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BINDING OF PIRPROFEN TO HUMAN SERUM ALBUMIN STUDIED BY DIALYSIS AND SPECTROSCOPY TECHNIQUES

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Abstract—The interaction of pirprofen with human serum albumin (HSA) was investigated by equilibrium dialysis and spectroscopic (UV absorption, fluorescence, CD, NMR) techniques. It was found that HSA binds pirprofen nonstereospecifically. The binding of pirprofen depends upon the N–B conformational change of albumin. Chloride ions appear to displace the drug from its binding site. The thermodynamic parameters suggest that the interaction may be explained by electrostatic as well as hydrophobic forces. The absorption spectral changes which accompanied the binding of pirprofen to HSA implied that the aromatic portion of drugs was inserted into the hydrophobic crevice in the protein, while the carboxyl group of the drug interacted with a cationic site on the albumin surface. The NMR data indicated that the pyrroline ring and propionic acid parts may be the major binding site for HSA. A specific binding site for pirprofen on the HSA was found to be site II, benzodiazepine site, using fluorescence probes and drug markers. In addition, from the binding data with modified HSA, it seems that Tyr-411 is specifically involved in pirprofen binding.

The binding of pirprofen (Fig. 1) to human serum albumin (HSA) has been quantitatively investigated [1]. However, the published result has mainly shown the percentage binding (98.6% bound) [1]. In addition, the binding mode and the location of the drug binding site on HSA molecule have not been determined. A range of acidic non-steroidal anti-inflammatories including flufenamic acid and ibuprofen are bound to site II on HSA molecule [2–5]. These findings lead us to expect that the primary binding site of pirprofen may be classified to site II. However, some anti-inflammatory drugs such as phenylbutazone share the same binding site with warfarin (site I drug) [2–5]. Thus, the present study was undertaken to identify the binding site of pirprofen on HSA and to elucidate the binding mechanism of the drug to HSA.

MATERIALS AND METHODS

Materials. HSA fraction V (lot No. 54F-9344) was obtained from Sigma Chemical Co. (St Louis, MO). Pirprofen and its enantiomers were donated by Ciba Geigy Co. (Summit, NJ). Ibuprofen (Kaken Pharmaceutical Co., Tokyo, Japan), digitoxin (Mitsubishi Yuka Pharmaceutical Co., Ibaraki, Japan) and chlorpropamide (Toyama Kagaku Co., Toyama, Japan) were used as supplied. 7-Anilinocoumarin-4-acetic acid (ACA) was a generous gift from Prof. Goya of Kumamoto University. All other materials were reagent grade, and all solutions were prepared in deionized and distilled water. All the buffers used were prepared with sodium phosphate dibasic and sodium phosphate monobasic. The pH values were checked at 25° with a suitably standardized pH meter.

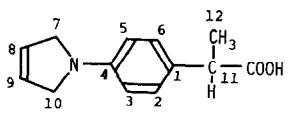

Fig. 1. Structural formula of pirprofen 2-[3-chloro-4(3-pyrrolin-yl)-phenyl]-propionic acid.

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Apparatus. Circular dichroism (CD) measurements were made with a JASCO model J-50A spectropolarimeter (Tokyo, Japan) in cells of pathlength 5 or 10 mm. All solutions were scanned from a wavelength at which no induced optical activity was observed. The induced ellipticity was defined as the ellipticity of the drug albumin mixture minus the ellipticity of the albumin alone at the same wavelength and is expressed in degrees.

Absorption and absorption difference spectra were recorded with a Hitachi 556S dual-wavelength spectrophotometer (Tokyo, Japan). Difference spectra were measured by using a pair of 10 mm split-compartment-tandem cuvette.

Fluorescence measurements were made with a Hitachi 650-60 fluorescence spectrophotometer (Tokyo, Japan). The excitation wavelength was 340–375 nm, and the emission was read at 430–480 nm.

^{1}H-NMR were recorded on a JNM-GX 270 spectrometer (270 MHz) (Tokyo, Japan) at 20 ± 0.5°. The ionic strength of the solution was maintained at a constant value of 0.154 by means of KD_2PO_4 and Na_2DPO_4, and the pD was maintained at 7.4. Pirprofen was used at concentrations of 2.5 to 20.0 × 10^{-3} M while the concentration of HSA was fixed at 5 × 10^{-5} M. The transversal relaxation rates ($1/T_2$) were calculated from the expression

$(1/T_2) = \pi\, r_{1/2}$, where $r_{1/2}$ is the full-line width at half-maximum peak height. For each signal at the first exchange limit for a single binding site on the macromolecule [6]

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

where $1/T_{2obs}$, $1/T_{2free}$, $1/T_{2bound}$ are the observed transversal relaxation rate, relaxation rate of free and bound pirprofen and α is the fraction bound to HSA. T_{2bound} is shown as the average of T_2 on some binding sites, based on the assumption that the characteristics of the primary and the secondary binding site of pirprofen to HSA are almost the same. In the case of $[Drug]_t \gg [HSA]_t$, then

$$\alpha = \frac{n_1 K_1 [HSA]_t}{1 + K_1 [Drug]_t} + \frac{n_2 K_2 [HSA]_t}{1 + K_2 [Drug]_t}$$

α is calculated at each ratio $[HSA]_t/[Drug]_t$, by using n and K values supplied by dialysis techniques.

The $1/T_{2bound}$ and the ratio T_{2free}/T_{2bound} were estimated from the plots of $1/T_{2obs}$ versus α by the method of least-squares.

Preparation of HSA derivatives. The modification of the lone tryptophan residue in HSA was performed according to Koshland *et al.* [7]. HSA was dissolved in 10 M urea adjusted to pH 4.4 by acetic acid, and a 1100-fold molar excess of 2-hydroxy-5-nitrobenzyl bromide was added, and then the reaction mixture was shaken occasionally. After 2 hr the insoluble hydrolyzed reagent was separated by centrifugation. The supernatant was dialyzed against water for 60 hr and then lyophylized. Another HSA sample was treated similarly but without 2-hydroxy-5-nitrobenzyl bromide and used as a control. The degree of modification was determined by UV absorbance according to the method of Karkhanis *et al.* [8], using the equation

$$\frac{\text{number of tryptophans}}{\text{mole of HSA}} = \frac{A_{410} \times 66500 \times E^{1\%}_{280}}{18700\,(A_{280} - 0.14\,A_{410})}$$

The degree of modification was 1.1 residue per mole HSA.

The tyrosine residues in HSA were modified by the method of Sokolovsky *et al.* [9]. HSA was dissolved in 0.05 M Tris buffer adjusted to pH 8.0, and 3.2-fold molar excess of tetranitromethane was added. The reaction mixture was shaken for 2 hr at 25 ± 1°, dialyzed against distilled water for 60 hr, and then lyophylized. The degree of tyrosine residue modification was calculated from the UV absorbance at 428 nm, and the degree of modification was 1.7 residue per mole HSA.

Equilibrium dialysis. The dialysis experiments were performed in a Sanko Plastic dialysis cell (Fukuoka, Japan). In the apparatus, the chambers are divided with Visking membrane. HSA solution (2 ml) was poured into the one compartment and 2 ml of pirprofen solution into the opposite. Adsorption of pirprofen onto membranes was negligible and the volume of the solutions on either side of the membrane stayed constant during the dialysis procedure. After the 12 hr dialysis, the free concentration of pirprofen was determined from the UV absorbance at 253 nm.

RESULTS AND DISCUSSION

Binding parameters

The induced CD spectra of pirprofen and its enantiomers bound to HSA are shown in Fig. 2. The changes in Cotton effects observed with binding to HSA are similar in signal and wavelength position for both enantiomers. The induced ellipticities at 320 nm for various ratios of pirprofen to HSA were plotted (not shown). Following the method of Rosen [10], a tangent to the plot of induced ellipticity against drug/HSA was drawn, this gives an estimation of free and bound fraction of the drug. Scatchard plots of the dialysis and CD data obtained at pH 7.4 are shown in Fig. 3. The dialysis data were analyzed assuming two independent classes of binding sites with a nonlinear least-squares curve-fitting procedure [11]. The best fit was obtained with the binding data $n_1 = 0.9$, $K_1 = 3.9 \times 10^5\ M^{-1}$ and $n_2 = 3.9$, $K_2 = 0.8 \times 10^4\ M^{-1}$. These values agree fairly with those obtained for the interaction of other 2-arylpropionic acid non-steroidal anti-inflammatory drugs including tolmetin with HSA [12]. In addition, the binding of *d*- and *l*-pirprofen to HSA was examined, and the calculated binding parameters are summarized in Table 1. The binding constants between *d*- and *l*-isomers for primary and secondary sites are almost the same which indicates that the high- and low-binding sites lack stereoselectivity. These results agree with the findings that no differences in plasma concentrations and clinical effects were observed for pirprofen enantiomers [13]. On the other hand, Scatchard plots (Fig. 3) obtained

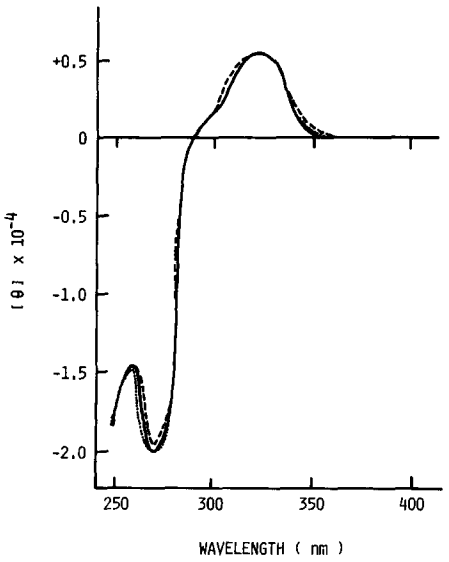

Fig. 2. CD spectra of the pirprofen–HSA complex at pH 7.4 (1/15 M phosphate buffer) and 25°: ———, *dl*-pirprofen; – – – –, *d*-pirprofen; . . . . . , *l*-pirprofen. The following concentrations were employed: HSA, 1.5×10^{-5} M; pirprofen, 1.5×10^{-4} M.

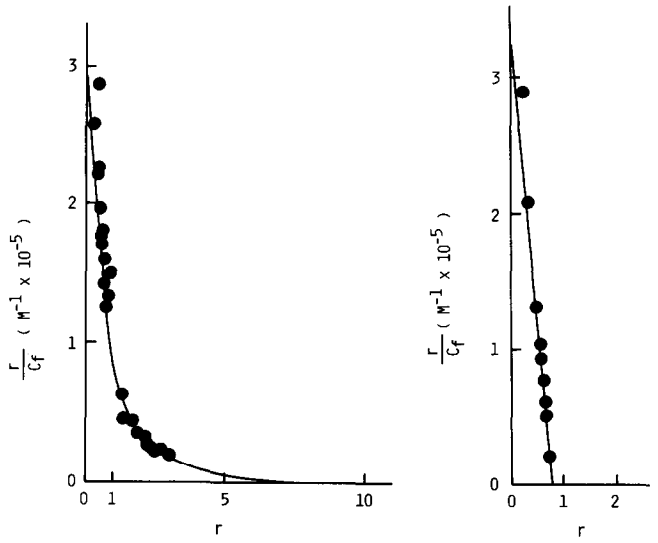

Fig. 3. Scatchard plot of data obtained from equilibrium dialysis (left, 37°) and CD (right, 25°) on pirprofen–HSA binding at pH 7.4 (1/15 M phosphate buffer). The line for dialysis data was calculated using $n_1 = 0.9$, $K_1 = 3.9 \times 10^5\ M^{-1}$; $n_2 = 2.9$, $K_2 = 8 \times 10^3\ M^{-1}$. The line for CD data was calculated using $n_1 = 0.8$, $K_1 = 4.3 \times 10^5\ M^{-1}$.

from CD data indicate one binding site for HSA, a result which differs from that obtained by dialysis. This suggests that the CD technique detects only the primary binding sites which generates induced CD spectra. In fact, the primary binding constant by the CD method is in reasonable agreement with that obtained by the dialysis method.

Effect of pH and chloride ion on the binding

Figure 4 shows the effect of pH on the ellipticity induced by the binding of pirprofen to HSA. The drug-to-HSA ratio of 5.0 was used because a single binding site on HSA contributes to the induced CD at these concentrations. The observed ellipticity of the pirprofen–HSA complex decreased significantly on raising the pH. Similarly, the binding constants decreased with increasing pH (Table 2). The pH dependence of the induced ellipticities and the binding constants can be explained by the N–B transition [14–16], rather than the changes in the degree on ionization of the pirprofen, because the pK_a values of pirprofen have been reported to be 3.3 and 4.3 [17] and so the drug exists as the anionic form over the pH region of these investigations. Fleitman and Perrin [18] have recently reported the involvement of N–B transition for the interaction of 2-arylpropionic acid, non-steroidal anti-inflammatory drugs, with HSA.

It is well-known that the N–B transition is affected by chloride and calcium ions, particularly by calcium ion [14, 15]. So, the effect of chloride ion on the ellipticity of the pirprofen–HSA complex was examined. Chloride ion apparently caused the decrease in the ellipticity at each pH measured (Fig. 4). It should be noted that the effect of chloride ion is greater for the N-form rather than the B-form. This suggests that N-form in HSA is more sensitive to the effect of chloride ion. The effect of calcium ion, which alters the conformation of HSA, was also examined in the present system. However, unfortunately, no clear effect could be observed because of solubility problems due to calcium phosphate formation. It is also possible that the reduced ellipticity in the presence of chloride ion is due to displacement.

Binding mechanism

The thermodynamic parameters were determined from a van't Hoff plot (Table 3). However, at each temperature investigated only binding constants for the high-affinity site were estimated (i.e. at $r < 0.5$) in order to simplify matters, because in the clinical

Table 1. Binding parameters for the binding of pirprofen and its enantiomers to HSA at pH 7.4 and 37° measured by equilibrium dialysis

Pirprofen	n_1	K_1 ($M^{-1} \times 10^{-5}$)	n_2	K_2 ($M^{-1} \times 10^{-4}$)
d	0.9 ± 0.2	4.2 ± 1.5	3.4 ± 0.2	1.0 ± 0.3
dl	0.9 ± 0.3	3.9 ± 0.9	2.9 ± 0.9	0.8 ± 0.4
l	0.9 ± 0.2	4.1 ± 1.1	3.5 ± 0.1	1.1 ± 0.2

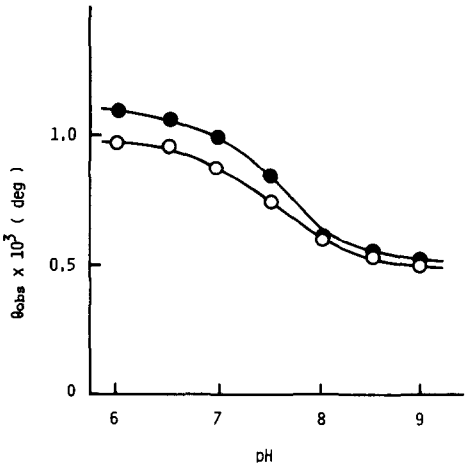

Fig. 4. Observed ellipticity of pirprofen–HSA complexes in the absence and the presence of NaCl at 320 nm as a function of pH: ●, without NaCl; ○, with NaCl. The following concentrations were employed: HSA, 3×10^{-5} M; pirprofen, 3×10^{-4} M; NaCl, 0.2 M; 1/15 M phosphate buffer.

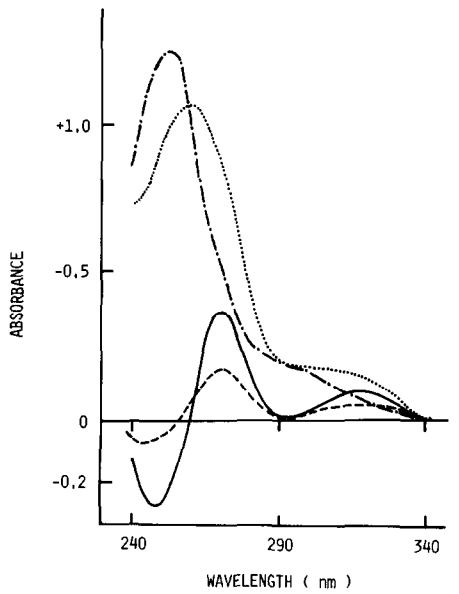

Fig. 5. UV absorption spectra and pirprofen in the absence and the presence of HSA, and the difference spectra: —·—·—, pirprofen alone; ....., pirprofen + HSA; ———, [pirprofen-HSA] − [pirprofen] difference spectrum; – – – –, [pirprofen-CTAB] − [pirprofen] difference spectrum. The following concentrations were employed: pirprofen, 1×10^{-4} M, HSA, 1.5×10^{-5}; CTAB, 0.05%, 1/15 M phosphate buffer.

situation, it is likely that only the primary site is involved in the binding process. The binding constants increased with the rise in temperature and from Table 3, it can be seen that the large positive entropy makes a much greater contribution to the free energy term for HSA. This suggests that the nature of the interaction is largely electrostatic, that is, the anionic part of the pirprofen molecule interacts with the cationic parts of the HSA molecule and the large positive entropy is the main source of the free energy variation (ΔG) [19]. However, interestingly, pirprofen showed remarkable ability to displace hydrophobic organic molecules such as ibuprofen and dansyl-L-proline from HSA binding site (see below). In addition, chloride ion competed with pirprofen.

The effect of HSA on the UV absorption spectrum of pirprofen was examined and compared with the spectrum of pirprofen in the presence of detergent. The UV absorption spectrum of pirprofen was shifted to a longer wavelength with a decrease in molar absorptivity (Fig. 5). Similar spectral change was observed when pirprofen was dissolved in a solution containing the cationic detergent cetrimide. However, no influence on the absorption spectrum of pirprofen was seen at cetrimide concentrations below the critical micellar concentration and in the presence of anionic detergent or non-ionic detergent. Interestingly, the shape and the location of the UV difference peak generated by the binding of pirprofen to HSA closely resembles that in cetrimide micelle (Fig. 5). Therefore, the similarity of the two difference spectra suggests that the microenvironment of pirprofen bound to HSA is similar to that in a cetrimide micelle. That is, the HSA binding site for pirprofen consists of a cationic site on the surface of the albumin molecule with a hydrophobic patch to accommodate the aromatic ring [20]. This hypothesis is consistent with the type of interaction suggested by the thermodynamic parameters.

Binding sites for pirprofen

Figure 6 shows the variation of $1/T_{2obs}$ for all peaks of pirprofen as a function of concentration ratio at the constant concentration of HSA (5×10^{-5} M). These results can be explained by assuming that the broadening of the proton signals was produced by a specific interaction of pirprofen with HSA, because $1/T_{2obs}$ decreased with increasing pirprofen concentration. The effect was particularly remarkable on

Table 2. Effect of pH on binding parameters of pirprofen to HSA at 37° measured by equilibrium dialysis

pH	n_1	K_1 ($M^{-1} \times 10^{-5}$)	n_2	K_2 ($M^{-1} \times 10^{-4}$)
6.5	0.7 ± 0.2	7.3 ± 4.5	3.8 ± 0.2	1.2 ± 0.3
7.4	0.9 ± 0.3	3.9 ± 0.9	2.9 ± 0.9	0.8 ± 0.4
8.5	1.4 ± 0.3	2.1 ± 1.8	3.7 ± 0.2	1.0 ± 0.3

Table 3. Thermodynamic parameters of pirprofen–HSA system obtained from equilibrium dialysis

Temperature (°K)	ΔG (kJ·mole^{-1})	ΔH (kJ·mole^{-1})	ΔS (J·°K^{-1}·mole^{-1})
280	−26.21		
290	−29.03	83.4	381
300	−31.28		
310	−32.91		

the protons of the pyrroline ring (H-7, 8, 9, 10) and the propionate portion (H-11, 12) (Fig. 6). The marked effect of the relaxation rate on a characteristic portion of the molecule suggests the existence of a specific intermolecular interaction differing from such nonspecific effects as an increase in viscosity. In theory, the transversal relaxation rate of proton i is related to the correlation time T_c by the following equation [21]

$$(1/T_2)_i = Af(T_c) \sum_j 1/r^6{}_{ij}$$

where A is constant, $f(T_c)$ is a function of drug correlation time, and r_{ij}^6 is the interaction distance. Thus the ratio T_{2free}/T_{2bound} allows for both variation of drug correlation time and changes in interaction, when binding take place. 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 the HSA macromolecule as a consequence of a stronger or new interatomic interaction.

Table 4 shows the free and bound relaxation rate and T_{2free}/T_{2bound}. The largest increase in the T_2 ratio was obtained on the protons of the propionate portion (H-11, 12). The T_2 ratio of the protons of the chlorbenzene ring was very small compared with those of the pyrroline ring and the propionate portion. These results indicate that the pyrroline ring and the propionate portion of pirprofen are the major binding site to HSA. Consideration of the thermodynamic parameters, absorption spectral data together with the NMR data suggest that the pyrroline ring is inserted into the hydrophobic crevice in the albumin while the propionate portion interacts with a cationic site on the HSA surface.

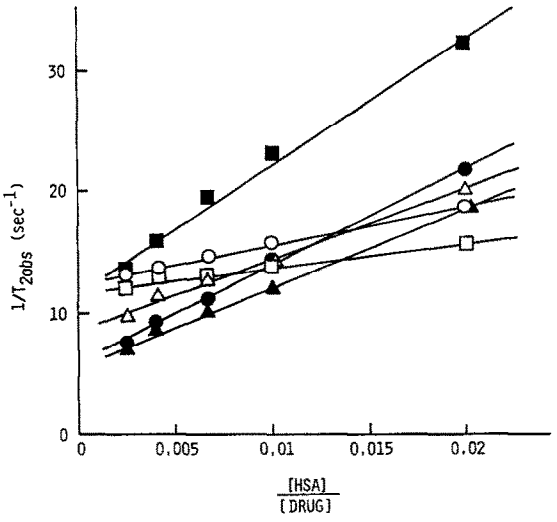

Fig. 6. Effect of HSA on relaxation rate of each proton of pirprofen: □, H-2; ○, H-5.6; ■, H-7.10; △, H-8.9; ▲, H-11; ●, H-12. The following concentrations were employed: HSA, 5×10^{-5}; pirprofen, 2.5–20 $\times 10^{-3}$ M; 1/15 M phosphate buffer (pD 7.4).

Location of the drug binding site on HSA

The specific drug binding sites on HSA have been established as sites I, II and III, in other words, as warfarin, benzodiazepine and digitoxin sites by several workers [2–5]. However, site III (digitoxin) has not been investigated as much as site I and II. In order to identify the pirprofen binding site on HSA, competitive displacements were carried out using the fluorescence probe of the known binding sites on HSA [2]. As shown in Fig. 7, pirprofen markedly displaced dansyl-L-proline (a site II probe), whereas 5-dimethyl-aminonaphthalene-1-sulfonamide (a site I probe) and 7-anilinocoumarin-4-acetic acid (a site III probe) were not displaced by pirprofen.

Furthermore, the effect of drugs on the induced ellipticity of the pirprofen—HSA complex was investigated to determine the specificity of the pirprofen binding site. All the drugs had shown negligible extrinsic Cotton effects in the experimental conditions of Fig. 8. Ibuprofen (a site II drug) gave significant displacement of pirprofen, whereas chlorpropamide (a site I drug) and digitoxin (a site III) did not displace any pirprofen under the present experimental conditions. Therefore, these displacement data clearly indicate that the primary binding site of pirprofen on HSA is site II, indole or benzodiazepine site.

Table 4. Free and bound relaxation rate and T_{2free}/T_{2bound} ratios for each proton of pirprofen

	Relaxation rate (sec^{-1})					
	2	5.6	7.10	8.9	11	12
$1/T_{2free}$	11.8	12.4	11.5	8.8	5.9	5.8
$1/T_{2bound}$	63.8	98.0	300.3	163.7	178.6	225.3
T_{2free}/T_{2bound}	5.4	7.9	26.0	18.7	30.4	39.1

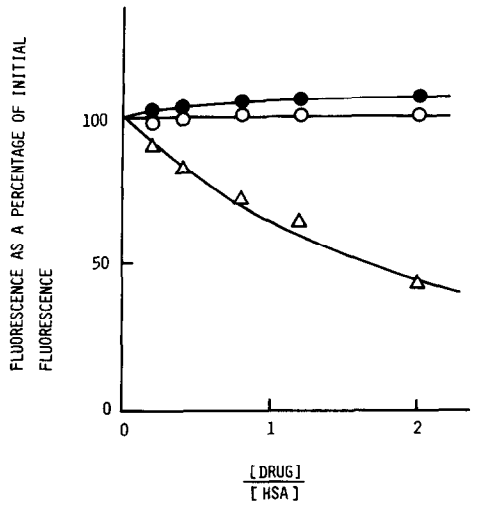

Fig. 7. Pirprofen-induced changes in fluorescence of probes bound to HSA: ●, 5-dimethyl-aminonaphthalene-1-sulfonamide; ▲, dansyl-L-proline; ○, 7-anilinocoumarin-4-acetic acid. The fluorescence of solutions containing 1×10^{-5} M HSA and 5×10^{-6} M probe was measured before and after the addition of drug.

Evidence from several laboratories [22, 23] suggests that the tyrosine residues are involved in the benzodiazepine binding site and that the lone tryptophan residue is part of the warfarin binding site. Thus, modifications of both were used to investigate whether the lone tryptophan residue and/or the tyrosine residue of HSA molecule are involved in the specific binding site of pirprofen. The modification of only 1.7 tyrosine residues markedly reduced the binding constants of high-affinity as well as low-affinity for pirprofen. On the other hand, the binding of pirprofen was little affected by the modification of the tryptophan residue. Some recent findings [24, 25] have demonstrated that Tyr-411 is most likely the reactive tyrosine of HSA. In the current work, only 1.7 of the 18 tyrosine were modified. Thus, it seemed that Tyr-411 is specifically involved in the pirprofen binding site of HSA. The involvement of other amino acid residues such as His-146 and Lys-194 in addition to Tyr-411 is conceivable, however, and so further investigation of binding to modified HSA is needed.

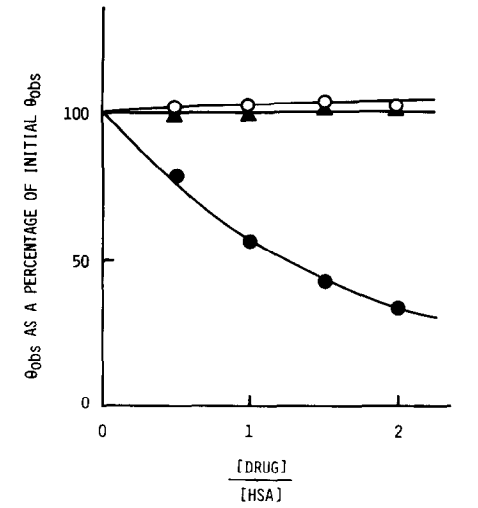

Fig. 8. Effect of drugs on observed ellipticity for pirprofen–HSA complex: ○, chlorpropamide; ●, ibuprofen; ▲, digitoxin. The θ_{obs} of solution containing 6×10^{-5} M HSA and 6×10^{-5} M pirprofen was measured before and after addition of drugs at 320 nm.

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