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

MECHANISM OF BINDING STUDIED BY DIALYSIS AND SPECTROSCOPIC METHODS

MOHAMMED HABIBUR RAHMAN, TORU MARUYAMA, TOMOKO OKADA, KEISHI YAMASAKI and MASAKI OTAGIRI*

Faculty of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862, Japan

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Abstract—The binding of carprofen, a non-steroidal anti-inflammatory drug of the aryl propionic acid class [2-(6-chlorocarbazole)propionic acid], and its enantiomers to human serum albumin (HSA) has been studied by dialysis and spectroscopic methods. Binding parameters obtained by different methods were in close agreement. The binding of carprofen to HSA by both fluorescence and equilibrium dialysis (ED) methods is characterized by two sets of association constants [$K_1 = 5.1 \times 10^6\ M^{-1}$ (fluorescence) and $3.7 \times 10^6\ M^{-1}$ (ED), $K_2 = 3.7 \times 10^5\ M^{-1}$ (fluorescence) and $1.3 \times 10^5\ M^{-1}$ (ED)]. The *S*(+)-enantiomer of carprofen showed slightly higher affinity for HSA than its corresponding antipode by both methods. Different analyses of the binding to HSA suggested the presence of one high affinity site and five to seven low affinity sites for carprofen and its enantiomers on HSA. Fluorescence displacement data implied that carprofen primarily binds to site II, the benzodiazepine site, while the low affinity site of carprofen is site I, the warfarin site. Circular dichroism data suggested different mechanisms for the high affinity and the low affinity binding of carprofen to HSA. The data are consistent with the major part of the binding energy at site II being electrostatic and hydrophobic interactions, whereas for the low affinity binding, hydrophobic interactions. Binding was exothermic, entropy driven and spontaneous, as indicated by the thermodynamic analyses. From binding data with chemically modified HSA derivatives, it is likely that tyrosine, lysine and histidine residues are especially involved in carprofen binding to HSA, and it is most likely that the high affinity binding of carprofen is located in the N-terminal part of domain III or that section of protein plus the C-terminal part of domain II of the HSA molecule. When the binding of carprofen to HSA was compared to the binding of carprofen methyl ester to HSA ($K = 0.1 \times 10^6\ M^{-1}$), the carboxyl group of carprofen was found to play an important role especially in the high affinity binding of carprofen to HSA. The high affinity of carprofen to HSA was independent of the conformational changes on HSA caused by N–B transition.

Carprofen, a propionic acid derivative [2-(6-chlorocarbazole) propionic acid] is a chiral non-steroidal anti-inflammatory drug (NSAID†) marketed as the racemate with the therapeutic benefit ascribed mainly to the active *S*(+)-enantiomer as are various other 2-aryl propionic acids [1]. Most NSAIDs show a high degree of binding to albumin, and their binding to plasma proteins is a primary determinant of their pharmacokinetic properties [2]. With the exception of aspirin, all the NSAIDs are lipid soluble, weakly acidic and extensively bound especially to human serum albumin (HSA), the most abundant protein in plasma.

There are many reports on the binding of different NSAIDs to HSA *in vivo* and *in vitro* [3–9]. From these studies, the existence of two specific binding sites for NSAIDs on HSA has been proposed. Almost all NSAIDs appear to interact primarily with one of two loci, site I and site II, which are also called the warfarin binding site, and the benzodiazepine binding site, respectively [10, 11]. Although a few exceptions are known, the concept of site I and site II has proven its value in classification of drugs according to binding characteristics and seems to be useful for prediction of drug competition [11]. NSAIDs possessing a carboxyl group at the end of the extended hydrophobic molecule, e.g. ibuprofen, flufenamic acid and pirprofen, mainly bind to site II; site I-bound NSAIDs are azapropazone, phenylbutazone, indomethacin and diflumisal which possess bulky heterocyclic compounds with a negative or positive charge localized in the middle of the molecule [9, 10]. Though general articles regarding the pharmacokinetic implications of plasma proteins and tissue binding of a wide variety of drugs can be found [12–14], there is still a great paucity of information regarding the protein binding characteristics of NSAIDs as a class of drugs.

Carprofen is presently undergoing extensive clinical evaluation as a non-steroidal anti-inflammatory agent. The possible stereoselective dis-

* Corresponding author: Prof. Masaki Otagiri, Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, Kumamoto University, Kumamoto 862, Japan. Tel. (81) 96 344 2111 ext. 4147, 4148; FAX (81) 96 362 7690.

† Abbreviations: NSAID, non-steroidal anti-inflammatory drug; HSA, human serum albumin; CPM, carprofen methyl ester; CD, circular dichroism; ED, equilibrium dialysis; ACA, 7-anilinocoumarin-4-acetic acid; DNSA, dansyl-L-asparagine; DNSS, dansylsarcosine; CTAB, cetyltrimethylammonium bromide; SLS, sodium lauryl sulfate; PLE, polyoxyethylene lauryl ether; HNBB, 2-hydroxy-5-nitrobenzyl bromide; TNM, tetranitromethane; SA, succinic anhydride; MB, methylene blue.

position of carprofen is of interest since it is a racemic compound with a chiral center at the α carbon position [15]. Whitlam and Brown [16] reported extremely high binding (>99%) of racemic carprofen to 1% HSA using the ultrafiltration method. Recently, Iwakawa *et al.* [17] identified different binding sites for carprofen and its acyl glucuronide metabolite on the HSA molecule. However, they found that the maximum number of saturable binding sites of carprofen on the HSA molecule was two, but our preliminary experiments revealed that the number of high affinity binding sites for carprofen binding to HSA was one. Furthermore, for carprofen, these reports are not sufficient to define the binding of carprofen to HSA in detail.

Due to the conflicting results and paucity of information regarding binding of carprofen to HSA, the present work was undertaken to study the interaction of carprofen and its enantiomers with HSA by both direct and indirect methods. We also tried to characterize the binding mode of carprofen to HSA by comparing it with that of carprofen methyl ester (CPM), in terms of both thermodynamic and extra thermodynamic parameters.

MATERIALS AND METHODS

Materials

HSA was donated by the Chemo-Sera-Therapeutic Research Institute (Kumamoto, Japan). It was defatted with activated charcoal according to the procedure described by Chen [18] but with the following modification. The HSA solution was acidified with 0.1 M H_2SO_4 instead of HCl. H_2SO_4 was used instead of HCl as SO_4^{2-}, in contrast to Cl^-, has not been reported to bind to HSA [19]. Carprofen was purchased from Nippon Roche K.K. (Tokyo, Japan). CPM was synthesized by methylation of carprofen by the following procedure: first, the carboxylate was converted to carbonyl chloride, and then an aliquot of this reactant was slowly dropped into methanol containing pyridine. Excess of methanol and pyridine was removed under high vacuum and the residue obtained was repeatedly treated with benzene to remove completely traces of impurities. The solid product was recrystallized from isopropyl alcohol. The yield was about 91%. CPM was obtained as white crystals. The purity and identity of the compound were established before beginning the experiment. The preliminary purity of the compound was determined by TLC. The elemental analysis for C, H and N was within ±0.4% of the theoretical values. The structure of the compound was assigned on the basis of i.r., NMR and mass spectral data analysis. 7-Anilinocoumarin-4-acetic acid (ACA) was a generous gift from Prof. Goya of Kumamoto University. Dansyl-L-asparagine (DNSA) and dansylsarcosine (DNSS) were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.) and from the Tokyo Kasei Kogyo Co. (Tokyo, Japan), respectively. Cetyltrimethylammonium bromide (CTAB), sodium lauryl sulfate (SLS) and Brij35 were obtained from Wako Pure Chemical Industries (Osaka, Japan). CTAB and SLS were gently recrystallized twice by CCl_4 and an ethanol–ether mixture, respectively. Polyoxyethylene lauryl ether (PLE) was purified from commercially available Brij35 according to the procedure of Ikeda *et al.* [20]. 2-Hydroxy-5-nitrobenzyl bromide (HNBB), tetranitromethane (TNM), methylene blue (MB) and succinic anhydride (SA) were obtained from Nacalai Tesque Inc. (Kyoto, Japan). All other chemicals were of analytical grade. All the buffers used were prepared with sodium phosphate dibasic and sodium phosphate monobasic salts.

Preparation of HSA derivatives

The MB-catalysed photooxidation of HSA (MB-treated HSA) was carried out in a similar manner to that of Yamagata *et al.* [21]. In brief, the reaction mixture containing HSA (500 mg) and 25 mL of 0.01% MB in 0.067 M phosphate buffer (pH 7.4) was irradiated by visible light from a distance of 30 cm with a 200 W spot light at 37°. The changes in activity were corrected for those of the control HSA. The samples for histidine (His) analyses were passed through a Sephadex G-25 column (0.9 × 11 cm) to remove the MB and buffer salts and lyophilized. The lyophilized protein was then hydrolysed with 1.0 mL of glass-distilled 6 N HCl at 105° for 24 hr in a sealed evacuated test tube. The hydrolysate was dried *in vacuo* over NaOH pellets. The increase of absorption at about 250 nm was taken as evidence of the absorption of some photooxidation products of His residues.

SA-catalysed succinylation of HSA was carried out according to the procedure of Gounaris and Perlmann [22]. To the mixture of HSA (500 mg) and 20 mL of 0.1 M sodium chloride solution adjusted to pH 8.0 with 0.5 M $NaHCO_3$, SA (50 mg) was slowly added at 15° and the pH was maintained at between 7.5 and 8.5 by 1 N NaOH. At 30 min after the last addition of SA, the reaction mixture was passed through a Sephadex G-25 column. Protein fractions were collected and dialysed against distilled water for 60 hr, and then freeze-dried. The unreacted lysine residues (Lys) were determined by trinitrobenzene sulfonic acid procedure of Haynes *et al.* [23].

The single tryptophan residue (Trp) of HSA was modified by HNBB by the procedure of Khoshland *et al.* [24]. The modification of tyrosine residues (Tyr) was performed by TNM as described by Sokolovsky *et al.* [25]. CD, fluorescence and SDS–PAGE methods were used to check the conformation of HSA derivatives.

Apparatus and methods

Physicochemical properties of carprofen and CPM. The pK_a and PC values for carprofen were as reported by Schmitt and Guentert [26]. Solubility measurements were carried out according to the method of Higuchi and Connors [27]. Excess amounts of carprofen or CPM were added to phosphate buffer (pH 7.4) and the suspensions were shaken at 25°. After equilibrium was reached, an aliquot was centrifuged and filtered through a membrane filter (pore size 0.45 μm, Toyo Scientific Co. Ltd, Tokyo, Japan). A 0.5 mL aliquot of the sample solution was diluted to the required extent and then analysed by a HPLC system as described later. The molecular

sizes of carprofen and CPM were measured using the Corey–Pauling–Kolthun model.

Fluorescence measurements. Fluorescence measurements were made on a Jasco FP-770 fluorometer (Tokyo, Japan). The intrinsic fluorescence of HSA was obtained at 290 nm. Fluorescence titrations were carried out as follows: HSA solution (20 or 2 μM, 3 mL) was titrated by successive addition of drugs (to give a final concentration of 0.1–1.5×10^{-5} M), and the fluorescence intensity was measured (excitation 320 nm and emission 360 nm). The total volume was less than 3 mL + 20 μL; corrections of protein concentration were not made as they were insignificant. The fluorescence, given as relative fluorescence (F), increased successively with increasing concentrations of carprofen and its enantiomers in a solution of HSA. For substances like carprofen, which fluoresce much more intensely when bound than as the free ligand, the fraction of carprofen bound, α, was determined by using the following equation [28]:

$$\alpha = \frac{F_p - F}{F_b - F}$$

where F_p and F are fluorescence intensities of a given concentration of carprofen in a solution with a low protein concentration and in a solution without any protein, respectively. F essentially represents background fluorescence. F_b is the fluorescence intensity of the same concentration of the fully bound probe. The latter is taken to be the fluorescence intensity of the probe in the presence of excess protein. Such a treatment will yield good values of α provided the fluorescence intensity of the bound substrate is a linear function of its concentration.

Fluorescence probe displacement. The percentage of displacement of probe was determined using the following equation according to the method of Sudlow *et al.* [10]:

$$\frac{F_1 - F_2}{F_1} \times 100$$

where F_1 and F_2 are the fluorescences of probe plus HSA without and with drug, respectively. In brief, the fluorescence of the probe plus HSA (1:1, 3 μM each) was measured at 25° before and after the addition of drug (1.5–9 μM). The intrinsic fluorescence of HSA was obtained at 290 nm. Within the limit of sensitivity, the excitation wavelength was chosen to ensure the least possible absorption of incident light. The emission wavelength was chosen to give maximum fluorescence of the probe bound to HSA with insignificant fluorescence in buffer. The probes which were chosen had either a single binding site or two sites with widely separated dissociation constants. The probe to HSA ratio was kept to 1:1 in order to keep the non-specific binding of probes to a minimum.

CD measurements. CD measurements were carried out on a Jasco J-600 spectropolarimeter (Tokyo, Japan), using a 10 mm cell. All solutions were scanned from wavelengths at which no induced optical activity was observed.

Equilibrium dialysis (ED). ED experiments were performed using a Sanko plastic dialysis cell (Fukuoka, Japan). The two cell compartments were separated by Visking cellulose membrane. The 1.5 mL mixture of HSA (50 μM) and drug (10–500 μM) was poured into one compartment and 1.5 mL of buffer was poured into the opposite compartment. After 13 hr dialysis at specified temperatures, free carprofen concentrations were assayed 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 (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 samples were injected onto the column using a 250 μL loop. The flow rate was 1 mL/min. The measurements were carried out with excitation at 290 nm and emission at 365 nm.

For the separation of the unbound drug (carprofen and CPM) species from both native and modified HSA, the ED method as described above was used with little modification. The samples containing 1×10^{-5} M solution (1.5 mL) of protein and drug were dialysed against an equal amount of 0.067 M phosphate buffer, pH 7.4. The quantitative determination of the unbound drug was determined by the HPLC method as described above, except that the same mobile phase but at a different ratio (A:B, 37:63, v/v) was used for the quantitative determination of CPM.

Statistical differences between ligand binding to native HSA and to the other chemically modified HSA preparations were tested by Student's t-test.

Data analysis

The binding parameters were calculated by linear regression when the Scatchard plot [29] was a straight line. When the Scatchard plot was curved the binding parameters were obtained from the following expression using a non-linear squares computer program (MULTI program) [30]:

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

where r is the number of moles of bound drug per mole of protein, $[D_b]$ and $[D_f]$ are the bound and free 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.

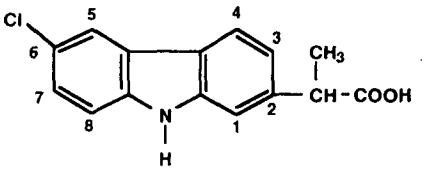
RESULTS

The chemical structures and the different physicochemical properties of carprofen and CPM are shown in Table 1.

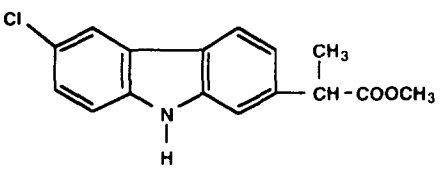
Binding of carprofen and its enantiomers, and CPM to HSA

When the fixed concentration of HSA was titrated with increments of carprofen, the binding exhibited triphasic extrinsic Cotton effects with all positive maxima at 295, 255 and 345 nm at the 1:1 drug to HSA ratio, but with still higher ratios (3:1, 5:1), the shape of the CD spectrum was drastically changed

Table 1. Chemical structures and physicochemical properties of carprofen and CPM



Carprofen



CPM

Parameter	Carprofen	CPM
Solubility (M) (25°)*	5.8×10^{-4}	8.4×10^{-5}
PC (pH 7.4, octanol–water)†	40	—
pK_a	4.43	—
Molecular size‡		
Width (Å)	21.1	23.8
Depth (Å)	10.0	11.4

* Estimated in 0.154 M phosphate buffer (pH 7.4) at 25°.
† Partition coefficient.
‡ Estimated by Corey–Pauling–Kolthun model, which prefers to space filling model.
— Not determined.

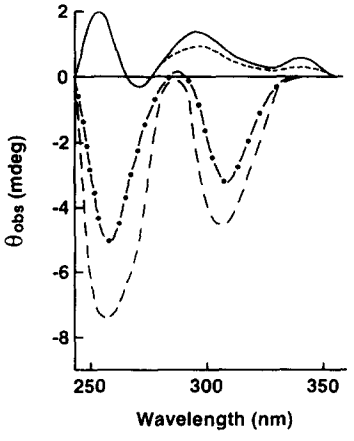


Fig. 1. CD spectra of the carprofen–HSA complex at different drug to HSA ratios at pH 7.4 and 25°. Drug to HSA (30 μM) ratios, 0.5 (----), 1.0 (——), 3.0 (–●–) and 5.0 (— —), were measured in a 10 mm cell.

by exhibiting polyphasic extrinsic Cotton effects with large negative ellipticities at 305 and 255 nm without any isosbestic point (Fig. 1). This indicates that the induced Cotton effects observed herein were not based on a single mechanism under the experimental conditions. Furthermore, this differing behaviour of carprofen bound to HSA at different molar ratios prevented estimation of binding parameters of carprofen by the CD technique.

Scatchard analysis of the fluorescence data showed a non-linear line, suggesting the presence of two classes of binding sites for the binding of carprofen and its enantiomers to HSA. The binding of carprofen and its enantiomers was also directly monitored by the ED method. From the dialysis data also, two successive saturable processes were observed when carprofen and its enantiomers were bound to HSA. As a representative example, Scatchard analyses of carprofen binding to HSA by both the fluorescence and ED methods are shown in Fig. 2. The best fitting values for the binding parameters obtained by both the fluorescence and ED methods shown in Table 2. Due to the extremely low solubility of CPM in aqueous solution, the binding of CPM was only determined by the fluorescence methods and the binding characteristics obtained by linear curve fitting (Fig. 2) suggest that CPM was bound to only one class of binding sites. Results obtained are summarized in Table 2.

Job's plots [31] were constructed for carprofen and its enantiomers bound to HSA by maintaining the total concentration of drugs plus protein at a constant value of 1×10^{-6} M. As a representative example, the Job's plot for the carprofen–HSA system is shown in Fig. 3. The inflection point gives a value of near 0.5, which corresponds to the number of binding sites, N = 1.

Identification of binding sites

In order to identify the location of binding sites of carprofen and its enantiomers on HSA, site marker displacement experiments were carried out using fluorescent probes which specifically bind to known sites on HSA. As illustrated in Fig. 4, carprofen remarkably displaced DNSS, a marker for site II [32]. Carprofen also displaced DNSA, a site I marker [32], moderately, whereas the fluorescence of ACA, a site III marker [33], was not affected by carprofen binding. Both enantiomers of carprofen showed similar behaviour. In contrast, CPM had no influence on the extrinsic fluorescence of the site II marker, but showed some influence on the extrinsic fluorescence of the site I marker by displacing it from its binding site to a noticeable extent (Fig. 4).

Difference UV absorption spectra

The effect of HSA on the UV absorption spectra

Table 2. Binding parameters of carprofen- and CPM–HSA complexes at pH 7.4 and 25°

	Fluorescence				ED			
	n_1	K_1 ($\times 10^6$ M^{-1})	n_2	K_2 ($\times 10^5$ M^{-1})	n_1	K_1 ($\times 10^6$ M^{-1})	n_2	K_2 ($\times 10^5$ M^{-1})
RS(±)-carprofen	0.9 ± 0.1	5.1 ± 0.2	7.3 ± 0.3	3.7 ± 0.3	1.2 ± 0.1	3.7 ± 0.3	4.0 ± 0.3	1.3 ± 0.1
S(+)-carprofen	1.1 ± 0.1	5.3 ± 0.2	8.7 ± 0.3	1.7 ± 0.1	1.3 ± 0.1	4.7 ± 0.2	4.5 ± 0.3	1.4 ± 0.1
R(−)-carprofen	0.8 ± 0.1	4.7 ± 0.2	7.9 ± 0.4	4.6 ± 0.3	1.1 ± 0.1	3.5 ± 0.2	4.8 ± 0.4	1.4 ± 0.1
CPM	0.9 ± 0.1	0.1 ± 0.0	—	—	—	—	—	—

Each value is the mean ± SD of data from three experiments.
— Could not be determined.

of carprofen and CPM was examined and compared with the spectra of the drugs in the presence of detergents having different properties. The binding of carprofen to HSA resulted in a shift of their absorption spectra towards a longer wavelength (data not shown). As shown in Fig. 5, the difference spectrum in the binding of carprofen to HSA is characterized by two negative maxima at 288 and 300 nm and two positive maxima at 265 and 313 nm. The absorption of carprofen produced similar spectra when the carprofen molecule was dissolved in a solution containing the cationic detergent CTAB and the non-ionic detergent PLE. A different spectrum was produced when carprofen interacted with the anionic detergent, SLS. However, no influence on the absorption spectrum of carprofen was seen at CTAB, PLE or SLS concentrations below the critical micelle concentrations. On the other hand, CPM produced similar spectra with all kinds of detergent and HSA (data not shown).

Effect of pH and temperature on the binding of drugs

The effect of pH on the CD ellipticity of the complexes of carprofen and its enantiomers with HSA was examined. Though prominent changes in CD ellipticities due to the high affinity binding of carprofen to HSA as a function of pH were not observed, the negative CD ellipticity due to the low affinity binding of carprofen was found to be increased with the rise in pH without changing of the signs of the Cotton effects (Fig. 6). Binding parameters for the carprofen–HSA complex estimated by the fluorescence method at several pH values also validated the above results by showing that the association constant and number of binding sites for the high affinity binding of carprofen to HSA were not significantly affected by a pH change, but those of the low affinity binding were affected noticeably by the change in pH (Table 3). However, temperature did not have any noticeable effects on the CD spectra of the carprofen–HSA complex (data not shown).

Thermodynamics analysis

Thermodynamic parameters were calculated by the usual procedure using linear van't Hoff plots at three specified temperatures, 10°, 25° and 40°, and the results are summarized in Table 4. The van't Hoff curves by plotting ln nK against $1/T$ are straight lines (Fig. 7), indicating that ΔH values are constant for the reaction over the experimental temperature range. Thermodynamic parameters given here were evaluated at both high affinity and low affinity binding sites. The formation of carprofen–HSA complexes at the high affinity binding site was accompanied by zero enthalpy and a positive value for entropy, while at the low affinity binding site, the formation of the complex was an exothermic reaction accompanied by negative enthalpy and a positive entropy value.

Binding of drugs to HSA derivatives

The reaction products of HSA with HNBB, TNM, SA and MB were assayed by procedures described in Materials and Methods. The results, in terms of per cent reacted residues with native HSA taken as

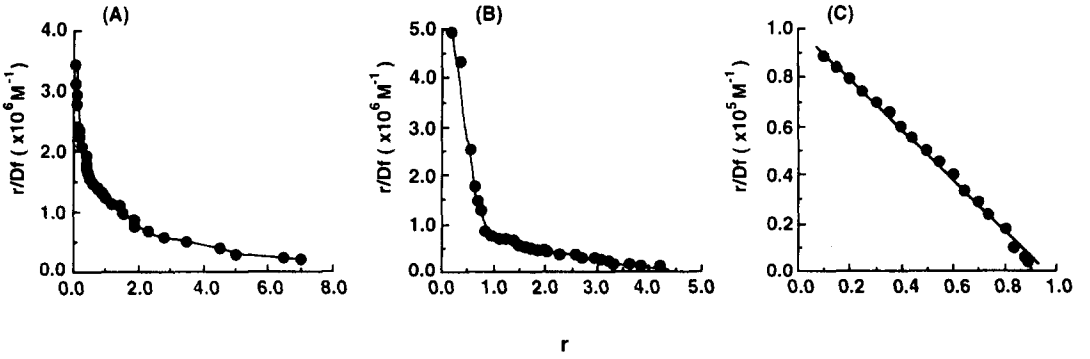


Fig. 2. Scatchard plots of carprofen binding to HSA by ED (A) and fluorescence (B), and CPM binding to HSA by fluorescence (C) at pH 7.4 and 25°.

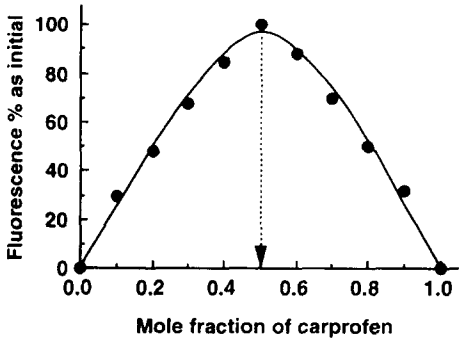


Fig. 3. Job's plot of relative fluorescent intensities as a function of mole fraction of carprofen. The total concentration of HSA + carprofen was kept constant at 1 μM.

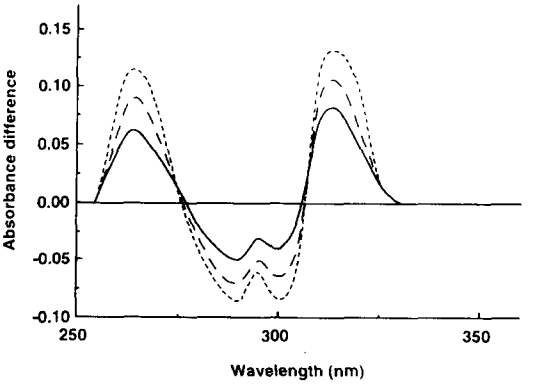


Fig. 5. Difference UV absorption spectra of carprofen bound to HSA and detergents at pH 7.4 and 25°. (——) Drug–HSA system, (----) drug–CTAB system, (——) drug–PLE system. The following concentrations were employed: drug, 10 μM; HSA, 10 μM; detergent, 0.05%; phosphate buffer, 0.067 M.

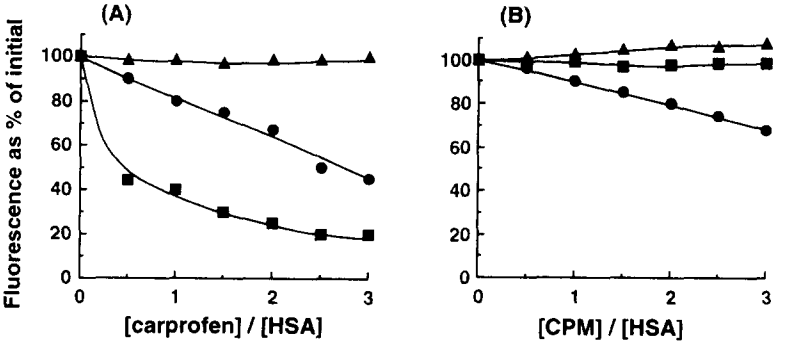


Fig. 4. Effect of carprofen (A) and CPM (B) on fluorescence of site marker probes. HSA and probes were at 3 μM in 0.067 M phosphate buffer (pH 7.4). Mean data of four experiments are shown. Variance of data is less than 6%. Site marker probes used were: DNSA (●), site I area marker; DNSS (■), site II area marker; ACA (▲), site III area marker.

0%, are shown in Table 5. It was confirmed that Trp, Tyr, Lys and His were especially modified by HNBB, TNM, SA and MB, respectively. Figure 8 shows the carprofen and CPM free fractions for binding to native, nitrated, succinylated, HNBB-treated and MB-treated HSA. Modification of Tyr and Lys moderately reduced the binding of carprofen; no binding change was observed by Trp modification. However, His modification reduced the binding of carprofen to the greatest extent by increasing the

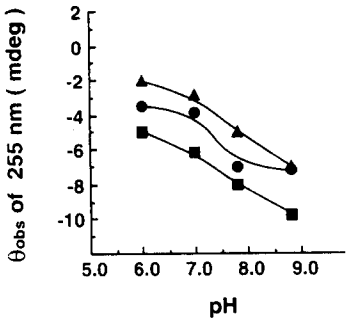


Fig. 6. Effects of pH on the induced CD ellipticity of carprofen and its enantiomers bound to HSA at a fixed wavelength (255 nm) and 25°. *R*(−)-CP(■), *RS*(±)-CP(●), *S*(+)-CP(▲). Following concentrations were used: carprofen, 9×10^{-5} M; HSA, 3×10^{-5} M.

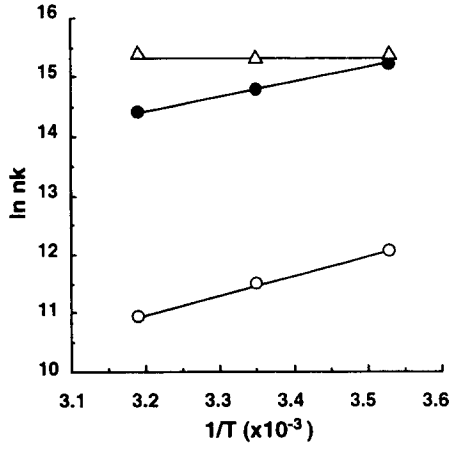


Fig. 7. van't Hoff plots for binding of carprofen and CPM to HSA. (△) For high affinity binding of carprofen to HSA, (●) for low affinity binding of carprofen to HSA, (○) for binding of CPM to HSA.

Student's *t*-test the free fractions for the binding of carprofen to His-, Tyr- and Lys-modified HSA were significantly different from those obtained for the binding of carprofen to native HSA. However, in the case of CPM, the free fraction of CPM bound to Trp-modified HSA was only statistically significant from that obtained for the binding of CPM to native HSA.

DISCUSSION

Binding ability

Initially, we attempted to estimate the binding parameters of carprofen and its enantiomers to HSA by both direct (ED) and indirect (CD, fluorescence) methods. However, as previously mentioned, the estimation of binding parameters by the CD method was unsuccessful. As can be seen in Table 2, binding parameters for the primary binding sites are in close agreement between different methods. CD results, however, indicated the heterogeneous binding processes for carprofen to HSA, suggesting that carprofen and its enantiomers are optically active at both their high affinity and low affinity binding sites. The non-linearity obtained by Scatchard plots obviously indicated the presence of two classes of binding sites. As shown in Table 2, *S*(+)-carprofen had slightly higher binding affinity for HSA over its corresponding antipode. The binding parameters of CPM were determined only by the fluorescence method because of its extreme low solubility in aqueous solutions, and the results are also given in Table 2. As can be seen in Table 2, the number of high affinity binding sites for carprofen and its enantiomers bound to HSA is approximately one. To establish further the value of high affinity binding sites, a Job's plot (Fig. 3) was carried out for the carprofen–HSA system. The inflection point was near 0.5, suggesting that the value of high affinity binding site corresponds to 1. Therefore, it is reasonably certain that the maximum number of high affinity binding sites for carprofen on the HSA molecule is one. This result, however, is in contrast to the findings reported by Iwakawa *et al.* [17], who studied the binding of carprofen and its enantiomers to HSA by an ultrafiltration method and estimated the number of saturable binding sites for carprofen

Table 3. Binding parameters of carprofen to HSA at 25° and various pH values as determined by ED

pH	n_1	K_1 (× 10^6 M^{-1})	n_2	K_2 (× 10^5 M^{-1})
6.5	0.8 ± 0.0	3.5 ± 0.1	4.4 ± 0.1	1.3 ± 0.1
7.5	0.9 ± 0.1	3.4 ± 0.2	5.5 ± 0.2	3.9 ± 0.1
8.5	0.9 ± 0.1	3.5 ± 0.2	6.8 ± 0.2	5.5 ± 0.1

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

free fraction more than two times as compared to native HSA. In contrast, though modification of Lys, His and Tyr did not impart any influence on the free fraction of CPM bound to HSA, modification of Trp moderately increased the free fraction of CPM. According to calculations performed by

on HSA as two. Though the source of the discrepancy is not very clear, it might reside in the following facts: first, these authors have used an ultrafiltration method, while our investigations were performed with fluorescence and ED methods. Second, they used a different HSA concentration to ours for the

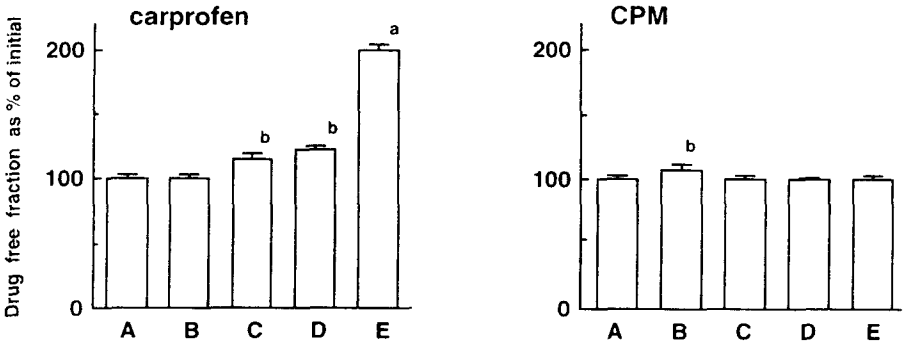


Fig. 8. Binding of carprofen and CPM to native and modified HSA at pH 7.4 and 25°. (A) Native HSA, (B) HNBB-treated HSA, (C) TNM-treated HSA, (D) SA-treated HSA, (E) MB-treated HSA. The following concentrations were used: protein, 10 μM; carprofen and CPM, 10 μM. Each column and bar represents the mean ± SD of data from three experiments. a, $P < 0.01$ in His-modified HSA vs native HSA (for carprofen); b, $P < 0.05$ in Tyr- and Lys-modified HSA vs native HSA (for carprofen) and Trp-modified HSA vs native HSA (for CPM).

Table 4. Thermodynamic parameters for carprofen- and CPM–HSA interactions at pH 7.4

Temperature (°K)	Carprofen						CPM		
	For high affinity binding			For low affinity binding					
	ΔG	ΔH	ΔS	ΔG	ΔH	ΔS	ΔG	ΔH	ΔS
283	−35.4			−30.0			−28.0		
298	−37.3	0	128	−31.0	−11.2	104	−28.8	−14.4	53
313	−39.0			−32.1			−29.5		

Table 5. Percentage reacted amino acid residues in modified HSA

Modified HSA	Reacted amino acid (%)			
	Trp (1)	Tyr (18)	Lys (59)	His (16)
HNBB-treated	93	0.0	2,3	1.5
TNM-treated	0.0	10	0.0	0.0
SA-treated	0.0	3.2	64	0.0
MB-treated	60	6.8	2.0	10

The number in parentheses represents the number of amino acids per HSA molecule.

estimation of binding parameters. Many workers have reported that drug protein binding parameters paradoxically depend on the HSA concentrations used [34, 35]. Third, the presence of any contaminants (even though in very small amounts) might also be the cause of the difference in the number of binding sites, as contamination by a competitive ligand has been given as an explanation for the inverse dependence of the binding parameters of another ligand on the protein concentration [36]. Moreover, in a recent study performed in our laboratory, it has been found that the ratio of mercapt albumin and non-mercapt albumin in commercial albumin differs from source to source. This difference might also be responsible for the difference in the number of binding sites.

Location and nature of binding sites

Figure 4 shows the effects of carprofen and its enantiomers on the fluorescence of DNSA (site I probe), DNSS (site II probe) and ACA (site III probe). The results of fluorescence experiments clearly indicate that site II (benzodiazepine site) is the high affinity binding site and site I (warfarin site) is the low affinity site for carprofen, similar to the results for other NSAIDs. By contrast, our fluorescence measurements could indicate that CPM binds only to site I (warfarin site). CPM gave rise to a strong negative Cotton effect around 290 nm when bound to HSA. At different molar ratios of CPM to HSA, the intensity of the CD spectrum of the CPM–HSA complex was changed without changing the sign of the Cotton effect (figure not shown), suggesting further that CPM might bind to HSA via a single mechanism. Fehske and co-workers [37, 38] from the binding studies of drugs to both native and modified HSA suggested that the Tyr residues are involved in the diazepam binding site (site II) and the lone Trp residue is a part of the

warfarin binding site. On the other hand, Vestberg *et al.* [19] and Kragh-Hansen *et al.* [39] in their recent works have shown the involvement of Lys and aspartic acid in the binding of drugs to site II by using different albumin variants.

In our study, the derivatives of HSA were prepared by chemical modification of native protein with HNBB, TNM, SA and MB in order to have information about the binding site of carprofen and its enantiomers. Our data indicated that His especially, and to some extent Tyr and Lys play an important part in constructing the binding site of carprofen and its enantiomers. All these data indicate that a positive charge might be located in or near the high affinity binding site of carprofen and its enantiomer. At pH 7.4, the amino group of Lys exists in ionized form; therefore, the cation of the amino acid may be part of an attachment for the carboxyl group of carprofen. Bos *et al.* [40] have tried to locate a high affinity binding site for diazepam in the albumin molecule by investigating interaction between the drug and the same large peptic and tryptic fragments by using CD and ED methods. They found that the main part of the primary part of the primary binding site is located in domain III. On the basis of the results of the present study and the data that have already been reported in previous studies, it can be inferred that the binding site of carprofen and its enantiomers may be located in the N-terminal part of domain III or that section of protein plus the C-terminal part of domain II, and the CPM binding site is most probably located in domain II. This finding is also supported by He and Carter [41] who have recently solved the three-dimensional structure of crystalline HSA at a resolution of 2.8 Å and have shown in the stereo view of HSA that the binding sites of so-called site II drugs are located in sub-domain III A and those of site I drugs in sub-domain II A. However, due to the paucity of information within this area, the exact location of the high affinity binding site of carprofen and its enantiomers cannot be predicted. The binding of CPM probably involves the participation of the Trp amino acid as shown by the increase in the free concentration of CPM during binding to Trp-modified HSA as compared to native HSA.

Binding mode

There are essentially four types of non-covalent interaction that could play a role in ligand binding to proteins. These are hydrogen bonds, van der Waals forces, hydrophobic bonds and electrostatic interactions [42, 43]. In order to obtain such information, the implications of the present results have been discussed in conjunction with thermodynamic characteristics obtained for carprofen and CPM binding. The thermodynamic parameters for binding of carprofen by HSA given in Table 4 show that for high affinity binding, ΔH and ΔS are zero and positive, whereas for the low affinity binding sites, ΔH and ΔS are negative and positive. The high affinity binding of HSA for carprofen was unaffected by rise in temperature, in contrast to many protein–ligand interactions [44]. The binding process was always spontaneous as evidenced by the negative sign of the ΔG values. For typical hydrophobic interactions, both ΔH and ΔS are positive, while negative enthalpy and entropy changes arise from van der Waals forces and hydrogen bonding formation in low dielectric media [45]. Negative enthalpy might however play a role in electrostatic interactions, but for actual or true electrostatic interactions, ΔH is expected to be very small or almost zero [46]. Therefore, the high affinity binding of carprofen to HSA might involve electrostatic interaction strongly as evidenced by the zero value of ΔH (Table 4) due to the presence of the carboxyl group in the propanoate portion of the structure. This conclusion agrees well with the previous finding reported by Maruyama *et al.* [9] which suggested that the carboxyl group was very vital for the binding of suprofen, another NSAID of the aryl propionic acid class, to HSA. Furthermore, it is found that ΔS is positive for both high and low affinity binding of carprofen by HSA and that the major contribution to ΔG arises from the ΔS term rather than from ΔH. Consequently the binding process is entropically driven. However, the thermodynamic parameters of the carprofen–HSA complex cannot be explained on the basis of a single intermolecular force model. It is more likely that electrostatic interaction along with hydrophobic interaction play a significant role in the high affinity binding of carprofen by HSA, while low affinity binding of carprofen by HSA is mainly based on hydrophobic interaction, which categorically indicates that hydrophobic interaction in the low affinity binding of carprofen to HSA should be more important than in its high affinity binding. However, van der Waals interactions as a consequence of hydrophobic interaction might also play an important role in the binding of carprofen to HSA. The high affinity binding of carprofen is more entropically driven than its low affinity binding to HSA. The possibility of unfolding of the protein molecule during the binding process because of high positive ΔS values can be rejected as unfolding of proteins requires the breaking or bending of several bonds resulting in an endothermic reaction [47]. However, an electrostatic interaction is not expected in the case of CPM binding due to the lack of a carboxyl group. Therefore, though the ΔS value obtained from the CPM binding is small, it is based mainly on hydrophobic interactions, which are more important for CPM binding than for carprofen binding. Apart from the hydrophobic interactions, van der Waals interactions as a consequence of hydrophobic interactions may also be involved in CPM binding as indicated by the negative value of ΔH. The high negative value of ΔH for CPM binding suggests that CPM binding to HSA is much more enthalpically driven than carprofen binding.

The binding of carprofen and CPM to HSA had very distinct features, which indicates that the mechanisms of binding of these two compounds to HSA differ. Figure 5 represents the UV difference spectra of carprofen in the presence of HSA and different detergents. The binding of carprofen to HSA is characterized by two positive maxima at 265 and 313 nm and two negative maxima at 288 and 300 nm. A similar spectral pattern was also observed in the case of the binding of carprofen to CTAB and

PLE. However, with PLE, negative peaks at 288 and 300 nm were slightly shifted to longer and shorter wavelengths, respectively, with a decrease in molar absorptivity. As carprofen is an amphipathic molecule, carprofen is likely to be adsorbed to the polar–non-polar interface of CTAB in such a way that the carboxyl group is located near the aqueous layer and can interact with the tertiary ammonium moiety of the CTAB molecule, while the remaining hydrophobic portion of the carprofen molecule (carbazole ring) is directed towards the hydrophobic centre. This indicates that the HSA binding site for carprofen probably consists of a cationic site on the surface of the HSA molecule with a hydrophobic patch to accommodate the carbazole ring. This hypothesis is consistent with the type of interaction suggested by the thermodynamic parameters and is also supported by the results published earlier from this laboratory [8, 9] in the case of two other NSAIDs, pirprofen and suprofen. On the other hand, similar difference spectra produced by CPM with HSA and other detergents suggest that electrostatic interaction is not involved in the binding of CPM to HSA. Considering the thermodynamic parameters and UV difference spectra, we regard the binding mode of carprofen and CPM to HSA to be as follows: the hydrophobic side chain of the carprofen molecule, especially the carbazole ring, is inserted deeply in the hydrophobic crevice, while the carboxyl group of the propanoate portion interacts with a cationic sub-site located at or near the hydrophobic surface of HSA. By contrast, the whole CPM molecule is inserted into a hydrophobic patch on HSA, suggesting that hydrophobic interactions almost exclusively govern the binding of CPM to HSA.

HSA is said to undergo conformational changes in the physiological pH range (pH 6–9), commonly regarded to as the N–B transition [48, 49]. At pH 6, HSA is almost entirely in the neutral form, whereas at pH 9 it is almost entirely in the basic form. When the protein is in the B-conformation, it has fewer protons bound than when it is in the N-conformation. N–B transition does not seem to affect the high affinity binding of carprofen to HSA as HSA binding of carprofen was found to be barely affected by raising the pH from 6.5 to 8.5. This indirectly indicates that the ionized form or carprofen prefers to bind at site II. This conclusion agrees with the structural features defined by Sudlow *et al.* [10] which suggests that the drugs which bind to site II are aromatic carboxylic acids, largely ionized at physiological pH. However, though N–B transition was not found to play any role in the high affinity binding of carprofen to HSA it showed some effects on the low affinity binding of carprofen, suggesting that the nature of binding of carprofen to high affinity and low affinity sites differs. This finding is in contrast to the previous report published from this laboratory regarding the binding of suprofen to HSA [9], where both high and low affinity binding were found to be independent of the N–B transition. This probably indicates a difference in the binding mode of carprofen and suprofen to HSA, even though they belong to the same class of drug.

In conclusion, though the binding of carprofen and its methyl derivative, CPM is quantitatively similar, their binding mode and location of binding sites are significantly different, implying that the carboxyl group of carprofen plays a vital role in determining the mode and location of binding sites of carprofen to HSA. The number of high affinity binding sites of carprofen and its enantiomers is one, and the *S*(+)-enantiomer of carprofen shows slightly higher binding affinity for HSA than its corresponding antipode. The low affinity binding, but not high affinity binding, of carprofen to HSA is dependent on the structural fluctuations accompanying N–B transition. These data provide a more complete picture of the binding of carprofen to HSA, and provide a basis for a more detailed study of the pharmacokinetics of not only carprofen but also other NSAIDs of the same group.

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