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Determinants of benzodiazepine brain uptake: lipophilicity versus binding affinity

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Abstract. Factors influencing brain uptake of benzodiazepine derivatives were evaluated in adult Sprague Dawley rats ($n=8$–10 per drug). Animals received single intraperitoneal doses of alprazolam, triazolam, lorazepam, flunitrazepam, diazepam, midazolam, desmethyldiazepam, or clobazam. Concentrations of each drug (and metabolites) in whole brain and serum 1 h after dosage were determined by gas chromatography. Serum free fraction was measured by equilibrium dialysis. In vitro binding affinity (apparent K_i) of each compound was estimated based on displacement of tritiated flunitrazepam in washed membrane preparations from rat cerebral cortex. Lipid solubility of each benzodiazepine was estimated using the reverse-phase liquid chromatographic (HPLC) retention index at physiologic pH. There was no significant relation between brain:total serum concentration ratio and either HPLC retention ($r=0.18$) or binding K_i ($r=-0.34$). Correction of uptake ratios for free as opposed to total serum concentration yielded a highly significant correlation with HPLC retention ($r=0.78$, $P<0.005$). However, even the corrected ratio was not correlated with binding K_i ($r=-0.22$). Thus a benzodiazepine's capacity to diffuse from systemic blood into brain tissue is much more closely associated with the physicochemical property of lipid solubility than with specific affinity. Unbound rather than total serum or plasma concentration most accurately reflects the quantity of drug available for diffusion.

Key words: Benzodiazepines – Binding affinity – Lipid solubility – Brain uptake – Metabolites – Pharmacokinetics

Well defined, objective criteria for quantitating or predicting the clinical potency of the various benzodiazepine derivatives are not currently available. This is of importance for the assignment of "comparable" dosages (or comparable plasma concentrations) of different benzodiazepines for therapeutic purposes or in comparative preclinical studies. For example, some benzodiazepines, such as triazolam, are administered clinically in very low doses (i.e., 0.25–0.5 mg) and achieve correspondingly low plasma concentrations (0.5–5.0 ng/ml), whereas others, such as temazepam, require much higher doses (i.e., 15–30 mg), resulting in higher plasma concentrations (50–600 ng/ml), but producing comparable clinical effects (Greenblatt et al. 1983a). However, estimates of clinically "equivalent" dosages and plasma concentrations of two such drugs are generally based on experience and impression rather than objective criteria. A second important problem is the need to evaluate the contribution of endogenously-formed metabolites to overall clinical activity of a parent or precursor benzodiazepine. For a number of benzodiazepine derivatives, administration of therapeutic doses leads to the formation of one or more metabolites that appear in systemic plasma in significant amounts (Klotz et al. 1980; Greenblatt et al. 1982a, 1983a, b; Guentert 1984; Greenblatt and Shader 1985). Since the quantities of these metabolites, as well as their time-course of appearance and disappearance, may differ considerably from those of the parent compound, criteria for assessment of the activity of these metabolites is needed.

Differing intrinsic affinities of the various benzodiazepines for their postulated pharmacologic receptor at least partly underlie these differences (Tallman et al. 1980; Müller 1981; Skolnick and Paul 1982; Miller et al. 1987a, b). Nonetheless, before a benzodiazepine derivative can have access to its receptor site, it must reach the extracellular water surrounding the cellular components containing the functional receptor. The delivery of drug to the receptor site is achieved via the systemic circulation, which brings the drug to the cerebral circulation from which it must diffuse into brain tissue. The extent of benzodiazepine diffusion from systemic blood into brain, and the factors influencing this diffusion, have received relatively little attention. The present study evaluated the extent of uptake of a series of benzodiazepines into mammalian brain, and assessed some physicochemical and molecular factors influencing this process.

Methods

Procedures for animal experiments. Experimental animals were adult male Sprague Dawley rats (150–340 g) housed under diurnal lighting conditions with free access to food and water. Animals received a single intraperitoneal dose of the following benzodiazepines: alprazolam, triazolam, lorazepam, flunitrazepam, diazepam, midazolam, desmethyldiazepam, or clobazam (Table 1). Each drug was administered to eight to ten different animals, and each animal

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Table 1. Summary of benzodiazepines administered and measured in serum and brain

Administered	Dose (mg/kg)	Present in serum and brain	Reference to analytic method
Alprazolam	2.5	-Alprazolam	Greenblatt et al. 1981b, 1983d
Triazolam	1.25	-Triazolam	Greenblatt et al. 1981b, 1983d
Lorazepam	2.5	-Lorazepam	Greenblatt et al. 1978
Flunitrazepam	1.25	-Flunitrazepam	Greenblatt et al. 1982c
Diazepam	10.0	-Diazepam -Desmethyldiazepam -Oxazepam	Greenblatt et al. 1980
Midazolam	50.0	-Midazolam -1-Hydroxy midazolam[1] -4-Hydroxy midazolam	Arendt et al. 1984
Desmethyldiazepam	12.5	-Desmethyldiazepam -Oxazepam	Greenblatt et al. 1980
Clobazam	50.0	-Clobazam -Desmethylclobazam	Greenblatt 1980

1 Also termed 1-hydroxymethyl midazolam

received only one drug. Animals were sacrificed by decapitation 1 h after drug administration. The whole brain was immediately dissected free, weighed, and stored at −30° C until the time of assay. A sample of blood was simultaneously obtained from the carotid artery. The blood was allowed to clot, centrifuged, and the serum separated and frozen at −40° C until the time of assay.

Analysis of benzodiazepines in serum and brain. Whole brain samples were mechanically homogenized in 0.9% sodium chloride using a glass-glass homogenizer. Whole brain concentrations of each benzodiazepine and its metabolites were determined by electron-capture gas-liquid chromatography after addition of a suitable internal standared (Table 1). Previous studies have demonstrated that benzodiazepine concentrations in cerebral cortex are within 5% of those measured in whole brain (Miller LG, Greenblatt DJ: unpublished data). Serum concentrations were analyzed by similar methods.

Following measurement of total serum benzodiazepine concentrations, the remaining serum was pooled into 3 aliquots. After addition of tracer amounts of radiolabelled benzodiazepine derivatives, samples were subjected to equilibrium dialysis at 37° C for 18 h (Moschitto and Greenblatt 1983). The extent of serum protein binding was determined as the ratio of radioactivity present in the dialysate divided by that remaining in the serum. The free concentration of each benzodiazepine was determined as the product of the total concentration multiplied by the free fraction. Previous studies have demonstrated that benzodiazepine free fraction is independent of total concentration (Moschitto and Greenblatt 1983).

Determination of binding affinity. Cerebral cortices from adult male Sprague Dawley rats (150–175 *g*) were homogenized in 20 volumes (w/v) of ice cold 0.32 M sucrose using a Teflon-glass homogenizer. The homogenate was centrifuged at 1000 *g* for 10 min at 4° C. The supernatant was recentrifuged at 20000 *g* for 20 min at 4° C, and the pellet resuspended in 20 volumes of ice cold 50 mM Tris-HCl buffer (pH = 7.4) with a Polytron (Brinkman Instruments). This procedure was repeated twice before the pellet was frozen at −20° C. Just prior to assay, the frozen membranes were resuspended in 20 volumes of ice cold 50 mM Tris buffer and recentrifuged at 20000 *g* for 15 min at 4° C. This procedure was repeated 3 times before diluting the membrane with 50 mM Tris HCl buffer (pH = 7.4) to yield a membrane suspension with a protein concentration of approximately 2 mg/ml). The protein concentration was measured by the Lowry method. Membrane suspension aliquots (0.2 ml) were incubated for 60 min at 0° C with approximately 1 nM (^{3}H)-flunitrazepam (specific activity: 92.3 Ci/mM; New England Nuclear, Boston, MA), in 0.5 ml of an incubation medium which contained 50 mM Tris-HCl buffer (pH = 7.4) and various concentrations of the benzodiazepines to be tested. After reaching equilibrium (60 min), the samples were filtered under vacuum through Whatman GF/B filters and immediately washed twice with 5 ml ice cold buffer. Radioactivity was measured by liquid scintillation spectroscopy. K_i values were derived from IC_{50} values according to Cheng-Prusoff equation:

$$K_i = \frac{IC_{50}}{1 + S/K_d}$$

where S is the concentration of (^{3}H)flunitrazepam and K_d is the apparent dissociation constant (0.85 nM) calculated by Scatchard analysis in separate experiments (McPherson 1983). Each K_i value is the mean of two to three determinations.

Determination of liquid chromatographic (HPLC) retention. Retention time on a HPLC system (Arendt et al. 1983; Greenblatt et al. 1983c) was determined using a reverse-phase C_{18}-microBondapack column (Waters Associates, Milford, MA). The mobile phase was methanol-acetonitrile-0.001 M sodium acetate buffer (25:25:50), having a final pH of 7.4. The mobile phase flow rate was 1.5 ml/min. Methanolic solutions of the pure benzodiazepines were sequentially injected, with column effluent quantitated by ultraviolet detection at 254 nm. All analyses were performed at room temperature.

HPLC retention times for individual drugs were ex-

Table 2. Summary of benzodiazepine lipophilicity, receptor affinity, and brain uptake

Drug	HPLC retention index[1]	Receptor K_i	Brain:total serum concentration ratio	Free fraction in serum	Brain:unbound serum concentration ratio
Midazolam	1.544	0.44	2.52	0.074	33.91
Diazepam	1.000	9.57	3.34	0.133	26.05
Desmethyldiazepam	0.793	5.58	3.14	0.142	22.18
1-OH midazolam	0.713	2.23	0.85	0.103	8.29
Triazolam	0.643	0.47	5.48	0.239	19.52
4-OH midazolam	0.593	6.61	0.93	0.215	4.28
Alprazolam	0.544	4.24	0.92	0.349	2.62
Lorazepam	0.482	1.64	2.56	0.157	16.01
Oxazepam	0.453	11.53	3.52	0.171	20.30
Clobazam	0.397	222.5	2.25	0.377	5.96
Flunitrazepam	0.310	1.66	1.15	0.193	5.97
Desmethylclobazam	0.289	2843.0	2.30	0.289	7.95

1 Relative to diazepam

pressed as the ratio to the retention time for diazepam, yielding a retention index.

Statistical analysis. For each benzodiazepine derivative in each animal, the ratio of the whole brain concentration to the total concentration in serum, as well as the whole brain to unbound (free) serum concentration, was calculated. The mean uptake ratio for each drug (and metabolites when appropriate) was then calculated across the eight to ten animals that received that individual drug. Mean uptake ratios were then assessed by correlational analysis in relation to the HPLC retention index as well as to the apparent K_i value.

Results

Table 2 shows values of each variable for each drug. There were wide variations among drugs in lipophilicity based on the HPLC retention index (from midazolam, 1.54, down to desmethylclobazam, 0.29), in binding K_i (from midazolam, 0.44, and triazolam, 0.47, up to desmethylclobazam, 2843.0), in serum free fraction (midazolam, 0.07, up to clobazam, 0.38) and in brain to unbound serum concentration ratio (midazolam, 33.9, down to alprazolam, 2.6).

Linear regression analysis indicated no significant relationship between whole brain:total serum concentration ratio versus HPLC retention index ($r=0.18$) or versus binding K_i ($r=-0.34$). However, correction of uptake ratios for the free as opposed to total serum concentration yielded a highly significant relationship between whole brain:unbound serum uptake ratio and HPLC retention index ($r=0.78$, $P<0.005$; Fig. 1). Again, there was no significant relationship between binding K_i and brain:unbound serum uptake ratio ($r=-0.22$).

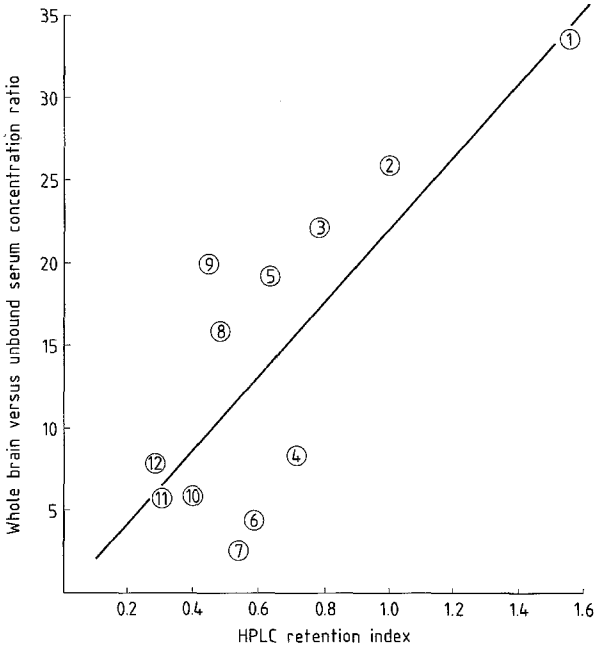

Fig. 1. Relation between in vitro lipophilicity based on the HPLC Retention Index and the whole brain versus unbound serum concentration ratio for 12 benzodiazepines. Line was determined by linear regression analysis (see Table 2 for individual values). $r=0.78$; $P<0.005$; 1 = Midazolam; 2 = Diazepam; 3 = Desmethyldiazepam; 4 = 1-Hydroxy Midazolam; 5 = Triazolam; 6 = 4-Hydroxy Midazolam; 7 = Alprazolam; 8 = Lorazepam; 9 = Oxazepam; 10 = Clobazam; 11 = Flunitrazepam; 12 = Desmethylclobazam

Discussion

Differences among benzodiazepines in the intensity of pharmacodynamic action at any given dose or serum concentration depend in part on differences in their affinity for the functional receptor (Tallman et al. 1980; Müller 1981; Skolnick and Paul 1982; Miller et al. 1987a, b). However, drug molecules must first become available to extracellular water surrounding the receptor before the drug-receptor interaction is possible (Borea and Bonora 1983). The HPLC retention index is an in vitro measure of benzodiazepine lipophilicity which in previous studies has been a better predictor of in vivo benzodiazepine distribution than the octanol:-buffer partition coefficient (Arendt et al. 1983, Greenblatt et al. 1983c). The high correlation of this HPLC retention index and the ratio of whole brain divided by free serum concentrations suggests that the physicochemical property of lipid solubility is closely associated with a given benzodiazepine's capacity to diffuse from the systemic circulation into brain tissue. Also of critical importance is the extent of benzodiazepine binding to serum protein, inasmuch as only the unbound concentration in serum of plasma is available for diffusion out of the vascular system to peripheral

Table 3. Evaluating possible clinical importance of benzodiazepine metabolites

	Quantitative importance	Lipophilicity (HPLC Retention Index)	Brain: free serum ratio	Receptor K_i
I. Diazepam and metabolite desmethyldiazepam				
Parent drug: diazepam	+	1.00	26.1	9.6
Metabolite: desmethyldiazepam	+	0.79	22.8	5.6
II. Clobazam and metabolite desmethylclobazam				
Parent drug: clobazam	+	0.40	6.0	222.5
Metabolite: desmethylclobazam	+ +	0.29	8.0	2843.0
III. Midazolam and metabolite 1-hydroxy and 4-hydroxy midazolam				
Parent drug: midazolam	+	1.54	33.9	2.23
Metabolite: 1-hydroxy midazolam	+	0.71	8.3	2.23
Metabolite: 4-hydroxy midazolam	±	0.59	4.3	6.61

tissues (Greenblatt et al. 1982b; Sellers et al. 1982). Although the correlation between HPLC retention and *unbound* uptake ratio was high, there was no significant correlation with *total* serum benzodiazepine uptake ratio.

Thus the objective assignment of comparable dosages or plasma concentrations of different benzodiazepines, or the assessment of the importance of endogenously formed metabolites, should involve the simultaneous consideration of molecular as well as physicochemical factors. Since specific binding affinity as well as tissue uptake characteristics of benzodiazepines are highly comparable between rats and humans (Sieghart et al. 1985; Scavone et al. 1987), data from one species may in part be extrapolated to the other. Evaluation of the importance of the metabolic products of three benzodiazepines – diazepam, clobazam and midazolam – serves to illustrate the process. During multiple-dose therapy with diazepam, steady-state serum or plasma concentrations of its metabolite desmethyldiazepam are equal to, or slightly exceed, those of diazepam, indicating quantitative importance of this metabolic product (Eatman et al. 1977; Greenblatt et al. 1981a, 1984; Abernethy et al. 1983; Ochs et al. 1983). Desmethyldiazepam lipophilicity is close to that of diazepam, the brain: unbound serum concentration ratio for the two compounds is similar, and the binding affinity of desmethyldiazepam actually exceeds that of diazepam (Table 3). Taken together, these data indicate that desmethyldiazepam most probably does contribute to overall clinical activity during chronic therapy with diazepam. A second example is the benzodiazepine clobazam. During chronic treatment with clobazam, its demethylated metabolite (desmethylclobazam) is also of quantitative importance, with steady-state desmethylclobazam concentrations exceeding those of clobazam by twofold or more (Greenblatt et al. 1983e; Ochs et al. 1984). The brain:unbound serum concentration ratios of clobazam and desmethylclobazam are similar, but the binding affinity of desmethylclobazam is more than tenfold lower than that of clobazam (Table 3), making it unlikely that desmethylclobazam adds an important component to the overall clinical action of clobazam. Finally the benzodiazepine midazolam has two hydroxylated metabolites (Table 1). Following administration of midazolam to humans, serum or plasma concentrations of 1-hydroxymidazolam are similar to those of the parent compound, while concentrations of 4-hydroxy midazolam are considerably lower (Arendt et al. 1984; Reves et al. 1985). Due to their lower lipophilicity, both the 1- and 4-hydroxy metabolites have considerably lower brain:free serum uptake ratios than that of midazolam, and both metabolites likewise have reduced binding affinity (higher K_i) compared to the parent drug (Table 3). Therefore these metabolites are unlikely to be clinically important.

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