CHIRALITY 11:115–120 (1999)

Stereoselective Allosteric Binding Interaction on Human Serum Albumin Between Ibuprofen and Lorazepam Acetate

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ABSTRACT The effect of ibuprofen enantiomers on the stereoselective binding of 3-acyloxy-1,4-benzodiazepines to human serum albumin (HSA) was studied using both native and Sepharose-immobilized protein. (S)-Lorazepam acetate exhibited considerably enhanced binding, especially in the presence of (+)-(S)-ibuprofen. The phenomenon is an indication of cooperative allosteric interaction between different binding sites during multiple cobinding of two ligands. *Chirality 11:115–120, 1999.* © 1999 Wiley-Liss, Inc.

KEY WORDS: chiral chromatographic separation; benzodiazepines; plasma protein

Stereoselective plasma protein binding of chiral drug molecules administered in racemic form results in uneven enantiomeric composition of the unbound molecules. Besides quantitative differences the enantiomers may take part in different kinds of binding interactions during simultaneous binding with other drugs or endogenous ligands. Recently, Oravcová et al.[1] reviewed drug-protein binding studies including stereoselective aspects. Human serum albumin (HSA) is the most abundant, thus the most studied serum protein.[2] Although the existence of two main ligand binding regions[3,4] on HSA was confirmed by X-ray studies,[5] there are several indications that simultaneous binding of two ligands may have individual characteristics due to induced conformational changes of the protein.[6] The most remarkable allosteric interactions have been found for chiral ligands. While diazepam can be used successfully [4,7,8] as a specific marker of site II, certain 3-substituted 1,4-benzodiazepines were found[9–13] to exhibit significantly enhanced binding of the (S)-enantiomers in the presence of some other ligands bound to site I.

The high affinity binding of ibuprofen [2-(4-isobutylphenyl)-propionic acid] on HSA also occurs[4,8] on site II. Both enantiomers have high affinity and the binding of the (R)-enantiomer is stronger.[14,15] Binding interaction studies performed by high performance liquid chromatography (HPLC) on HSA-based stationary phase indicated[16] that ibuprofen enantiomers competitively displaced (S)-oxazepam hemisuccinate, leaving the weakly bound (R)-benzodiazepine unaffected. Similar interaction experiments with some other benzodiazepines suggest[17] that the selective displacement of the (S)-enantiomers does not correspond to simple competition. Systematic binding studies of ibuprofen enantiomers performed with the same method concluded[15] that, besides the common binding site, (S)-ibuprofen has some other major binding region. Ibuprofen enantiomers were also found[14] to exert an unusual effect on the binding of carprofen enantiomers to HSA; instead of the expected competitive displacement, an altered binding mechanism of carprofen could be detected.

In this work, we studied the influence of ibuprofen enantiomers on the stereoselective binding of some 3-acyloxy-1,4-benzodiazepines (Table 1) by using both native and Sepharose-immobilized HSA. Both methods, i.e., chiral analysis of the ultrafiltrates of solutions containing the racemate and the protein, and affinity chromatography, have been used successfully to reveal enantioselective interactions.[18,9]

MATERIALS AND METHODS

Chemicals were obtained from the following sources: (+)-(S)-ibuprofen was kindly provided by K.M. Williams (St.Vincent's Hospital, Sydney, Australia); (-)-(R)-ibuprofen (RBI, Natick, MA); *rac*-oxazepam and lorazepam esters and warfarin enantiomers (cf. refs. 9 and 12); and HSA (Fraction V powder) and diazepam (Sigma, St. Louis, MO).

Ultrafiltration of solutions containing racemic benzodiazepine ligand, (R)- or (S)-ibuprofen and HSA in Ringer buffer, pH 7.4 were performed in an Amicon MPS-1 system using YMT30 membranes. Free fractions of the benzodiazepine enantiomers were determined by direct HPLC analysis of the initial racemic ligand solutions as well as the corresponding ultrafiltrates, using Chiral-AGP or Chiral-HSA columns.

HPLC studies were performed using a system composed of a Jasco PU-980 pump with a Rheodyne 7125 injector (20 µl loop), a Jasco 975 UV-VIS detector at 225 or 240 nm and Barspec chromatographic software. The stationary phases

Contract grant sponsor: Hungarian Academy of Sciences; Contract grant numbers: OTKA 15833 and 26039.
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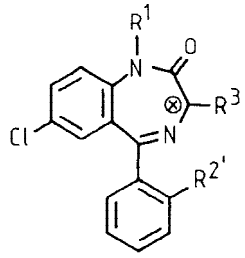
Received for publication 18 September 1997; Accepted 2 June 1998

TABLE 1. Structure of benzodiazepines investigated



No.	Compound	R^1	R^3	$R^{2'}$
1	Lorazepam acetate	H	$OCOCH_3$	Cl
2	Lormetazepam acetate	CH_3	$OCOCH_3$	Cl
3	Oxazepam acetate	H	$OCOCH_3$	H
4	Temazepam acetate	CH_3	$OCOCH_3$	H
5	Lorazepam hemisuccinate	H	$OCO(CH_2)_2COOH$	Cl
6	Oxazepam hemisuccinate	H	$OCO(CH_2)_2COOH$	H

applied: Chiral-AGP (100 × 4.0 mm i.d.) and Chiral-HSA (50 × 4.0 mm i.d.) columns with the corresponding guard columns (10 × 3.0 mm i.d.) from ChromTech AB, Hägersten, Sweden. Chromatographic conditions are given in Figure 1. Elution orders (R,S) were checked previously.[19] The presence of ibuprofen in the ultrafiltrate did not interfere.

Affinity chromatography on HSA-Sepharose gel was performed following the method of Lagercrantz et al.[20] HSA in 1% concentration was immobilized on CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden). The gel was filled into a glass column (12 mm i.d.), the elution was made by Ringer buffer containing 0.01% sodium azide, and the flow rate was about one ml/min. The experiments were performed in duplicates on a short column (V_o = 5 ml) as well as on a longer column (V_o = 11 ml). After regeneration of the column (removal of ibuprofen by washing with 1% HSA solution, followed by 0.1 M NaCl and buffer) the results were reproducible, with only slight shifts (±2 ml) in the elution volume values. For binding interaction studies, ibuprofen was dissolved in the eluent buffer. Ligand samples of 2–5 µg were applied in 10–20 µl ethanol. Elution volumes were detected by UV or by radioactive liquid scintillation counting of collected fractions. Elution orders (R,S) had been proved in previous studies.[9,12]

RESULTS

Ultrafiltration

Ultrafiltrations of solutions containing racemic ligands (**1, 5,** and **6**) and HSA were performed in the absence and in the presence of ibuprofen enantiomers, and the free fractions were determined by chiral HPLC. The results, summarized in Table 2, indicate that while the binding of (R)-benzodiazepines was unchanged, the free fractions of the (S)-enantiomers were subjected to various effects. In the presence of ibuprofen the free fraction of (S)-oxazepam hemisuccinate was almost doubled, indicating displacement. While it is in accordance with the previous findings,[16] lorazepam esters exhibited different behavior: (S)-lorazepam hemisuccinate was not displaced by (R)-ibuprofen and its binding was even increased slightly in the presence of (S)-ibuprofen. The free fraction of (S)-lorazepam acetate showed considerably enhanced binding provoked by both ibuprofen enantiomers. The opposite effect of ibuprofen on the free fractions of (S)-oxazepam

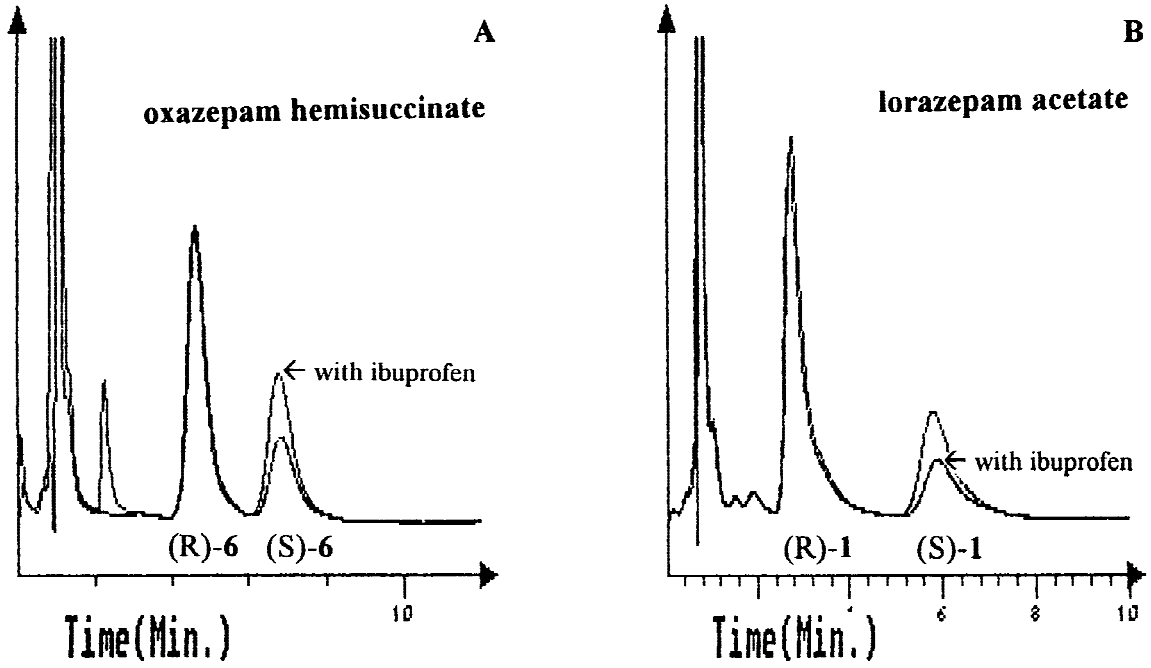


Fig. 1. Chiral-HPLC analysis of ultrafiltrates. **A:** *rac*-**6** (26 µM) and HSA (47 µM); effect of 60 µM (S)-ibuprofen; column: Chiral-AGP, mobile phase: 0.01 M phosphate buffer pH 7.0 with 3% acetonitrile flow rate, 0.9 ml/min. **B:** *rac*-**1** (44 µM) and HSA (90 µM); effect of 60 µM (S)-ibuprofen; column: Chiral-HSA, mobile phase: 0.01 M phosphate buffer pH 7.0 with 10% 2-propanol flow rate, 0.9 ml/min.

TABLE 2. Effect of ibuprofen enantiomers on the free fractions of benzodiazepine enantiomers (α_R and α_s) in HSA solution of racemic ligand*

	Oxazepam hemisuccinate c_{rac} = 26 μM c_{HSA} = 47 μM		Lorazepam hemisuccinate c_{rac} = 48 μM c_{HSA} = 90 μM		Lorazepam acetate c_{rac} = 44 μM c_{HSA} = 90 μM	
	α_R	α_S	α_R	α_S	α_R	α_S
Control	0.91	0.30	0.57	0.40	0.60	0.32
With 60 μM (S)-ibuprofen	0.93	0.51	0.55	0.32	0.62	0.17
With 60 μM (R)-ibuprofen	0.91	0.58	0.55	0.39	0.62	0.20

*Data are average of two parallels, with ± 0.02 SD. HSA, human serum albumin; SD, standard deviation.

hemisuccinate and (S)-lorazepam acetate can be seen in Figure 1.

Binding Interaction Studies on HSA-Sepharose Gel

The unexpected enhancing effect of ibuprofen on the binding of (S)-lorazepam acetate was confirmed by the chromatographic technique on HSA-Sepharose gel (c_{HSA} ~ 150 μM), when the sample was *rac*-[^{14}C]lorazepam acetate (trace amount) and the ibuprofen enantiomers (100 μM) were applied in the eluent. The chromatograms (Fig. 2) indicate considerably increased retention of the second peak belonging to (S)-lorazepam acetate; the enhancing effect of (S)-ibuprofen was stronger.

To study the structural requirements of the irregular binding interaction, similar experiments were performed with five other benzodiazepine esters. Elution volumes are summarized in Table 3. In accordance with the ultrafiltration, it was found that the interaction was highly dependent on the benzodiazepine substitution. In the presence of ibuprofen, the elution volumes of the (R)-enantiomers showed only slight decreases, whereas the binding of the (S)-benzodiazepines indicated various behavior:

- Enhanced binding is restricted to (S)-lorazepam acetate. This effect is totally reversed if the molecule has R^1 : CH_3 substitution (cf. **1** and **2**).
- The chromatogram of oxazepam acetate (**3**) did not show significant change in the presence of ibuprofen, but its analogue, having R^1 : CH_3 substituent (**4**) showed the regular displacement behavior, i.e., the originally high binding stereoselectivity of temazepam acetate disappeared in the presence of ibuprofen.
- Comparison of the two negatively charged hemisuccinates (**5** and **6**) reflects the ultrafiltration results, i.e., whereas (S)-oxazepam hemisuccinate is displaced by ibuprofen enantiomers, the binding of (S)-lorazepam hemisuccinate ($R^{2\prime}$: Cl) is practically unchanged.

A systematic chromatographic study of ligands **1, 2, 5,** and **6** was performed on a longer HSA-Sepharose column, varying the concentration of ibuprofen enantiomers over the range 0–100 μM. The results shown in Figure 3 indicate only a slight decrease for the binding of (R)-benzodiazepines, but drastic binding interactions of at least

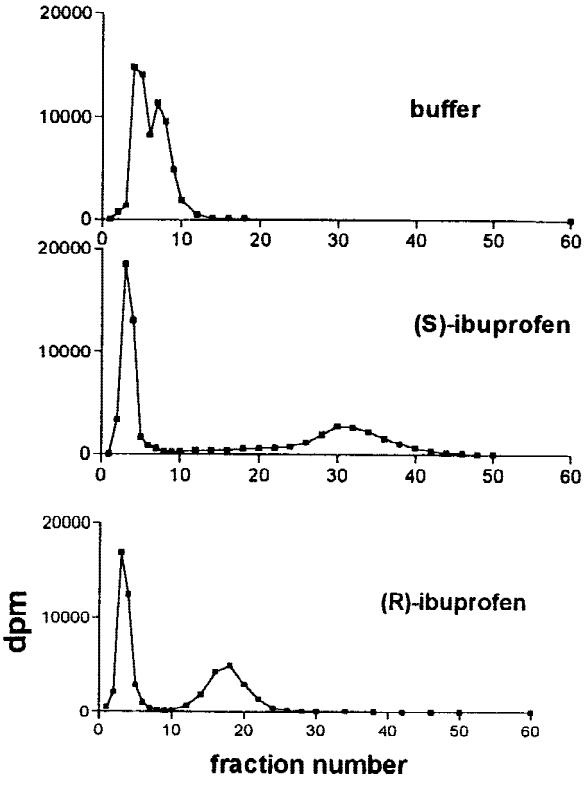


Fig. 2. Radiochromatogram of *rac*-[^{14}C]lorazepam acetate on an HSA-Sepharose column (V_o:5 ml; $V_{fraction}$:2.5 ml). Elution was made by buffer as well as by 100 μM (S)- and (R)-ibuprofen solutions.

TABLE 3. Influence of ibuprofen enantiomers in the eluent on the elution volumes of benzodiazepine enantiomers (V_R and V_S in ml) obtained on an HSA-Sepharose colume V_o:5 ml)*

	Control		With 100 μM (S)-ibuprofen		With 100 μM (R)-ibuprofen	
Sample	V_R	V_S	V_R	V_S	V_R	V_S
1[a]	10	17	7	75	7	45
2[a]	9	23	7	7	7	7
3	8	28	6	29	7	30
4[a]	7	52	7	7	7	7
5	11	24	7	26	7	19
6	8	40	7	15	6	12

*HSA, human serum albumin.
[a]Measured also with radioactive ligand.

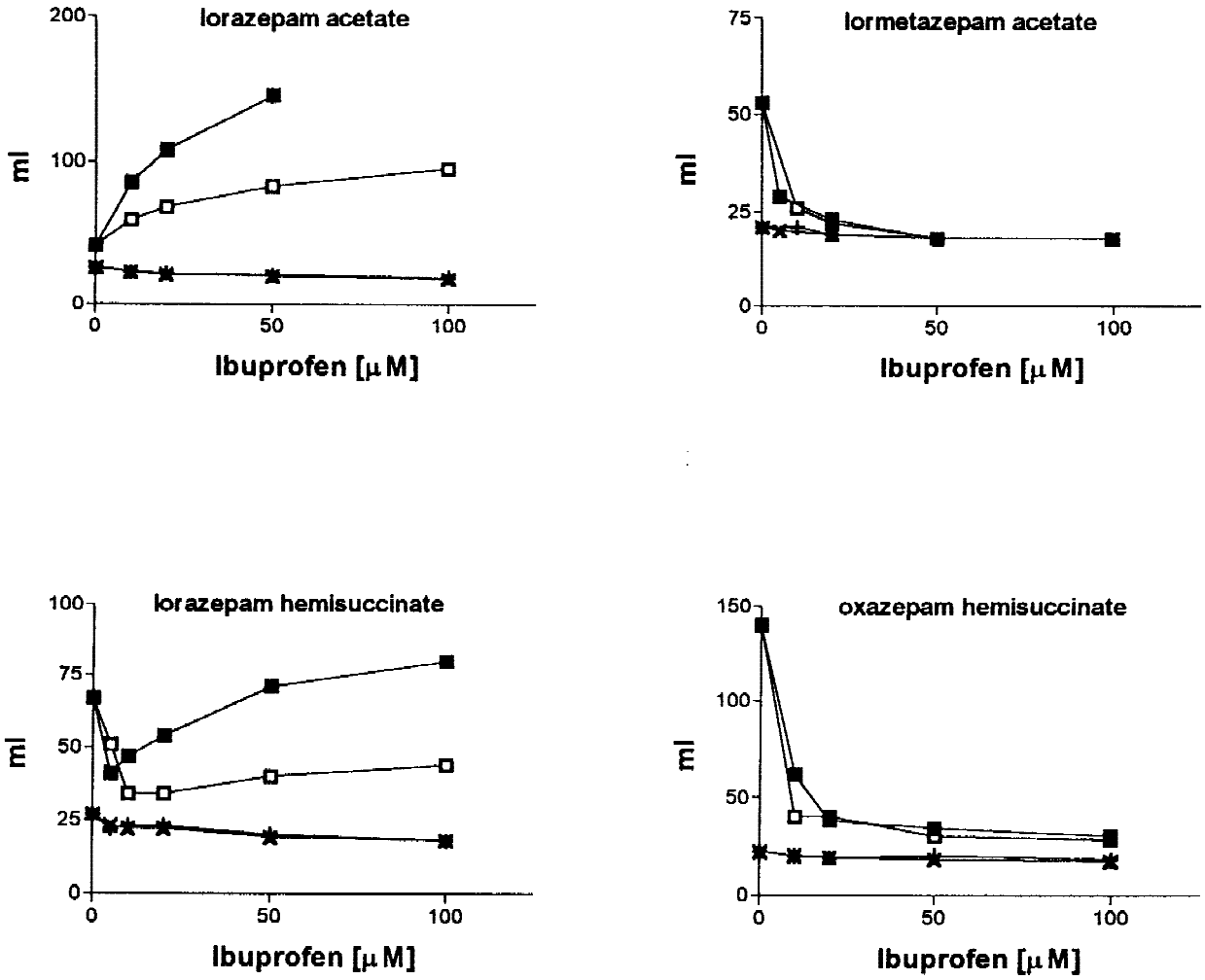


Fig. 3. Effect of ibuprofen enantiomers in the eluent on the elution volumes of benzodiazepine (Bzd) enantiomers obtained on an HSA-Sepharose column (V_o:11 ml). ■, (S)-Bzd and (S)-ibuprofen; □, (S)-Bzd and (R)-ibuprofen; X, (R)-Bzd and (S)-ibuprofen; +, (R)-Bzd and (R)-ibuprofen.

three different characters between (S)-benzodiazepines and ibuprofen enantiomers:

- The binding of (S)-lorazepam acetate increases progressively with increasing ibuprofen concentration; the effect of (S)-ibuprofen is more pronounced.
- The displacement of (S)-lormetazepam acetate and (S)-oxazepam hemisuccinate can be achieved by low ibuprofen concentrations, suggesting competitive displacement.
- (S)-lorazepam hemisuccinate showed discontinuous change with increasing ibuprofen concentration. It is interesting that while displacements could be brought about by 10 μM (S)-ibuprofen, it could not be detected by 100 μM of the same additive. (The decreasing effect of (R)-ibuprofen was somewhat stronger.) This character of (S)-**5** seems to be a mixed effect of **1** and **6;** displacement can be detected at low ibuprofen concentrations, which is compensated by enhanced binding at higher concentrations.

DISCUSSION

The presented results prove that instead of simple competition, ibuprofen enantiomers exert a complex effect on the HSA binding of the (S)-enantiomers of 3-acyloxy-1,4-benzodiazepines. The overall effect depends on all three substitutions studied (R^1, R^3, and $R^{2'}$).

Regular competitive displacement takes place when the substitution pattern in the benzodiazepine is as follows: 1. $R^1 = CH_3$ or 2. $R^1 = H$, $R^{2'} \neq Cl$ and R^3 has anionic character. The irregular behavior is connected with the $R^{2'} = Cl$ substituent or the nonanionic character of the acyloxy group at the chiral centre (R^3). This phenomenon is abolished by the $R^1 = CH_3$ substituent. The requirements of enhanced binding are fulfilled in case of (S)-lorazepam acetate ($R^{2'} = Cl$, $R^1 = H$, and R^3 is nonanionic).

The enhanced binding of (S)-lorazepam acetate provoked by ibuprofen, preferably by the (S)-enantiomer, is a manifestation of cooperative binding interaction between different ligand binding sites on HSA. Since there is evidence proving that the main binding site of diazepam and

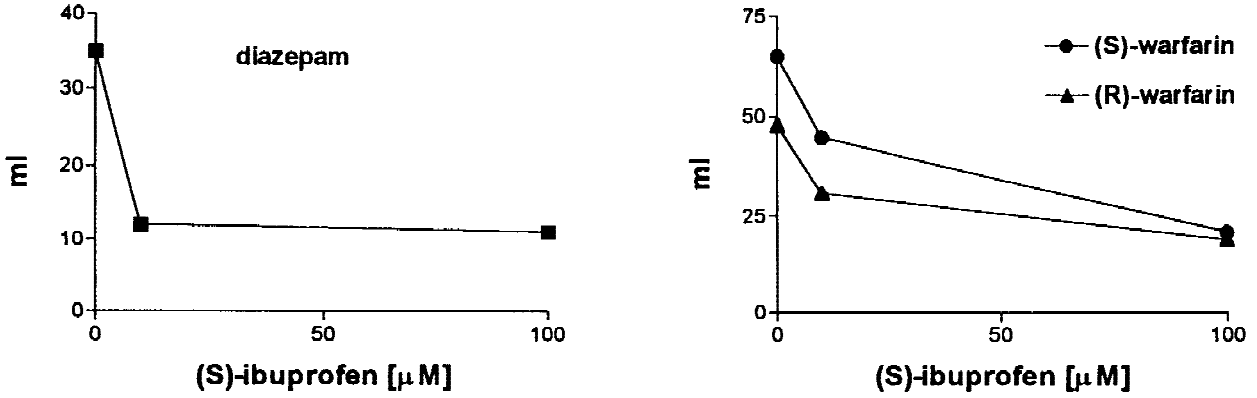


Fig. 4. Influence of (S)-ibuprofen in the eluent on the elution volumes of diazepam as well as (R)- and (S)-warfarin, obtained on an HSA-Sepharose column (V_o: 5 ml)

(S)-benzodiazepines as well as that of ibuprofen enantiomers is common (site II), there must be some other binding site or sites involved.

In principle, both ligands can have such an additional binding site. In case of ibuprofen there is evidence for the existence of secondary sites both for the binding of racemic ibuprofen,[21,22] and even for the enantiomers.[14]

It is notable that the cooperative allosteric interaction found with ibuprofen shows strikingly similar structural requirements for the benzodiazepine molecule as found previously with coumarins[9,12] and bilirubin.[10] Since those are typical site I ligands, it can be assumed that ibuprofen bound to this site provokes the enhanced binding of (S)-lorazepam acetate. The effect of ibuprofen on the markers of the two main sites was checked experimentally. Figure 4 shows the elution volumes of diazepam as well as warfarin enantiomers on HSA-Sepharose column in the presence of (S)-ibuprofen (both enantiomers acted similarly). It can be seen that ibuprofen does displace warfarin, but not in a direct competitive way.[4] Indirect competition was also reported[8] between ibuprofen and another site I drug, phenprocoumon.

The multiple effect of ibuprofen would also explain the mixed character found for lorazepam hemisuccinate: competitive displacement for the common primary site in which the ionic interaction is important,[21] accompanied by induced binding of the benzodiazepine due to allosteric modification of the protein conformation.

When two ligands do not seem to influence the free fractions of each other, there can be the sum of opposing effects. There is CD evidence for the complex involvement of multiple binding sites on HSA, e.g., during the simultaneous binding of carprofen-ibuprofen[14] or indomethacin-probenecid.[23] Lorazepam acetate proved to be a good indicator molecule to reveal chiral allosteric interactions between binding sites on HSA.

ACKNOWLEDGMENTS

We thank Kenneth M. Williams for providing the (+)-(S)-ibuprofen sample. For the synthesis of the benzodiazepines, we are indebted to Drs. Zs. Tegyey and E. Simon-Trompler. Skillful technical assistance by Mrs. I. Kawka is acknowledged.

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