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The Role of Substituents in the Hydrophobic Binding of the 1,4-Benzodiazepines by Human Plasma Proteins

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SUMMARY

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The effect on the extent of plasma protein binding by varying the substituents on the basic benzodiazepine molecule has been studied. Hansch lipophilic substituent constants were calculated for each substituent on the molecule. A correlation was found between the degree of protein binding and changes in the lipophilic nature of the molecule as measured by the summation of its lipophilic substituent constants. Protein binding increased with the increasing lipophilic character of substituents in positions 1, 2, 3, 4, 7, and 4'. The electronic character of substituents in position 1, 3, or 7, as expressed by their Hammett constant (σ), was also found to correlate with the observed protein binding. Changes in protein binding with respect to various 2'-substituents were not related to the lipophilic nature of the 2'-substituents but may be the result of changes in the spatial orientations of the benzodiazepine molecule in relation to its albumin binding loci. It was concluded that the major factor in determining the extent of 1,4-benzodiazepine binding to human plasma proteins is the degree of lipophilicity of the molecule. Substituents which affect the conformation of the molecule, such as those in position 2', also affect the extent of binding.

INTRODUCTION

Plasma proteins, particularly albumin, have the ability to form reversible complexes with a wide variety of organic ligands. Albumin has been shown to possess multiple binding sites, the number, nature, and affinity of which vary with the nature of the binding ligand (1).

Structural alteration of the drug may significantly affect its physicochemical nature and consequently alter the degree to which it interacts with plasma proteins. Changes in the extent to which a compound binds to plasma proteins may influence its pharmacological response by altering its unbound concentration in plasma water (2).

There have been a number of attempts to determine the physicochemical nature of the drug-protein binding forces (3, 4). Several authors have recently reported quantitative correlation of protein binding with drug hydrophobicity (5-9).

At present little has been reported on the nature of the interactions between the 1,4-benzodiazepines, a class of widely used psychotropic agents, and plasma proteins. A study was therefore conducted to investigate the relationship between the chemical structure of the benzodiazepines and their degree of binding to plasma proteins.

MATERIALS AND METHODS

Experimental Design

The effect of drug structure on plasma binding was investigated by studying a series of benzodiazepine analogues. Single

substitutions to the basic benzodiazepine structure were made at positions 1, 2, 3, 4, 7, 2′, or 4′, while holding all other groups constant (see Table 1). The degree of binding to human plasma for each structure was determined using equilibrium dialysis.

Heparinized whole blood obtained by venous puncture from three normal adults was centrifuged at 2500 rpm for 15 min at 5°. The plasma obtained was pooled, divided into 10-ml aliquots, and stored at −40°. All protein binding determinations were carried out using this plasma and a benzodiazepine concentration of 5 μg/ml, which was at the upper limit of physiological concentration and afforded, even for highly bound compounds, a detectable dialysate concentration. This drug concentration was well below the plasma binding capacity, which was found to show little or no dependence on benzodiazepine concentration up to 15 μg/ml, as previously reported by van der Kleijn (10). Binding measurements were conducted at pH 7.4, at which all the compounds were in an unionized form.

Equilibrium Dialysis

Dialysis membranes (dialysis tubing 0.625 inch in diameter, Fisher Scientific Company) were prepared prior to use by soaking them at 95° in three changes of distilled water. The tubing was knotted at one end, and 2.5 ml of plasma, pH 7.4, containing 5 μg/ml of drug, were added to the dialysis sac. The sac was suspended in a 20 × 150 mm test tube containing 5 ml of buffer solution (0.01 M phosphate buffer, pH 7.4, and 0.15 M NaCl), sealed, and incubated with constant agitation in a water bath at 37° for 16 hr. All determinations were carried out in triplicate. Controls were run to determine the extent of drug binding to the dialysis membrane. No appreciable binding was found. At the end of the incubation period the two compartments were separated and the concentration of drug in each compartment was determined.

Determination of Drug Concentration

Radiolabeled compounds. Radiolabeled compounds were checked for purity by their migration with authentic standards in suitable thin-layer chromatographic systems. All radiolabeled compounds used had a radiochemical purity of at least 98% except C-3027, which was 93%. When radiolabeled compounds were available the concentrations of drug in the two compartments after equilibrium dialysis were determined by liquid scintillation spectrometry. Dialyzed plasma (0.5 ml) and buffer dialysate (1.0 ml) were counted in 10 ml of Aquasol (New England Nuclear Corporation), using a Packard model 3380 liquid scintillation spectrometer, Counting efficiency was determined by the external standard channels ratio technique.

Nonradiolabeled compounds. When nonradiolabeled compounds were used, quantitation of drug was achieved by differential pulse polarography (11). The entire contents of the dialysis sac and the buffer compartment were assayed separately. Each sample was buffered with 10 ml of borate buffer, pH 9.0, and extracted with two 10-ml aliquots of diethyl ether. The ether extracts were combined, dried under nitrogen, dissolved in 2 ml of 0.1 N HCl, and assayed by differential pulse polarography. Compounds not stable in 0.1 N HCl were quantitated using a supporting electrolyte of 0.1 M phosphate buffer, pH 7.0.

Data Analysis

The extent to which each benzodiazepine was bound to plasma proteins was expressed as the percentage bound in the dialysis sac at equilibrium and defined by

$$\% \text{ bound} = \frac{\text{concn inside sac} - \text{concn in dialysate}}{\text{concn inside sac}} \times 100$$

The mean and standard deviation of the mean for triplicate determinations were calculated for each compound.

To correlate changes in the extent of protein binding with changes in the physicochemical properties of the molecule, particularly its lipophilic character, the free energy-related Hansch substituent constant π was determined for all substituent groups (X) in the benzodiazepine series by the relationship (12).

$$\pi = \log P_X - \log P_H$$

where P_H is the partition coefficient of the reference benzodiazepine structure and P_X that of a benzodiazepine derivative differing from the reference structure by the X substituent (see Table 2). In the few cases in which octanol/water partition coefficients[1] were not determined, π-substituent constants were taken as reported for phenoxyacetic acid by Fujita *et al.* (12) and for aliphatic groups as reported by Leo, Hansch, and Elkins (13). By definition, the more lipophilic the substituent, the greater its π value.

RESULTS AND DISCUSSION

The largest group of compounds in this series contained different substituents at position 7, hydrogen at position 1, and a carbonyl group in position 2. The extent of protein binding varied widely, from 99.4% to 19.8%, with respect to the nature of the substituent in position 7 (Table 1).

A plot of the extent to which each compound is bound to plasma proteins (percentage bound) as a function of the Hansch lipophilic substituent constant (π) calculated for each substituent in position 7 is given in Fig. 1.

The methods of least squares and stepwise regression analysis were used to evaluate the coefficients in Eqs. 1 and 2. The analysis was carried out on a Honeywell 6080 computer, using the BMD02R stepwise regression program of the Health Sciences Computing Facility, University of California, Los Angeles.

	r	r^2	n	
$\%B = 30.32\pi + 76.76$	0.861	0.741	26	(1)
$\%B = -21.67\pi^2 + 33.43\pi + 88.54$	0.983	0.965	26	(2)

where r = multiple correlation coefficient, n = number of data points, and $\%B$ = percentage bound.

The addition of the second-degree term to Eq. 1 yields Eq. 2, which results in an excellent correlation ($r = 0.983$) and accounts for 96.5% of the variance (r^2) in the data.

The plot in Fig. 1 indicates that the change in extent of protein binding seen with changes in the position 7 substituent is highly correlated with the lipophilic nature of the substituent. The greater the lipophilic nature of the substituent, the greater the extent of protein binding. It is particularly interesting that protein binding decreases through the halogen series from iodine to fluorine, a finding which also correlates with the increase in charge density on the halogen through this series and the consequent increase in the bound aqueous envelope surrounding the halogen moiety.

Within the framework of the few analogues which were available to be tested, the correlation between the extent of protein binding and the degree of substituent lipophilicity, similar to that found for position 7 substituents, was also found to hold for substituents in position 2, 3, or 4. It appears that binding is affected by more than a single substituent in the molecule. C-2092, possessing two hydrophilic groups, a 2-position carbonyl, and a 4-position *N*-oxide, was found to be 77.2% bound. Upon removal of the 2-position carbonyl or the 4-position *N*-oxide, the resultant compounds, C-5167 and C-2180, exhibited increases in binding, to 93.6% and 96.9%, respectively (Table 1). C-3453, which contains two hydrophilic groups, a 7-nitro and a 2-position carbonyl, was found to be 81.3% bound. However, removal of either or both hydrophilic groups increased the extent of protein binding to approximately 98% (C-4963, C-2807, and C-5031).

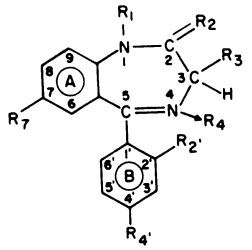
Methyl substitution for hydrogen at position 7 in C-2921 resulted in an increase in protein binding from 92.7% to 97.3% in C-2748. However, methyl substitution for hydrogen at position 1 in C-3059 and C-2925 to yield the 1-methyl analogues C-3453 and C-4556, respectively, did not result in a statistically significant change in their protein binding (Table 1). One possible explanation of this difference in influence between the 1- and 7-position aliphatic substitutions may lie in the relationship of position 1 with respect to the over-all

[1] Octanol/water partition coefficients were determined by P. Seiler by measuring ultraviolet absorption after partitioning between mutually saturated solvents (personal communication, 1972).

TABLE 1

Plasma protein binding and chemical structure of benzodiazepines

The percentage binding is reported as the mean and standard deviations of three determinations. All compounds used in this study were obtained from the Organic Chemistry Department, Hoffmann-La Roche.



Compound	R_1	R_2	R_3	R_4	R_7	$R_{2'}$	$R_{4'}$	Bound	Generic name
								%	
C-9000	H	O	H		I	H	H	99.4 ± 0.1	
C-2832	H	O	H		Br	H	H	98.6 ± 0.2	
C-2748	H	O	H		CH_3	H	H	97.3 ± 0.1	
C-2180	H	O	H		Cl	H	H	96.9 ± 0.2	Nordiazepam
C-2921	H	O	H		H	H	H	92.7 ± 0.6	
C-3059	H	O	H		NO_2	H	H	88.9 ± 3.0	
C-3061	H	O	H		F	H	H	86.1 ± 1.2	
C-3053	H	O	H		OH	H	H	54.7 ± 4.7	
C-3072	H	O	H		NH_2	H	H	19.8 ± 12.7	
C-2092	H	O	H	→ O	Cl	H	H	77.2 ± 2.0	Demoxepam
C-6789	H	O	OH		Cl	H	H	95.7 ± 0.6	Oxazepam
C-2925	H	H_2	H		Cl	H	H	99.4 ± 0.0	
C-5167	H	H_2	H	→ O	Cl	H	H	93.6 ± 0.3	
C-3350	H	O	H		Br	—[a]	H	52.3 ± 1.3	Bromazepam
C-3027	H	O	H		Cl	Cl	H	94.9 ± 1.0	
C-4023	H	O	H		NO_2	Cl	H	85.4 ± 1.5	Clonazepam
C-3367	H	O	H		Cl	F	H	96.1 ± 0.2	
C-3344	H	O	H		NO_2	H	Cl	95.2 ± 2.7	
C-4556	CH_3	H_2	H		Cl	H	H	99.3 ± 0.1	Medazepam
C-5031	CH_3	H_2	H		H	H	H	98.9 ± 0.1	
C-4963	CH_3	H_2	H		NO_2	H	H	98.4 ± 0.4	
C-2807	CH_3	O	H		Cl	H	H	98.3 ± 0.6	Diazepam
C-3453	CH_3	O	H		NO_2	H	H	81.3 ± 4.0	
C-2922	C_2H_5	O	H		Cl	H	H	95.3 ± 0.5	
C-3438	CH_3	O	H		Cl	F	H	97.8 ± 0.2	
C-4200	CH_3	O	H		NO_2	F	H	83.4 ± 2.4	Flunitrazepam
C-2750	CH_2CH_2OH	O	H		Cl	F	H	64.7 ± 1.9	

[a] In this compound the phenyl ring at position 5 is replaced by a pyridyl ring with the nitrogen at position 2'.

structure of the benzodiazepine molecule. The methyl group at position 1 is *para* to the 7-position substituent on the A benzene ring; therefore electron-withdrawing groups in position 7 could affect the electron distribution of a methyl group in position 1, resulting in its hyperconjugation. This localization of a partial positive charge on the methyl group in position 1 would lower the lipophilic nature of the 1-position methyl group, resulting in the observed lack of increase in binding.

The difference in the physicochemical nature of the 1- and 7-position methyl groups can be seen from their respective lipophilic substituent constants (Table 2). The methyl group in position 7 (π = 0.51) is lipophilic with respect to hydrogen and would be expected to raise the total lipophilic character of the molecule and thus

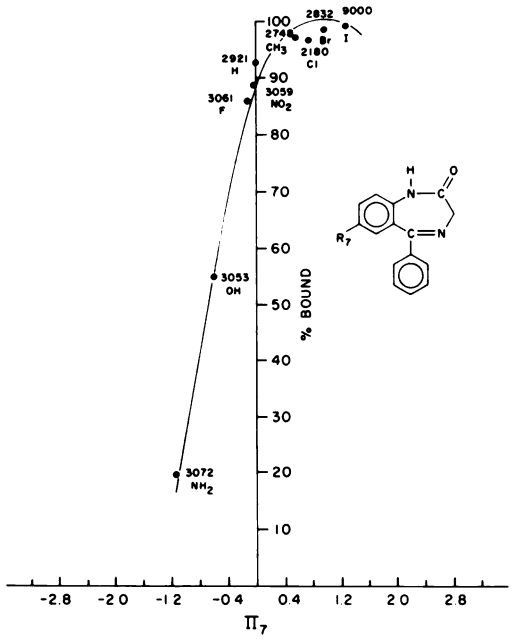


FIG. 1. *Extent of plasma protein binding for benzodiazepines having a hydrogen at position 1, a carbonyl at position 2, and a variable group at position 7 as a function of the lipophilic substituent constant π of the 7-position substituent*

The curves shown in this figure and Fig. 2 were obtained by computer fit to a parabolic model, $\%B = a_2\pi^2 + a_1\pi + a_0$, which is used to illustrate the correlation between binding and lipophilicity. It is not intended to imply that the actual physical relationship between these parameters is parabolic.

the extent to which it is bound to plasma proteins. However, the 1-position methyl group is actually slightly hydrophilic with respect to hydrogen ($\pi = -0.07$), and when substituted for hydrogen would not result in an increase in the lipophilic character of the molecule and resultant protein binding.

To incorporate the effect of functional group interdependence on the extent of protein binding, the percentage binding was plotted as a function of the total lipophilic nature of the molecule, as measured by the summation of the lipophilic substituent constants for substituents in positions 1, 2, 3, 4, and 7 ($\Sigma\pi_{1,2,3,4,7}$) (Fig. 2).

Regression analysis was used to derive Eq. 3.

$$\%B = -13.08\pi^2 - 2.36\pi + 102.19 \quad (r = 0.921,\ r^2 = 0.848,\ n = 52) \quad (3)$$

The multiple correlation coefficient of 0.921 indicates a very good correlation between the change in extent of protein binding and the total lipophilic character of the molecule as measured by the lipophilic substituent constant for 1-, 2-, 3-, 4-, and 7-position substituents. The correlation accounts for 84.8% of the variance in the data.

The degree of protein binding does not appear to be influenced by the lipophilic nature of the halogen substituent in position 2′. Substitution of fluorine for hydrogen in position 7 was found to lower the extent of protein binding (C-2921, 92.7%; C-3061, 86.1% bound). However, fluoro substitution for hydrogen in position 2′ of C-2180 had no effect on the extent of protein binding (C-3367, 96.1%; C-2180, 96.9% bound). Chloro substitution in position 7 increased the extent of binding with respect to the hydrogen substituent, from 92.7% in C-2921 to 96.9% bound in C-2180. However, upon 2′-chloro substitution of C-2180 and C-3059, a decrease in protein binding was observed in the resulting compounds, C-3027 and C-4023 (Table 1). Protein binding expressed as a function of the lipophilic substituent constants for compounds with 2′-substituents resulted in a poor correlation (Eq. 4), which accounted for only 17.0% of the variance in the observed percentage binding.

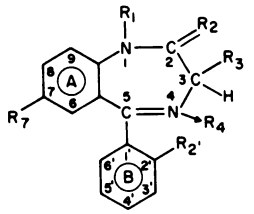
$$\%B = 16.80\pi^2 + 40.88\pi + 107.42 \quad (r = 0.412,\ r^2 = 0.170,\ n = 20) \quad (4)$$

The binding of benzodiazepines with 2′-substituents appears not to be related to their lipophilic character but may be governed by changes in the spatial orientation of the B benzene ring. Bulky halogen groups in position 2′ would restrict the free rotation of the B benzene ring, thus forcing it out of the plane of the remaining ring system (15). If the benzodiazepine protein binding site is to some degree stereospecific, as indicated by the recent work of Müller and Wollert (16), this rotational restriction may prevent the optimal orientation of the benzodiazepine B ring in relation to its albumin binding loci (17). Forcing the B benzene ring out of the plane of the remaining ring system would

TABLE 2

Hansch and Hammett constants for benzodiazepine substituents

Values of π were calculated from octanol/water partition coefficients as described in the text. Values of σ were taken from Jaffé (14).



Substituent	Lipophilic constant π						σ
	R_1	R_2	R_3	R_4	R_7	$R_{2'}$	
H	0.00	0.00	0.00		0.00	0.00	0.00
CH_3	−0.07				0.51		−0.17
C_2H_5	0.29						−0.15
C_2H_4OH	0.17						
C=O		−1.21[a]					
OH			−0.68		−0.61		−0.36
O				−1.58			
I					1.26[b]		0.28
Br					0.94[b]		0.23
Cl					0.74	0.23	0.23
F					−0.14	−0.24	0.06
NO_2					−0.06		0.78
NH_2					−1.18		−0.66

[a] From Leo *et al.* (13).

[b] From Fujita *et al.* (12).

also result in substantial changes in the total molecular electronic configuration by reducing the resonance interaction of the B benzene ring with the remaining ring system.

This hypothesis is supported by observation of the binding of a series of 7-nitrobenzodiazepines. The binding of C-4023, possessing a 2′-chlorine, increased from 85.4% to 95.2% when the chlorine was moved from position 2′ to 4′ (C-3344). In C-3344 the 4′-chlorine does not interfere with the free rotation of the B benene ring, so that the increase in molecular lipophilicity due to chlorine substitution is not suppressed by rotational restriction, as in C-4023. In contrast to 2′-chlorine substitution, substitution of chlorine for hydrogen in position 4′ resulted in an increase in lipophilicity and protein binding, as was found for 7-position chlorine substitution; protein binding of C-3059 increased from 88.9% to 95.2% in the 4′-chloro derivative (C-3344).

To determine whether the protein binding of the benzodiazepines could be described equally well by the electronic character of the substituents, the extent of protein binding was regressed against the summation of the Hammett substituent constants σ for substituents at positions 1, 3, and 7. All Hammett substituent constants were obtained from the compilation of Jaffé (14). The resulting equation, Eq. 5,

$$\%B = -81.72\sigma^2 + 47.92\sigma + 92.68 \quad r = 0.885 \quad r^2 = 0.782 \quad n = 52 \quad (5)$$

indicates that the plasma protein binding of the benzodiazepines can be adequately described ($r = 0.885$) by the Hammett substituent constant σ. However, the lipophilic constant π resulted in a slightly better correlation (Eq. 3; $r = 0.921$).

Expansion of Eq. 3 to the form

$$\%B = a_0 + a_1\pi + a_2\pi^2 + a_3\sigma^2 + a_4\sigma$$

was used to incorporate both electronic and lipophilic substituent character into

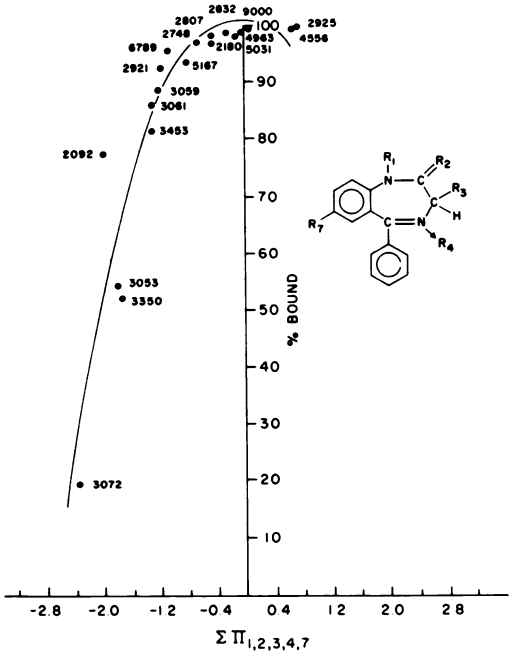


FIG. 2. *Extent of plasma protein binding for benzodiazepines as a function of summation of the lipophilic substituent constant π for substituents in positions 1, 2, 3, 4, and 7*

the correlation with plasma protein binding. The regression of the extent of protein binding against the summation of the lipophilic substituent constants for substituents at positions 1, 2, 3, 4, and 7 and the summation of the Hammett substituent constants for substituents at positions 1, 3, and 7 resulted in Eq. 6.

$$\%B = -7.15\pi^2 + 1.17\pi - 38.89\sigma^2 + 24.39\sigma + 99.50 \quad (6)$$

$r = 0.964$, $r^2 = 0.930$, $n = 52$

The inclusion in the equation of a term for the electronic character of the substituents resulted in a significant increase (F-test) in the multiple correlation coefficient, from 0.921 in Eq. 3 to 0.964 in Eq. 6, and accounted for an additional 7.2% of the variation in the observed percentage bound.

Partial differentiation of Eq. 6 with respect to π and σ reveals that protein binding increases positively with increases in the total lipophilic character of the substituents up to a limiting π value of 0.08214, and with their increasing electron-attracting character to a limiting σ value of 0.3135, after which further increases in π and σ are not reflected in increased protein binding.

Our findings indicate that the binding of the benzodiazepines is highly influenced by the lipophilic character of their substituents and that the albumin binding loci are of a hydrophobic nature. However, a good correlation was also observed between protein binding and the electronic character of substituents as expressed by their Hammett constants. An increase in the electrophilic character (σ) of the substituent may lead to reduced availability for hydrogen bonding of the lone-pair electrons at positions 1 and 2 in the benzodiazepine molecule. Presumably this would result in greater lipophilicity and increased protein binding of the benzodiazepine. Correlation between increased lipophilicity and substituent electrophilic character has been reported previously (13).

Experimental support is given to the speculation that the benzodiazepine-protein interaction is also dependent upon the proper orientation of the B benzene ring with respect to the benzodiazepine nucleus and the albumin binding site.

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