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OCCURRENCE OF PHARMACOLOGICALLY ACTIVE BENZODIAZEPINES IN TRACE AMOUNTS IN WHEAT AND POTATO

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Abstract—Aqueous acid extracts of wheat grains and of potato tuber were found to contain a series of compounds displaying a high affinity to the central type benzodiazepine receptor (BZR) in mammalian brain. Further analysis using different HPLC systems, as well as mass spectrometry and gas chromatography combined with mass spectrometry lead to the identification of compounds belonging to the classical 5-phenyl-1,4-benzodiazepinones. In wheat grains diazepam, *N*-desmethyldiazepam, delorazepam, deschloro-diazepam, delormetazepam, lormetazepam and isodiazepam were identified, while potato tuber contained diazepam, *N*-desmethyldiazepam, delorazepam, lorazepam and delormetazepam. The concentration of the benzodiazepines (BZ) was in the low ppb range. Their biosynthesis most probably takes place in the plant tissue. The availability of BZs in plant nutritives points to a possible source for the previously reported presence of BZ in brain and peripheral tissues of several animal species and man.

The synaptic action of the neurotransmitter γ-aminobutyric acid (GABA)† in the central nervous system can be modulated in a bidirectional fashion by compounds acting at the benzodiazepine receptor (BZR), an allosteric modulatory site of the $GABA_A$ receptor complex. Agonists of the BZR which encompass the clinically useful tranquillizing benzodiazepines (BZ), enhance the effect of GABA and thereby reduce the level of anxiety, vigilance, muscle tension and the likelihood of convulsions. Inverse agonists of BZR reduce the effect of GABA and consequently show pharmacological effects opposite to those of agonists [1–3]. Because of this unique modulatory mechanism by synthetic compounds, the BZR was presumed to be uniquely suited also for the physiological regulation of anxiety, sleep and muscle tension through endogenous ligands of the BZR, of which tranquillizing as well as excitatory examples were postulated to exist. Recently, the benzodiazepines *N*-desmethyldiazepam and diazepam were found to be present in trace amounts in the brain [4–8] and peripheral organs [8] of animals and man not exposed to BZ-treatment, which raised the possibility that BZ themselves might act as endogenous ligands. We report here that major nutritives contain a variety of benzodiazepines with high affinity to the BZR. This finding provides a possible source for the presence of benzodiazepines in various organs of animals and man.

MATERIALS AND METHODS

Extracts of grains of wheat. Wheat was randomly collected from fields up to 600 km apart. Grains from a defined strain of wheat (Arina) were obtained from Maag AG, Dielsdorf, Switzerland, including samples of strain Arina, which were not treated with either herbicides or fungicides. After stirring 50 g of grains of wheat in a plastic beaker in 50 ml of bidistilled water at room temperature for 1–2 min, 50 ml of 2 N hydrochloric acid were added. Fifteen minutes later the pH was adjusted to 4.8 by adding 50 ml of 2 N sodium hydroxide followed by centrifugation at 20,000 *g* for 20 min. The supernatant was loaded onto a C-18 Sep-pak cartridge (Waters Associates), which had been rinsed several times with bidistilled water and methanol. The external surface of cartridges was carefully washed in order to avoid adhesive C-18 material to interfere with the radioligand binding assay to be performed with the eluted samples. After washing the column with 10 ml of water, the cartridge was eluted with 1 ml of methanol. The eluate was evaporated to dryness *in vacuo* and dissolved in 1.0 ml of 10% acetonitrile in H_2O ("crude extract") to be used for radioligand binding and HPLC analysis.

In some experiments, the supernatant (about 130 ml) was extracted by shaking with 40 ml of ethyl acetate in a separation funnel. The organic layer was evaporated to dryness *in vacuo* and the residue was dissolved in 1 ml of 10% acetonitrile in H_2O ("crude extract").

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† Abbreviations used BBI, benzodiazepine-binding inhibition; BZ, benzodiazepine; BZR, benzodiazepine receptor; DE, diazepam equivalent; GABA, γ-aminobutyric acid; HPLC, high performance liquid chromatography; GC, gas chromatography; MS, mass spectrometry.

Extracts of potatoes. Potatoes were purchased from different local plantations and analyzed soon after the harvesting in 1986. After peeling, 20 g of tuber marrow, which is considered to be sterile, were homogenized in 50 ml of bidistilled water using an Ultra-Turrax homogenizer (2 min, at low speed). After adding 50 ml of 2 N hydrochloric acid the sample was stirred for 15 min and the pH adjusted to 4.8 by adding 50 ml of 2 N sodium hydroxide. All subsequent steps were the same as described above for the wheat samples.

HPLC analysis. Aliquots of crude extracts were chromatographed on a C-18 Nucleosil reversed phase HPLC column (particle size 5 μm, Macherey and Nagel, Düren, F.R.G.) equilibrated with 10% acetonitrile, 0.1% trifluoroacetic acid in H_2O (eluent A). The column was developed with a 0–60% linear gradient of 90% acetonitrile, 10% H_2O 0.1% trifluoroacetic acid (eluent B) over 60 min (ACN system). In order to alter the elution profile, a second C-18 column was developed with a linear gradient of 45% tetrahydrofuran, 45% acetonitrile, 10% H_2O, 0.1% trifluoroacetic acid (eluent A) and 5% tetrahydrofuran, 5% acetonitrile, 90% H_2O, 0.1% trifluoroacetic acid (eluent B) referred to as the THF system. Fractions of 1 min or single peaks (UV monitoring) were collected, brought to dryness and dissolved in 50–200 μl of 10% acetonitrile. Reference compounds were passed through the HPLC-column only after an entire crude extract had been analysed (5–6 HPLC runs). Before reloading, the column was rinsed by at least 3 blank runs of which the last one was monitored for the lack of BBI activity (see below). When the retention times found for the two different HPLC systems appeared to be identical with the retention times of a known BZ reference, samples were subjected to mass spectrometry or gas chromatography–mass spectrometry with selected ion monitoring.

Benzodiazepine binding inhibitory (BBI) activity. Crude extracts and all of the HPLC fractions were tested for their ability to inhibit the binding of the BZ antagonist flumazenil [9] ^{3}H-Ro 15-1788 (71.4 Ci/mmol, New England Nuclear) to the BZR in rat brain membranes [10]. HPLC fractions exhibiting at least 30% displacement (BBI activity) were collected, brought to dryness and stored at 4° until further processing by the second HPLC-system, by MS or GC/MS. The amount of displacing agent was assessed in terms of the amount of diazepam which causes the same % of displacement. It was expressed as ng diazepam equivalents (DE).

Mass spectrometry (MS). For MS analysis [8] those HPLC fractions were selected which contained only a single UV peak containing high activity in the BBI assay.

Gas chromatography–mass spectrometry (GC MS). A Hewlett-Packard mass specific detector (HP 5970) coupled via an open split interface to a capillary gas chromatograph (HP 5890) equipped with a crosslinked column HP Ultra-1 (length 12.5 m, ϕ 0.2 mm) was used to identify the BZ by the selected ion monitoring technique (SIM, cycle time 0.5 sec; electron ionisation at 70 eV). The GC oven was programmed with a rate of 25°/min from 90° up to 290°. Sample aliquots of 5 μl were injected in splitless mode. HPLC-fractions with retention times corresponding to *N*-desmethyldiazepam, lorazepam and delorazepam, were silylated using a solution of *N*-methyl - *N* - (tert - butyldimethylsilyl) - trifluoroacetamide in ethyl acetate 1/10 (v/v) and left for 1 hr at ambient temperature. Five to ten peaks of the mass spectrum (including the natural carbon and chlorine isotope pattern) considered to be characteristic for the BZ or their derivatives were monitored. Fragmentation pattern and retention times were checked by injecting 1 μl of solutions containing 10, 1 and 0.1 ng of the reference compound. Prior to the unknown sample, 4 μl of a blank solution (ethyl acetate with silylating agent or a HPLC fraction containing none of the BZs looked for) were injected to prove the absence of any memory effect in the gas chromatography system. The compounds were identified by comparing their retention times and the pattern of the selected peaks with those of reference benzodiazepines. Duplicate or triplicate analysis of the samples was carried out. Since no internal standard was used, only a rough estimate of the BZ content could be made.

RESULTS

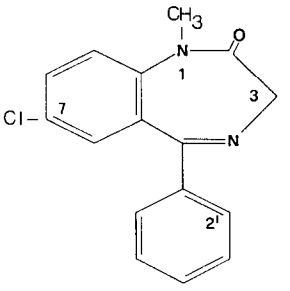
Material which inhibited the binding of ^{3}H-Ro 15-1788 to the BZR was found in crude extracts of samples of randomly collected grains of wheat as well as in wheat (strain Arina) which had not been subjected to plant protective chemicals (Table 1). When expressed in terms of diazepam equivalents

Table 1. BBI activity in crude extracts (Sep-pak) of grains of wheat from different sources

Sample No.*	Strain	Treatment with herbicide/fungicide	BBI activity† (ng DE/50 g grains)
1	Arina	Foxtril/Corbel Top and Cycocel Extra	80–100
2	Arina	Foxtril/none	160–180
3	Arina	None/none	200–220
4	Unknown	Unknown	120–140
5	Unknown	Unknown	140–160
6	Unknown	Unknown	180–190
7	Unknown	Unknown	160–190

* Samples 1–3 were from Maag AG, Dielsdorf, Switzerland, the others were freshly harvested from random locations up to 600 km apart.

† BBI activity was estimated in at least 3 samples.



DIAZEPAM

Compound	IC 50 nmol	Estimation of content in 50 g Wheat: Radioligand binding (ng DE)	50 g Wheat: GC/MS (ng)	20 g Potato: Radioligand binding (ng DE)	20 g Potato: GC/MS (ng)
deschlorodiazepam	370	-	5	-	-
N-desmethyldiazepam	9.2	20	20	10	10
diazepam	8.6	10	10	5	5
7-deschloro-2'-chlorodiazepam	3.8	10	5	-	-
lorazepam (N-desmethyl-3-hydroxy-2'-chlorodiazepam)	1.6	-	-	5	1
delorazepam (N-desmethyl-2'-chlorodiazepam)	1.8	20 - 50	10	10 - 20	5
lormetazepam (3-hydroxy-2'-chlorodiazepam)	1.4	10	2	-	-
2'-chlorodiazepam	1.6	5	1	5	1

Fig. 1. Summary of BZs found in various fractions of HPLC extracts from wheat grains and from potato. The amounts of BZ refer to 50 g of wheat grains (strain Arina not treated with herbicides or pesticides) or 20 g of potato tuber estimated by their BBI activity (ng DE) and by GC-MS analysis (ng). The values obtained with both methods were in the same range. Nearly all BZ show a very high affinity to BZR as indicated by the IC_{50} values of the synthetic BZ in ^{3}H-Ro 15-1788 binding. Compounds which were not identified in the respective plant extracts are indicated by —.

the amount of displacing material was in the low ppb range (Table 1). When the crude extracts were analyzed by HPLC distinct fractions could be identified containing material which inhibited radioligand binding to the BZR.

In order to identify this material chemically, the extracts of grains of wheat which had not been treated with plant protective chemicals were subjected to GC-MS analysis. This analysis was performed with those HPLC fractions (Fig. 2b) which contained a high benzodiazepine binding inhibitory activity (BBI activity) and showed a retention time corresponding to a known benzodiazepine. Those HPLC peaks which showed a retention time similar to the respective reference BZ, were analyzed by GC-MS with selective ion monitoring. By comparing the GC retention time and the characteristic fragmentation pattern with synthetic references the following compounds were identified: *N*-desmethyldiazepam (Fig. 5), diazepam (Fig. 6), lormetazepam (2'-chloro-3-hydroxydiazepam, Fig. 10), delorazepam (2'-chloro-*N*-desmethyldiazepam, Fig. 9), 2'-chlorodiazepam (Fig. 11), the 2'-chloro isomer of diazepam (Fig. 7) and deschlorodiazepam (Fig. 12). All of these compounds belong to the classical 5-phenyl-1,4-benzodiazepinones, of which diazepam is the therapeutically most relevant representative. Apart from deschlorodiazepam all compounds showed a high affinity to the BZR *in vitro* with half maximal displacement of ^{3}H-flumazenil in the low nM range (Fig. 1).

The amount of benzodiazepine present in a HPLC fraction was estimated by its potency to displace ^{3}H-flumazenil from the BZR in brain membranes as compared to a known amount of diazepam. These values of diazepam equivalents were converted to ng of the particular BZ using the ratio of the IC_{50} values of the BZ in question to that of diazepam. The values calculated from the radioligand binding data corresponded to the amount of the BZ estimated from the GC-MS analysis. The concentrations of BZ were in the range of 0.02–0.4 μg per kg of dry wheat grains (Fig. 1). In some cases crude extracts of grain of wheat Arina were prepared also by ethyl acetate extraction instead of purification via Sep-pak. The aqueous supernatant contained amounts of active material similar to that obtained after Sep-pak purifications. However, the HPLC eluate profile showed less interfering material in ethyl acetate extracts than in Sep-pak-treated samples.

Since the presence of benzodiazepines in wheat was unexpected, potatoes as another major nutritive were analysed. From extracts (ethyl acetate procedure) of tuber marrow of potatoes HPLC fractions could be isolated which showed inhibition of ^{3}H-flumazenil binding to brain membranes (Fig. 2c). In such fractions the following benzodiazepines were identified by GC-MS: lorazepam (3-hydroxy-2'-chloro-*N*-desmethyldiazepam; Fig. 8) and, as in wheat samples, *N*-desmethyldiazepam, diazepam, 2'-chlorodiazepam and delorazepam (spectra not shown).

Since the HPLC fraction which contained delorazepam appeared to be a single peak, a MS analysis was attempted in this particular case. Fraction 15, showing a retention time of delorazepam, of the HPLC eluate in the acetonitrile system (Fig. 2c), was rechromatographed on HPLC using the tetrahydrofuran system (Fig. 3). One fraction (No. 14) contained nearly all of the BBI activity (Fig. 3),

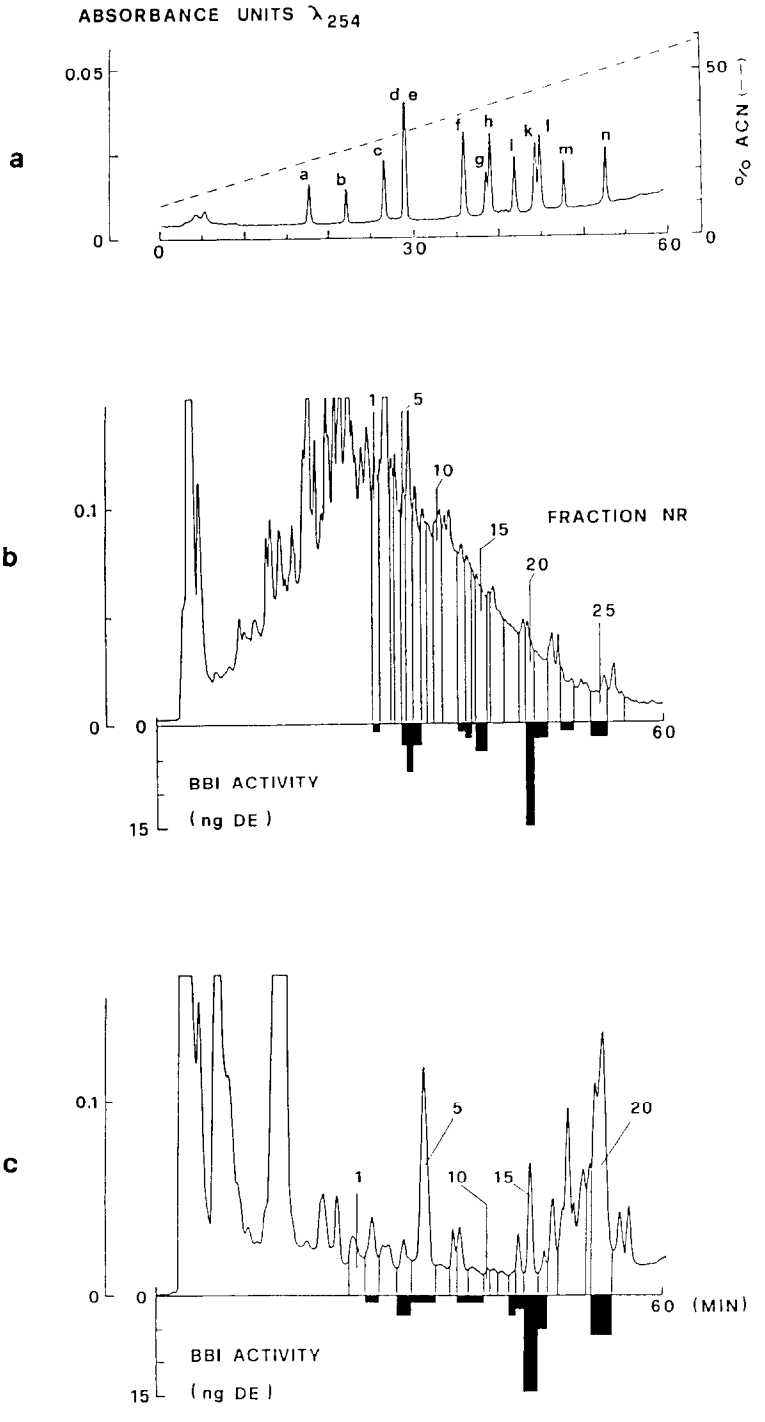


Fig. 2. HPLC elution pattern [acetonitrile (ACN) system] of extracts from grains of wheat (Fig. 2b; Sep-pak extract from 10 g of grains from strain Arina untreated) and potatoes (Fig. 2c; ethyl acetate extract from 5 g of potato tuber). Fractions of variable sizes were collected (1–25) and tested in ^{3}H-Ro 15-1788 binding. The BBI activity is shown as downward column under the corresponding HPLC fraction and expressed in terms of DE. The most active peaks were rechromatographed in the tetrahydrofurane (THF) system (Fig. 3) before analysis by gas chromatography-mass spectrometry. The HPLC retention times of BZ references (Fig. 2a) are given for comparison [*N*-desmethyl-deschlorodiazepam (a), deschlorodiazepam (b), deschloro-3-hydroxydiazepam (c), nordiazepam (d), *N*-desmethyl-isodiazepam (e), diazepam (f), 7′-deschloro-2′-chlorodiazepam (g), oxazepam (*N*-desmethyl-3-hydroxydiazepam) (h), lorazepam (i), delorazepam (k), temazepam (3-hydroxydiazepam) (l), lormetazepam (m), 2′-chlorodiazepam (n)].

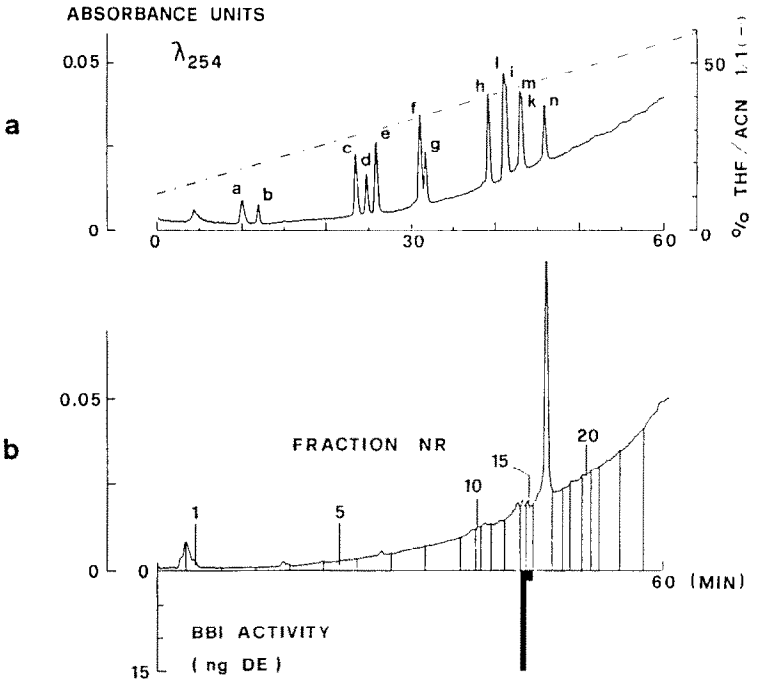


Fig. 3. Rechromatography of the HPLC fraction No. 15 of potato extracts (Fig. 2c) in a HPLC tetrahydrofurane system (b). Almost the entire BBI activity eluted in fraction 14 which was subjected to mass spectrometry (Fig. 4). The retention times of BZ references (see Fig. 2a) are given for comparison (a).

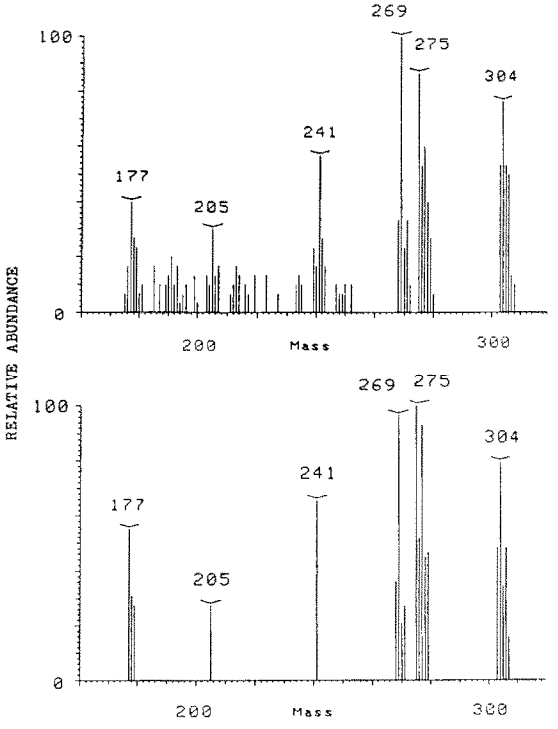


Fig. 4. Mass spectrometric identification of delorazepam in an HPLC fraction from a potato ethyl acetate extract. The relevant fragmentation profile including the isotope pattern of natural chlorine (top) obtained from the HPLC fraction No. 14 (elution profile Fig. 3b) corresponded to the spectrum obtained with 10 ng delorazepam (bottom). Samples were introduced directly into the ionisation chamber after deposition onto the tip of a glass rod from a methanolic solution (MS-9; 70 eV, ion source temperature 250°).

which again eluted with the retention time of delorazepam. In this fraction delorazepam was identified by MS (Fig. 4). Thus, apart from lorazepam, all the benzodiazepines identified in potato also occurred in the wheat samples, suggesting a wide distribution of these compounds in plant nutritives.

The concentrations of benzodiazepines in the HPLC fractions of potato extracts were assessed as described above for the wheat samples and were found to vary between 0.04 and 0.1 μg/kg of potato tuber (Table 1).

In order to avoid any contamination in the extraction and detection procedure, a solvent blank without plant tissue was processed in the same glass ware and columns which were used later for the extraction and analysis of the plant tissue. In HPLC fractions from such blank runs, no displacement of ^{3}H-flumazenil binding could be detected.

Among the benzodiazepines identified in plants three were monochlorinated and four dichlorinated. It was therefore tested whether a chlorination of benzodiazepines could occur during the extraction with 1 N HCl (15 min, room temp.). When 5 ng of ^{3}H-diazepam (1.25 μCi, 90 Ci/mmol, New England Nuclear; purified on HPLC before use) was added to the extraction solution for 20 g of potato, only ^{3}H-diazepam could be detected in the HPLC analysis of the extract. No radioactivity was found in the fraction corresponding to 2'-chlorodiazepam. Thus, additional chlorination of mono-chlorinated BZ due to the extraction procedure can be excluded.

DISCUSSION

We have found that wheat and potatoes contain a series of 5-phenyl-1,4-benzodiazepinones (Fig. 1).

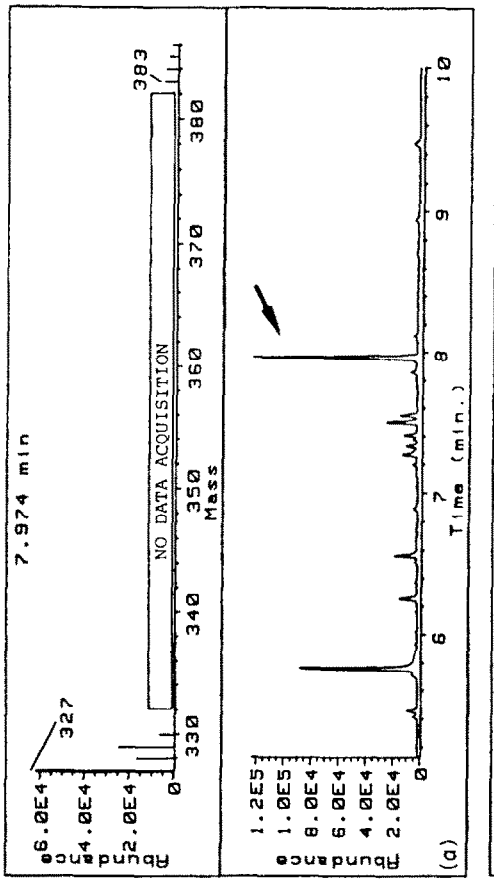


Fig. 5. Identification of nordiazepam by GC-MS analysis in wheat extracts in HPLC fraction (retention time 29 min). After silylation the GC retention time (a, lower panel, arrow) and the relative intensities of the characteristic peaks chosen for SIM (a, upper panel) were identical to those obtained from 1 ng *N*-desmethyl-diazepam-tBDS (b, lower and upper panel, respectively) (tBDS = tertiary butyl dimethylsilyl).

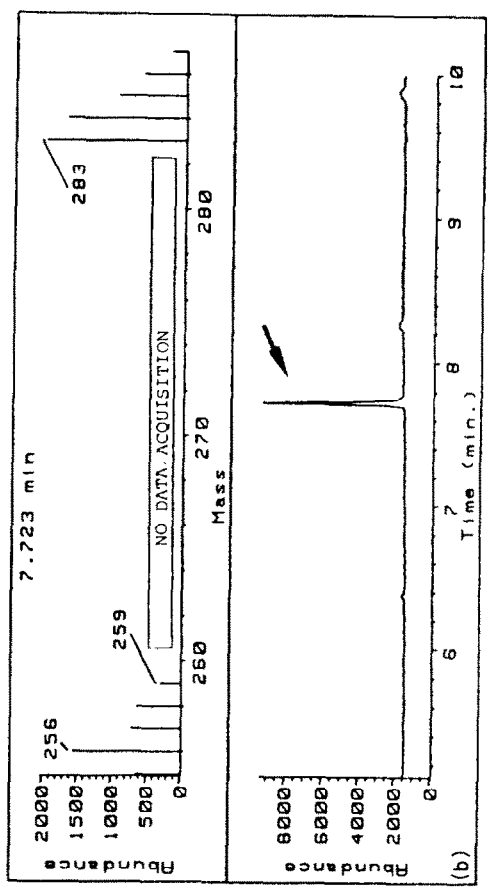


Fig. 6. Identification of diazepam by GC-MS analysis in HPLC fraction (36 min) of wheat extracts. The GC retention time (a, lower panel, arrow) and the relative intensities of the characteristic peaks chosen for SIM (a, upper panel) were identical to those obtained with 1 ng diazepam (b, lower and upper panel, respectively).

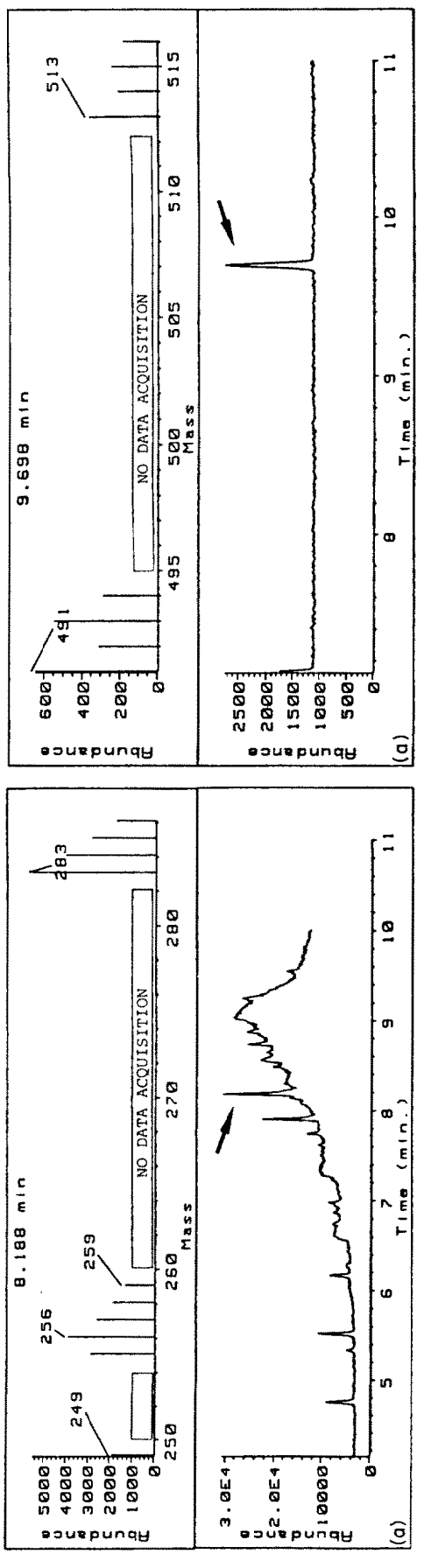


Fig. 7. Identification of 7'-deschloro-2'-chlorodiazepam by GC-MS analysis in HPLC fraction (38 min) of wheat extracts. The GC retention time (a, lower panel, arrow) and the relative intensities of the characteristic peaks chosen for SIM (a, upper panel) were identical to those obtained with 200 pg 7'-deschloro-2'-chlorodiazepam (b, lower and upper panel, respectively).

Fig. 8. Identification of lorazepam by GC-MS analysis in HPLC fraction (42 min) of potato extracts. After silylation the GC retention time (a, lower panel, arrow) and the relative intensities of the characteristic peaks chosen for SIM (a, upper panel) were identical to those obtained with 1 ng lorazepam-tBDS (b, lower and upper panel, respectively).

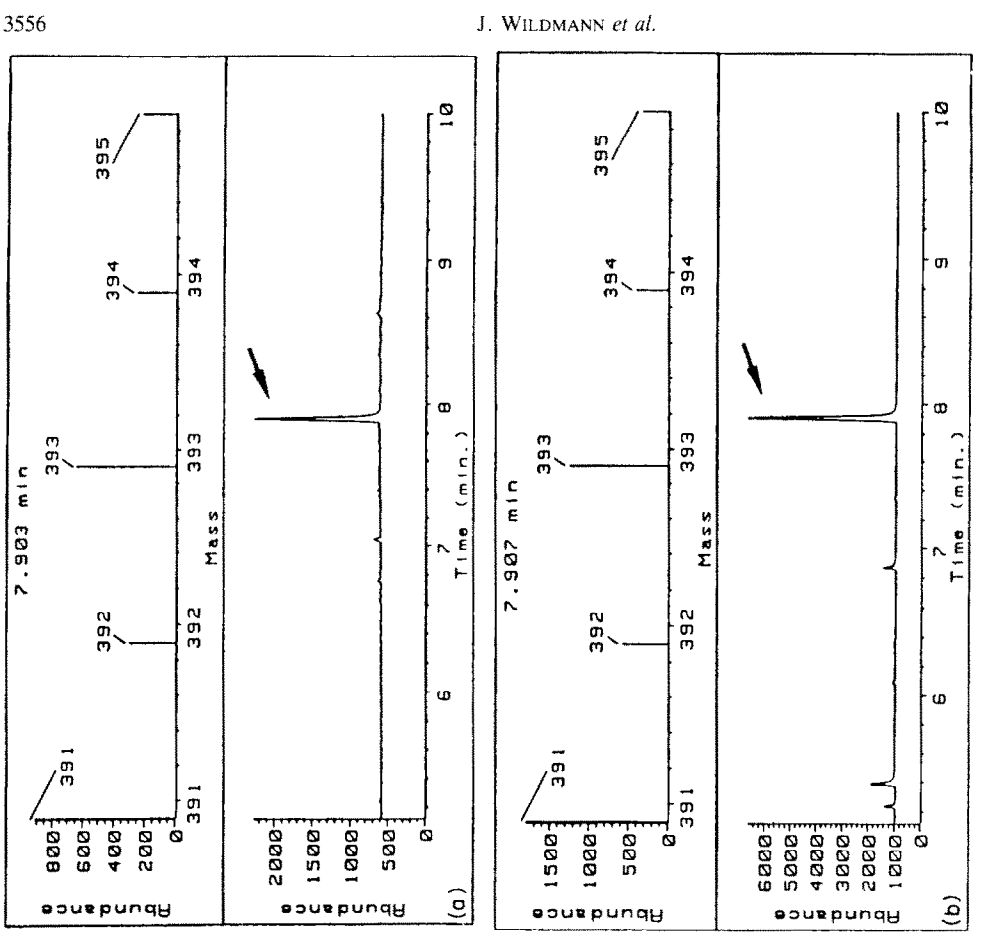


Fig. 9. Identification of delorazepam by GC-MS analysis in HPLC fraction (45 min) of wheat extracts. After silylation the GC retention time (a, lower panel, arrow) and the relative intensities of the characteristic peaks chosen for SIM (a, upper panel) were identical to those of 1 ng delorazepam-tBDS (b, lower and upper panel, respectively).

Fig. 10. Identification of lormetazepam by GC-MS analysis in HPLC fraction (48 min) of wheat extracts. After silylation the GC retention time (a, lower panel, arrow) and the relative intensities of the characteristic peaks chosen for SIM (a, upper panel) were identical to those of 1 ng lormetazepam-tBDS (b, lower and upper panel, respectively).

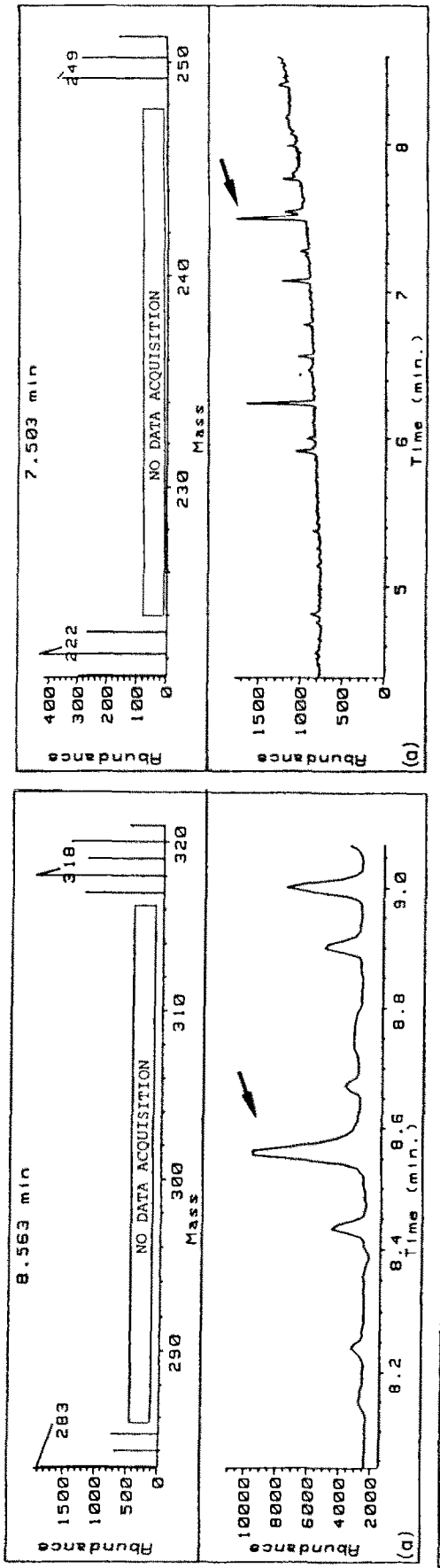


Fig. 11. Identification of 2'-chlorodiazepam by GC-MS analysis in HPLC fraction (53 min) of wheat extracts. The GC retention time (a, lower panel, arrow) and the relative intensities of the characteristic peaks chosen for SIM (a, upper panel) were identical to those of 1 ng deliormetazepam (b, lower and upper panel, respectively).

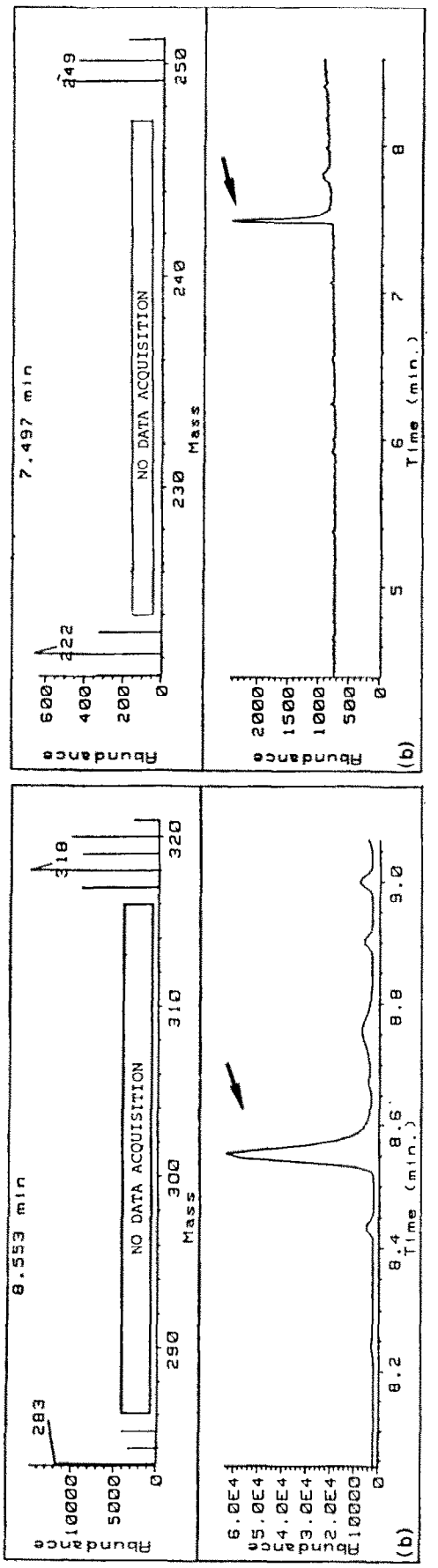


Fig. 12. Identification of deschlordiazepam by GC-MS analysis in HPLC fraction (23 min) of wheat extracts. The GC retention time (a, lower panel, arrow) and the relative intensities of the characteristic peaks chosen for SIM (a, upper panel) were identical to those of 200 pg deschlordiazepam (b, lower and upper panel, respectively).

The compounds were identified by their high affinity to the BZR, their retention times in HPLC systems, their retention times in GC and analysis by MS. These benzodiazepines are presumably not of microbial origin. Although microorganisms are known to synthesize complex benzodiazepine derivatives [11, 12] such as the antibiotic anthramycin [13] and the cholecystokinin-antagonist asperlicin [14], microbial BZ interacting with BZR have not been described. Furthermore, these BZ were found in potato marrow which is considered to be sterile. Plants have the enzymatic prerequisites to produce chlorinated compounds [15–17]. These considerations do not completely exclude microorganisms as a site of synthesis; however, a high rate of microbial synthesis would be required to furnish the plant tissue with BZ by diffusion.

It is most surprising that the chemical structures of some of the benzodiazepines found in plants are identical to those of benzodiazepine drugs used in the current therapy of anxiety, insomnia, muscle spasms and epilepsy (e.g. diazepam, lorazepam, lormetazepam, delorazepam, Fig. 1). Their main representative, diazepam, was chemically synthesized 30 years ago without prior knowledge of the occurrence of this chemical entity in plants. The presence of its target site, the BZR [18, 19], in the central nervous system was likewise unknown at the time.

Recently, *N*-desmethyldiazepam was identified in the brain of rats and man [7]. In another study, diazepam and *N*-desmethyldiazepam were found in brain and adrenals of rats [8], suggesting a physiological presence of these and possibly other benzodiazepines in higher organisms. The occurrence of pharmacologically active BZ in plant nutritives now could provide an explanation for the presence of benzodiazepines in animals and man. Our findings do not support the notion that the benzodiazepines found in brain tissue are endogenously synthesized.

The amount of pharmacologically active benzodiazepines which can be ingested by animals or man on a diet containing wheat and potato seem to be well below pharmacologically active doses. While a single therapeutic dose of diazepam in man ranges from 5 to 20 mg, 1 kg of wheat contains only a few μg of pharmacologically active benzodiazepines (Fig. 1). It will be of interest to study whether the chronic intake of trace amounts of benzodiazepines from plant diets can lead to their accumulation in the brain. In brains of drug free rats amounts of 5–10 μg/kg ($2–4 \times 10^{-8}$ M) of benzodiazepines (diazepam + *N*-desmethyldiazepam) have been found [8].

The industrial synthesis of benzodiazepines used in therapy is unlikely to contribute to their occurrence in plants. The spectrum of benzodiazepines found in plants does not correlate with the prevalence of marketed benzodiazepines. For instance, dichlorinated BZ are available only recently but occur in plants in amounts comparable to those of diazepam which has been available over 30 years. Furthermore isodiazepam and deschlorodiazepam are not marketed and are not metabolites of commercially available BZs. In addition, N-desmethyldiazepam was found in the brain of persons who died before the introduction of benzodiazepines into therapy [7].

It should be noted that the radioreceptor assay procedure by which the plant extracts were screened selected for pharmacologically active benzodiazepines. The occurrence of pharmacologically inactive benzodiazepines in plants cannot be excluded.

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