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BINDING OF SUPROFEN TO HUMAN SERUM ALBUMIN

ROLE OF THE SUPROFEN CARBOXYL GROUP

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Abstract—The binding of suprofen (SP), a non-steroidal anti-inflammatory drug of the arylpropionic acid class, and its methyl ester derivative (SPM) to human serum albumin (HSA) was studied by dialysis and spectroscopic techniques. In spite of the remarkable differences in the physicochemical properties of SP andSPM, the binding of each molecule to HSA was quantitatively very similar. Thermodynamic analysis suggests that the interaction of SP with HSA may be caused by electrostatic as well as hydrophobic forces, whereas the interactions with SPM may be explained by hydrophobic and van der Waals forces. Similarities in the difference UV absorption spectra between ligand–detergent micelle and –HSA systems indicate that the SP and SPM molecules are inserted into a hydrophobic crevice on HSA. The same studies suggest that the carboxyl group of SP interacts with a cationic sub-site which is closely associated with the SP binding site. Proton relaxation rate measurements indicate that the thiophen ring and propanoate portion of the SP molecule is the major binding site for HSA. The locations of SP and SPM binding sites were identified by using fluorescence probes which bind to a known site on HSA. The displacement data implied that SP primarily binds to Site II, while the high affinity site of SPM as well as low affinity site of SP are at the warfarin binding site in the Site I area. From binding data with chemically modified HSA derivatives, it is likely that highly reactive tyrosine (Tyr) and lysine (Lys) residues, which may be Tyr-411 and Lys-195, are specifically involved in SP binding. In contrast, these two residues are clearly separated from the SPM binding site. The binding of SP and SPM is independent of conformational changes on HSA that accompany N–B transition. There is evidence that the carboxyl group may play a crucial role in the high affinity binding processes of SP to HSA.

Non-steroidal anti-inflammatory drugs (NSAIDs‡) are one of the most frequently prescribed and co-administered group of drugs in the clinical field. Most NSAIDs strongly interact with human serum albumin (HSA), the most abundant protein in plasma [1–3]. Thus, it is well established that HSA binding is very important in the pharmacokinetics, pharmacodynamics and toxicology of NSAIDs. Consequently, many reports have appeared on the binding of various NSAIDs to HSA *in vivo* as well as *in vitro* [4–9]. From these studies, it has been concluded that NSAIDs bind to two primary sites on the HSA molecule, namely Site I, also called the azapropazone site, and Site II, also called the benzodiazepine binding site [10–12]. Interestingly, NSAIDs which bind to Site II possess a carboxyl group at the end of extended hydrophobic molecules, e.g. ibuprofen, flufenamic acid and carprofen. On the other hand, Site I-bound NSAIDs are bulky heterocyclic compounds with a negative or positive charge localized in the middle of the molecule, e.g. azapropazone, phenylbutazone and GP53,633.

Recently, Iwakawa *et al.* [13] identified the different binding sites of carprofen and its acyl glucuronide metabolite on the albumin molecule. On the basis of these findings, we have assumed that Site II recognises the carboxylate portion of NSAIDs in the binding process. Furthermore, some NSAID prodrugs, which have masked carboxyl groups blocked by esterification and amidation, have been designed to avoid adverse effects such as damage to the gastrointestinal mucosal membrane. These modifications may affect the physiological distribution of the prodrug as compared to the parent compound. However, there is as yet little information on the binding of NSAID prodrugs to plasma protein.

In this study, we have used suprofen (SP), one of the 2-arylpropionic acid NSAIDs, and its methyl ester (SPM), as model compounds to elucidate the role of the carboxyl group of NSAIDs in binding to HSA. The binding of SP and SPM to HSA was studied by spectroscopic and equilibrium dialysis techniques. The chemical structures and physicochemical properties of SP and SPM are summarized in Table 1.

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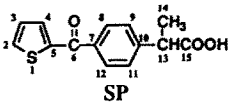
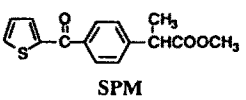
‡ Abbreviations: NSAID, non-steroidal anti-inflammatory drug; HSA, human serum albumin; SP, suprofen; SPM, suprofen methyl ester; CD, circular dichroism; ACA, 7-anilinocoumarin-4-acetic acid; *p*-ABE, *n*-hexyl-*p*-aminobenzoate; DNSA, dansyl-L-asparagine; DNSP, dansyl-L-proline; CTAB, cetyltrimethylammonium bromide; SLS, sodium lauryl sulfate; PLE, polyoxyethylene lauryl ether; HNBB, 2-hydroxy-5-nitrobenzyl bromide; TNM, tetranitromethane; SA, succinic anhydride.

Table 1. Chemical structures and physicochemical properties of SP and SPM



SP



SPM

	SP	SPM
Solubility* (25°)	5.4×10^{-4} M	7.4×10^{-5} M
DC (pH 7.4, *n*-hexane)†	0.01	130
pK_a	3.91	—
Molecular size‡		
Width (Å)	13.6	15.2
Depth (Å)	7.2	8.4

* Estimated in 0.154 M phosphate buffer (pH 7.4) at 25°.

† Distribution coefficient.

‡ Estimated by Corey–Pauling–Kolthun model, which refers to space filling model.

MATERIALS AND METHODS

Materials. HSA was donated by the Chemo-Sera-Therapeutic Research Institute (Kumamoto, Japan). It was defatted with activated charcoal in solution at 0°, acidified with H_2SO_4 to pH 3 and then freeze-dried. SP was supplied by the Taiyo Pharmaceutical Industry Co. (Takayama, Japan). SPM was synthesized by methylation of SP 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. 7-Anilinocoumarin-4-acetic acid (ACA) was a generous gift from Prof. Goya of Kumamoto University. *n*-Hexyl-*p*-aminobenzoate (*p*-ABE) was kindly provided by Prof. Takadate of Daiichi College of Pharmaceutical Sciences. Dansyl-L-asparagine (DNSA) and dansyl-L-proline (DNSP) were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.) and from the Tokyo Kasei Kogyo Co. (Tokyo, Japan). 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.* [14]. 2-Hydroxy-5-nitrobenzyl bromide (HNBB), tetranitromethane (TNM) and succinic anhydride (SA) were obtained from Nacalai Tesque Inc. (Kyoto, Japan). Di-sodium deuterophosphate (Na_2DPO_4) and potassium di-deuterophosphate (KD_2PO_4) were purchased from E. Merck (Darmstadt, Germany).

All other chemicals were of analytical grade. All the buffers used were prepared with sodium phosphate dibasic and sodium phosphate monobasic.

Apparatus and method. Fluorescence measurements were made on a Jasco FP-770 fluorometer (Tokyo, Japan). The intrinsic fluorescence of HSA was obtained by excitation at 295 nm. Fluorescence titrations were carried out as follows: 2×10^{-6} M HSA solution was titrated by successive additions of drug stock solution (to give a final concentration of $0.5–1.5 \times 10^{-5}$ M). The bound fraction of drug was determined as described by Halfman and Nishida [15].

Circular dichroism (CD) measurements were made on a Jasco model J-500A spectropolarimeter (Tokyo, Japan), using 5 and 10 mm cells at 25°. The induced ellipticity was defined as the ellipticity of the drug–HSA mixture minus the ellipticity of HSA alone at the same wavelength and is expressed in degrees. The free and bound concentrations of drug were calculated according to the method of Rosen [16], as described earlier.

Absorption spectra were recorded with a Shimadzu UV-240 spectrophotometer (Kyoto, Japan). Difference absorption spectra were measured by using a pair of 10 mm split-compartment tandem cuvettes. ^{1}H-NMR spectra were recorded on a NMR spectrometer (JEOL JNM FX-270) (Tokyo, Japan) at 20° ± 0.5. pD was adjusted to 7.4 by means of 0.067 M KD_2PO_4 and Na_2DPO_4. SP was used at a concentration of $3.5–12.5 \times 10^{-3}$ M while HSA was fixed at 1.0×10^{-4} M. Transversal relaxation rates ($1/T_2$) were calculated from the equation: $1/T_2 = \pi \cdot \Delta_{\nu 1/2}$ where $\Delta_{\nu 1/2}$ is the full line width at half-maximum height. For each signal at first exchange limit for a single binding site on the macromolecule:

$$\frac{1}{T_{2\text{obs}}} = \frac{1}{T_{2\text{free}}} + \alpha\left(\frac{1}{T_{2\text{bound}}} - \frac{1}{T_{2\text{free}}}\right) \qquad (1)$$

where α is the bound fraction of SP and $T_{2\text{obs}}$, $T_{2\text{free}}$, $T_{2\text{bound}}$ are the observed, free and bound relaxation rate of SP, respectively. An α value was calculated at each ratio of HSA and SP([HSA]t/[SP]t) by using data (K, the apparent association constant and N, the apparent number of binding sites) supplied by a dialysis technique. The $1/T_{2\text{bound}}$ and stabilization ratio ($T_{2\text{free}}/T_{2\text{bound}}$) were estimated from the plots of $1/T_{2\text{obs}}$ versus α by the least-squares method.

Equilibrium dialysis experiments were performed using 3 mL Sanko Plastic dialysis cells (Fukuoka, Japan) and a visking membrane. Aliquots of various ratios of SP–HSA mixture were dialysed at 25° for 6 hr against the same volume of buffer solution. Adsorptions of SP and SPM onto membrane or apparatus were negligible, since no adsorptions were detected by measuring the drug concentrations of both compartments in the equilibrium dialysis experiments without HSA. After equilibrium was reached, the free concentration of SP was determined by HPLC. The HPLC system consisted of a Hitachi 655A-11 pump and Hitachi 655A 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 23% acetonitrile and 30 mM phosphate buffer (pH 7.7). The detector was set at 275 nm with a sensitivity of 0.005 absorbance units full scale.

All the binding parameters were estimated by fitting the experimental data to the following equation using a non-linear least-squares computer program (MULTI program) [18]:

$$r = \frac{[D_b]}{[P_t]} = \sum_{i=1}^{m} \frac{N_i K_i [D_f]}{1 + K_i [D_f]} \qquad (2)$$

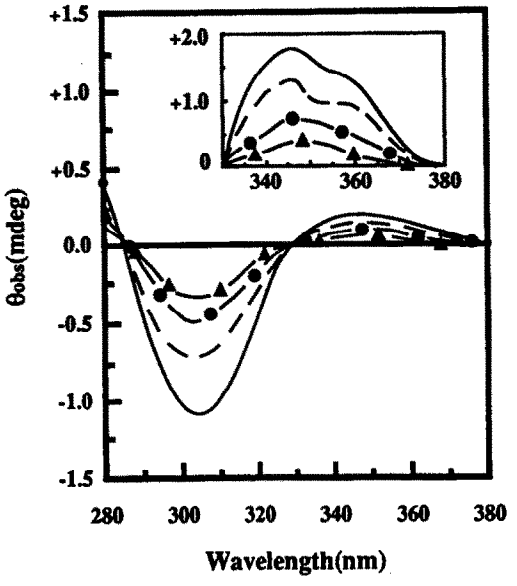


Fig. 1. Observed ellipticity of SP–HSA complex at various drug to protein ratios (D/P) at pH 7.4 and 25°. Drug to HSA (5.0×10^{-5} M) ratios, 0.2 (▲), 0.4 (●), 1.0 (– – –) and 2.0 (——) were measured in two cells of 2 and 20 mm for the normal and amplified version (inset), respectively.

where r is the number of moles of bound drug molecule per mole 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 association constant and the number of binding sites for the ith class of binding sites.

Preparation of HSA derivatives. The modification of HSA with either HNBB or TNM was performed on the basis of the procedures of Koshland *et al.* [19] and Sokolovski *et al.* [20], respectively, as described earlier. The modified percentages were calculated by monitoring the absorbance at 280 and 410 nm for the tryptophan residue (Trp) [21], and at 428 nm for the tyrosine residue (Tyr). The succinylation of HSA was carried out based on procedure of Gounaris and Perlmann [22]. HSA (500 mg) was dissolved in 20 mL of 0.1 M NaCl solution adjusted to pH 8.0 with 0.5 M $NaHCO_3$. Succinic anhydride (50 mg) was slowly added to the protein solution 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 succinic anhydride, 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 were freeze-dried. The unreacted lysine residues (Lys) were determined with the trinitrobenzene sulfonic acid procedure of Hanes *et al.* [23]. The conformation of HSA derivatives was checked by CD and fluorescence measurement and SDS–PAGE. No significant structural alternation or polymerization was observed as compared with native protein. For separation of the unbound drug species, the micropartition system MPS-3 (Amicon Corp., Danvers, MA, U.S.A) was used. One milliliter of sample containing 1×10^{-5} M of protein and drug was centrifuged at 1000 g for 10 min at 25°. The unbound drug concentration to the filtrate was determined by the same procedure described in the equilibrium dialysis section above, except that a different mobile phase was used for SPM [methanol: phosphate buffer (pH 7.4) = 68:32].

RESULTS

Determination of binding parameters

The binding of SP and SPM to HSA induced biphasic Cotton effects by exhibiting a negative maximum at 305 nm and a positive band centered at 350 nm. When the fixed concentration of HSA was titrated with increments of SP, the extrinsic ellipticities of these peaks increased with the isosbestic point at 285 and 330 nm (Fig. 1). A linear relationship was observed between the increment of ellipticities at 305 and at 350 nm (data not shown). This relationship indicates that the induced Cotton effects observed herein were based on a single mechanism under these conditions. Accordingly, the induced

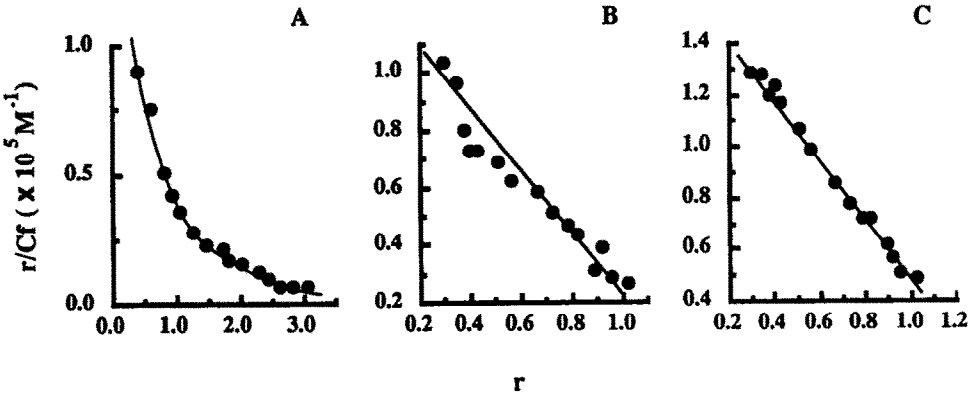


Fig. 2. Scatchard plots of SP binding to HSA at pH 7.4 and 25° determined by equilibrium dialysis (A), CD (B) and fluorescence quenching (C).

Table 2. Binding parameters for SP- and SPM–HSA complexes at pH 7.4 and 25°

Methods	SP				SPM	
	N_1	K_1 ($\times 10^5\,M^{-1}$)	N_2	K_2 ($\times 10^5\,M^{-1}$)	N	K ($\times 10^5\,M^{-1}$)
Equilibrium dialysis	1.05 ± 0.18	1.40 ± 0.20	2.90 ± 0.34	3.76 ± 0.58	—	—
CD	1.09 ± 0.38	1.50 ± 0.52	—	—	—	—
Fluorescence quenching	1.12 ± 0.06	1.00 ± 0.11	—	—	0.95 ± 0.23	1.22 ± 0.18

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

ellipticities at 350 nm were plotted against various ratios of SP to HSA, and then the free and bound fractions of SP were calculated.

The binding of SP and SPM to HSA quenched the intrinsic fluorescence of the single tryptophan in HSA (Trp-214). This phenomenon might be the result of radiationless energy transfer between Trp-214 and the ligands bound to the molecule. In this case, the amount of quenching would be directly proportional to the amount of SP and SPM bound to HSA. Therefore, the fluorescence intensities were plotted as a function of ligand concentration at a fixed HSA concentration to calculate the bound molar ratios of ligand to HSA.

The binding of SP to HSA was also directly monitored by the equilibrium dialysis method. From the dialysis data, two successive saturable processes were observed when SP was bound to HSA, as shown by the Scatchard plot in Fig. 2A. On the other hand, linear Scatchard plots were obtained from quenching and CD data (Fig. 2B and C). The best fitting values for the binding parameters are summarized in Table 2. The binding parameter for the primary binding site was the same by all three methods. The binding constant of SP to the primary binding site was somewhat lower ($10^5\,M^{-1}$) than of other NSAIDs reported previously (10^5–$10^6\,M^{-1}$) [10–13].

The quantitative analysis for the binding of SPM was carried out using the fluorescence quenching method only, for reasons that will be given later on. The Scatchard plot obtained from this method showed a straight line for SPM binding to HSA, which indicates a single class of binding sites (Fig. 2C). Interestingly, the binding parameter for the SPM–HSA complex was very similar to that for the SP–HSA complex.

Thermodynamic analysis

From the temperature dependence of binding constants it is possible to calculate values for the thermodynamic functions involved in the binding process. First, the association constants of both SP and SPM to HSA at three specified temperatures (25°, 35°, 45° for SP and 35°, 40°, 45° for SPM) were estimated. Then, by plotting the association constants according to the van't Hoff equation, the thermodynamic parameters were determined from linear van't Hoff plots and are presented in Table 3. The thermodynamic parameters given here were only evaluated at the primary binding site because only the fluorescence quenching technique was used for these measurements. As shown in Table 3, the formation of SP- and SPM–HSA complexes is an exothermic reaction accompanied by negative enthalpy changes (ΔH) and positive entropy changes (ΔS). Consequently, both binding processes are entropically as well as enthalpically driven. The contribution of $T\Delta S$ to free energy variation (ΔG) for SPM binding was higher than that for SP.

Difference UV absorption spectra of drugs

The effect of HSA on the UV absorption spectra of SP and SPM was examined and compared with the spectra of the drugs in the presence of detergents having different properties. The binding of SP and SPM to HSA resulted in a shift of their absorption spectra toward a longer wavelength (data not shown). The difference spectrum for the binding of SP to HSA was characterized by two negative maxima at 265 and 315 nm, while only a negative maximum at 320 nm was generated by the formation of the SPM–HSA complex, as shown in Fig. 3. The

Table 3. Thermodynamic parameters for SP- and SPM–HSA interaction at pH 7.4

Temperature (°K)	SP				SPM			
	ΔG (kJ/mol)	ΔH (kJ/mol)	ΔS (J/°K · mol)	$T\Delta S/\Delta G$ (%)	ΔG (kJ/mol)	ΔH (kJ/mol)	ΔS (J/°K · mol)	$T\Delta S/\Delta G$ (%)
298	−28.8	−15.2	45.6	47.2				
308	−29.4				−28.9			
313					−29.0	−11.4	56.2	63.4
318	−30.8				−29.2			

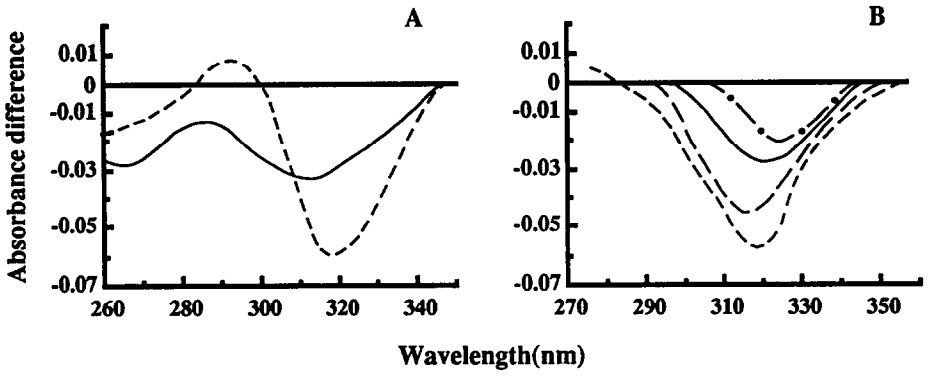


Fig. 3. Difference UV absorption spectra of SP (A) and SPM (B) bound to HSA and detergents at pH 7.4 and 25°. (——) Drug–HSA system; (- - - -) drug–CTAB system; (– – –) drug–PLE system; (●) drug–SLS system. The following concentrations were employed: drug, 10 mM; HSA, 10 mM; detergent, 0.05%; phosphate buffer, 0.067 M.

Table 4. Free and bound relaxation rate and stabilization ratio (T_{2free}/T_{2bound}) for each proton of SP

	Relaxation rate (sec^{-1})						
	2	3	4	8, 12	9, 11	13	14
$1/T_{free}$	9.5	9.7	7.4	10.3	7.2	6.5	5.7
$1/T_{bound}$	369.4	394.5	306.7	208.0	178.8	112.5	179.9
T_{2free}/T_{2bound}	38.9	40.9	41.6	20.2	24.7	17.2	31.7

absorption spectrum of SP was also shifted to a longer wavelength when the SP molecule was dissolved in a solution containing the cationic detergent CTAB (Fig. 3A). Interestingly, this difference spectrum was qualitatively similar to that observed when SP was bound to HSA. However, no influence on the absorption spectrum of SP was seen at CTAB concentrations below the critical micelle concentration or in the presence of the anionic detergent SLS or non-ionic detergent PLE. On the other hand, all three kinds of micelle caused similar spectral changes for SPM as compared with that of HSA (Fig. 3B).

Proton relaxation rate experiment

The ^{1}H-NMR spectra of SP were measured in the absence and presence of HSA to obtain information on the nature of the sites in the complexation process. The same experiments could not be performed for SPM due to its poor solubility to D_2O. Broadening of the proton signals of SP occurred in the presence of HSA. However, this effect was reduced with an increased SP concentration (data not shown). To analyse this phenomenon quantitatively, free and bound relaxation rates and stabilization ratios were calculated for each proton. These results are summarized in Table 4. Interestingly, T_2 values of all peaks were decreased by binding of SP to HSA, but the extent of this effect varied between different portions of the SP molecule.

Identification of binding site

In order to identify the location of the SP and SPM binding sites on HSA, site marker displacement experiments were carried out using a fluorescence probe which specifically binds to a known site on HSA. As shown in Fig. 4, SP remarkably displaced DNSP, which is a marker for Site II [10], via a competitive mechanism, whereas the fluorescence of DNSA (Site I marker) [10] and ACA (Site III marker) [24] bound to HSA was not affected by SP binding. The effect of SP on the fluorescence of the *p*-ABE (a marker for the warfarin site in the Site I area)* –HSA complex exhibited a complicated pattern (see Fig. 4A): the fluorescence intensity of the *p*-ABE–HSA complex was initially enhanced by additional SP up to a 1:1 molar ratio for HSA, and then gradually decreased with an increase in SP concentration. On the other hand, SPM only caused a net decrease in the fluorescence of *p*-ABE bound to HSA, but other probes were not displaced by SPM (Fig. 4B).

* Otagiri M, Miyoshi T, Maruyama T and Takadate A, Characterization of warfarin binding site in Site I area on human and rat serum albumin, manuscript in preparation.

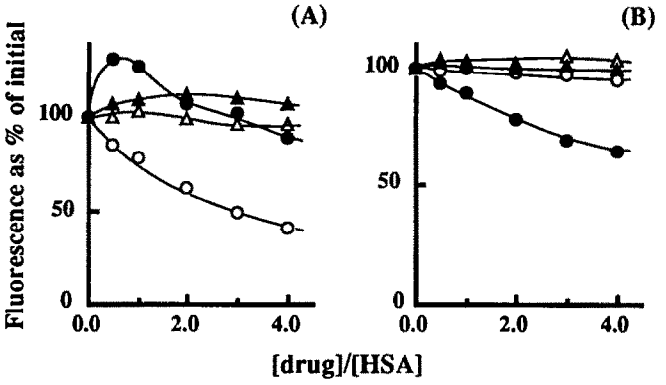


Fig. 4. Effect of SP (A) and SPM (B) on fluorescence of site marker probe. HSA and probes were 5 mM in 0.067 M phosphate buffer (pH 7.4). The mean data of three experiments are shown. Variance of data is less than 8%. Site marker probes used were: *p*-ABE (●; warfarin binding site marker in Site I area), DNSA (▲; Site I area marker), DNSP (○; Site II area marker), ACA (△; Site III area marker).

Table 5. Percentage of reacted amino acid residues in the modified HSA

	Reacted amino acid (%)		
Modified HSA	Trp(1)	Tyr(18)	Lys(59)
HNBB-treated	93	0	2.3
TNM-treated	0	10	0
SA-treated	0	3.2	64

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

Binding of drugs to HSA derivatives

The reaction products of HSA with HNBB, TNM and SA were assayed by the procedures described in Materials and Methods. The results, expressed in terms of per cent reacted residues, with native HSA taken as 0%, are shown in Table 5. It was confirmed that Trp, Tyr and Lys were specifically modified by HNBB, TNM and SA, respectively. Figure 5 shows SP and SPM binding to native, nitrated, succinylated and HNBB-treated HSA. The SP binding was most strongly inhibited by the nitration of only two out of 18 Tyr of HSA. The modification of Lys moderately reduced the binding of SP, while no binding change was observed by the Trp modification. On the other hand, the binding of SPM was altered by neither Tyr nor Lys modification; however, Trp-modified HSA showed some tendency to inhibit the binding of SPM.

Effect of pH on the binding of drugs

The effect of pH on the ellipticity of the SP– and SPM–HSA complexes was examined. Changes in ellipticity as a function of pH could not be observed for both the SP– and SPM–HSA complexes. In order to confirm the above results, the binding parameters for the SP–HSA complex were estimated by the dialysis technique at several pH values. As indicated by the CD data, both the association constants and the number of binding sites were not significantly affected by pH.

DISCUSSION

In plasma, HSA and α_1-acid glycoprotein are the major carrier proteins for drugs [25, 26]. In spite of the fact that both proteins carry a net negative charge at physiological pH, HSA strongly binds acidic drugs rather than basic drugs, whereas α_1-acid glycoprotein is the main plasma protein for binding basic drugs. This suggests the importance of negative charge on drugs for binding to HSA. Interestingly, long-chain aliphatic compounds which possess a negatively charged portion other than a carboxyl group, e.g. sulfate and sulfonate group, exhibit different binding behavior [27, 28]. In the case of NSAIDs, as described in the introduction, the high affinity binding site of NSAIDs seems to be different between carboxylic and non-carboxylic compounds. These findings suggest the importance of the carboxyl group of the ligand for binding to HSA.

This study was performed to determine the role of the carboxyl group in binding to HSA, and revealed the difference in binding characteristics between SP and SPM. Their intentions were analysed by Scatchard plots in order to characterize binding ability, binding mode, location and nature of the binding site.

Binding ability

We attempted to estimate the binding parameter by direct (equilibrium dialysis) and indirect (fluorescence, CD) methods. Unfortunately, the binding experiments of SPM could not be performed using equilibrium dialysis for the following reasons: (a) SPM has a poor solubility to aqueous solution (see Table 1); (b) HSA has been proposed to exhibit weak esterase-like activity [29, 30], such that HSA may cause hydrolysis of SPM at high SPM

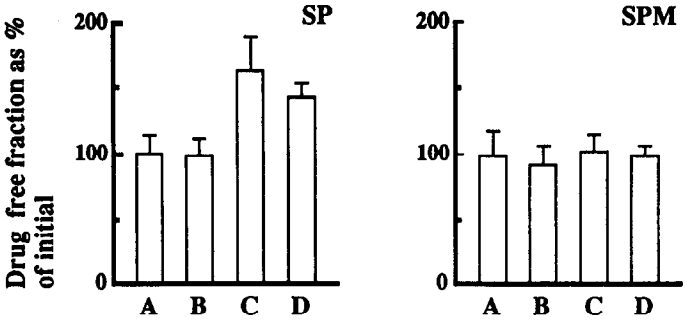


Fig. 5. Binding of SP and SPM to native and modified HSA at pH 7.4 and 25°. (A) Native HSA; (B), HNBB-treated HSA; (C), TNM-treated HSA; (D), SA-treated HSA. The following concentrations were employed: protein, 10 mM; SP and SPM, 10 mM. Each column and bar are the mean ± SD of data from five experiments.

concentrations during a long incubation time. Thus, we used the fluorescence quenching technique to estimate the binding parameters for SPM to HSA because this method can be carried out rapidly at low ligand and protein concentrations. It was confirmed that SPM was stable under our experimental conditions.

The binding data obtained from spectroscopic techniques only detected the primary binding site for the SP–HSA system. However, the Scatchard plot obtained from dialysis data was characterized by heterogeneous binding processes. This inconsistency means that SP molecules are optically inactive at a second class of binding sites. In fact, the binding parameters for the primary binding site are in excellent agreement between methods. As shown in Table 1, the partition coefficient of SPM is more than 10 times greater than that of SP. Since it has been proposed that the binding strength of ligands to HSA is generally correlated with hydrophobicity of ligands [31–33], we expect that the binding constant of SPM to HSA would be considerably larger than that of SP. However, the binding parameters of SPM and SP were very similar. This unpredicted result not only suggests the significant contribution of the carboxyl group in the binding mechanism, but also suggests differences in the binding region between SP and SPM on the HSA molecule.

Binding mode

The binding of any ligand to biological macromolecules always involves intermolecular forces whose variety is rather limited and which contribute to both affinity and specificity [34]. In order to obtain such information, thermodynamic analyses were performed for SP and SPM binding. In these experiments, the formation of SP- and SPM–HSA complexes was accompanied by negative ΔH and positive ΔS values. Neméthy and Scheraga [35], Timasheff [36] and Ross and Subramanian [37] have characterized the sign and magnitude of the thermodynamic parameter associated with various individual kinds of interaction that may take place in protein association processes, as described below. From the point of view of water structure, a positive ΔS value is frequently taken as evidence for hydrophobic interactions. Furthermore, specific electrostatic interactions between ionic species in aqueous solution are characterized by a positive value of ΔS and a negative ΔH value. In contrast, van der Waals interactions introduced as a consequence of the hydrophobic effect are one of the most important factors contributing to the observed negative values of ΔH and ΔS. Accordingly, it is not possible to account for the thermodynamic parameters of SP- and SPM–HSA complexes on the basis of a single intermolecular force model, as can be expected from their structures. It is more likely that hydrophobic, electrostatic and van der Waals interactions are involved in their binding processes. However, ionic interactions can not be expected for SPM binding due to the lack of a carboxyl group. Thus, the ΔS value obtained from the SPM system is based mainly on hydrophobic interactions. In other words, hydrophobic interaction for SPM binding should be more important than in the case of the SP–HSA complex. In fact, the binding of SPM to HSA is more entropically controlled than SP binding. However, thermodynamic parameters upon protein association processes are also affected by altering solvent structure and by changing the low frequency vibrational mode of the protein structure [38–40]. More insight into the mechanism of binding can be gained by measuring ΔH as a function of temperature.

SP and SPM showed almost identical UV absorption spectra in buffer solution. However, the binding of SP and SPM to HSA caused distinct features in their difference spectra. The former was characterized by two negative maxima, while the latter showed only one negative peak. This difference indicates that the binding mechanisms of SP and SPM are somewhat different from each other. To clarify these differences, the same measurements were performed in the presence of cationic, anionic and non-ionic detergent micelles, because the detergent micelles have been used as a simple model of a binding site on a protein and membrane [41–43], due to endowing a hydrophobic region with a charged moiety.

In the case of SP, only the CTAB micelle, which is charged positively, caused an effect which is similar to HSA. Since SP is an amphipathic molecule, the SP molecule is likely to be adsorbed to the apolar–polar interface of the CTAB micelle in such a manner that the carboxyl group is located at the aqueous layer and can interact with the quaternary ammonium moiety, while the remaining hydrophobic side chain is directed towards the hydrophobic centre. On the other hand, similar difference spectra for the HSA–SPM complex are generated by all kinds of micelle system. It clearly suggests that the charged portion of the detergent is not involved in the binding of SPM to the detergent. Thus, SPM is more likely to partition into the hydrophobic interior of micelles than be embedded in the hydrocarbon–water interface. These data would suggest that the HSA binding site for SP and SPM is made up of a hydrophobic patch to accomodate the heterocyclic ring and a cationic centre at or near the SP binding site.

Nuclear magnetic relaxation techniques were used to identify the portion of the SP molecule involved in complexation with HSA. The proton signals of the SP molecule were broadened during complex formation. Particularly large stabilization ratios were assigned to the protons of the thiophen ring and methyl group in the propanoate portion as compared with that of the benzene ring. The stabilization ratio means that both variation in the SP correlation time and changes in the interaction occur when binding takes place [44, 45]. The former effect involves a theoretically constant value of this ratio for each proton, the latter effect results in an increase in the T_2 ratio, especially for protons that get close to HSA as a consequence of a stronger or new intermolecule interaction.

These results suggest that the thiophen ring and methyl group in the propanoate portion are rigidly immobilized in the binding site, while the benzene ring retains its freedom of motion to a much greater extent. This assumption is supported by the generation of an extrinsic Cotton effect for the SP–HSA interaction because, if the SP–HSA complex is fixed by a one point electrostatic attachment of a carboxylate, it would allow the chromophore to rotate freely. No optical activity would be expected under such conditions.

Considering the thermodynamic analysis, together with the UV absorption and NMR spectra, we regard the binding mode of SP and SPM to HSA to be as follows: the hydrophobic side chain of the SP molecule, especially the thiophen ring and methyl group of the propanoate portion, is deeply inserted into the hydrophobic crevice, while the carboxyl group interacts with a cationic subsite located at or near the hydrophobic surface of HSA. On the other hand, the SPM molecule is inserted into a hydrophobic patch on HSA, creating an interaction similar to an interaction with a membrane.

Location and nature of binding site

The results of fluorescence probe experiments indicate that Site II is the primary binding site for SP, similar to the result for other carboxylic NSAIDs, while SPM specifically interacts with the warfarin site in the Site I area. The SP binding to Site II simultaneously changes the microenvironment of the *p*-ABE binding site until saturation of SP binding at the high affinity site, and then further SP molecules directly displace the *p*-ABE molecules bound to the warfarin site. Thus, SP also binds to the warfarin site with low affinity. Similar phenomena have been reported for the allosteric interaction of warfarin and oleate ion with HSA by Chakrabarti [46], and for the positive energy coupling of dansylsarcosine or sulfobromophthalein on the warfarin–HSA interation by Sjöholm *et al.* [11].

The above observations suggest that the carboxyl group is needed for the high affinity of SP to Site II, while the heterocyclic ring as a basic structure of SP and SPM is favoured for binding to the warfarin site. Very recently, He and Carter [47] solved the three dimensional structure of HSA. On the basis of this molecular configuration, the high affinity binding site of SP and SPM may be located within subdomain IIIA and IIA of HSA, respectively.

Derivatives of HSA were prepared by partial chemical modification of native protein with HNBB, TNM and SA to obtain information about the structure of the SP and SPM binding site. It is well known that some amino acid residues are specifically involved in the drug binding site, e.g. Tyr-411, Lys-195 and His-146 in Site II [48, 49] and Trp-214 in a part of the Site I area [50]. Our data indicate that Tyr and Lys take up an important part of the SP binding site, as expected from the result of the fluorescence probe experiment. A positive charge is probably located in or near the high affinity site of SP. At pH 7.4 the amino group of Lys exists in ionized form so that this cation may be a point of attachment for the carboxyl group of SP. Several investigations have demonstrated that some reactive Lys residues are modified by both endogenous and exogeneous substances during disease states and aging, e.g. glycation [51, 52]. Therefore, it is possible that such modification may be increased by the free concentration of SP.

In contrast to SP, there seems to be no amino acid residue particularly participating in the binding of SPM. This assumption does not contradict the proposed binding mode of SPM, wherein the binding of SPM is not specific, as in partitioning between aqueous and hydrophobic phases. However, it is also possible to interpret our data by saying that the amino acid residues which form the SPM binding site may not be modified under our experimental conditions. Thus, further specific modifications at different positions will be needed to clarify the amino acid composition of the SPM binding site.

In HSA, proton-linked conformational changes occur in the physiological pH range (pH 6–9), commonly referred to as the N–B transition [53, 54]. The N and B forms of HSA can bind ligands with different affinities, for instance, warfarin and diazepam prefer to bind to the B form [55, 56], whereas pirprofen and benoxaprofen prefer to bind to the N form [9, 57]. Even though SP and SPM share the binding site with the above drugs, the N–B transition does not seem to affect their binding behavior. It is likely that the binding sites of SP and SPM are independent of structural fluctuations accompanying the N–B transition.

We cannot explain this phenomenon from our limited data.

The results presented demonstrate that the binding of SP and SPM to HSA is quantitatively very similar, though their binding mode and the locations of the binding sites differ from each other. Therefore, it is concluded that the carboxyl group is necessary for the binding of SP to Site II on HSA. These findings will not only aid understanding of the NSAID–enzyme and –receptor interactions, but may also be useful for the design of new NSAIDs.

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