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Interaction of Protizinic Acid with Human Serum Albumin: Site-to-Site Displacement of Protizinic Acid by Ibuprofen

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Abstract

The binding of protizinic acid [(10-methyl-7-methoxy-2- phenoxythiazinyl)-2-propionic acid, PA], a non-steroidal anti-inflammatory drug, to human serum albumin (HSA) was studied by dialysis and spectroscopic methods. A scatchard analysis of equilibrium dialysis (ED) data and the results of site marker displacement experiments suggest that PA predominantly binds to site II or the benzodiazepine site on HSA, although the drug also has a low affinity for site I. Circular dichroism (CD) spectra of PA bound to HSA in low drug to HSA and high drug to HSA ratios were completely different, indicating that the extrinsic mechanism responsible for the high and low affinity of PA to HSA are probably different. In the presence of ibuprofen, a site II-specific drug, the CD spectra of the PA-HSA complex (0.5:1) changed to that for a high ratio (5: 1) of the drug to HSA in the absence of ibuprofen. This characteristic change in the CD spectra of the PA-HSA complex indicates that PA is displaced from a high to low affinity binding site after it is displaced by ibuprofen. ED findings also indicate site-to-site displacement of PA, i.e., when PA is displaced from the high affinity site, it re-binds to its low affinity site.

Keywords: ***Protizinic acid; Ibuprofen; Serum albumin; Displacement; Site-to site transfer; Circular dichroism.***

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Introduction

Human serum albumin (HSA), the most abundant protein in serum, functions as carrier of drugs, and consists of three homologous domains (I, II and III), each of which contains two subdomains, A and B. HSA reversibly binds many endogenous and exogenous compounds and contains two major drug-binding sites, site I and site II, where are located in subdomains IIA and IIIA respectively [1-4].

Most non-steroidal anti-inflammatory drugs (NSAIDs) show a high degree of binding to HSA, and their binding to plasma proteins is a primary determinant to their pharmacokinetic properties [5-9]. Almost all hydrophobic NSAIDs that contain a carboxyl group bind to site II, one of the specific drug binding sites on HSA. We previously proposed an interesting model, referred to as site-to-site displacement, which is based on the observation that carprofen, 2-(6-chlorocarbazole) propionic acid, in the presence of ibuprofen, a site II-specific drug, underwent site-to-site displacement from its high to a low affinity site [10]. In this continuing investigation, we examined the issue of whether or not the binding of protizinic acid, (10-methyl

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7-methoxy-2-phenoxythiazinyl)-2-propionic acid, a tricyclic NSAID, is inhibited by ibuprofen through a site-to site displacement mechanism.

Materials and Methods

Materials

HSA (fraction V) was a gift from the Chemo-Sera-Therapeutic Research Institute (Kumamoto, Japan). It was defatted with activated char-coal using methodology originally described by Chen [11] but with minor modifications as described below. [10] Dansyl-L-asparagine (DNSA) and dansylsarcosin (DNSS) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and Tokyo Kasei Kogyo Co. (Tokyo, Japan), respectively. Ibuprofen (Kaken Pharmaceuticals Co., Tokyo, Japan), PA and warfarin (Eisai Co., Tokyo, Japan) were obtained as pure substances from the respective manufacturer. All other reagents were of analytical grade. All buffers used were prepared with sodium phosphate dibasic and sodium phosphate monobasic salts.

Circular Dichroism (CD) measurements

CD measurements of PA-HSA complexes at different drug to HSA ratios (0.5: 1 to 5:1) (10 µM) were carried out on a Jasco J-600 spectropolarimeter (Tokyo, Japan), using a 10-mm cell at pH 7.4 and 25°C. Ibuprofen was used in different ibuprofen to HSA (l0 µM) ratios (0 to 5) to measure its effect on the induced CD spectra of the PA-HSA system (0.5: 1) at pH 7.4 and 25°C. Induced CD ellipticity is defined as the CD of the drug HSA alone at the same wavelength, and is expressed in degrees.

Equilibrium Dialysis (ED)

ED experiments were performed using 2 ml Sanko plastic dialysis cells (Fukuoka, Japan). The two cell compartments were separated by a Visking cellulose membrane. For determining the binding characteristics by ED, aliquots (1.5 ml) of various ratios of drug-HSA mixtures (HSA, 40 µM: drug, 20-400 µM) were dialyzed at 25°C for 13 h against the same volume of buffer solution. It was demonstrated that, at the concentration used, there was no significant adsorption of the drug to the dialysis membrane. Further control experiments with protein-free solutions showed that Visking membranes were fully permeable to all drugs and that equilibrium was established within the designated period of time. After equilibrium was reached, the free concentration of PA was determined by HPLC. The HPLC system consisted of a Hitachi 655A-11 pump and either a Hitachi 655A variable UV monitor or a Hitachi Fl000 variable fluorescence monitor. A column of Li Chrosorb RP-18 (Cica Merk, Tokyo, Japan) for the drugs was used as a stationary phase. The mobile phase consisted of acetonitrile and deionized water (65:35 v/v). ED experiments were performed at different conditions for the site-to-site displacement study. The free fraction (%) of PA (20 µM) bound to HSA (40 µM, 1:2) upon the addition of ibuprofen or warfarin ([drug]/ [HSA] = 05) was determined.

Treatment of the data

Binding parameters were estimated by fitting the experimental data to the following equation using a nonlinear least squares computer program (MULTI) [12].

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

Where r is the number of moles of bound drug molecules per mole of protein. [Db] and [Df] are the concentrations of bound and unbound drug, respectively, and [Pt] is the total protein concentration. Ki and ni are the association constant and the number of binding sites for the ith class of binding sites, respectively. A simple competition between two drugs A and B for identical protein binding sites was analyzed by the following equations [1]:

$$\frac{[P_A]}{[P_t]} = \frac{K_a[A_f]}{1 + K_a\left[A_f\right] + K_b\left[B_f\right]} \quad (2)$$

$$\frac{[P_B]}{[P_t]} = \frac{K_b[B_f]}{1 + K_b\left[B_f\right] + K_a\left[A_f\right]} \quad (3)$$

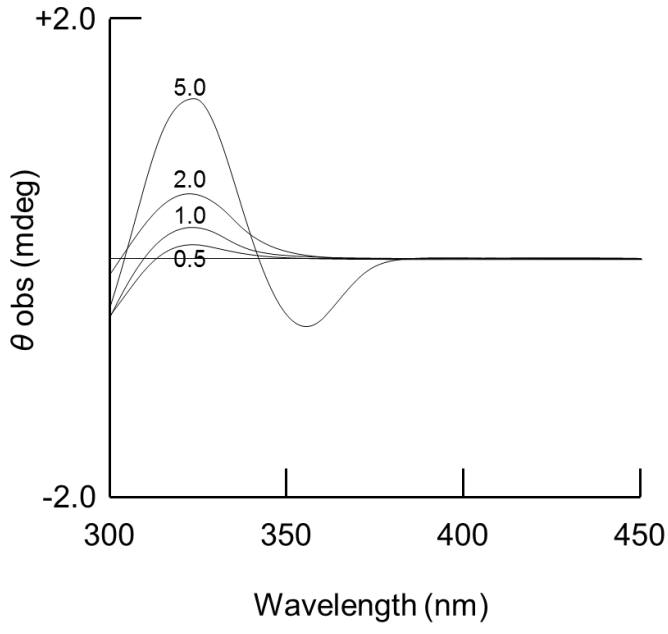
Where: Ka = the association constant for drug A, Kb = the association constant for drug B, [Af] = concentration of free drug A, [Bf] = the concentration of free drug B, [PA] = the concentration of bound drug A, [PB] = the concentration of bound drug B.

Results

Interaction of PA with HSA

CD spectra of PA bound to HSA at low drug to HSA and high drug to HSA ratios were completely different (**FIG. 1**), indicating that the mechanisms responsible for the high and low affinity binding of PA to HSA are probably different. PA at a molar ratio 0.5: 1 with HSA produced a positive CD band at 325 nm. When this molar ratio was increased to 2: 1, the amplitude of the CD band at 325 nm showed a net increase, without any other changes in the CD spectrum. When the molar ratio of the drug to HSA was further increased to 5: 1, prominent changes in both the shape and amplitude of the CD spectrum were observed with a new negative CD band at about 352 nm and a further increase in the amplitude of the CD band at 325 nm.

FIG. 1. **CD spectra of PA-HSA complexes at different drug to HSA ratios at pH7.4 and 25°C. Number indicates the ratio of the drug to HSA (10 µM).**



A scatchard analysis of the ED data as shown in **FIG. 2** suggests the presence of at least two classes of binding sites for the binding of PA to HSA. The best fit values assuming two classes of binding sites, are shown in TABLE 1.

FIG. 2. **Scatchard plot of PA binding to HSA by ED at pH7.4 and 25°C.**

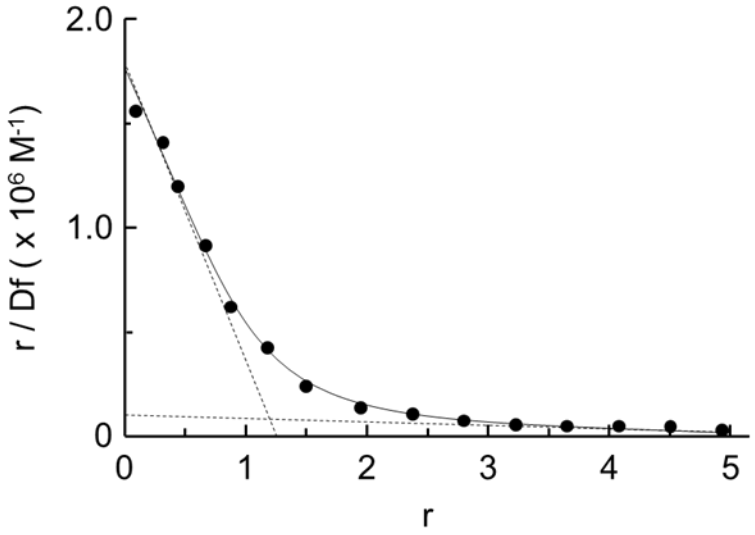


TABLE 1. **Binding parameters for protizinic acid bound to HSA as determined by ED at pH 7.4 and 25 °C.**

Drug	**n_1**	**K_1 (x 10^6 M^{-1})**	**n_2**	**K_2 (x 10^6 M^{-1})**
Protizinic acid	1.21 ± 0.2	1.74 ± 0.3	5.36 ± 0.5	0.05 ± 0.0

When site marker displacement experiments were carried out using fluorescent probes, PA very efficiently displaced both DNSS and DNSP, two site-II specific probes, from their binding site, while only moderate displacements of DNSA and warfarin, two site I-specific probes, were observed (data not shown).

Site-to site displacement of PA

When ibuprofen (ibuprofen: HSA=1:1) was added, the CD spectrum **(FIG. 3)** of the PA-HSA complex (0.5:1) was similar to the CD spectrum for the PA-HSA complex (5: 1) in the absence of ibuprofen, as shown in **FIG. 1.**

FIG. 3. **Effect of ibuprofen on the induced CD spectra of the PA-HSA system (0.5: 1) at pH7.4 and 25°. Ibuprofen to HSA (10 µM) ratios- 0 (opened circle), 1.0 (open squares), 2.0 (closed squares) and 5.0 (closed circles) were employed.**

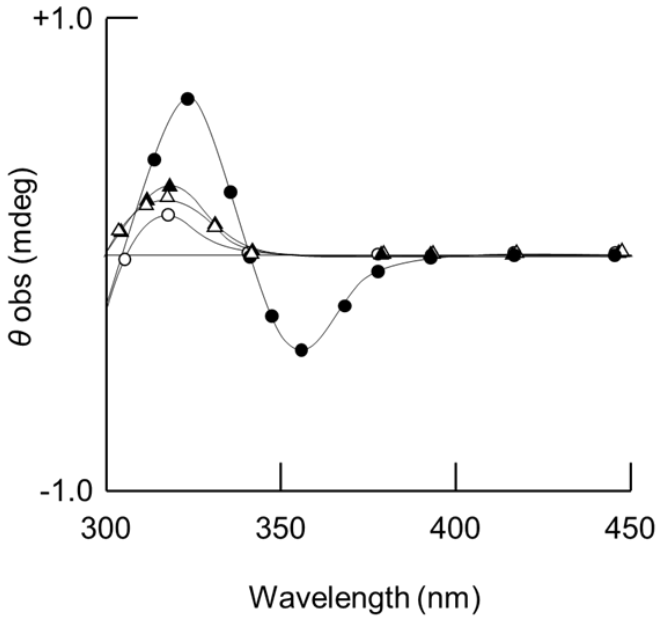
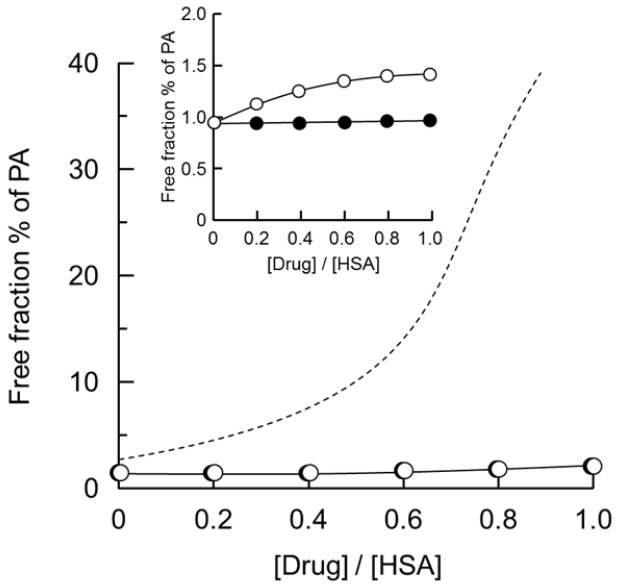


FIG. 4. illustrates the change in the free fraction (%) of PA caused by the presence of ibuprofen or warfarin at 7.4. The free fraction of PA was increased from 0.8% to 1.4% when ibuprofen, a site II-specific drug, was present. However, no change in the free fraction of PA was observed in the presence of warfarin, a site I-specific drug, at a ratio 1:1 to HSA.

FIG.4. **Free fraction (%) of PA bound to HSA (1: 2) upon the addition of ibuprofen (open circles), or warfarin (closed circles). Theoretical curve (dotted line) assuming a simple competition between PA and ibuprofen at site II on HSA. The following concentrations were used: HSA, 40 µM; PA, 20 µM**.



Discussion

Although more than two decades have passed since the discovery of PA as a potent NSAID, little is known regarding the characteristics of its binding to HSA: PA is capable of binding a multitude of chemically different ligands, and contributes significantly to their transport, distribution and metabolism. In the present study, the interactions of PA with HSA were evaluated by both CD and ED. Since PA is not optically active and HSA does not produce any Cotton effects at the wavelengths used, there is little doubt that the observed Cotton effects are extrinsic in origin. In addition, no cotton effects were observed in the CD spectra of ibuprofen bound to HSA under these experimental conditions. The abnormal behavior of the CD spectra of PA prevented the binding parameters of PA to HSA to be estimated by the CD method and hence, only ED was used in this estimation, since it provides both accurate and reproducible in vitro estimates of drug-protein binding, provided that time-dependent shifts in both the pH and volume can be avoided or taken into account during the data analysis. [13]. In the case of the drug, the non-linearity of the Scatchard plots indicated the presence of at least two classes of binding sites. As shown in **TABLE 1**, PA is characterized by a high association constant for HSA. The association constant for the high affinity binding site (K_1) is high (1.74×10^6 M^{-1}), in the range of values reported for many other NSAIDs [14-18]. The association constant for the second site (K2) was about 35 times lower (0.05×10^6 M^{-1}) than that of the primary association constant. From a preliminary study using the probe displacement method, PA was found to be predominantly bound to site II or the benzodiazepine site on HSA but the drug also has a low affinity for site I or the warfarin site. The binding characteristics of PA to HSA were found to be similar to many other NSAIDs that contain carboxylic acid groups, as previously reported by us [14].

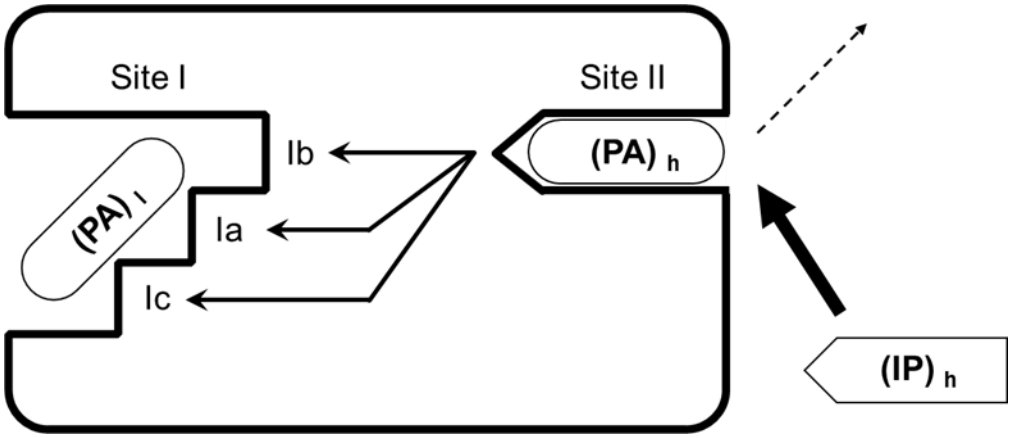
The CD spectra of PA bound to HSA at low and high drug to protein ratios were characteristically very different. Ibuprofen changed the CD spectra of the PA-HSA complex (0.5:1) compared to that obtained at a high ratio (5: 1) of the drug to HSA in the absence of ibuprofen. This characteristic change can be explained by allosteric modification of the PA binding site on HSA by the ibuprofen molecule. However, an increase in the temperature or pH had a negligible effect on CD spectrum of the PA-HSA complex, suggesting that the alteration in the CD spectra was not due to a micro environmental change in the PA binding site on HSA or an accompanying conformational change in the HSA molecule. It is also possible that the PA molecule bound to site II forms complexes with PA or ibuprofen molecules through stacking or electron transfer. However, this possibility can be ruled out because no self-association of PA was detected in the aqueous solution (without HSA solution) under these experimental conditions. In addition, PA and ibuprofen share a common binding site on HSA and therefore the two ligands cannot simultaneously bind to HSA.

The qualitative results obtained from CD data can be explained with the help of ED experiments. The free fraction of PA was increased by the addition of ibuprofen, but the experimental data did not fit the theoretical curve assuming a simple competition between two drugs A and B for identical high affinity sites on the HSA molecule. Moreover, warfarin, a site 1-specific drug, did not generate any increase in the free fraction of PA (**FIG. 4**). All NSAIDs bind to either site I or site II with high association constants ($K > l0^5$ M^{-1}). NSAIDs that contain a carboxylic group that is located a distance from the aromatic ring via an extended chain primarily bind to site II on HSA. On the other hand, NSAIDs that are bulky heterocyclic compounds with a negative or positive charge localized in the middle of the molecule bind to site I on HSA [14] . Therefore,

the results obtained in **FIG 4** suggest that PA is displaced from the high affinity site and then re-binds to the low affinity site. In this case, some of the low affinity sites may correspond to site I, which consists of at least three regions (site Ia (warfarin region), site Ib (azapropazone or phenylbutazone region) and site Ic (p-aminobenzoate region)) [2].

The large deviation between the experimental value and the theoretical curve can be explained by this rationalization to some extent. From the above discussion - it can be concluded that PA undergoes site-to-site displacement from its high affinity site (site II) to its low affinity site (site I) in the presence of ibuprofen, a drug that binds selectively to site II (**FIG. 5**). Thus, the marked CD spectral change of the PA-HSA system in the presence of ibuprofen can be explained by a site-to-site displacement mechanism: the development of a negative band is obviously due to the secondary binding of PA to HSA that had been displaced from the high affinity site and then rebound to its low affinity site. PA undergoes a modified displacement, referred to as site-to-site displacement, after being displaced from its high to its low affinity site by a type II-specific drug such as ibuprofen. The concept of site-to-site displacement in relation to drug-protein reactions was first reported by us for carprofen [7].

FIG. 5**. Proposed model of PA-ibuprofen interaction on HSA. (PA) h: PA at the high affinity binding site on HSA, (PA) l: PA at the low affinity binding site on HSA, (IP) h: ibuprofen at the high affinity binding site on HSA.**



If two drugs of the same type that bind to the same sites on a protein molecule are administered at the same time, competition at the protein binding level could occur: the drug with the higher affinity would be expected to bind preferentially, displacing the other drug or inhibiting its binding. From a pharmacokinetic view point, it is important to accurately measure the free drug concentration as competitive phenomena which are readily demonstrable *in vitro* and may not explain the pharmacokinetic properties. Because of the site-to-site displacement, the free concentration of PA does not fit the theoretical curve after being competitively displaced by ibuprofen. Therefore, a quantitatively significant difference between the free concentrations of the displaced drug caused by ibuprofen with or without site-to-site displacement would be expected. For highly bound drugs like PA or carprofen 99% or more of which bind to HSA, an increase in the free fraction of only 1% would result in a doubling of the amount of the drug available for pharmacological activity. Thus, a quantitative change of even 1% in binding might exert a substantial effect on the disposition of displaced drugs that bind at a level of 99% or more. Because of this, care must be taken in calculating the free concentration of drugs such as PA which show site-to-site

displacement in the presence of another drug. The results of this study are important and provide a basis for a more detailed study of the pharmacokinetics of, not only PA but other NSAIDs of the same group.

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