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DETECTION OF DESMETHYLDIAZEPAM AND DIAZEPAM IN BRAIN OF DIFFERENT SPECIES AND PLANTS*

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Abstract—Recent data suggest that desmethyldiazepam (DD), a major metabolite of several benzodiazepines (BZD), might be of natural origin. Therefore we tried to quantify DD and diazepam (D) in animals during maturation (e.g. hen, chicken, eggs), in brain of species at different evolutionary stages e.g. salmon, frog, monitor/reptile, rat, cat, dog, deer, bovine) including newborn and adult humans. Since low concentrations of DD (range 0.01–0.04 ng/g wet wt) and D (range 0.005–0.02 ng/g) could be measured in different species by sensitive and specific mass spectrometry (GC–MS), we analysed also several plants (e.g. maize corn, lentils, potatoes, soybeans, rice, mushrooms). Again, DD and D could be detected in low amounts (0.005–0.05 ng/g) in some plant products. This would suggest that DD and D might be of natural origin and incorporated via the foodchain into the animal and human body. The biological role or clinical relevance of these intriguing findings need still to be elucidated.

Previously, desmethyldiazepam (DD) was identified in human, bovine and rat brains by specific analytical methods including mass spectrometry [1]. These surprising and intriguing findings have been recently confirmed in case of rat brain and adrenals by Wildmann *et al.* [2]. In addition to DD the latter found diazepam (D) not only in rat organs but also in the extract of wheat grains and potato tuber [2, 3]. It is therefore likely that benzodiazepines (BZD) are components of our diet, but the site and mode of their biosynthesis are still unknown.

Since the pharmacological and clinical actions of BZD are mediated by specific receptors [4, 5] which are present in vertebrate but not in invertebrate species [6], the evolutionary appearance of "natural" BZD is of great interest. We tried to assess this problem by exact quantification (applying mass spectrometry (MS) using stable isotope dilution techniques) of DD and D in the brain of animals during maturation and species at different evolutionary stages. In addition, food (plant products) as a potential source of these BZD was also investigated.

METHODS

Extraction and purification. Brain material from freshly killed animals was either obtained from local slaughterhouses (bovine, hen, chicken), breeding institutes for laboratory animals (rat, frog, monitor), grocery stores (salmon) or from dead wild animals, given for pathological examination to a veterinary institute. The brain samples were frozen at −20° up to analysis for a period no longer than 2 months. All plant material was obtained from local grocery stores and stored in fresh or dried form.

Total brain tissues (10 g wet weight) of each animal species namely bovine, deer, dog, cat, rat (N = 5), hen (N = 10), 1-day-old chicken (N = 35), monitor/reptile (N = 3), frog (N = 40), salmon (N = 8) were homogenized in 20 ml of bidistilled water. Likewise, three human brain samples, one from a 22-year-old male subject killed in an accident and two from stillborns, all provided by the Pathology Department of our hospital were also processed. Blood measurements and anamneses revealed that no BZD had been taken by the adult subject or by the mother of one of the two stillborns in the immediate past. To the homogenized samples 15 ng deuterated DD (d_5-DD) or 10 ng deuterated D(d_5-D) were added as internal standards. After proteolysis with subtilopeptidase A (1 mg/g brain; 90 min at 60°) extraction at pH 11 was performed twice with 10 ml ethyl acetate. The organic extract was concentrated under N_2 at 50° to 3 ml and reextracted twice with 3 ml of 2N HCl. Following pH adjustment to 9 (with 5 N NaOH) the aqueous phase was applied to an open solid phase column (RP C-18, Baker-system) which was prewashed with 10 ml methanol and 5 ml water. The first fraction (5 ml water) was discarded and the subsequent methanol fractions (2 × 2 ml) were combined and evaporated. The residue was dissolved in 500 μl mobile phase, centrifuged at 10,000 *g* and the clear supernatant concentrated to 100 μl. The same procedure was adopted for plasma samples (20 ml) obtained from 4 different healthy male volunteers (age range 25–35 years) and for material (10–20 g) obtained from different plants.

HPLC-conditions. Purified biological samples (100 μl) were injected onto a C_{18}-reverse phase column (200 × 4 mm, particle size 5 μm; Machery–Nagel, Düren, F.R.G.) and eluted (flow 1 ml/min) with a mixture of 0.01 M KH_2PO_4, pH 7.4 (366 ml),

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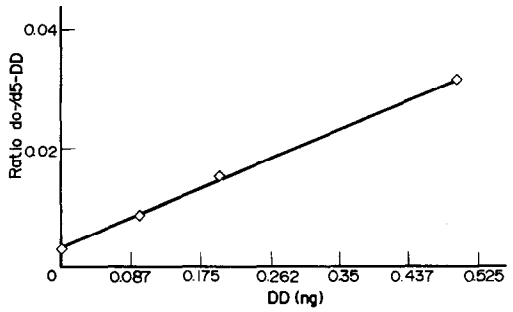

Fig. 1. Calibration curve of bovine brain homogenate spiked with increasing amounts of DD and measured by GC–MS.

methanol (275 ml), acetonitrile (256 ml) and tetrahydrofuran (18 ml). According to UV-monitoring (λ = 242 nm) the retention times of DD and D were evaluated and the corresponding fractions between 8–10 min and 10.5–13 min collected, respectively. After evaporation the residues were dissolved in ethyl acetate, centrifuged and the clear supernatant completely dried for GC–MS analysis (see below) or dissolved in 100 μl tris buffer for the radio receptor assay (RRA).

RRA. Crude synaptosomal membranes from total rat brain were used as BZD-binding protein (0.02 mg protein/500 μl assay volume). ^{3}H-flumazenil (0.5 nmol/sample volume) was used as labelled ligand. Competitive binding with the purified biological samples was accomplished by incubation for 1 hr at 37°. Bound and free radioactivity were separated at 0° by vacuum filtration through Whatman GF/B filter discs. The filter-bound radioactivity was determined by liquid scintillation counting. Calibration curves (ratio of bound to total radioactivity, corrected for nonspecific binding determined in the presence of 10 μM DD vs concentration of unlabelled DD) were linear in the range from 1–50 DD ng/ml.

Mass spectrometry. The purified HPLC-fraction of DD was redissolved in 10 μl *bis*-(trimethylsilyl)-trifluoroacetamide (BSTFA)-reagent to form the trimethylsilyl (TMS)-derivative, whereas the evaporated HPLC-fraction of D was redissolved in 10 μl ethyl acetate. Identification and quantification were performed in negative ion chemical ionization mode (NICI) using methane as reactant gas. The gas chromatograph–mass spectrometer was a Hewlett–Packard Model 5985A additionally equipped for negative ion acquisition. The samples were analysed after separation on a 24 m SE-30 crosslinked capillary column (0.32 mm I.D., 1.05 μm film thickness; Hewlett–Packard) which was directly coupled to the ion source. The 100% relative abundance ions m/z 234 and 239 for TMS-DD and the internal standard (d_5-DD-TMS) respectively were recorded in selected ion monitoring mode (SIM). For D and d_5-D the ions m/z 283 and 288, respectively, were monitored. (Conditions: injection temperature, 280°; temperature program 150° to 320°, heating rate 30°/min, 1 min after injection; interface temperature, 320°; ion source temperature, 200°; ionization energy, 130 eV; sample volume injected, 3–7 μl aliquots.) The detection limit was about 10 pg DD or D per injected reference sample.

Quantification. According to the RRA-conditions BZD-like activity could be expressed only in DD-equivalents. From the tracings in the SIM-mode ratios of the area under the curve (AUC) of the DD

Table 1. Concentrations (ng/g wet weight) of desmethyldiazepam (DD) and diazepam (D) in brain of different species as quantified by radio-receptor-assay (RRA) or by gas chromatography–mass spectrometry (GC–MS) with stable isotope dilution technique (all values represent mean values of at least 2 measurements)

	DD		D
Biological sample	RRA (DD-equivalents)	GC–MS	GC–MS
Salmon	n.m.	0.015	0.005
Frog	n.m.	0.025	0.018
Monitor (reptile)	n.m.	0.024	0.019
Rat	n.m.	0.005	0.007
Cat	0.042	0.008	0.006
Dog	0.027	0.006	0.006
Deer	0.093	0.017	traces
Bovine	0.168	0.040	traces
Hen	0.075	0.014	b.d.l.
Chicken	0.042	0.009	b.d.l.
Egg-white	n.m.	0.007	b.d.l.
Adult man	n.m.	0.008	traces
Stillborn	n.m.	0.51 (2.24)*	traces
Human plasma (range N = 4)	n.m.	0.003–0.01	b.d.l.

n.m.: not measured; b.d.l.: below detection limit of 0.005 ng/ml.
* Mother received 10 mg D (im) 12 hr before delivery.

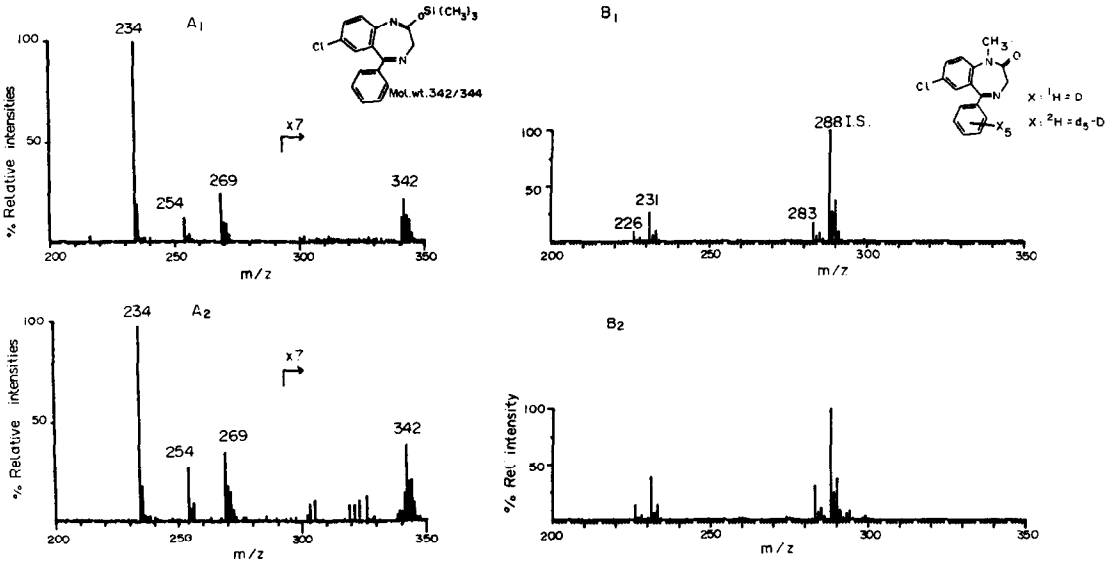

Fig. 2. Partial mass spectra in negative ion chemical ionization mode. A: Desmethyldiazepam as TMS-derivative; A_1: Reference compound:desmethyldiazepam; A_2: Biological sample: extract of salmon brain; B: Diazepam (D); B_1: Reference compound:D (1ng) and d_5-D (5ng); B_2 Biological sample: extract of soy beans spiked with 5ng of d_5-D.

(or D) to d_5-DD (or d_5-D) peaks (retention time 5.7 min for TMS-DD and 6.5 min for D) were calculated (after correction for the 0.2% amount of DD or D in d_5-DD or d_5-D, respectively) and multiplied by the concentration of the added internal standard.

Materials. Reference substances of DD and D were kindly provided by Hoffmann-La Roche Grenzach (F.R.G.); d_5-DD and d_5-D were purchased from Sigma Chemical Co. (U.K.); Subtilopeptidase A was obtained from Boehringer (Mannheim, F.R.G.); ^{3}H-flumazenil was purchased from New England Nuclear (Boston, MA). All reagents and solvents used were at least of analytical grade or of special HPLC-purity.

RESULTS

Analytics

Recovery of DD and D from various brain and plant samples averaged 55 and 57%, respectively. When various amounts (0.1–5 ng/sample) of DD were spiked to homogenized bovine brain the generated calibration curve was linear, but exhibited a distinct *y*-intercept, indicating the presence of endogenous DD (see Fig. 1). Injecting solvent or BSTFA-reagent after DD- and D-standards or biological samples showed no memory or carry over effect of the GC–MS system. The detection limit of our assay for DD and D was between 0.03 and 0.05 ng/10 g wet sample weight.

Identification and quantification

According to the results of the RRA BZD-like material could be observed after extraction and purification of the homogenized brain samples. However, the calculated DD-equivalents were about 5-fold higher than the DD-concentrations measured by the specific GC–MS technique (see Table 1) indicating the possible presence of either other BZDs or cross-reacting BZD-like material or both. There was a good correlation between both assay methods ($r = 0.96$; $P < 0.005$).

When the mass spectrum of TMS-DD was compared with that of purified and derivatized biological extract of bovine or salmon brain, identity in all specific and typical mass peaks was observed (see Fig. 2). When the purified brain extracts were quantified by SIM (m/z of 239 for d_5-DD-TMS and m/z 234 for TMS-DD; see Fig. 3) low concentrations in the range of 0.005 and 0.04 ng/g wet weight could be measured. Blank values (background noise) determined for 20 ml tap water and tris buffer, containing all reagents and solvents used in the extraction procedure, were lower than the detection limit (see Fig. 3). Thus laboratory contaminations were very unlikely.

Following the identical procedure DD could be identified as TMS-derivative and quantified also in minute amounts in human plasma and different plant materials (see Tables 1 and 2).

With the same GC–MS technique low levels of D were observed in most animal brains (see Table 1) as well as some plant materials investigated (see Table 2). Again the different GC–MS tracings (see Figs 2 and 3) indicated identity between the chemical reference of D and the purified biological material.

DISCUSSION

There is accumulating evidence that DD and D represent natural products. As has been documented in two previous studies by specific mass spectrometry, D and its biologically active metabolite are present in brain of mammalian species, such as bovine, rat and even man [1, 2]. Our study not only confirms these interesting results but also extends

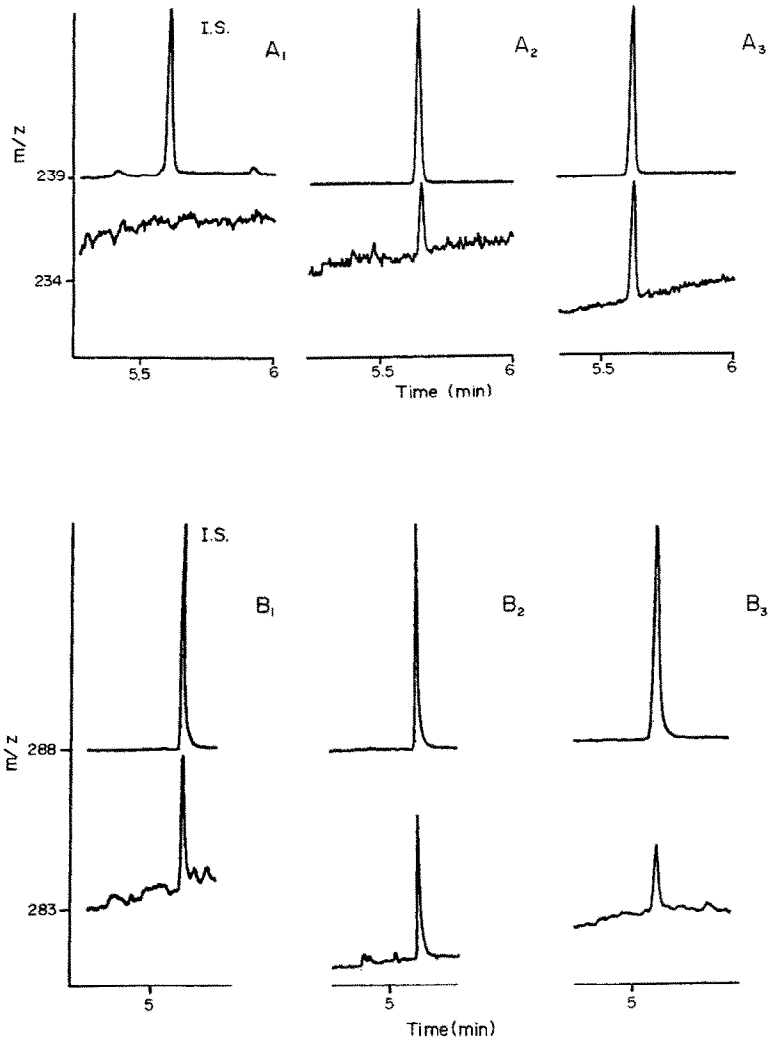

Fig. 3. GC–MS tracings in negative ion chemical ionization mode. A: Desmethyldiazepam (DD) as TMS-derivative; A_1: Tris buffer blank + 15 ng of d_5-DD; A_2: Extract of 10 g rat brain + 15 ng of d_5-DD; A_3: Reference standard of 100 pg of DD and of 2.25 ng d_5-DD; B: Diazepam (D); B_1: Standard of 40 pg D and of 1 ng d_5-D; B_2: Extract of 10 g soy beans spiked with 10 ng of d_5-D; B_3: Extract of 10 g chicken brain spiked with 10 ng of d_5-D.

Table 2. Concentration range (ng/g) of desmethyldiazepam (DD) and diazepam (D) in extracts of different plants as quantified by gas chromatography–mass spectrometry with stable isotope dilution technique

Plant products	DD	D
Potato tuber	b.d.l.	0.003–0.01
Brown lentils	0.008–0.02	b.d.l.
Yellow soy beans	0.004–0.006	0.006–0.05
Unpealed rice	0.003–0.004	0.006–0.05
Maize corn	0.005–0.015	b.d.l.
Mushrooms*	b.d.l.	0.002–0.003

b.d.l.: below detection limit of 0.003 ng/ml.
* *Agaricus bsalliota prunnescens*.

them to several mammalian and non-mammalian species. Since DD and D could be detected also in fish, frog and monitor (reptile), it appears that the brain of a wide range of animals contains these BZD. According to our comparative measurements indicating higher brain levels by RRA than by GC–MS (see Table 1), one could speculate that additionally other BZDs or at least cross-reacting BZD-like material are present in these tissues.

However, since the values generated by specific GC–MS analysis appear more reliable we have applied this technique also to human plasma and plant materials. When exogenous DD or D was spiked to different biological samples a linear and proportionate increase (see also Fig. 1) in the BZD content was measurable and when the added amount was subtracted the levels were identical to the results obtained in parallel with unspiked material. This indicates that during the purification procedure both BZD and their corresponding internal standards

were well extracted from the biological samples and that the SIM-analyses measured DD or D accurately and specifically.

In human plasma we could quantify only DD in very low concentrations (0.003–0.01 ng/ml) if 20 ml were extracted (see Table 1). Therefore it is understandable that recently no DD was detected in serum of different volunteers by a less sensitive (lower limit 1 ng/ml) GC-assay [7]. Thus, depending on the applied analytical methods and their sensitivity limits different conclusions might be drawn.

It is noteworthy that our observed brain concentrations (range 0.05–0.04 ng/g wet weight) were considerably lower than those reported by Sangameswaran *et al.* [1] (6–600 ng/g) and Wildmann *et al.* [2] (3–5 ng/g). In one study [2] Wildmann *et al.* added tritiated D to a sample of rat cortex for testing the recovery of DD only up to the HPLC-separation; in the other studies [1, 3] no internal standard at all was used. Normally, a suitable internal standard should be added to all biological samples and transferred through the complete and final analyses. This can best be accomplished by stable isotope dilution technique which was applied to each of our biological samples. Therefore the observed discrepancy could be most likely attributed to differences in methodology.

Another simple calculation should be considered. CSF/plasma concentration ratios of BZD range normally between 0.02 and 0.05 [8]. Based on the plasma protein binding of DD (97.6%) [9] and assuming that DD in the brain is in equilibrium with the free form in the blood, brain levels of unbound DD in the range of 3–600 ng/g would theoretically result in plasma levels which should have been detected in blank plasma by specific GC-methods with electron capture detection (sensitivity limit around 1 ng/ml). Neither in our laboratories [9] nor in others [7] could DD be detected in human plasma with such methods. Thus, it is conceivable that our measured low brain levels might represent a somewhat more realistic picture, especially if one remembers that concentrations between 3 and 30 ng/g tissue are obtained after therapeutic doses of BZD [10]. Similarly, we measured in the brain of one stillborn a DD level of 2.24 ng/g wet weight 12 hr after the delivering mother had received 10 mg D. In the brain of a BZD-free adult man only 0.008 ng DD/g and traces of D were found. In another stillborn whose delivering mother claimed not to have taken any BZD a higher level of DD (0.51 ng/g) could be detected.

Likewise, the reported "endogenous" brain levels between 3 and 600 ng DD/g [1, 2] would implicate that some pharmacological biological activity should have been noted in those species. However, it might also be possible that the constant exposure to DD has induced tolerance.

According to the recent studies of Wildmann *et al.* [2, 3] and our results DD and D were also detected in different plants which serve animals and man as natural food. Whereas Wildmann *et al.* [2, 3] estimated DD and D (0.1–0.4 ng/g) in wheat grains and potato tuber by GC–MS, we detected DD (0.005–0.02 ng/g) in lentils, soy beans, rice and maize corn and D (0.003 to 0.05 ng/g) in potatoes, soy beans, rice and mushrooms (see Table 2). The origin and/or the biosynthesis of both BZD are still unknown. It remains to be investigated whether animals are able to synthesize them or whether exogenous sources (e.g. microorganisms, plants) account for the presence of minute amounts in the body of different species. Based on our measurements in hen, chicken and egg-white (see Table 1) DD appears very early during ontogenesis or maturation and it is likely to be incorporated via the food chain. In this context, it is noteworthy that cyclopeptine, a benzodiazepine alkaloid can be formed from anthranilic acid, L-phenylalanine and L-methionine by *penicillium cyclopium* [11]. Similarly, antramycin which is a bacterial product of *Streptomyces refuineus*, contains the basic 1,4-benzodiazepine structure [12].

In all studies it was tried rigorously to exclude laboratory contaminations, therefore evidence is accumulating that BZD present in animals and plants are of natural origin. The crucial question is whether or not the brain levels of DD or D play any biological role or are of clinical significance. Our detected concentrations of DD or D were much lower 0.02–0.15 pmol) than their K_D-values of *in vitro* binding studies (10–20 nmol). This would indicate that they neither act as nutritionally relevant ligands nor induce direct pharmacological effects.

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