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THE BINDING OF ARYL CARBOXYLIC ACID DERIVATIVES TO HUMAN SERUM ALBUMIN—A STRUCTURE–ACTIVITY STUDY

SAÏK URIEN,* PHILIPPE D'ATHIS† and JEAN-PAUL TILLEMENT*
*Département de Pharmacologie, Faculté de Médecine de Créteil, 8 rue du Général Sarrail, 94010 Créteil, France
and
†Département d'Informatique Médicale, Faculté de Médecine de Dijon, Hôpital du Bocage, 6 rue Paul Thénard, 21000 Dijon, France

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Abstract—The binding interactions of some aryl carboxylic acid derivatives have been examined by circular dichroism and fluorescence spectroscopy. With specific probes, we have shown that the seven ligands under study bind primarily to the benzodiazepine site on HSA. Their association constants are in the range of 10^5–10^6 M^{-1} as found by spectropolarimetric titration, and appear to be closely related to some chemical features. It is concluded that the binding is enhanced by the lengthening of the carbon chain substituent with a terminating carboxyl moiety and by halogen substitution in the aromatic rings. It is further shown that hydrophilic substitutions such as hydroxyl or ketone groups in the carbon chain substituent will decrease the binding.

Many xenobiotic compounds have been shown to bind in blood to human serum albumin (HSA). The binding properties of the protein are actually explained by the presence of several binding regions [1, 2] and most drugs having carboxylate moieties or other electronegative centra bind to at least two well-defined binding sites of varying specificity [3]. But only few studies are available about the possible relationships between the structure of the ligands and their affinity to HSA. Sjödin *et al.* [4] have studied the HSA binding of a series of benzodiazepines derivatives. In this study, the complexity of the molecules did not permit the authors to draw any clear and simple relationships between chemical structure and binding to HSA. Other studies have suggested that hydrophobicity is an important determinant of ligand binding to albumin [5]. Finally, recent studies have shown that the chain length of fatty acids is a determining factor for the location and the affinity of the albumin binding [6].

In the present work, we have examined the binding characteristics of an homologous series of anionic ligands differing by their substituents on the aromatic rings (Table 1). The binding parameters and the location of the binding sites have been investigated by circular dichroism and fluorescence spectroscopy.

MATERIAL AND METHODS

Human serum albumin. HSA (A-1887, Sigma) was dissolved 15 μM in M/15 phosphate buffer pH 7.4 for all experiments.

Drugs. Itanoxone, orthochlorophenyl-4 phenyl hydroxyacetic acid (F1803), (orthochlorophenyl-4 phenyl) acetic acid (F2006), chloro-2′ *p*-biphenyl carboxylic acid (F1893), *p*-biphenylyl-4 oxo-4 methyl-2 butyric acid (F1594), flurbiprofen and fenbufen were provided by P. F. Médicament (Castres, France), Boots-Dacour (Paris, France) and Lederle (Paris, France).

Optical absorption spectroscopy. Absorption spectra and difference spectra were measured with a JY 201 Jobin–Yvon spectrophotometer. All difference spectra were obtained by using matched quartz split-compartment cells with each compartment of path-length 0.4375 cm.

Circular dichroism (CD) spectra. They were recorded at room temperature using a Jobin–Yvon Mark III dichrograph equipped with a Nicolet 1171 signal averager. Rectangular cells with path-lengths of 0.5–1 cm were used to optimize the measuring conditions and to keep the absorbance below 1.4. CD is expressed in term of the molar dichroic absorbance, $\Delta\varepsilon$, based on the HSA concentration.

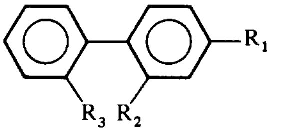
Spectropolarimetric titration. They were performed at wavelengths in the u.v range, at 285, 290, 260 and 275 nm for F1594, itanoxone, flurbiprofen and fenbufen respectively, and at 255 nm for F1803, F2006 and F1893. The ligands were added as microliter volumes of concentrated stock solutions in the cells containing the HSA solution.

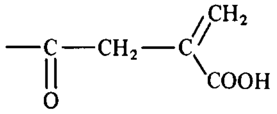
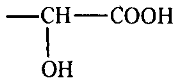
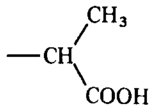
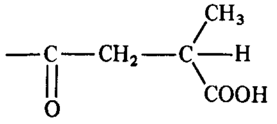
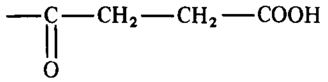
Fluorescence measurements. Dansylsarcosine was obtained from Sigma Chemical Company and warfarin from Merrell. The fluorescence of solutions containing probe (1.5 μM) and HSA (15 μM) was measured at room temperature before and after the addition of the compounds to concentrations of 15, 30 or 45 μM. The wavelengths of exciting light were 315 and 350 nm and the fluorescence was measured at 375 and 475 nm for warfarin and dansylsarcosine respectively. Fluorescence measurements were made in an Aminco SPF-500 spectrofluorometer.

Data analysis. The change in the CD difference spectrum at a given wavelength amounts to a measurement of the fractional saturation of HSA

Correspondence should be addressed to Dr. S. Urien or to Pr. J.-P. Tillement at Departement de Pharamacologie, Faculté de Médecine de Créteil, 8 rue de Général Sarrail, 94010 Créteil, France.

Table 1. Chemical structures of the studied compounds.



Compound	Substituents other than H atoms		
	R_1	R_2	R_3
Itanoxone	 $-C(=O)-CH_2-C(=CH_2)-COOH$		$-Cl$
F1803	 $-CH(OH)-COOH$		$-Cl$
F2006	$-CH_2-COOH$		$-Cl$
F1893	$-COOH$		$-Cl$
Flurbiprofen	 $-CH(CH_3)-COOH$	$-F$	
F1594	 $-C(=O)-CH_2-C(CH_3)(H)-COOH$		
Fenbufen	 $-C(=O)-CH_2-CH_2-COOH$		

with the ligand at a particular ligand concentration. The difference in the molar dichroic absorbance, $\delta(\Delta\varepsilon)$, is the sum of the contribution of the primary and secondary sites, if any. According to Roosdorp and Sjöholm [7], the general equation used is:

$$\delta(\Delta\varepsilon) = \Sigma e_i \,.\, X_i(T) \qquad (1)$$

where e_i is the proportionality constant and $X_i(T)$ is the bound ligand as a function of the total ligand concentration (T) for the ith class of binding sites. Using the Newton–Raphson algorithm method, the function $X_i(T)$ can be obtained by solving the equations:

$$X_i(T) = \frac{K_i \,.\, n_i\,(T - \Sigma\, X_j(T))}{1 + K_i\,(T - \Sigma\, X_j(T))} \qquad (2)$$

where K_i, n_i and R are the association constant, the number of binding sites and the total concentration of receptor respectively, and $\Sigma\, X_j(T)$ is the sum of the bound species to the different sites. The estimation of the binding parameters was then performed by fitting the data to equation (1) with an iterative non-linear regression program using a Gauss–Newton algorithm [8].

RESULTS

Identification of the binding sites.

All ligands investigated form complexes with HSA in such a way that the CD spectra of these complexes are substantially different from the spectrum of HSA alone. The binding of a drug to a protein generally induces new Cotton effects in the wavelength region where the drug exhibits absorption bands. The good correlation between the wavelength positions of the CD bands with maxima and shoulders of the u.v. difference spectra of each ligand (compare Fig. 1 to Figs 2 and 3) suggests that the observed Cotton effects are extrinsic in the origin [9].

Figure 2 shows the change produced by the binding of itanoxone to HSA. At the lower concentration, itanoxone is essentially bound to the primary site, inducing a positive Cotton effect at about 295 nm. When the concentration was increased to a 2- to a 3.66-fold molar excess, itanoxone was bound to a secondary site, giving a negative Cotton effect with maximum at 270 nm. The difference spectra of the F2006, F1803, F1893, F1594 derivatives and fenbufen–HSA complexes (Fig. 3) are quite distinct

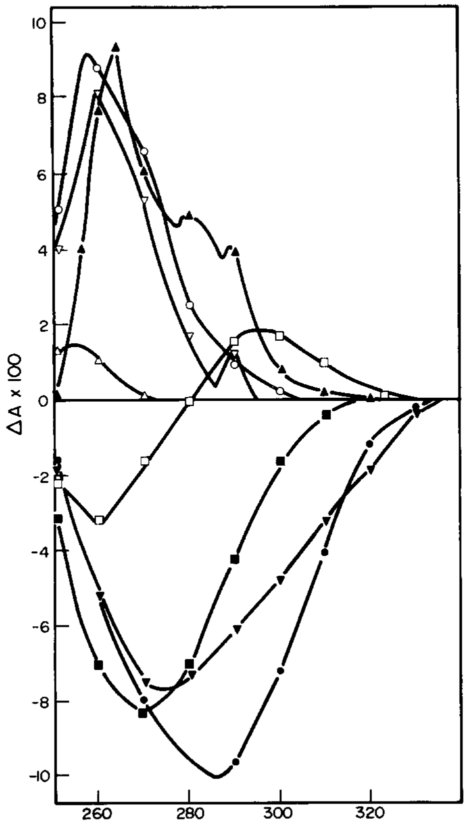


Fig. 1. Ultraviolet difference spectra generated on the binding of the following ligands to HSA: itanoxone at molar ratios of 1/1 (□) and 3/1 (■), and F2006 (○), F1893 (△), F1803 (▽), F1594 (●), fenbufen (▼) and flurbiprofen (▲) at molar ratios of 1/1. The HSA concentration was 15 μM in phosphate buffer, pH 7.4.

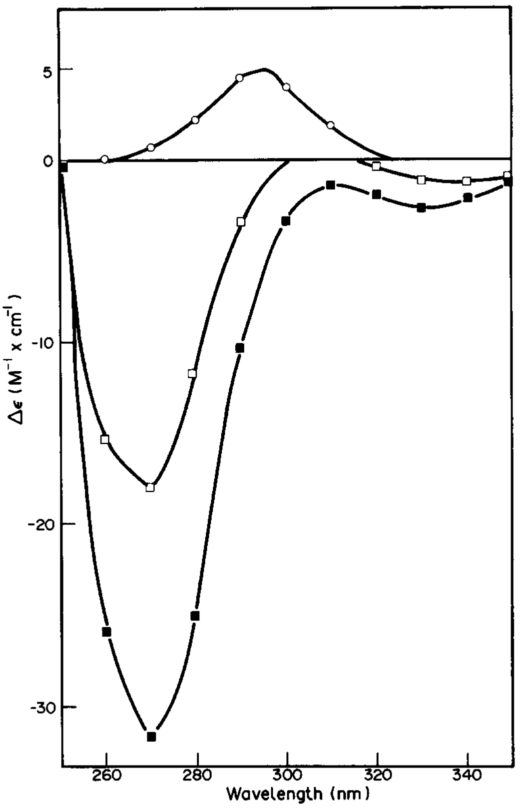


Fig. 2. Difference CD spectra of itanoxone-HSA 1/1 (○), 2/1 (□), 3.66/1 (■) after subtraction of the HSA spectrum. Experimental conditions: HSA 15 μM in phosphate buffer, pH 7.4, cell pathlength, 1 cm, room temperature.

from those observed with itanoxone. Below 300 nm, the Cotton effects induced by the halogenated compounds add positive band at about 255–260 nm to that of HSA (Fig. 3) and the difference CD spectra shapes are concentration-independent. On the other hand, the binding of the nonhalogenated compounds of the series, F1594 and fenbufen, induces optical activity with a large negative band at 280 nm and a small positive band at 320–330 nm, and the different CD spectra shapes were also concentration-independent.

To locate the itanoxone binding sites, three drugs, chlordiazepoxide, flurbiprofen and azapropazone, were used as probes. Their CD difference spectra when bound to HSA are depicted in Fig. 4. It has been demonstrated earlier that chlordiazepoxide and flurbiprofen bind to the diazepam site, while azapropazone binds to the warfarin binding area on HSA [1, 3]. Their association constants are high, in the range of 10^6 M^{-1} [3, 4]. All these probes affect the CD spectra obtained from the binding of itanoxone to HSA. Evidently, itanoxone does not show any positive band when added to HSA in equimolar amount or in a 3-fold excess, if flurbiprofen or chlordiazepoxide is present (Fig. 5). Comparing Fig. 2 to Fig. 5, it is obvious that the positive band induced by itanoxone at equimolar amount is blocked by either flurbiprofen or chlordiazepoxide. The strong negative band at 270 nm depicted in Fig. 5 originates from the interaction of itanoxone with its second site. At higher concentration of itanoxone (3-fold molar excess), the negative CD band from the second site will dominate completely. Consequently, one may assume that itanoxone shares its primary binding site with flurbiprofen and chlordiazepoxide. Correspondingly, Fig. 5 shows that azapropazone blocks the second binding site of itanoxone, since only the positive CD band originating from the primary site is induced at equimolar concentration of itanoxone. At higher concentration (3-fold molar excess), the negative CD band at 270 nm appears, indicating that a fraction of the added itanoxone bound to its secondary binding site by displacing azapropazone.

We identified the binding sites of the other compounds under study by fluorescence measurements. Figure 6 shows the effects of these compounds on the fluorescence of warfarin (marker for the azapropazone/warfarin binding site) and dansylsarcosine (marker for the benzodiazepine binding site). All the compounds caused a marked displacement of dansylsarcosine. By contrast, they did not involve any significant change in the HSA-bound

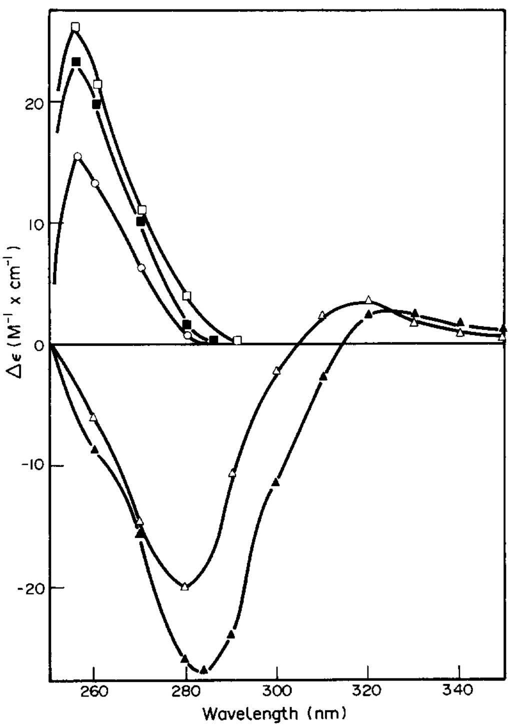


Fig. 3. Difference CD spectra of fenbufen-HSA (△), F1594-HSA (▲), F2006-HSA (□), F1803-HSA (○) and F1893-HSA (■) at molar ratios of 1/1 after subtraction of the HSA spectrum. The experimental conditions were the same as in Fig. 2.

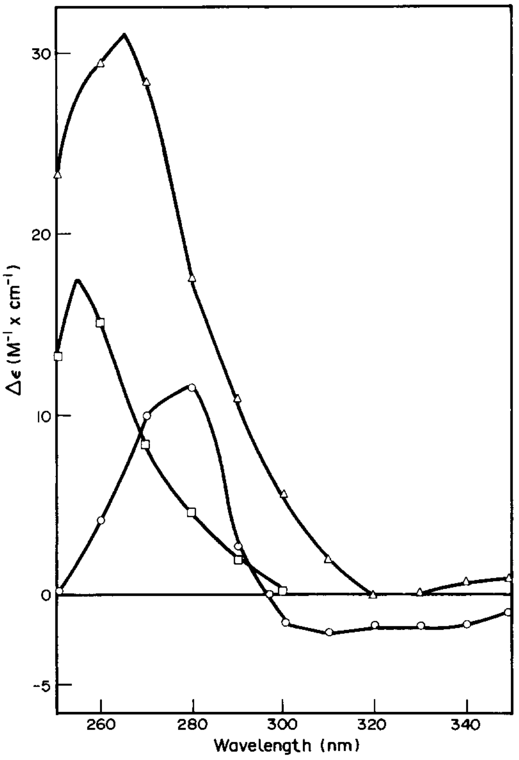


Fig. 4. Difference CD spectra of flurbiprofen-HSA (□), chlordiazepoxide-HSA (△) and azapropazone-HSA (○) at molar ratios of 1/1 after subtraction of the HSA spectrum. Experimental conditions as in Fig. 2.

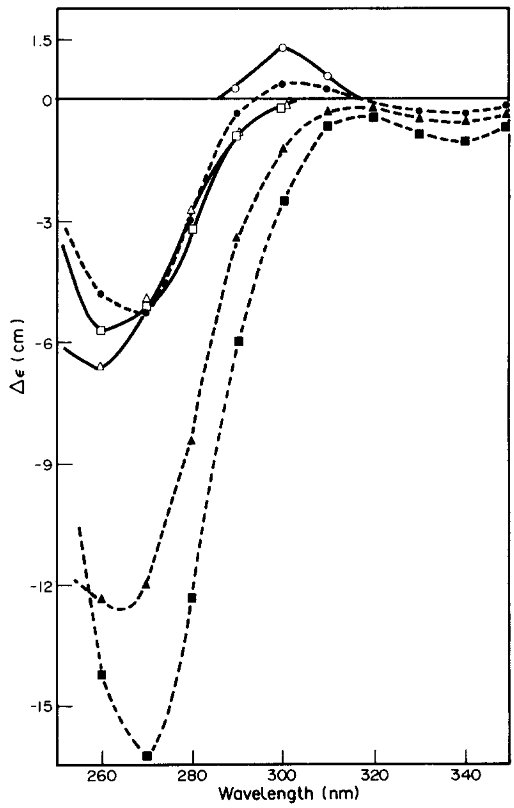


Fig. 5. The CD difference spectra obtained with itanoxone-HSA in the presence of the following binding sites probes: azapropazone (○●), chlordiazepoxide (□■) or flurbiprofen (△▲) after subtraction of the contribution from each corresponding probe-HSA spectrum. The molar ratios of itanoxone-probe-HSA were 1/1/1 (empty symbols, solid lines) and 3/1/1 (plain black symbols, dashed lines). The experimental conditions were the same as in Fig. 2. Scale setting, 2 × 10^{-6} mm^{-1}.

warfarin fluorescence, except itanoxone which caused a 25% fluorescence reduction at the final 3/1 concentration ratio of ligand to HSA.

Quantitative binding studies

Table 2 summarizes the binding parameters obtained for these compounds. The Cotton effects in-

Table 2. Binding characteristics for the studied compounds. In this table, the binding parameters of itanoxone interaction with its first binding site are given. All ligands except itanoxone bind to one type of site, which is common with the first binding site of itanoxone. The values in parenthesis are standard deviations

Compound	n	K (M^{-1} × 10^{-6})
Itanoxone	0.78 (± 0.05)	2.26 (± 1.22)
F 1803	0.78 (± 0.12)	0.93 (± 0.22)
F 2006	0.56 (± 0.02)	2.77 (± 0.73)
F 1893	0.83 (±0.04)	0.69 (± 0.13)
Flurbiprofen	0.68 (± 0.03)	3.46 (± 1.10)
F 1594	0.78 (± 0.09)	0.54 (± 0.27)
Fenbufen	1.05 (± 0.10)	0.19 (± 0.05)

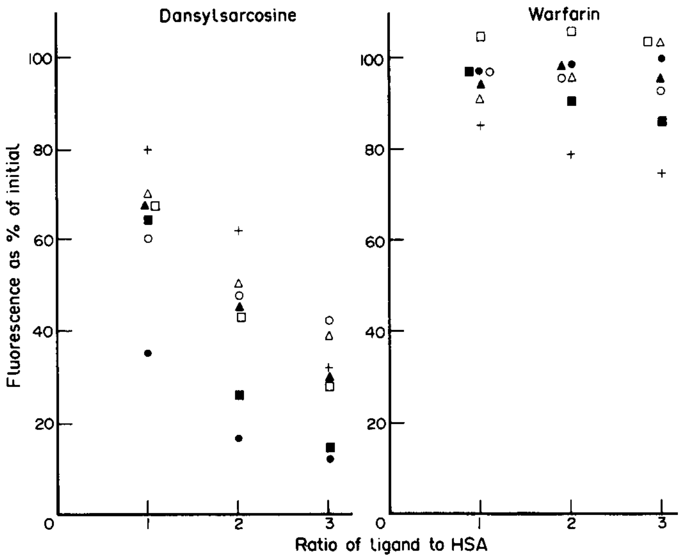


Fig. 6. Effects of the ligands on the fluorescence of probes selective for the azapropazone/warfarin binding site (warfarin) or the benzodiazepine binding site (dansylsarcosine). HSA was used at a concentration of 15 µM in phosphate buffer pH 7.4. Warfarin and dansylsarcosine concentrations were 1.5 µM. Fluorescence was measured at 475 or 375 nm with excitation at 350 or 315 nm for dansylsarcosine and warfarin respectively. Fluorescence is expressed as a percentage of that before addition of displacing drug. Itanoxone (+), F1893 (■), F2006 (□), F1803 (○), F1594 (▲), fenbufen (△) and flurbiprofen (●).

duced by the ligands were large enough to allow the estimation of the binding constants by a meaningful numerical analysis. The results demonstrate that all the ligands bind to one type of site except itanoxone which exhibits two successive binding processes (Fig. 7) corresponding to two separate binding classes. Comparing the ligands, they all exhibit a common structure consisting of two aromatic rings with or without one halogen substituent and a carbon side chain (R_1). So, for these ligands, we examined the relationships between the binding parameters and the atomic composition (C and O) of R_1 with reference to the presence or absence of one halogen substituent. A first statistical analysis of our data showed that the oxygen substituents impaired the binding, the effect of carbonyl oxygen being twice that of the hydroxyl oxygen, whereas carbon atoms of R_1 and halogen substituents improved the binding. Thus, multiple linear regression equations were derived relating primary association constants of the ligands to the R_1 number of carbons plus the number of halogens (positive contributions), and whether

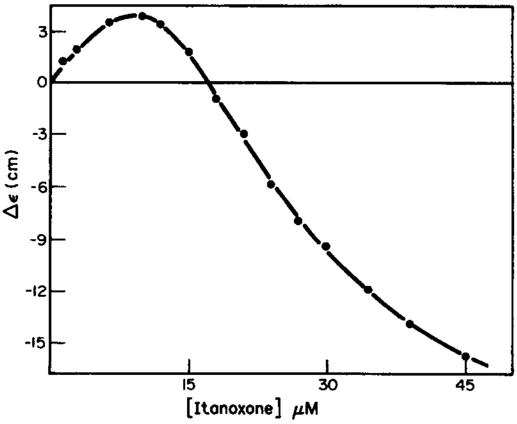


Fig. 7. Spectropolarimetric titration of HSA with itanoxone at 290 nm. The curve was drawn with the following parameters: $n_1 = 0.78$, $K_1 = 2.26 \times 10^6\,M^{-1}$, $e_1 = 0.89\,cm.M^{-1}$ and $n_2 = 0.93$, $K_2 = 0.102 \times 10^6\,M^{-1}$, $e_2 = -2.62\,cm.M^{-1}$. HSA 15 µM in phosphate buffer, pH 7.4, cell path-length, 1 cm, scale setting, $2 \times 10^{-6}\,mm^{-1}$.

Table 3. Regression statistics for the relationship between the affinity constant and the atomic composition of R_1 with reference to the presence or the absence of one halogen substituent. The ordinate intercept was not found significant. *r*: coefficient of correlation; *t* value: Student's *t*-test value obtained by dividing the partial regression coefficient by its corresponding standard error; P: probability, degree of significance

Variable number of	Partial regression coefficient	Standard error	*t* value	P value
(C + Cl or F)	0.825	0.095	8.684	<0.001
(>C=O + >C—OH)	−3.127	0.569	5.495	<0.01

Multiple $r = 0.930$.

they had either one carbonyl oxygen (= 1) or one hydroxyl oxygen (= 0.5) (negative contributions). The multiple regression (see Table 3) shows that the number of carbon or halogen atoms enhance the binding, while it is lessened by substituents with an oxygen atom.

DISCUSSION

All the compounds interact with HSA in such a way that large amplitude CD bands are generated. The mechanisms to be considered as the source of CD bands have been discussed in detail by Strickland [10]. Two main mechanisms were considered by Hood *et al.* [11] for CD bands of this size:

(a) immobilization of a chromophore within the molecule in a stable chiral conformation with CD generated by an intermolecular polarizability mechanism localized essentially within the chromophoric ligand;

(b) interaction of amino acid residues with the chromophores of the ligand, including the possibility of coupling between the strongly allowed electronic transitions of the chromophore and those of nearby amino acids.

From the CD difference spectra, it should be possible to identify the chomophores involved in the binding. For flurbiprofen, F1893, F2006 and F1803, the positive CD bands centered at 255 nm should originate from the $\pi \rightarrow \pi^*$ transitions of the substituted aromatic rings. For itanoxone interaction with its primary site, the positive CD band observed around 290 nm should be assigned mainly to a pertubation in the $n \rightarrow \pi^*$ transition of the R_1 carbonyl group, as reported earlier for the interaction of phenylbutazone with HSA [12]. Fenbufen, F1594 and itanoxone (interaction with its secondary site) give rise to large negative CD bands at 280 and 270 nm which may be ascribed to the $\pi \rightarrow \pi^*$ transitions of the aromatic rings. A characteristic feature of these three difference CD spectra is the small band around 315–320 nm. It is missing in the spectra obtained with the other compounds lacking the carbonyl group. Thus, this band might originate from the $n \rightarrow \pi^*$ transition of the carbonyl group of R_1, as shown earlier for some benzodiazepines [4].

We have demonstrated that all the compounds under study share one primary binding site on HSA, that is, the diazepam site. Evidently, the structure of the different binding sites of a macromolecule varies from site to site, meaning that the interaction of different sites with a ligand will lead to qualitative and quantitative differences in the binding. The asymmetric conformation of the site around the chromophore will be different, which may lead to different extrinsic Cotton effects [13].

Itanoxone gave rise to positive Cotton effects when bound to its primary site on HSA, while binding to the secondary site induced a strong negative Cotton effect. Similar findings have already been reported for the binding of oxyphenylbutazone [14] and of indomethacin [13]. In these two studies, the induced Cotton effects change sign when titrating HSA by these drugs. Consequently, the events taking place at a certain site may be investigated according to the different Cotton effects induced.

The specificity of at least two binding sites on HSA, namely the azapropazone/warfarin site [1, 15], also called site 1 [16], and the diazepam site [1, 15], also called site 2 [16], is now well-documented for anionic drugs. Azapropazone, and flurbiprofen and chlordiazepoxide are bound to just their primary site, the azapropazone/warfarin and diazepam sites respectively, when present in equimolar amounts relative to HSA. In this study, we have shown that these probes block the formation of the specific Cotton effects from interaction of itanoxone with its two specific binding sites on HSA.

The studies on displacement of fluorescent probes selective for the azapropazone/warfarin binding site, warfarin and the benzodiazepine binding site, dansylsarcosine [16], indicate clearly that the ligands bind to the benzodiazepine site, sharing the primary binding site of itanoxone. Using only fluorescence probe studies, it would have been a little doubtful whether itanoxone bound primarily to the benzodiazepine or to the azapropazone/warfarin binding site, since itanoxone is the sole ligand of the series that was capable to diminish the warfarin-bound fluorescence to a significant extent. These results agree with the structural features defined by Sudlow *et al.* [16] which allow ligand binding to one of these two specific sites:

(a) drugs which bind to the benzodiazepine site (site II) are aromatic carboxylic acids, largely ionized at physiological pH. The configuration of these molecules is generally extended and the negative charge is specifically located at one end of the molecule, away from the non polar region;

(b) drugs which bind to the azapropazone/warfarin binding site are more bulky heterocyclic molecules with the negative charge more centered in the molecule.

Since these ligands share one common binding site on HSA, we examined the relationships between the structures of these ligands and their binding parameters to HSA. For all the compounds, the estimation of the binding parameters gave a good fit with the model and program used. In the case of flurbiprofen, our results for the binding constants are close to those reported by Kober and Sjöholm [3]. The major observation of this study is the linear increase in the association constant (K) with increasing number of carbons of R_1. By contrast, the introduction in R_1 of hydrophilic substituents such as carbonyl or hydroxyl groups weakens the binding. Similar increases in the association constant with increasing chain length has been previously reported for fatty acids [5, 6]. It has been suggested that the primary HSA or BSA binding site for fatty acids of 8–10 carbons is the diazepam site, and that the lengthening of the carbon chain provides more efficient interactions with the binding site because the cationic and the hydrophobic areas of the site are separated by a distance such that a longer molecule may have more hydrophobic interactions [6]. Furthermore, the weakening of the binding by the introduction of hydrophilic substituents near the aromatic ring, where strong hydrophobic interactions are expected to take place, is in good agreement with this hypothesis.

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