Mode of Interaction of Loop Diuretics with Human Serum Albumin and Characterization of Binding Site

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Purpose. The purpose of this study was to investigate the binding mechanism of loop diuretics with HSA and to characterize the binding site on HSA.
Methods. Quantitative analysis of potential interaction between ligands bound to HSA was performed by equilibrium dialysis and data for binding of the two ligands to HSA were analyzed on the basis of a theoretical model of simultaneous binding of two ligands.
Results. The binding of loop diuretics is dependent upon the N-B transition, conformational change of albumin. Furthermore, from the results of binding of the drugs to modified HSA, the lysine residue seems to be involved in the binding of loop diuretics to HSA.
Conclusions. Analysis using models describing independent, competitive, cooperative and anti-cooperative binding led to the conclusion that loop diuretics bind to site I, particularly to the warfarin region on HSA.

KEY WORDS: warfarin binding region; loop diuretics; human serum albumin; binding site; displacement.

INTRODUCTION

Loop diuretics such as furosemide are widely used because they produce a good diuretic response in patients with edema due to congestive heart failure, hepatic cirrhosis, or renal disease. These drugs bind exclusively or almost exclusively to albumin (1). The results of in vitro displacement experiments have demonstrated that many acidic drugs including warfarin bound at site I on human serum albumin (HSA) competitively inhibit the interaction of furosemide with the binding site on HSA (2,3). However, the mode of interaction of these drugs with HSA as well as the binding site on HSA have not been fully elucidated. Better understanding of the molecular interactions and the binding sites involved might help to predict drug interactions because these loop diuretics are frequently coadministered with different drugs such as antibiotics and coumarin anticoagulant drugs. Previously, we reported that both hydrophobic and electrostatic interactions are the driving forces for the binding of benzothiadiazide diuretics to HSA (4). Thus, the present study was undertaken to investigate the binding mechanism of loop diuretics with HSA and to characterize the binding site on HSA.

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MATERIALS AND METHODS

Materials

HSA (essentially fatty acid-free albumin) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Furosemide and piretanide were gifts from Hoechst Japan Co. (Tokyo, Japan). Bumetanide was supplied by Sankyo Co. (Japan). The chemical purity of different loop diuretics was above 99% based as determined by HPLC. 7-Amino-4-methylcoumarin-3-acetic acid (AMCA) was a generous gift from Prof. Goya of Kumamoto University. Dansylsarcosin (DNSS) and dansyl-L-asparagine (DNSA) were purchased from the Sigma Chemical Co.. Potassium warfarin (Eisai Co., Tokyo, Japan), ibuprofen (Kaken Pharmaceuticals Co., Tokyo, Japan), diazepam (Nippon Roche K.K., Tokyo, Japan) and phenylbutazone (Ciba Geigy Co., Summit, NJ, U.S.A.) were obtained as pure substances from the respective manufacturer. All other reagents were of analytical grade. All the buffers used were prepared with sodium phosphate dibasic and sodium phosphate monobasic salts.

Methods

Preparation of HSA Derivatives

The lone tryptophan (Trp) residue, lysine (Lys) residues, and tyrosine (Tyr) residues were modified as described previously (4). The conformation of HSA derivatives was checked by circular dichroism (CD), fluorescence measurements and SDS-PAGE. No significant structural alterations were observed as compared with the native protein.

Fluorescence Method

Fluorescence measurements were made using a Jasco FP-770 (Tokyo, Japan). The percentage of displacement of probe was determined using the following equation according to the method of Sudlow et al. (5):

$$\frac{F_1 - F_2}{F_1} \times 100 \tag{1}$$

where, F_1 and F_2 are the fluorescence intensities of probe plus HSA without and with the drug, respectively. The fluorescence intensities of the probe plus HSA (1:5, 2 μM:10 μM) was measured at 25°C before and after the addition of drugs (5–40 μM).

Ultrafiltration Method

Ultrafiltration experiments were performed using Tosoh plastic ultrafiltration (Kanagawa, Japan). Aliquots of various ratios of drug (0–400 μM)-HSA (60–120 μM) mixture (0.9 ml) were centrifuged at 3300 rpm for 15 minutes at 25°C Adsorptions of drugs or probes onto the membrane or apparatus were negligible. No protein leakage was detected during the experiment. After equribirum was reached, the free concentration of drug was determined by HPLC.

For the separation of unbound drug species from both native and differently modified forms of HSA, ultrafiltration method as described above was used with little modification. The samples containing 20 μM solution (0.9 ml) of protein

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and drug were dialyzed by ultrafiltration at pH 7.4 at 25°C. The quantitative determination of unbound drug was performed by the ultrafiltration method as described above. Both drug and displacer concentrations were determined by HPLC, in a system consisting of a Shimadzu SPD-6A pump, Shimadzu LC-6A UV detector and Shimadzu system controller SCL-6A. A column of LiChrosorb RP-select B (Cica Merck, Tokyo, Japan) for all compounds was used as stationary phase. For the assay of furosemide, bumetanide, piretanide, DNSA, DNSS and warfarin, a UV monitor was used. The UV wavelengths were 229 nm, 229 nm, 229 nm, 229 nm, 229 nm and 285 nm for furosemide, bumetanide, piretanide, DNSA, DNSS and warfarin, respectively. The mobile phases consisted of 35 mM phosphate buffer (pH4.6)-acetonitrile-methanol (58:30:12 v/v) for furosemide, bumetanide, piretanide, DNSA and DNSS, and of 11 mM phosphate buffer (pH 5.1)-acetonitrile (60:40 v/v) for warfarin. Bound concentration was calculated by the following equation.

$$D_b = \text{total drug concentration (before ultrafiltration) } (D_t) - \text{drug concentration in filtrated fraction } (D_f) \quad (2)$$

The displacement patterns were estimated by monitoring the changes in the bound fraction which were calculated by the following equation.

$$f_b = \frac{D_b}{D_f + D_b} \quad (3)$$

The percentage of displacement of loop diuretic was determined according to following equation.

$$p(\%) = \frac{f_{b1} - f_{b2}}{f_{b1}} \times 100 \quad (4)$$

where f_{b1} and f_{b2} are the bound fractions of loop diuretic without ligand and with drug displacer, respectively.

CD and UV Spectroscopic Experiments

CD experiments were performed on a Jasco J-720 spectropolarimeter (Tokyo, Japan) using 5 mm cells at 25°C. Loop diuretic solutions, all at 20 μM, were used in the CD experiments. HSA was also used as a 20 μM solution. Induced ellipticity was defined as the ellipticity of drug-HSA mixture minus the ellipticity of HSA alone within the same wavelength region and was expressed in degrees. Difference spectra (UV spectrum (drug-HSA mixture)—UV spectrum (drug)—UV spectrum (HSA)) were measured by using a pair of 10mm split compartment-tandem cuvette.

Data Analysis

All binding parameters were estimated by fitting the experimental values to the following Scatchard equation using a nonlinear squares computer program (MULTI program) (6),

$$r/D_f = nK - rK \quad (5)$$

where r is the number of moles of bound drug per mole of protein, n is the number of binding sites per protein molecule, K is the association constant, and D_f is the free drug concentration.

The binding of two ligands is sometimes very complicated and cannot be explained on the basis of simple competition alone. In the present study, on the basis of 1:1 complex formation by all compounds to HSA, we treated data according to the assumption that the interaction of two ligands simultaneously bound to HSA had taken place according to the interaction models proposed by Kragh-Hansen (2).

RESULTS

Binding of Loop Diuretics to HSA

Scatchard analysis of the ultrafiltration data showed a nonlinear curve, indicating the presence of at least two classes of sites for the binding of loop diuretics to HSA. The best fitting values for the binding parameters obtained by the ultrafiltration method are shown in Table I. The binding parameters of loop diuretics are in good agreements with those in the literature (7).

Moreover, the binding of loop diuretics to HSA was examined using CD and UV spectroscopy. The binding of furosemide to HSA generated biphasic extrinsic Cotton effects in the wavelength region from 250 to 300 nm. In addition, the UV difference spectrum of furosemide bound to HSA was observed (data not shown). Two other drugs, piretanide and bumetanide, also caused the induced CD spectra accompanied with the UV difference spectra following binding of the drugs to HSA. However, CD data obtained for the three systems were different from each other in the location of the peaks and magnitude of ellipticity (θobs = 6290 (mdeg) at 263 nm, θobs = −5740 (mdeg) at 283 nm for furosemide; θobs = 8430 (mdeg) at 336 nm for piretanide; θobs = −2720 (mdeg) at 286 nm for bumetanide). These differences can be explained on the basis of different spatial orientation of the drugs within the binding cavities.

Next, the effects of pH on binding of loop diuretics to HSA were studied. The binding fractions for all the systems increased with the rising of pH (data not shown).

Table I. Binding Parameters of Loop Diuretics to HSA as Determined by Ultrafiltration at pH 7.4 and 25°C

	Present Data				Literature Data				
Ligand	n_1	$K_1(\times 10^4 M^{-1})$	n_2	$K_2(\times 10^4 M^{-1})$	n_1	$K_1(\times 10^4 M^{-1})$	n_2	$K_2(\times 10^4 M^{-1})$	Reference
Furosemide	1.0	19.0	3.5	3.0	1.29	20.7	7.57	0.237	(8)
					1.0	17.0	4–5	0.96	(9)
Bumetanide	1.0	16.0	3.6	2.6	1.22	11.0	6.54	0.415	(8)
Piretanide	1.0	9.5	2.8	2.2	1.06	9.5	5.72	0.166	(8)

Table II. Displacement Data of Loop Diuretics Determined by Ultrafiltration at pH 7.4 and 25°C

Displacer Drugs	Displacement of Bound Ligands (%)		
	Furosemide	Bumetanide	Piretanide
Warfarin (Site I)	11.2	11.5	23.0
Phenylbutazone (Site I)	11.2	9.9	22.5
DNSA (Site I)	6.6	9.0	9.5
Ibuprofen (Site II)	2.2	3.8	7.2
DNSS (Site II)	5.9	6.0	3.6

The following concentrations were used: [HSA] = 6×10^{-5}M, [Ligands] = 3×10^{-5}M, [Displacer drugs] = 4×10^{-5}M for furosemide, bumetanide and piretanide.

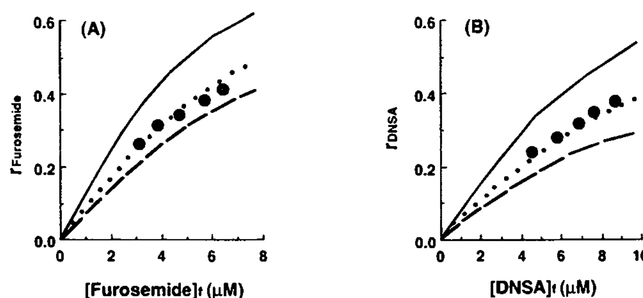

Fig. 2. Binding of furosemide in the presence of DNSA (A) and vice versa (B) to HSA at pH 7.4 and 25°C. (A), Binding of furosemide (35–55 μM) to HSA (120 μM) in the presence of DNSA (60 μM); (B), Binding of DNSA (35–55 μM) to HSA (120 μM)in the presence of furosemide (60 μM). ●, Experimental values; ----------, Theoretical curve assuming competitive binding; ———, Theoretical curve assuming independent binding; . . . . ., Theoretical curve assuming anti-cooperative interaction between furosemide and DNSA (χ = 0.32).

Displacement Studies by Fluorescent Probe and Ultrafiltration Methods

To identify the location of the binding site of furosemide on HSA, site marker displacement experiments were carried out using fluorescent probes which specifically bind to known sites on HSA. Furosemide markedly displaced warfarin, a marker for site I. Furosemide also displaced DNSS, a site II marker, moderately, whereas the fluorescence of AMCA, a site III marker, was not affected by furosemide binding.

We further performed detailed displacement experiments with loop diuretics using typical site I and site II drugs by measurement of free concentration using an ultrafiltration method. The results are summarized in Table II. All the drugs tested were significantly displaced by site I ligands compared with site II ligands except DNSS.

Binding of Furosemide to HSA in the Presence of Site-specific Probe and Vice Versa

Mutual displacement experiments were carried out and data were analyzed on the basis of a theoretical model of simultaneous binding of two ligands. Figure 1 represents the binding of furosemide to HSA in the presence of warfarin, a site I-specific probe (Fig. 1A), and vice versa (Fig. 1B). In both cases, the observed data fit the theoretical curve well, assuming competitive binding between furosemide and warfarin as predicted from the results shown in Table II.

The binding data of furosemide in the presence of DNSA apparently fit the theoretical curve assuming anti-cooperative binding and were very close to the theoretical curve assuming competitive binding as shown in Fig. 2A. Figure 2B shows the results of the converse experiments. Figure 3A illustrates the binding of furosemide to HSA in the presence of a site II-specific probe, dansylsarcosine (DNSS). The experimental data for the binding of furosemide to HSA in the presence of DNSS seemed to fit well to the theoretical curve assuming anti-cooperative binding of the drug and were also close the theoretical curve assuming independent binding of the two drugs. Figure 3B shows the results of the converse experiments. Furthermore, binding experiments between loop diuretics were carried out to confirm whether loop diuretics bind to the same or different sites on HSA. As shown in Fig. 4, a competitive interaction was observed for furosemide-piretanide.

Binding of Loop Diuretics to HSA Derivatives

To investigate which amino acids are involved, the binding of loop diuretics to chemically modified HSA was examined by

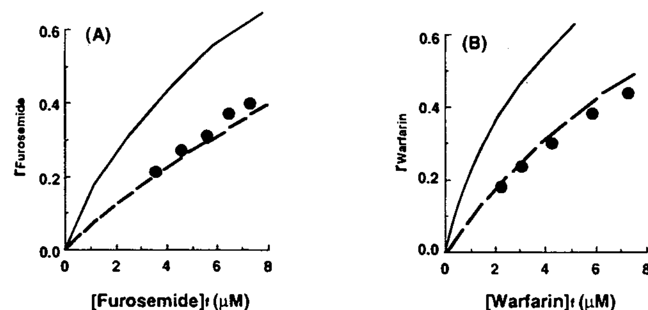

Fig. 1. Binding of furosemide in the presence of warfarin (A) and vice versa (B) to HSA at pH 7.4 and 25°C. (A), Binding of furosemide (35–55 μM) to HSA (120 μM) in the presence of warfarin (60 μM); (B), Binding of warfarin (35–55 μM) to HSA (120 μM) in the presence of furosemide (60 μM). ●, Experimental values; ---------, Theoretical curve assuming competitive binding; ———, Theoretical curve assuming independent binding.

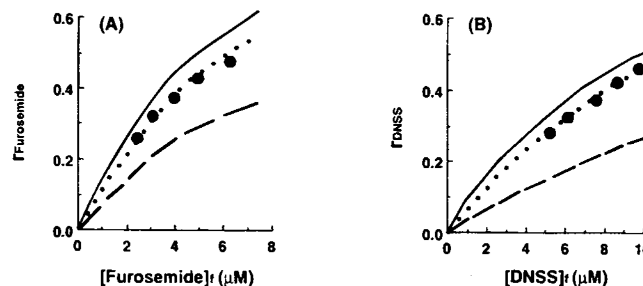

Fig. 3. Binding of furosemide in the presence of DNSS (A) and vice versa (B) to HSA at pH 7.4 and 25°C. (A), Binding of furosemide (35–55 μM) to HSA (120 μM) in the presence of DNSS (60 μM); (B), Binding of DNSS (35–55 μM) to HSA (120 μM) in the presence of furosemide (60 μM). ●, Experimental values; ----------, Theoretical curve assuming competitive binding; ———, Theoretical curve assuming independent binding; . . . . ., Theoretical curve assuming anti-cooperative interaction between furosemide and DNSS (χ = 0.83).

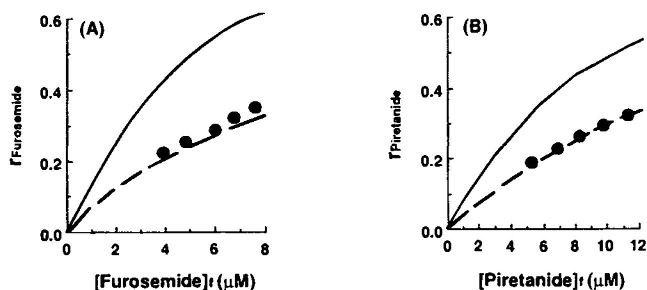

Fig. 4. Binding of furosemide in the presence of piretanide (A) and vice versa (B) to HSA at pH 7.4 and 25°C. (A), Binding of furosemide (30–50 μM) to HSA (120 μM) in the presence of piretanide (100 μM); (B), Binding of piretanide (30–50 μM) to HSA (120 μM)in the presence of furosemide (60 μM). ●, Experimental values; ----------, Theoretical curve assuming competitive binding; ———, Theoretical curve assuming independent binding.

ultrafiltration. Results of chemical modification of HSA were the same as those previously reported (4). Free drug fraction following loop diuretic binding to Lys-modified HSA ($p < 0.01$) was increased significantly over that with native HSA. However, Trp-, and Tyr-modified HSA showed no increase in free concentration of the three drugs (data not shown).

DISCUSSION

Mode of Interaction of Loop Diuretics with HSA

Serum protein binding of loop diuretics was almost exclusively due to their binding to HSA. It is generally considered that hydrophobic interactions play an important role in binding of small organic molecular ligands to albumin. However, the primary as well as secondary binding constants for these drugs were almost the same (Table I), although their lipophilicities were different (partition coefficient values (1-octanol/pH 7.4 phosphate buffer): 0.05 for furosemide; 0.08 for bumetamide; 0.28 for piretanide). This suggests that the binding cannot be explained only by hydrophobic interaction. In fact, interaction of furosemide with cetyltrimethyl ammonium bromide (CTAB), a cationic detergent, produced a UV difference spectrum similar to that bound to HSA (data not shown). However, no UV difference spectrum of furosemide was induced at CTAB concentrations below the critical micellar concentrations or in the presence of the anionic detergent sodium lauryl sulfate. Similar spectral patterns were also observed for bumetanide and piretanide when they were dissolved in albumin or CTAB solution. This implies that the HSA binding site for loop diuretics consists of a cationic site on the surface to accommodate the aromatic ring. Moreover, it suggests that the 3-carboxybenzene sulfonamide part of loop diuretics is involved in the binding, although the compounds used here were limited.

It is well known that within the pH range from 6–9, HSA exists in two conformational states, the so-called N-form and B-form (8). The pH dependence of binding may be due to changes in albumin rather than in the degree on ionization of furosemide, as suggested by the pKa values of furosemide ($pka_1 = 3.8$; $pka_2 = 7.5$). Despite the differences in the pKa values of the three drugs, the favorable binding in the B form seems to support this hypothesis.

Binding Site of Loop Diuretics on HSA

From the preliminary displacement experiments using fluorescent probes and ultrafiltration, it was revealed that warfarin was markedly displaced by loop diuretics, suggesting that these molecules share a common binding site on HSA. However, these displacement methods cannot fully explain the type of drug binding in the presence of other drugs. The binding of two ligands to HSA can be classified according to the ligand-binding model proposed by Kragh-Hansen (1) as independent binding, competitive binding, cooperative binding and anti-cooperative binding. So, the binding data were analyzed using this model. As can be seen in Fig. 2, the binding of furosemide in the presence of warfarin and vice versa were competitive, suggesting the validity of the previous finding obtained by preliminary displacement experiments that furosemide binds to site I on HSA. Fehske et al. (3) proposed that site I consists of two regions, called the warfarin and azapropazone regions. Very recently, we also found data (9) to support Fehskes' idea. For this, we characterized the binding site of loop diuretics in greater detail. Firstly, the binding of furosemide in the presence of DNSA was examined because DNSA binds the same site as azapropazone or phenylbutazone. Interaction of furosemide-DNSA or DNSA-furosemide indicated that the two ligands were not competitive but anti-cooperative contrary to the expectations from the data shown in Table II. However, the χ value ($\chi = 0.32$) was rather small. This suggests that the two binding sites may be close. In fact, our previous results regarding the interaction of warfarin-DNSA and DNSA-warfarin (9) suggested that the warfarin and azapropazone regions (or DNSA) might be in the same vicinity. Site I consists of at least three regions (warfarin, azapropazone and p-aminobenzoate region), which are partially overlapping (9). The binding of furosemide was almost independent with respect to that of the site II-specific probe DNSS. Finally, we characterized the mechanism of the interaction in the furosemide-piretanide system (Fig. 4) and it was found to be completely competitive.

Based upon the results of X-ray analysis reported by Carter and Ho (10), site I appears to be located in hydrophobic cavities of subdomain IIA on HSA. The involvement of Lys residues in the binding of the loop diuretics to HSA was strongly expected from the binding experiments using modified HSA. At least, thirty Lys residues were modified in these experiments. Lys-199 located in the subdomain IIA is considered to be the major site of conjugation with drugs such as aspirin (11). Interestingly, the reactivity of Lys-199 may be attributed to its unusually low pKa of 7.9 (12), which can be rationalized based upon the close interaction of Lys-199 with His-242 which may play an important role in the N-B transition of HSA. This implies that Lys-199 may indirectly participate in N-B transition through the interaction with His-242. In fact, pH had hardly any effect on the binding of furosemide to Lys-modified-HSA (Free fraction ratio; F(pH 6.5)/F(pH 7.4) = 1.02; F(pH 8.5)/F(pH 7.4) = 0.98) compared with natural HSA (Free fraction ratio; F(pH 6.5)/F(pH 7.4) = 1.05; F(pH 8.5)/F(pH 7.4) = 0.79). Therefore, these limited results suggest that Lys-199 might be involved in the binding of loop diuretics. It is also possible that the modification of Lys residues may be forced to take up preferably one conformation, the N- or B- conformation. According to crystal structure of HSA, the subdomains located in drug binding sites, such as site I and II, share a common interface which stabilized by both hydrophobic and salt bridge interactions. Moreover, Bos et al. (13) proposed that during N-B transition albumin molecule altered from a "heart shaped" structure to a more open ellipsoid

structure. Therefore, if binding subdomain no longer share a common interface due to modification of Lys residues located in site I and/or site II, the drug binding may be pH independent.

In conclusion, loop diuretics bind to site I, particularly to the warfarin region of HSA. Very recently, we found that 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF), one of the uremic toxins, was strongly bound to site I, the warfarin region on HSA (14). This compound is largely responsible for the impaired binding of many site I drugs such as warfarin and glibenclamide. Interestingly, Lim et al. (15) recently proposed a complex drug interaction mechanism, a "cascade" effect, on ligand displacement reaction for CMPF-fenclofenac-thyroxine interaction. Therefore, the data presented will be useful for prediction of drug interaction.

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