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Evaluation of D-amino acid levels in rat by gas chromatography–selected ion monitoring mass spectrometry: no evidence for subacute toxicity of orally fed D-proline and D-aspartic acid

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Abstract

Sprague–Dawley rats received deionized water (controls) during 28 days or drinking water with added D-proline, L-proline, D-aspartic acid or L-aspartic acid corresponding to a mean daily load of approximately 50 mg amino acid enantiomer kg^{-1} body weight. Parameters indicating the physiological status (food intake and body weight, glutamic-oxalic-transaminase, glutamic-pyruvic-transaminase, alkaline phosphatase, urea and creatinine in serum, and creatine and osmomolality of urine) were determined. After 28 days the weights of the supposed target organs of toxicity (kidney, liver, brain, thymus) were determined and organs were inspected for macroscopic and microscopic alterations. No pathological changes in the organs were observed and no signs of subacute toxicity (liver, kidney) were found. In serum, homogenates of liver, kidney and brain, and in part, in urine, the amounts of D-amino acids (D-AAs) were quantitatively determined using chiral phase capillary gas chromatography-selected ion monitoring mass spectrometry. Significant levels of certain D-AAs (Ala, Pro, Ser, Asx, Glx, Orn and Lys) were already detectable in kidney and liver homogenates and serum of controls. In brain homogenates the highest amounts among the D-AAs were found for D-Ser (up to 382 nmol g^{-1}), moderate amounts for D-Ala, D-Asx and D-Glx, and, in a few cases, trace amounts for D-Orn and D-Lys (1–2 nmol g^{-1}). D-Pro was not detected either in the brains of controls or in the brains of animals loaded with D-Pro. Feeding with D-Pro resulted in a 20–30 fold increased renal excretion of D-Pro at the end of the experiment. Continuous feeding with D-Asp did not increase renal excretion of this enantiomer, but in the serum, higher amounts (0.8–4.0 μmol^{-1}) were determined in comparison to the control group (0.3–0.9 μmol^{-1}). Feeding with D-Pro led to an increase of this enantiomer in serum (1.3–10.5 μmol^{-1}). Feeding with D-Asp did not increase its amounts in brain homogenates (38 and 43 nmol g^{-1}) in comparison to controls.

Keywords: D-Amino acids; D-Proline; D-Aspartic acid

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1. Introduction

The nutritional value and possible toxic effects of D-amino acids (D-AAs), the mirror images of the biologically dominating L-amino acids (L-AAs), have been investigated and intensively discussed for about half a century [1–4]. We agree, however, with the recent, most authoritative reviews [5,6] that, somewhat unexpectedly, currently no clearcut and general answer can be given to these questions for all common D-AAs. This is attributed to the various objectives (e.g., nutritional, medical, toxicological, neurochemical aspects) under which the respective experiments were carried out and, in particular, to the greatly varying responses, depending strongly on the conditions of administration and amounts of AAs used, as well as on the sex [3], species, strains, and even genotypes of experimental animals [7]. Thus, in feeding studies, animals usually have free access to diets supplemented with pure AA enantiomers or racemic mixtures of both enantiomers, used either alone or in mixtures with other AAs, whereas in toxicological studies oral loading via a stomach tube, or intravenous (i.v.) or i.p. administration of relatively high amounts of enantiomers is common.

Since L-Pro is not an indispensable AA, reports on the nutritional or physiological relevance of its D-enantiomer are rather scarce in the literature. Therefore, a communication by Kampel et al. [8] reporting that continuous uptake of relatively low amounts of D-Pro in drinking water for a month led to severe pathophysiological changes of the liver and kidney of rats attracted our attention. This caused us to repeat these experiments using D- and L-Pro and to determine quantitatively the amounts of D- and L-amino acids in blood serum, liver, kidney, brain and urine of rats serving as controls and those fed with D- and L-Pro. We also included D- and L-Asp in the feeding experiments since a decrease of body and liver weights of rats was reported [9,10] as well as growth retardation in chicks when their diets were supplemented with D-Asp [11]. This might be of interest since racemic mixtures of Asp or the pure L-enantiomers are widely used in electrolyte supplements.

2. Experimental

2.1. Chemicals

For amino acids (AAs) standard abbreviations were used: Ala, alanine; Asx, (Asp+Asn); Gaba, γ-aminobutyric acid; Glx, (Glu+Gln); Leu, leucine; Lys, lysine; Met, methionine; Nle, norleucine; Orn, ornithine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Tyr, tyrosine; Val, valine. Other abbreviations: GC–SIM-MS, gas chromatography–selected ion monitoring mass spectrometry; bw, body weight.

AAs used for feeding (D-Asp, Lot No. 89F 5024; L-Asp, Lot No. 128 F 0066; D-Pro, Lot No. 109 F, and L-Pro, Lot No. 50H 0324) were supplied by Sigma, St. Louis, MO, USA.

For amino acid (AA) quantification, an AA standard solution (1 nmol μl^{-1}) (Pierce, Rockford, IL, USA) was fortified with 1 nmol μl^{-1} L-Orn and L-Nle (from Sigma).

Dowex 50 WX8 cation-exchanger, practical grade, particle size 200–400 mesh (0.037–0.075 mm), purchased from Serva (Heidelberg, Germany), was washed several times with threefold distilled water, 1 *M* NaOH and 4 *M* HCl before use and the final eluate was tested for the absence of amino acid enantiomers by GC–SIM-MS (see below). Hydrochloric acid (32%), trichloroacetic acid (TCA), dichloromethane (DCM), aqueous ammonia (25%), diethyl ether and 2-propanol (2-PrpOH) were from Merck, Darmstadt, Germany and of pro analysi (p.a.) grade; acetyl chloride (synthetic grade) was from Merck; pentafluoropropionic acid anhydride (PFPAA) was from Pierce.

2.2. Kits for determining clinical parameters

Commercially available test kits were used for determining alkaline phosphatase (No. 415 278, Boehringer, Mannheim, Germany), glutamic-oxalic-transaminase (GOT) (Merckotest series No. 12150, Merck, Darmstadt, Germany), glutamic-pyruvic-transaminase (GPT) (Merckotest No. 12166), urea (Merckotest No. 14359), creatinine (Merckotest No. 3385). The osmomolality of urine was determined

via freezing point depression (Osmometer Model DM 8901, Vogel, Giessen, Germany).

2.3. Experimental animals

Female SPF Sprague–Dawley rats (Interfauna, Tuttlingen, Germany) with an initial body weight of 140±10 g ($\bar{x}$±S.D.) and of approx. 6 weeks age were randomly allocated to five groups (designated A, B, C, D, and E) of seven animals each (designated A1–A7, B1–B7, C1–C7, D1–D7, and E1–E7). The animals were kept in Macrolon® cages (3–4 rats per cage) under standardized conditions (room temperature: 22–24°C; relative humidity: 50–70%; ca. 12 room air changes h^{-1}; 12 h light–dark cycles).

2.4. Feeding of rats

Feed pellets [standard chow No. 1324, consisting of grain, coarse soybean meal and 3% fish meal, fortified with 3.8 μmol DL-Met g^{-1} feed by the manufacturer (Altromin, Lage, Germany)] and drinking water (bottle of 250 ml per cage) were offered ad libitum. The control group A received deionized water, whereas the drinking water of groups B, C, D, and E was fortified with 0.033% (w/v) of D-Pro, L-Pro, D-Asp, and L-Asp, respectively. Assuming a daily water consumption of 30 ml and a mean body weight (bw) of 200 g (related to the whole experimental period) this would correspond to a daily load of approximately 50 mg AA kg^{-1} bw (dosage used by Kampel et al. [9]). Treatment was performed for 28 days. Body weight, food and water consumption were monitored once weekly. Urine was collected in metabolic cages on days 0 and 21 during 6 h after loading the rats with 25 ml demineralized water kg^{-1} bw by gavage (to increase urine production). The volume of urine was determined, samples were acidified by addition of 1 ml of 1 *M* HCl and stored at −20°C until analysis. On day 28 the animals were bled under pentobarbital anesthesia (6 mg per 100 g bw, i.p.) and blood was taken from the Arteria abdominalis. Blood was allowed to clot for 30 min, then serum was prepared by centrifugation (1000 *g*; 5 min). On autopsy all organs were carefully inspected and the fresh weights of liver, left kidney, brain and thymus were determined.

2.5. Histology

The left kidney and two sections of the liver (peripheral and central) were taken from each animal and fixed in Bouin's solution. Three sections (4 μm) of each tissue sample were prepared and stained with periodic acid–Schiff's reagent, azan, or hematoxylin–benzopurpurin using standard procedures. The sections were coded, randomized and evaluated under blind conditions by two independent observers.

2.6. Statistics

Randomization was performed using standard tables with randomly arranged figures. Since several parameters did not reveal homogeneity of variances or normal distribution it was decided to generally apply the Kruskall–Wallis test, followed, if indicated, by the Wilcoxon rank-sum/Mann–Whitney U-test [12]. The level of significance was set at 95%. Data are presented as the arithmetic means with standard deviations. Statistics software SAS [13] for personal computers was used for all calculations presented in Table 1.

2.7. Treatment of physiological samples and isolation of amino acids

2.7.1. Tissue samples

To 200–400 mg amounts of fresh tissue samples (kidney, liver, brain) appropriate amounts (60–100 nmol) of L-Nle as internal standard, and 10 ml of cold (4°C) TCA (6%, w/v) were added. Tissues were homogenized at 0°C using an Ultra Turrax homogenizer Model TP 18/2 N (Janke and Kunkel, Staufen, Germany). The homogenate was centrifuged at 1600 *g* (20 min), the supernatant was collected and the remaining precipitate was again homogenized with addition of 5 ml TCA and centrifuged. The supernatants were combined and extracted with diethyl ether (3×20 ml) in order to remove TCA. The aqueous solution (pH 2.5) was passed through a column packed with Dowex 50 WX8 cation-exchanger (bed size 10×1 cm). The resin was washed with threefold distilled water (15 ml) and AAs were desorbed with 4 *M* aqueous ammonia (20 ml). The

Table 1
General toxicological data of rats serving as controls (A) or subacutely loaded with amino acids (B–E), approx. 50 mg kg^{-1} body weight per day via the drinking water

	Control (A)	D-Pro (B)	L-Pro (C)	D-Asp (D)	L-Asp (E)
Food (g)	21.5±1.5	22.5±2.3	26.6±4.2	22.8±0.8	21.8±2.1
Drinking water (ml)	38.8±8.5	38.3±3.2	44.0±7.2	40.9±5.1	42.2±9.2
Body weight (g)	226±17	234±23	265±37*	235±11	224±27
Kidney (g)	0.78±0.11	0.80±0.09	0.87±0.14	0.77±0.05	0.74±0.07
Liver (g)	9.7±0.9	10.5±1.7	12.0±1.7*	10.1±0.5	10.2±1.3
Brain (g)	1.22±0.06	1.23±0.08	1.22±0.05	1.18±0.06	1.19±0.06
Thymus (g)	0.51±0.06	0.62±0.07*	0.63±0.13	0.57±0.06	0.54±0.11
Serum					
GOT (U l^{-1})	53±10	56±5	60±9	58±17	51±11
GPT (U l^{-1})	38±7	41±8	53±17*	45±13	46±19
Alkaline phosphatase (U l^{-1})	183±48	209±50	199±61	244±49	229±84
Urea (mg dl^{-1})	42±5	44±4	42±5	40±4	38±5
Creatinine (mg dl^{-1})	0.44±0.05	0.44±0.03	0.44±0.03	0.44±0.06	0.43±0.05
Urine					
Creatinine (mg dl^{-1})	23.7±6.5	29.5±17.0	41.9±11.2*	29.8±12.8	36.2±41.6
Osmolality (mosmol kg^{-1})	660±251	788±536*	1147±295*	860±335	911±834

Data are presented as the arithmetic means±S.D., $n=7$ per group. Amino acids in parenthesis (i.e., D-Pro, L-Pro, D-Asp, L-Asp) behind rats B–E in all Tables refer to the respective AA enantiomer loaded.
*: $P<0.05$ versus control.

eluate was evaporated to dryness in vacuo (in all cases 30–40 mbar at a bath temperature of 40°C), the residue was dissolved in 0.1 *M* HCl (1 ml) and 50-μl volumes were used for derivatization for GC–MS analysis.

2.7.2. Blood

To 0.5 ml volumes of serum, 50 nmol of L-Nle and 100 μl of aqueous 5-sulfosalicylic acid (10%; w/v) were added. The mixture was centrifuged at 1600 *g* and the supernatant was passed through a Dowex cation-exchange column as described above (bed size 5×0.5 cm). The resin was washed with threefold distilled water (3 ml) and AAs were eluted with 4 *M* aqueous ammonia (5 ml). The eluate was evaporated to dryness in vacuo, the residue was dissolved in 0.1 *M* HCL (0.5 ml) and 50-μl volumes were used for derivatization.

2.7.3. Urine

Urine samples (1–5 ml) were adjusted to pH 2.0, if necessary, with the addition of 4 *M* HCl. Amounts of 50 nmol of L-Nle were added and the solution was passed through a Dowex cation-exchanger column (bed size 5×1 cm). The resin was washed with distilled water (10 ml) and AAs were eluted with 4 *M* aqueous ammonia (10 ml). The eluate was evaporated to dryness in vacuo, the residue was dissolved in 0.1 *M* HCl (1 ml) and 50-μl volumes were used for derivatization.

The internal standard L-Nle was prepared as a 1 m*M* aqueous solution and suitable amounts were added to samples.

2.8. Determination of free D-amino acids in rat chow

A sample of rat chow (800 mg) was suspended in 70% aqueous ethanol (5 ml) and sonicated for 20 min at ambient temperature. The mixture was centrifuged (1600 *g*, 20 min), the supernatant was collected and the precipitate was extracted twice in the same manner. Supernatants were combined, evaporated to dryness (40°C, 30–40 mbar) and the residue was dissolved in 0.1 *M* HCl (5 ml). A volume (1 ml) was passed through a membrane filter (Anotop 10; 0.2 μm pore size, Merck) and aliquots

of 50 μl and 250 μl, respectively, were used for GC–MS and for quantitative AA analysis by ion-exchange chromatography.

2.9. Derivatization of amino acids for investigation by GC–SIM-MS

Samples of 50 μl of AA solutions (see above) were transferred into 1-ml Reacti-Vials (Wheaton, Millville, NJ, USA) and dried in a stream of nitrogen at ambient temperature. Amino acids were converted into their *N(O)*-pentafluoropropionyl 2-propyl esters by subsequent treatment with HCl in 2-propanol and PFPAA. Excess of reagents was removed in a stream of nitrogen, the residue was dissolved in DCM (10–20 μl) and samples of 0.2 μl were analysed by GC–MS. Racemization of AA during derivatization was ruled out by use of appropriate blanks.

2.10. Gas chromatography–selected ion monitoring mass spectrometry (GC–SIM-MS)

For GC–MS a Shimadzu GC-17A gas chromatograph and a QP-5000 mass spectrometer (Shimadzu, Kyoto, Japan) were used. Chromatographic runs were monitored with Shimadzu Class 5000 software. A Chirasil-L-Val fused-silica capillary column (25 m×0.25 mm I.D.; Chrompack, Middelburg, The Netherlands) was used for separating AA enantiomers. Carrier gas was helium at an inlet pressure of 5 kPa; purge flow 3 ml min^{-1}; flow-rate 0.5 ml min^{-1}; injector and detector temperature 250°C; split ratio 1:30. Temperature program was 70°C for 5 min, then at 2.5°C min^{-1} to 100°C, then at 3.5°C to 190°C and 15 min at 190°C. The pressure program was 5 kPa for 5 min, then 0.2 kPa min^{-1} to 7.9 kPa, then 0.3 kPa min^{-1} to 15 kPa in 10 min.

Electron impact mass spectra were measured at an acceleration energy of 70 eV. For selected ion monitoring, appropriate ion sets were selected and characteristic mass fragments (*m/z*) of AA derivatives were used: Ala (190), Val (218), Thr (203), Ile (232), Pro (216), Leu (190), Ser (189), Nle (232), Gaba (232), Asp (234, 262), Met (203, 221), Phe (91, 148), Glu (202, 230), Tyr (253, 310) Orn (216), and Lys (230).

2.11. Quantification of amino acids

Response factors (F) of amino acids in relation to L-Nle in an equimolar standard solution were determined at each measurement session. The amount of each AA was calculated according to the equation

$$c = 1/F \times A_{AA}/A_{IS} \times C_{IS}$$

were c = amount (nmol) of amino acid enantiomer to be determined, F = response factor of amino acids in equimolar standard, A_{AA} = peak area of amino acid enantiomer to be determined, A_{IS} = peak area of L-Nle determined in sample and C_{IS} = amount (nmol) of L-Nle added to original sample.

Relative amounts of D-AA (referred to as %D in the text) were determined from electronically integrated peak areas of the sum of (D+L)-AA according to the equation: %D = 100 D/(D+L).

Due to the high analytical expenditure, not all samples could be analysed. Instead, randomly chosen samples were studied as indicated in Tables 2–4.

3. Results

3.1. Treatment-related effects in rats, histology

No signs of general toxicity were detected in any group during the 28-day observation period. In Table 1 are compiled the data of controls (group A) in comparison to groups B, C, D, and E treated with the pure enantiomers of D-Pro (group B), L-Pro (group C), D-Asp (group D), and L-Asp (group E) (n = 7 of each group); numbers behind letters (e.g., A1) refer to individual animals. In comparison to the controls neither D-Pro nor D-Asp nor L-Asp significantly affected food and water consumption, the development of body and organ weights, or serum parameters. A slight increase of thymus weight and minor changes in urine parameters in the D-Pro group B are probably not biologically significant. Six of 12 parameters were significantly affected following the treatment with L-Pro (increase of food consumption and of body weight, increase of liver weight and GPT, increased urinary creatinine levels and osmolality); however, it is not certain that these minor changes are actually biologically significant. In addition, no subacute toxicity was observed for the

Table 2
Relative amounts of D-amino acids [%D = 100×D/(D+L)] and absolute amounts (nmol g^{-1} wet organ) of D-amino acids (D-AA) in the liver and kidney of rats

D-AA	Liver						Kidney					
	A1 (Control group)		B1 (D-Pro group)		D1 (D-Asp group)		A1 (Control group)		B1 (D-Pro group)		D1 (D-Asp group)	
	%D	nmol g^{-1}	%D	nmol g^{-1}	%D	nmol g^{-1}	%D	nmol g^{-1}	%D	nmol g^{-1}	%D	nmol g^{-1}
Ala	0.3	20	0.3	27	0.3	25	0.2	25	0.2	25	0.1	20
Pro	0.1	2	0.3	4	0.2	2	0.3	20	0.3	11	0.1	3
Ser	1.0	25	1.2	39	1.2	42	0.3	24	0.3	16	0.6	43
Asx	1.5	9	2.3	15	3.4	44	1.3	60	1.3	43	11.8	494
Glx	0.3	16	0.3	12	0.3	14	0.3	35	n.d.	n.d.	n.d.	n.d.
Orn	n.d	n.d	0.7	5	0.5	5	1.0	7	0.9	4	0.8	10
Lys	n.d.	n.d.	0.3	4	0.2	3	0.2	13	0.2	8	0.2	23

A1 = control; B1 = fed with D-Pro; D1 = fed with D-Asp; numbers refer to individual rats.
n.d. = not detectable, detection limit approximately 1 nmol D-AA g^{-1} wet organ.

amounts of 27 mg of D-Met per kg body weight resulting from DL-Met fortified standard rat chow as exemplified for control group A from the data of Table 1 and Table 5.

The histological evaluation of renal and hepatic tissues did not reveal any treatment-related pathological alterations in any of the 35 rats. It should be noted that the evaluation was done under blind conditions by two independent observers. Micrographs of rat No. B4, belonging to the group treated with D-Pro, are presented in Fig. 1a (kidney) and 1b (liver).

3.2. D-Amino acid levels in kidney and liver

For the determination of free D- and L-AAs in biological samples, AAs were extracted and converted into their volatile *N(O)*-pentafluoropropionyl 2-propyl esters. The enantiomers were separated on a fused-silica capillary column coated with the chiral stationary phase (Chirasil-L-Val), characterized by selected ion monitoring (SIM)–mass spectrometry (MS) and quantified using L-Nle as an internal standard. Representative chromatograms of the pattern of AA enantiomers determined in hepatic and renal tissue homogenates of rats exposed to D-Asp are shown in Fig. 2a and Fig. 2b, respectively.

The amounts of D-AAs determined in the liver and kidney of rats of the control group (A1) and those fed with D-Pro (B1) and D-Asp (D1) are presented in Table 2. As can be seen, the D-enantiomers of Ala, Pro, Ser, Asx, Glx, Orn, and Lys were detectable in liver (Fig. 2a) and kidney (Fig. 2b). (Note that under the acidic conditions used for the isolation of AAs

Table 3
Relative (%D) and absolute (nmol g^{-1}) amounts of D-amino acids in rat brain homogenates

D-AA	A1 (Control group)		A2 (Control group)		B1 (D-Pro group)		B2 (D-Pro group)		D1 (D-Asp group)		D2 (D-Asp group)	
	%D	nmol g^{-1}	%D	nmol g^{-1}	%D	nmol g^{-1}	%D	nmol g^{-1}	%D	nmol g^{-1}	%D	nmol g^{-1}
Ala	0.8	12	0.5	9	0.4	12	0.4	9	0.5	11	0.5	11
Pro	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ser	10.6	160	9.8	98	13.7	280	12.5	219	13.6	382	13.6	381
Asx	0.4	32	0.3	19	0.4	42	0.4	34	0.5	38	0.6	43
Glx	0.2	23	n.d.	n.d.	0.2	41	0.2	23	0.2	37	n.d.	n.d.
Orn	1.3	1	n.d.	n.d.	n.d	n.d.	n.d.	n.d.	1.0	1	n.d.	n.d.
Lys	0.4	2	n.d.	n.d	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

A1, A2 = controls; B1, B2 = animals fed with D-Pro; D1, D2 = animals fed with D-Asp.

Table 4
Relative (%D) and absolute (μmol l^{-1}) amounts of D-amino acids (D-AA) in blood serum of rats

D-AA	%D	μmol $^{-1}$	%D	μmol $^{-1}$	%D	μmol $^{-1}$	%D	μmol $^{-1}$	%D	μmol $^{-1}$
Control group (A)										
	A1		A2		A3		A4		A5	
Ala	2.9	9.2	3.5	7.8	2.2	0.7	4.2	5.2	3.1	12.2
Pro	0.2	0.3	0.2	0.2	0.2	0.5	0.2	0.3	0.2	0.5
Ser	0.5	0.5	0.4	0.4	0.7	1.2	1.0	0.5	0.5	0.8
Asx	1.3	0.4	1.4	0.3	1.7	0.9	1.6	0.5	1.6	0.6
Met	6.2	0.4	3.5	0.2	7.3	0.4	7.6	0.6	6.7	0.6
Orn	0.3	0.2	0.2	0.2	0.4	0.5	2.0	0.1	2.1	0.2
Lys	n.d.	n.d.	n.d.	n.d.	0.2	0.5	0.6	1.3	0.8	0.2
D-Pro group (B)										
	B1		B2		B3		B4		B5	
Ala	4.7	16.6	3.2	9.0	4.4	9.0	3.5	19.1	2.0	10.8
Pro	3.2	5.8	2.4	3.6	1.4	1.3	3.4	10.3	4.2	10.5
Ser	0.5	0.7	1.0	1.2	0.8	0.7	1.1	1.9	0.7	2.3
Asx	1.8	0.6	1.9	0.4	2.0	0.4	1.6	0.7	0.9	0.7
Met	6.6	0.5	6.6	0.3	4.8	3.4	10.0	1.3	2.0	1.5
Orn	0.7	0.3	0.5	0.2	0.4	0.2	0.4	0.3	n.d.	n.d.
Lys	0.3	0.6	0.2	0.4	0.2	0.2	0.2	0.9	0.2	0.1
D-Asp group (D)										
	D1		D2		D3		D4		D5	
Ala	3.3	11.8	2.6	12.6	2.8	9.8	1.8	12.3	2.3	11.3
Pro	0.3	0.5	0.3	0.8	0.4	0.6	0.1	n.c.	0.3	0.5
Ser	0.8	1.9	0.8	2.2	0.9	1.7	0.7	1.9	1.0	2.2
Asx	7.2	4.0	6.1	3.9	7.8	2.6	3.4	0.8	4.9	1.7
Met	5.3	2.5	8.3	3.2	4.9	1.3	7.9	1.8	4.8	1.5
Orn	0.4	0.2	0.3	0.4	0.3	0.1	0.2	0.1	0.3	0.2
Lys	0.2	0.4	0.1	0.1	n.d.	n.d.	0.1	0.2	0.2	0.4

A = control group; B = fed with D-Pro; D = fed with D-Asp. Sera of individuals 1–5 were analyzed.
n.c. = not calculable; N.D. = not detectable; for other abbreviations see Table 2.

Table 5
Absolute amounts of free, ethanol extractable (D + L)- and D-amino acids (AA), and relative amounts (%) of D-amino acids in the rat chow used

D-AA	(D + L)-AA (μmol g^{-1})	%D	D-AA (nmol g^{-1})
Asp	2.88	2.0	58
Ser	0.73	3.6	26
Glu	4.86	2.2	107
Ala	3.76	3.6	135
Met	3.95	48.4	1910
Phe	0.76	2.4	18
Orn	0.06	13.4	8
Lys	0.69	2.2	15
Pro	2.25	1.7	38

and for the derivatization for GC–MS, Asn and Gln, if present, are hydrolyzed to Asp and Glu which are determined. The sum of Asp + Asn and Glu + Gln are designated as Asx and Glx, respectively, in the Tables). As can be seen in Table 2, in the controls (group A) amounts of 2–20 nmol D-Pro g^{-1} liver or kidney were already detectable. The amounts of D-Pro did not change drastically in the liver and kidney of rats fed with D-Pro (group B). In contrast, feeding with D-Asp (group D) resulted in a moderate increase in liver [44 nmol g^{-1} (3.4%); control 9 nmol g^{-1} (1.5%)] and a drastic increase in kidney levels [494 nmol g^{-1} (11.8%); control 60 nmol g^{-1} (1.3%)]; percent values in parentheses in the text refer to relative amounts of D-AAs with respect to the sum of D- and L-enantiomers.

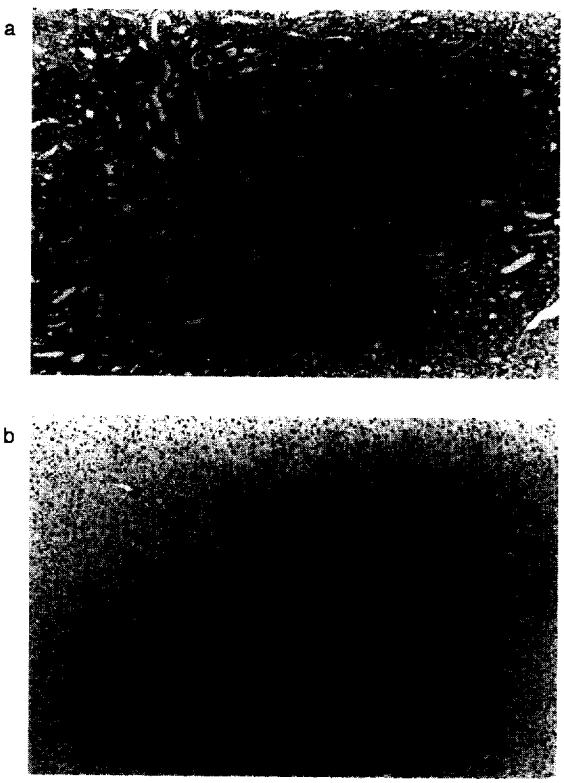

Fig. 1. Histology of the left liver and kidney of rat B4 after treatment with D-Pro (ca. 50 mg kg^{-1} body weight per day) via the drinking water during 27 days. Renal tissue (a, 25-fold magnification) does not show any alterations typical for parenchymatous damage, necrosis or dystrophic alterations of the tubulus epitheli or the glomerula. Hepatic tissue (b, 25-fold magnification) shows normal configurations without any fibrotic processes of the periportal area and no necrosis.

3.3. D-Amino acids in brain homogenates

D-AAs determined in brain homogenates of rats of the control group and those fed with D-Pro and D-Asp are listed in Table 3. No D-Pro was detected in brain homogenates of controls and rats fed with this enantiomer (detection limit approximately 1 nmol D-Pro g^{-1}). Feeding with D-Asp did not increase its amounts (38 and 43 nmol g^{-1}) in comparison to controls (19 and 32 nmol g^{-1} and the group fed with D-Pro (34 and 42 nmol g^{-1}). In agreement with the literature [14,15] the highest amounts of D-Ser were detected in brains, ranging from 98–382 nmol g^{-1}. In addition, however, moderate amounts of D-Ala (9–12 nmol g^{-1}), D-Asx (19–43 nmol g^{-1}), and D-Glx (0–41 nmol g^{-1}) were determined and, in a few cases, trace amounts of D-Orn and D-Lys (1–2 nmol g^{-1}).

3.4. D-Amino acids in blood serum

The relative and absolute amounts of D-AAs in blood serum of each of the five rats of the control group A (Nos. A1–A5), of the group B fed with D-Pro (B1–B5), and of the group D fed with D-Asp (D1–D5) were determined with GC–SIM-MS and are presented in Table 4. D-Pro levels in the serum of the controls amounted to 0.2–0.5 μmol l^{-1} serum and increased to 1.3–10.5 μmol l^{-1} in the D-Pro group B. D-Asx levels of the control group A amounted to 0.8–4.0 μmol l^{-1} serum in rats fed with D-Asp (group D). As shown in representative chromatograms, the D-enantiomers of Ala, Pro, Ser, Asx, Met, Orn, and Lys were already detectable in the serum of control group A (Fig. 3a) as well as in group B fed with D-Pro (Fig. 3b), and group D fed with D-Asp (Fig. 3c).

It is worth noting that Hashimoto et al. [14], also using chiral phase GC combined with electron impact (EI) mass spectrometry, could detect D-Asp in rat periphery, but not in the kidney, liver, brain, and serum. This might be attributed to the higher specificity of the GC–SIM-MS used in this work, resulting in much lower background noises in chromatograms.

3.5. D-Amino acids in urine

Urine samples were exclusively investigated for the occurrence of D-Pro and D-Asp, respectively. Feeding with D-Pro resulted in an approximately 20–30-fold increase of renally excreted D-Pro: from 0.9 μmol l^{-1} (4.4%) to 28.7 μmol l^{-1} (42.2%) in rat B1, and from 13.9 μmol l^{-1} (18.8%) to 256.1 μmol l^{-1} (71.2%) in rat B4. Oral application of D-Asp, however, led to an increase of relative, but not of absolute amounts of D-Asp in urine: from 10.3 μmol l^{-1} (19.7%) to 6.0 μmol l^{-1} (24.3%) in rat D1, and from 10.4 μmol l^{-1}(19.4%) to 5.5 μmol l^{-1} (20.3%) in rat D4.

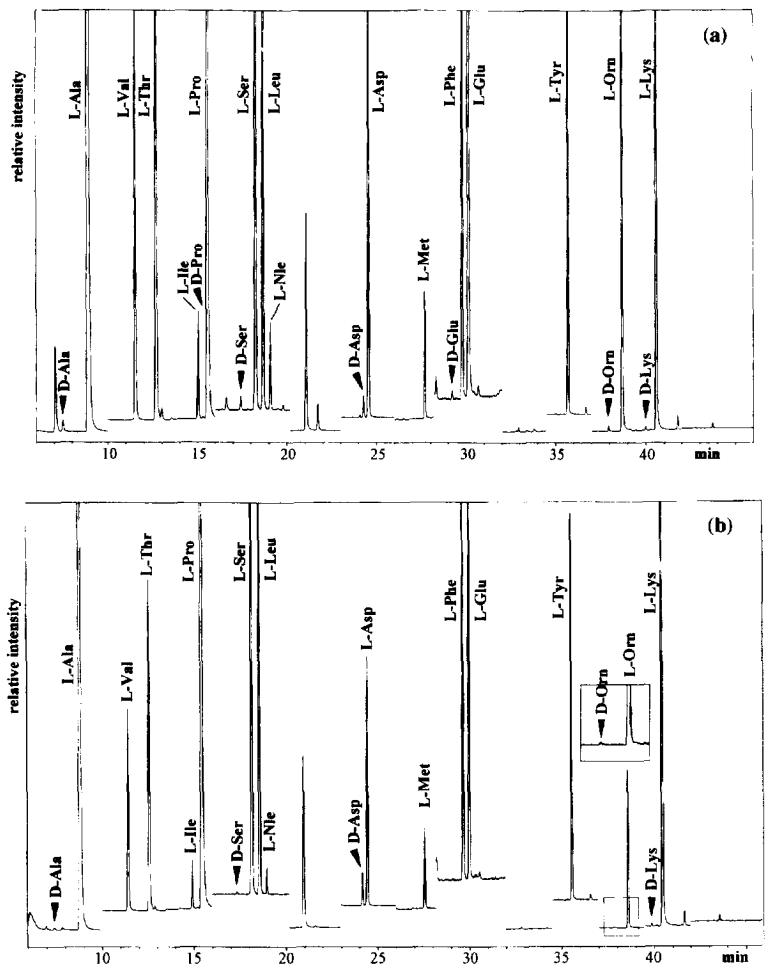

Fig. 2. GC–SIM-MS of D- and L-amino acids in TCA extracts of (a) liver and (b) kidney of rat D1; arrows in all chromatograms show positions of D-amino acids; L-Nle = internal standard. For quantitative data of all chromatograms see Tables; signal at 21 min = GABA.

3.6. Free D-amino acids in rat chow

The amounts of free, ethanol extractable D-AAs in the rat chow used are presented in Table 5. The high amounts of 1910 nmol g^{-1} D-Met determined are attributed to fortification of feed with racemic DL-Met. However, significant amounts of other free D-AAs (8–135 nmol g^{-1} feed) were also detectable.

4. Discussion

The study was primarily designed and clinical parameters were chosen according to the work of Kampel et al. [8] in order to confirm the reported hepatotoxic and nephrotoxic effects of D-Pro to rats. D- and L-Asp were included in the experiments as retardations of weights of rats [10] and chickens [11] have been reported on administration of D-Asp. Furthermore, salts of L-Asp or the racemic mixture DL-Asp are widely used in mineral supplements.

In contrast to Kampel et al. [8] we did not find that the serum parameters for GOT, GPT, alkaline phosphatase, and creatinine were elevated significantly in the groups fed with D-Pro as compared to untreated controls or to rats fed with L-Pro (Table 1).

Similarly, no pathological alterations like fibrosis or necrosis of liver cells or severe tubular lesions of

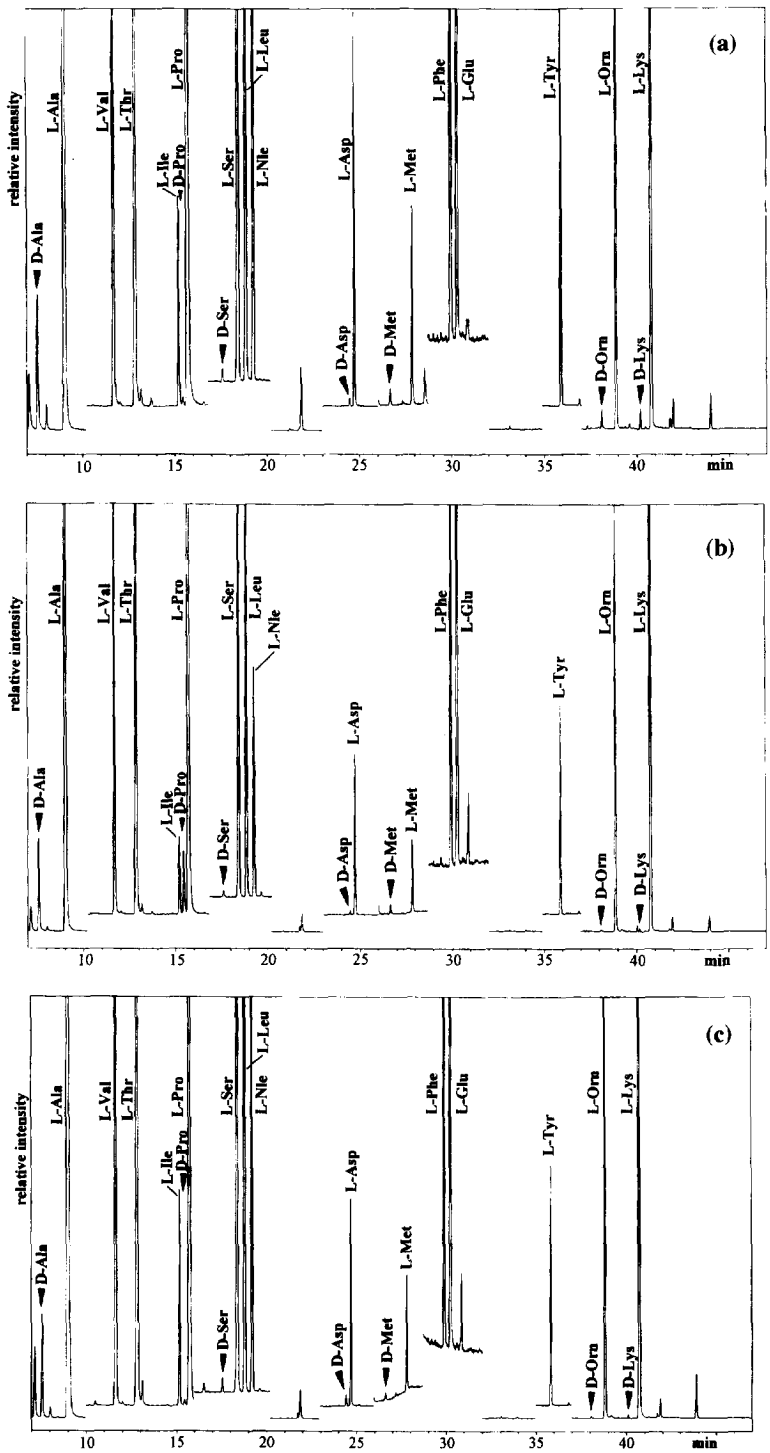

Fig. 3. GC–SIM-MS of D- and L-amino acids in blood serum of rats; (a) = control (rat A4), (b) fed with D-Pro (rat B4), (c) fed with D-Asp (rat D5); designations in parentheses characterize individual rats.

renal structures were found in rats treated with D-Pro (Fig. 1). Whereas Kampel et al. [8] could not detect D-Pro in liver and kidney homogenates and in serum and urine before and after feeding animals with D-Pro, we found that low but significant amounts of D-Pro, among other D-AAs, are present in liver and kidney (Fig. 2a,b; Table 2) and serum (Fig. 3a, Table 4) of the control group of rats. The amount of D-Pro increased significantly in blood serum (Fig. 3b) and drastically in urine on administration of D-Pro; the amounts of renally excreted D-Pro exceeding even L-Pro (see Section 3.5). However, D-Pro was not detected in the brain homogenates of control rats or in the brains of those rats fed with D-Pro (Table 3).

Consequently, we cannot confirm the reports on the toxicity of D-Pro to rats or the analytical data on the absence of D-Pro in the serum, tissues, and urine of control and D-Pro treated rats. These differences might be explained by the various strains or genotypes of the rats used [7] and composition of rats chow (see Section 2). Differences in the gut flora and coprophagous behaviour of rats should also be taken into account. Furthermore, since levels of D-Pro were not detected in kidney, liver, serum and urine of rats by Kampel et al. [8], it might be possible that an analytical methodology not allowing the sensitive determination and effective separation of Pro enantiomers in complex matrices, was employed.

These results are also of interest since from autoradiographic studies on the incorporation of ^{3}H-DL-Pro, administered i.p. in amounts of 200 mg kg^{-1} bw, the presence of a relatively specific D-proline oxidase in the pars recta of the proximal tubule of the rat kidney was postulated [16]. Furthermore, loading of mice i.p. with ^{3}H-D-Pro (amounts not given) was reported to lead to a drastic increase of protein-bound radioactivity in the cerebellum in comparison to the administration of ^{3}H-L-Pro [17]. Intraventricular injection of D-Pro together with sodium chloride into chicks brain was reported to cause lethal convulsive activity, while use of D-Pro with replacement of saline by water, or administering L-Pro under the same conditions, exerted no effects [18]; for other actions of D-Pro see references cited by Kampel et al. [8]. To draw conclusions from these experiments with regard to human health problems which might arise from orally consumed D-Pro seems unjustified without definite experimental proof. D-Pro is common in blood plasma of man [19] and animals, it is partly renally excreted in its unmetabolized form and effectively oxidized by D-amino acid oxidase to α-keto-δ-amino-valeric acid which is further metabolized [2].

Administration of D- and L-Asp did not result in significant changes of the parameters presented in Table 1. However, loading with D-Asp led to a drastic increase of its amounts in kidney homogenates (Table 2). In liver homogenates moderate amounts of D-Asp could be determined despite the presence of D-aspartate oxidase in these organs [20] which is capable of converting D-Asp into oxalacetic acid [2]. D-Asx was determined in brain homogenates of control rats (32 and 19 nmol g^{-1}) as well as those fed with D-Asp (38 and 43 nmol g^{-1}) or D-Pro (42 and 34 nmol D-Asx g^{-1}) (Table 3). Thus, feeding with D-Asp did not increase its amount in rat brains.

In serum (Table 4), however, a moderate increase of D-Asp was found on feeding with this enantiomer in comparison to the control group. Koyuncuoglu et al. [9,10] reported a decrease in body and liver weights of rats who had free access to diets and drinking water enriched with D- and (D+L)-Asp. An analogously treated, food-deprived control group, however, showed only a decrease in liver triglyceride contents. No histopathological changes were reported. The authors conclude that D-Asp decreases food and fluid intake in rats and that the effects caused by D-Asp can be antagonized to a certain extent by L-Asp. As these experiments were conducted for only 1 week and since much higher amounts of D-Asp were administered via drinking water, the results of Koyuncuoglu et al. [9,10] are not directly comparable with ours. With regard to the species-dependence of D-AA utilization [5], it is also not justified to draw conclusions from moderate or low growth depression effects of DL-Asp in chicks [11] or rats [21,22] with regard to mammalia in general.

Also, from a physiological point of view, an until recently underestimated continuous uptake of dietary free D-AAs with foods and feeds certainly contributes to the apparent omnipresence of D-AAs in animals [23,24], food [25], and plants [26]. This should be considered when adverse health effects of common D-AAs are extrapolated from experimental

animals to man. It is also known that D-AAs are effectively metabolized by enzymes such as D-AA oxidases, D-AA transaminases, or D-AA reductases [27]. As a result of developments in chiral AA analysis it also became evident that the D-enantiomers of most physiological L-AAs are renally excreted by mammalia [28,29]. The reported nephrotoxicity of D-Ser to rats [3,30] is strongly dependent on its mode of administration and the amounts applied [4]. Although the standard rat chow we used contained 26 nmol free D-Ser g^{-1} feed as well as other D-AAs (see Table 5), no toxic effects were observed in the experiments described.

In conclusion, GC–MS reveals that significant amounts of certain D-AAs are permanently present in serum, liver, kidney, brain, and urine of rats. No evidence was found for subacute toxicity of orally fed D-Pro and D-Asp applied in amounts of 50 mg kg^{-1} body weight for 4 weeks.

5. Declaration

The authors declare that the experiments comply with the Principles of Animal Care, publication No. 86-23, revised 1985, of the National Institute of Health. Permission to perform the experiments was given by the local authorities (Regierungspräsidium Stuttgart, No. A 75/90 PT).

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