Study of Interaction of Pranoprofen with Human Serum Albumin: Binding Properties of Enantiomers and Metabolite

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The interaction of pranoprofen, pranoprofen glucuronide and pranoprofen methylester with human serum albumin (HSA), was investigated by equilibrium dialysis and spectroscopic techniques. The binding affinities of pranoprofen glucuronide and pranoprofen methylester to HSA were found to be almost the same, although they were remarkably small as compared to that of the parent compound, pranoprofen. Pranoprofen and pranoprofen methylester showed stereoselective affinities to HSA. It was found from the competitive displacement experiments using the fluorescent probes that the specific binding site for pranoprofen was site II, the diazepam site, and that the binding sites of pranoprofen glucuronide and pranoprofen methylester were site I, the warfarin site. In addition, from the binding data with modified HSA, it seemed that tyrosine-411 was specifically involved in the pranoprofen binding. The absorption spectral changes which accompanied the binding of pranoprofen and pranoprofen methylester to HSA or detergents implied that the HSA binding site of pranoprofen consisted of a cationic site on the surface of the albumin molecule with a hydrophobic region to accommodate the aromatic ring and that the binding site for pranoprofen methylester seemed to occupy a wide hydrophobic area. These limited data indicated differences in the location and microenvironments of binding sites for pranoprofen and its glucuronide on the HSA molecule.

Keywords — pranoprofen; pranoprofen glucuronide; pranoprofen methylester; stereoselectivity; binding site; human serum albumin; microenvironment; metabolite

Introduction

Pranoprofen or α -methyl-5*H*-[1]benzopyrano[2,3-*b*]pyridine-7-acetic acid (Fig. 1) is a nonsteroidal anti-inflammatory drug (NSAID) which is one of 2-arylpropionic acid derivatives having a chiral center and is clinically effective in rheumatoid arthritis as a mixture of *S*(+)- and *R*(-)-pranoprofen.¹⁾ Most of NSAIDs are extensively bound to plasma proteins, particularly to albumin.²⁾ Pranoprofen, similar to other arylpropionic acids, was also found to be highly bound to plasma protein.³⁾ However, only the binding percentage of pranoprofen to serum was reported. Furthermore, it is well known that NSAIDs are excellent probes for the classification of drug binding sites on human serum albumin (HSA).⁴⁻⁶⁾ For example, the arylpropionic acids including ibuprofen and pirofen are bound to site II (diazepam site), while the bulky hetero aromatic molecules such as phenylbutazone share the same binding site with warfarin (site I). This indicates that the carboxylic group attached to the chiral carbon of arylpropionic

acids may bind to site II, while the ester forms of arylpropionic acid or the hetero aromatic molecules without carboxylic acid group may bind to site I. In fact, recently the differences in the binding regions between enantiomers and the glucuronide metabolites of carprofen on HSA have been demonstrated.⁷⁾ Thus, the present work was undertaken to identify and characterize the binding site of pranoprofen and its metabolite, pranoprofen glucuronide, and to elucidate the binding mechanism of the drug and its metabolite to HSA. In addition, the binding of pranoprofen methylester, as a substitute for pranoprofen glucuronide, to HSA was used in our experiment because of the instability of pranoprofen glucuronide in the present experimental conditions.

Fig. 1. Chemical Structure of Pranoprofen

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Experimental

Materials — HSA fraction V was supplied by Chemo-Sera-Therapeutic Research Institute (Kumamoto, Japan) and purified by the method of Chen.⁸⁾ The molecular weight of HSA was assumed to be 66500. Pranoprofen and its enantiomers were donated by Yoshitomi Pharmaceutical Industries Ltd. (Fukuoka, Japan). The optical purities of *S*(+)- and *R*(-)-pranoprofen were 99.7 and 99.4%, respectively. Pranoprofen glucuronide was collected from human urine after oral administration of pranoprofen by the method of Arima.⁹⁾ Pranoprofen methylester was synthesized by adding pranoprofen chloride into methanol according to the conventional method. Fluorescence probes, dansyl-L-arginine for site I and dansyl-L-proline for site II, were obtained commercially. All other materials were reagent grade, and the buffers used were prepared with monobasic sodium phosphate using deionized distilled water. The pH (pH7.4) of buffer was checked at 25 °C with a suitable standard pH meter. Human serum was prepared after centrifugation at 3000 r.p.m. × 10 min of fresh blood collected from healthy volunteers.

Preparation of HSA Derivatives — The modification of the lone tryptophan residue was performed according to Koshland *et al.*¹⁰⁾ The tyrosine residue in HSA was modified by the method of Sokolovsky *et al.*¹¹⁾ The modification of the lysine residue was carried out by the method of Gounaris *et al.*¹²⁾ The degree of the modification of tryptophan, tyrosine and lysine residues of HSA was determined spectrophotometrically. The results are shown in Table I.

UV Absorption — Absorption and absorp-

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

Fluorescence — Fluorescence measurements were made using a Jasco FP-70 fluorescence spectrophotometer (Tokyo, Japan) at 25 °C. The fluorometric titrations were as follows: Protein solution (2 μM, 3 ml) was titrated by the successive addition of pranoprofen and pranoprofen methylester (to a given final concentration of 0.1–8 μM), and the fluorescence intensity of protein was measured at excitation 290 nm and emission 340 nm, while in the case of pranoprofen glucuronide, each step of the total titration was done separately within 30 s to escape the possibility of degradation of pranoprofen glucuronide. Thirty μl of drug solution was the maximum amount used in the titration which was negligible to make any volume correction for protein concentration. At the selected wavelength the drugs did not contribute to the fluorescence. The fraction of drug bound, *X*, was determined according to Weber and Young¹³⁾:

$$X = \frac{F_o - F_b}{f_a \times F_o}$$

where F_b and F_o are the fluorescence intensities of protein in a solution with given concentration of drug and without drug, respectively, and f_a is the maximum fraction of quenchable fluorescence. The value of f_a was between 0.80–0.84. F_b was determined for a wide range of concentration.

Ultrafiltration — An ultrafiltration experiment was performed using a micropartition system MPS-1 (Amicon Corp., Danvers, MA, U.S.A.) fitting a membrane filter with a molecular weight cut off value of 30000. The 1×10^{-5} M of pranoprofen and its derivatives were dissolved in protein solution (1×10^{-5} M, pH 7.4). One ml of sample was immediately applied to the sample reservoir and centrifuged at $2000 \times g$ for 10 min at 0–4 °C. After centrifugation, the filtrate was acidified with 0.1 M citrate

TABLE I. Percentage of Reacted Amino Acid Residues in the Modified HSA

Modified HSA	Reacted amino acid		
	Trp (1 ^{a)})	Tyr (18 ^{a)})	Lys (59 ^{a)})
Trp modified HSA	93.0	—	2.3
Tyr modified HSA	—	10.0	—
Lys modified HSA	—	3.2	64.0

a) Number of amino acids per HSA molecule.

buffer (pH 3.0) to prevent hydrolysis of pranoprofen glucuronide. The unbound concentrations of pranoprofen, pranoprofen methylester and pranoprofen glucuronide were determined by HPLC. The HPLC system consisted of a Hitachi 655A-11 pump and a Hitachi 655A variable wavelength UV monitor (275 nm) using a LiChrosorb RP-18 (Cica Merck, Tokyo, Japan). The mobile phase composed of acetonitrile–0.14% phosphoric acid (9:11, v/v) and a flow rate was 1.0 ml/min. Adsorption of pranoprofen, pranoprofen glucuronide and pranoprofen methylester on the membrane was negligible.

Equilibrium Dialysis (ED) — The ED experiments were performed in a Sanko Plastic dialysis cell (Fukuoka, Japan) for 10 h at 37 °C. In the apparatus, the chambers were divided by Visking membrane (MW cut off value, 12000–14000). HSA solution (2 ml, 30 μ M), dissolved in phosphate buffer (pH 7.4), was poured into one compartment and 2 ml of racemic pranoprofen or each enantiomer solution (5–180 μ M) into the opposite. Adsorption of pranoprofen onto membranes was negligible and the volume of solution on either side of the membrane was constant during dialysis. The free concentration of pranoprofen was determined from the UV absorbance data at 270 nm. Degradation of pranoprofen and racemization of each enantiomer were not observed during dialysis.

Results and Discussion

Binding of Pranoprofen to Serum and Serum Protein

It is generally considered that HSA binds weakly acidic drugs, while α_1 -acid glycoprotein (AGP) is the main serum protein for the binding of basic drugs.^{14–16} Therefore, first of all, the bindings of pranoprofen to serum and serum proteins (HSA, AGP and γ -globulin) were examined by an ultrafiltration method in order to elucidate its binding characteristics to serum proteins (Table II). Pranoprofen showed a much higher binding affinity to HSA as compared with AGP and γ -globulin. In fact, binding of pranoprofen to HSA and serum was found to be almost the same. This clearly indicated that

TABLE II. *In Vitro* Binding of Racemic Pranoprofen to Human Serum and Serum Protein Fractions by Ultrafiltration

Fraction	Binding percentage
Human serum	99.3
HSA (4%)	99.7
γ -Globulin (1.4%)	6.5
α_1 -Acid glycoprotein (0.1%)	2.3

[pranoprofen] = 1×10^{-5} M.

the serum protein binding of pranoprofen was mainly due to its binding with HSA. So, the binding mode of pranoprofen and its metabolite, pranoprofen glucuronide with HSA was further examined in more detail.

Binding Affinity and Binding Site of Pranoprofen, Pranoprofen Glucuronide and Pranoprofen Methylester

It has been reported that a metabolite can displace the parent drug from its binding site(s) on serum proteins.¹⁷ However, information concerning the displacement of metabolites from their binding sites by the parent drugs has been very limited. We have previously demonstrated that sulfadimethoxine and its major metabolite, *N*⁴-acetylsulfadimethoxine, share the same binding site on HSA.¹⁸ On the other hand, Iwakawa *et al.*⁷ have recently reported that the binding area of carprofen may be different from that of carprofen glucuronide, the main metabolite of carprofen. Of course, we need to know the binding sites as well as binding affinity to protein, when we consider the possibility of the displacement of parent compounds by their metabolites. However, pranoprofen glucuronide seems to be extremely unstable in plasma or albumin solution, as the degradation half-life for the pranoprofen glucuronide in plasma at 37 °C was only 24 min. In addition, the pranoprofen glucuronide may not be as stable even in the solid state. In fact, 35% of pranoprofen glucuronide in a solid state was found to be degraded in a week at room temperature. Therefore, a substitute compound was to be chosen instead of pranoprofen glucuronide which would show the binding characteristics similar to pranoprofen glucuronide. We had the expectation as described in the introduction that the carboxylic acid group

TABLE III. *In Vitro* Binding Percentage of Pranoprofen, Pranoprofen Glucuronide, and Pranoprofen Methylester, to HSA by Ultrafiltration at pH 7.4 and 0–4 °C

	Binding percentage
Pranoprofen	75.1
Pranoprofen glucuronide	22.6
Pranoprofen methylester	24.9

[albumin] = [drug] = 1×10^{-5} M.

attached to chiral carbon of arylpropionic acids may bind to site II, while the ester compounds of arylpropionic acid or the bulky molecules without a carboxylic group may bind to site I on HSA. Thus, we synthesized pranoprofen methylester and it was evaluated as a good substitute for pranoprofen glucuronide in terms of binding characteristics (binding percentage, binding site *etc.*) when bound to HSA even though the physical properties (*e.g.* molecular weight, partition coefficient, solubility *etc.*) of these two compounds were quite different.

Binding experiments were carried out by ultrafiltration for 10 min of centrifugation at 0–4 °C to prevent the hydrolysis of pranoprofen at an elevated temperature. Direct analysis of pranoprofen and pranoprofen

glucuronide concentrations indicated that less than 2% of glucuronide was degraded under present experimental conditions. The binding percentage of pranoprofen methylester was almost the same as that of the pranoprofen glucuronide (Table III). However, these bindings were much lower as compared to that of the parent compound, the pranoprofen. Furthermore, in order to identify the binding sites for pranoprofen glucuronide and pranoprofen methylester on HSA, the competitive displacement studies were carried out using fluorescence probes at 25 °C. As shown in Fig. 2, pranoprofen markedly displaced dansyl-L-proline (site II probe), whereas it caused little displacement of dansyl-L-arginine (site I probe). By contrast, pranoprofen glucuronide and pranoprofen methylester displaced dansyl-L-arginine more than dansyl-L-proline and the displacement was statistically significant even though the degree of probe displacements by these two compounds was smaller than that which was observed in pranoprofen, probably because of their low binding to HSA (Table III). The displacement data indicated that the primary binding site of pranoprofen was site II whereas the binding sites for pranoprofen glucuronide

Fig. 2. Displacement of Site I (A) and Site II (B) Probes from HSA by Pranoprofen, Pranoprofen Methylester and Pranoprofen Glucuronide at 25 °C

●, pranoprofen; ○, pranoprofen methylester; ▲, pranoprofen glucuronide.

[HSA] = [probe] = 2.0×10^{-6} M.

Fig. 3. Scatchard Plots of *R*(-)-, *S*(+)- and *RS*(±)-Pranoprofen-HSA Interaction at pH 7.4 and 37 °C
 (●), *R*(-)-pranoprofen-HSA system; (■), *S*(+)-pranoprofen-HSA system; (▲), *RS*(±)-pranoprofen-HSA system.

and pranoprofen methylester were probably site I. The above results also supported the validity of using for pranoprofen methylester as a substitute compound for pranoprofen glucuronide in terms of binding characteristics.

Enantioselectivity of Binding

Scatchard plots of the binding data of *R*(-)-, *S*(+)- and *RS*(±)-pranoprofen obtained at pH 7.4 using an equilibrium dialysis technique are shown in Fig. 3. The data analyzed assuming two independent classes of binding sites are listed in Table IV. The *S*(+)-isomer of pranoprofen showed higher binding than the *R*(-)-isomer, similar to other tricyclic NSAIDs such as carprofen.⁷⁾ Similar enantioselectivity for pranoprofen methylester was also observed. However, the binding affinity for each ester was significantly small as compared with pranoprofen, as expected from the data shown

in Table III. In addition, there was no significant difference between the binding parameters of two enantiomers. These data suggested that the carboxylic group of pranoprofen was important for binding to site II on HSA, and that the absolute configuration of the carboxylic group might regulate the binding. The only one binding site was found for pranoprofen methylester which differed from pranoprofen. This difference was probably due to the fact that different methods were used for the binding of these two compounds to HSA. In the case of pranoprofen methylester, fluorescence quenching but not dialysis technique was used because of its poor solubility in the used buffer and the technique detected only the primary binding site by quenching the lone tryptophan residue in HSA. However, it is also possible that a single binding site for pranoprofen methylester exists on HSA.

TABLE IV. Binding Parameters for Pranoprofen and Pranoprofen Methylester Systems

	Pranoprofen ^{a)}				Pranoprofen methylester ^{b)}	
	n_1	$K_1 (\times 10^5 \text{ M}^{-1})$	n_2	$K_2 (\times 10^5 \text{ M}^{-1})$	n	$K (\times 10^4 \text{ M}^{-1})$
<i>S</i> (+)-Form	0.70	12.2	3.30	0.14	1.01	3.77
<i>RS</i> (±)-Form	0.58	12.3	3.14	0.23	0.99	3.28
<i>R</i> (-)-Form	0.43	14.7	2.77	0.25	1.00	2.95

n , number of binding sites; K , binding constant, as determined from Scatchard plots.

a) Measured by equilibrium dialysis method.

b) Measured by quenching method.

In fact, Iwakawa *et al.*⁷⁾ demonstrated the different numbers of binding sites for carprofen and its glucuronide.

Characterization of Binding Site

Fehske and his coworkers,^{19,20)} from the binding studies of drugs to both native and modified HSA suggested that the tyrosine residues are involved in the diazepam binding site (site II) and that the lone tryptophan residue is a part of the warfarin binding site. Thus, the lysine modified HSA in addition to tryptophan and tyrosine modified HSA were used to investigate by an ultrafiltration method which amino acid residues in HSA played an important role in the binding of pranoprofen and pranoprofen glucuronide to HSA. The modification of approximately two tyrosine residues markedly reduced the binding of pranoprofen (Fig. 4). In addition, the binding of pranoprofen to the lysine-modified HSA was significantly decreased although the reduction of binding percentage was small as compared to that observed by the modification of tyrosine. On the other hand, the modification of tryptophan had little affect on the binding of pranoprofen to HSA. This clearly suggested that the tyrosine and lysine residues were involved in the primary binding site of pranoprofen. Particularly, tyrosine-411 was conceivable for the

specific binding of the drug from some reports.^{21,22)} However, the exact location of the lysine residue was not elucidated from the limited data. In glucuronide and methylester, unfortunately, we could not find any specific amino acid for the binding of these compounds to HSA, as illustrated in Fig. 4. This might indicate the difference in the microenvironment of the binding sites between the parent compound and its metabolite. The idea was also supported by the UV spectral data.

Figure 5 shows UV difference spectra of pranoprofen and pranoprofen methylester in the presence of HSA or detergents. In the case of pranoprofen, a positive peak around 255 nm and two negative peaks around 274 nm and 285 nm were generated by the binding to HSA. A similar spectral pattern was also observed in cetyltrimethylammonium bromide (CTAB), a cationic detergent, solution although the intensities of the absorption difference spectra were different. Moreover, UV difference spectra of pranoprofen were induced when pranoprofen was dissolved in a solution containing polyoxyethylene lauryl ether (PLE), a nonionic detergent, and the spectral shapes were clearly different from those when bound to HSA. However, no influence on the absorption spec-

Fig. 4. Drug Free Fraction of Pranoprofen (A), Pranoprofen Methylester (B) and Pranoprofen Glucuronide (C) to Native and Modified HSA by Ultrafiltration at 0–4 °C

(1), native HSA; (2), Lys modified HSA; (3), Trp modified HSA; (4), Tyr modified HSA.

Concentration: pranoprofen and its derivatives, native and modified HSA = 1×10^{-5} M.

Fig. 5. UV Absorption Difference Spectra of Pranoprofen (A) and Pranoprofen Methylester (B) Bound to HSA and Detergents

(—), HSA; (---), CTAB; (···), PLE; (-·-·), SLS.

Concentration: HSA, pranoprofen and pranoprofen methylester = 1.5×10^{-5} M, detergents = 2.0×10^{-3} M.

trum of pranoprofen was observed at a CTAB concentration below the critical micellar concentration and in the presence of sodium lauryl sulfate (SLS), an anionic detergent. Therefore, the similarity of the two difference spectra observed for binding to HSA and CTAB implied that the microenvironment of pranoprofen bound to HSA was similar to that in CTAB micelle. That is, the site II for pranoprofen on HSA consisted of a cationic site on the surface of the albumin molecule with a hydrophobic region to accommodate the aromatic ring. In sharp contrast, pranoprofen methylester generated similar peaks when bound to either HSA or detergents. This suggested that the pranoprofen methylester molecule interacted only with the hydrophobic parts of HSA or detergents. So, there is strong reason to consider that pranoprofen methylester was incorporated into the hydrophobic region of the HSA molecule during binding.

In conclusion, the data presented here suggested that there were differences in the location and microenvironments of the binding sites on the HSA molecule for pranoprofen and its metabolite, pranoprofen glucuronide.

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