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Brain concentrations of benzodiazepines are elevated in an animal model of hepatic encephalopathy

(benzodiazepine receptors/fulminant hepatic failure/diazepam/*N*-desmethyldiazepam/mass spectroscopy)

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ABSTRACT **Brain extracts from rats with hepatic encephalopathy due to thioacetamide-induced fulminant hepatic failure contained 4- to 6-fold higher concentrations of substances that inhibit radioligand binding to benzodiazepine receptors than corresponding control rat extracts. Both isocratic and gradient-elution HPLC indicated that this inhibitory activity was localized in 3–8 peaks with retention times corresponding to deschlorodiazepam, deschlorolorazepam, lorazepam, oxazepam, diazepam, and *N*-desmethyldiazepam. The presence of diazepam and *N*-desmethyldiazepam was confirmed by mass spectroscopy. Both mass spectroscopic and radiometric techniques indicated that the concentrations of *N*-desmethyldiazepam and diazepam in brain extracts from encephalopathic rats were 2–9 and 5–7 times higher, respectively, than in control brain extracts. While benzodiazepines have been identified previously in mammalian and plant tissues, this report demonstrates that concentrations of these substances are increased in a pathophysiological condition. These findings provide a rational basis for the use of benzodiazepine receptor antagonists in the management of hepatic encephalopathy in humans.**

Hepatic encephalopathy (HE) is a complex neuropsychiatric disorder arising from the metabolic abnormalities associated with acute or chronic liver failure (1). It has been proposed that an increase in the activity of γ-aminobutyric acid (GABA)-containing pathways contributes to the manifestations of this disorder (2). Studies of visual evoked responses in the galactosamine-treated rabbit model of HE due to fulminant hepatic failure (FHF) (3) and in rats with HE due to thioacetamide (TAA)-induced FHF (4) indicate that central nervous system (CNS) GABAergic tone is increased in these models of HE. Electrophysiologic studies of single cerebellar Purkinje neurons in the rabbit model of HE further support this hypothesis. These neurons are hypersensitive to depression by the GABA receptor agonist muscimol and the benzodiazepine (BZ) receptor agonist flunitrazepam, but not to the α-adrenergic receptor agonist phenylephrine (5). In contrast, BZ receptor antagonists increase the spontaneous firing rate of these neurons and reverse their hypersensitivity to muscimol at concentrations that do not affect the firing rates of control neurons (5). Furthermore, BZ receptor antagonists correct the abnormalities in visual evoked responses associated with HE while transiently reversing the behavioral manifestations of this syndrome in both the rabbit and rat models of this disorder (3, 4).

These observations indicate that the increase in "GABAergic tone" found in HE may be mediated by the BZ receptor, possibly as a consequence of an increase in the concentration or availability of a BZ receptor ligand with agonist (BZ-like) properties (6, 7). Elevated concentrations of substances that competitively and reversibly inhibit radioligand binding to BZ receptors have recently been found in extracts of brain and peripheral tissues from the TAA-treated rat model of HE (8). We have further purified the brain extracts from this model and now report that the substances which inhibit radioligand binding to BZ receptors exhibit chromatographic properties characteristic of BZs. Diazepam and *N*-desmethyldiazepam were chemically identified in these extracts by mass spectroscopy and were present in significantly elevated concentrations in the brains of rats with HE.

METHODS

Animals. The rat model of HE due to TAA-induced FHF was used throughout this study (4). Adult (180–225 g) male Sprague–Dawley rats (Taconic Farms) were maintained in a 12-hr light/dark cycle (lights on at 0700) with free access to rat chow (National Institutes of Health open formula no. 1 rat chow) and water. FHF was induced by the daily intraperitoneal injection of TAA (600 mg/kg in 0.9% NaCl, 5 ml/kg) for 3 consecutive days, along with supportive therapy. Vehicle-treated controls and rats in stage III–IV HE were used in these studies.

Extraction Procedure. Rats were decapitated according to guidelines of the American Association for the Accreditation of Laboratory Animal Care. The brains were rapidly removed and placed in ice-cold isotonic sucrose. Brains were extracted by a modification of the techniques of Osselton (9) and Foerster *et al.* (10). Tissues were homogenized in 4 volumes (vol/wt) of 1 M Tris base (pH 10.9) with alkaline protease (1 mg/g of tissue) by using a Brinkmann Polytron (setting 6.5, 30 sec). Internal standards for monitoring the extraction efficiency of BZs were then added to the homogenate: prazepam (100 ng) was the internal standard for the quantitation of materials by HPLC/radioreceptor assay; diazepam-d_5 and *N*-desmethyldiazepam-d_5 (50, 100, or 200 ng) were used for quantitation by HPLC/mass spectroscopy. The homogenate was then incubated in a water bath with continuous shaking for 1 hr at 55–60°C. At the end of the incubation, the pH was adjusted to 9 with 1 volume of saturated Na_2CO_3. The homogenate was extracted by stirring with 8 volumes of 1-chlorobutane for 20 min, after which the samples were centrifuged (15,000 × *g*, 10 min, 0–4°C) and the organic layer was removed. This step, as well as all subsequent steps, was repeated once. The 1-chlorobutane was then extracted by stirring with 4 volumes of 1 M HCl for 20 min

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Abbreviations: BZ, benzodiazepine; FHF, fulminant hepatic failure; GABA, γ-aminobutyric acid; HE, hepatic encephalopathy; TAA, thioacetamide; TFA, trifluoroacetic acid.

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to remove basic compounds. The aqueous layer was retained and washed once against 8 volumes of 1-chlorobutane. The pH of the aqueous phase was then adjusted to 9 with concentrated ammonium hydroxide to free the bases, then extracted against 8 volumes of 1-chlorobutane. The organic layer was retained, and the 1-chlorobutane was evaporated under vacuum at 50°C. Three to five grams of tissue was routinely extracted for quantitation by radioreceptor assay, whereas 19–30 g of brain was extracted for mass spectral analysis. All glassware was siliconized and was either discarded or acid-washed after use.

Chromatography. Extracts were purified using two HPLC techniques. Samples were applied to either a Novapak C_{18} reverse-phase cartridge in a RCM-100 radial compression module or a μBondapak C_{18} (7.8 × 300 mm) reverse-phase column (Waters/Millipore). Samples applied to the Novapak column were eluted isocratically with 40% 10 mM NH_4-CO_2H/60% methanol (pH 4) at a rate of 2 ml/min for 30 min. Fractions were collected every 18 sec. Absorbance was monitored at 229 nm. The column was then washed with 30 ml of 100% methanol and reequilibrated with 30 ml of the mobile phase. The second elution technique used the μBondapak column and a gradient of 90% trifluoroacetic acid (TFA, 0.143% vol/vol)/10% acetonitrile (solution A) and 10% TFA/90% acetonitrile (solution B), developed at a rate of 0–60% solution B over 120 min at a flow rate of 1 ml/min. Fractions were collected every minute. At the end of each run, the column was washed with 120 ml of 10% TFA/90% acetonitrile and reequilibrated with 60 ml of 90% TFA/10% acetonitrile. All fractions were dried under vacuum at 45°C and stored at −20°C until assayed.

Quantitative Analysis. Extracts were analyzed by a radioreceptor assay or by mass spectroscopy. Radioreceptor analysis was performed on each dried fraction by using [^{3}H]Ro 15-1788 as a ligand (8). The retention times of fractions that inhibited radioligand binding by 10–90% under these standard assay conditions were compared to those of diazepam and *N*-desmethyldiazepam standards. The amount of inhibitory material in each fraction was quantitated by constructing competition curves for known concentrations of these standards assayed under identical conditions. The minimum quantity of diazepam and *N*-desmethyldiazepam reliably detected by this radioreceptor assay was 0.3 and 0.9 ng per assay, respectively. The recovery was determined using the ratio of the known extraction efficiency of the particular BZ to the observed efficiency of the prazepam internal standard. The concentration of material determined by the radioreceptor assay was then corrected for this recovery. The mean extraction efficiency for 10–100 ng of diazepam, *N*-desmethyldiazepam, and prazepam from 1–2 g of control rat brain was 68 ± 7.2% (n = 13), 68 ± 4.8% (n = 13), and 16 ± 5.3% (n = 11), respectively. When the total quantity of inhibitory activity was expressed in "diazepam equivalents," normalization of the quantity of BZ based on extraction efficiency was not used.

Mass spectroscopic analysis was performed using an LKB 2091 spectrometer in the electron-impact mode with 70-eV ionizing voltage and 50-μA ionizing current. The source was maintained at 290°C. The sample was admitted by a direct insertion probe and rapidly heated to 300°C.

Materials. [^{3}H]Ro 15-1788 (specific activity, 78 Ci/mmol; 1 Ci = 37 GBq) was obtained from New England Nuclear. Ro 14-7437, Ro 7-1986, deschlorodiazepam, deschlorolorazepam, diazepam, and *N*-desmethyldiazepam were gifts from Peter Sorter (Hoffmann–La Roche). Oxazepam was a gift from Ayerst Pharmaceuticals. Prazepam, lorazepam, diazepam-d_5, *N*-desmethyldiazepam-d_5, TAA, and alkaline protease were purchased from Sigma. 1-Chlorobutane was purchased from Fluka. Water, methanol, and acetonitrile (Fisher) and TFA (Pierce) for mobile phases were of HPLC grade.

RESULTS

Several fractions obtained from reversed-phase HPLC of control extracts inhibited [^{3}H]Ro 15-1788 binding (Figs. 1*A* and 2). Fractions eluted with the TFA/acetonitrile gradient contained low levels of inhibitory activity, which had reten-

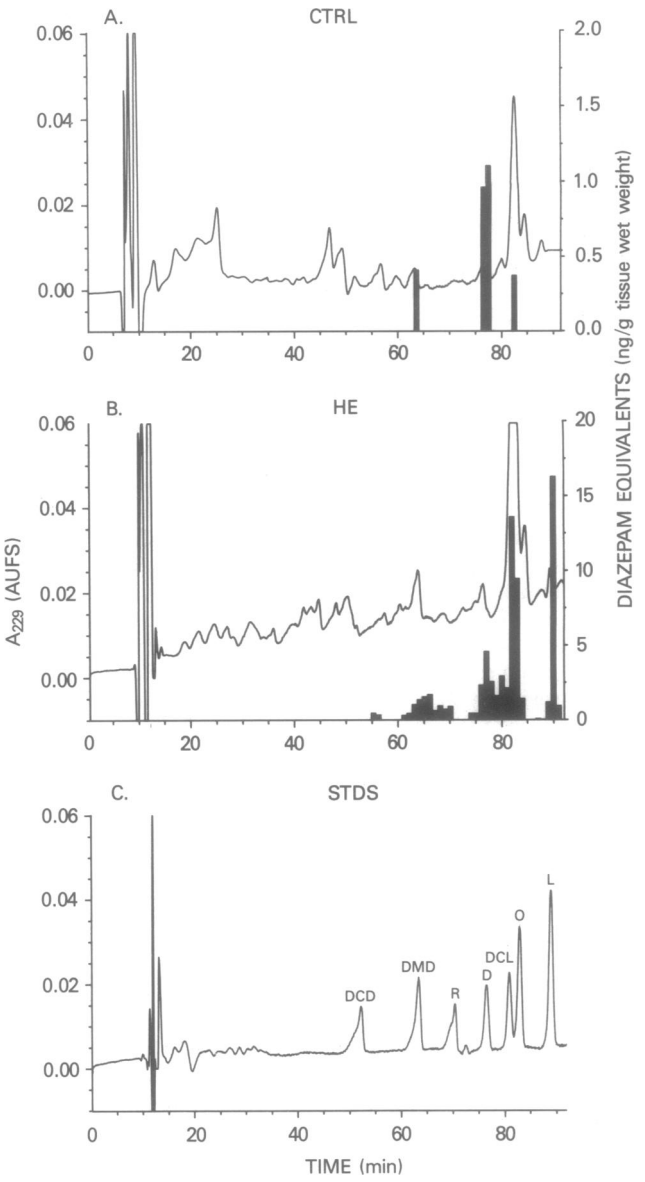

FIG. 1. Representative chromatograms of extracts from control (CTRL) rat brains (19.7 g) (*A*) and the brains (21.6 g) of TAA-treated rats with HE (*B*). The elution profile of BZ standards (STDS, 200 ng each) is also shown (*C*). Materials were eluted with a TFA/acetonitrile gradient. The BZ standards included deschlorodiazepam (DCD; retention time, 52 min), *N*-desmethyldiazepam (DMD; 64 min), Ro 7-1986 (R; 71 min), diazepam (D; 77 min), deschlorolorazepam (DCL; 81 min), oxazepam (O; 83 min), and lorazepam (L; 89 min). UV absorption was monitored at 229 nm (left ordinate AUFS, absorbance units full-scale). The black bars in *A* and *B* represent the inhibition of [^{3}H]Ro 15-1788 binding to BZ receptors expressed as diazepam equivalents (in ng/g of tissue wet weight) in each fraction (right ordinate). Radiometric quantitation was performed on 100-μl aliquots of each 1-ml fraction. The concentrations of *N*-desmethyldiazepam and diazepam were determined to be 0.17 ng/g (fraction 64) and 3.2 ng/g (fractions 76 and 77) in the control rat chromatogram (*A*). The total inhibitory activity was 6.7 ng/g. *N*-Desmethyldiazepam and diazepam concentrations in extracts from TAA-treated rats were 6.7 ng/g (fractions 61–66) and 12 ng/g (fractions 74–78), respectively (*B*). Total inhibitory activity in this brain extract from rats with HE was 72 ng/g.

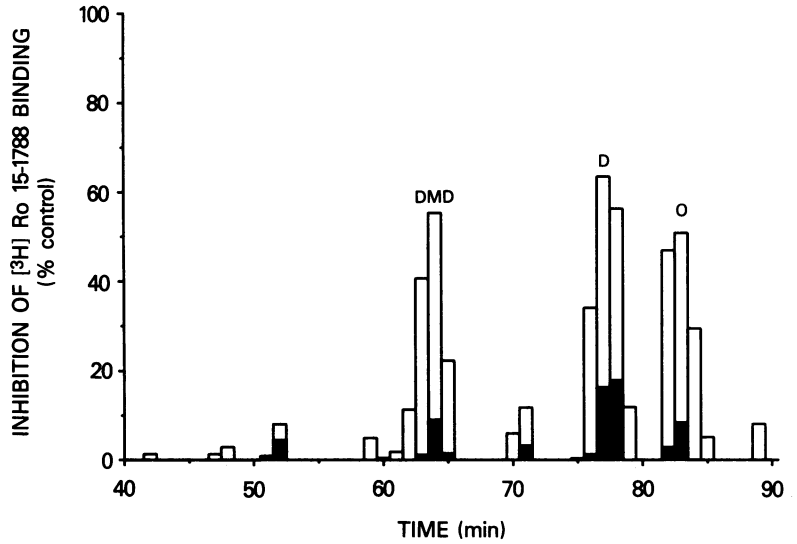

FIG. 2. Comigration of inhibitory fractions from control rat brain extracts (filled bars) with control rat brain extracts spiked with 100 ng of *N*-desmethyldiazepam (DMD), diazepam (D), and oxazepam (O) (open bars). The brain extracts were eluted with a TFA/acetonitrile gradient. Fraction activity is represented as the percentage inhibition of [^{3}H]Ro 15-1788 binding to cerebral BZ receptors by dried 100-μl aliquots of HPLC column fractions. Approximately 19.5 g of control rat brains was extracted for each group.

tion times of 66 ± 0.3, 79 ± 0.3, and 85 ± 0.4 min (mean ± SEM, n = 9–17) corresponding to *N*-desmethyldiazepam (66 ± 0.7 min), diazepam (78 ± 0.5 min), and oxazepam (85 ± 0.4 min, n = 5) standards, respectively (Fig. 1 *A* and *C* and Fig. 2). The prazepam internal standard was eluted with a retention time of 107 ± 0.1 min. The identity of these fractions as BZs was verified by addition of 100-ng quantities of *N*-desmethyldiazepam and diazepam to control brain homogenates, followed by extraction, chromatography, and assay (Fig. 2). The concentrations of *N*-desmethyldiazepam and diazepam found in control rat brain extracts by using this elution gradient followed by a radioreceptor assay were 0.9 ± 0.4 and 2.2 ± 0.6 ng/g of tissue, respectively (Table 1). Fractions from buffer extracts (no tissue) and mobile-phase residues had no effect on the radioligand binding assay.

Extracts from rats with HE contained a larger number of fractions with inhibitory activity than controls (Fig. 1*B*). In addition to fractions corresponding to *N*-desmethyldiazepam, diazepam, and oxazepam (Fig. 1 *B* and *C*), these extracts contained additional active fractions with retention times of 56 ± 0.5, 82 ± 0.4, and 90 ± 0.3 min, corresponding to deschlorodiazepam (56 ± 0.7 min), deschlorolorazepam (83 ± 0.4 min), and lorazepam (90 ± 0.5 min), respectively. All active fractions corresponding to these retention times were not found in every extract from encephalopathic rats. In addition, the retention times of several other active fractions did not correspond to those of any of the BZ standards available for analysis. However, fractions comigrating with diazepam and *N*-desmethyldiazepam were observed in extracts from encephalopathic rats at concentrations approximately 5 and 9 times higher, respectively, than in control extracts (Table 1). The concentration of inhibitory materials in all fractions (expressed as diazepam equivalents in ng/g of tissue) was 4- to 5-fold higher in encephalopathic rats than in control rats (Table 1).

Quantitatively similar results were obtained when extracts were fractionated using isocratic elution and a reverse-phase column with different retention characteristics. This procedure resulted in an elution order and retention times for BZs that were different from those obtained using the gradient elution. The mean retention times for *N*-desmethyldiazepam, diazepam, and prazepam standards on a Novapak column with NH_4CO_2H/methanol elution were 9.6 ± 0.2, 11 ± 0.1, and 27 ± 1.0 min, respectively. The concentrations of *N*-desmethyldiazepam and diazepam in control extracts assayed with the radioreceptor assay were 1.7 ± 1.1 and 37 ± 2.0 ng/g of tissue, with retention times of 9.8 ± 0.2 and 11.9 ± 0.1 min, respectively (Table 1). Extracts of encephalopathic rat brains contained concentrations of *N*-desmethyldiazepam and diazepam (retention times, 9.4 ± 0.1 and 11.9 ± 0.1 min) that were 5- and 7-fold higher than in controls. The total inhibitory activity (expressed in diazepam equivalents) was 6-fold higher in encephalopathic rat brains than in controls (Table 1).

Table 1. Diazepam and *N*-desmethyldiazepam in brain extracts from control rats and from TAA-treated rats with HE

Method	*N*-Desmethyldiazepam, ng/g		Diazepam, ng/g		Total diazepam equivalents, ng/g	
	Control	HE	Control	HE	Control	HE
Gradient HPLC	0.9 ± 0.4	8.5 ± 3.2†	2.2 ± 0.6	11 ± 3.9*	15 ± 4.6	58 ± 13†
Isocratic HPLC	1.7 ± 1.1	8.4 ± 3.0*	3.7 ± 2.0	26 ± 6.4†	15 ± 6.6	84 ± 10‡
Mass spectroscopy	3.1 ± 0.9	7.4 ± 1.3*	3.2 ± 0.2	17 ± 1.6‡		

Extracts from 4–5 g of brain either were applied to a μBondapak C_{18} reverse-phase column and eluted with a 0.143% TFA/acetonitrile gradient of 0–60% acetonitrile developed over 120 min (gradient HPLC) or were applied to a Novapak C_{18} column and eluted isocratically with 40% 10 mM NH_4CO_2H/60% methanol, pH 4, for 30 min (isocratic HPLC). Fractions were taken every 60 or 18 sec, respectively, dried, and assayed for their ability to inhibit radioligand binding to BZ receptors. Mean retention times for the active fractions were correlated with retention times for *N*-desmethyldiazepam, diazepam, and prazepam standards. The recovery of these substances from the extract was based on the ratio of the mean extraction efficiency of diazepam and *N*-desmethyldiazepam and on the observed extraction efficiency of the prazepam standard in the preparation. Total inhibitory activity was determined for all active fractions in the extract and expressed as diazepam equivalents (ng/g of tissue wet weight) without normalization for extraction efficiency. When mass spectroscopy was used, ≈20 g of tissue was extracted and analyzed by the gradient technique described. Diazepam-d_5 and *N*-desmethyldiazepam-d_5 (50–200 ng) were added to the extracts as internal standards. Fractions corresponding to the retention times of the standards were dried into sample cups for mass spectroscopy. Values are the mean ± SEM of 4–11 observations. Symbols: *, $P < 0.05$; †, $P < 0.01$; ‡, $P < 0.001$ compared to control extracts, determined by Student's t test (one-tailed).

The identity and concentration of the substances constituting two of the peaks eluted using gradient HPLC techniques were confirmed by mass spectroscopy (Fig. 3). *N*-Desmethyldiazepam was found to be a component of fractions collected at 58–67 min, while diazepam was a component of fractions obtained at 71–79 min (Fig. 2). In control extracts, the concentrations of *N*-desmethyldiazepam and diazepam determined by this technique were 3.1 ± 0.9 ng/g and 3.2 ± 0.2 ng/g, respectively (Table 1). Mass spectroscopic quantitation of *N*-desmethyldiazepam and diazepam in extracts from encephalopathic rats indicated concentrations that were 2- and 5-fold greater, respectively, than in controls.

DISCUSSION

Electrophysiological and behavioral findings in both rat and rabbit models of HE due to FHF (3–6, 11) strongly suggest that an increase in GABAergic tone contributes to the manifestations of this syndrome. In particular, specific, high-affinity BZ receptor antagonists excite Purkinje neurons, reverse their hypersensitivity to depression by a GABA-mimetic in encephalopathic rabbits (5), and improve the neurologic status of both animal models of HE and humans with HE (3, 4, 12–14). These findings are most readily explained by an increase in the concentration or availability of a substance(s) with agonist (BZ-like) properties. This hypothesis is supported by the demonstration of elevated concentrations of a substance(s) with BZ-like properties in the brain and peripheral tissues of rats with TAA-induced FHF (8), rabbits with galactosamine-induced FHF (15, 16) and the cerebrospinal fluid and plasma of patients with HE (7, 17). However, the chemical identity of these substance(s) was not determined.

Subsequent HPLC separation of brain extracts from TAA-treated rats with HE and control animals resolved several fractions that inhibit radioligand binding to BZ receptors. Using two techniques that separate these substances with markedly different retention times, we found that many, but not all, of these fractions comigrated with known BZs. Identical profiles of fractions containing inhibitory substances were not detected in every extract. Since the origin of these compounds is unknown, the reasons for this variability are speculative. However, this variability may reflect individual differences in gut flora, dietary content of the compounds or their precursors, or the severity and duration of the encephalopathy. Nonetheless, substances comigrating with the *N*-desmethyldiazepam and diazepam standards were consistently present. Mass spectroscopic analysis confirmed the identity of two of these unknown substances as *N*-

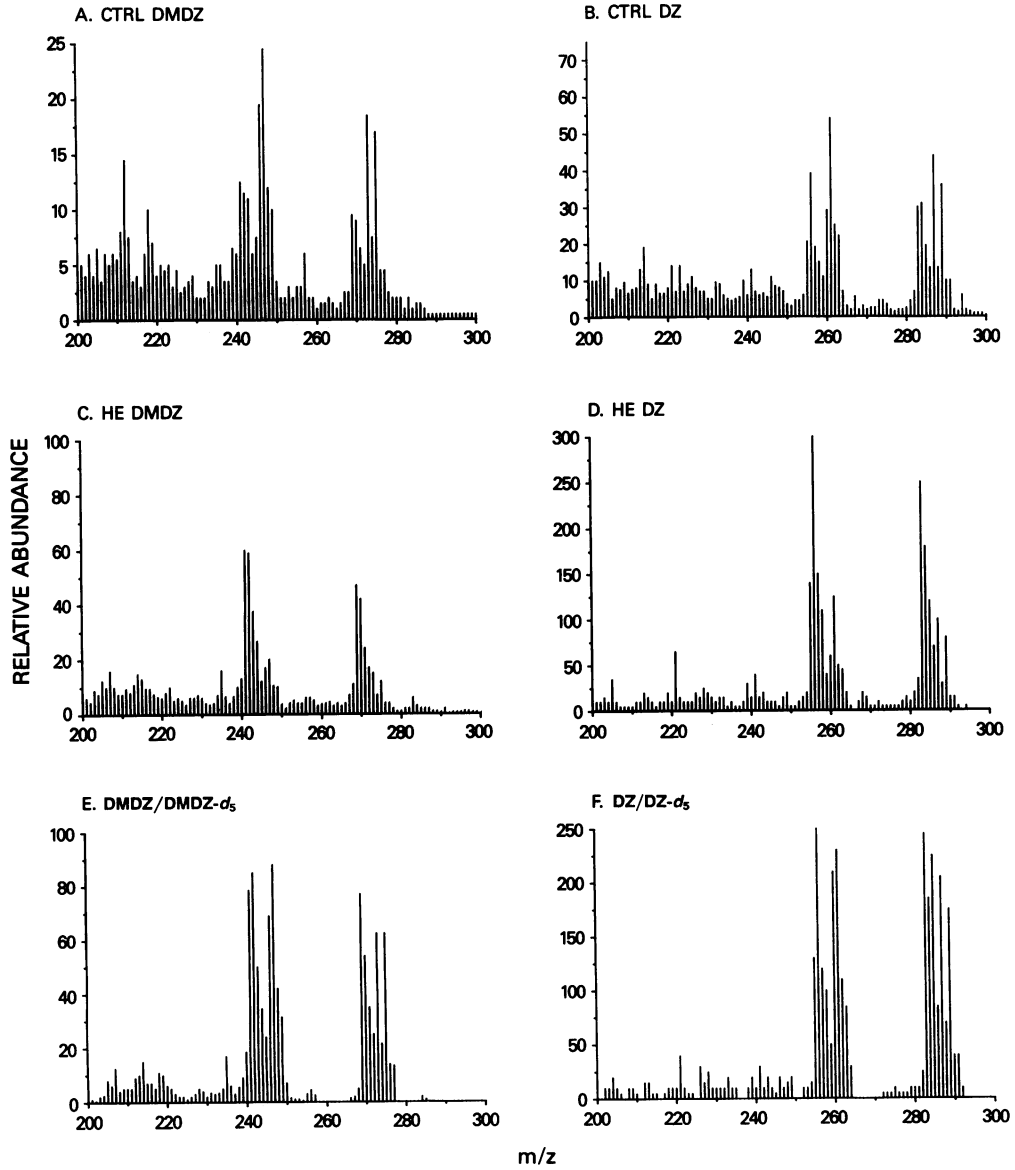

FIG. 3. Mass spectra of fractions comigrating with *N*-desmethyldiazepam (DMDZ) and diazepam (DZ) from an extract of 20.6 g of control (CTRL) rat brain (*A* and *B*, respectively), an extract of 20.7 g of brain from rats with HE (*C* and *D*), and pure standards (*E* and *F*). The standards in *E* and *F* each contained 100 ng of undeuterated and deuterated BZ dissolved in ethanol and dried in the sample cup. In *A* and *B*, 100 ng of *N*-desmethyldiazepam-d_5 or diazepam-d_5 was used as internal standard. In *C* and *D*, 50 ng of *N*-desmethyldiazepam-d_5 or 200 ng of diazepam-d_5 was used, respectively, as internal standard.

desmethyldiazepam and diazepam. While the presence of these substances in mammalian brain has been reported (18–20), elevated concentrations of BZs have not previously been shown to be associated with a pathophysiologic state.

The concentrations of BZs in extracts of control rat brains (1–3 ng/g) are consistent with levels previously reported in normal brains (18, 19). In contrast, the concentrations of diazepam and *N*-desmethyldiazepam were increased 2- to 9-fold in brain extracts from rats with HE. The variability in the concentrations of these substances in HE may reflect, in part, the wide range of BZ concentrations in control brains, a phenomenon that has been observed previously (18, 20). In addition to the elevated concentrations of diazepam and *N*-desmethyldiazepam present in brain extracts of rats with HE, the overall concentration of HPLC-purified substances inhibiting radioligand binding to BZ receptors (expressed in diazepam equivalents) was elevated 4- to 6-fold compared to controls (Table 1, Fig. 1). These increased levels are comparable to the 3-fold increase in inhibitory activity found in encephalopathic rat brains extracted with acidified methanol (8), which is consistent with the ability of both basic and acidic media to extract hydrophobic compounds such as the BZs.

It is unclear whether the brain concentrations of BZ receptor agonists are elevated sufficiently to account for all of the neuropsychiatric manifestations of HE. The whole-brain concentrations of *N*-desmethyldiazepam and diazepam after the administration of diazepam at 1.3 mg/kg (a behaviorally active but nonhypnotic dose) to normal rats are 6 and 12 times higher, respectively (21), than the corresponding brain concentrations in encephalopathic rats reported here. Nonetheless, autoradiographic studies indicate that a significant regional heterogeneity in the distribution of BZ receptor agonists exists in the brains of animals with HE, such that local concentrations of BZ receptor agonists may be much higher than whole-brain concentrations (15). In these studies, radioligand binding to BZ receptors in the cortex and cerebellum of animals with HE was reduced by 22% and 42%, respectively (15), which is similar to the level of BZ receptor occupation observed following the administration of diazepam at 4–18 mg/kg (an anticonvulsant and hypnotic dose) to normal rats (22, 23). Moreover, the ability of BZ receptor antagonists to correct both the electrophysiological and behavioral manifestations of HE in animal models (3–5) and humans (12–14) indicates that the concentrations of BZ receptor agonists found in liver failure appear sufficient to produce some of the behavioral manifestations of HE. These effects may be further enhanced by the other metabolic changes that frequently accompany HE, such as increased GABA concentrations (24) and/or altered glutamate metabolism (25).

The origin of the BZs detected in control and encephalopathic rat brains is unknown. However, dietary sources may be important, since BZs have been reported in a number of plant and animal foodstuffs (26). We have isolated active fractions that exhibit retention times identical with known BZs in extracts of the chow fed to the rats used in the present experiments (data not shown). Differences in the types of BZs as well as their concentrations in dietary sources may also contribute to the variability observed in BZ concentrations in the rat brain. However, in view of the ability of fungi to synthesize BZs (27), the possible contribution of gut flora to the production of naturally occurring BZs in mammals must be considered. Together, gut flora and diet may be responsible for the presence of BZs in animal brains. Thus, the presence of increased levels of BZs in HE suggests that therapeutic modalities directed at decreasing the effective concentrations of these compounds at the BZ receptor, such as the administration of BZ receptor antagonists (12–14), may be a useful supplement to conventional therapies (28) for the management of HE in humans.

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