Binding of Carprofen to Human and Bovine Serum Albumins

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The binding of carprofen (CP) to human serum albumin (HSA) and bovine serum albumin (BSA) was compared using equilibrium dialysis method. The affinity of CP for the primary binding site was BSA > HSA. However, the number of primary binding sites (n_1) was 1.94 on HSA, considerably greater than that on BSA (0.79). The displacement of the binding of CP to HSA and BSA was studied in the presence of phenylbutazone (PB, site I marker), ibuprofen (IB, site II marker) or 2,3,5-triiodobenzoic acid (TIB, both site I and II marker). The binding of CP to HSA was altered by PB, IB and TIB, while the binding of CP to BSA was not changed by PB, although it was reduced by IB and TIB. These results suggested that, for the binding of CP, HSA has two major sites (site I and site II), whereas BSA has a single primary site (site II). The binding characteristics of 2-anthracenecarboxylic acid with HSA and BSA were found quite similar to those of CP. Thus, it seemed that long, planar compounds with a carboxyl group at one end can bind to site I and site II on HSA, but only to site II on BSA. The difference between HSA and BSA in the affinity of site I may be due to the difference in the basic and hydrophobic amino acid residues.

Keywords protein binding; carprofen; human serum albumin; bovine serum albumin; binding site; equilibrium dialysis

The binding of drugs to serum albumin is an important pharmacokinetic property because it greatly influences their distribution, excretion and pharmacological effect in the body.[1] On human serum albumin (HSA), there are specific binding sites called site I (warfarin site) and site II (diazepam site), which were characterized by Sudlow *et al.*[2] and Sjöholm *et al.*[3] Recently, the two binding regions on HSA were revealed by crystallography.[4] Since there is an approximately 80% homology of the amino acid sequences between HSA and bovine serum albumin (BSA),[5–7] BSA is thought to have binding sites similar to those of HSA.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used in clinical therapy. It is well known that 2-arylpropionic acid NSAIDs including ibuprofen (IB)[8] and ketoprofen[9] are bound to site II. In the present study, the binding of carprofen (CP), which is also a 2-arylpropionic acid NSAID, with HSA and BSA was measured by equilibrium dialysis (ED) method, and the identification and characteristics of the binding sites for CP were investigated using several displacers. Contrary to expectations, it was found that, for the binding of CP, HSA has two major sites, site I and site II, whereas BSA has a single primary site, site II.

Experimental

Materials HSA (fraction V; Sigma Chemical Co., St. Louis) and BSA (fraction V; Armour Pharmaceutical Co., Kankakee) were used. The molecular weight was assumed to be 67000 for both albumins, and their concentration was determined by measuring the absorbance at 280 nm using $E_{1\,cm}^{1\%}=5.30$[10] and 6.67[11] for HSA and BSA, respectively. CP and 2,3,5-triiodobenzoic acid (TIB) were purchased from Sigma Chemical Co., St. Louis. 2-Anthracenecarboxylic acid (2-ACA) and 9-anthracenecarboxylic acid (9-ACA) were obtained from Tokyo Kasei Kogyo (Tokyo). Phenylbutazone (PB) was generously provided by Nihon Ciba-Geigy Co. (Tokyo), and IB by Kaken Seiyaku Co. (Tokyo). All other reagents used were commercial products of special grade. All final solutions were made with 0.15 M Tris–HCl buffer of pH 7.4.

ED Method Using 3.0 ml dialysis cells devised by Goto *et al.*,[12] sample solutions (3.0 ml) were shaken for 16 h at 25±1 °C in a thermostatic environment. Spectrapor 1 dialysis membrane (Spectrum Medical Inc., Los Angeles) was used after being boiled four times in distilled water. The decrease in the unbound drug content was measured, and the binding parameters were calculated. Adsorption of drugs on the membrane was negligible. The wavelengths of excitation were 298, 366, and 365 nm and the fluorescence was measured at 365, 430, and 415 nm for CP, 2-ACA, and 9-ACA, respectively. The fluorescence measurements were made in a Hitachi MPF-4 fluorescence spectrophotometer. The concentration of albumins was 5.0×10^{-6} M. Each point in the Scatchard plots[13] represents the mean value of four or five experiments.

Calculation The binding parameters were calculated by linear regression when the Scatchard plot was linear. When the plot was curvilinear, the data were fitted to Karush's equation[14] by a nonlinear least squares method (modified Marquardt, Gauss-Newton, or Simplex method). The calculation of binding parameters was performed with an NEC PC-9801 personal computer.

Results and Discussion

Binding of CP to HSA and BSA The binding data for each albumin were analyzed by means of Scatchard plots. Figure 1 illustrates the bindings of CP ($3.0—60.0\times10^{-6}$ M) to HSA and to BSA (both 5.0×10^{-6} M). The plots for HSA and BSA are both curvilinear, suggesting the presence of at least two independent types of binding site.

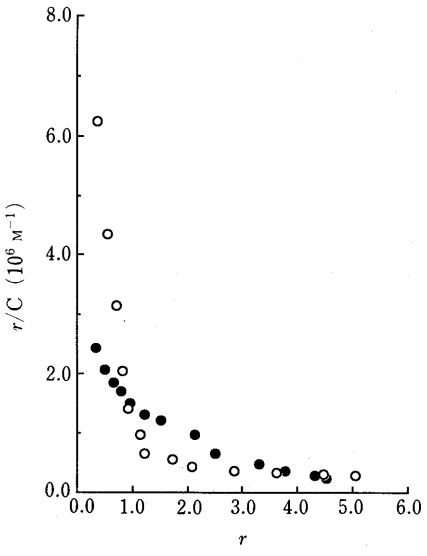

Fig. 1. Scatchard Plots for the Binding of CP to Serum Albumin
●, HSA; ○, BSA.

TABLE I. Influence of Displacer Drugs (5.0×10^{-5} M) on Binding Parameters for CP with HSA and BSA (5.0×10^{-6} M)

Drug	HSA						BSA					
	n_1	K_1 (10^6 M^{-1})	$\log n_1K_1$	n_2	K_2 (10^4 M^{-1})	$\log n_2K_2$	n_1	K_1 (10^6 M^{-1})	$\log n_1K_1$	n_2	K_2 (10^4 M^{-1})	$\log n_2K_2$
CP	1.94	1.08	6.32	4.68	7.50	5.55	0.79	15.55	7.09	14.18	2.23	5.50
+PB	0.95	2.48	6.37	6.58	5.40	5.55	0.74	15.39	7.06	24.63	0.92	5.36
+IB	1.15	0.87	6.00	10.63	2.35	5.40	—	—	—	10.40	3.99	5.62
+TIB	—	—	—	4.00	13.25	5.72	0.53	9.93	6.72	16.39	1.26	5.51

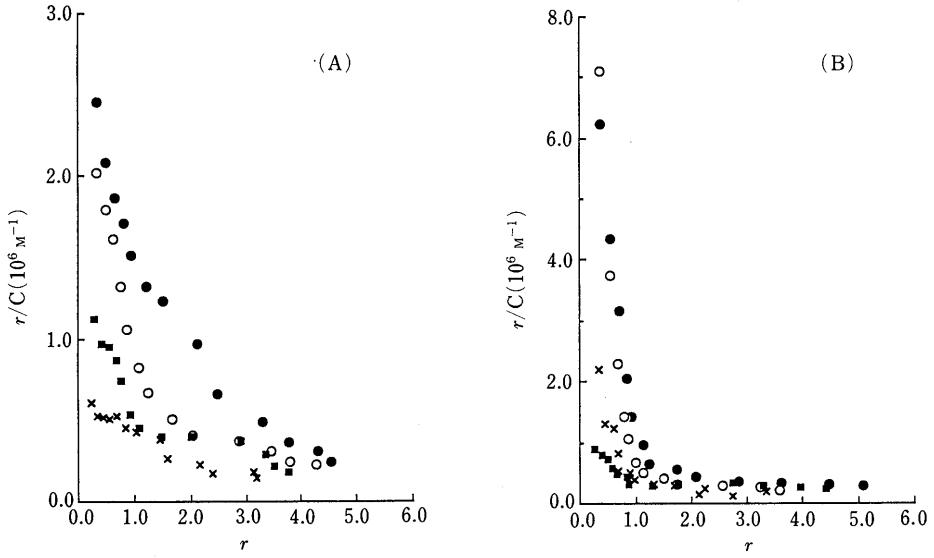

Fig. 2. Scatchard Plots for the Binding of CP Alone or in Combination with Displacer Drugs to HSA (A) and BSA (B)
●, CP alone; ○, in the presence of PB; ■, in the presence of IB; ×, in the presence of TIB.

The calculated binding parameters are listed in Table I.

The value of the primary binding constant (K_1) for HSA (1.08×10^6 M^{-1}) agreed with those reported by Whitlam and Brown[15] and Iwakawa *et al.*[16] The apparent number of primary binding sites (n_1) for HSA (1.94) was not in accord with that[15] reported. The n_1 value of CP was also different from those of other 2-propionic acid derivatives reported previously.[8,9]

The value of the primary binding affinity ($\log n_1K_1$) for BSA was higher than that for HSA. The n_1 value for BSA was about 1, differing from that for HSA. These results suggest that CP binds to HSA through a mechanism different from that for the binding to BSA. The degree of coincidence of the amino acid sequences of HSA and BSA is approximately 80%.[5-7] In spite of the high degree of sequence similarity, there were differences in binding characteristics for CP to HSA and BSA.

Identification of the Binding Sites Two specific binding sites have been established on the HSA: site I and site II.[2,3] Matsushita *et al.*[17] and Panjehshahin *et al.*[18] reported that BSA has binding sites with similar properties ot those of site I and site II on HSA. To identify the binding sites for CP on HSA and BSA, competitive binding studies were performed using three marker drugs: PB (site I marker), IB (site II marker), and TIB (site I and site II marker). Figure 2 shows Scatchard plots for bindings of CP to HSA and BSA in the presence of the three marker drugs. The binding parameters calculated are listed in Table I. The n_1 value of CP for HSA was reduced to about one half in the presence of PB and IB, and disappeared in the presence of TIB. In the presence of PB, the unbound fraction of CP increased and it is apparent that CP was displaced by PB. The K_1 value was increased because of the unchanged intercept of Scatchard plot; the reason is uncertain. Thus, it is quite apparent that CP binds to both site I and site II on HSA.

However, closer examination of Fig. 2A shows that the r/C value is markedly reduced in the presence of IB at a low r region. On the other hand, in the presence of PB, the r/C value is slightly reduced at a low r region, but is greatly decreased with an increase of the r value. This suggests that CP binds mainly to site II at a low concentration. However, the binding of CP to site I increases with an increase of CP concentration. Iwakawa *et al.*[16] reported that, with HSA, the binding of CP was reduced in the presence of diazepam (site II marker) but was not affected in the presence of warfarin (site I marker). So we reexamined the competitive binding of CP (HSA: 5.0×10^{-6} M, CP: 3.0—50.0×10^{-6} M) in the presence of warfarin (5.0×10^{-5} M) using ED at 25 °C. Our result under the experimental conditions described above was that the n_1 value of CP for HSA was decreased to about one half in the presence of warfarin; this result was similar to that in the presence of PB. Thus, it is apparent that the binding of CP to HSA is displaced in the presence of site I binding drugs including PB and warfarin. This does not agree with

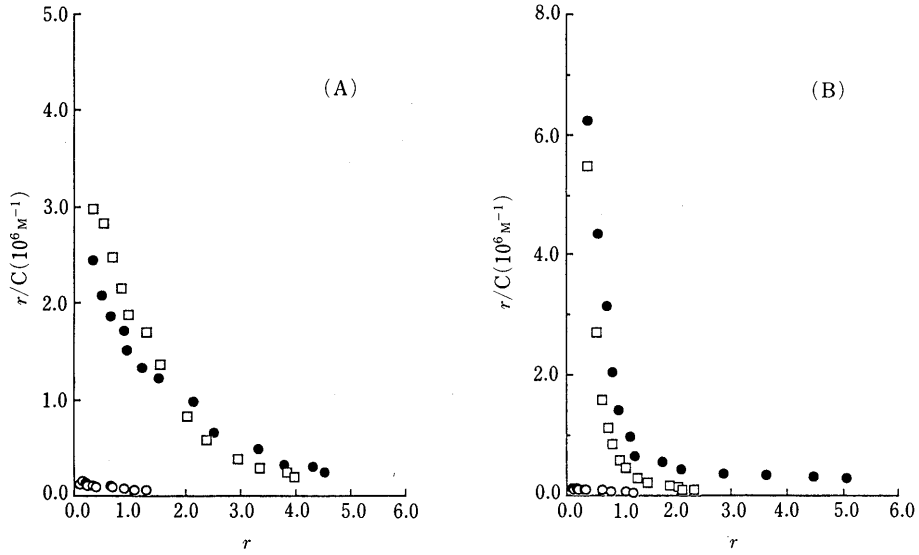

Fig. 3. Scatchard Plots for the Binding of Drugs to HSA (A) and BSA (B)
●, CP; □, 2-ACA; ○, 9-ACA.

TABLE II. Parameters for Binding of Drugs ($2.0—60.0 \times 10^{-6}$ M) with HSA and BSA (5.0×10^{-6} M)

Drug	HSA						BSA					
	n_1	K_1 (10^6 M^{-1})	$\log n_1K_1$	n_2	K_2 (10^4 M^{-1})	$\log n_2K_2$	n_1	K_1 (10^6 M^{-1})	$\log n_1K_1$	n_2	K_2 (10^4 M^{-1})	$\log n_2K_2$
CP	1.94	1.08	6.32	4.68	7.50	5.55	0.79	15.55	7.09	14.18	2.23	5.50
2-ACA	1.98	1.71	6.53	4.81	3.86	5.27	0.67	15.86	7.03	2.42	8.45	5.31
9-ACA	—	—	—	2.24	5.80	5.11	—	—	—	2.26	5.03	5.06

the results of Iwakawa and his colleagues. The discrepancy is probably due to their displacement experiment in which the measurement was done only at one low concentration of CP.

On the other hand, the binding of CP to BSA was markedly reduced by IB and TIB, but not by PB. Thus, it is suggested that CP binds to the IB binding site (referred to site II), but not to the PB binding site (referred to site I).

Characterization of the Binding Sites on HSA and BSA The binding behavior of CP to HSA differs from that of other 2-propionic acid derivatives. In order to characterize the binding sites, bindings of 2-ACA and 9-ACA, which are both long planar molecules with a carboxyl group similar to CP, were measured with HSA and BSA by ED method, and the results were compared. Figure 3 shows plots for binding of CP, 2-ACA, and 9-ACA to both albumins. The Scatchard plots for 2-ACA to HSA and BSA are curvilinear, suggesting the presence of at least two independent types of binding sites. The plot of 9-ACA appears to be linar, suggesting a single class of weak binding sites. The calculated binding parameters are listed in Table II. The n_1 values of 2-ACA with HSA and BSA, like those of CP, were about 2 and 1, respectively. As shown in Fig. 3, however, a very poor binding at lower drug concentration was observed for 9-ACA with HSA and BSA compared to the binding for CP and 2-ACA. This indicates that the primary binding site for 9-ACA does not exist on the albumins.

To determine the binding sites for 2-ACA on HSA and BSA, displacement studies were carried out using the three marker drugs. Figure 4 shows Scatchard plots for binding of 2-ACA to HSA and BSA in the presence of PB, IB, and TIB. The calculated binding parameters are listed in Table III. With HSA, the n_1 value of 2-ACA was decreased to about one half in the presence of PB and IB, and disappeared in the presence of TIB. In BSA, as shown in Fig. 4B, the binding of 2-ACA was clearly reduced by IB and TIB, but not by PB. Thus, it can be said that 2-ACA binds to both site I and site II on HSA, and binds to site II on BSA.

Wanwimolruk *et al.*[19] reported that a hydrophobic cleft about 16 Å deep and 8 Å wide exists in site II on the HSA molecule. Matsushita *et al.*[17] indicated that a similar site II cleft (10—15 Å deep) also exists on the BSA molecule. CP and 2-ACA, both capable of binding to site II, have molecular lengths within this range. For 9-ACA, the binding affinity was much lower than those for CP and 2-ACA. This is probably because the anthracene ring of 9-ACA cannot be positioned so that it fits the hydrophobic cleft on the albumin molecule when the carboxyl group at position 9 interacts with the basic amino acid residue at the entrance to site II.

He and Carter recently crystallographically determined the three dimensional structure of HSA to a resolution of 2.8 Å.[4] Site II is thought to exist in subdomain IIIA. We found the specific binding sites for CP and 2-ACA to be site I and site II on HSA. In site II, the carboxyl

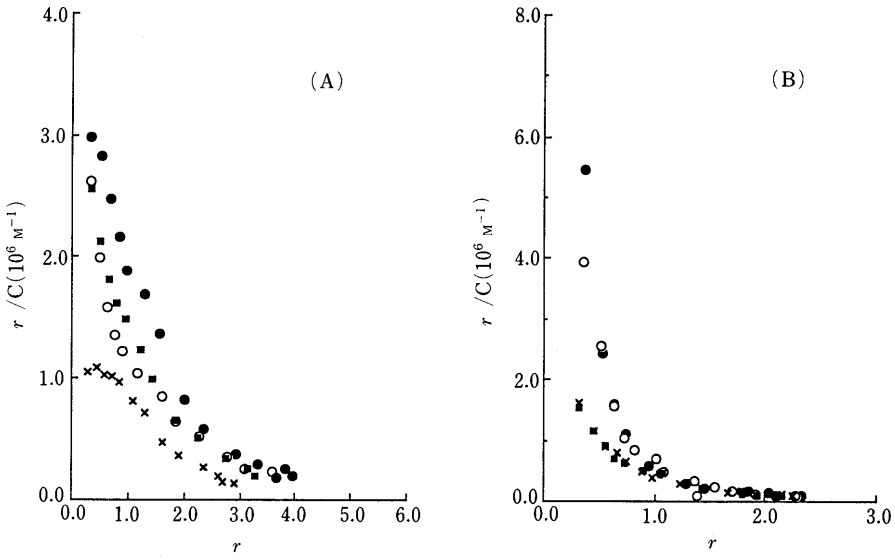

Fig. 4. Scatchard Plots for the Binding of 2-ACA Alone or in Combination with Displacer Drugs to HSA (A) and BSA (B)
●, 2-ACA alone; ○, in the presence of PB; ■, in the presence of IB; ×, in the presence of TIB.

TABLE III. Influence of Displacer Drugs (1.0×10^{-5} M) on Binding Parameters for 2-ACA with HSA and BSA (5.0×10^{-6} M)

Drug	HSA						BSA					
	n_1	K_1 (10^6 M^{-1})	$\log n_1K_1$	n_2	K_2 (10^4 M^{-1})	$\log n_2K_2$	n_1	K_1 (10^6 M^{-1})	$\log n_1K_1$	n_2	K_2 (10^4 M^{-1})	$\log n_2K_2$
2-ACA	1.98	1.71	6.53	4.81	3.86	5.27	0.67	15.86	7.03	2.42	8.45	5.31
+PB	0.94	2.54	6.38	3.93	11.72	5.66	0.72	9.22	6.82	1.86	12.31	5.36
+IB	1.18	2.27	6.43	3.15	13.28	5.59	0.62	3.25	6.30	1.86	14.10	5.42
+TIB	—	—	—	3.11	37.04	6.06	0.84	2.44	6.31	2.90	4.10	5.08

group of CP may be caught by the basic amino acid residues, and the planar aromatic ring may then fall into a hydrophobic pocket. However, since CP and 2-ACA also bind to site I, planar hydrophobic compounds with a suitable molecular length and an acidic group at one end may also fit a hydrophobic pocket of site I on HSA. In the case of BSA, CP and 2-ACA bind only to site II. Accordingly, it seems that the environment of site I on BSA, such as the depth and width of the hydrophobic pocket, are different from that on HSA.

As we have described, there is a difference of the binding sites for CP and 2-ACA between HSA and BSA. Site I on HSA is thought to exist in subdomain IIA (Lys199–Glu292).[4] Consideration of the amino acid sequences of HSA and BSA suggests that the difference of the binding characteristics is caused by the variation in polar and hydrophobic amino acid residues between HSA and BSA. For polar residues, Lys199, Ala215, Arg222, and Thr243 of HSA correspond to Arg197, Ser213, Lys220, and Lys241 of BSA, respectively. For the hydrophobic residues, Leu203 and Phe211 of HSA correspond to Ile201 and Leu209 of BSA. This difference may also affect the binding behavior of drugs to albumins.

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