2362, 0.2087 and 0.0986, respectively. The sum of the contents of the three D-amino acids in a plasma sample correlated with the serum creatinine level of the subject. No D-amino acid was shown to be present in the plasma proteins.
Key words. D-Alanine; D-proline; D-serine; glomerulonephritis; plasma.

It has long been believed that the naturally occurring amino acids have an L-configuration in mammals[1]. Only D-aspartate is known to accumulate in metabolically stable proteins in human tissues, e.g., teeth[2,3], eye lens[4], and myelin[5], as a result of spontaneous chemical racemisation. As for free amino acids, no D-amino acid but D-aspartate[6] has been reported to be present in vertebrate. However, considerable amounts of free neutral D-amino acids were detected in plasma from patients with glomerulonephritis[7], and in samples of tissues free from the action of D-amino acid oxidase, i.e., the serum, kidney, liver, brain, heart and lung of a mutant mouse lacking D-amino acid oxidase[8], using a method[9] for microdetermination of D-amino acids.

In the present study, the plasma samples were analyzed by sensitive reversed-phase high-performance liquid chromatography (HPLC)[10] to identify the D-amino acids. As a result, the presence of free D-serine, D-alanine and D-proline was clearly demonstrated. The plasma proteins were also investigated for the presence of D-amino acids in an attempt to seek the source of the three D-amino acids.

Materials and methods

Plasma samples were collected from 11 patients with glomerulonephritis (5 females and 6 males, aged 22 to 79) and 7 normal subjects (6 females and 1 male, aged 20 to 76) after about 15-h starvation. The patients were in a hospital, and were not under medication with any antibiotics that might contain D-amino acids. Serum creatinine was determined with a kit (Shionogi, Osaka, Japan) by the Jaffé reaction.

Preparation of amino acids. To 0.1 ml of plasma, 0.5 ml of cold trichloroacetic acid (TCA) solution was added to make a final concentration of 5% (w/v). After centrifugation at 3000 × g for 5 min, the supernatant fraction was applied to a Dowex 1 × 8 (acetate form; Muromachi Chemicals, Tokyo, Japan) column (4.0 × 0.5 cm ID). The effluent was used as the free amino acid fraction. The TCA-precipitation fraction of the plasma, and albumin (from human sera, Sigma, St. Louis, MO, USA) were hydrolyzed in 6 M HCl at 110 ± 0.2 °C for 6 h.

Resolution of D- and L-enantiomers. The precision of the method for resolution of D- and L-enantiomers is described elsewhere[10]. Briefly; the effluents and hydrolysates were mixed with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA, Pierce, Rockford, IL, USA) according to the method of Marfey[11] to produce FDAA-derivatized amino acids, i.e., diastereomers. The FDAA amino acids were analyzed by HPLC for the resolution of D- and L-enantiomers. HPLC was performed using a reversed-phase column, Nova-Pak C18 (150 × 3.9 mm ID, Waters, Milford, MA, USA), and a Tosoh (Tokyo, Japan) gradient HPLC system. Each FDAA amino acid, isolated beforehand by two-dimensional thin-layer chromatography, was applied to the column and eluted with a linear gradient from 10 to 40% acetonitrile in 50 mM triethylamine-phosphate buffer (pH 3.5) in 45 min at a flow rate of 1.0 ml/min at 22 °C. The eluate was monitored at 340 nm on a D-2500 Chromato-Integrator (Hitachi, Tokyo, Japan). Amounts of D- and L-enantiomers were obtained using the peak areas given by the Chromato-Integrator and the standard curves[10].

Chemicals used were chromatography or analytical grade.

Results and discussion

Free serine, threonine, glutamine, asparagine, alanine, proline, methionine, tyrosine, valine, isoleucine, leucine and phenylalanine in the plasma were analyzed for the presence of D-enantiomers. The HPLC pattern of a patient's plasma sample is depicted in figure 1. The HPLC method clearly resolved D- and L-enantiomers of FDAA amino acids, demonstrating that serine, alanine and proline contained the D-enantiomers, but other amino acids contained no D-enantiomers.

The present method was able to quantify 20–50 pmol of a D-amino acid in plasma, in the presence of a large excess of the corresponding L-isomer[10]. Experiments using authentic D- or L-amino acids showed that no chem-

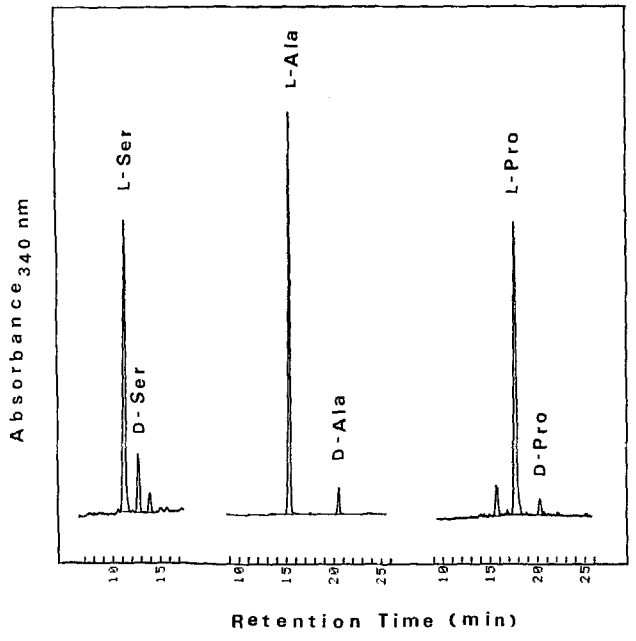

Figure 1. Reversed-phase HPLC of FDAA-derivatized serine, alanine and proline from patient plasma. Chromatograms of a typical sample are shown.

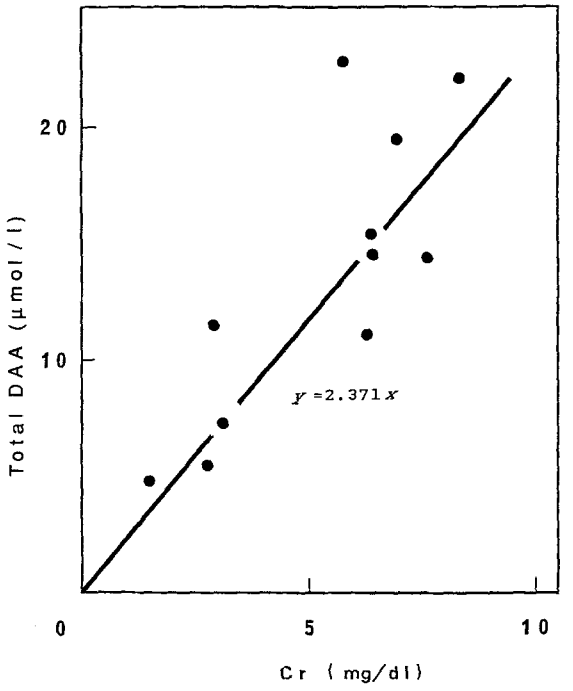

Figure 2. Total D-amino acids (DAA, μmol/l) vs serum creatinine level of samples from patients with glomerulonephritis. The samples were the same as in table 1.

ical racemisation occurred during derivatization with FDAA or the other procedures used.

Table 1 represents the D- and L-enantiomer contents of free serine, alanine and proline in plasma samples, and the D/L ratios, as well as serum creatinine level, age and sex of the subjects. The highest contents of D-enantiomers of serine, alanine and proline were observed in the samples from subjects Nrs 5, 1 and 3, respectively. The sum of the contents of the three D-amino acids is given as 'total DAA' in the table. As shown in figure 2, the total DAA indicates a strong positive correlation ($n = 11$, $r = 0.8327$, $p < 0.005$) with serum creatinine level, suggesting that the total DAA may provide a pathological marker for the degree of kidney dysfunction.

As a result of HPLC analyses of the protein fractions, no D-isomers of threonine, methionine, tyrosine, valine, leucine, isoleucine and phenylalanine were detected. Table 2 shows the D/L ratios of serine, alanine, proline, glutamate and aspartate in hydrolysates of the plasma proteins. None of the D/L ratios of the five amino acids is higher than the value for albumin. The positive values of D/L ratios especially for aspartate and glutamate residues in albumin are ascribable to chemical racemisa-

Table 1. Contents (μmol/l plasma) of free neutral amino acids and the D/L ratios in human plasma. *, Normal subject; N.D, not determined; total DAA, the sum content of D-serine, D-alanine and D-proline in the plasma.

				Ser			Ala			Pro			Total
Subject	Cr (mg/dl)	Age	Sex	L	D	D/L	L	D	D/L	L	D	D/L	DAA
1	8.3	61	m	46.4	6.25	0.1347	69.0	14.40	0.2087	53.0	1.50	0.0283	22.15
2	7.6	59	f	48.8	5.55	0.1137	68.5	4.50	0.0657	70.5	4.35	0.0617	14.40
3	6.9	73	m	31.8	3.85	0.1211	82.5	8.65	0.1048	71.0	7.00	0.0986	19.50
4	6.4	67	m	56.0	5.35	0.0956	165.0	5.80	0.0352	161.0	3.40	0.0211	14.55
5	6.4	22	m	59.5	13.40	0.2252	118.5	1.60	0.0135	77.5	0.40	0.0052	15.40
6	6.3	61	m	34.6	3.25	0.0939	108.5	3.30	0.0304	75.5	4.55	0.0603	11.10
7	5.8	57	f	27.8	6.56	0.2360	198.0	11.67	0.0589	60.6	4.59	0.0757	22.82
8	3.1	79	f	60.5	4.15	0.0686	149.5	1.45	0.0097	77.0	1.70	0.0221	7.30
9	2.9	51	f	57.0	4.45	0.0781	125.5	4.80	0.0382	66.0	2.25	0.0341	11.50
10	2.8	43	f	34.6	2.70	0.0780	121.0	1.30	0.0107	121.0	1.50	0.0124	5.50
11	1.5	75	m	58.0	1.85	0.0319	181.0	2.55	0.0141	47.3	0.40	0.0085	4.80
12*	ND	76	m	87.6	0.72	0.0082	94.2	1.11	0.0118	150.0	0.69	0.0046	2.52
13*	ND	75	f	92.4	1.76	0.0190	124.0	0.25	0.0020	41.2	0.26	0.0063	2.26
14*	ND	45	f	76.0	0.42	0.0055	208.0	0.28	0.0013	59.0	0	0	0.69
15*	ND	40	f	105.0	0.54	0.0051	211.0	0.57	0.0027	62.7	0	0	1.11
16*	ND	40	f	95.4	1.19	0.0125	207.0	0.28	0.0014	68.5	0	0	1.47
17*	ND	20	f	89.8	0.86	0.0096	195.0	0	0	104.0	0.62	0.0060	1.48
18*	ND	20	f	94.0	1.33	0.0141	168.0	1.77	0.0105	44.3	0	0	3.10

Table 2. D/L Ratios of amino acids in hydrolysates of human plasma. The TCA-precipitation fraction was hydrolyzed in 6 M HCl, at 110 ± 0.2 °C for 6 h. The plasma were obtained from patients with glomerulonephritis as well as from normal subjects. * The subject number is the same as in table 1.

Subject*	Ser	Ala	Pro	Glu	Asp
1	0.0032	0.0034	0.0126	0.0128	0.0213
4	0	0.0065	0.0090	0.0113	0.0144
7	0.0051	0.0057	0.0100	0.0122	0.0175
8	0	0.0047	0.0097	0.0130	0.0207
13	0	0.0006	0.0058	0.0130	0.0219
14	0.0015	0.0036	0.0128	0.0140	0.0267
15	0.0028	0.0084	0.0127	0.0141	0.0219
16	0.0041	0.0066	0.0135	0.0133	0.0239
17	0.0047	0.0110	0.0141	0.0115	0.0219
18	0.0029	0.0128	0.0114	0.0141	0.0230
Albumin	0.0023 ± 0.0019	0.0037 ± 0.0003	0.0085 ± 0.0014	0.0101 ± 0.0013	0.0198 ± 0.0018

tion caused by acid hydrolysis. The reason why human serum albumin was employed as the control protein for racemisation rates was that albumin is the most dominant serum protein, and it contains no D-amino acids (Nagata et al., unpublished results). Hence, the result in table 2 indicates that the plasma proteins contain no D-amino acid residues. The D-amino acids observed in the present study, therefore, seem unlikely to have been released from the plasma proteins. Experiments with mice indicate that the D-amino acids probably did not originate from the tissue proteins of kidney and liver either (Nagata et al., unpublished results). Microorganisms in the gut, and food, appear to be the possible sources. However, this is a matter which requires further study. The physiological role of free D-amino acids found in mammals is not known, whereas the importance of D-amino acids in functional peptides such as frog dermorphin [12] and snail achatin [13] has been reported. Administration of D-serine to rats induced acute necrosis of the proximal straight tubules that was accompanied by proteinuria, glucosuria, and aminoaciduria [14]. Therefore, it is conceivable that the large amounts of free D-serine observed in some patients could cause deterioration.

The present results suggest the metabolic role of human D-amino acid oxidase. The enzyme is known to catalyse oxidative deamination of free neutral D-amino acids in vitro, and to be located in large quantities in the renal proximal tubular cells [15]. Involvement of the enzyme in elimination of free neutral D-amino acids has been demonstrated in mutant mice lacking the enzyme [8]. D-amino acid oxidase in the human kidney may catabolize free D-serine, D-alanine and D-proline in the blood stream. This view gains support from the facts that a strong positive correlation was observed between the total DAA and the serum creatinine level, and that a high creatinine level indicates a low filtration rate of blood in the nephrons.

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