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# Stereochemical Aspects of Benzodiazepine Binding to Human Serum Albumin. II. Quantitative Relationships between Structure and Enantioselective Retention in High Performance Liquid Affinity Chromatography

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**SUMMARY**

Previously determined retention data for a series of benzodiazepine (BDZ) derivatives, comprising nine achiral compounds, four single enantiomers, and 18 individual isomers of nine racemates, on a chiral stationary phase based on immobilized human serum albumin (HSA) were analyzed to define quantitative relationships between structure and enantiospecific retention. Structural parametrization of the agents was done by means of hydrophobic fragmental constants and electronic and steric parameters obtained by computational chemistry methods. A structural descriptor was identified, a submolecular measure of polarity about the stereogenic center, that accounted for the stronger electrostatic interactions of the second-eluting enantiomer with the HSA chiral stationary phase. Quantitative structure-enantiospecific retention relationships were derived for both enantiomeric series and for achiral compounds, and structural requirements for binding to HSA were determined. Two types of binding sites were postulated. For BDZs in the *P*-conformation, binding to HSA involved a hydrophobic region with steric restrictions. For BDZs in the *M*-conformation, a hydrophobic region was also involved, as well as a cationic region that interacted electrostatically with carbon C(*3*) of the diazepine system and substituents at that carbon. These differences lead to different binding patterns for BDZ enantiomers and provide a rationalization for the diversified behavior of individual BDZs that was observed in previous displacement studies.

Recently, an approach has been developed for the study of the mechanisms of and structural requirements for HSA-drug interactions, based on the use of a HPLC CSP, consisting of immobilized HSA (the HSA-CSP) (1–3). Because the immobilization process does not appear to affect the binding properties or the conformational mobility of the protein, the HSA-CSP offers what may be termed a "high performance chiral liquid affinity chromatographic" tool for the examination of the interactions between drugs and HSA.

It should be emphasized that HSA, like all proteins, is a polymer of chiral subunits (L-amino acids). When the enantiomers of a chiral compound bind to this protein, its innate chirality results in the formation of diastereomeric complexes of differing energies. When the protein is used as a CSP, this property is reflected in the chromatographic resolution of optical antipodes. This chromatographic approach readily allows the study of enantioselective protein-binding phenomena, which are often extremely difficult to study by conventional means. For instance, the enantioselective binding of compounds that have rapid racemization half-lives (including many of the chiral BDZs), may be impossible to detect using standard techniques. In addition, established equilibration methods for the study of enantioselective drug-protein interactions require substantial amounts of previously separated enantiomers, which are not required in the chromatographic studies.

Using linear free energy relationships (4), retention data can be quantitatively related to solute structural parameters. Models generated in this manner can be used to gain insight into the mechanism of retention on the HSA-CSP, without a need to identify explicitly the full structural and topographical characteristics of the constant interacting chemical entity (i.e., HSA).

Quantitative structure-retention relationships have been reported for various sets of solutes chromatographed in a variety of nonchiroselective gas and liquid chromatographic systems (5). However, it has been much more difficult to get satisfactory

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**ABBREVIATIONS:** HSA, human serum albumin; CSP, chiral stationary phase; HPLC, high performance liquid chromatography; BDZ, 1,4-benzodiazepine; QSAR, quantitative structure-activity relationship; QSERR, quantitative structure-enantioselective retention relationship.

correlations between chiroselective measures of retention and structural descriptors of solutes, i.e., QSERRs; even in the case of a homologous series of solutes, the enantiomeric separation factor was observed to have a complex nonlinear dependence on the length of a linear alkyl substituent (6).[2] Wolf *et al.* (7) have succeeded in describing the retention of the second-eluting enantiomers of a group of alicyclic racemates on cellulose triacetate; retention was related to the probability of the molecules assuming a flat conformation and to the negative electrostatic potential around the chiral center. Our preliminary studies on the chromatography of a series of chiral BDZs on the HSA-CSP resulted in the derivation of statistically significant regression equations relating retention to a few structural descriptors (8).

Because the binding of a drug to HSA may have considerable influence on its biological activity, QSERRs derived from retention data obtained on the HSA-CSP can be of direct pharmacokinetic and pharmacodynamic relevance. Recently, interest in the determination of QSARs for the biological activities of chiral compounds has increased considerably (9, 10). However, problems in obtaining quantitative, numerically expressed, differences between enantiomers on one hand and the lack of reliable quantitative measures of bioactivity of the individual enantiomers on the other still limit QSAR studies of chiral agents. Thus, the common practice is that the chirality of the agents subjected to QSAR studies is not taken into consideration.

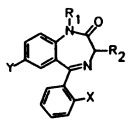
The series of test drugs studied in this work were all BDZs. As far as interactions of the agents with proposed BDZ receptors in the mammalian central nervous system are concerned, several structure-activity relationship studies have been carried out (11–13). There was also a report (14) on quantitative relationships between hydrophobic constants of substituents and plasma protein binding of a series of achiral BDZs that contain several small substituents in position 7 of the fused benzene ring. Of relevance to the present work is the paper by Wanwimolruk *et al.* (15) on structural requirements of organic acids for binding to the site on HSA at which the BDZs are thought to bind. Although certain chiral BDZs have long been known to interact in a highly enantioselective manner with HSA (e.g. Ref. 16), the influence of structure upon enantioselective interactions has not been directly addressed. With a set of enantiospecific data, supported by the results of displacement studies (17), we have undertaken the following study to get insight into the molecular mechanism of chromatographic separations on HSA-CSP and, thus, into the mechanism of drug-protein binding.

## Materials and Methods

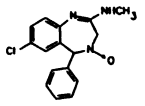
Structures of the BDZ derivatives studied are presented in Fig. 1, and the description of the procedure for chromatographic determinations is given in the accompanying work (17). Logarithms of HPLC capacity factors for the first-eluting enantiomers (log $k'_P$), for the second-eluting enantiomers (log $k'_M$), and for the achiral solutes (log $k'_{AC}$) are collected in Table 1.

**Parameterization of structure of BDZs.** Hydrophobicity of individual structural fragments of BDZ derivatives was characterized by means of fragmental hydrophobic constants (18, 19). The most significant hydrophobicity descriptor was obtained by adding the fragmental

[2] W. H. Pirkle, J.-P. Chang, and J. A. Burke. Contribution of specifiable hydrophobic interactions to chiral recognition. Submitted for publication.

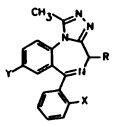


Compounds 2-15

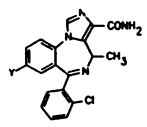


Chlordiazepoxide

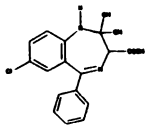
| | Compound | $R_1$ | $R_2$ | X | Y |
|---|---|---|---|---|---|
| 1 | Chlordiazepoxide | See above | - | - | - |
| 2 | (R,S)-Oxazepam H. | H | $OCO(CH_2)_2COO^-$ | H | Cl |
| 3 | Nitrazepam | H | H | H | $NO_2$ |
| 4 | Flunitrazepam | $CH_3$ | H | F | $NO_2$ |
| 5 | Clonazepam | H | H | Cl | $NO_2$ |
| 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



Clorazepate

| | 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 |
| 22 | Clorazepate | See above | - | - |

**Fig. 1.** Structures of BDZ analogues studied.

constant of the substituent at position 7 in the fused benzene ring and the fragmental constant of the substituent at position 2′ of the phenyl functionality.

Molecular geometry optimization and electron charge distribution within the molecules were calculated by the MOPAC procedure (20) within the molecular modeling package InsightII (Biosym Technologies, Inc., San Diego, CA). Calculations were done on an IBM RISC System 6000 computer (IBM Corporation, Austin, TX). Individual structural descriptors used eventually in structure-activity relationship studies are defined under Results and listed in Table 1.

**Analysis of quantitative structure-HSA binding relationships.** Structural descriptors and logarithms of HPLC capacity factors were mutually related by means of multiparameter regression analysis, using the CSS package (StatSoft, Inc., Tulsa, OK) run on a personal computer. Standard and stepwise regression methods were applied. The requirements for meaningful correlation analysis were observed, and the relationships derived were checked to avoid chance correlations (21). The QSERR analysis was done separately for the first-eluting enantiomers, for the second-eluting enantiomers, and for the achiral derivatives.

## Results

The structures of the 22 compounds studied are given in Fig. 1 and include nine achiral solutes, nine racemic mixtures, and four single enantiomers. Individual enantiomers of racemic mixtures were well separated using the HPLC conditions, except for compound (*RS*)-Ro 11 3128/002. Retention on the HSA-CSP observed for the single enantiomers (*S*)-Ro 23 0983,

TABLE 1
**HPLC retention parameters and structural data of BDZs under study**

| No.[a] | log $k'_P$[b] | log $k'_M$[c] | log $k'_{AC}$[d] | $P_{SM}$[e] | $f_{X+Y}$[f] | C(3)[g] | W[h] Å |
|---|---|---|---|---|---|---|---|
| 1 | | | 0.8645 | 0.0849 | 1.05 | 0.1035 | 9.30 |
| 2 | 0.8512 | 1.8938 | | 1.8635 | 1.05 | 0.2785 | 8.74 |
| 3 | | | 0.6243 | 0.0703 | 0.06 | 0.0960 | 8.54 |
| 4 | | | 0.4857 | 0.0609 | 0.20 | 0.0882 | 9.63 |
| 5 | | | 0.7679 | 0.0680 | 0.77 | 0.0966 | 8.67 |
| 6 | | | 1.0614 | 0.0635 | 1.76 | 0.0977 | 8.69 |
| 7 | | | 1.0969 | 0.0634 | 1.05 | 0.0979 | 8.59 |
| 8 | | | 1.1216 | 0.0578 | 1.05 | 0.0933 | 9.56 |
| 9 | 0.7672 | 0.9745 | | 0.6120 | 1.76 | 0.2388 | 9.76 |
| 10 | 0.8068 | 0.9360 | | 0.5953 | 1.76 | 0.2425 | 8.71 |
| 11 | 0.6561 | 1.0261 | | 0.7049 | 1.05 | 0.2451 | 8.60 |
| 12 | 0.5224 | 1.1793 | | 0.6113 | 1.05 | 0.2353 | 9.49 |
| 13 | 0.3892 | | | 0.0675 | −0.18 | 0.0549 | 10.02 |
| 14 | 0.6628 | | | 0.0600 | 1.19 | 0.0624 | 8.64 |
| 15 | 0.7193 | 0.7193 | | 0.0633 | 0.77 | 0.0651 | 8.70 |
| 16 | 0.2648 | 0.4533 | | 0.5862 | 1.05 | 0.3169 | 10.50 |
| 17 | | | 0.4200 | 0.0784 | 1.05 | 0.1722 | 10.29 |
| 18 | | | 0.6243 | 0.0730 | 1.76 | 0.1725 | 10.26 |
| 19 | 0.3838 | | | 0.0484 | 1.19 | 0.1379 | 10.24 |
| 20 | 0.7404 | | | 0.0320 | 1.19 | 0.1309 | 9.20 |
| 21 | 1.0523 | 1.1156 | | 0.0441 | 1.76 | 0.1184 | 9.22 |
| 22 | 0.9715 | 1.3992 | | 1.0745 | 1.05 | 0.0916 | 8.14 |

[a] Compounds are numbered as in Fig. 1.
[b] Logarithm of capacity factor of the first-eluting enantiomer.
[c] Logarithm of capacity factor of the second-eluting enantiomer.
[d] Logarithm of capacity factor of achiral solutes.
[e] Submolecular polarity parameter (see text for definition).
[f] Sum of hydrophobic constants of substituents *X* and *Y*.
[g] Electron excess charge on carbon $C_3$ of the diazepine system.
[h] Molecular width.

(*S*)-Ro 14 8935, (*S*)-Ro 11 5073, and (*S*)-Ro 23 1117 was independently identified (17, 22, 23) as being due to the first, less retained isomer, i.e., capacity factor is $k'_P$.

In the analysis of QSERRs for chiral BDZs, the logarithms of capacity factors corresponding to both the first peak (log $k'_P$) and the second peak (log $k'_M$) were considered. Retention parameters of the achiral agents (log $k'_{AC}$) were analyzed independently. The three groups of the capacity factor-dependent variables are collected in Table 1, along with the structural descriptors for the solutes.

The starting hypothesis was that the retention of the first-eluting enantiomers of the chiral compounds (i.e., $k'_P$) was due to nonspecific hydrophobic interactions with structurally featureless regions of HSA-CSP. The retention of the second-eluting enantiomer was assumed to be due to the summation of the nonspecific interactions observed for the first peak and enantiospecific interactions with a specific binding site, presumably site II.

Initially, the correlation between log $k'_P$ and log $k'_M$ for the set of nine agents for which we had both retention values was examined. The correlation was very poor (correlation coefficient $r = 0.5831$). Attempts to improve the correlation by introducing additional variables into the regression equation eventually succeeded when a submolecular polarity parameter, $P_{SM}$, was considered. The parameter was determined as follows (Fig. 2). Electron excess charges, determined by MOPAC (20), on the individual atoms of the substituents at carbon C(*3*) were analyzed. The charge difference between the hydrogen atom at position C(*3*) and the most negatively charged atom in the other substituent was calculated. The charge difference was multiplied by the distance (in Å) between these two atoms. Description of the capacity factor of the second-eluting enantiomer, $k'_M$, in terms of $k'_P$, in combination with the parameter $P_{SM}$ was very good:

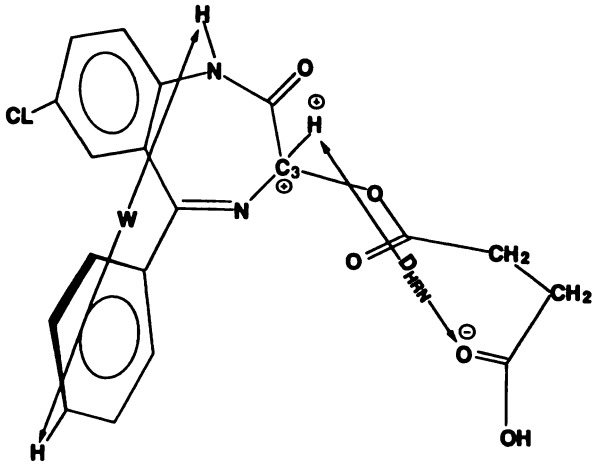



**Fig. 2.** Determination of structural descriptors used in the QSERR analysis. Polarity parameter $P_{SM} = \Delta \times D_{HRN}$, where $\Delta$ = (excess charge on hydrogen at $C_3$) − (excess charge on most negatively charged atom in substituent R), and $D_{HRN}$ = distance between the two atoms; C(*3*) denotes excess charge on carbon $C_3$; *W* is the width of the molecule along the phenyl substituent.

$$\log k'_M = 0.0467 + 0.8994(\pm 0.2678) \log k'_P + 0.5410(\pm 0.1167) P_{SM} \quad (1)$$

$t = 3.36$ and $p \leq 1.5 \times 10^{-2}$ for the log $k'_P$ term, $t = 4.64$ and $p \leq 3.6 \times 10^{-3}$ for the $P_{SM}$ term, and $n = 9$, $r = 0.9252$, $F = 17.83$, and $p < 10^{-3}$ for the whole equation; the values in parentheses are the standard deviations of the regression coefficients, $t$ is $t$ test value, $p$ is the significance level of individual variables and the whole equation, $n$ is the number of data points used to derive the regression equation, $r$ is the correlation coefficient, and $F$ is the $F$ test value. The evident outlier of the regression was (*S*)-temazepam, for which the calculated log $k'_M$ was much

lower than that experimentally observed. Excluding temazepam, we obtained eq. 2:

$$\log k'_M = -0.1494 + 1.0974(\pm 0.1621) \log k'_P + 0.5466(\pm 0.0666)\, P_{SM} \quad (2)$$

$t = 6.77$ and $p \leq 1.1 \times 10^{-3}$ for the $\log k'_P$ term, $t = 8.21$ and $p \leq 4.4 \times 10^{-4}$ for the $P_{SM}$ term, and $n = 8$, $r = 0.9801$, $F = 60.93$, and $p < 3.1 \times 10^{-4}$ for the whole equation. There is no intercorrelation between $\log k'_P$ and $P_{SM}$ ($r = 0.08$ and 0.07 in the case of data used in eqs. 1 and 2, respectively).

Within the set of chromatographic parameters studied, there were 13 data points quantifying retention of the first-eluting enantiomers. We tested the applicability of various structural descriptors for description of $\log k'_P$, eventually attaining the following statistically significant "best" equation:

$$\log k'_P = 2.4790 + 0.1834(\pm 0.0791)\, f_{X+Y} - 0.2779(\pm 0.0551)\, W \quad (3)$$

$t = 2.32$ and $p \leq 0.043$ for the $f_{X+Y}$ term, $t = -3.95$ and $p \leq 2.7 \times 10^{-3}$ for the $W$ term, and $n = 13$, $r = 0.8448$, $F = 12.46$, and $p < 2 \times 10^{-3}$ for the whole equation. The terms in eq. 3 denote (Fig. 2; Table 1) the sum of the hydrophobicity of the substituent at position 7 in the fused benzene ring plus that of the substituent at position 2′ of the phenyl substituent ($f_{X+Y}$) and the width of the molecule, as measured from the extremity of the phenyl substituent ($W$). There is no intercorrelation between $f_{X+Y}$ and $W$ ($r = 0.17$).

With the set of eight $\log k'_M$ data points (temazepam excluded) for the second-eluting enantiomers, the stepwise regression analysis resulted in the following equation:

$$\log k'_M = 0.5558 + 0.8354(\pm 0.1540)\, P_{SM} + 0.3645(\pm 0.1987)\, f_{X+Y} - 2.6904(\pm 0.9317)\, C(3) \quad (4)$$

$t = 5.42$ and $p \leq 6 \times 10^{-3}$ for the $P_{SM}$ term, $t = 1.83$ and $p \leq 0.14$ for the $f_{X+Y}$ term, $t = -2.89$ and $p \leq 4.5 \times 10^{-4}$ for the C(3) term, and $n = 8$, $r = 0.9384$, $F = 9.83$, and $p < 0.026$ for the whole equation. The variable C(3) is the excess electronic charge on carbon $C_3$ of the diazepine system. Intercorrelations for the pairs of variables $P_{SM}$ versus $f_{X+Y}$, $P_{SM}$ versus C(3), and $f_{X+Y}$ versus C(3) are $r = 0.25$, 0.44, and 0.14, respectively.

Regressing logarithms of capacity factors ($\log k'_{AC}$) of achiral solutes against the structural descriptors listed in Table 1, we obtained the following relationship:

$$\log k'_{AC} = 1.2208 + 0.3742(\pm 0.0962)\, f_{X+Y} - 7.0681(\pm 1.6632)\, C(3) \quad (5)$$

$t = 3.89$ and $p \leq 8 \times 10^{-3}$ for the $f_{X+Y}$ term, $t = -4.25$ and $p \leq 6 \times 10^{-3}$ for the C(3) term, and $n = 9$, $r = 0.8893$, $F = 11.34$, and $p < 0.009$ for the whole equation. Intercorrelation between $f_{X+Y}$ and C(3) is $r = 0.47$.

Logarithms of capacity factors measured for achiral solutes ($\log k'_{AC}$) were compared with the values calculated by eq. 5, as well as the values produced by eq. 1, derived for the first-eluting enantiomers of chiral agents. The data are collected in Table 2.

TABLE 2

**Logarithms of capacity factors of achiral BDZs determined experimentally and calculated using eqs. 5 and 3**

| No.[a] | $\log k'_{AC}$ | | |
|---|---|---|---|
| | Observed | Calculated by eq. 5 | Calculated by eq. 3 |
| 1 | 0.8645 | 0.8821 | 0.6456 |
| 3 | 0.6243 | 0.5647 | 0.6295 |
| 4 | 0.4857 | 0.6722 | 0.4178 |
| 5 | 0.7679 | 0.8261 | 0.7314 |
| 6 | 1.0614 | 1.1888 | 0.9087 |
| 7 | 1.0969 | 0.9217 | 0.8002 |
| 8 | 1.1216 | 0.9542 | 0.5889 |
| 17 | 0.4200 | 0.3966 | 0.4299 |
| 18 | 0.6243 | 0.6601 | 0.5666 |

[a] Compounds are numbered as in Fig. 1.

## Discussion

The mechanism by which drugs bind to HSA has long been a subject of speculation and controversy. It is believed that some drugs are bound within cylindrical domains of albumin, which possess a hydrophobic interior and a polar exterior (24–29). Thus, the binding would depend on the hydrophobic, steric, and electrostatic properties of the drug molecule. Several specific binding sites for drugs and endogenous compounds on HSA have been postulated. Assignment of the binding of particular xenobiotics to such sites was done by determining the structural requirements for drugs believed to bind to a given site. The conclusions concerning the structural parameters of importance for binding to HSA were drawn based on qualitative, rather than quantitative, comparison of small sets of drugs.

Some quantitative structure-HSA-binding relationships for BDZs were reported in 1976 by Lucek and Coutinho (14). The agents studied differed mostly in the substituent at position 7 of the fused benzene ring and were achiral, except for one compound. For this particular set of BDZs, the extent of protein binding was a function of molecular hydrophobicity, which, in turn, depended on the hydrophobic character of the substituent at position 7 of the fused benzene ring.

Semiquantitative structure-activity studies of the binding of chiral 2-arylpropionic acid nonsteroidal anti-inflammatory agents to HSA have been reported by Wanwimolruk *et al.* (15). Because BDZs are assumed to bind at this site, the findings of Wanwimolruk *et al.* (15) are also of interest in the present context. Their results suggest that the binding site is a hydrophobic cleft, about 16 Å long and about 8 Å wide, with a cationic group located near the surface. However, Wanwimolruk *et al.* (15) did not take into account the possibility of enantioselectivity in the binding to HSA of the chiral solutes they studied. This is despite the fact that enantioselectivity has long been known to occur in the binding of chiral drugs to HSA (16).

When considering the structure-binding relationships of BDZs, it is important to note that achiral BDZs exist as an equimolar mixture of two conformers, the *M*- and *P*-forms (Fig. 3). Each of the enantiomers of BDZs asymmetrically substituted at C(3) exists in only one of the possible conformations. The *M*-conformer, selectively adopted by the *S*-enantiomer of compounds substituted with an oxygen atom at C(3) or the *R*-enantiomer of those substituted with a carbon atom, is the form that binds more strongly to HSA (22, 29–31). In addition, circular dichroism studies of chiral and achiral BDZs have indicated that there are separate binding sites for the *M*- and *P*-conformers and that achiral BDZs bind to HSA in the *M*-conformation (29). The existence of separate BDZ binding sites was supported by studies of the chromatographic resolution of (*R*)- and (*S*)-oxazepam hemisuccinate on the HSA-CSP (2).

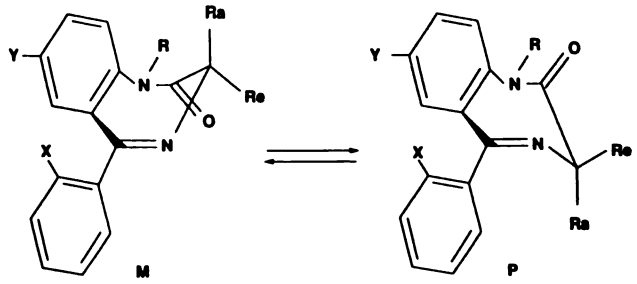



**Fig. 3.** *M*- and *P*-conformations of 1,4-benzodiazepine-2-ones. $R_a$, axial; $R_e$, equatorial orientation of substituents.

The latter results were also verified by ultrafiltration studies (2).

Eqs. 1 and 2 derived here suggest that some interactions of the enantiomers of the BDZs with HSA-CSP are common for both isomers. The greater retention of the second-eluting enantiomers is due to additional specific interactions. These additional interactions are evidently of a polar electrostatic nature. The submolecular polarity parameter, $P_{SM}$, is concerned with the area of the molecule that is the source of its chirality. Thus, the input of $P_{SM}$ to retentive interactions with HSA-CSP for the second-eluting enantiomer alone, and its lack of importance in the binding of the first-eluting enantiomer, can be rationalized. The parameter $P_{SM}$ may be treated as a measure of a submolecular local dipole.

The correlations obtained in eqs. 1 and 2 are very good. Their poor description of the behavior of temazepam does not diminish their adequacy. It may be that some special steric fit between (*S*)-temazepam and the binding site leads to its increased retention. Alternatively, (*S*)-temazepam may be bound at additional sites on the HSA molecule, which are unimportant for the other members of this series. Atypical binding of temazepam by HSA-CSP was also observed by us in the preceding displacement studies (17).

The form of eq. 3 indicates that the binding of the first-eluting enantiomer of the BDZs is not simply due to hydrophobic attraction. The importance of the width constraint implied by the parameter *W* suggests that there is a steric specificity involved in the binding of the first-eluting enantiomers. The form of eqs. 1 and 2 might suggest that there is a single binding site for both BDZ enantiomers on HSA, to which the second-eluting enantiomer is able to bind more tightly. However, the evidence from displacement studies (2, 17) is that there are different binding sites for the enantiomers of the BDZs; compounds that cause very large displacement of the second-eluting enantiomer have very little effect on log $k'_P$.

In view of the relationships derived here, these binding sites must possess some common structural features. The properties of the binding site to which the first-eluting enantiomer binds are revealed in eq. 3. The equation proves that the most important positive input to the binding of the first-eluting enantiomer is provided by the hydrophobicity of substituents *X* and *Y*. It is not the hydrophobicity of the whole molecule but that of a specific submolecular region that affects binding. Thus, the aromatic fragments of the phenyl-BDZ molecule may play the role of a hydrophobic "anchor" at the BDZ binding sites.

The other significant structural descriptor in eq. 3 is molecular width, as measured from the extremity of the phenyl substituent at C(*5*) to the furthest atom of the substituent at N(*1*) or C(*2*). It provides a negative input to retention, proving the existence of steric restriction.

The two-parameter eqs. 1, 2, and 3 imply the necessity of using several structural descriptors to calculate the retention of the second-eluting enantiomer in terms of nonempirical parameters. The small number of available log $k'_M$ data points limits the possibilities of deriving significant QSERR equations. However, the three-parameter regression equation obtained here by the stepwise approach is significant at the 97% significance level. According to eq. 4, the retention of the second-eluting enantiomers is determined by the polarity parameter, $P_{SM}$, the fragmental hydrophobicity, $f_{X+Y}$, and the excess charge on carbon C(*3*). The most significant binding descriptor for the more retained enantiomer is the submolecular polarity parameter, $P_{SM}$, which was meaningless in the description of log $k'_P$.

Thus, the binding site on HSA-CSP for the more retained enantiomer may be assumed to comprise a hydrophobic anchoring area and a cationic site. The greatest difference between the binding sites of the first- and second-eluting enantiomers seems to be the extent and the charge density of the cationic area at the exterior part of the binding site. In the case of the nonenantioselective binding site, that area must be either missing or relatively small and bearing limited charge. The existence of a highly charged cationic area on the enantioselective binding site explains the pronounced displacing efficiency of carboxylic acids observed previously (17).

Equation 5, derived for achiral solutes, demonstrates that retention of this class of BDZs is positively affected by hydrophobicity of aromatic moieties, whereas it is negatively affected by excess charge on carbon $C_3$. Thus, eq. 5 is similar to eq. 4, which describes retention of the more retained enantiomer of chiral BDZs. The difference is that in eq. 5 there is no polarity parameter, $P_{SM}$. The lack of significance of $P_{SM}$ in eq. 5 is due to the fact that, in symmetrically substituted BDZs, this parameter approaches zero.

As evident from Table 2, eq. 5 is a better predictor of retention for the achiral solutes than is eq. 3, which was derived for the first-eluting enantiomer of the chiral solutes. The fact that achiral BDZs are not retained solely due to the mechanism responsible for the retention of the first-eluting enantiomers is especially evident in the case of diazepam and desmethyldizaepam, which have been shown by circular dichroism studies to bind predominantly in the *M*-conformation (29).

Based on the equations derived in this study, a model may be proposed for the structural requirements of the two postulated modes of BDZ binding to HSA (Fig. 4). According to eq. 3, the BDZs appear to bind within hydrophobic cavities, and substituents at N(*1*), C(*2*), and C(*5*) would then provide spatial orientation of the BDZ molecules within this cavity. Steric limitations suggest that the hydrophobic cavity has definite boundaries. In addition, the steric features at the stereogenic center, carbon C(*3*), appear to play no role in this binding mode (Fig. 4I).

The binding mode of the second-eluting enantiomer, as described by eq. 4, involves hydrophobic electrostatic interactions. Thus, in addition to a hydrophobic cavity, there must be a cationic region in close proximity (Fig. 4II). For the BDZs in the *M*-conformation, the electrostatic repulsion between the excess positive charge on carbon C(*3*) and the cationic site on the protein surface appears to be more than offset by the

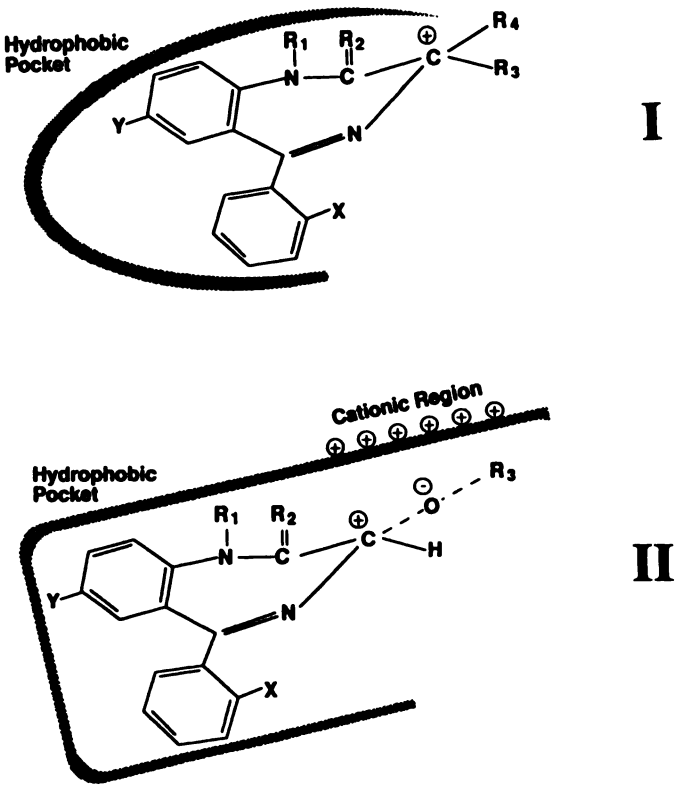



**Fig. 4.** Model for the structural requirements of the two postulated types of BDZ binding to HSA. *I*, binding of BDZs in the *P*-conformation; *II*, binding of BDZs in the *M*-conformation.

attraction of a negatively charged atom within the substituent at C(*3*) and the same area. In the case of the lesser retained enantiomer, the electrostatic repulsion between carbon C(*3*) and the cationic area and steric hindrance due to the *P*-conformation may prohibit binding at this site.

In conclusion, it should be stressed that the new generation of HPLC CSPs, as represented by immobilized HSA, offer a unique research tool for molecular pharmacology. Chromatographic processes are dynamic in character, as are the processes in living biological systems. Thus, the information obtained from HPLC on the HSA-CSP has the informative value of data obtained from biological experiments, as well as the quantitative precision of chemical measurements. HPLC stationary phase materials based upon other immobilized biopolymers, such as enzymes, receptors, and ion channels, should produce rapid advances in molecular pharmacology.

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