Biochemical Pharmacology, Vol. 46, No. 10, pp. 1733–1740, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

STUDY OF INTERACTION OF CARPROFEN AND ITS ENANTIOMERS WITH HUMAN SERUM ALBUMIN—II

STEREOSELECTIVE SITE-TO-SITE DISPLACEMENT OF CARPROFEN BY IBUPROFEN

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(*Received* 11 *May* 1993; *accepted* 12 *August* 1993)

Abstract—The site-to-site displacement of carprofen, a site II-specific drug, bound to human serum albumin (HSA) by ibuprofen, another site II-specific drug, was qualitatively and quantitatively studied by circular dichroism (CD) and equilibrium dialysis (ED). Carprofen gives rise to different CD spectra at lower (1:1) and higher (3:1) molar ratios to HSA, indicating different mechanisms for the binding of this drug to its high and low affinity sites on HSA. Ibuprofen at a 5:1 molar ratio to HSA displaces carprofen at a molar ratio of 1:1 to HSA from its high affinity binding site (site II) to its low affinity site (site I), as shown by production of the CD spectrum similar to that obtained in the case of the carprofen–HSA complex at a molar ratio 3:1. As revealed by the ED experiments, the free fraction of carprofen at a molar ratio of 1:2 to HSA (2×10^{-5} M) was not initially increased by the addition of ibuprofen at a lower concentration, but at a higher concentration (6×10^{-5} M), the free fraction was increased by only 90%. When site I was sufficiently blocked by a site I-specific drug like warfarin or phenylbutazone (6×10^{-5} M), there was about a 4-fold increase in the free fraction of carprofen caused by ibuprofen. This site-to-site displacement demonstrated by carprofen was found to be stereospecific as indicated by the highest interaction between the *S*(+)-enantiomers of carprofen and ibuprofen. Moreover, the displacement of carprofen occurred at the azapropazone region rather than the warfarin region of site I on HSA.

The pharmacokinetic properties of exogenous as well as endogenous compounds can be influenced by binding to human serum albumin (HSA†) in a reversible manner [1, 2]. To evaluate interactions between drugs, it is essential to be able to identify binding sites [3]. Non-steroidal anti-inflammatory drugs (NSAIDs) are distinguished as a class by the high degree to which they bind to plasma protein, especially albumin. Plasma protein binding properties are considered to be the primary determinants of the pharmacokinetic properties of the NSAIDs. Therefore, any alteration or change in the HSA binding of NSAIDs might lead to a change in the pharmacokinetic properties of the NSAIDs.

As previously shown [4], carprofen binds with a high association constant ($K_1 > 10^6\,M^{-1}$) but low capacity to site II (benzodiazepine site) and with a low association constant ($K_2 > 10^5\,M^{-1}$) but high capacity to site I (warfarin site) on HSA. In that study we further showed that carprofen at a lower ratio to HSA (1:1) gave the circular dichroism (CD) spectrum with three positive maxima at 255, 295 and 345 nm, whereas at a higher ratio (3:1), it gave a completely different CD spectrum having two big negative maxima at 255 and 305 nm, in addition to the positive Cotton effect at 345 nm. This suggests that the induced Cotton effects due to carprofen binding to HSA cannot be explained on the basis of a single mechanism. The primary binding might give a positive Cotton effect, whereas strong negative Cotton effects are probably due to the binding of carprofen to its low affinity site on HSA. Due to this, the determination of binding parameters of carprofen to HSA by CD was not feasible. Furthermore, our preliminary investigation revealed that in the presence of ibuprofen, another site II-specific drug, carprofen even at a lower ratio to HSA (1:1) gave a CD spectrum similar to that obtained at a higher ratio in the absence of ibuprofen. This suggests that carprofen even in a lower ratio to HSA (1:1) can bind to its lower affinity site after being displaced by another site II-specific bound drug like ibuprofen, and this unusual displacement has been tentatively referred to as a site-to-site displacement phenomenon. This finding leads us to investigate this overall unusual site-to-site displacement phenomenon of carprofen by ibuprofen by CD and equilibrium dialysis (ED) using different site-specific drugs/probes. We have also tried to show whether stereospecificity plays a role in such interactions and thus the pharmacokinetic implications.

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† Abbreviations: NSAID, non-steroidal anti-inflammatory drug; HSA, human serum albumin; CD, circular dichroism; ED, equilibrium dialysis.

MATERIALS AND METHODS

Materials

HSA (fraction V, lot no. 36F-9333) was donated

by the Chemo-Sera-Therapeutic Research Institute (Kumamoto, Japan). The M_r of HSA was assumed to be 66,500 and it gave only one band in SDS–PAGE. Carprofen and its enantiomers were used as supplied by the manufacturer, Nippon Roche K.K. (Tokyo, Japan), and were at least 99% pure. Drugs for the displacement investigations, sodium warfarin (Eisai Co., Tokyo, Japan), phenylbutazone (Ciba-Geigy Co., Summit, NJ, U.S.A.) and ibuprofen and its enantiomers (Kaken Pharmaceutical Co., Tokyo, Japan), were used as supplied by the manufacturers. All other materials were of reagent grade and solutions were prepared in deionized and distilled water.

Methods

All solutions were prepared in 0.067 M phosphate buffer at pH 7.4 and 25 ± 1°.

CD. CD spectra were measured on a Jasco J-600 spectropolarimeter (Tokyo, Japan). CD spectra of carprofen and its enantiomers (30 μM) bound to HSA (30 μM) were measured using a 10 mm cell at 25°. Ibuprofen (30–150 μM) was used in the displacement of carprofen bound to HSA. The induced ellipticity was defined as the ellipticity of the drug–HSA mixture minus the ellipticity of HSA alone at the same wavelength and was expressed in degrees.

ED. ED experiments were performed with Sanko plastic dialysis cells (Fukuoka, Japan). The two cell compartments were separated by Visking cellulose membrane with the M_r cut off at 6000. ED displacement experiments were carried out under different conditions. In one case, HSA (20 μM, 1.5 mL) was dialysed against equal volumes of carprofen (10 μM) without or with addition of site-selective displacers (5–60 μM). In another case, 1.5 mL of HSA (20 μM) plus warfarin/phenylbutazone (60 μM) was dialysed against the same volumes of carprofen (10 μM) with or without the addition of ibuprofen (5–60 μM). Adsorption of any drugs onto membrane or apparatus was negligible, since no adsorption was detected by measuring the drug concentrations of both compartments in the ED experiments without HSA. After 12 hr of dialysis at 25°, in all cases, the concentration of free carprofen was determined by HPLC. The HPLC system consisted of a Hitachi 655A-11 pump and Hitachi F1000 variable wavelength fluorescence monitor. A column of LiChrosorb RP-18 (7 μm) (Cica Merck, Tokyo, Japan) was used as the stationary phase. The mobile phase consisted of acetonitrile (A) and deionized water (B) (A:B, 35:65, v/v). The detector was set at 290 and 365 nm for excitation and emission wavelength, respectively. The sample was injected onto the column with a 250 μL loop. The flow rate was 1 mL/min.

The binding parameters of ibuprofen and its enantiomers bound to HSA were estimated by ED. Briefly, aliquots of various ratios of ibuprofen and its enantiomers (10–500 μM) bound to HSA (50 μM) (1.5 mL) were dialysed at 25° for 13 hr against the same volumes of phosphate buffer solution (pH 7.4). In the preliminary experiments, the adsorption of ibuprofen onto the membrane was found to be negligible. After equilibrium was reached, the free concentration of ibuprofen was determined by HPLC. The HPLC system consisted of a Hitachi 655A-11 pump and a Hitachi 665A variable wavelength UV monitor. A column of LiChrosorb RP-18 (7 μM, Cica Merck, Tokyo, Japan) was used as the stationary phase. The mobile phase consisted of acetonitrile (A), deionized water (B) and phosphoric acid (C) (A:B:C, 275:225:05). The detector was set at 220 nm with a sensitivity of 0.005 absorbance units full scale. The sample was injected onto the column with a 20 μL loop. The flow rate was 1 mL/min.

Data treatment. Binding parameters were estimated by fitting the experimental data to the following equation using a non-linear least squares computer program (MULTI program) [5]:

$$r = \frac{[D_b]}{[P_t]} = \sum_{i=1}^{m} \frac{n_i K_i [D_f]}{1 + K_i [D_f]} \quad (1)$$

where r is the number of moles of bound drug molecules per mole of protein. $[D_b]$ and $[D_f]$ are the bound and unbound drug concentration, respectively, and $[P_t]$ is the total protein concentration. K_i and n_i are the association constant and the number of binding sites for the ith class of binding sites, respectively.

A simple competition between two drugs A and B for identical protein binding sites was analysed by the following equations [6]:

$$\frac{[P_A]}{[P_t]} = \frac{K_a[A_f]}{1 + K_a[A_f] + K_b[B_f]} \quad (2)$$

$$\frac{[P_B]}{[P_t]} = \frac{K_b[B_f]}{1 + K_b[B_f] + K_a[A_f]} \quad (3)$$

$$[A_t] = [P_A] + 2[A_f] \quad (4)$$

$$[B_t] = [P_B] + 2[B_f] \quad (5)$$

where: K_a = association constant for drug A, K_b = association constant for drug B, $[A_f]$ = concentration of free drug A, $[B_f]$ = concentration of free drug B, $[P_A]$ = concentration of bound drug A, $[P_B]$ = concentration of bound drug B, $[A_t]$ = total concentration of drug A, $[B_t]$ = total concentration of drug B, $[P_t]$ = total concentration of protein. Because $[P_t]$, K_a, K_b, $[A_t]$ and $[B_t]$ are known, it is possible to calculate theoretically $[A_f]$, $[P_A]$, $[B_f]$ and $[P_B]$ from Eqns 2–5. Thus the theoretical value of free fractions of compound A (f_a) and compound B (f_b) can be calculated as follows:

$$f_a(\%) = \frac{[A_f]}{[P_A] + [A_f]} \times 100 \quad (6)$$

$$f_b(\%) = \frac{[B_f]}{[P_B] + [B_f]} \times 100. \quad (7)$$

RESULTS

The chemical structures of the compounds used in this study are shown in Fig. 1.

Binding parameters

The binding parameters of ibuprofen bound to

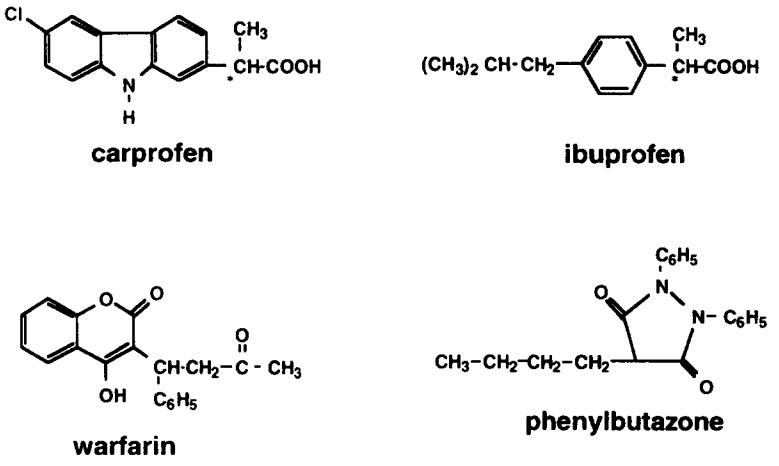

Fig. 1. Chemical structures of the compounds used in this study. *Chiral centre.

Table 1. Binding parameters of ibuprofen bound to HSA as measured by ED at 25° and pH 7.4

Ibuprofen	n_1	K_1 ($\times 10^6$ M^{-1})	n_2	K_2 ($\times 10^5$ M^{-1})
RS(±)	1.1 ± 0.1	2.5 ± 0.2	4.2 ± 0.3	1.5 ± 0.1
S(+)	1.2 ± 0.1	2.0 ± 0.2	4.5 ± 0.4	1.8 ± 0.1
R(−)	1.4 ± 0.1	3.0 ± 0.2	4.7 ± 0.2	1.9 ± 0.2

Each value is the mean ± SD of data from three experiments.

HSA as determined by ED are summarized in Table 1. Two successive saturable processes were obtained for the binding of ibuprofen to HSA. As can be seen in Table 1, the high affinity association constant of *R*(−)-ibuprofen was slightly higher than that of its corresponding antipode.

Interaction studied by CD

CD spectra of the carprofen–HSA complex at different drug to HSA (30 μM) ratios, as shown in our previous paper [4], indicated that carprofen at the ratio 1:1 to HSA produced positive Cotton effects only with the maxima at 255, 295 and 345 nm, whereas at the ratios 3:1 and 5:1, in addition to the positive Cotton effect at around 345 nm, two large negative Cotton effects with the maxima at 255 and 305 nm were also observed. Upon the addition of ibuprofen (ibuprofen:HSA, 1:1), the signs of the Cotton effects of the CD spectrum of the carprofen–HSA complex at the ratio of 1:1 were almost reversed as indicated in Fig. 2. After further addition of ibuprofen (ibuprofen:HSA, 5:1), the carprofen–HSA complex (1:1) showed a CD spectrum similar to that obtained for the carprofen–HSA complex (3:1) in the absence of ibuprofen (Fig. 2).

Ibuprofen did not generate any measurable extrinsic Cotton effects with HSA at a wavelength longer than 255 nm under the given experimental conditions. In order to elucidate the mechanism of the sign changes of the extrinsic Cotton effects of the carprofen–HSA system, various effects on the induced Cotton effects of the carprofen–HSA (1:1) system were examined. With the rise of pH from 6.5 to 8.5, the observed ellipticity due to the high affinity binding site was found to be unaffected.

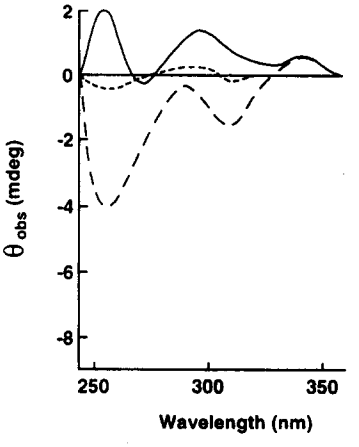

Fig. 2. Effect of ibuprofen on the induced CD spectra of the carprofen–HSA system (1:1) at pH 7.4 and 25°. Ibuprofen to HSA (30 μM) ratios 0 (——), 1.0 (----), 5.0 (——) were employed.

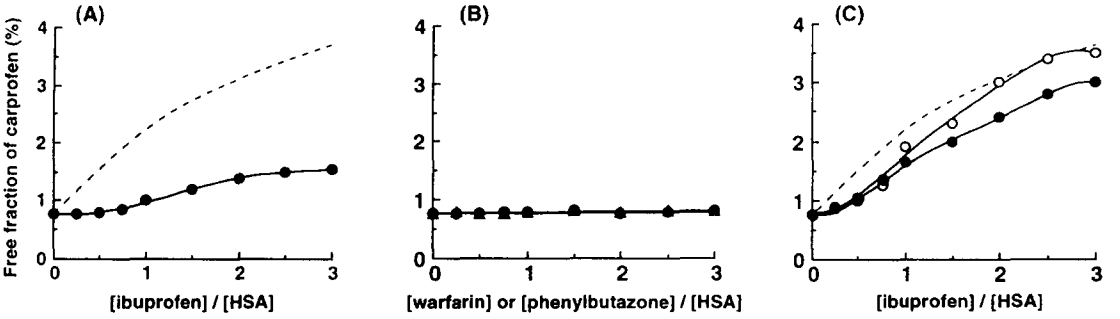

Fig. 3. Free fraction (%) of carprofen bound to HSA (1:2) in the presence of ibuprofen (A), warfarin (●) or phenylbutazone (▲) (B), and ibuprofen–warfarin (●) or ibuprofen–phenylbutazone (▲) (C). (----) Theoretical curve assuming competition between carprofen and ibuprofen. The following concentrations were used: HSA, 20 μM (A, B and C); carprofen, 10 μM (A, B and C); ibuprofen, 5–60 μM (A, C); warfarin or phenylbutazone, 5–60 μM (B) and 60 μM (C). Each value represents the average of three experiments ± SD (within the symbol). At 1:1 or a higher ratio of ibuprofen to HSA, the greater increase in the free fraction of carprofen in the presence of phenylbutazone was statistically significant ($P < 0.01$) compared to that obtained in the presence of warfarin.

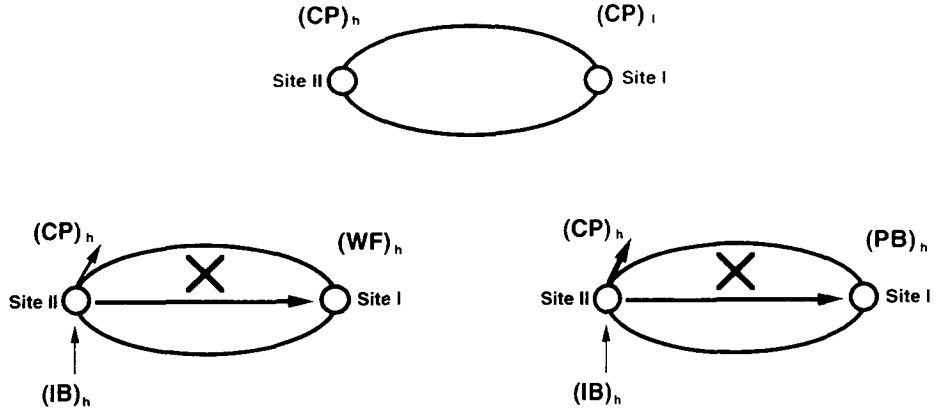

Fig. 4. Proposed models of the carprofen–HSA–ibuprofen interactions in the presence and absence of warfarin/phenylbutazone.

Temperature also had no effect on the CD spectrum of the carprofen–HSA system (data not shown).

Interaction studied by ED

Figure 3 shows the change in free fractions of carprofen (10 μM) bound to HSA (20 μM) in the presence of different site-specific drugs, ibuprofen, warfarin and phenylbutazone. As observed in Fig. 3, the free fraction of carprofen was not changed by the addition of ibuprofen up to 10 μM; however, by the addition of further ibuprofen (60 μM), the free fraction of carprofen was increased from 0.8% to 1.5%. Warfarin or phenylbutazone (60 μM) showed no influence on the free fraction of carprofen bound to HSA in the ratio 1:2. However, when so-called site I was blocked by a sufficient amount (60 μM) of either warfarin or phenylbutazone, ibuprofen (60 μM) increased the free fraction of carprofen bound to HSA (1:2) from 0.75% to 2.8% and 3.5%, respectively. The free fraction of carprofen was increased to a greater extent when site I was blocked by phenylbutazone than when by warfarin. Figure 4 shows the proposed models of the carprofen–warfarin–ibuprofen and carprofen–phenylbutazone–ibuprofen interactions when they simultaneously bind to HSA.

Stereospecific interaction of carprofen with ibuprofen

Effect of ibuprofen on the CD of carprofen and its enantiomers bound to HSA. The relative CD intensities (%) of carprofen and its enantiomers bound to HSA were increased to various extents upon the addition of ibuprofen as indicated in Fig. 5. The relative CD intensity of the *S*(+)-carprofen–HSA complex was increased to the highest extent (145%) by ibuprofen, followed by the CD intensity of the *RS*(±)-carprofen–HSA system (120%). There was no visible increase in the CD intensity of the *R*(−)-carprofen–HSA system upon the addition of ibuprofen.

Effect of ibuprofen and its enantiomers on the CD of the S(+)-carprofen–HSA complex. On the basis of the results obtained in Fig. 5, effects of ibuprofen and its enantiomers on the relative CD intensity of

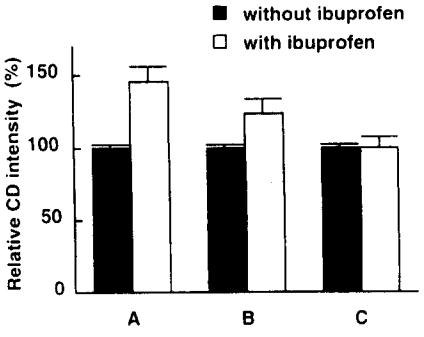

Fig. 5. Effects of ibuprofen on the relative CD intensity (%) of carprofen and its enantiomers bound to HSA (1:1) at 255 nm. (A) *S*(±)-Carprofen–HSA system, (B) *RS*(±)-carprofen–HSA system, (C) *R*(−)-carprofen–HSA system. The following concentrations were used: HSA, 30 μM; carprofen, 30 μM; ibuprofen, 150 μM. Each column and bar represents the mean ± SD of data from three experiments.

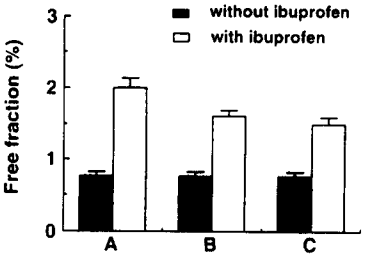

Fig. 7. Effects of ibuprofen on the free fraction (%) of carprofen and its enantiomers bound to HSA (1:2) as determined by ED at pH 7.4 and 25°. (A) *S*(+)-Carprofen–HSA system, (B) *RS*(±)-carprofen–HSA system, (C) *R*(−)-carprofen–HSA system. The following concentrations were used: HSA, 20 μM; carprofen, 10 μM; ibuprofen, 60 μM. Each bar and column represents the mean ± SD of data from three experiments.

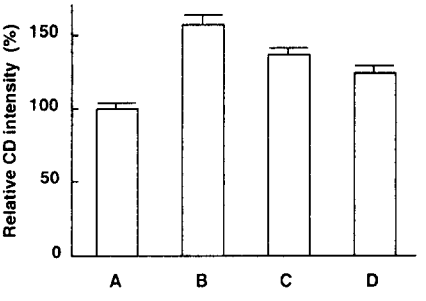

Fig. 6. Effects of ibuprofen and its enantiomers on the relative CD intensity (%) of the *S*(+)-carprofen–HSA system (1:1) at 255 nm. (A) Without ibuprofen or its enantiomers, (B) with *S*(+)-ibuprofen, (C) with *RS*(±)-ibuprofen, (D) with *R*(−)-ibuprofen. Same concentrations as in Fig. 5 were used. Each column and bar represents the mean ± SD of data from three experiments.

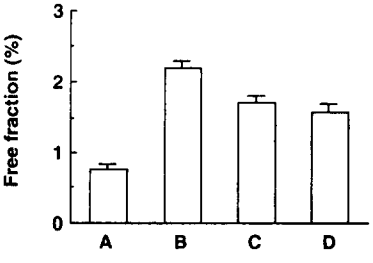

Fig. 8. Effects of ibuprofen and its enantiomers on the free fraction (%) of *S*(+)-carprofen bound to HSA (1:2) as determined by ED at pH 7.4 and 25°. (A) Without ibuprofen or its enantiomers, (B) with *S*(+)-ibuprofen, (C) with *RS*(±)-ibuprofen, (D) with *R*(−)-ibuprofen. Same concentrations as in Fig. 7 were used. Each bar and column represents the mean ± SD of data from three experiments.

the *S*(+)-carprofen–HSA complex were studied. As shown in Fig. 6, the relative CD intensity of the *S*(+)-carprofen–HSA system, considered as 100%, was increased to the following extents upon the addition of ibuprofen and its enantiomers: *S*(+)-ibuprofen (156%) > *RS*(±)-ibuprofen (140%) > *R*(−)-ibuprofen (125%).

Effect of ibuprofen on the free fraction of carprofen and its enantiomers. To elucidate further the stereospecific interaction between ibuprofen and carprofen obtained by CD, the effects of ibuprofen on the free fraction of carprofen and its enantiomers were studied by ED. The free fractions of carprofen and its enantiomers were increased upon the addition of ibuprofen. As shown in Fig. 7, the free fractions of carprofen and its enantiomers (0.75%) were increased to the following extents upon the addition of ibuprofen: *S*(+)-carprofen (1.90%), *RS*(±)-carprofen (1.66%) and *R*(−)-carprofen (1.50%).

Effect of ibuprofen and its enantiomers on the free fraction of S(+)*-carprofen.* Figure 8 shows the free fraction of *S*(+)-carprofen bound to HSA upon the addition of ibuprofen and its enantiomers. It is easily seen (Fig. 8) that the free fraction of *S*(+)-carprofen was almost trebled by *S*(+)-ibuprofen, whereas the smallest increase of the free fraction of *S*(+)-carprofen was caused by the addition of *R*(−)-ibuprofen.

Figure 9 shows the schematic model of stereospecific displacement of carprofen bound to HSA in the presence of ibuprofen.

DISCUSSION

Carprofen, a highly lipophilic NSAID, has a very high primary association constant to HSA ($>10^6$ M^{-1}) in the range of values reported for other NSAIDs. Our previous study [4] could indicate that carprofen binds with high affinity to site II (benzodiazepine site) and with weaker affinity to site I (warfarin site) on HSA.

It is expected from classical competitive dis-

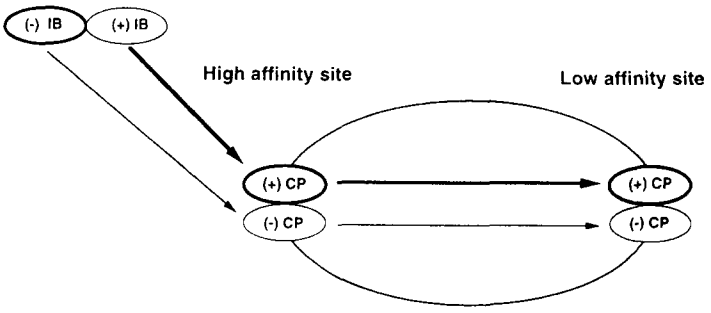

Fig. 9. Schematic representation of the stereospecific site-to-site displacement of carprofen bound to HSA by ibuprofen. CP, carprofen; IB, ibuprofen.

placement experiments that when another site II-specific bound drug like ibuprofen with a high association constant is concomitantly administered with carprofen, significant increases in the concentration of unbound carprofen should result. However, this does not happen in practice, in the case of carprofen binding to HSA, as shown in our present study.

In our previous study [4], we showed that the CD spectra of the complexes of carprofen and HSA at molar ratios 1:1 and 3:1 were quite distinct and spectropolarimetric titration showed that carprofen at a low molar ratio to HSA gave rise to a positive Cotton effect at around 255, 295 and 345 nm when bound to its primary site on HSA, while binding to the secondary site induced two strong negative Cotton effects at around 255 and 305 nm. This result suggests that at a lower molar ratio to HSA, carprofen exclusively binds to site II, whereas at a higher molar ratio to HSA (3:1), the binding also takes place at the secondary binding site and thus produces two large negative Cotton effects. Ibuprofen, at a molar ratio of 5:1 with HSA, caused a dramatic change in both signs and amplitudes of the CD bands of the carprofen–HSA complex at a molar ratio 1:1 (Fig. 2). Ibuprofen gave rise to the CD spectrum of the carprofen–HSA complex at a molar ratio 1:1 similar to that obtained in the case of the carprofen–HSA complex at a molar ratio 3:1. At the lowest drug to HSA molar ratio (0.5:1), when carprofen was mainly bound to its primary site, two positive maxima centered at 290 and 340 nm were induced. Thus development of the negative bands is obviously due to the secondary binding of carprofen to HSA. Our ED experiments (Fig. 3) could indicate that the free fraction of carprofen was not increased by the addition of ibuprofen, a site II-specific drug, up to 1×10^{-5} M, suggesting that displaced carprofen might have been rebound to its low affinity site. However, upon the further addition of ibuprofen (6×10^{-5} M), the free fraction was found to be increased by 90%, but the experimental data did not fit the theoretical curve assuming competition (Fig. 3). At the same molar ratio (1:2) of carprofen to HSA, warfarin or phenylbutazone even at the molar ratio 3:1 to HSA failed to demonstrate any effect on the free fraction of carprofen (Fig. 3). This further validates that carprofen at this particular molar ratio (1:2) to HSA exclusively binds to its high affinity site. When site I was blocked by a sufficient amount (6×10^{-5} M) of either warfarin or phenylbutazone, ibuprofen at the same molar ratio to HSA (3:1) caused an almost 4-fold increase in the free fraction of carprofen from its high affinity binding site. Interestingly, the increase in the free fraction was even higher when site I was blocked by phenylbutazone than by warfarin. This increase in the free fraction of carprofen caused by ibuprofen (at 1:1 or a higher ratio of ibuprofen to HSA) when site I was blocked by phenylbutazone than by warfarin was statistically significant as indicated by Student's t-test ($P < 0.01$). Moreover, when site I was blocked by phenylbutazone rather than warfarin, the experimental data were more close to the theoretical curve assuming competition. Within so-called site I, the presence of two sub-areas, namely the warfarin area and azapropazone area, is clearly seen [7]. Thus, from the above discussion, two definite conclusions can be drawn, carprofen shows site-to-site displacement by being displaced from its high affinity site (site II) to its low affinity site (site I) in the presence of ibuprofen, a site II-selective bound drug, and within so-called site I, the azapropazone area is the more favoured area over the warfarin area for the rebinding of displaced carprofen. In other words, secondary binding of carprofen mainly takes place in the azapropazone area of site I. We are referring to this site-to-site displacement as modified competitive displacement of carprofen by ibuprofen, since the observed curve of the free fraction of carprofen by ibuprofen did not fit the theoretical curve assuming competition (Fig. 3).

Schematic representations of the proposed binding models on the basis of interactions of carprofen–phenylbutazone–ibuprofen and carprofen–warfarin–ibuprofen are shown in Fig. 4. Carprofen possesses two classes of binding sites: the high affinity binding site and the low affinity binding site which are also called the site I and site II binding sites on the HSA molecule. When site I is blocked by a sufficient amount of either phenylbutazone or warfarin, carprofen should not be displaced from site II (high affinity binding site) to site I (low affinity binding site), thereby upon the addition of ibuprofen, an

increase in the free fraction of carprofen was observed.

The increase in the free fraction of carprofen by ibuprofen from site II to site I is more prominent in the presence of phenylbutazone as indicated by a bold arrow (Fig. 4) than in the presence of warfarin. This indicates that displacement of carprofen by ibuprofen occurs favourably to the azapropazone region of site I of the HSA molecule.

Stereoselective interaction

It is well established that the anti-inflammatory actions of the profen NSAIDs reside in their *S*(+)-enantiomers [8]. As carprofen and ibuprofen both contain a chiral centre and exhibit optical activity, and thus exist as pairs of (relatively) readily separable stereoisomers (Fig. 1), we were interested to see whether this site-to-site displacement phenomenon of carprofen caused by ibuprofen was stereospecific or not. Thus the overall interaction was studied considering both racemate and the enantiomers of both compounds separately, with the help of CD and ED experiments. Figure 5 shows the effect of ibuprofen on the CD intensity of the binding of carprofen and its enantiomers to HSA. Considering the CD intensity of the *RS*(±)-carprofen–HSA complex without ibuprofen as 100%, the CD intensities of the *S*(+)-carprofen- and *RS*(±)-carprofen–HSA complexes were found to be increased to almost 150% and 125%, respectively, whereas the CD intensity of the *R*(−)-carprofen–HSA complex was unaffected upon the addition of ibuprofen, implying that among these interactions, the interaction between racemate ibuprofen and *S*(+)-carprofen was the strongest. Furthermore, from the effects of ibuprofen and its enantiomers on the *S*(+)-carprofen–HSA complex as shown in Fig. 6, it was observed that the *S*(+)-enantiomer of ibuprofen had the highest interacting activity with the *S*(+)-enantiomer of carprofen. To substantiate the validity of this finding, the effects of ibuprofen and its enantiomers on the free fraction of carprofen and its enantiomers bound to HSA were studied with the ED technique. When the racemate ibuprofen was taken into consideration, ibuprofen caused the highest increase and the lowest increase of the free fractions of *S*(+)-carprofen and *R*(−)-carprofen, respectively, when bound to HSA (Fig. 7). Furthermore, as shown in Fig. 8, when the enantiomers of ibuprofen were considered separately, the *S*(+)-enantiomer of ibuprofen increased the free fraction of the *S*(+)-enantiomer carprofen bound to HSA to the highest extent. These results indicate that the concept of site-to-site displacement of carprofen bound to HSA in the presence of ibuprofen is most likely to be stereospecific.

It is interesting to note that the association constants of carprofen and its enantiomers, and ibuprofen and its enantiomers when bound to HSA separately are of the following order. For carprofen: $S(+) > RS(\pm) > R(-)$ [4]; for ibuprofen: $R(-) > RS(\pm) > S(+)$ (Table 1). On the basis of the association affinity of the enantiomers of carprofen and ibuprofen to HSA, the interaction between *R*(−)-carprofen and *R*(−)-ibuprofen should be the strongest. However, this was not the case. It is possible that stereospecificity plays a more important role than the binding affinity for the protein molecule in this particular respect, hence the interaction between the *S*(+)-enantiomers of ibuprofen and carprofen was the strongest among all the possible enantiomeric interactions.

In summary, the data presented based on the studies carried out by both CD and ED are depicted by the schema in Fig. 9 where by: carprofen is stereospecifically displaced by ibuprofen from its high affinity to its low affinity binding site, and the interaction between *S*(+)-carprofen and *S*(+)-ibuprofen was the strongest among all possible enantiomeric interactions.

Pharmacokinetic significance

If two drugs of the same type which bind to the same sites are administered at the same time, competition at the protein binding level may ensue: the drug with higher affinity, binding preferentially, displaces the other drug or inhibits its binding. Since NSAIDs have a relatively small volume of distribution (V_d) and are restrictivelty cleared, an increase in the free concentration may cause a significant decrease in $T_{1/2}$ if the intrinsic clearance remains constant.

Though a positive correlation between clearance and plasma free fraction has not been reported for any NSAIDs, it has been reported for some other drugs [8–11], and correlation between clearance and free fraction would be expected for NSAIDs. Therefore, from a pharmacokinetic view point, it is important to measure free drug concentration accurately as competitive phenomena which are readily demonstrable *in vitro* may not be of sufficient accuracy to explain the pharmacokinetic properties. Because of the so-called site-to-site displacement, the free concentration of carprofen does not fit the theoretical curve as it should after being competitively displaced by ibuprofen. Therefore, there will be a quantitatively significant difference between the free concentrations of carprofen caused by ibuprofen with or without the site-to-site displacement. For a highly bound drug like carprofen, whose binding to HSA is 99% or above, an increase of only 1% of the free fraction doubles the amount of drug available for pharmacological activity. Therefore the quantitative change of even 1% in binding might exert a profound effect on the disposition of carprofen bound 99%. So care must be exercised in calculating the free concentration of drugs like carprofen which shows site-to-site displacement in the presence of another drug like ibuprofen. Furthermore, although the differences between drug enantiomers in affinity for plasma proteins are not so great as the differences between enantiomers in their affinities for a given pharmacological receptor, these differences in protein binding can be important particularly when the enantiomers are stereospecifically displaced by another drug causing an increase in the free concentration of a particular enantiomer over another, as happens in the case of the interaction between carprofen and ibuprofen. Moreover, as stereoselective interaction of some drugs with plasma protein binding site is an important aspect in characterizing the specificity, selectivity and saturation of these binding sites [12–14], it might

also help in predicting the interaction of carprofen with HSA in more detail. However, it is too early to draw definite conclusions about the actual changes in pharmacokinetic behaviour of carprofen in the presence of such stereoselective site-to-site displacement caused by ibuprofen when bound to HSA.

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