Characterization of Binding Site of Uremic Toxins on Human Serum Albumin

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The interaction of uremic toxins including indole-3-acetic acid (IA), indoxyl sulfate (IS), hippuric acid (HA) and 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF) with human serum albumin (HSA) has been investigated by three methods of fluorescent probe displacement, ultrafiltration and equilibrium dialysis. The binding parameter of CMPF was found to have the strongest affinity (10^7 M^{-1}) among all the uremic toxins studied. Competitive experiment based on the method of Kragh-Hansen suggested that IA, IS and HA bind to site II, whereas CMPF binds to site I. The present limited data indicated that the four uremic toxins caused inhibition to any endo- or exogenous substances on HSA.

Key words human serum albumin; uremic toxin; binding site; competitive displacement; protein binding; renal disease

It is well known that plasma protein binding of endogenous and exogenous substances is affected in a disease state,^{1,2)} especially, in chronic renal disease.³⁾ Many endogenous substances accumulated in the body responsible for reduced renal function and some of them also reduce the serum binding of drugs.^{4,5)} Researchers have reported several compounds such as indoxyl sulfate (IS), indole-3-acetic acid (IA), hippuric acid (HA) and 3-carboxy-4-methyl-5-propyl-2-furan propanoic acid (CMPF) (Fig. 1), generally called "uremic toxins," which inhibit drug protein binding and were isolated and determined in uremic serum.^{6–8)} These uremic toxins are not removed by hemodialysis^{9,10)} and their concentrations remain higher for at least 90 d even after a successful kidney transplantation.¹¹⁾ These compounds are largely responsible for the decrease in binding of many drugs, *e.g.* L-tryptophan,¹²⁾ diazepam,¹³⁾ warfarin,¹⁴⁾ salicylate and furosemide¹⁵⁾ in uremic serum, and the concomitant increase in unbound fraction of a drug may cause side effects in renal disease. However, it is not clear from these reports whether or not interaction between uremic toxins and drugs was generated by competitive or allosteric displacement.

As human serum albumin (HSA) in serum is the most important drug carrier protein^{16,17)} and binds more strongly to acidic drugs than basic to drugs, most uremic

toxins seem to bind to HSA. HSA is considered to have at least three specific binding sites for high-affinity binding of drugs, sites I, II, and III.^{18–20)} He and Carter revealed crystal structures of some albumins, human²¹⁾ and horse,²²⁾ and they suggested that HSA has binding sites of compounds within specialized cavities in subdomains IIA and IIIA, which are consistent with site I and site II, respectively. Thus, interaction between accumulated uremic toxins and administered drugs on HSA will be generated. So far, binding site of uremic toxins were deduced from the extent of binding inhibition of site marker drugs. However, displacement data have not been quantitatively analyzed using a competitive and allosteric interaction model.

In the present study, we characterized the binding site of four uremic toxins on HSA based on the theoretical model described by Kragh-Hansen.²³⁾ This was performed by indirect method, fluorescence displacement method, and direct methods, ultrafiltration and equilibrium dialysis, using warfarin (WF), dansylsarcosine (DNSS) as specific site marker probes on HSA.

MATERIALS AND METHODS

Materials HSA was donated by the Chemo-Sera-Therapeutic Research Institute (Kumamoto, Japan). It

indole-3-acetic acid (IA)

indoxyl sulfate (IS)

hippuric acid (HA)

3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF)

Fig. 1. Chemical Structures of Uremic Toxins in This Study

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was defatted with activated charcoal in solution at 0 °C, acidified with H₂SO₄ to pH 3 and then freeze-dried. The molecular mass of HSA was assumed to be 66500 Da. HSA gave only one band on SDS-PAGE. DNSS (Sigma Chemical Co., St. Louis, MO, U.S.A.), IS, IA, HA (Nacalai Tesque, Inc., Kyoto, Japan), and WF (Eisai Co., Tokyo, Japan) were obtained as pure substances from manufacturers. CMPF was synthesized according to the method of Pfordt *et al.*²⁴⁾ and the structure of the compound was assigned on the basis of IR, NMR and mass spectral data analysis. The purity of the compound was determined by HPLC.

All other chemicals were of analytical grade. All solutions were prepared in deionized and distilled water. Phosphate buffer (0.067 M, pH 7.4) was prepared with sodium phosphate dibasic and sodium phosphate monobasic and used exclusively in this study.

Fluorescent Probe Displacement Fluorescence measurements were carried out using a Jasco FP-770 fluorometer (Tokyo, Japan). The intrinsic fluorescence of HSA was obtained at 290 nm. Within the limits of sensitivity, an excitation wavelength was chosen to ensure the least possible absorption of incidental light. Emission wavelengths were chosen to give the maximum fluorescence of the probe bound to HSA, with insignificant fluorescence in the buffer. Probes chosen had either a single binding site or two sites with widely separated dissociation constants. The probe to HSA ratio was kept at 1:10 in order to keep the non-specific binding of probes to a minimum. The percentage of displacement of the probe was determined according to the method of Sudlow *et al.*¹⁸⁾:

$$\frac{F_1 - F_2}{F_1} \times 100 \quad (1)$$

where F_1 and F_2 represent the fluorescence of the probe plus HSA without the drug and with the drug, respectively.

Determination of Ligand Binding Parameters A quantitative analysis of potential interaction between ligands bound to HSA was performed by two direct methods, ultrafiltration and equilibrium dialysis. The ultrafiltration was applied in an Amicon MPS-1 micropartition system with YMT ultrafiltration membrane. One-ml samples were ultrafiltered at 25 °C at 2000 rpm for 40 min. The equilibrium dialysis was performed using 2-ml plastic dialysis cell (Sanplatec Co., Osaka, Japan). Two cell compartments were separated by Visking cellulose membranes. Aliquots (1.5 ml) of ligand-HSA complexes at various ratios were dialyzed against the same volume of buffer at 25 °C for 12 h. Free ligand concentrations were measured by HPLC; HPLC system consisted of a Hitachi L-6200 intelligent pump and either a Hitachi F-1050 fluorescence spectrophotometer or Hitachi L-4000 UV detector. A column of LiChrosorb RP-18 (Cica Merck, Tokyo, Japan) was used as a stationary phase. The mobile phase consisted of acetate buffer (0.2 M, pH 4.5)–acetonitrile (93:7 v/v, for HA; 65:35 v/v, for IA and IS; and 40:60 v/v, for DNSS and WF) and acetate buffer (0.2 M, pH 7.4)–acetonitrile–acetic acid (60:40:0.5 v/v, for CMPF). HA and CMPF were detected by UV at 240 nm and 261 nm, respectively. DNSS, WF, IA and IS were detected by the fluorescence monitor. The excitation and

emission wavelengths were at 310/550 nm, 300/400 nm and 280/375 nm for DNSS, WF and IA, respectively. IA has the same excitation and emission wavelength as IS.

In the ultrafiltration method, bound ligand concentration (C_b) was calculated by the following equation:

$$C_b = \text{total ligand concentration (before ultrafiltration)} (C_t) \\ - \text{ligand concentration in filtrated fraction} (C_f) \quad (2)$$

where C_t and C_f are total and free ligand concentration, respectively.

In case of equilibrium dialysis, the bound ligand concentration (C_b) was calculated by estimation of free ligand concentration (C_f) according to the following equation:

$$C_b = \text{total ligand concentration (before equilibrium dialysis)} (C_t) \\ - \text{ligand concentration in buffer compartment} (C_f) \times 2 \quad (3)$$

Data Analysis: All binding parameters were estimated by fitting the experimental values to the following Scatchard equation using a non-linear squares computer program (MULTI program)²⁵⁾:

$$r = \sum_{i=1}^I \frac{n_i K_i C_f}{1 + K_i C_f} \quad (4)$$

where r is the number of moles of bound drug per mole of protein, n_i and K_i are the number of binding sites per protein molecule and the association constant, respectively, in the i th binding class.

Interaction Mode between Two Ligands Bound to High-Affinity Site on HSA Ligand-HSA mixtures at various ratios including a fixed concentration of a potential displacer were dialyzed against the same volume of buffer or ultrafiltered. Determination of concentrations of bound and free ligand was done as described above. The binding of two ligands to the protein is sometimes complicated and cannot be explained by a simple competition interaction alone. Therefore, in order to estimate the mode of interaction between two ligands simultaneously bound to HSA, the data were analyzed using the method of Kragh-Hansen.²³⁾ For this type of dialysis the association constants for high-affinity sites were used, and the concentrations of the ligands must be set in such a way that the mole of protein is less than 0.6 to minimize ligand binding to any low-affinity sites.

A simple competition between two drugs A and B for identical protein binding sites was analyzed by the following equations:

$$r_A = \frac{[PA]}{[P]} = \frac{K_A[A_f]}{1 + K_A[A_f] + K_B[B_f]} \quad (5)$$

$$r_B = \frac{[PB]}{[P]} = \frac{K_B[B_f]}{1 + K_A[A_f] + K_B[B_f]} \quad (6)$$

$$[A_t] = [PA] + [A_f] \quad (7)$$

$$[B_t] = [PB] + [B_f] \quad (8)$$

where K_A and K_B are the association constants of ligands A and B, respectively, and $[A_f]$ and $[B_f]$ are the concentrations of the free ligands. $[PA]$ and $[PB]$ are the concentrations of bound ligands, $[A_t]$ and $[B_t]$ are the total concentration of the ligands, and $[P_t]$ is the total

concentration of HSA. With knowledge of the concentration of the total ligands, the association constant of the ligands, and the concentration of HSA, it is possible to calculate theoretically the r values by the above equation.

Independent binding of ligands A and B can be quantitated as follows:

$$r_A = \frac{[PA]}{[P]} = \frac{K_A[A_f]}{1 + K_A[A_f]} \quad (9)$$

$$r_B = \frac{[PB]}{[P]} = \frac{K_B[B_f]}{1 + K_B[B_f]} \quad (10)$$

In certain cases ligand displacement did not follow a competitive binding scheme. In these cases a coupling constant (χ), represented by $\chi = K_{BA}/K_A$ or $\chi = K_{AB}/K_B$, was estimated. K_{BA} is the association constant of ligand A in the presence of ligand B, and K_{AB} is that of ligand B in the presence of ligand A. If A and B bind independently to HSA, then $\chi = 1$; i.e. K_{BA} is equal to K_A and K_{AB} to K_B . $\chi > 1$ and $0 < \chi < 1$ indicate cooperative and anticooperative binding, respectively. Competitive displacement between two ligands is represented by $\chi = 0$. The value of χ was determined as follows.

The total protein concentration is equal to the sum of the concentrations of [P], [PA], [PB] and [BPA]:

$$[P_t] = [P] + [PA] + [PB] + [BPA] \quad (11)$$

where [P] is the free concentration of HSA.

This relationship is transformed into

$$[P_t] = [P] + K_A[P][A_f] + K_B[P][B_f] + \chi K_A K_B [P][A_f][B_f] \quad (12)$$

The concentration of bound A is given by

$$[PA] = [A_t] - [A_f] = K_A[P][A_f] + \chi K_A K_B [P][A_f][B_f] \quad (13)$$

From Eqs. 12 and 13 the following equation was obtained:

$$\chi = \frac{([A_t] - [A_f])(1 + K_A[A_f] + K_B[B_f]) - [P_t]K_A[A_f]}{K_A[A_f]K_B[B_f]([P_t] - [A_t] + [A_f])} \quad (14)$$

Since $[P_t]$, $[A_t]$, $[A_f]$, K_A and K_B are known, the value of χ can be easily obtained.

RESULTS

Identification of Binding Site of Uremic Toxins by Displacement Fluorescence Method To identify the binding site of uremic toxins on HSA, site marker displacements were performed using representative fluorescent probes, WF (site I probe) and DNSS (site II probe) based on the method of Sudlow *et al.*¹⁸⁾ As shown in Fig. 2, WF for site I specific probe was significantly displaced by CMPF alone, however, IA and IS caused a little enhancement of the fluorescence of WF-HSA complex (Fig. 2A). DNSS, on the other hand, which is already known as site II marker fluorescent probe was remarkably decreased by IA and IS, but to different extents. The effect of CMPF on the fluorescence of DNSS-HSA complex exhibited unique behavior (Fig. 2B). The fluorescence intensity of the complex was initially increased by additional CMPF up to a 1:1 ratio for HSA. There were almost no differences in the fluorescence changes of any of the site marker probes observed by HA.

Fig. 2. Effect of Uremic Toxins on the Fluorescence Intensity of WF (A) and DNSS (B) Bound to HSA

Uremic toxins used were IA ($\blacktriangle$), IS ($\circ$), HA ($\bullet$) and CMPF ($\blacklozenge$). Following concentrations were used: HSA, 20 μ M; WF and DNSS, 2 μ M.

Table 1. Binding Parameters of WF and DNSS to HSA as Measured by Direct Methods at pH 7.4 and 25 $^{\circ}$ C Relative to Literature Values

Probe	Present data		Literature data		Ref. No.
	n	K ($\times 10^5 \text{ M}^{-1}$)	n	K ($\times 10^5 \text{ M}^{-1}$)	
WF	1	3.0 ^{a)} 3.6 ^{b)}	1	3.5	26
DNSS	1	14.5 ^{a)} 15.6 ^{b)}	—	—	—

a) HSA; 40 μ M. b) HSA; 200 μ M. —, no literature data could be found.

Table 2. Binding Parameters of Uremic Toxins to HSA as Determined by Direct Methods at pH 7.4 and 25 $^{\circ}$ C

Uremic toxin	n_1	K_1 ($\times 10^5 \text{ M}^{-1}$)	n_2	K_2 ($\times 10^3 \text{ M}^{-1}$)
IA	1	2.1	5	0.8
IS	1	16.1	3	8.3
HA	1	0.1	7	0.3
CMPF	1	130.5	2	33.4

Determination of Binding Parameters To confirm the results obtained by the fluorescence displacement method, binding between uremic toxins and the fluorescent probes was further examined by direct method, ultrafiltration or equilibrium dialysis. Before performing the competition studies, the individual binding of the probes was studied. The number of high-affinity binding site was one in all cases and the primary association constants were in the order of 10^4 – 10^6 M^{-1} (Table 1). The individual binding of uremic toxins was monitored by the direct method and the n_i and K_i values, obtained by non-linear least squares method, are summarized in Table 2. It was found that the number of high-affinity binding sites for each uremic toxin was one and the primary association constants were in the order of 10^4 – 10^7 M^{-1} ; especially, CMPF strongly bound to HSA. The results, summarized in Table 2, were used for analysis of interaction mode.

Binding between High-Affinity Binding of WF (Site I Maker Probe) and Uremic Toxins The bindings between the site I maker probe, WF, and the four uremic toxins

Fig. 3. WF (a) (d), IA (b), IS (c), HA (e) and CMPF (f) Binding to HSA in the Presence of Another Ligand at pH 7.4 and 25 °C

(a) Binding of WF (8–18 μM) to HSA (40 μM) in the presence of IA (24 μM) (▲) or IS (20 μM) (○). (b) Binding of IA (8–18 μM) to HSA (40 μM) in the presence of WF (20 μM) (▲). (c) Binding of IS (4–24 μM) to HSA (40 μM) in the presence of WF (24 μM) (○). (d) Binding of WF (40–140 μM) to HSA (200 μM) in the presence of HA (140 μM) (●) or CMPF (80 μM) (△). (e) Binding of HA (40–140 μM) to HSA (200 μM) in the presence of WF (100 μM) (●). (f) Binding of CMPF (60–120 μM) to HSA (200 μM) in the presence of WF (140 μM) (△). —, theoretical curves assuming independent binding of two ligands; ---, theoretical curves for WF (d) and CMPF (f) binding assuming competition between the two ligands for a common binding site on HSA.

on HSA were investigated. As shown in Fig. 3 (upper), the binding of WF in the presence of IA or IS (a) and the binding of them in the presence of WF (b, c) were in agreement with the theoretical curves assuming the independent binding between uremic toxins and WF. A similar finding (Fig. 3 lower) was observed in the binding of HA-WF and this, too, was clearly independent (d, e). Thus, IA, IS and HA did not interact with site I but they may share a common binding site on HSA. Moreover, the binding of WF in the presence of CMPF and the binding of CMPF, which significantly decreased the intensity of WF in the fluorescence method, was examined in the presence of WF (d, f). As expected, the binding of CMPF and WF exhibited a competitive manner.

Binding between High-Affinity Binding of DNSS (Site II Marker Probe) and Uremic Toxins Figure 4 (upper) represents the binding of DNSS in the presence of IA or IS (a) and the binding of IA or IS in the presence of DNSS (b, c). The binding of both IA-DNSS and IS-DNSS on HSA was in a competitive manner, fitting well to the theoretical curves assuming the competitive displacement between them. As shown in Fig. 4 (lower), the different binding patterns between HA- and CMPF-DNSS were observed and the binding of DNSS in the presence of HA was similar to the curve assuming the displacement, but the binding of CMPF did not fit the competitive curve. The two converse experiments (e, f) suggested the same results.

Relation between High-Affinity Binding of Uremic

Toxins Binding experiments among uremic toxins were carried out to confirm whether these uremic toxins bind to the same or different sites on HSA. As shown in Fig. 5 (a, b), competitive interaction was observed for IA-IS. Therefore, the binding site of IA was the same site, site II, as that of IS. In contrast, no interaction between CMPF and IS was observed (c, d).

DISCUSSION

It is well known that plasma protein binding of many drugs^{4,12-14,26} and endogenous substances³ are decreased in renal disease, especially in uremia. Some investigators, firstly, proposed that the reduced binding of drugs to plasma protein are partly due to change of albumin concentration.²⁷ However, as the impaired drug binding to uremic plasma occurred despite its being a normal concentration, hypoalbuminaemia altering albumin concentration alone can not fully explain this.⁵ Other authors postulated other factors to account for the observed behaviors: (1) the structural change of albumin molecule and (2) accumulation of endogenous binding inhibitors in uremic plasma. Firstly, the structural change is unlikely to be the most important factor, because the binding to carbamylated albumin, which is due to urea in renal disease, cannot be restored fully by treatment of activated charcoal.²⁸ Thus, the most widely accepted explanation for the impaired binding to uremic plasma is that endogenous substances act as inhibitors of drug

Fig. 4. DNSS (a) (d), IA (b), IS (c), HA (e) and CMPF (f) Binding to HSA in the Presence of Another Ligand at pH 7.4 and 25 °C

(a) Binding of DNSS (4–14 μM) to HSA (40 μM) in the presence of IA (24 μM) ($\blacktriangle$) or IS (20 μM) ($\circ$). (b) Binding of IA (4–16 μM) to HSA (40 μM) in the presence of DNSS (20 μM) ($\blacktriangle$). (c) Binding of IS (2–18 μM) to HSA (40 μM) in the presence of DNSS (20 μM) ($\circ$). (d) Binding of DNSS (30–100 μM) to HSA (200 μM) in the presence of HA (180 μM) ($\bullet$) or CMPF (100 μM) ($\triangle$). (e) Binding of HA (20–80 μM) to HSA (200 μM) in the presence of DNSS (100 μM) ($\bullet$). (f) Binding of CMPF (60–120 μM) to HSA (200 μM) in the presence of DNSS (100 μM) ($\triangle$). —, theoretical curves assuming independent binding of two ligands; ---, theoretical curves for DNSS (a), IA (b) binding assuming competition between the two ligands for a common binding site. - - - - , theoretical curves for DNSS (a), IS (c) binding assuming competition between the two ligands for a common binding site. ·····, theoretical curves for DNSS (d), HA (e) binding assuming competition between the two ligands for a common binding site.

Fig. 5. IA (a), IS (b) (c), and CMPF (d) Binding to HSA in the Presence of Another Ligand at pH 7.4 and 25 °C

(a) Binding of IA (6–16 μM) to HSA (40 μM) in the presence of IS (20 μM) ($\blacktriangle$). (b) Binding of IS (4–14 μM) to HSA (40 μM) in the presence of IA (20 μM) ($\circ$). (c) Binding of IS (40–90 μM) to HSA (200 μM) in the presence of CMPF (80 μM) ($\circ$). (d) Binding of CMPF (60–120 μM) to HSA (200 μM) in the presence of IS (100 μM) ($\triangle$). —, theoretical curves assuming independent binding of two ligands; ---, theoretical curves for IA (a), IS (b) binding assuming competition between the two ligands for a common binding site.

binding.^{4,29,30}) Many accumulated endogenous substances is responsible for reduced renal function in chronic renal disease,⁸⁻¹⁰) but many are still unidentified. Researchers have reported several compounds, generally called 'uremic toxins', IS, IA, HA and CMPF, which were isolated and characterized in uremic serum (Fig. 1).^{6,7}) The resources of indole compounds and HA in serum are better understood. IA and IS are derived from intestinal bacteria metabolism of tryptophan. HA, glycine conjugate of benzoic acid, is derived primarily from aromatic amino acids by the metabolism of intestinal bacteria. How CMPF is produced in the body, however, is not known. Evidence has suggested that furan fatty acids are common constituents of diet and are also produced by degradation of longer side chain furan fatty acids.^{31,32}) These uremic toxins are not removed by hemodialysis and their concentrations remain increased for at least 90 d even after a successful kidney transplantation.¹¹) These compounds are largely responsible for the decrease in binding of endo- and exogenous substances, L-tryptophan, diazepam, warfarin, salicylate¹²⁻¹⁵) and thyroxine,³³) in uremic serum, and the concomitant increase in unbound fraction of compounds may cause side effects in renal disease.

There are many proteins in plasma, especially HSA^{16,17}) and α_1 -acid glycoprotein (AGP),³⁴) which are important drug carrier proteins. HSA binds to acidic drugs more than to basic drugs, while, AGP interacts with basic drugs. A number of isolated uremic toxins are acidic compounds, and it is believed that these might strongly bind to HSA. In fact, uremic toxins did not bind to AGP (data not shown).

There is a small number of distinct drug binding sites on HSA.³⁵) Sudlow *et al.*¹⁸) classified these sites into two specialized categories, which were denoted site I and site II, from their experiments based on interaction with fluorescent probes. More recently, He and Carter²¹) revealed the three dimensional structure of human by X-ray crystallographic study at a resolution of 2.8 Å, and suggested that HSA has binding sites of compounds within hydrophobic cavities in subdomains IIA and IIIA, which are consistent with site I and site II, respectively.

To characterize the binding site of the four uremic toxins on HSA, the fluorescent probe displacement was studied based on the method of Sudlow.¹⁸) The fluorescent probes used in this study, WF and DNSS, are bound to site I and site II, respectively. The results of the fluorescent probe experiments (Fig. 2) indicated that the high-affinity binding site of CMPF is site I, whereas IA and IS specifically interact with the site II marker probe. The CMPF binding to site I may simultaneously change the microenvironment of site II until saturation of CMPF binding to high-affinity site, site I, and then the site II probe may be displaced by CMPF which binds to low-affinity binding site. As shown in Table 2, CMPF has one high-affinity binding site and two low-affinity binding sites on HSA, and both values are much stronger than those of other uremic toxins. However, the association constant of CMPF in this study was one-order higher than the values calculated by Mabuchi²⁹) and Henderson³⁰) previously. The differences from values of those investigators are probably due to the distinct conditions which influence ligand binding such as

albumin lots (differences in ratio of mercaptoalbumin and non-mercaptoalbumin as well as fatty acid content), temperature, salt condition of buffer and techniques.

It is possible that the low-affinity binding site of CMPF influences site II or the benzodiazepine site. The present results of CMPF using fluorescent probes were supported by the report that warfarin and other site I drugs were more readily displaced by CMPF than site II drugs such as L-tryptophan.³⁶) Uremic toxins might be mainly bound to primary binding sites on HSA *in vivo*, and thus it is of significance to consider the primary binding sites of ligands on the HSA.

To confirm the results obtained by fluorescent probe displacement, which is an indirect quantitative method, required detailed investigation of the interactions at the primary binding sites between fluorescent probes and uremic toxins by the direct method, ultrafiltration or equilibrium dialysis. Thus, we performed competition experiments proposed by Kragh-Hansen²³) for the exact binding site of uremic toxins relative to the site marker fluorescent probes, WF and DNSS. These fluorescent probes in this study have one high-affinity binding site and the primary association constants were in excellent agreement with the literature values.³⁷) Competitive interaction was only observed between WF and CMPF (Fig. 3, lower), while the other compounds did not interact with WF but exhibited independent binding. As shown in Fig. 4, the binding of CMPF in the presence of DNSS and the binding of DNSS in the presence of CMPF was independent, as expected from the results in Figs. 2 and 3 (d, f). The three compounds also revealed that their binding site is site II since competitive interaction was observed between DNSS, specifically bound to site II, and the three uremic toxins. Finally, we attempted to identify further the interaction mechanism for the IA-IS and IS-CMPF systems (Fig. 5). The interaction between IA and IS was completely competitive, while no interaction was observed between IS and CMPF. These observations confirmed that IA and IS possess the same primary binding site, site II on HSA and that CMPF has a binding site distinct from these compounds. Thus, CMPF and IS bind independently to the high-affinity binding site on HSA and CMPF binds to site I.

The binding of HA is weak compared with other uremic toxins, but its accumulation in uremic serum is greater (approaching about 1 mM) in renal disease.³⁸) HA more significantly affects site I than site II in serum,¹⁴) thus it was thought that the binding site of HA is site I. However, the present result clearly showed that HA binds to site II on HSA. Therefore, the displacement of HA observed by a site I drug can be explained by allosteric interaction rather than competitive displacement. Interestingly, IS or IA was displaced competitively by high concentrations of HA.

In conclusion, IA, IS and HA primarily bind to site II on HSA, whereas the primary binding site of CMPF is site I. The former may possess the same binding site as endogenous substances such as medium fatty acids,³⁹) L-tryptophan¹²) and thyroxine,³³) while the latter may share the same binding site as bilirubin^{3,40}) and prostaglandin.¹⁶) Therefore, the interaction of those

endogenous substances with uremic toxins may increase the free fraction of uremic toxins and consequently cause increase of the free fraction of a drug.

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