Amino Acids (1994) 6: 205–211

Amino
Acids

Quantification of D-amino acids in human urine using GC–MS and HPLC

Short Communication

H. Brückner[1], **S. Haasmann**[1], and **A. Friedrich**[2]

[1] Institute of Food Technology, University of Hohenheim, Stuttgart, Federal Republic of Germany
[2] State Veterinary Laboratory Stuttgart, Stuttgart, Federal Republic of Germany

Accepted September 6, 1993

Summary. The relative amounts of free D-amino acids (D-AA) in the urine of seven healthy volunteers (age 27 to 49 years) were determined using chiral phase (Chirasil-L-Val) capillary gas chromatography in conjunction with selected ion monitoring mass spectrometry. The absolute amounts of free D-AA were determined by pre-column derivatization of the amino acids with *o*-phthaldialdehyde and *N*-isobutyryl-L-cysteine followed by high-performance liquid chromatographic separation and fluorescence detection of the isoindol derivatives formed. The following most abundant D-AA were found (highest and lowest absolute and relative amounts): D-Ser (379.8 – 30.1 μMol/L; 56.5 – 19.0%), D-Ala (53.8 – 7.6 μMol/L; 19.6 – 5.7%), D-Thr (5.8 – 0.25 μMol/L; 3.4 – 1.0%), D-Val (3.7 – 0 μMol/L; 4.2 – 0%), and D-Phe (3.5 – 0.35 μMol/L; 4.8 – 1.4%).

Keywords: Amino acids – Amino acid enantiomers – Chiral analysis – Chirasil-L-Val – Gas chromatography – Mass spectrometry – Selected ion monitoring – D-Serine – D-Alanine

Introduction

It has been recently reported that trace amounts of hydrophobic D-amino acids (D-Trp, D-Phe, D-Tyr and possibly D-Leu) are detectable in the urine of healthy volunteers (Armstrong et al., 1991). We have found that relatively high amounts of hydrophilic D-amino acids (D-AA) such as D-Ala and D-Ser are present in food (Brückner and Hausch, 1989), bacteria (Brückner et al., 1993) and human blood plasma (Brückner and Hausch, 1993). Following the assumption that these D-AA are renally excreted we investigated a number of urine samples of volunteers using chiral phase gas chromatography – mass spectrometry and

high-performance liquid chromatography and found D-Ser and D-Ala to be the most abundant in urine.

Materials and methods

Urine

Random urine samples were provided from healthy male volunteers of the laboratory staff (age 27 to 49 years). Volunteers were not under treatment with peptide or amino acid drugs or subject to a special diet. Urine samples were immediately frozen at −18°C and thawn prior to investigation.

Isolation and derivatization of amino acids

For investigation by gas chromatography – mass spectrometry (GC–MS) 10 ml aliquots of urine samples were adjusted to pH 2 by addition of 0.01 M HCl and passed through a Dowex 50 WX 8 cation-exchanger (200–400 mesh, analytical grade, from Serva, Heidelberg, Germany; bed volume 5 × 1 cm). The ion-exchanger was washed with bidistilled water (60 ml), AA were eluted with 4 N aqu. ammonia (30 ml), the eluent was evaporated to dryness *in vacuo*, the residue was dissolved in 1 ml 0.1 M HCl and the solution was transferred into a 1 ml "reacti vial" (Wheaton, Milleville, NJ, USA). AA were converted into *N(O)*-trifluoroacetyl 2-propyl esters by subsequent treatment with trifluoroacetic anhydride and HCl in 2-propanol as described previously (Brückner et al., 1993). Blank analyses were carried out in order to detect a possible contamination of reagents.

For quantification of AA by HPLC, to 450 µl aliquots of the urine samples, 50 µl of sulfosalicylic acid (30%, w/v)) were added, proteines which had been precipitated were removed by centrifugation at 4000 × *g* and 2 µl aliquots of the supernatant were investigated.

Instruments

For gas chromatography – mass spectrometry (GC–MS) a Hewlett-Packard MS-Engine (HP 59827A) with HP 5890 Series II gas chromatograph and the MS ChemStation (HP-UX series) with the X Window System MS software were used. An electron acceleration energy of 70 eV was used. The column was a Chirasil-L-Val capillary column (25 m × 0.25 mm I.D.; Chrompack, Middelburg, The Netherlands); carrier gas was helium at an inlet pressure of 80 kPa (flow rate 0.7 $mlmin^{-1}$). The temperature of the injector was 230°C; samples were injected in the split mode, splitting ratio was approx. 1:30. In the selected ion monitoring (SIM) mode 20 ions were simultaneously monitored each at a dwell time of 50 msec. The individual amino acid enantiomers were evaluated by extracting single ion traces. For HPLC a model 1090 instrument equipped with a fluorescence detector (Hewlett Packard, Waldbronn, Germany) was used. For the quantification of the AA enantiomers pre-column derivatization of AA with *o*-phthaldialdehyde (OPA) – *N*-isobutyryl-L-cysteine (IBLC) was carried out as described previously in detail (Brückner et al., 1992). The diastereomeric isoindol derivatives formed were separated on a 250 mm × 4 mm ID column packed with Shandon Hypersil 5 µm (Shandon Scientific Ltd., Runcorn, Cheshire, U.K.). The relative fluorescence of the derivatives was measured at 230 nm (excitation) and 445 nm (emission). For the quantification of urine amino acids an external amino acid standard was used.

Results

The absolute and relative amounts of D-amino acids detected in the urine of all volunteers are displayed in Table 1. The absolute amounts were determined

Table 1. Absolute (μMol/L) and relative [%D = 100 * D/(D + L)] amounts of the most abundant D-amino acids (D-AA) in human urine

D-AA	1 30y μMol/L (mean ± S.D.)	%D	2 34y μMol/L (mean ± S.D.)	%D	3 49y μMol/L (mean ± S.D.)	%D	4 27y μMol/L (mean ± S.D.)	%D
Ser	379.8 ± 5.1	56.5	241.4 ± 6.6	25.0	221.2 ± 22.3	51.5	218.4 ± 1.1	43.0
Ala	36.6 ± 0.4	5.8	44.6 ± 1.1	6.2	53.8 ± 5.7	14.6	22.3 ± 1.9	5.7
Thr	3.8 ± 0.1	1.4	3.8 ± 0.1	1.1	2.5 ± 0.8	2.3	2.8 ± 0.1	1.5
Val	2.7 ± 0.1	1.3	2.5 ± 0.1	1.7	1.0 ± 0.1	1.4	1.1 ± 0.1	1.6
Phe	2.1 ± 0.0	1.5	3.2*	2.4	1.6 ± 0.3	1.8	1.9*	1.4

D-AA	5 35y μMol/L (mean ± S.D.)	%D	6 34y μMol/L (mean ± S.D.)	%D	7 27y μMol/L (mean ± S.D.)	%D
Ser	178.7 ± 2.0	37.5	50.7 ± 4.8	19.0	30.1 ± 1.1	40.5
Ala	27.4 ± 0.8	7.2	31.2 ± 2.4	19.6	7.6 ± 0.1	16.1
Thr	5.8 ± 0.1	2.8	3.0 ± 0.3	3.4	0.25 ± 0.0	1.0
Val	3.7 ± 0.2	4.2	n.d.	—	0.25 ± 0.0	2.9
Phe	3.5 ± 0.1	4.8	0.7 ± 0.1	2.5	0.35 ± 0.0	4.0

S.D. standard deviation (n = 3, n* = 2), *y* age of volunteer in years; *n.d.* not detected

by HPLC and fluorescence detection of the isoindol derivatives formed by reaction of AA with OPA – *N*-isobutyryl-L-cystein, the relative amounts were calculated by comparing the ratios of the most abundant fragment ions using GC–MS. The following results are obtained: D-Ser (379.8 – 30.1 μMol/L), D-Ala (53.8 – 7.6 μMol/L), D-Thr (5.8 – 0.25 μMol/L), D-Val (3.7 – 0 μMol/L), and D-Phe (3.5 – 0.35 μMol/L). The relative amounts of D-AA were D-Ser (56.5 –

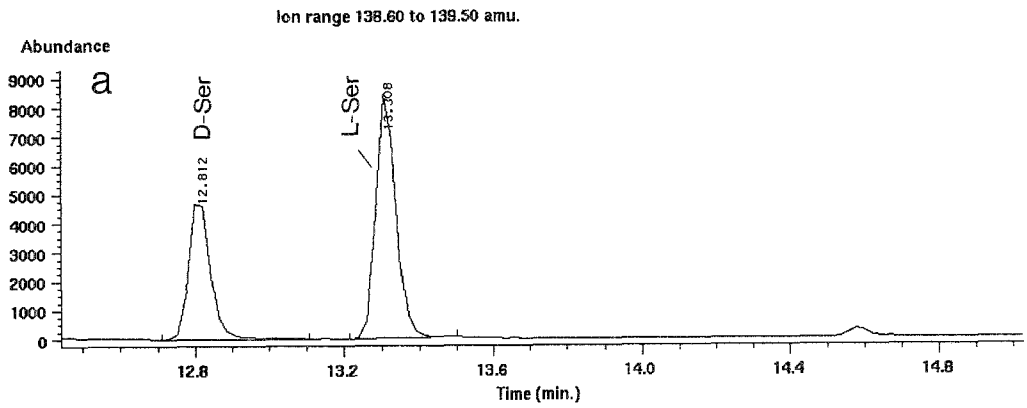

Fig. 1. Selected ion monitoring (SIM) chromatograms and electron impact mass spectra (MS) of the most abundant D-amino acids determined in the urine of volunteer no. 5; **a** SIM at m/z 139 of D- and L-Ser, **b** MS of D-Ser, **c** MS of L-Ser; **d** SIM at m/z 140 of D- and L-Ala, **e** MS of D-Ala, **f** SIM at m/z 153 of D- and L-Thr, **g** MS of D-Thr, **h** SIM at m/z 168 of D- and L-Val, **i** MS of D-Val, **j** SIM at m/z 190 of D- and L-Phe, **k** MS of D-Phe

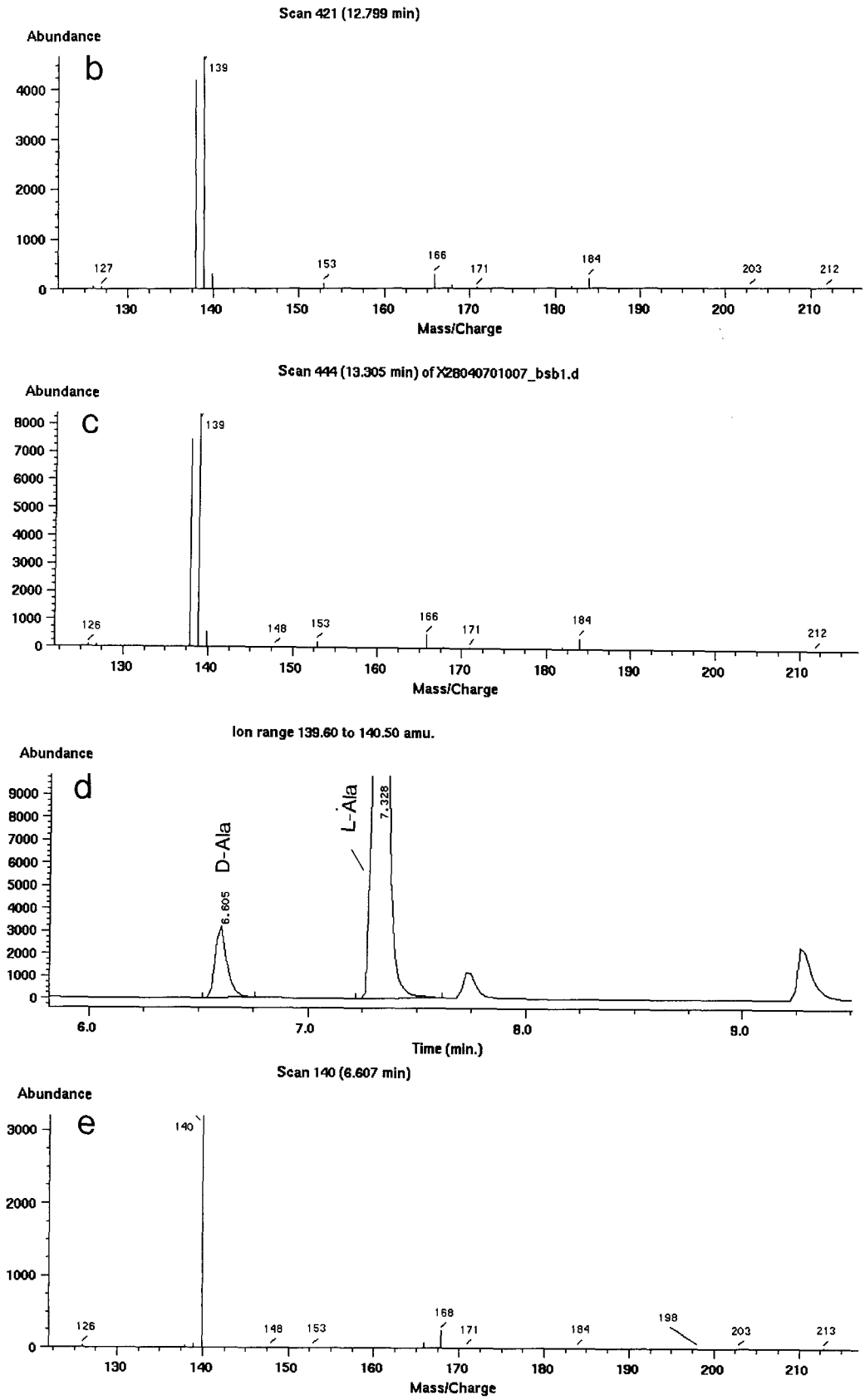

Fig. 1 (continued)

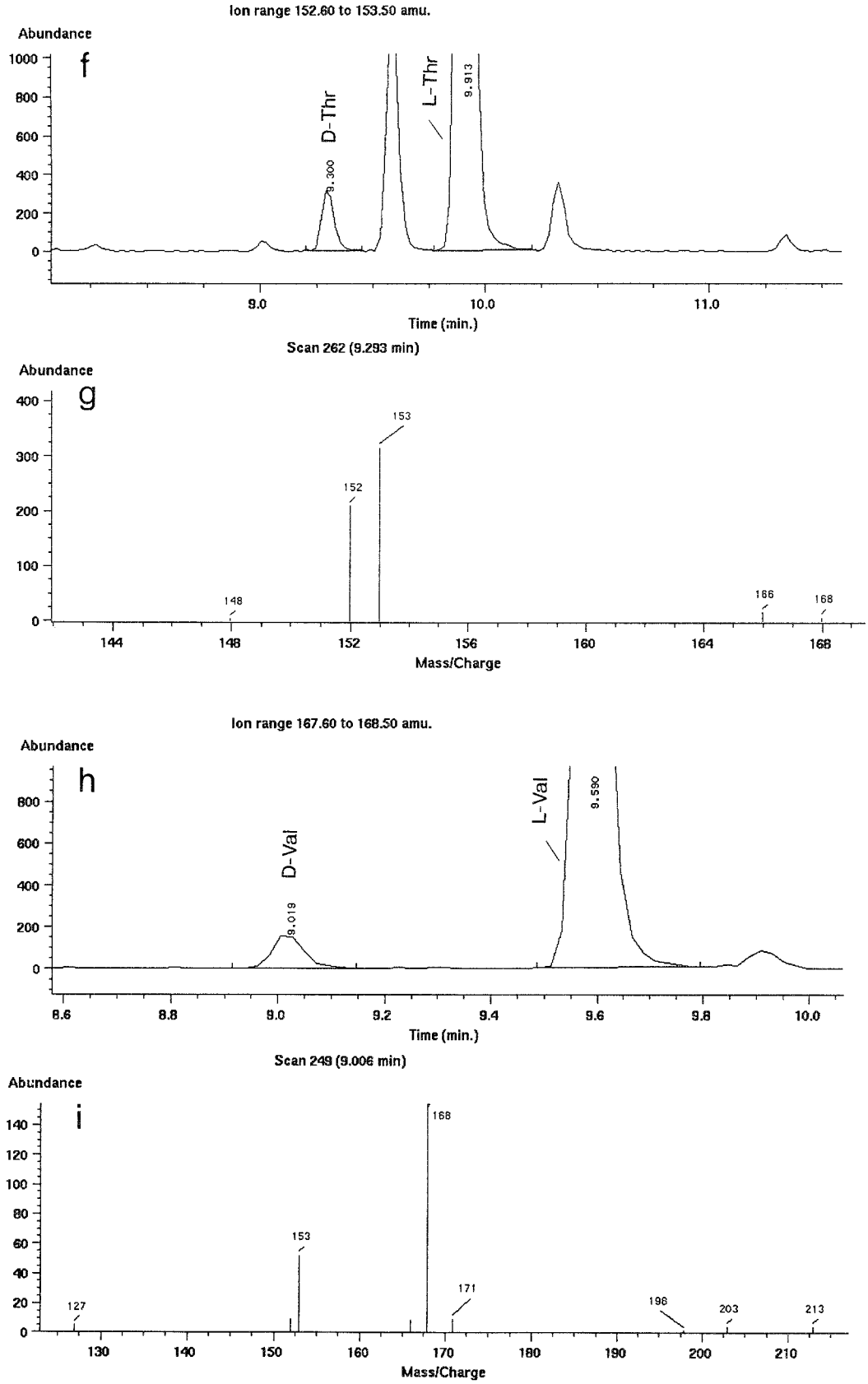

Fig. 1 (continued)

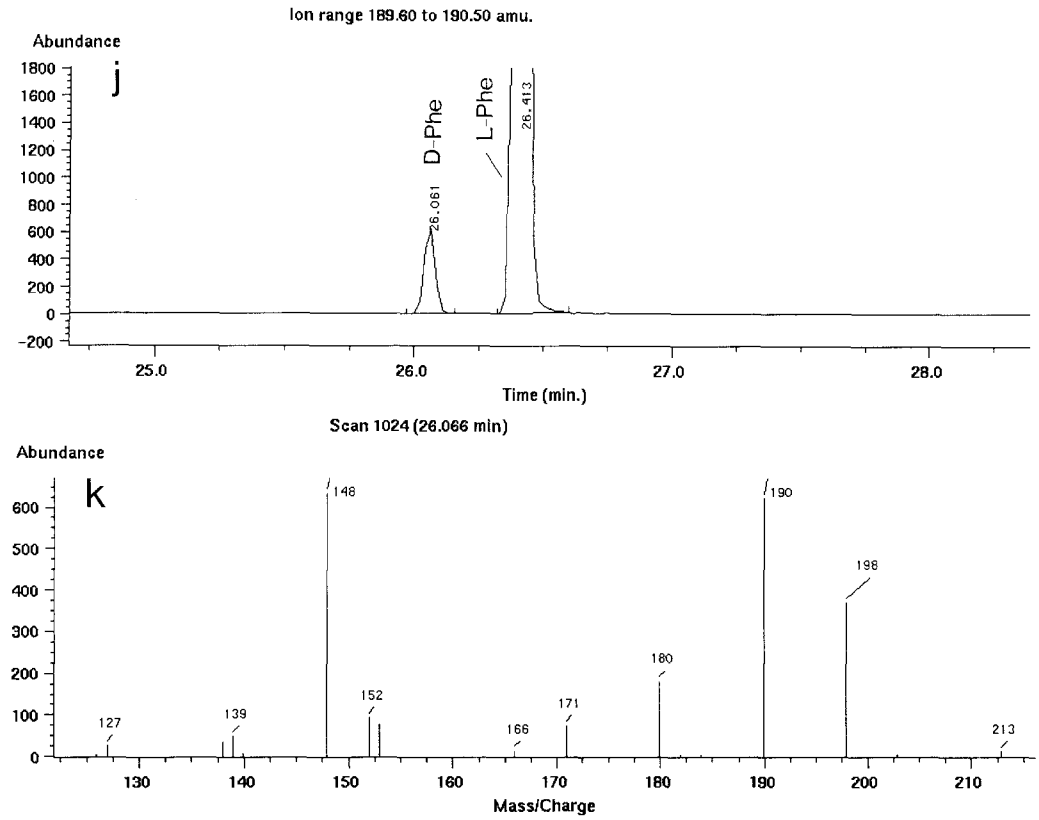

Fig. 1 (continued)

19.0%), D-Ala (19.6 – 5.7%), D-Thr (3.4 – 1.0%), D-Val (4.2 – 0%) and D-Phe (4.8 – 1.4%).

SIM chromatograms of the above derivatized AA found in the urine of volunteer no. 5 are shown in Fig. 1a–k. For selected ion monitoring the most intensive, specific fragment ions of the *N(O)*-trifluoroacetyl amino acid 2-propyl esters were used: Ser (m/z = 139), Ala (m/z = 140), Thr (m/z = 153), Val (m/z = 168), Phe (m/z = 190).

Discussion

It is shown that GC on a chiral stationary phase together with SIM MS is a highly suitable and specific method for the detection of D-AA in human urine (see Fig. 1). D-Ser, D-Ala, D-Thr, D-Phe and, with the exception of volunteer no. 6, D-Val were found as the most abundant D-AA in the urine of seven volunteers. In particular the discovery of very high absolute and relative amounts of renally excreted D-Ser is an unexpected result. From the data in Table 1 an excretion of 40 – 19 mg D-Ser per liter urine of volunteers no. 1–5 has been calculated.

Trace amounts of hydrophobic D-AA (D-Phe, D-Tyr, D-Trp, and possibly D-Leu) have been determined in human urine using combined reversed-phase and chiral HPLC (Armstrong et al., 1991). The undetection of the high amounts

of hydrophilic AA, D-Ser and D-Ala, can be attributed to the methodology employed.

Although the origin of the relative high amounts of D-Ser in urine remains speculative it is worth noting that significant amounts of free D-Ser have been found in certain foods and in lactic bacteria (Brückner et al., 1993).

It would be of interest to explore the physiological relevance of the type and amounts of D-AA being renally excreted with respect to age, race, sex, nutrition and health status. The data obtained might be useful for a deeper insight into the biochemistry of D-AA occurring in the free or peptide-bonded form in the human body (Dunlop et al., 1986; Fisher et al., 1991; Payan et al., 1992; Nagata et al., 1992; Brückner and Hausch, 1993).

References

Armstrong DW, Duncan JD, Lee SH (1991) Evaluation of D-amino acid levels in human urine and in commercial L-amino acid samples. Amino Acids 1: 97–106

Brückner H, Hausch M (1989) Detection of free D-amino acids in food by chiral phase capillary gas chromatography. J High Resol Chromatogr 12: 680–684

Brückner H, Hausch M (1993) Gas chromatographic characterization of free D-amino acids in the blood serum of patients with renal disorders and of healthy volunteers. J Chromatogr 614: 7–17

Brückner H, Jaek P, Langer M, Godel H (1992) Liquid chromatographic determination of D-amino acids in cheese and cow milk. Implication of starter cultures, amino acid racemases, and rumen microorganisms on formation, and nutritional considerations. Amino Acids 2: 271–284

Brückner H, Becker D, Lüpke M (1993) Chirality of amino acids of microorganisms used in food biotechnology. Chirality 5: 385–392

Dunlop DS, Neidle A, McHale D, Dunlop DM, Lajtha A (1986) The presence of free D-aspartic acid in rodents and man. Biochem Biophys Res Comm 141: 27–32

Fisher GH, D'Aniello A, Vetere A, Padula L, Cusano GP, Man EH (1991) Free D-aspartate and D-alanine in normal and Alzheimer brain. Brain Res Bull 26: 983–985

Nagata Y, Masui R, Akino T (1992) The presence of free D-serine, D-alanine and D-proline in human plasma. Experientia 48: 986–988

Payan IL, Chou S-J, Fisher GH, Man EH, Emory C, Frey WH (1992) Altered aspartate in Alzheimer neurofibrillary tangle. Neurochem Res 17: 187–191

Authors' address: Prof. Dr. H. Brückner, Institute of Food Technology, University of Hohenheim, Garbenstrasse 25, D-70599 Stuttgart, Federal Republic of Germany.

Received August 16, 1993

Verleger: Springer-Verlag KG, Sachsenplatz 4–6, A-1201 Wien. – Herausgeber: Prof. Dr. Gert Lubec, National Heart & Lung Institute, Biochemistry, Manresa Road, London SW3 6LR, U.K. – Redaktion: Sachsenplatz 4–6, A-1201 Wien. – Satz und Umbruch: Asco Trade Typesetting Limited, Hong Kong; Offsetdruck: Novographic, Ing. W. Schmid, Maurer-Lange-Gasse 64, A-1238 Wien. – Verlagsort: Wien – Herstellungsort: Wien. Printed in Austria.