Plasma Binding and Transport of Diazepam across the Blood-Brain Barrier

No Evidence for In Vivo Enhanced Dissociation

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Abstract

The tissue uptake of extensively plasma-bound compounds is reportedly inconsistent with the conventional free-drug hypothesis limiting transport to unbound moiety in rapid intracapillary equilibrium with bound complex. Instead, protein-mediated/cell surface enhancement of dissociation has been postulated to occur in the microvasculature. This possibility was investigated by studying the passive transport of diazepam across the blood-brain barrier. Microdialysis probes placed within the vena cava and brain cortex were used to directly compare steady-state, interstitial unbound diazepam levels in both Wistar and genetically analbuminemic rats. The absence of albumin in the latter increased the unbound fraction of diazepam by almost fivefold; however, in both groups, the ratio of unbound concentrations in brain and blood at equilibrium was equal to unity. If enhanced dissociation occurred in the microvasculature, then the unbound brain level should have been greater than that in the systemic circulation. It is probable that earlier findings suggestive of protein-mediated transport reflect a nonequilibrium phenomenon. Comparison of the extent of diazepam's in vivo binding in blood by microdialysis to that estimated in vitro using conventional equilibrium dialysis with microcells showed good agreement, thus validating a widely accepted assumption of equivalency of these two values.

Introduction

Many endogenous compounds, drugs, and other xenobiotics are reversibly bound to plasma proteins, often to a significant extent. However, tissue uptake is frequently rapid and efficient despite the small fraction of available unbound ligand. Conventionally, such uptake is thought to be limited to the unbound moiety with the intracapillary binding equilibrium maintained by rapid dissociation of the ligand-protein complex. However, the validity of this free-drug (hormone) hypothesis has been challenged by recent experimental observations on the transport of certain highly bound ligands. For example, the hepatic uptake of a number of organic anions (1–5), steroids (6, 7), thyroxine (8, 9), and drugs (10) is greater than predicted by their in vitro binding characteristics, and the rate of transport correlates better with the albumin-bound concentration than the unbound level. The brain extraction of drugs such as valproic acid (11) and benzodiazepines (12) as well as steroids (7, 13–15) and thyroid hormones (7) has also been interpreted to indicate a role for albumin in transmembrane transport beyond that associated with the simple binding interaction.

In many instances, the discordancy between the conventional concept and experimental observations of a protein-mediated effect may be accounted for, at least operationally, if the binding affinity of the ligand is smaller in vivo than that measured in vitro (2–4, 7, 9–15). Cell surface–mediated alterations in protein conformation resulting in enhanced dissociation (9, 16) or the presence of local inhibitors of binding within the microvasculature (9) have been suggested as possible biochemical mechanisms for the phenomenon. Direct experimental support for such a hypothesis is, however, lacking, as the protein-mediated interpretation is based on the use of kinetic models whose inherent assumptions may not be valid (17, 18).

In principle, enhanced in vivo dissociation may be demonstrated, provided that only the unbound ligand is transported and its concentration can be determined within tissue water, because at equilibrium such a level should be identical to that at the capillary surface (19). Accordingly, transport consistent with the conventional hypothesis would result in an unbound tissue level equal to that measured in systemic blood, whereas a higher concentration would indicate enhanced dissociation in the microvasculature. This study investigates these two possibilities by examining the transport of diazepam across the blood-brain barrier of the rat; a passive process which appears to exhibit a protein-mediated effect that may be accounted for by enhanced dissociation (12). The results provide direct evidence in support of the conventional free-drug hypothesis. Furthermore, the data suggest that the previous interpretations of enhanced in vivo dissociation may be a result of nonequilibrium conditions.

Methods

Experimental design. Normal adult Wistar rats (n = 6) weighing between 271 and 311 g (Harlan Sprague-Dawley Inc., Indianapolis, IN) and fed regular laboratory chow and water ad lib. were used. A similar sized group of Nagase analbuminemic rats, kindly supplied by Dr. J. Roy Chowdhury (Liver Research Unit, Albert Einstein College of Medicine, New York) weighing 210–306 g, were also investigated. On the day of study, each animal was anesthetized with sodium pentobarbital (45 mg/kg i.p.) and both femoral veins were cannulated with PE-50 tubing. Anesthesia was maintained throughout the remainder of the study by constant rate infusion into the left femoral vein of sodium pentobarbital (0.2 mg/kg per min) in Ringer's solution containing 5

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mM Hepes, pH 7.4. Body temperature was monitored by a rectal probe and maintained at 37°C by a heating pad. A microdialysis probe was then surgically placed in the inferior vena cava and subsequently another was placed in the cortex of the brain. Infusion ($3.712 \times 10^7 \pm 1.59 \times 10^5$ dpm/ml, mean±SD) of [*N-methyl*-^{3}H]diazepam (New England Nuclear, Boston, MA; sp act 76.7 Ci/mmol, > 99% purity by TLC) in 5 mM Hepes-buffered Ringer's solution (pH 7.4) was then begun through the left femoral vein cannula. For the first 60 min the infusion rate was held constant at 100 μl/min and then it was reduced to 16 μl/min and maintained at this value for the remainder of the experiment. Blood samples were periodically obtained from the right femoral vein cannula at various times after beginning the slower rate of infusion. At the end of the experiment, the animal was decapitated, a heparinized blood sample was collected, and the brain cortical tissue was excised. Both the blood and brain samples were stored at −20°C before analysis. The unbound fraction of diazepam in blood (f_B^u)[1] and brain tissue (f_T^u) were calculated from the ratio of the respective microdialysis perfusate concentration and the total drug level.

Placement of microdialysis probes. Microdialysis probes (4 mm dialysis membrane tip, 20 mm cannula length, 0.64 mm OD, molecular mass cut-off 15,000 D) were obtained from Carnegie Medicine AB/Bioanalytical Systems Inc. (West Lafayette, IN). After laparotomy, one probe was placed in the iliolumbar vein through a small incision made ~ 4 mm from its junction with the inferior vena cava. The probe tip was gently pushed into the vena cava and kept in place by ties around the steel cannula shaft, and then stitched to the abdominal muscle. Throughout placement and the remainder of the study, the probe was continuously perfused with phosphate buffer solution (pH 7.4) (22 mM Na_2HPO_4, 5.6 mM $NaH_2PO_4 \cdot H_2O$, and 58.5 mM NaCl) at 2 μl/min using a microinjection pump, (model CMA 100; Carnegie Medicine AB/Bioanalytical Systems Inc.). Another probe was placed in the brain cortex using a stereotaxic instrument (model BR-2; Carnegie Medicine AB/Bioanalytical Systems Inc.). After exposure of the frontal and parietal bones of the skull, a 2-mm hole was drilled 3.2-mm anterior and 1.15 mm lateral to the bregma. A steel rod with the same external diameter as the probe was then lowered 5 mm deep into the brain and withdrawn using the micromanipulator. The probe was placed in the hole and its position was maintained so that the dialysis tip was embedded within the cortical tissue. Artificial cerebrospinal fluid (126.5 mM NaCl, 27.5 mM $NaHCO_3$, 2.4 mM KCl, 0.5 mM KH_2PO_4, 1.1 mM $CaCl_2$, 0.85 mM $MgCl_2$, 0.5 mM Na_2SO_4, 5.9 mM glucose, pH 7.4) was continuously perfused through the probe at a rate of 2 μl/min using the microinjection pump. The perfusates from both microdialysis probes were collected every 20 min into small conical vials containing 20 μl 5 mM Hepes-buffered Ringer's solution (pH 7.4) and stored at 4°C until analyzed.

Calibration of microdialysis probe. To relate the concentration of [^{3}H]diazepam in the collected perfusate to that in the particular fluid surrounding the dialysis tip, the efficiency of recovery of each microdialysis probe was determined before use. This was accomplished by perfusing the probe at 2 μl/min with the appropriate solution and placing its tip into various solutions of [^{3}H]diazepam in 5 mM Hepes-buffered Ringer's solution, pH 7.4, ranging in concentration from 5 to 150 dpm/μl. After a 60-min equilibration period, 20-min samples were collected and the radioactivity in both the perfusate and bathing solution was determined by liquid scintillation counting. In the majority of studies, calibration of the probes was also performed subsequent to their recovery from the animal after killing.

Analysis of [^{3}H]diazepam. The concentration of [^{3}H]diazepam in blood, brain tissue, and microdialysis probe perfusate samples was determined by liquid scintillation after reverse-phase HPLC separation of unchanged drug from its metabolites. Briefly, 2 μg of unlabeled diazepam in 25 μl methanol and 1 ml saturated sodium borate solution, pH 9.5, was added to 0.1 ml sample; with brain tissue, a 10% wt/vol homogenate (0.5 ml) in artificial cerebrospinal fluid was used. The mixture was extracted twice with 5 ml toluene/isopropyl alcohol (96:4) and the combined organic layers evaporated to dryness under nitrogen at 50°C. After reconstitution with 100 μl methanol, the extract was chromatographed using a 5-μm, octadecyl 25 cm × 4.6 mm Ultrasphere column (Beckman Instruments Inc., Fullerton, CA) with spectrophotometric detection at 230 nm. The mobile phase consisted of acetonitrile/water (1:1) at a flow rate of 1 ml/min. The eluent corresponding to the diazepam peak was collected into a scintillation vial and evaporated under nitrogen at 50°C; 5 ml Aquasol (New England Nuclear) was added and radioactivity was determined by scintillation counting using external standardization. Assay recovery was determined relative to the peak height response of the added unlabeled diazepam. In the case of probe perfusate samples, the organic extraction step was omitted and chromatography was performed after the addition of internal standard.

Total radioactivity in blood was also determined by solubilizing a 50–100-μl sample with 0.5 ml Protosol (New England Nuclear)/ethanol (1:2) at 60°C for 1 h followed by the addition of 0–5 ml hydrogen peroxide solution (30% vol/vol) and heating for a further 30 min. After cooling, the sample was mixed with 15 ml Biofluor (New England Nuclear) and 0.5 ml 0.5 N HCl and analyzed by liquid scintillation counting.

In vitro protein binding. The in vitro binding of diazepam in blood and plasma obtained at the end of the study was determined using [2-^{14}C]diazepam (Amersham Corp., Arlington Heights, IL; sp act 52.3m Ci/mmol, > 99% purity by TLC). Labeled drug to produce a concentration of 62.5×10^3 dpm/ml, equivalent to 0.15 μg/ml, was added to the heparinized blood sample and incubated at 37°C for 15 min, an aliquot of plasma was then harvested. The unbound concentration of [^{14}C]diazepam was determined at 37°C in both blood and plasma using the described microdialysis technique; the calibrated probe was perfused at 2 μl/min for 60 min with pH 7.4 phosphate buffer before serial collection of 20-min samples. Radioactivity in aliquots of the perfusate and also the blood and plasma samples was then determined by liquid scintillation counting. Plasma binding was also determined by equilibrium dialysis at 37°C with semimicro cells and a membrane (Spectrapor Spectrum Medical Industries, Inc., Los Angeles, CA; molecular mass cut-off, 12,000–14,000 D) as previously reported (20).

Results

The relative recovery of [^{3}H]diazepam across the microdialysis membrane typically ranged from 15 to 25% for individual probes and was independent of the bathing fluid concentration over the range 5 to 150 dpm/μl. Moreover, the recovery measured after probe implantation, use, and removal was similar to that determined before the in vivo study, regardless of tissue location. Good agreement was also found between the binding of [^{14}C]diazepam in plasma obtained at the end of the study using the microdialysis technique and the more conventional approach based on equilibrium dialysis with semimicro cells. For example, the unbound fraction in Wistar rats was 0.123±0.006 (mean±SD) by microdialysis compared with a value of 0.127±0.023 using the more conventional technique. Similar correspondence (ratio of unbound fractions, 0.950±0.127) was also noted with plasma obtained from analbuminemic rats.

Blood concentrations of total radioactivity increased to a varying degree throughout the two-step intravenous infusion period, presumably because of the accumulation of radiolabeled metabolites. However, the concentrations of HPLC-separated radioactivity, i.e., unchanged diazepam, attained a steady state in both rat strains ~ 1 h after beginning the slow

1. *Abbreviations used in this paper:* f_B^u, unbound fraction of diazepam in blood; f_T^u, unbound fraction of diazepam in tissue.

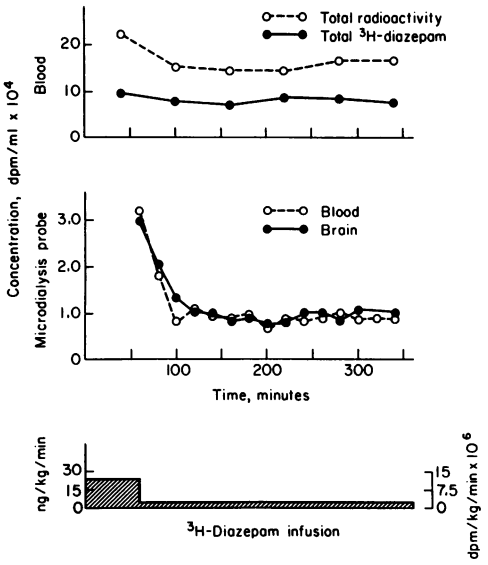

Figure 1. Blood and microdialysis probe perfusate concentrations during intravenous infusion of [^{3}H]diazepam to a representative Wistar rat (zero time corresponds to the beginning of the rapid (loading) infusion period).

infusion and this condition was maintained throughout the remainder of the study (Fig. 1). The average steady-state blood concentration of total [^{3}H]diazepam during the 120–300-min period was almost threefold higher ($P < 0.001$, two-tailed, unpaired t test) in Wistar than analbuminemic rats (Table I). On the other hand, the steady-state unbound level of [^{3}H]-diazepam given by the perfusate concentration from the microdialysis probe located in the inferior vena cava was ~ 1.5–2-fold higher ($P < 0.002$) in the analbuminemic animals (Table I). Accordingly, clearance from the blood (infusion rate/steady-state blood concentration) was more efficient with respect to total (unbound plus bound) diazepam in the analbuminemic rats (17.82±2.50 ml/min vs. 6.59±1.23 ml/min, $P < 0.001$) but less rapid when unbound drug was considered (33.05±9.92 vs. 60.95±13.42 ml/min, $P < 0.003$).

Table I. Steady-State Disposition Characteristics (Mean±SD) of Unchanged [^{3}H]Diazepam in Wistar and Analbuminemic Rats after Intravenous Infusion

	Wistar rat $n = 6$	Analbuminemic rat $n = 6$
Total blood concentration (*dpm/μl*)	113.82±16.55	42.65±5.94
Total brain concentration (*dpm/μl*)	274.56±51.45	435.93±126.68
Brain/blood concentration ratio for total drug	2.41±0.43	10.60±3.67
Microdialysis perfusate (unbound) concentration from blood probe (*dpm/μl*)	12.65±2.46	20.88±3.59
Microdialysis perfusate (unbound) concentration from brain probe (*dpm/μl*)	12.37±2.33	20.80±4.23
Microdialysis perfusate concentration ratio, brain/blood	0.982±0.039	0.995±0.41
Unbound in blood, in vivo, f_B^u (%)	11.07±1.60	50.02±10.70
Unbound blood, in vitro (%)	14.72±2.06	43.13±5.69*
Unbound in brain, f_T^u (%)	4.54±0.70	5.04±1.38
Unbound in blood/unbound in brain (%)	2.46±0.51	10.70±3.86

* $n = 3$.

The perfusate concentration from the brain microdialysis probe fluctuated and fell to a varying extent during the period immediately after placement and the beginning of intravenous infusion of [^{3}H]diazepam. However, within 60–90 min after reducing the intravenous infusion rate the level had stabilized and remained constant for the remainder of the experiment (Fig. 1). Similar to the unbound [^{3}H]diazepam level in the blood, the brain perfusate concentration was higher in analbuminemic than Wistar rats ($P < 0.003$). Importantly, however, no significant difference was found between the microdialysis probe perfusate (unbound) concentrations of [^{3}H]diazepam in blood and brain (Table I). The concentration ratio was close to unity at each individual sampling time during steady state in every animal studied, regardless of strain (Table I, Fig. 2).

Comparison of [^{3}H]diazepam binding based on the perfusate concentration from the microdialysis probe in the vena cava and the total drug level in blood indicated that the unbound fraction was about fivefold higher in the analbuminemic animals (Table I). Similar in vitro values for binding were also obtained using the microdialysis technique and [^{14}C]-diazepam added to heparinized blood after termination of the study (Table I). Because the binding of [^{3}H]diazepam to brain tissue was similar in the two rat strains, the reduced plasma binding in the analbuminemic rats resulted in the total [^{3}H]-diazepam brain level being greater, and the brain/tissue concentration ratio was higher and similar to the ratio of the unbound fractions in blood and brain (Table I).

Discussion

According to the conventional free-drug hypothesis, the tissue uptake of plasma-bound ligands by passive transport depends on the concentration gradient of unbound moiety between the capillary endothelial surface and tissue water (19). Moreover,

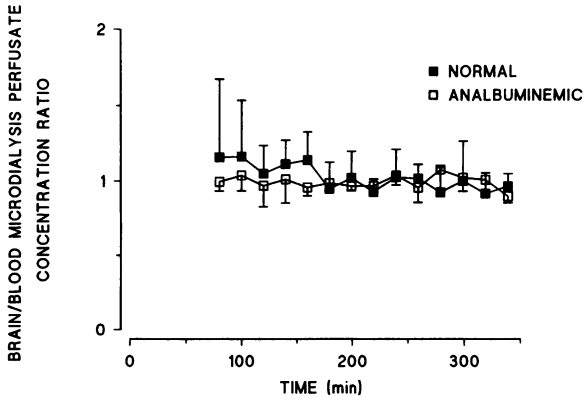

Figure 2. Ratio of brain to blood microdialysis probe perfusate concentrations (mean±SD) during constant rate intravenous infusion of [^{3}H]diazepam to Wistar and analbuminemic rats ($n = 6$).

rapid dissociation of ligand-protein complex maintains binding equilibrium that is the same throughout the vasculature and can be determined by appropriate in vitro techniques such as equilibrium dialysis. Because of the unique characteristics of the brain capillary endothelium, the translocation of drugs and certain hormones across the blood-brain barrier has been considered a classic example of such transport (21). In the case of diazepam, the similarity of cerebrospinal fluid concentrations to those of estimated unbound drug in systemic plasma is consistent with this conventional concept (22–24). However, data based on measurement of diazepam's unidirectional brain extraction by the tissue sampling, single carotid artery injection technique could not be explained on this basis. Uptake appeared to be greater than expected and the discordancy became greater as the level of albumin increased and unbound fraction decreased (12). Similar findings using the same procedure have also been reported with other compounds (7, 11, 13–15). Operationally, the experimental observations can be accounted for if the ligand's affinity for albumin in vivo is less than that measured in vitro, which could occur if dissociation of bound complex within the microvasculature is different from that elsewhere in the circulation (7, 9–15). A similar protein/cell surface–mediated enhanced dissociation has also been postulated to be involved in the hepatic transport of various highly bound anions and drugs (2–4, 7, 10).

Under equilibrium conditions the unbound concentration in brain tissue should be equal to that at the capillary surface (19); thus, enhanced dissociation in the microvasculature would result in a higher unbound brain level than that measured in systemic blood. In the case of diazepam, the expected difference should be $\sim$ 25-fold (12). The use of microdialysis probes placed in the vena cava and brain cortex permitted direct measurement of the magnitude of this concentration difference. In this technique, buffer solution perfusing through a hollow fiber is separated from surrounding tissue fluid by a semipermeable membrane. Small molecules dialyze across the membrane and are subsequently measured in the effluent perfusate (25, 26). Any initial tissue damage associated with probe placement appears to be rapidly repaired and the concentration of the measured compound in the perfusate then reflects that in the interstitial fluid in the immediate vicinity of the dialysis tip (25). The good agreement between estimates of the extent of in vitro binding by microdialysis and equilibrium dialysis suggest that this concentration corresponds to unbound drug. Further support for this assumption is provided by the excellent agreement between the ratio of unbound fractions in blood and brain to the brain to blood concentration ratio for total drug measured at the end of the study. If equilibrium is achieved between unbound drug in tissue and that in emergent venous blood, then these two ratios should be identical (27).

The steady-state conditions in both measured fluids allowed the effects of dynamic and transient processes dependent on unbound diazepam to be disregarded, including any minimal metabolism of the drug within the brain (28). Moreover, the maintenance of steady state for several hours provided ample opportunity for equilibrium to be attained between kinetically distinct compartments within blood or brain. In this regard, binding of diazepam to the extremely small amounts of albumin or other proteins present in brain extracellular fluid (21) is likely to be of negligible significance. Assuming that steady-state equilibrium was achieved and that intratissue translocation is limited to unbound drug, then the concentration of this moiety would be the same throughout the brain. Accordingly, the experimental observation that the unbound diazepam was the same in brain interstitial fluid as that in systemic blood is a major finding, as this is the expected result predicted by the free-drug hypothesis. Another important finding was that the in vitro binding of diazepam in blood, as estimated by microdialysis, was similar to that measured in vivo in the vena cava. Countless studies have been performed based on the assumption of such equivalency but this is perhaps the first to directly establish its validity.

Discordancy between the conclusion of this study and that reported earlier (12) may be accounted for by important differences in experimental design. The steady-state condition is not likely to be contributory because the unidirectional brain extraction of diazepam was found to be unchanged by prior establishment of a constant blood level of unlabeled drug (12). However, differences in the degree of equilibrium present in the brain capillary blood are probably critical. After rapid injection of a small bolus containing ligand and albumin into the carotid artery (12), axial mixing has been shown to be $< \sim 5\%$ and of negligible importance (29). On the other hand, complicated radial gradients of unbound ligand, albumin-bound ligand, and ligand-free albumin probably exist. Minimally, these involve the bulk-flow solution and a functional unstirred layer at the endothelial surface. Physical streaming of the injectate could add further to such heterogeneity (30). Only a few seconds are available for equilibrium to be achieved during the single passage of the bolus through the brain microvasculature. By contrast, infusion of diazepam into the peripheral circulation over several hours provides excellent opportunity for complete equilibrium to be attained and diazepam binding to be the same throughout all of the brain capillary. Importantly, the transport of unbound ligand under nonequilibrium conditions appears theoretically to be facilitated as a function of the albumin concentration and in such a way that the uptake of total ligand is similar to that which would occur if enhanced dissociation was present (31). Probably this nonequilibrium effect also accounts for the apparent protein-mediated uptake of other compounds by the brain (7, 11, 13–15) and liver (6–9) using the single-pass, rapid injection technique, and may be a possible factor with other experimental approaches (1–5, 10).

Albumin is virtually absent in genetically analbuminemic rats (32) and a decrease in binding of diazepam would be anticipated because normally this is the major plasma binding protein to which the drug binds in a concentration-independent fashion (33). Surprisingly, the extent of binding (50%) was still relatively high, indicating interactions with the elevated plasma levels of compensatory proteins (32). The observation that the unbound brain tissue to blood concentration ratio was the same in analbuminemic rats as that in the Wistar strain would appear to argue against a functional role of any albumin receptor in the brain uptake process (34–37). Potential quantitative interstrain differences in the determinants of diazepam's disposition, such as free intrinsic clearance (27) and the possible involvement of extrahepatic metabolism did not allow further pharmacokinetic interpretation of the observed differences in diazepam's clearance rates in the two groups of rats.

In conclusion, direct studies of the transport of diazepam across the blood-brain barrier under equilibrium conditions have shown that the process is consistent with the conventional, free-drug hypothesis. Enhanced dissociation of the di-

azepam-albumin complex within the microvasculature does not occur, and an earlier conclusion to this effect probably reflects nonattainment of true equilibrium within the brain microvasculature under the particular experimental conditions used. The presence of a similar phenomenon may also account for other reports of protein-mediated transmembrane transport in the brain, liver, and other organs.

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