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Circular Dichroism Simulation Shows a Site-II-to-Site-I Displacement of Human Serum Albumin-Bound Diclofenac by Ibuprofen

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ABSTRACT **Purpose:** The purpose of this study was to confirm the hypothesis that a site-II-to-site-I displacement takes place when some nonsteroidal anti-inflammatory drugs are displaced by another drug from their high-affinity binding site to a site of lower affinity on human serum albumin (HSA). **Methods:** Diclofenac, sodium salt, was used as a representative example because of its prominent reversal of the Cotton effect. Effects of site-specific drugs on the free fraction of diclofenac were determined by equilibrium dialysis, and effects on induced circular dichroism (CD) of diclofenac bound to HSA were studied by CD and CD simulation techniques. **Results:** Ibuprofen, a site-II-specific drug, altered the CD spectrum of the diclofenac-HSA complex at a molar ratio of 0.5 : 1 to that obtained at a higher ratio (5:1) without ibuprofen. The induced CD spectrum obtained in the presence of ibuprofen was very similar to one that assumed that all diclofenac displaced from its high-affinity binding site (site II) became rebound to a lower-affinity site (site I). The rebinding could be influenced by a free energy linkage between the two sites which would make site I (or parts thereof) more suitable for diclofenac binding. **Conclusion:** We have confirmed the existence of a site II-to-site I displacement, which is very striking and pharmacologically important, because the concentration of unbound drug being displaced is much lower than expected for a competitive mechanism.

KEYWORDS: Human serum albumin, Diclofenac, Ibuprofen, Site II-to-site I displacement, Circular dichroism simulation.

ABBREVIATIONS: HSA, human serum albumin; NSAID, nonsteroidal anti-inflammatory drug; CD, circular dichroism; ED, equilibrium dialysis; DNSA, dansyl-L-asparagine; DNSS, dansylsarcosine.

INTRODUCTION

The pharmacokinetic properties of exogenous and endogenous compounds can be influenced by reversible binding to human serum albumin (HSA), which is thought to be one of the primary determinants of the pharmacokinetic properties of drugs [1-4]. Therefore, when evaluating interactions between drugs, it is important to be aware of possible identities of their binding sites on the protein, because any alteration in drug-binding to HSA, including binding of the nonsteroidal anti-inflammatory drugs (NSAIDs), could lead to a change in pharmacokinetic properties. In a previous report, we proposed an interesting model for such interactions, which we referred to as site-to-site displacement. This model was based on the observation that carprofen, a carboxyl group containing site II-specific NSAID, underwent a site-II-to-site-I displacement when displaced from its high-affinity site to its low-affinity site in the presence of ibuprofen, another site-II-specific NSAID [5]. Although Chamouard *et al*. [6] reported a similar phenomenon on the basis of circular

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dichroism (CD) studies of the binding of diclofenac in the presence of another site II-specific drug, available CD simulation data do not confirm this phenomenon. A CD simulation technique was used to confirm the hypothesis that the reversal of the CD spectra of carprofen and diclofenac by ibuprofen was due to site-to-site displacement. Diclofenac was used as a representative example, because the reversal of the sign of the Cotton effect for this compound is prominent, and because a large difference exists between the high-affinity and low-affinity association constants.

MATERIALS AND METHODS

Materials

HSA (fraction V) was donated by the Chemo-Sera-Therapeutic Research Institute (Kumamoto, Japan) and was defatted with activated charcoal using the method originally described by Chen [7] but with some modifications as described previously [8]. Dansyl-L-asparagine (DNSA) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and dansylsarcosin (DNSS) was purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Ibuprofen (Kaken Pharmaceuticals Co., Tokyo, Japan), diclofenac, sodium salt, (Taiyo Pharmaceutical Industry, Takayama, Japan), and warfarin, potassium salt, (Eisai Co., Tokyo, Japan) were obtained as pure substances from their manufacturers. All other reagents were of analytical grade. The buffer was prepared with sodium phosphate dibasic and sodium phosphate monobasic salts.

CD measurements

CD measurements of the diclofenac-HSA complex at different drug-to-HSA ratios (0.2:1 to 5:1) (40 µM protein) were carried out on a Jasco J-600 spectropolarimeter (Tokyo, Japan), using a 10 mm cell at pH 7.4 and 25°C. Different ratios (0 to 5) of ibuprofen to HSA (40µM) were used to measure the effect of that drug on the induced CD spectra of the diclofenac-HSA system (0.5:1) at pH 7.4 and 25°C. In all cases the medium contained 67 mmol/L sodium phosphate as a buffer. The induced CD is defined as the CD of the drug-HSA mixture minus the CD of HSA alone at the same wavelength and is expressed as ellipticity in degrees.

Equilibrium dialysis (ED)

ED experiments were performed using a 2-mL Sanko plastic dialysis cell (Fukuoka, Japan). The two cell compartments were separated by a Visking cellulose membrane. To determine binding, aliquots (1.5 mL) of various ratios of drug-HSA mixtures (HSA, 40 µM; drug, 20-400 µM) were dialyzed at 25°C for 13 hours against the same volume of buffer solution (67 mmol/L sodium phosphate)., Adsorption of the drugs to the dialysis membrane and cell was insignificant at the concentrations used.. Further control experiments, using protein-free solutions, showed that the Visking membranes were fully permeable to the drugs, and that equilibrium was established within the designated period of time. After equilibrium was reached, the free concentrations of diclofenac and ibuprofen were determined by high-performance liquid chromatography (HPLC). The HPLC system consisted of a Hitachi 655A-11 pump and a Hitachi 655A variable UV monitor. A column of LiChrosorb RP-18 (Cica Merk, Tokyo, Japan) was used as a stationary phase for the analyses. The mobile phase consisted of acetonitrile and deionized water (65:35 vol/vol) with diclofenac and of acetonitrile and 30 mmol/L phosphate buffer, pH 7.4 (77:23 vol/vol) with ibuprofen.

ED was also used for drug displacement studies. In one type, the free fraction (%) of diclofenac (20 µM) added to HSA (40 µM), in the presence of ibuprofen ([ibuprofen]/[HSA] = 0-5), was determined in the absence and presence of warfarin (20 µM) or phenylbutazone (20 µM**).** In another type, the free fraction (%) of diclofenac (20 µM) added to HSA (40 µM), was determined after addition of warfarin or phenylbutazone ([warfarin]/[HSA] or [phenylbutazone]/[HSA] = 0-5).

Treatment of Data

Binding parameters were estimated by fitting a theoretical curve based on the following equation to the experimental data by using MULTI, a non-linear least squares computer program [9].

$$r = \frac{C_b}{P_t} = \sum_{i=1}^{j} \frac{n_i K_i C_f}{1 + K_i C_f} \qquad (1)$$

In this equation, r is the number of moles of drug bound per mole of protein. C_b and C_f are the bound and unbound drug concentrations, respectively, and P_t is the total protein concentration. K_i is the association constant and n_i is the number of binding sites for the *i*th class of binding sites.

A simple competition between two drugs for identical protein binding sites was analyzed using the methodology described by Kragh-Hansen [2].

CD simulation

The mathematical calculations for the CD simulation that were used in this study are given below.

$$\Theta_{obs} = C_1[\Theta_1] + C_2[\Theta_2] + C_3[\Theta_3] + C_4[\Theta_4] + C_5[\Theta_5] + C_6[\Theta_6]$$

$$= \sum_{i=1}^{j} C_i[\Theta_i] = C_h[\Theta_h] + C_l[\Theta_l] \qquad (2)$$

where Θ_{obs} is the total CD ellipticity, C_i and $[\Theta_i]$ are the concentration of bound drug and the molar ellipticity for the *i*th class of binding sites, respectively. $[\Theta_h]$ is the molar ellipticity at the high-affinity site, and $[\Theta_l]$ is the mean molar ellipticitiy at low-affinity sites, whereas C_h and C_l are the concentrations of the drug bound at the high- and low-affinity sites, respectively. For a theoretical calculation of C_h and C_l, the following equation was used.

$$r = \frac{n_h K_h C_f}{1 + K_h C_f} + \frac{n_l K_l C_f}{1 + K_l C_f} = \frac{C_b}{P_t} \qquad (3)$$

In Equation 3, n_h and n_l are the numbers of high and low affinity binding sites, and K_h and K_l are the high and low affinity association constants. In this relationship, $n_h K_h C_f / (1 + K_h C_f)$ represents C_h and $n_l K_l C_f / (1 + K_l C_f)$ represents C_l.

It is known that:

$$C_b = C_t - C_f \qquad (4)$$

where C_t is the total drug concentration.

Equation 3 can be transformed to

$$r = \frac{n_h K_h (C_t - C_b)}{1 + K_h (C_t - C_b)} + \frac{n_l K_l (C_t - C_b)}{1 + K_l (C_t - C_b)} = \frac{C_b}{P_t} \qquad (5)$$

After rearrangement, Equation 5 can be expressed as follows.

$$wC_b^3 + xC_b^2 + yC_b + z = 0 \qquad (6)$$

where

$$w = K_h K_l$$

$$x = -(n_h K_h K_l P_t + n_l K_h K_l P_t + 2K_h K_l C_t + K_h + K_l)$$

$$y = 2n_h K_h K_l P_t C_t + 2n_l K_h K_l P_t C_t + K_h K_l C_t^2 + n_h K_h P_t + n_l K_l P_t + K_h C_t + K_l C_t + 1$$

$$z = -(n_h K_h K_l P_t C_t^2 + n_l K_h K_l P_t C_t^2 + n_h K_h P_t C_t + n_l K_l P_t C_t)$$

Using MULTI, the bound concentration (C_b) can be calculated from Equation 6. Inserting the value for C_b into Equation 5 gives the values for C_h and C_l, because P_t and C_t are known. The values of C_h and C_l can be used in Equation 2. At a fixed drug-to-HSA molar ratio, it is possible to measure the observed ellipticity. Since the theoretical values of C_h and C_l are known, the values of Θ_{obs} for other drug-to-HSA ratios can be obtained for the corresponding wavelength. By entering two corresponding values of C_h, C_l and Θ_{obs} into Equation 2, $[\Theta_h]$ and $[\Theta_l]$, which are unknown, can be calculated for the corresponding wavelength. However, for accuracy, the mean values of $[\Theta_h]$ and $[\Theta_l]$ were obtained from the values of $[\Theta_h]$ and $[\Theta_l]$ derived from several drug-to-HSA ratios at the corresponding wavelength. Using the values for $[\Theta_h]$ and $[\Theta_l]$ and the values for C_h and C_l (from Equation 2) at the corresponding wavelength, a theoretical CD spectrum can be constructed. The values of $[\Theta_h]$ and $[\Theta_l]$ used are shown in **Table 1.**

In the case of displacement of two drugs binding to one site, the value of the concentration of bound drug (A_b) for the displaced one can be theoretically calculated in the following manner.

$$r_A = \frac{K_A A_f}{1 + K_A A_f + K_B B_f} = \frac{A_b}{P_t} \quad (7)$$

$$r_B = \frac{K_B B_f}{1 + K_B B_f + K_A A_f} = \frac{B_b}{P_t} \quad (8)$$

where K_A and A_f are the association constant and the free concentration, respectively, for drug A (displaced drug), whereas K_B and B_f are the association constant and the free concentration, respectively for drug B (displacing drug).

Equation 7 can be written as

$$r_A = \frac{K_A a}{1 + K_A a + K_B b} = \frac{A_t - a}{P_t} \quad (9)$$

where $a=A_t-A_b$.

Equation 8 can be written as

$$r_B = \frac{K_B b}{1 + K_B b + K_A a} = \frac{B_t - b}{P_t} \quad (10)$$

where $b=B_t-B_b$.

Equation 10 can be transformed to Equation 11.

$$b = \frac{K_A a^2 + ga - A_t}{K_B (A_t - a)} \quad (11)$$

where $g=K_AP_t-K_AA_t-1$.

Equation 10 can also be written as Equation 12

$$K_Bb^2+(K_BP_t-K_BB_t+K_Aa+1)b-B_t(1+K_Aa)=0 \quad (12)$$

Now, Equation 12 can be transformed by entering the expression for b

$$ha^4 + ia^3 + ja^2 + ka + 1 = 0 \quad (13)$$

Table 1. Values of [h] and [l] used for CD simulation

Wavelength (nm)	***Molar ellipticity (mdeg·cm^2/dmol)***	
	$[\Theta_h]$	$[\Theta_l]$
250	-1.35	9.59
255	-1.67	11.82
260	-2.23	15.77
265	-3.25	22.97
270	-4.77	33.76
275	-6.40	45.24
280	-6.91	48.84
285	-6.67	47.13
290	-5.19	36.69
295	-3.40	24.01
300	-1.17	8.24
305	0.41	-2.91
310	1.82	-12.86
315	2.26	-15.94
320	2.02	-14.23
325	1.41	-9.95
330	0.88	-6.17
335	0.46	-3.26
340	0.15	-1.03
345	0.00	0.00
350	0.00	0.00

where,

$$h = K_A K_B (g + K_A A_t - 1 - K_B P_t + K_B B_t - K_B B_t)$$

$$i = K_B g^2 + K_B (K_A A_t - K_B P_t + K_B B_t - 1) g - 2K_A K_B A_t + K_A K_B A_t (1 + K_B P_t - K_B B_t) + K_A K_B A_t - K_B^2 B_t (1 - 2K_A A_t)$$

$$j = K_B A_t (1 + K_B P_t - K_B B_t - 2) g - K_A K_B A_t^2 + K_B A_t (1 + K_B P_t - K_B A_t) - K_B^2 A_t B_t (K_A A_t - 2)$$

$$k = K_B A_t^2 \{1 - K_B B_t - (1 + K_B P_t - K_B B_t)\}$$

Since the values of h, i, j and k are constants, the value for A_b can be calculated from the value derived from Equation 13. In the case of displacement of drug A by drug B, A_b represents C_h plus C_l.

For simplification of the CD simulations, we assumed that one drug (A) is displaced by another drug (B) and that 100% of the displaced drug rebinds to its low-affinity site or sites. This means $C_h = A_b$ in the absence of displacer, and $C_l = A_b$ in the presence of the displacer. If no displacement occurs, C_l is equal to zero. However, given that displacement actually occurred, the value of Θ_{obs} was calculated from Equation 2, using $[\Theta_h]$ and $[\Theta_l]$ for the corresponding wavelength and the values of C_h and C_l. In later experiments, we can obtain theoretically simulated CD curves by plotting the Θ_{obs} values against corresponding wavelengths.

RESULTS AND DISCUSSION

Interaction of diclofenac with HSA

Identical concentrations of HSA were used for all the CD and ED experiments. The CD spectra of the complexes of different ratios of diclofenac and HSA are shown in **Figure 1.**

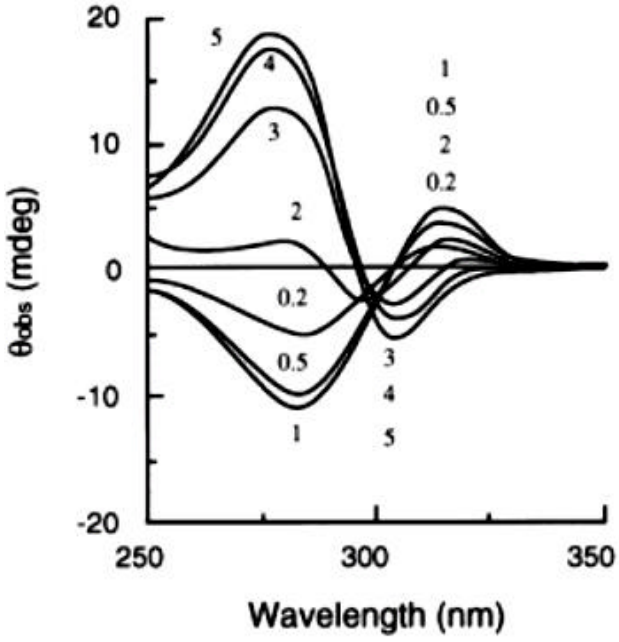

Figure 1. CD spectra of diclofenac-HSA complexes at different drug to HSA ratios at pH 7.4 and 25°C. The numbers indicate the molar ratio of drug to HSA (40 μ M).

Since diclofenac itself is not optically active, and HSA does not give rise to any Cotton effects at the wavelengths used, there can be no doubt that the observed Cotton effects are extrinsic in origin. In all cases, large amplitude CD bands were generated, when diclofenac had bound to HSA. At the lowest molar ratio of drug to HSA (0.2:1) when diclofenac was largely bound to its primary binding site, a negative band, centered at 282 nm, and a weaker positive band, centered at 315 nm, were induced. Also at slightly higher diclofenac-to-HSA ratios (0.5:1 and 1:1), strong negative Cotton effects were observed at 282 nm. In addition, the shapes of the CD spectra were qualitatively the same. However, at a higher ratio of diclofenac to HSA (2:1), a situation in which binding to the low-affinity sites also takes place, a dramatic change in the CD spectrum was generated, resulting in the production of a new positive band around 280 nm and a small negative band at 300 nm. The amplitude of the band at 315 nm was also reduced. At still higher ratios of diclofenac to HSA (3:1,4:1, and 5:1), the same pattern in the CD spectra was obtained, showing a positive maximum at about 280 nm and a negative maximum at about 310 nm. This unequivocally indicates that binding of diclofenac to its high and low affinity binding sites on HSA shows completely different Cotton effects due to differences in the spatial orientation in these sites.

The binding parameters, determined by ED, for the interaction of diclofenac with HSA are included in **Table 2**.

Table 2. Binding parameters for diclofenac and ibuprofen bound to HSA, as determined by equilibrium dialysis, at pH 7.4 and 250C

Drug	n_h	K_h (10^6 M^{-1})	n_l	K_l (10^4 M^{-1})
Diclofenac	1.0	3.3 ± 0.7	4.9 ± 0.2	5.4 ± 0.5
Ibuprofen	1.0	3.3 ± 0.4	4.8 ± 0.3	5.5 ± 0.2

The high-affinity binding site for diclofenac is placed in site II [10]. Fluorescence displacement experiments show the effect of diclofenac on the fluorescence of different site-specific probes. The drug caused a dramatic decrease in HSA-bound DNSS fluorescence and a weaker effect in the case of HSA-bound warfarin fluorescence (data not shown).

Site-to-site displacement of albumin-bound diclofenac

The effect of increasing the ibuprofen-to-HSA ratio on the CD spectrum of the diclofenac-HSA complex (0.5:1) is shown in **Figure 2.**

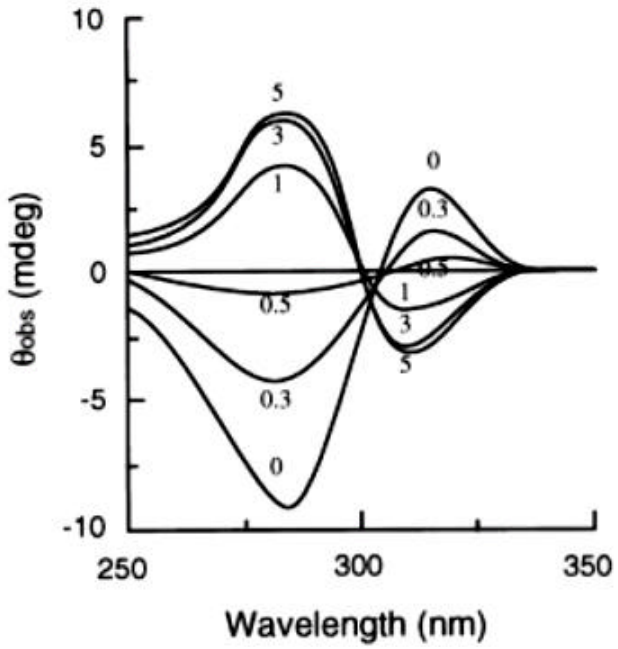

Figure 2. The effect of different concentrations of ibuprofen on the induced CD spectra of a diclofenac-HSA system (0.5: 1) at pH 7.4 and 25°C. The following concentrations were used: HSA, 40 μ M; diclofenac, 20 μ M. The ibuprofen to HSA molar ratios were 0, 0.3, 0.5, 1, 3, and 5.

In the presence of a high concentration of ibuprofen (ibuprofen:HSA 5:1), the sign of the Cotton effect at 290 nm was completely reversed. This spectrum is similar to those obtained for the diclofenac-HSA complexes (3:1 or 5:1) in the absence of ibuprofen (**Figure 1**). Ibuprofen itself showed no measurable CD spectrum under the present experimental conditions.

The effects of different concentrations of ibuprofen on the free fraction (%) of diclofenac-HSA at a molar ratio of 1:2, in the absence and presence of a constant concentration of warfarin or phenylbutazone, were determined by ED and are shown in **Figure 3**.

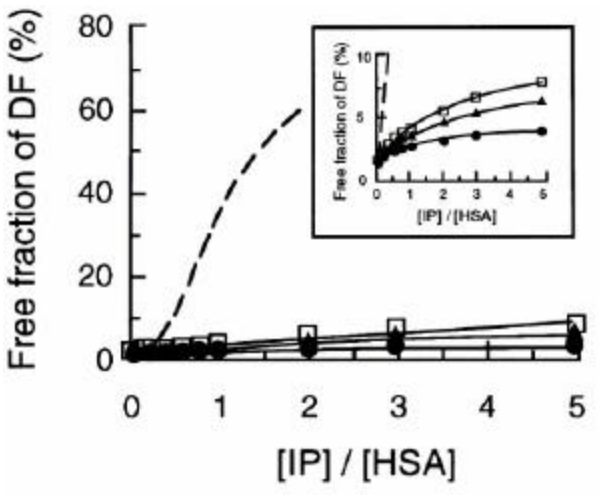

Figure 3. The free fraction (%) of diclofenac (DF) added to HSA (1:2) in the presence of different concentrations of ibuprofen (IP) in the absence (●) or in the presence of a constant concentration of warfarin (▲) or phenylbutazone (☐). (---):

A theoretical curve assuming a simple competition between diclofenac and ibuprofen at site II on HSA without rebinding of diclofenac to the low-affinity site(s). The following concentrations were used: HSA, 40μ M; diclofenac, 20 μ M; warfarin, 20 μ M and phenylbutazone, 20μ M.

In the absence of warfarin and phenylbutazone the free fraction of diclofenac increased from 0.80 % to approximately 3% with the gradual addition of ibuprofen (Insert, **Figure 3**). However, the experimentally determined free fractions were much lower than those calculated (broken curve) assuming simple competition between the two drugs for the same high-affinity site on HSA. This result suggested that diclofenac displaced from its high-affinity binding site could rebind to another site. **Figure 3** also shows that the free fractions of diclofenac caused by ibuprofen are higher when warfarin or phenylbutazone is present in a relatively low concentration.

The increments of the free fractions of diclofenac were higher in the presence of phenylbutazone than in the presence of warfarin (**Figure 3**). The same pattern was observed throughout the course of displacement. Basically the same effect was observed in the absence of ibuprofen (**Figure 4**).

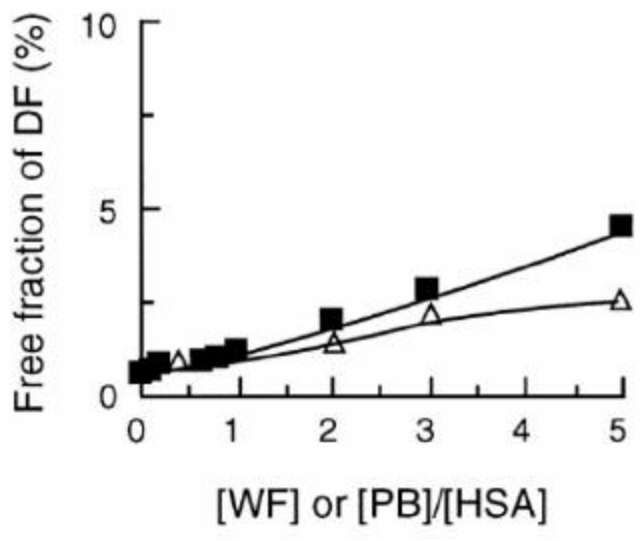

Figure 4. The free fraction (%) of diclofenac (DF) added to HSA (1:2) in the presence of different concentrations of warfarin (WF) (△) or phenylbutazone (PB) (■). The following concentrations were used: HSA, 40 μ M; diclofenac, 20 μ M.

Circular dichroism (CD) simulation

To further establish the validity of the site-to-site displacement (see Material and Methods for details), CD curves were constructed for the diclofenac HSA complex (0.5:1), in the presence and absence of ibuprofen, by using the observed ellipticity at both the high- and low-affinity sites ([Θ] multiplied by the respective concentration).

Figure 5 shows the results of the CD simulation. Curve a was obtained from the experimental data in the absence of ibuprofen, and curve b was obtained from this data in the presence of ibuprofen; curves a' and b' are the theoretical curves for the same conditions. Curve b" denotes the theoretical curve in the presence of ibuprofen, assuming no rebinding of displaced diclofenac.

CONCLUSION

The nonlinearity of the Scatchard plots (data not shown) suggested the presence of at least two classes of binding sites for diclofenac and ibuprofen on HSA. As shown in **Table 2**, diclofenac exhibited a high association constant (K_h=3.3 x 10^6 M^{-1}) for

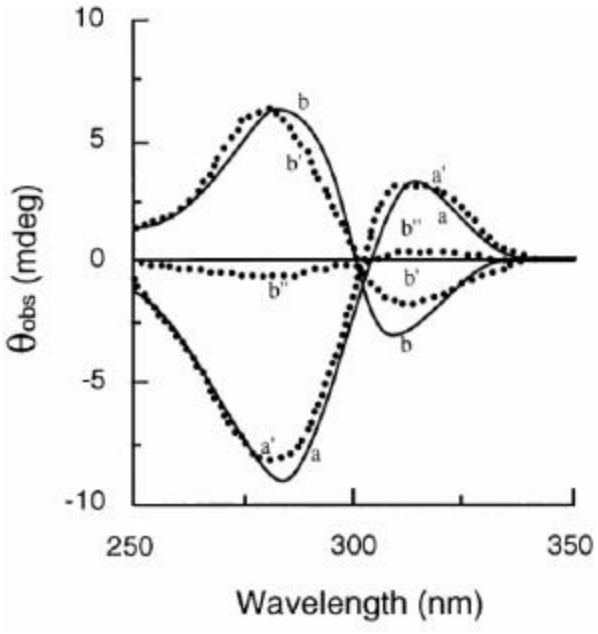

Figure 5. CD spectra of a diclofenac-HSA complex (0.5:1) in the presence and absence of ibuprofen at pH 7.4 and 25°C. Curves a and b (solid lines) are the experimentally obtained CD curves for the diclofenac-HSA system in the absence and presence of ibuprofen, respectively. Curves a' and b' (dotted lines) are the CD curves of the diclofenac-HSA system in the absence and presence of ibuprofen, respectively, as obtained from the theoretical values assuming a site-to-site displacement. Curve b" (dotted line) is the CD curve of the diclofenac-HSA system in the presence of ibuprofen as obtained from the theoretical values, assuming no site-to-site displacement. The following concentrations were used in the models: HSA, 40μ M; diclofenac, 20μ M; ibuprofen, 0μ M (a, a') and 200μ M (b,b',b").

the high-affinity site on HSA, similar to that of ibuprofen. Furthermore, diclofenac has a more than 10-fold lower association constant (K_l=5.4 x 10^4 M^{-1}) for the low-affinity sites, a finding that is in general agreement with the results reported earlier by Chamouard *et al.* [6]. Diclofenac, like carprofen and ibuprofen, binds to site II with a very high association constant.

The CD spectra of diclofenac bound to HSA at low and high ratios of drug to protein were very different. The single high-affinity binding site on HSA might almost totally contribute to the induced

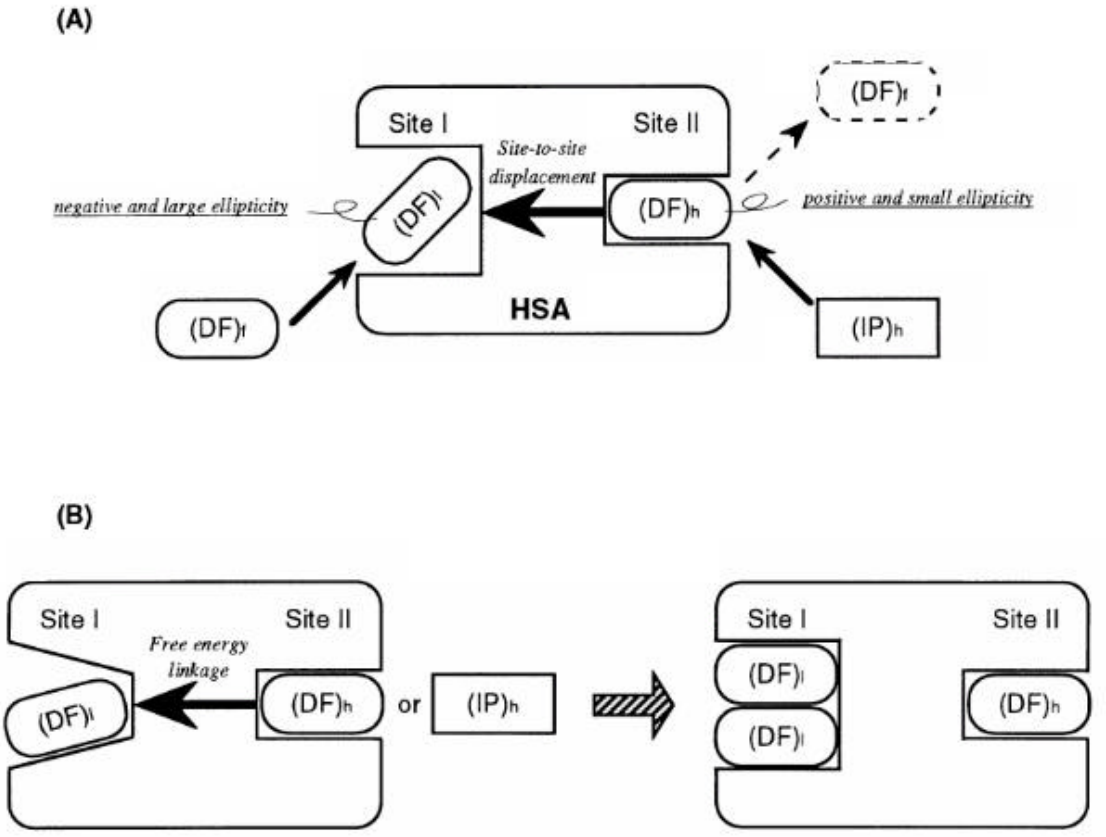

Figure 6. Proposed models for diclofenac-ibuprofen interaction on HSA. (A) : Site-to-site displacement from site II to site I. (B): Free energy linkage between the sites. (DF)h : diclofenac at high-affinity binding site on HSA, (DF)l : diclofenac at a low-affinity binding site on HSA, (IP)h : ibuprofen at high-affinity binding site on HSA, (DF)f: free concentration of diclofenac.

CD at a low ratio of drug to protein (1:2). However, at higher ratios several low-affinity binding sites also contribute to the extrinsic CD, and it may be that their contribution to CD could be larger than that of the high-affinity site. Ibuprofen altered the CD spectrum of the diclofenac-HSA complex (0.5:1) to those which were obtained at higher drug-to-HSA ratios (for example, 5:1) in the absence of ibuprofen. Results of the ED experiments were helpful in analyzing the mechanism of generation of these experimentally determined CD data. Although, the ED experiments were not as useful in explaining the CD data, their results can be used to make some predictions about site-to-site displacement. For example, at low ratios of drug to albumin, most of the diclofenac molecules might bind to the high-affinity site and ibuprofen could at first be bound to the free HSA molecules (**Figure 6A**).

The following ibuprofen molecules being bound displace the diclofenac bound to its high-affinity site and, finally , most of the displaced diclofenac molecules rebind to the low-affinity site. An additional displacement of diclofenac is observed in the presence of low concentrations of warfarin and phenylbutazone. The finding that phenylbutazone causes a larger displacement than warfarin might be due to the differences in association constants (warfarin:~2.7 x 10^5 M^{-1} < phenylbutazone:~1.2 x 10^6 M^{-1}). These results support the proposal that diclofenac is being rebound at site I.

A priori, it is also possible that the characteristic CD spectra of the diclofenac-HSA-ibuprofen system could be due to allosteric modifications introduced by the bound ibuprofen molecule. However, increases in temperature (data not shown) and pH (**Table 3**) failed to cause a significant change in the CD spectrum of the diclofenac-HSA complex, suggesting that the alteration in the CD spectra was not a result of microenvironmental changes in the diclofenac binding site on HSA.

Table 3. Effect of pH on observed ellipticity (Θ_{obs}) of albumin-bound diclofenac at the two maxima

pH	Q_{obs}	
	λ_{max}(282nm)	λ_{max}(315nm)
6.5	-11.5	4.97
7.0	-11.1	4.98
7.4	-11.1	4.95
8.3	-10.8	4.85

It is also possible that the diclofenac molecule bound to site II on HSA could form a complex (a diclofenac-HSA-ibuprofen ternary complex or a diclofenac-HSA-diclofenac ternary complex) with another diclofenac or ibuprofen molecule through stacking or electron transfer. However, this is not possible for diclofenac and ibuprofen to simultaneously bind to HSA, because they have a common binding site. In addition, according to spectrophotometric studies performed at 278 nm, no self-association of diclofenac takes place in aqueous solution (without HSA) under the study conditions because the absorbancy coefficient (8.3×10^3 $M^{-1} \times cm^{-1}$) was found to be constant in the concentration range 2×10^{-6} M - 1×10^{-4} M.

To confirm our hypothesis that the reversal of the CD spectra of diclofenac bound to HSA in the presence of ibuprofen was due to site-to-site displacement, we used a CD simulation technique. **Figure 5** compares the experimentally determined CD curves with the curves based on theoretical values, obtained using the CD simulation technique. Curve a (solid line) is the CD curve for the diclofenac-HSA complex at a 5:1 drug-to-HSA ratio. Curve a' (dotted line) represents a CD curve obtained from the theoretical values. When the effect of ibuprofen, as a displacer, was taken into consideration, curve b (solid line) was obtained from the experimental data. The theoretical values elicited curve b' (dotted line), which closely resembles the curve derived from the experimental data, further suggesting that the reversal of the Cotton effect sign in the presence of ibuprofen is very likely due to a site-to-site displacement of diclofenac by ibuprofen. If there had been no rebinding of diclofenac, the theoretically calculated CD curve would resemble curve b", which is very different from curve b. These data further support the hypothesis that diclofenac shows site-to-site displacement in the presence of ibuprofen.

Although it is possible that all of the displaced drug from site II simply rebinds to another site, our proposal might be a little too simple, because the rebinding could be affected by a free energy linkage between ibuprofen bound to site II and diclofenac bound to site I. The free energy linkage between the two sites could result in conformational changes in site I (or parts thereof), rendering it more suitable for diclofenac binding (**Figure 6B**) (for reviews on this topic, see [2,3]). A finding supporting this proposal is that ibuprofen causes an enhancement of the binding of the site I-drug warfarin [12]. However, this hypothesis based upon the free energy linkage cannot alone explain the CD data. Therefore, it is reasonable to hypothesize that site-to-site displacement from site II to site I as well as free energy linkage between the sites caused the CD spectra. However, we cannot clarify the relative magnitudes of the above factors in detail from these limited data.

For drugs such as diclofenac and carprofen, whose binding to HSA is in excess of 99%, an increase in the free fraction of only 1% doubles the amount of drug available for pharmacological activity. Therefore, a quantitative change of even 1% in binding can be expected to have an effect on its disposition. Thus, care should be exercised in calculating the free concentration of drugs that show site-to-site displacement in the presence of other drugs, since the results can be misleading.

In conclusion, we have confirmed the existence of a significant siteII-to-siteI displacement, which is pharmacologically important. When studying drug-drug interactions, more specifically drug displacements, the possibility of site-to-site displacement should be considered, because it can

cause significant differences between the free concentrations of a displaced drug. Although the results presented here are significant, a more detailed study, including in vivo experiments, is needed to predict the actual changes in pharmacokinetic properties caused by this site-to-site displacement.

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