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Stereochemical Aspects of Benzodiazepine Binding to Human Serum Albumin. I. Enantioselective High Performance Liquid Affinity Chromatographic Examination of Chiral and Achiral Binding Interactions between 1,4-Benzodiazepines and Human Serum Albumin

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SUMMARY

The displacement of a series of 1,4-benzodiazepine (BDZ) drugs from a chiral stationary phase, based upon human serum albumin, for high performance liquid chromatography was investigated. The different displacement patterns obtained using various mobile phase additives could not be interpreted in terms of binding of the solutes to a single site. The observations were better described by considering the attachment of the BDZs to several loci on the protein. Two main mechanisms of binding were discerned, a nonstereoselective mode, which affected all solutes and seemed to occur at a large number of locations on the protein, and a highly stereoselective mode, which involved only one enantiomer of chiral BDZs and presumably one conformation of certain achiral solutes. The stereoselective binding mode encompassed at least four different sites, each of which displayed slightly different structural requirements. It is suggested that the nomenclature currently used to describe drug binding to human serum albumin may be misleading. Rather than the use of site I or site II, it may be preferable to adopt the terms type I and type II binding, according to the displacement patterns of the compound concerned. This approach would retain the conceptual simplicity of the current notation, while avoiding misleading implications of the exact molecular locus of binding.

The ability of HSA to bind a wide range of drugs and endogenous compounds has been recognized for more than a halfcentury. Such binding is believed to occur at three to six specific ligand binding sites (1–3). The majority of solutes that bind to HSA appear to do so at either, or both, of two principal sites, i.e., a site to which warfarin binds and a site that appears to bind solutes such as diazepam. Sudlow *et al.*, (1) have referred to the warfarin and the BDZ binding sites as site I and site II, respectively, the nomenclature that will be used in the present report.

Site I is usually characterized as a broad "binding area," possibly composed of a number of subsites at which azapropazone and bilirubin, for example, bind (4). Site II, on the other hand, is more typically described as a small, sharply defined, receptor-like site, mainly due to the high enantioselectivity it occasionally exhibits (5). Although it is difficult to identify structural features that dispose a solute to bind at a particular site, compounds that bind to site I often are bulky heterocyclic anions, such as warfarin and azapropazone, that have the charge situated in the middle of the molecule (6). Site II, on the other hand, seems to favor compounds with extended structures that are either electronically neutral or bear a negative charge at one end of the molecule (7). In addition to the BDZs, the 2-arylpropionate nonsteroidal anti-inflammatory drugs and medium chain (C_6 to C_{12}) free fatty acids are thought to bind at site II (1–3).

There has been a great deal of interest in the binding of BDZs to HSA, particularly the influence of the stereochemistry of the BDZs on such binding. Many chiral BDZs display impressive degrees of enantioselectivity in their interactions with the protein (8).

The majority of BDZ-HSA binding studies have worked from the premise, based upon the results of competition studies, that these compounds bind to site II. In turn, these compounds, especially diazepam, are often used as characteristic "markers" for this site (1–3). However, these assumptions are questionable in the face of certain experimental observations. For instance, whereas (*S*)-warfarin exerts a cooperative enhancement in the

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ABBREVIATIONS: HSA, human serum albumin; HPLC, high performance liquid chromatography; BDZ, 1,4-benzodiazepine; MPA, mobile-phase additive; CSP, chiral stationary phase.

binding of particular BDZs (often in an enantiospecific fashion), the binding of most BDZs is unaffected by warfarin (9–11). In addition, several studies that have used Scatchard-type analyses of the binding of BDZs to HSA have revealed binding to at least two different sites, which sometimes exhibit quite similar affinity constants (8, 12, 13). The precise nature and location of these different sites are not known.

Such apparent inconsistencies prompted us to examine further the binding of BDZs to HSA and, in particular, to study the influence of the stereochemistry of these compounds upon such binding. To this end, we have used a CSP for HPLC, based upon immobilized HSA (the HSA-CSP). Previous studies (11, 13–16) have demonstrated that observations made using the HSA-CSP directly reflect properties of the free protein, such as competitive displacement phenomena (14), cooperative and anticooperative binding interactions (11, 16), and inherent enantioselectivity (13–16). The HSA-CSP is, therefore, an effective tool in the examination of ligand-HSA interactions.

A consequence of the innate chirality of HSA is its ability to bind enantiomers with different affinities. Thus, when a racemic compound, such as a chiral BDZ, is chromatographed on a HPLC stationary phase containing HSA, the two enantiomers may migrate separately through the column, depending upon the enantioselectivity of the protein for that particular compound. This property of the HSA stationary phase permits the examination of the behavior of individual enantiomers in the presence of different competitors. The enantioselective binding data generated benefit from chromatographic precision and reproducibility, which are much greater than those obtained when conventional techniques for the study of protein binding are used. In addition, because the amount and state of the immobilized protein do not change between experiments, very small differences in binding affinity can be measured precisely.

The use of enantioselective chromatography provides an additional significant advantage over other techniques. Because enantiomers have identical physical properties, both will be equally affected by nonspecific effects, for instance, by adsorption onto the support matrix or by variations in mobile phase composition. Any change that affects one enantiomer more than the other can, therefore, be attributed solely to the chiral constituents of the chromatographic system, i.e., the protein and, if applicable, the compounds added to the mobile phase.

The chromatographic approach can, therefore, provide extremely subtle information on the processes that the BDZs undergo when binding to HSA. To perform enantioselective BDZ-HSA binding studies by using conventional nonchromatographic techniques would be very difficult, if not impossible, due to the need for large quantities of the resolved enantiomers and the fact that these compounds may undergo rapid racemization in aqueous solution.

This study reports the effects on the retention of chiral and achiral BDZs, on the HSA-CSP, resulting from the addition of a range of ligands to the mobile phase. The ligands were compounds known, or suspected, to affect the binding of compounds at site II on HSA. This "high performance chiral liquid affinity chromatographic" approach is able to reveal subtle stereoselective interactions between solutes binding to the immobilized biopolymer (17). The effects of varying the mobile phase concentrations of diazepam and (*R*)-ibuprofen on the enantioselective retention of BDZs were also determined. This approach was able to provide semiquantitative information on the binding processes.

Materials and Methods

Chemicals. The BDZs Ro 23-0983/001, Ro 11-3128/002, Ro 14-8935/000, Ro 11-5073/000, Ro 23-1117/000, and Ro 23-3880/000 were generously provided by Hoffman-La Roche, Inc. (Nutley, NJ). Alprazolam, 4-hydroxyalprazolam, triazolam, and the individual enantiomers of ibuprofen were kindly supplied by Upjohn Laboratories (Kalamazoo, MI). L-Tryptophan, lauric acid, and the remaining BDZs were obtained from Sigma Chemical Co. (St. Louis, MO). Octanoic acid was obtained from the Aldrich Chemical Company (Milwaukee, WI).

Chromatography. Chromatography was carried out isocratically, using a modular HPLC system that consisted of a Spectroflow 400 pump, 480 injector module equipped with 20-μl loop, 783 programmable absorbance detector (all from ABI Analytical, Ramsey, NJ), and a DataJet integrator (Spectra-Physics, San Jose, CA). The column temperature was regulated using a CH-30 jacket (FIAtron Laboratory Systems Inc., Oconomowoc, WI).

The HSA-CSP (15 cm × 4.6 mm, inner diameter) was prepared as previously described (15), by Shandon Scientific PLC (Runcorn, Cheshire, UK).

The mobile phases used in these studies were based on sodium dihydrogen phosphate-disodium hydrogen phosphate (100 mM, pH 6.90), modified with 5% (v/v) propan-1-ol. The flow rate was 1.0 ml/min throughout. Column temperature was maintained at 28 ± 0.1°. When competitors were added to the mobile phase, the required amount was generally first dissolved in the alcohol fraction, before addition of the buffer. All mobile phases were filtered (0.45-μm filter) and degassed, by ultrasonication, immediately before use. Detection of the solutes was by their UV absorbance at 233 nm. The injected amount of each solute was 0.5 μg for the racemic compounds and 0.25 μg for the nonchiral solutes.

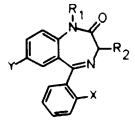
The chromatographic parameters measured were capacity factor [k', which is defined as $(t_R - t_0)/t_0$, where t_R is the retention time of the solute of interest and t_0 is that of an unretained solute (water)] and α (which is the ratio of the capacity factors of the second- and the first-eluting enantiomers), for the chiral BDZs.

Each of the BDZs (compounds **1–21**; Fig. 1) was chromatographed, in duplicate, under the conditions described above, and its capacity factor was determined. These data provided the "base-line" behavior of the BDZs on the HSA-CSP. The BDZs were rechromatographed under these standard conditions approximately midway through the series of experiments and again after their completion. In this way, it was verified that compounds added to the column mobile phase had not permanently affected the immobilized protein.

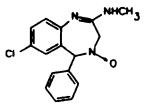
Various compounds, known or suspected to affect the binding of solutes at site II on HSA, were sequentially, and individually, added to the mobile phase. The MPAs, and their concentrations, were as follows: (*R*)-ibuprofen (10 μM), (*S*)-ibuprofen (10 μM), L-tryptophan (100 μM), octanoic acid (10 μM), dodecanoic acid (10 μM), diazepam (50 μM), delorazepam (50 μM), and chlordiazepoxide (50 μM). The different concentrations were used to reflect the different binding affinities of the various additives.

Modified mobile phases were pumped through the column until a "breakthrough profile" was observed at the detector. When a stable detector base line was obtained, indicating that equilibration had been established, the BDZs were reinjected. Except for the addition of the competitor, all conditions were identical to those used initially, so that any change in k' could be ascribed solely to the presence of the compound added to the mobile phase.

Data analysis. The effects of increasing concentrations of diazepam and (*R*)-ibuprofen, added to the mobile phase, on the retention behavior of the BDZs were analyzed according to eq. 1 (16):

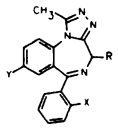


Compounds 2-15

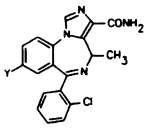


Chlordiazepoxide

	Compound	R_1	R_2	X	Y
1	Chlordiazepoxide	See above	-	-	-
2	(R,S)-Oxazepam H.	H	$OCO(CH_2)_2COO^-$	H	Cl
3	Nitrazepam	H	H	H	NO_2
4	Flunitrazepam	CH_3	H	F	NO_2
5	Clonazepam	H	H	Cl	NO_2
6	Delorazepam	H	H	Cl	Cl
7	Desmethyldiazepam	H	H	H	Cl
8	Diazepam	CH_3	H	H	Cl
9	(R,S)-Lormetazepam	CH_3	OH	Cl	Cl
10	(R,S)-Lorazepam	H	OH	Cl	Cl
11	(R,S)-Oxazepam	H	OH	H	Cl
12	(R,S)-Temazepam	CH_3	OH	H	Cl
13	(S)-Ro 14-8935/000	CH_3	CH_3	Cl	NH_2
14	(S)-Ro 23-0983/001	H	CH_3	Cl	F
15	(R,S)-Ro 11-3128/002	H	CH_3	Cl	NO_2



Compounds 16-19



Compounds 20 and 21

	Compound	R	X	Y
16	(R,S)-Alprazolam, 4-OH	OH	H	Cl
17	Alprazolam	H	H	Cl
18	Triazolam	H	Cl	Cl
19	(S)-Ro 11-5073/000	CH_3	F	Cl
20	(S)-Ro 23-1117/000	-	-	F
21	(R,S)-Ro 23-3880/000	-	-	Cl

Fig. 1. Structures of the solutes, and the numbering system, used in this study.

$$\frac{1}{(k' - X)} = \frac{V_m K_2[\mathrm{MPA}]}{K_3 m_L} + \frac{V_m}{K_3 m_L} \qquad (1)$$

In eq. 1, K_2 and K_3 represent the equilibrium constants for the binding of the MPA and BDZ at a common site, respectively, V_m is the void volume of the chromatographic system, and m_L is the number of available moles of binding site within the immobilized protein. The term X is the contribution to the capacity factor of the BDZ due to binding at site(s) at which the MPA does not bind or that are unaffected by the MPA.

Results

The capacity factors of the BDZs, determined under the controled conditions used, were found to vary by <1%. On the basis of this small degree of variation, it was possible to discern subtle interactions between the BDZs injected and the compounds added to the mobile phase. However, only changes in k' of 10% or greater were judged to be significant.

Twenty-one different BDZs were studied. These comprised nine achiral solutes, eight racemates (16 enantiomers), and four single enantiomers, giving a total of 29 different entities (Fig. 1). The suffix "a" or "b" appended to the compound number indicates the first- and second-eluting enantiomers of chiral BDZs, respectively.

Of the 29 compounds studied, only eight were consistently affected by the MPAs used. The first-eluting enantiomers of the chiral BDZs studied (compounds **2a, 9a, 10a, 11a, 12a, 13a, 14a, 15a, 16a, 19a, 20a**, and **21a**), as well as compounds **14, 17**, and **18**, were not affected ($\%\Delta k' < \pm 5\%$) by any of the solutes added to the mobile phase.

Effect of the enantiomers of ibuprofen and of L-tryptophan. Fig. 2 shows the influence of the enantiomers of ibuprofen and L-tryptophan, when added to the mobile phase, on the retention of several BDZs. Other than the eight BDZs shown in Fig. 2, all other compounds studied were not significantly affected by these MPAs.

Each enantiomer of ibuprofen caused a similar change in k' for compounds **1, 7, 8, 9b, 10b**, and **11b**. Compounds **12b** [(*S*)-temazepam] and **16b** [(*S*)-4-hydroxyalprazolam] were exceptions to this observation. For (*S*)-temazepam, the effect of (*R*)-ibuprofen was approximately 100% greater than that of the *S*-enantiomer. This situation was reversed in the case of (*S*)-4-hydroxyalprazolam, with (*S*)-ibuprofen having an approximately 3-fold greater effect than its antipode.

The concentration dependence of the displacement of each of the BDZs affected by (*R*)-ibuprofen was analyzed according to eq. 1 (16). Plots of $1/(k' - X)$ (see above) versus the concentration of (*R*)-ibuprofen in the mobile phase were linear up to MPA concentrations of 20 μM (Table 1). Above this concentration, additional amounts of (*R*)-ibuprofen continued to cause displacement of the BDZs, but the relationship was not described by eq. 1.

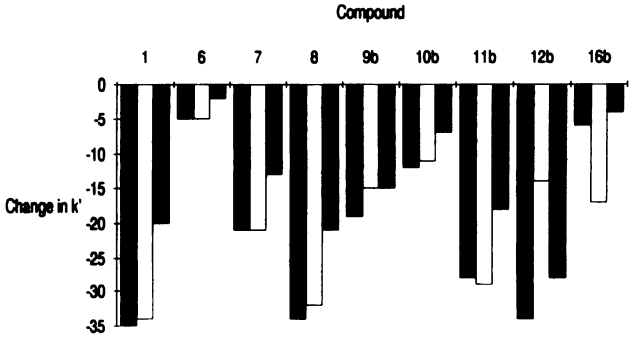


Fig. 2. Effect of (S)-ibuprofen (10 μM) (■), (*R*)-ibuprofen (10 μM) (□), and L-tryptophan (100 μM) (▨) on the capacity factors (k') of the solutes shown. The suffix "b" indicates that the compound is the enantiomer, of a racemate, that elutes second from the HSA-CSP.

TABLE 1

Parameters obtained from the study of the displacement of BDZs by (*R*)-ibuprofen (0–20 μM added to the chromatographic mobile phase)

Four or five experiments were performed in each case.

Compound	X[a]	K_2[b]	Binding[c]	r^2
		$\times 10^{-6}\ M^{-1}$	%	
1. Chlordiazepoxide	3.93	0.91	46	0.9999
7. Desmethyldiazepam	8.63	1.01	31	0.9999
8. Diazepam	7.11	1.12	46	0.9999
9b. (*S*)-Lormetazepam	7.10	1.60	25	1.0000
10b. (*S*)-Lorazepam	7.69	1.24	11	0.9999
11b. (*S*)-Oxazepam	7.40	1.80	30	0.9999
12b. (*S*)-Temazepam	9.79	2.03	35	0.9999

[a] *X* is the contribution to the k' of the BDZ, obtained in the absence of competitor, resulting from binding to site(s) unaffected by (*R*)-ibuprofen.

[b] K_2 is the binding affinity of (*R*)-ibuprofen for the site from which it displaces the BDZ. See text for derivation.

[c] Percentage of the overall binding of the BDZ due to attachment at the site from which it is displaced by (*R*)-ibuprofen.

The values of X (Table 1) were determined by iterative testing and indicated that approximately 50–90% of the binding of the BDZs studied occurred at sites that were unaffected by (*R*)-ibuprofen (16). For instance, only 11% of the overall binding of compound **10b** [(*S*)-lorazepam] was due to binding at sites from which it could be displaced by (*R*)-ibuprofen.

The binding affinity of (*R*)-ibuprofen at the site, or sites, from which it displaced the BDZs (K_2 in eq. 1) was approximately $1.4 \pm 0.4 \times 10^6$ M^{-1}. The binding affinities of the individual enantiomers of ibuprofen for HSA have not been reported, although the binding affinity of the racemate was determined by equilibrium dialysis to be 2×10^6 M^{-1} (18).

L-Tryptophan affected BDZ retention in a manner qualitatively similar to that of (*R*)-ibuprofen, although its effect at the concentration studied was consistently smaller (Fig. 2).

Effect of medium chain alkanoic acids. Fig. 3 depicts the displacement of BDZs by octanoic and dodecanoic acids. The capacity factors of the majority of the solutes studied were not modified by these compounds. The BDZs affected included those displaced by ibuprofen (Fig. 2), as well as compounds **3, 4, 5, 6, 13**, and **21b**.

Effect of other BDZs. The addition to the mobile phase of the BDZs chlordiazepoxide (compound **1**), delorazepam (**6**), and diazepam (**8**) significantly affected the retention of compounds **1, 6, 7, 8, 9b, 10b, 11b, 12b**, and **16b**, as illustrated in Fig. 4. As before, the other BDZs in Fig. 1 were not affected by the MPAs. Apart from delorazepam, the same compounds were displaced by addition of diazepam, chlordiazepoxide, or delorazepam to the mobile phase as were displaced by the enantiomers of ibuprofen. Delorazepam was not affected by either (*R*)- or (*S*)-ibuprofen, nor was it extensively displaced by the alkanoic acids. However, it was able to effect significant reductions in the k' of the BDZs shown in Fig. 4, except **10b** and **16b**.

Although the effects of adding diazepam to the mobile phase were studied up to 40 μM, the retention of compounds **1, 6, 7, 8, 9b, 10b, 11b, 12b**, and **15b** was affected in a linear manner (when plotted according to eq. 1) only up to a diazepam concentration of 1.3 μM (Table 2). Above this concentration of diazepam, linearity was lost for these compounds. However, the effect of diazepam on compound **15b** [(*R*)-Ro 11-3128] was linear throughout the concentration range studied (Table 2).

The binding affinity of diazepam (K_2 in eq. 1) at the site from which it displaced the BDZs listed in Table 2, except for **15b**, was approximately $7.2 \pm 0.5 \times 10^5$ M^{-1}. Diazepam has been reported (12) to bind at a single primary site, with an affinity of 4.7×10^5 M^{-1}, and to two secondary sites.

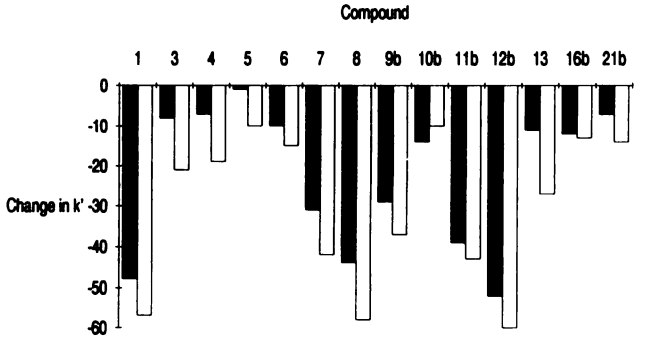


Fig. 3. Effect of octanoate (10 μM) (■) and dodecanoate (10 μM) (□) or the capacity factors (k') of the solutes shown.

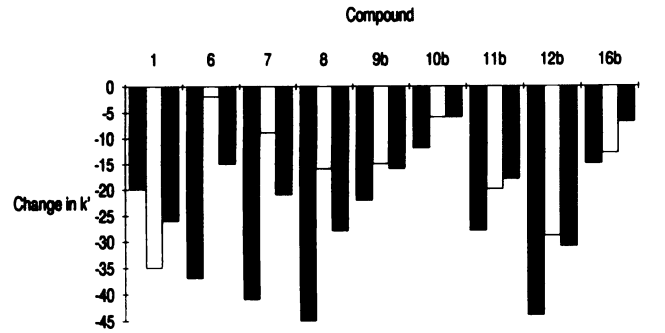


Fig. 4. Effect of diazepam (50 μM) (■), chlordiazepoxide (50 μM) (□), and delorazepam (50 μM) (▨) on the capacity factors (k') of the solutes shown.

Discussion

Although 21 BDZs were included in this study (Fig. 1), when the racemic mixtures used are considered this total becomes 29 distinct entities. Of this number, only eight compounds were consistently affected by the addition of solutes to the chromatographic mobile phase. This is despite the fact that the MPAs were selected because of their abilities to affect the binding of compounds to site II of HSA (1–3). Those BDZs that were affected by the various MPAs exhibited patterns of displacement that could not be accounted for by simple single-site binding.

It was not possible that the compounds that were not affected by the MPAs were simply bound to HSA too strongly to be displaced, because, generally, these compounds exhibited significantly lower retention on the HSA-CSP than did the eight compounds that were displaced. Also, the binding affinities of ibuprofen and the alkanoic acids for HSA are approximately 1 order of magnitude larger than those of the most highly bound BDZs (12, 18).

The first-eluting enantiomers of the chiral BDZs **9, 10, 11, 12, 15, 16**, and **21** were not affected by the MPAs studied, despite the fact that their opposite enantiomers were often

TABLE 2

Parameters obtained from the study of the displacement of BDZs by diazepam (0–1.296 μM added to the chromatographic mobile phase)

Six or seven experiments were performed in each case.

Compound	X^a	$K_2{}^b$	Binding[c]	r^2
		× 10^{-5} M^{-1}	%	
1. Chlordiazepoxide	4.70	6.4	60	0.9991
6. Delorazepam	10.88	7.8	12	0.9990
7. Desmethyldiazepam	8.90	7.2	28	0.9991
8. Diazepam	6.10	6.5	71	0.9996
9b. (*S*)-Lormetazepam	7.25	7.4	41	0.9981
10b. (*S*)-Lorazepam	7.55	7.6	16	0.9996
11b. (*S*)-Oxazepam	8.00	6.9	50	0.9995
12b. (*S*)-Temazepam	8.55	7.5	65	0.9997
15b. (*R*)-Ro 11-3128[d]	0	0.2	100	0.9999

[a] X is the contribution to the k' of the BDZ, obtained in the absence of competitor, resulting from binding to site(s) unaffected by diazepam.

[b] K_2 is the binding affinity of diazepam for the site from which it displaces the BDZ. See text for derivation.

[c] Percentage of the overall binding of the BDZ due to attachment at the site from which it is displaced by diazepam over this range of concentrations.

[d] Compound **15b** displayed linearity throughout the range of concentrations of diazepam studied (up to 40 μM).

TABLE 3

BDZ binding sites identified in this study, with ligands binding to each

IBU_{RS}	IBU_R	IBU_S	Delorazepam	7-NO_2
1	**12b**	**16b**	**(1)**[a]	**3**
7	(*R*)-Ibuprofen	(*S*)-Ibuprofen	**6**	**4**
8	L-Tryptophan	Octanoate	**(7)**	**5**
9b	Octanoate	Dodecanoate	**8**	**13**
10b	Dodecanoate		**(9b)**	**21b**
11b			**(11b)**	Octanoate
12b			**(12b)**	Dodecanoate
(*R*)-Ibuprofen				
(*S*)-Ibuprofen				
Octanoate				
Dodecanoate				
L-Tryptophan				

[a] Figures in parentheses are those compounds that were displaced by delorazepam, although the mechanism is unclear. For instance, whereas delorazepam was able to displace compound 1, the reverse was not true.

significantly affected by the same compounds. This observation would indicate that the individual enantiomers of the BDZs bind to different sites or, at least, one enantiomer binds to additional sites at which the other does not bind. This has already been reported for oxazepam hemisuccinate (14).

Ibuprofen and L-tryptophan are thought to bind to HSA at binding site II, along with the BDZs (1–3). However, only the BDZs shown in Fig. 2 were affected by these compounds.

The observations depicted in Fig. 2 may be explained by proposing that the enantiomers of ibuprofen bind to HSA at three distinct sites, at which certain BDZs also bind. At the first site (IBU_{RS}), the binding of ibuprofen exhibits very low, or no, enantioselectivity. This site is that to which compounds **1, 7, 8, 9b, 10b**, and **11b** bind. The second site (IBU_R) binds (*R*)-ibuprofen with a much greater affinity than its antipode. (*S*)-Temazepam binds to this site to a significant extent. At the final site (IBU_S), (*S*)-ibuprofen binds with significantly higher affinity than the *R*-enantiomer. It is this site to which compound **16b** binds (Table 3).

The existence of at least three different ibuprofen binding sites, with different enantioselectivities, is supported by competition studies on the HSA-CSP, using the individual enantiomers of ibuprofen as both solutes and MPAs.[2] Additional evidence for the existence of a nonenantioselective binding site for ibuprofen, IBU_{RS}, comes from studies performed using both free and immobilized HSA in which a single tyrosine residue (Tyr-411) had been acetylated. The acetylation of this residue, which is thought to be situated in site II, resulted in 50% reductions in the binding of both ibuprofen enantiomers, without any significant effect on enantioselectivity (13).

The data in Table 1 indicate that, whereas the affected BDZs were competitively displaced by (*R*)-ibuprofen from a particular site (or sites), they were also bound to other sites upon which ibuprofen had no effect, inasmuch as, for each of the compounds, the parameter X was not zero.

Based on the model described above for the binding of the enantiomers of ibuprofen, L-tryptophan may be said to displace BDZs from sites IBU_{RS} and IBU_R, inasmuch as it had an effect qualitatively similar that of (*R*)-ibuprofen. The quantitative difference between the effects of L-tryptophan and (*R*)-ibuprofen may be explained by the fact that both enantiomers of ibuprofen have binding affinities for HSA at least 2 orders of magnitude greater than that of L-tryptophan (12, 18).

Octanoate and dodecanoate had qualitatively similar, but quantitatively dissimilar, effects on most of the affected BDZs. Compound **16b** again displayed a different pattern of behavior, compared with the majority of the affected BDZs, supporting the assumption made above that this compound binds to a site (IBU_S) at which the others do not extensively bind. The effect of dodecanoate was generally more pronounced than that of octanoate, except for compound **10b**. The displacement by the alkanoic acids of six BDZs (including three substituted with a nitro group at position 7) that were not affected by the enantiomers of ibuprofen suggests the existence of an additional BDZ binding site, or sites. However, only octanoate and dodecanoate, of all the MPAs studied, were able to displace five of these six solutes (**3, 4, 5, 13**, and **21b**), and then to only a minor degree. The "nitro-BDZ" site (Table 3), therefore, appears to be a very low capacity/low affinity site.

The retention of delorazepam (compound **6**) on the HSA-CSP was not significantly affected by any of the non-BDZ MPAs studied, although it was strongly displaced by diazepam. This suggests the existence of an additional BDZ binding site, which is relatively unaffected by acidic solutes. Although delorazepam was strongly displaced by diazepam, it was unaffected by chlordiazepoxide (Fig. 4). However, delorazepam was able to effect a 26% displacement of chlordiazepoxide. This may be because the binding of delorazepam to this site is too strong to be overcome by chlordiazepoxide at the concentration used. Alternatively, the binding of delorazepam may exert an anticooperative effect on a separate site to which chlordiazepoxide binds.

It seems likely that the primary binding site of diazepam is that described above as IBU_{RS}, because the solutes that were affected by low concentrations of diazepam were also affected by both enantiomers of ibuprofen. Table 2 shows that, although delorazepam does bind to the primary binding site of diazepam, it does so to only a very small extent, which would explain why this compound was relatively unaffected by the enantiomers of ibuprofen.

The apparent binding affinity of diazepam at the site from which it displaced compound **15b** was $1.9 \times 10^4\ M^{-1}$. The fact that the parameter X was 0 for this compound (Table 2) indicates that **15b** binds to a single site. The 10-fold lower affinity of diazepam at this site, compared with that at the site from which it displaced the other BDZs, suggests that the sole binding site of **15b** is a secondary binding site for diazepam.

It would, therefore, appear that the binding of BDZs to HSA occurs at several different sites on the protein and that these sites fall into two groups, according to their stereochemical requirements. Sites of the first binding mode have some structural specificity, in that they have constraints on the molecular size that they can accommodate (19). However, it is likely that such sites are quite numerous, because none of the MPAs examined were able to displace compounds undergoing only this form of binding (i.e., the first-eluting enantiomers of the chiral solutes and compounds **14, 17**, and **18**), and they appear to be nonsaturable (20, 21).

The second form of binding of the BDZs to HSA is highly stereoselective. The competition studies described above indicate the existence of at least five sites of this type (Table 3). The BDZs are known to exist in two configurations of the

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diazepine ring, the *M*- and *P*-forms (8, 19). It is important to note that the eight compounds that underwent specific stereoselective binding included all of the stereoisomers of the BDZs that exist in the preferred *M*-conformation. Several of the achiral BDZs affected by the various MPAs used in this study have been shown, using spectroscopic techniques, to be able to adopt the same preferred *M*-conformation when binding to HSA (8). It, therefore, appears that the second mode of binding observed is actually specific for the *M*-conformation of the BDZs.

Presumably, it would be possible for both enantiomers of chiral BDZs to undergo binding to the low specificity sites, although, at the low concentrations injected, the enantiomer having the *M*-conformation may be expected to bind predominantly to the higher affinity, stereoselective sites.

The existence of a single BDZ binding site within HSA, therefore, appears highly questionable. The binding of BDZs, whether considered individually or as a group, is better described by binding at a number of comparable sites, each of which has slightly differing structural requirements.

It is suggested that, rather than describing a solute as binding to either site I or site II on the basis of competition studies, it may be more accurate to talk in terms of types, or modalities, of binding. For instance, the nomenclature "type I" and "type II" binding could be used to describe those compounds currently described as binding to site I or site II. In this way, questionable, and possibly misleading, pronouncements concerning the exact locus within the protein at which the solute binds may be avoided, and the practical benefits of the present system may be retained.

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