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DIRECT OBSERVATION OF A FLUORINATED ANTICONVULSANT IN BRAIN TISSUE USING ^{19}F-NMR TECHNIQUES

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Abstract—A fluorinated derivative of an anticonvulsant γ-butyrolactone [α-(1,1-difluoroethyl)-α-methyl-γ-butyrolactone; α-DFGBL] was synthesized as a probe for NMR spectroscopic observation of the drug in brain tissue. The fluorinated compound is an efficacious anticonvulsant in mice, and inhibits the specific binding of [^{35}S]*t*-butylbicyclophosphorothionate ([^{35}S]TBPS) to mouse brain membranes with a concentration dependence similar to that of the non-fluorinated compound α-ethyl-α-methyl-γ-butyrolactone. Quantitative ^{19}F-NMR spectroscopic studies, coupled with chromatographic measurements of drug tissue concentration, showed that virtually all of the α-DFGBL in brain was NMR-observable and that, following intraperitoneal injection, α-DFGBL rapidly achieved millimolar concentrations in brain. The ^{19}F-NMR spectra of a α-DFGBL in brain and liver tissue were broad (1–2 ppm) and complex, exhibiting multiple chemical shift features. The major chemical shift features in these spectra were assigned on the basis of differential extraction and comparison of ^{19}F spin–spin relaxation times (T_2s) and ^{19}F chemical shifts of α-DFGBL in tissue to those in pure solvents. The major feature at 10.4 ppm in the tissue spectra was assigned to a weakly polar, membrane-associated environment for the fluorinated compound, while the feature at 11.2 ppm was assigned to an aqueous environment for α-DFGBL. The drug was in slow exchange between these two environments in brain. In addition, the feature at lowest field (9.7–9.8 ppm) was identified as a water-soluble hydroxy-acid metabolite of α-DFGBL produced by the liver. These data indicate that γ-butyrolactone anticonvulsants achieve high concentrations in brain, where they exist in several, largely membrane-associated, environments. These findings are consistent with the purported action of the γ-butyrolactones as low-affinity modulators of γ-aminobutyric acid-A channels.

The α-substituted γ-butyrolactones (α-GBLs)§ and γ-thiobutyrolactones (α-TBLs) are anticonvulsant agents that protect animals against seizures induced by both pentylenetetrazole (PTZ) and maximal electroshock [1, 2]. Two lines of evidence suggest that these agents produce their pharmacological effects via interactions at the picrotoxin binding site of the γ-aminobutyric acid (GABA) receptor/chloride ionophore complex: First, the α-GBLs inhibit the specific binding of [^{35}S]*t*-butylbicyclophosphorothionate ([^{35}S]TBPS), a radioligand used to measure binding to the picrotoxin receptor [3], with IC_{50} values in the range of 10^{-4} to 10^{-3} M [4–6]. Second, electrophysiological experiments demonstrate that these agents modulate chloride conductance through GABA receptor/ionophore channels in voltage-clamped spinal cord motoneurons and hippocampal neurons [7–9]. These electrophysiologic effects are observed at drug concentrations of 10^{-6} to 10^{-4} M.

Our continued interest in studying the pharmacological properties of the substituted lactone derivatives led to the synthesis of a fluorinated analog of α-ethyl-α-methyl-γ-butyrolactone (α-EMGBL). We now report the synthesis of this compound, α-(1,1-difluoroethyl)-α-methyl-γ-butyrolactone (α-DFGBL), and its evaluation as an anticonvulsant and modulator of [^{35}S]TBPS binding. The introduction of fluorine into the drug molecule provided a way to non-invasively observe the drug in tissue using NMR spectroscopy. In the current study we utilized ^{19}F-NMR spectroscopy to examine several aspects of the pharmacology of the γ-butyrolactones. First, we measured brain concentrations of α-DFGBL to examine its target-organ-specific pharmacokinetics. Second, we examined the metabolism of α-DFGBL. Finally, by observing the chemical shifts of the diasterotopic fluorines of α-DFGBL in conjunction with ^{19}F relaxation

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§ Abbreviations: GBL, γ-butyrolactone; TBL, γ-thiobutyrolactone; α-DFGBL, α-(1,1-difluoroethyl)-α-methyl-γ-butyrolactone; TBPS, *t*-butylbicyclophosphorothionate; PTZ, pentylenetetrazol; α-EMGBL, α-ethyl-α-methyl-γ-butyrolactone; GABA, γ-aminobutyric acid; CD_{97}, convulsive dose in 97% of animals; ED_{50}, effective dose in 50% of animals; IC_{50}, concentration that inhibits the response by 50%; ED_{90}, effective dose in 90% of animals; and TD_{50}, toxic dose in 50% of animals.

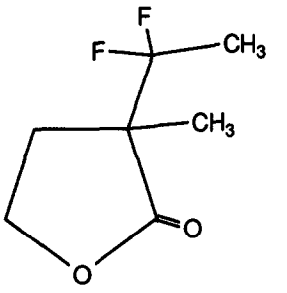
measurements, we made an initial characterization of some of the different chemical environments that this drug occupies in brain tissue.

MATERIALS AND METHODS

Materials. α-Acetyl-α-methyl-γ-butyrolactone and diethylaminosulfur trifluoride (DAST) were purchased from the Aldrich Chemical Co. (Milwaukee, WI). Reagent grade and HPLC grade solvents were obtained from the Baxter Healthcare Corp. (Muskegon, MI). Deuterated NMR solvents were purchased from Cambridge Isotope Laboratories (Woburn, MA). TLC was performed on Analtech (Newark, DE) precoated silica gel GF plates (250 mm). The β-EMGBL used as the internal standard in the GC determinations was synthesized in our laboratory [6] and exhibited satisfactory NMR, i.r. and mass spectroscopic data.

Chemical methods. The i.r. spectra were obtained (neat) using a Perkin–Elmer 1710 FT-IR spectrophotometer. ^{1}H-, ^{13}C- and ^{19}F-NMR spectra were obtained on a Varian XL-300 multinuclear spectrometer equipped with a 5-mm probe. Samples were dissolved in $CDCl_3$ and chemical shifts reported as δ values in parts per million (ppm). The chloroform resonance in proton (7.26 ppm) and carbon (77.0 ppm) spectra, and the difluoromethylene group resonance of methoxyflurane ($CHCl_2CF_2OCH_3$) in ^{19}F spectra (0.00 ppm), were used as internal standards. The multiplicity of peaks is defined by s (singlet), d (doublet), t (triplet), q (quartet), and m (multiplet). The relative peak heights of the resonances are reported as integers after the multiplicity. High resolution mass spectroscopic (HRMS) data were recorded using a VGZAB-SE double-focusing mass spectrometer. Accurate mass assignments were made by manual peak matching against appropriate reference ions in the spectrum of perfluorokerosene. Reverse-phase, preparative HPLC was performed on a Waters Prep LC/System 500A liquid chromatograph, using a PrepPAK-500/C18 cartridge and the product eluted with 3% acetone in water (0.2 L/min).

Synthesis of α-DFGBL. α-Acetyl-α-methyl-γ-butyrolactone (0.3 mmol) and DAST (0.3 mmol) were stirred in dichloromethane (100 mL) for 48 hr in a polyethylene bottle at room temperature [10]. Water was added slowly and the organic phase was dried over magnesium sulfate and concentrated *in vacuo*. The product was purified by preparative HPLC and extracted from the aqueous mobile phase with dichloromethane. The organic layer was dried over magnesium sulfate and concentrated to yield the product as a colorless oil. This oil was then vacuum distilled (b.p. = 42°; 0.2 mm Hg) to yield pure (> 99% by GC analysis) α-DFGBL (22.9 g, 48.4% yield): i.r. ν_{max} 1771 (C = O), 1213 (CF_2) 1193 (CF_2) cm^{-1}; ^{1}H-NMR 4.32 (m, 2H, γ-CH_2), 2.79 (m, 1H, β-CH_2), 2.08 (m, 1H, β-CH_2), 1.79 (t, 3H, —CH_3, $^{3}J_{HF}$ = 20 Hz), 1.39 (s, 3H, —CH_3); ^{13}C {^{1}H}-NMR 176.7 (m), 124.4 (t, $^{1}J_{CF}$ = 245.2 Hz), 65.4 (s), 49.4 (t, $^{2}J_{CF}$ = 26.2 Hz) 30.4 (m), 19.11 (m), 19.0 (t, $^{2}J_{CF}$ = 26.8 Hz); ^{19}F-NMR −10.6 (m, 1F, —CF_2, $^{2}J_{FF}$ = 250 Hz, $^{3}J_{HF}$ = 20 Hz), −10.2 (m, 1F, —CF_2, $^{2}J_{FF}$ = 250 Hz, $^{3}J_{HF}$ = 20 Hz); HRMS *m/z* 164.0636 ($C_7H_{10}F_2O_2$ requires 164.0648).



Hydrolysis of α-DFGBL. A solution of α-DFGBL in 10% ethanolic KOH was heated gently until TLC indicated that no starting material remained. Water was added, the ethanol was evaporated off under reduced pressure, and the pH was adjusted to 7.5–8.0 with dilute HCl. The residue was lyophilized to afford the crude hydroxy-acid which was diluted in D_2O for NMR analyses.

Anticonvulsant testing and radioligand binding assay. Drug screening was performed using methods described by Swinyard and Woodhead [11]. Briefly, the ability of α-DFGBL to protect 50% of animals from PTZ-induced seizures (ED_{50}) was tested in female CF1 mice weighing 20–25 g (6- to 8-weeks-old). The test compound was suspended in 30% polyethylene glycol and injected in a volume of 10 μL/g. The drug was administered i.p. 30 min prior to a challenge with a CD_{97} (convulsive dose in 97% of animals) dose of PTZ (85 mg/kg). PTZ was dissolved in normal saline and administered i.p. in a volume of 10 μL/g. Those mice which did not experience clonic seizures within 30 min of the convulsant challenge were considered protected. Vehicle-treated controls exhibited no signficant anticonvulsant activity. Neurotoxic effects were assessed using the rotorod toxicity test [12]. In this test, the mouse was placed on a 1-inch diameter rod rotating at 6 rpm. The animal was considered toxic if it fell from the rotating rod twice during the 10-min testing period. The ability of the test compound to displace [^{35}S]TBPS in radioligand binding assays (IC_{50}) was demonstrated using methods described previously [4, 13]. The ED_{50}, ED_{90}, TD_{50} (toxic dose in 50% of animals) and IC_{50} values reported were determined by $\log_{10}$ probit analysis [14].

Preparation of adipose, liver and brain tissue samples. Brain samples for relaxation experiments were prepared by administering an ED_{90} dose (400 mg/kg, i.p. injection) of the drug to CF1 strain mice. After 5 min, the animal was killed and the brain stem and cerebellum were removed by crude dissection. The remaining brain tissue was placed in a 1-mL syringe and injected through a No. 16 needle attached to polyethylene tubing into the bottom of a 5-mm NMR tube. The livers of the same animals were immediately excised and placed in an NMR tube using a similar technique. Finally, the retroperitoneal fat pads were dissected out of the same CF1 mice and similarly placed in a 5-mm NMR tube. All samples were kept at 4° prior to NMR analysis.

Quantitation of α-DFGBL in brain tissue using gas chromotography. For time–course experiments, a 275 mg/kg dose of α-DFGBL was administered i.p. into CF1 mice and, at the appropriate times (2, 10, 20 and 30 min), the animals were killed. Quantitation experiments using various doses of test compound (100, 175, 250, 325 and 400 mg/kg) were also performed, and the animals were killed and decapitated after 30 min (time of convulsant challenge in that assay). The brain stem and cerebellum were removed by crude dissection and the remaining tissue was immediately frozen on dry ice and later weighed. To avoid loss of extraction solvents and/or test compounds due to evaporation, further sample preparation was performed in a cold room at 4°. The brains were homogenized manually for 3 min with 10 vol. (by tissue weight) of a 2:1 mixture of chloroform and methanol containing 50 μM β-EMGBL as an internal standard. The homogenate was centrifuged for 30 min at 1500 *g*, and the lower phase was recovered and stored at 4° prior to GC analysis. Preliminary experiments ensured 100% recovery of the test compound. Analyses were performed with a Hewlett Packard 5890A GC on an Ultra 1 capillary column (0.2 mm i.d., 0.1 μm film thickness, 25 m length). Helium was used as the carrier gas (1 mL/min) and splitless injection was employed. Operating temperatures were: injection port, 150°; detector, 280°; oven temperature, 80°; programmed 80–120° at 50°/min. The detector response ratio for the internal standard and the test compound was obtained and its linearity validated over the concentration range of 0.025 to 0.225 mM. Brain concentration was determined from the ratio of the integration (HP 3393A integrator) of the flame ionization signal of the test compound and the internal standard with subsequent correction for detector response and tissue wet weight (specific gravity assumed to be 1.0).

Quantitation of α-DFGBL in brain tissue using ^{19}F-NMR. ^{19}F-NMR experiments were performed at 22–23° on a Varian-VXR multinuclear spectrometer operating at 470.3 MHz using a 5-mm probe. Tissue samples were prepared as follows: CF1 mice were injected i.p. with a 275 mg/kg dose of the test compound, and decapitated at the appropriate times. The brain stem was removed by crude dissection and the remaining tissue placed in a 3-mL syringe. Using a 22-gauge needle, the brain tissue was injected through a rubber septum into a 3-mm capillary. The capillary was sealed with clay and inserted into a 5-mm NMR tube containing an external standard solution of 2:1 d_8-toluene:toluene containing 1.42 mM methoxyflurane. The external standard served as both a chemical shift and concentration reference. Quantitation of the fluorinated drug in brain was performed as described by Evers *et al.* [15]. Briefly, the integration of the resonance of α-DFGBL in brain was compared to that of the difluoromethylene group of methoxyflurane in the external standard solution. The ratio of the two integration values was then corrected for the different cross-sectional areas of brain tissue (3 mm capillary) and external standard solution (5-mm NMR tube) exposed to the magnetic field, to obtain the drug concentration in the sample capillary. Finally, differences in tissue sample preparation were corrected for by measuring the ratio of the water resonance in brain to that of the methyl group of toluene in the external standard solution. Since brain tissue is 84% water by volume [16], these values were normalized to the same ratio obtained from a capillary containing 84% water. To obtain adequate signal-to-noise ratios, 500 transients were obtained for fluorine spectra and 16 transients for proton spectra.

Relaxation measurements. Longitudinal relaxation times (T_1) were measured by the inversion-recovery method (180°-τ-90°) [17] and reported values determined using a Varian software package. Transverse relaxation times (T_2) were determined using the Carr–Purcell–Meiboom–Gill pulse sequence (90°-τ-(180°-2τ)^n) [18] with an interpulse delay of 100 μsec and an equilibration delay of greater than 3 times T_1. Data were plotted as the natural logarithm of signal intensity as a function of echo evolution time. The T_2 values reported are the reciprocals of the negative slopes of the exponentials obtained from these plots. Exponential fitting of these curves was performed using the RS-1 software package on a VAX computer. Adequate signal-to-noise ratios were obtained by collecting 1200 transients at each echo evolution time for brain and liver samples, 128 transients for fat tissue and 64 transients for the solution studies.

Preparation of brain homogenates. CF1 mice were placed in a sealed chamber and deeply anesthetized with halothane in 100% oxygen. The animals were killed by decapitation, and the cerebral hemispheres and liver tissue were excised and weighed. The tissues were added to an equal volume of phosphate-buffered saline (PBS, pH 7.4; 20% in D_2O) and homogenized in a Tekmar tissue homogenizer. The homogenates were kept at 4° until used (< 6 hr) and then were placed in 5-mm NMR tubes either neat, or following 1:4 dilution in PBS. α-DFGBL was added to the homogenates (final concentration = 1 mM) as a 100 mM solution in dimethyl sulfoxide.

Isolation of the hydroxy-acid metabolite from brain and liver tissue extracts. A 400 mg/kg dose of α-DFGBL was administered i.p. to CF1 mice. The animals were decapitated 5 min after the injection. The brain and liver were removed by crude dissection and each of the tissues was homogenized manually for 3–5 min with 10 vol. (by tissue weight) or a 4:2:1 mixture of chloroform, methanol and water. The homogenate was centrifuged for 30 min at 1500 *g* and the upper and lower phases from both brain and liver extracts were recovered and stored at 4°. The organic solutions were analyzed directly, while the aqueous solutions were lyophilized and resuspended in D_2O for NMR analysis.

RESULTS

A fluorinated derivative of α-EMGBL, α-DFGBL, was synthesized and purified. Its structure was confirmed by ^{1}H-, ^{13}C- and ^{19}F-NMR spectroscopy and by mass spectrometry. Both the fluorinated drug and the parent drug were free of acute neurotoxic effects (maximum dose tested was 500 mg/kg) as assessed by the rotorod neurotoxicity test. α-DFGBL

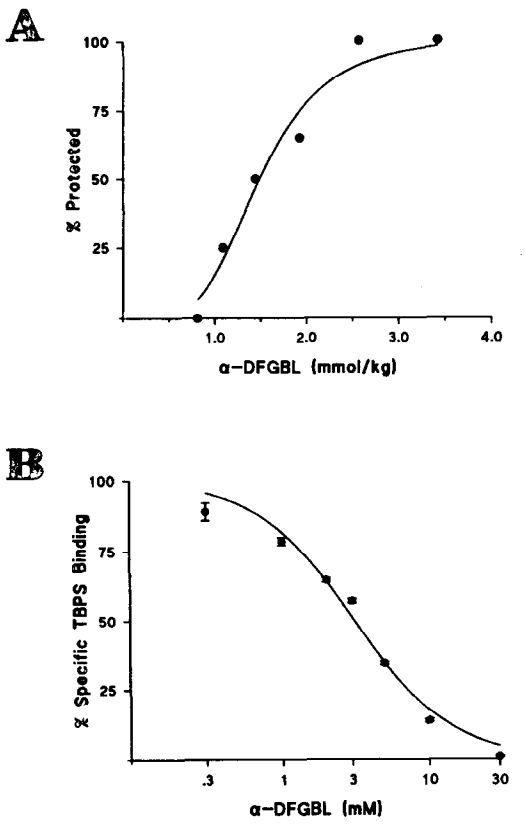


Fig. 1. Pharmacological effects of α-DFGBL. (A) Dose–response relationship for the anticonvulsant effect of α-DFGBL against PTZ-induced seizures. α-DFGBL was injected i.p. 30 min prior to challenge with a CD_{97} dose of PTZ (85 mg/kg). The ED_{50} as determined by $\log_{10}$ probit analysis was 245 mg/kg or 1.5 mmol/kg (95% fiducial limits of 216–278 mg/kg). (B) Concentration-dependent inhibition by α-DFGBL of specific [^{35}S]TBPS binding to rat cerebral cortical membranes. Each data point represents a mean value (± SEM)) of four experiments each performed in quadruplicate. The data points are fitted to the equation: $\text{Min} + (\text{Max} - \text{Min})/(1 + ((X/IC_{50})^{-N}))$ where X = the concentration of α-DFGBL, IC_{50} = the half-maximally effective concentration of α-DFGBL, and n is the Hill coefficient. The points are fit with IC_{50} = 3.1 mM and n = 1.32.

also was found to be an effective anticonvulsant against PTZ-induced seizures. As shown in Fig. 1A, the ED_{50} of α-DFGBL for preventing PTZ seizures was 1.5 mmol/kg (245 mg/kg). This value was very similar to the ED_{50} of the non-fluorinated compound against PTZ seizures (2.0 mmol/kg or 259 mg/kg), indicating that fluorination has little effect on its anticonvulsant activity.

Radioligand binding studies using 2 nM [^{35}S]TBPS as the ligand for the picrotoxin site on the $GABA_A$ receptor showed that α-DFGBL reduced specific TBPS binding in rat brain membranes with an IC_{50} of 3.1 mM and a Hill coefficient of 1.3 (Fig. 1B). This reduction of TBPS binding by α-DFGBL was due to an increase in the K_D for TBPS binding, not to a change in the maximal number of TBPS-binding sites (data not shown). The effects of α-DFGBL were similar to those of the non-fluorinated compound, α-EMGBL, which inhibits [^{35}S]TBPS binding with an IC_{50} of 2.3 mM [5]. These data are consistent with a competitive interaction between α-DFGBL and TBPS at the picrotoxin-binding site.

To determine if the high tissue concentrations of α-DFGBL required to alter TBPS binding and GABA channel function [19] are achievable *in vivo*, brain concentrations were measured after i.p. administration of 275 mg/kg of α-DFGBL. To ensure that there was not a significant component of "NMR-invisible" fluorinated drug in brain, tissue concentrations measured by quantitative ^{19}F-NMR spectroscopy were compared to concentrations measured by chemical extraction and GC. As shown in Fig. 2A, α-DFGBL rapidly reached a peak brain concentration of approximately 2 mM following i.p. administration. There was no significant difference between the concentrations measured by GC and by NMR methods, indicating that virtually all of the drug in brain was observable by NMR. Brain concentrations decayed in a bi-exponential fashion.

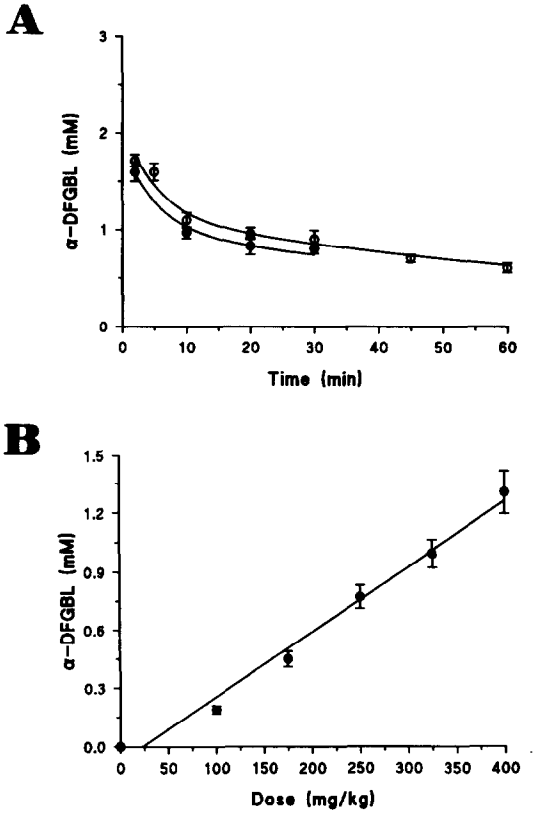


Fig. 2. Brain concentrations of α-DFGBL. (A) Time course of α-DFGBL concentration in brain tissue following i.p. administration of 275 mg/kg as determined by ^{19}F-NMR (○) and gas chromatography (●). Each time point represents the mean ± SEM) of three determinations. Both data sets were well fit by the sum of two exponentials. The NMR data was fit by the equation $y = 0.89e^{-0.19t} + 0.99e^{-0.01t}$ and the GC data to $y = 0.96e^{-0.19t} + 1.15e^{-0.01t}$. (B) Brain concentrations of α-DFGBL 30 min after i.p. administration of 100, 175, 250, 325 and 400 mg/kg doses, as determined by gas chromatography. The data are fitted by linear regression.

There was a rapid reduction in brain concentration (time constant of approximately 5 min), consistent with a redistribution phase, followed by a slow elimination phase (time constant of about 100 min). Concentrations were maintained in the range of 1 mM 30 min after i.p. administration, the time of anticonvulsant testing. Brain concentrations attained 30 min after administration of different doses of the drug (100, 175, 250, 325, and 400 mg/kg) were also examined using the GC method. The brain concentration of α-DFGBL was a relatively linear function of dose administered (Fig. 2B).

To examine the chemical environments in which α-DFGBL resides in tissue, ^{19}F-NMR spectra of α-DFGBL in several tissues and solvents were examined. Spectra of the drug in brain, liver and adipose tissue following *in vivo* drug administration, and in aqueous (0.9% NaCl) and chloroform solution are shown in Fig. 3. The chemical shifts of the diastereotopic fluorines were compared to that of the difluoromethylene of methoxyflurane ($CHCl_2$-CF_2-O-CH_3) to quantify the effects of electronic environment on chemical shift. In an aqueous solution, the resonances were centered at −10.4 and −11.7 ppm. In contrast, the fluorines of α-DFGBL in chloroform solution experienced a more similar magnetic environment as evidenced by chemical shifts of −10.2 and −10.6 ppm. Spectra of α-DFGBL in several other weakly polar solvents (acetone, dimethylacetamide, dimethylformamide) were similar to that obtained in chloroform; the fluorine resonances were between −10.1 and −10.5 ppm and the diasterotopic fluorines had similar ($\Delta < 0.2$ ppm) chemical shifts (spectra not shown). In highly non-polar solvents (hexane, decane) resonances of the two fluorines were separated by < 0.3 ppm, but had chemical shifts between −10.5 and −11 ppm (not shown). The drug in adipose tissue showed a single dominant feature (centered at approximately −10.2 ppm), which closely resembled the spectrum obtained in chloroform. This pattern suggests that α-DFGBL occupies a weakly polar environment in this tissue.

The spectra of α-DFGBL in brain and liver tissue were far more complex than the spectra obtained in solvents and in adipose. The features were broad ($\nu_{1/2} = 470$ Hz) and complex in shape with multiple chemical shift resonances. The prominent peak centered at −10.4 ppm in both liver and brain suggests a weakly polar, "chloroform-like" environment for the drug in both tissues. In addition to this major feature, the brain spectrum showed significant peaks at −11.2 and −9.8 ppm. The liver spectrum showed a major peak at −9.7 ppm, but no resolvable features at > −11.0 ppm. These chemical shifts suggest either that the drug exists in multiple chemical environments in the tissues (drug exchanges slowly between these environments on the NMR time scale), and/or that metabolites of the drug are present in tissue.

While chemical shift anisotropy can contribute to line broadening of fluorine features in a membrane environment [20], the broadness of the fluorine features in the tissue spectra could be explained in several other ways. The most likely explanation is that the drug is immobilized by its interaction with the tissue producing a short T_2 and a broad feature.

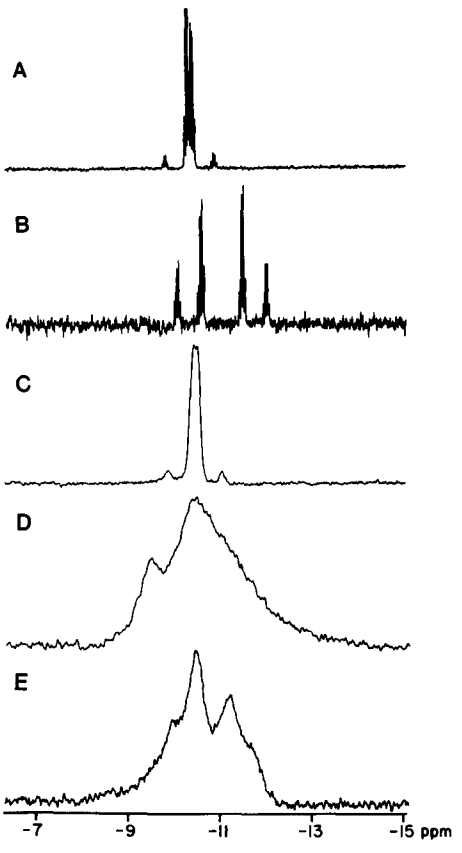


Fig. 3. ^{19}F-NMR spectra of α-DFGBL in chloroform (A), saline (B), adipose tissue (C), liver tissue (D) and brain tissue (E) recorded at 11.74 tesla on a Varian VXR multinuclear spectrometer operating at 470.3 MHz. All spectra are referenced to the difluoromethylene group of methoxyflurane. Representative parameters used in obtaining these spectra were as follows: 9 μsec pulse (90° flip angle); 0.4 sec acquisition time; interpulse delay > 3 times T_1; 2 Hz exponential line broadening for solution spectra and 20 Hz line broadening for tissue spectra.

Alternatively, the broadness could be explained by exchange broadening resulting from chemical exchange of the drug between multiple environments in the tissue. To examine the basis for the broad tissue features, and to determine whether the various chemical shift resonances were experiencing different motional environments, ^{19}F relaxation studies were performed. Both longitudinal (T_1) and transverse (T_2) relaxation measurements were made of α-DFGBL in brain and liver. For purposes of comparison, T_1 and T_2 were also measured in adipose tissue and in chloroform and aqueous solution.

All T_1 determinations were mono-exponential and showed no detectable variation in T_1 between the various chemical shift components of the drug resonances. As shown in Table 1, T_1 values were markedly shorter in brain and liver than in the other preparations studied.

T_2 measurements of α-DFGBL in adipose, chloroform and aqueous solutions were all characterized by mono-exponential signal decay, with T_2

Table 1. Longitudinal (T_1) and transverse (T_2) relaxation times of α-DFGBL in brain tissue, liver tissue, adipose tissue, and chloroform and saline solutions

Sample	T_1 (sec)	T_2 (msec)
Saline	3.2	>300
$CHCl_3$	3.1	>300
Adipose tissue	2.1	170
Liver tissue	1.5	4
		40
Brain tissue	1.0	3
		32

T_1 measurements were made using the inversion recovery technique. The T_2 values were determined using the Carr–Purcell–Meiboom–Gill pulse sequence.

values greater than 170 msec (Table 1). The spin-echo decay curves of α-DFGBL in brain and liver were non-linear (Fig. 4, left panel) and were well characterized by the sum of two exponential components (Table 1). The two T_2 values derived from exponential fitting of these decay curves indicated that two motionally distinct types of fluorines are present in these tissues. Examination of spectra obtained at various delay times in the spin-echo sequence showed that different fluorine resonances observed in brain (Fig. 4, right panel) and liver (not shown) had different T_2 values. Specifically, the feature at lowest field (−9.8 ppm) had a long T_2, whereas the more highly shielded features (−10.4 and −11.2 ppm) had short T_2 values. This indicates that either α-DFGBL exists in two segregated environments, characterized by distinct chemical shifts and T_2s, or that this drug is metabolized to a compound that experiences a less motionally-restricted environment.

Production of a metabolite seemed a likely possibility since blood and liver lactonases are known to metabolize γ-lactones [21]. The resulting hydroxy-acid would be expected to have different fluorine chemical shifts than the intact lactone. It would also be expected to be water soluble and, therefore, to be cytosolic and have a longer T_2. To test this hypothesis, water-soluble and chloroform-soluble compounds were differentially extracted from brain and liver tissue; ^{19}F spectra of these extracts were compared to spectra of α-DFGBL and to an authentic sample of the expected hydroxy-acid metabolite. Results of these experiments are shown in Fig. 5. Aqueous extracts of both liver and brain produced ^{19}F spectra that were virtually identical to those of the authentic hydroxy-acid sample. This indicates that the resonances at −9.6 to −9.8 ppm in the tissue spectra likely were due to a water-soluble metabolite of α-DFGBL. The organic extracts of brain and liver revealed ^{19}F spectra virtually identical to those of α-DFGBL in chloroform. (N.B. small differences between the spectra of organic tissue extracts and α-DFGBL in chloroform were due to methanol in

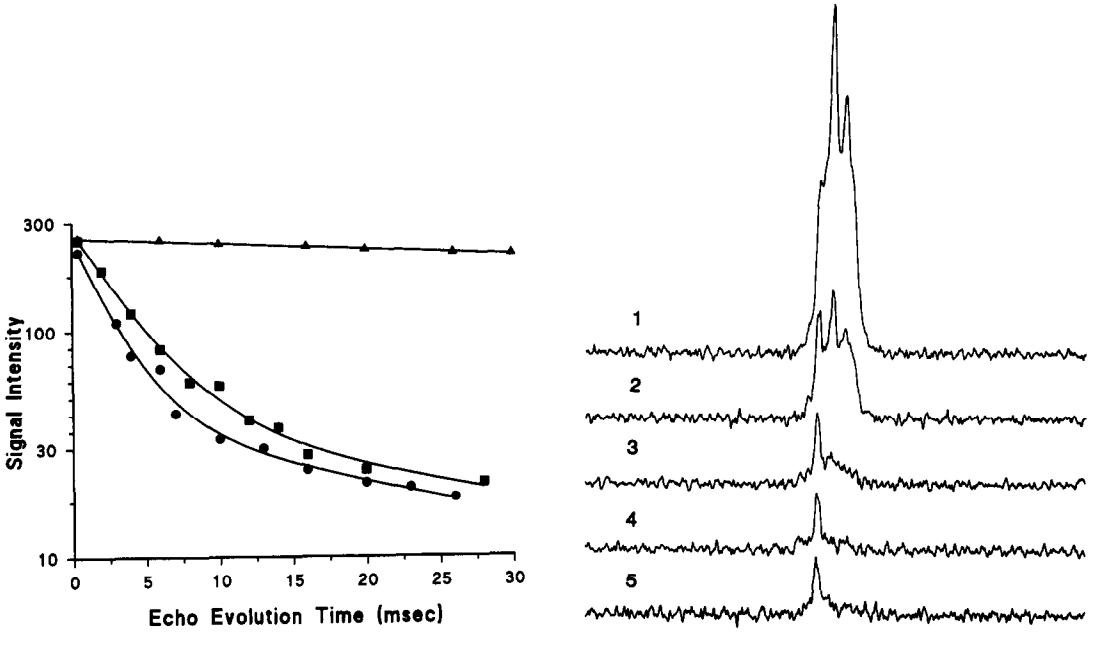


Fig. 4. ^{19}F-NMR spin-spin relaxation (T_2) measurements of α-DFGBL in adipose, liver and brain tissue. Measurements were made using the Carr–Purcell–Meiboom–Gill pulse sequence with an interpulse delay of 100 μsec, and an equilibration delay of greater than 3 times T_1. (Left panel) ^{19}F spin-echo decay curves for α-DFGBL in tissues. Signal intensities are plotted on a natural log scale as a function of echo evolution time. Data from adipose tissue (▲) are well fit to a mono-exponential decay with a T_2 value of 170 ± 10 msec. Data from liver (■) and brain (●) are well fit by the sum of two exponentials. For liver the curve is fit to $y = 236e^{-t/3.9} + 40e^{-t/40}$; for brain the data are fit to $y = 210e^{-t/2.8} + 40e^{-t/32}$. (Right panel) ^{19}F-NMR spectra of α-DFGBL in brain tissue obtained at various echo evolution times using the spin-echo pulse sequence used to measure T_2. The echo evolution times corresponding to spectra 1 through 5 are 0.4, 6, 10, 16 and 26 msec, respectively.

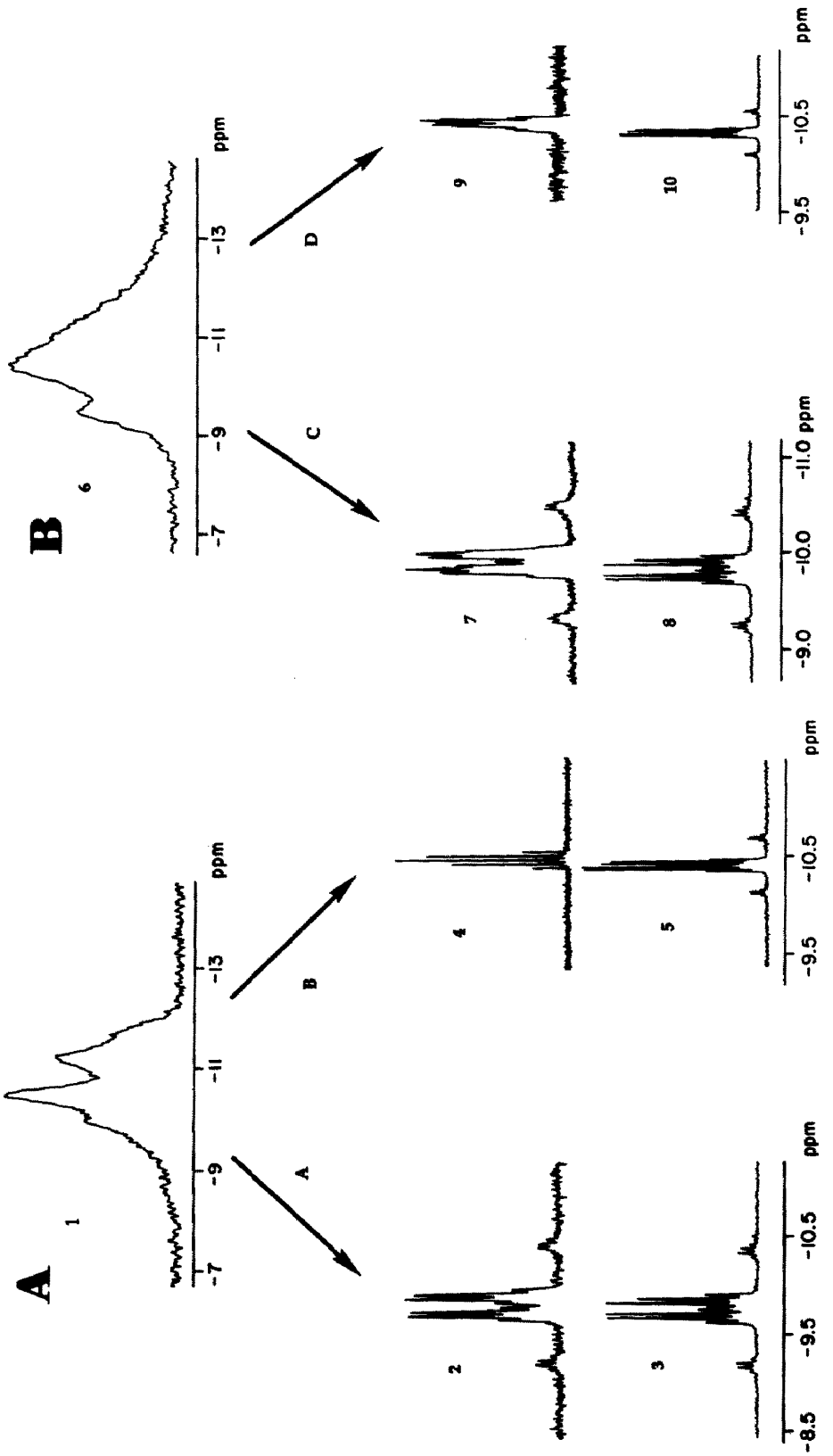


Fig. 5. Isolation of a water-soluble metabolite of α-DFGBL from brain and liver tissue. Mice were administered 400 mg/kg of α-DFGBL i.p. prior to excision of tissues and subsequent extraction. (A) Spectrum 1 is the ^{19}F-NMR spectrum of α-DFGBL in excised brain tissue. Aqueous extraction (route A) yielded the spectrum of the aqueous brain extract (2) with the spectrum of the hydroxy-acid metabolite in water (3) included for comparison. Organic extraction (route B) yielded the spectrum of the organic extract (4) with the spectrum of α-DFGBL in chloroform (5) shown for comparison. (B) Spectrum 6 is the ^{19}F-NMR spectrum of α-DFGBL in excised liver tissue. Route C yielded the spectra of the aqueous extract of liver tissue (7) with the spectrum of an authentic sample of the hydroxy-acid metabolite in water (8) shown for comparison. Route D yielded the spectrum of the organic extract of liver (9) with the spectrum of α-DFGBL in chloroform (10) shown for comparison. The small differences in the chemical shifts of the diastereotopic fluorines between spectra 4 and 5 and between spectra 9 and 10 were due to the fact that tissue extraction was in a $CHCl_3$:MeOH mixture whereas the authentic compound was observed in $CHCl_3$.

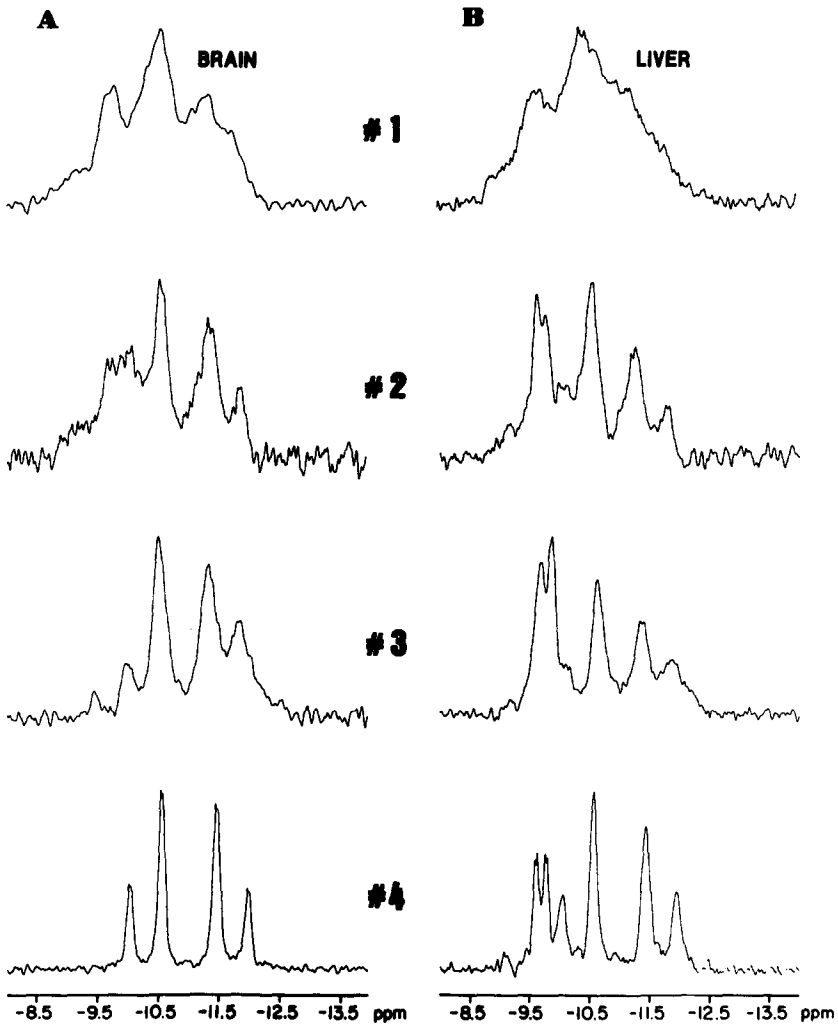


Fig. 6. ^{19}F-NMR spectra of α-DFGBL in homogenates of brain (A) and liver (B) tissue. At the top of each panel are the spectra of tissue excised from animals that had been administered 275 mg/kg of α-DFGBL i.p. Tissues from the animals receiving *in vivo* α-DFGBL were homogenized in an equal volume (w:v) of PBS. Spectra of these homogenates are shown in #2 panels. Homogenates were also prepared from animals that had received no drug; these homogenates were prepared on a 1:1:w:v basis with PBS and in some cases diluted an additional 4-fold with PBS (dilute homogenate). α-DFGBL was exogenously added to the homogenates at a final concentration of 1 mM. Spectra of undiluted homogenates are shown in #3 panels, and of dilute homogenates in #4 panels. All ^{19}F chemical shifts are referenced to the difluoromethylene group of methoxyflurane.

the extraction solution.) This indicates that the multiple broad features in the tissue spectra were not due to drug metabolites (assuming that all metabolites were fully extracted) and probably were the consequence of different chemical environments within the tissues.

To confirm that neither the −10.4 ppm nor the −11.2 ppm feature represents an α-DFGBL metabolite, and to determine the anatomic locus of lactonase activity, ^{19}F spectra were obtained of α-DFGBL added to homogenates of brain and liver tissue. These spectra were compared to those of intact (unhomogenized) tissue and tissue homogenates prepared following *in vivo* drug administration. Figure 6A shows spectra of α-DFGBL in intact brain tissue and in brain homogenates. The spectrum in intact brain showed ^{19}F resonances at −9.6 to −9.8 ppm, −10.3 to −10.5 ppm and −11.2 to −11.4 ppm; the resonance centered at −10.4 ppm dominated the spectrum. These features were also observed in the 1:1 homogenate of brain prepared following *in vivo* drug administration. The spectra of α-DFGBL exogenously added to brain homogenate showed the major peaks at −10.4 and −11.3 ppm, but only a very minor peak at −9.6 ppm. When this homogenate was diluted to 1:4 with PBS, the spectrum resembled that of α-DFGBL in water; the features at −10.5 and −11.4 ppm were of equal height and the feature at −9.6 to −9.8 ppm was not observable. Figure 6B shows spectra of α-DFGBL in intact liver tissue and liver homogenates. In the spectrum from intact tissue there were two, relatively

equal, major peaks centered at −9.5 ppm and −10.3 ppm. Spectra of homogenates prepared either following *in vivo* drug administration or with *ex vivo* addition of drug were virtually identical. The two major peaks observed in intact tissue remained; additionally a smaller feature at −11.2 ppm and a very small peak at −11.7 ppm were observed. The spectra of α-DFGBL added to dilute (1:4 in PBS) liver homogenate contained features at −9.6, −9.8, −10.0, −10.5, −11.4, and −11.9 ppm. This is consistent with superimposed spectra of α-DFGBL and its hydroxy-acid metabolite in water.

DISCUSSION

Our studies of the pharmacological properties of α-DFGBL demonstrated that this compound retains both anticonvulsant activity and the ability to inhibit TBPS binding at concentrations very similar to those observed for the unfluorinated compound. These results indicate that the changes in electronic and/or lipophilic character introduced by fluorination do not alter binding affinity or anticonvulsant potency significantly.

^{19}F-NMR spectroscopy initially was utilized to demonstrate that α-DFGBL could be observed in brain following *in vivo* administration, and to measure brain concentrations of the drug. Prominent ^{19}F-NMR features were observed in spectra of all tissues examined, demonstrating high tissue concentrations of NMR-observable drug. The virtually identical GC and NMR determinations of brain drug concentration demonstrate that there was no significant "NMR silent" compound. This is important since high affinity binding of drug molecules to large macromolecules could produce a very short ^{19}F T_2 value and, therefore, an unobservable signal. The absence of "NMR silent" α-DFGBL indicates either that a very small proportion of drug molecules in brain is bound to proteins, or that bound molecules are observable by virtue of their rapid exchange with other, observable, environments. This latter possibility is plausible, since the low affinity of these drugs for the picrotoxin site may imply a very short lifetime in the bound state.

As shown in Fig. 2A, α-DFGBL rapidly entered the CNS following peripheral administration, and reached millimolar peak concentrations. At the time of anticonvulsant testing, 30 min after peripheral administration, brain concentrations remained in the range of 0.5 to 1.5 mM (Fig. 2B). Are these brain concentrations high enough to produce the free drug concentrations required to produce effects on $GABA_A$ channels *in vitro*? If one assumes that 15% of brain volume is comprised of membrane (the brain is approximately 84% water by volume [16]) and that the membrane/water partition coefficient for α-DFGBL is 5 (similar to anticonvulsant barbiturates [22]), then a brain concentration of 1 mM would correspond to a free concentration of 0.63 mM. This is the same order of magnitude as the concentrations required to reduce TBPS binding and potentiate GABA-gated chloride currents [19]. It is thus plausible that the effects of α-DFGBL and other γ-butyrolactones on GABA currents that have been observed *in vitro* can account for the anticonvulsant effects of these drugs *in vivo*.

^{19}F-NMR spectroscopy was also used to examine the metabolism of α-DFGBL *in vivo* and to characterize the various chemical environments for α-DFGBL in tissue. Each of the three tissues examined had a unique and complex ^{19}F spectrum (Fig. 3), indicating that α-DFGBL exists in a variety of NMR-discernible, tissue-specific environments. The simplest of the tissue spectra examined was that of adipose. The relatively long ^{19}F T_2 in this tissue makes it unlikely that the drug is in the highly ordered environment of a lipid bilayer. It is most likely that the spectrum of α-DFGBL in adipose reflects partitioning of the drug into triglyceride micelles within the adipocytes. The chemical shift of α-DFGBL (−10.4 ppm for both fluorines) indicates that the drug resides in a weakly polar environment in this tissue. These data suggest that the drug preferentially resides near the polar head groups rather than deep within the hydrophobic core of the micelles.

The spectra of α-DFGBL in both liver and brain tissue contained a resonance at −9.6 to −9.8 ppm. This feature was distinguished from other features in the spectra by its longer T_2 value (32–40 msec), suggesting a water-soluble metabolite of α-DFGBL. Spectra of the aqueous extracts of brain and liver were virtually identical to the ^{19}F spectra of a pure sample of the hydroxy-acid derivative of α-DFGBL (Fig. 5). This provides strong evidence that the −9.6 to −9.8 ppm features in the tissue spectra represent an hydroxy-acid metabolite of α-DFGBL. To determine the site of metabolite production, homogenates of brain and liver were incubated with α-DFGBL, and examined by ^{19}F-NMR spectroscopy. These studies showed that the hydroxy-acid was produced in liver, but not in brain (Fig. 6). Lactone hydrolysis is most likely an enzymatic process since, first, metabolism of α-DFGBL was tissue specific. Second, incubation of the hydroxy-acid with liver homogenate resulted in significant production of the lactone (ring closure), whereas spontaneous ring closure did not occur at either pH 6.8 or pH 9.0. While it is unclear whether the hydroxy-acid metabolite of α-DFGBL has any pharmacologic activity or toxicity, the hydroxy-acid of α-EMGBL is not active in a hippocampal slice model of seizure activity [23].

^{19}F spectra of both liver and brain also had resonances in the area of −10.2 to −10.4 ppm. These features were produced by the parent compound and not by a drug metabolite, since ^{19}F spectra of organic extracts of these tissues were virtually identical to spectra of pure α-DFGBL in chloroform (Fig. 5). Based on the similarity of the chemical shift of the −10.3 ppm feature to those of α-DFGBL in chloroform and in adipose, it is likely that the −10.3 ppm feature represents a weakly polar environment for the drug. Unlike the −10.3 ppm feature in adipose tissue, the corresponding features in brain and liver were characterized by short (3–4 msec) T_2 relaxation times, suggesting a highly structured environment. A likely assignment for these features is a weakly polar region of the lipid bilayer, such as the area adjacent to the glycerol

backbones of phospholipids. It is unlikely that the drug resides in the hydrophobic core of the membrane since highly nonpolar solvents produce ^{19}F chemical shifts in the region of -10.5 to -11.0 ppm.

The ^{19}F spectrum of α-DFGBL in brain contained a feature at -11.2 ppm that was not observed in the other tissue spectra. Based on its chemical shift, it seems likely that this feature represents one of the diastereotoipic fluorines of α-DFGBL in a polar environment. (The other fluorine would be buried under the -10.3 ppm feature.) Since it is expected that half of the α-DFGBL in brain should be in the aqueous phase (assuming that brain is 16% membrane by volume and that the membrane/water partition coefficient for α-DFGBL is 5), it is likely that this feature represents α-DFGBL in water (cytosol and extracellular fluid). This aqueous drug must be in rapid exchange (on the relaxation time scale) with membrane-associated drug since the -11.2 ppm feature was characterized by a T_2 value of 3 msec (identical to the T_2 of the -10.3 ppm feature), and not the > 300 msec that would be expected of an aqueous site. This indicates that the lifetime (τ_B) of membrane-associated drug must be much shorter than the T_2 process ($\tau_B \ll 3$ msec) [24]. The exchange rate must, however, be slow relative to the chemical shift difference between membrane-associated and aqueous α-DFGBL, since features corresponding to the two environments had chemical shifts identical to those observed in pure solvents. This requires that:

$$\tau_B \cdot \Delta\omega \gg 1$$

where $\Delta\omega$ is the chemical shift difference between the two features [24]. Since $\Delta\omega$ was 2650 rad sec^{-1}, this means that τ_B must be $\gg 370$ μsec. Thus, the lifetime of α-DFGBL in brain membranes (τ_B) must be between 300 μsec and 3 msec.

Why was the feature at -11.2 ppm seen in brain but not in liver? Neither T_2 measurements nor chemical shift data provided any evidence of free α-DFGBL in liver. This suggests that the free concentration of drug must be low in liver, probably because of extensive partitioning into lipid membranes. A small feature at -11.2 ppm in the liver spectra may be obscured by the large, broad -10.4 ppm feature.

How do the NMR-characterized environments for α-DFGBL in brain tissue relate to the anticonvulsant effects of the drug? Based on the data from this study, it is plausible that free concentrations of α-DFGBL in brain are sufficiently high to produce effects on GABA-gated chloride conductances. While there is no evidence to support the preferential involvement of a specific NMR-defined environment in the pharmacologic effect of α-DFGBL, polar environments have been implicated in the actions of a variety of lipophilic drugs. For example, dihydropyridines have been shown to concentrate at the hydrocarbon core/water interface in biological membranes [25] and to diffuse along this plane [26]. Interactions in this plane might be a preferred path of access to the dihydropyridine binding site at the extracellular membrane surface [27]. Interactions with polar regions of the membrane have also been suggested as an important mechanism for optimizing ligand conformation and orientation for receptor binding [28–30]. The γ-butyrolactones are thought to interact at the picrotoxin binding site on the $GABA_A$ receptor. Picrotoxin is an uncharged but polar molecule that is likely to bind at a site at or near the aqueous/membrane interface. It is interesting to speculate that the weakly polar environment for α-DFGBL observed in brain may be an important pathway for presentation of the drug to its binding site.

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