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Effects of Drug Bindings on Esterase Activity of Human Serum Albumin. Dissociation Constants of the Complexes between the Protein and Drugs such as N-Arylanthranilic Acids, Coumarin Derivatives and Prostaglandins[1]

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The inhibition of esterase activity of human serum albumin (HSA) by the presence of several drugs (IN) was kinetically studied assuming a scheme of competitive binding to HSA between substrate, *p*-nitrophenyl acetate (NPA), and IN. The IN included N-arylanthranilic acids, coumarin derivatives and prostaglandins. The conditions of excessive concentrations of HSA and IN compared with NPA were employed to avoid complexities due to multiple active sites of HSA. For the solubilization of IN some organic solvents were added and the effects of the solvents on the reaction parameters of NPA with HSA were investigated. The dissociation constants of IN–HSA complexes were determined from the double reciprocal plots based on the scheme. The bulkiness of the substituents on phenyl groups of N-arylanthranilic acids enhanced the inhibition. Warfarin did not inhibit the reaction at all. Prostaglandins showed rather little inhibitions. From the comparisons of the dissociation constants obtained from this kinetic method with the literature binding constants from the conventional static methods, it was suggested that the esterase active site differed from the conventional binding sites on HSA.

Keywords——esterase activity of human serum albumin; competitive inhibition of esterase activity of albumin; Michaelis–Menten type complex; protein binding; dissociation constant of albumin–ligand complex; N-arylanthranilic acids; coumarin derivatives; prostaglandins; reaction of *p*-nitrophenyl acetate with albumin; acetylated human serum albumin

There have been a large number of studies on drug–albumin interactions, among which the competitive binding between two drugs to albumin is especially of interest for the elucidation of drug interactions and subsequent pharmacological effects.[3–7] Such binding might be affected by the second drug in extremely complicated manners such as the ratio of concentrations and the association constants of the two drugs, the competitive site of binding, the drug-induced change of the protein conformation and the displacement. The acetylation of human serum albumin (HSA) is caused by *p*-nitrophenyl acetate (NPA) and aspirin,[8–10] which is regarded as one of the drug-induced change of protein binding.

Means and Bender reported that HSA has multiple active sites responsible for the ester cleavage of NPA, one of which is rapidly acetylated by NPA than all others under the condi-

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2) Location: *Tanabe-dori, Mizuho-ku, Nagoya*, 467, *Japan*.
3) C.F. Chignell, *Mol. Pharmacol.*, **5**, 455 (1969).
4) J.J. Vallner, J.B. Perrin, and S. Wold, *J. Pharm. Sci.*, **65**, 1182 (1976).
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7) J.J. Vallner, *J. Pharm. Sci.*, **66**, 447 (1977).
8) J.T. Tildon and J.W. Ogilvie, *J. Biol. Chem.*, **247**, 1265 (1972).
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10) D. Hawkins, R.N. Pinkard, and R.S. Farr, *Science*, **160**, 780 (1968).

tion of excessive HSA concentration compared with that of NPA.[9] They proposed the reaction scheme as shown in Chart 1 and kinetically analyzed the reaction of NPA with HSA. In this scheme NPA·HSA and acetyl–HSA are the Michaelis–Menten type complex between NPA and HSA and the acetylated HSA by NPA, respectively. The rate constants of NPA·HSA and NPA are denoted by k_{cat} and k_0, respectively. NP and A indicate *p*-nitrophenol and acetic acid produced, respectively. K_S is the dissociation constant of NPA·HSA.

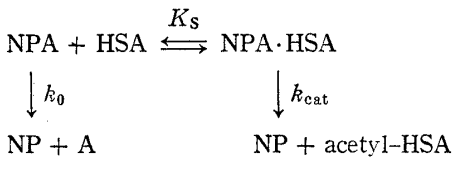

Chart 1

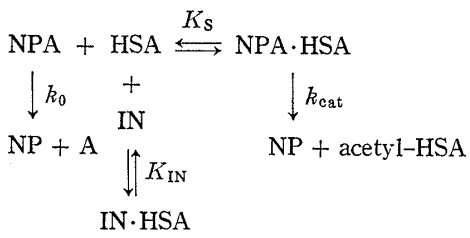

Chart 2

The reaction rate of NPA with HSA was decreased by the presence of second drugs (IN) such as N-arylanthranilic acids, coumarin derivatives and prostaglandins and also of the organic solvents used for the solubilization of IN. For the interactions of the second drugs with HSA, no report on their kinetic determinations has been found. The present study is concerned with the effects of the organic solvents on the reaction of NPA with HSA and with the determination of the dissociation constant, K_{IN}, of complex IN·HSA between HSA and IN, assuming a scheme of competitive albumin binding between the substrate and IN as shown in Chart 2. Also, the relations of the dissociation constants obtained from this kinetic method to the binding constants from the conventional static method found in the literatures are discussed briefly.

Experimental

Materials——Human serum albumin[11] was used after removing lipids according to the Chen's method.[12] The molecular weight of HSA was assumed as 69000 and the concentration of HSA was determined by the absorption at 278 nm using the extinction coefficient $E_{278}^{0.1\%}$ of 0.531.[9] Flufenamic acid, mefenamic acid and meclofenamic acid were the gifts from Sankyo Co. and recrystallized from ethanol. Diclofenac sodium was given from Fujisawa Pharmaceutical Co. Warfarin was given from Eisai Co. and prostaglandins $F_{2\alpha}$ and E_2 were the gifts from Ono Pharmaceutical Co. All other chemicals were commercially obtained and were of reagent grade.

Kinetic Procedure——The reaction of NPA with HSA in the absence and presence of the inhibitory drug (IN) was followed with time by assaying spectrophotometrically the resultant, *p*-nitrophenol, at 400 nm. Phosphate buffer of pH 7.0 was used as a reaction medium throughout and the temperature was 25°. Because of low solubilities of IN, 4% ethanol was added to the buffer for N-arylanthranilic acids and other related compounds, and 1% pyridine was used for warfarin and dicumarol. The reaction was started by the addition of NPA acetonitrile solution to the buffer solution in which HSA and IN were dissolved in advance. The final concentration of acetonitrile became 2%. The HSA concentration was approximately twice excessive compared to the initial NPA concentration (2.74×10^{-5} M). From the preliminary experiments the pseudo first order plot was found to be linear up to about 75% reaction completion under this condition.

11) Sigma Chem. Co., Fraction V, lot 47c-04421.
The purity of HSA was semi-quantitatively checked by the PTC method for N-terminal amino acid analysis. The equal amounts of HSA untreated and treated by the Chen's method were used for the analyses. For the sample before the purification two spots were found on the TLC plate. One was referred to leucine and the other might be referred to aspartic acid, which had almost equal color intensities. After the purification the spot due to leucine became significantly larger than that before the treatment, whereas the spot due to aspartic acid became quite small, which could be considered as negligible amount. The HSA after the purification had about twice esterase activity compared with HSA before the treatment. These results showed that the esterase activity was due to HSA itself.

12) R.F. Chen, *J. Biol. Chem.*, **242**, 173 (1967).

Then the pseudo first order analysis could be assumed for all the reactions of this study.

Determinations of the Dissociation Constants of the Complexes NPA·HSA and IN·HSA——According to Chart 1 the apparent first order rate constant of NP appearance in the absence of IN, k^0_{obs}, is expressed by the following equation.

$$k^0_{obs} = \frac{k_0 K_S + k_{cat}[HSA]}{K_S + [HSA]} \quad (1)$$

Equation (1) may be converted to equation (2), the double reciprocal form, known as Lineweaver–Burk plot.[13]

$$\frac{1}{k^0_{obs} - k_0} = \frac{K_S}{(k_{cat} - k_0)[HSA]_0} + \frac{1}{k_{cat} - k_0} \quad (2)$$

where concentration of HSA in equation (1) was approximated to the initial concentration of HSA, $[HSA]_0$, as would be shown in Fig. 1. The values of K_S and k_{cat} could be calculated from the slope divided by the intercept and from the intercept, respectively.

In the presence of IN the apparent first order rate constant, k_{obs}, of the reaction can be represented as follows;

$$k_{obs} = \frac{k_0 + \frac{k_0[IN]}{K_{IN}} + \frac{k_{cat}[HSA]_0}{K_S}}{1 + \frac{[IN]}{K_{IN}} + \frac{[HSA]_0}{K_S}} \quad (3)$$

One of the linear transforms of the equation (3) can be given by[14]

$$\frac{k_{cat} - k_{obs}}{k_{obs} - k_0} = \frac{K_S}{K_{IN}[HSA]_0}[IN]_0 + \frac{K_S}{[HSA]_0} \quad (4)$$

where [IN] in equation (3) could be represented as $[IN]_0$, the initial concentration of IN. The value of K_{IN} can be obtained from the value of (Intercept)/(Slope). The K_S value was also calculated by (Intercept) times $[HSA]_0$.

Results and Discussion

Reaction Parameters of NPA with HSA in the Presence and Absence of Organic Solvents.

Figure 1 shows the Lineweaver–Burk plots according to the equation (2), where the value of $1/(k^0_{obs} - k_0)$ in the ordinate was practically the reciprocal of k^0_{obs} since k_0 was small compared with k^0_{obs}. Good linear relationships between $1/(k^0_{obs} - k_0)$ and $1/[HSA]_0$ were obtained both in the presence and absence of the organic solvents, 2% acetonitrile and 4% ethanol. Table I lists the rate and dissociation constants obtained for the reaction of NPA with HSA. The values of k_{cat} were not affected by the presence of any organic solvents. The k_{cat} value was in agreement with that reported by Means and Bender.[9] It is seen that the esterase activity of HSA is very large, *i. e.* $k_{cat}/k_0 \fallingdotseq 10^4$. On the other hand, the values of K_S were increased by the presence of the organic solvents. Since the values of the intercepts as shown in Fig. 1 are identical for all of the three lines, the effects of the solvents on the reaction may be interpreted by the competitive inhibition of the active site on HSA between NPA and the solvents.

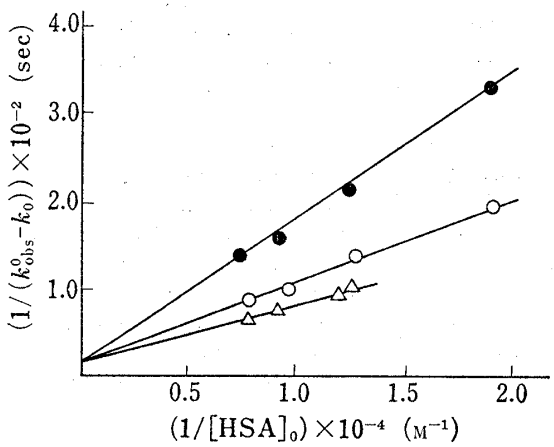

Fig. 1. Lineweaver–Burk Plots for Reaction of NPA with HSA

△: pH 7.0 phosphate buffer,
○: pH 7.0 phosphate buffer containing 2% acetonitrile,
●: pH 7.0 phosphate buffer containing 2% acetonitrile and 4% ethanol.

13) H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).
14) R.L. VanEtten, J.F. Sebastian, C.A. Clowes, and M.L. Bender, *J. Am. Chem. Soc.*, **89**, 3242 (1967).

Table I. Rate and Dissociation Constants for Reaction of NPA with HSA in the Absence and Presence of Organic Solvents.

Organic solvents	k_0 (sec^{-1})	k_{cat} (sec^{-1})	K_S (M)
No organic solvent	2.29×10^{-5}	1.39×10^{-1}	1.02×10^{-3}
2% Acetonitrile	1.36×10^{-5}	1.39×10^{-1}	1.34×10^{-3}
4% Ethanol and 2% acetonitrile	—	1.39×10^{-1}	2.33×10^{-3}
1% Pyridine and 2% acetonitrile	—	1.43×10^{-1}	3.61×10^{-3}

To evaluate the effects of the organic solvent on the dissociation constant, K_S, the dissociation constants newly assumed for each solvent-HSA complex were determined by the use of the equation (4) and listed in Table II. The K_S values in Table II calculated from the equation (4) were very close within 10% error to that in Table I, 1.02×10^{-3} M, determined separately from the equation (2) in the absence of the organic solvents. This may indicate that the nearby conformation of the esterase active site of HSA is not changed by the organic solvents over the range of the solvent concentrations applied in this study.

Table II. The Dissociation Constants of Assumed Organic Solvent-HSA Complexes

Organic solvents	K_{IN} (M)	K_S from Eq. (4) (M)
Acetonitrile	7.85×10^{-1}	9.92×10^{-4}
Ethyl alcohol	5.21×10^{-1}	9.27×10^{-4}
Pyridine	5.70×10^{-2}	1.06×10^{-3}

The values of K_S in Table I obtained in the presence of the mixed organic solvent were checked with respect to the solvent effects for both the acetonitrile–ethanol and acetonitrile–pyridine systems. In the presence of acetonitrile (AN) and ethanol (EA), the effects of the solvents on the binding of NPA to HSA may be represented as Chart 3.

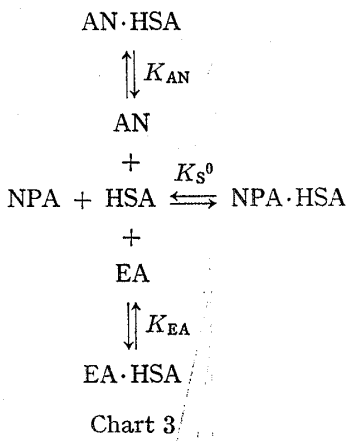

Chart 3

In Chart 3 AN·HSA and EA·HSA are the assumed complexes of AN and EA with HSA, respectively. K_S^0 is the intrinsic dissociation constant of NPA·HSA. K_{AN} and K_{EA} are the dissociation constants of AN·HSA and EA·HSA, respectively, which were previously determined and listed in Table II. The concentration of the HSA uncomplexed with the substrate NPA, $[HSA]_T$, can be given by the following equation (5).

$$[HSA]_T = [HSA]_0 - [NPA\cdot HSA]$$
$$= [HSA] + [AN\cdot HSA] + [EA\cdot HSA] \quad (5)$$

The apparent dissociation constant, K_S, of NPA·HSA obtained from equation (2) in the presence of the organic solvents may be expressed as follows;

$$K_S = \frac{[NPA][HSA]_T}{[NPA\cdot HSA]} = \frac{[NPA]\{[HSA]+[AN\cdot HSA]+[EA\cdot HSA]\}}{[NPA\cdot HSA]}$$
$$\fallingdotseq K_S^0\left(1+\frac{[AN]_0}{K_{AN}}+\frac{[EA]_0}{K_{EA}}\right) \quad (6)$$

In equation (6) the initial concentrations, $[AN]_0$ and $[EA]_0$, of AN and EA were used instead of [AN] and [EA], respectively, since the concentrations of AN and EA are much larger than those of NPA and HSA. This equation shows that the apparent dissociation constant, K_S,

in the presence of the mixed organic solvent can be calculated by use of the values of K_{AN}, K_{EA} and K_S^0 determined separately from the individual experiments. Inversely, the intrinsic dissociation constant, K_S^0, of NPA·HSA may be estimated from the values of K_S, determined from the equation (2) in the presence of the organic solvents, K_{AN} and K_{EA}. In the case of 2% acetonitrile and 4% ethanol system, the K_S value calculated from the equation (6) was 2.88×10^{-3} M. Meanwhile, the value of K_S determined from the plots in Fig. 1 was 2.33×10^{-3} M (see the third line in Table I). For the acetonitrile–pyridine system, the equation similar to equation (6) could be derived and the calculation of the K_S value gave the value of 3.74×10^{-3} M. The value of K_S determined from the equation (2) was 3.61×10^{-3} M (see the 4th line in Table I). These agreements between the calculated and determined K_S values suggest that the above assumption for the competitive inhibition of the organic solvents is appropriate.

The equation (6) indicates that if the concentrations of the organic solvents were fixed in a series of experiments, the K_S value obtained from the equation (2) or (4) in the presence of the solvents was considered to remain unchanged. Thus, the coexistence of the organic solvents did not prevent the following determination of the apparent dissociation constants of the complexes formed between HSA and competitive inhibitory drugs.

Dissociation Constants between HSA and Inhibitory Drugs

The effects of mefenamic acid and flufenamic acid on the apparent reaction rate constants of NPA with HSA were shown in Fig. 2. Both the drugs decreased significantly the apparent rate constants. The decrease in the rates may be caused by the competition of the active site of HSA between the inhibitory drug and NPA. Fig. 3 shows the plots according to the equation (4) for the results of Fig. 2. Linear relationships between $(k_{cat}-k_{obs})/(k_{obs}-k_0)$ and $[IN]_0$ were obtained for both mefenamic acid and flufenamic acid results. The value of K_{IN} was calculated from the intercept divided by the slope and the K_S value was obtained by the intercept of the line times $[HSA]_0$. The intrinsic dissociation constant, K_{IN}^0, between

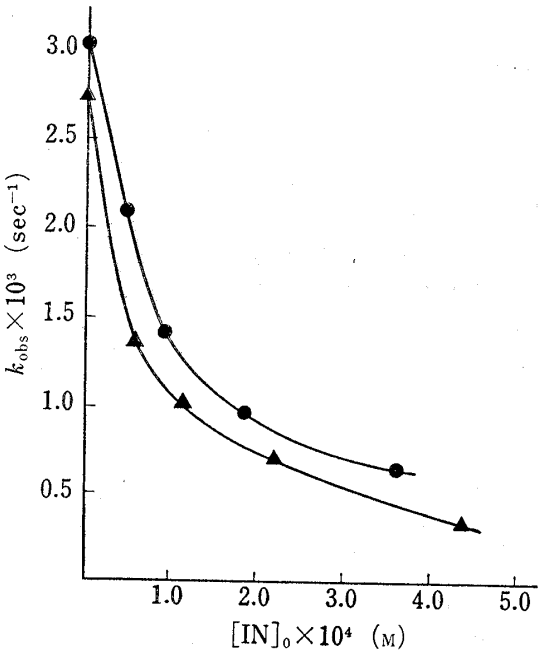

Fig. 2. Effect of Inhibitor Concentration on Apparent First Order Rate Constant for Reaction of NPA with HSA

●: mefenamic acid, ▲: flufenamic acid
pH 7.0 phosphate buffer containing 2% acetonitrile and 4% ethanol.

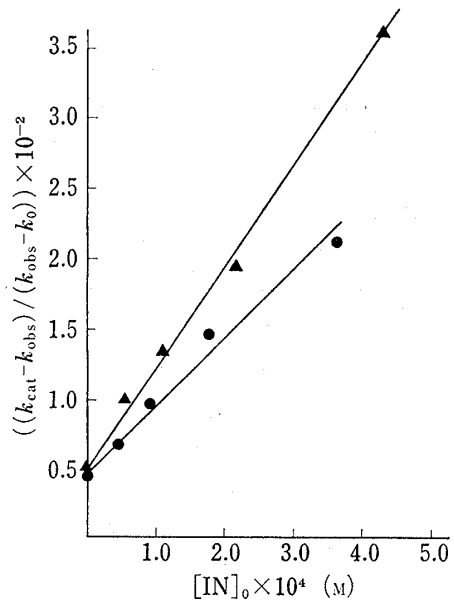

Fig. 3. Plots of $(k_{cat}-k_{obs})/(k_{obs}-k_0)$ versus Concentration of Inhibitor

●: mefenamic acid, ▲: flufenamic acid.

HSA and the inhibitory drugs can be related to the K_{IN} by the following equation (7), which is similar to the equation (6).

$$K_{IN} = K^0_{IN}\left(1+\frac{[AN]_0}{K_{AN}}+\frac{[EA]_0}{K_{EA}}\right) \quad (7)$$

Table III lists the values of K_{IN} and K_S determined from the plots as shown in Fig. 3 for N-arylanthranilic acids and the other related compounds. The values of K^0_{IN} and $K_S{}^0$ calculated from equation (7) and (6), respectively, were also included in Table III.

TABLE III. Dissociation Constants for Bindings of N-Arylanthranilic Acids and Other Related Compounds to HSA.

Drugs	K_{IN}[a] (M)	K^0_{IN}	K_S from Eq. (4)[a] (M)	$K_S{}^0$
Flufenamic acid 3′-CF_3	7.17×10^{-5}	2.54×10^{-5}	2.66×10^{-3}	9.42×10^{-4}
Mefenamic acid 2′,3′-CH_3	8.86×10^{-5}	3.14×10^{-5}	2.31×10^{-3}	8.18×10^{-4}
Meclofenamic acid 2′,6′-Cl, 3′-CH_3	9.34×10^{-5}	3.31×10^{-5}	2.43×10^{-3}	8.61×10^{-4}
N-Phenylanthranilic acid	9.98×10^{-5}	3.54×10^{-5}	2.54×10^{-3}	9.00×10^{-4}
Anthranilic acid	No inhibition		2.57×10^{-3}	9.10×10^{-4}
Diphenylamine 2-H	7.40×10^{-4}	2.62×10^{-4}	2.71×10^{-3}	9.60×10^{-4}
2,2′-Iminobis[benzoic acid] 2′-COOH	2.89×10^{-4}	1.02×10^{-4}	2.58×10^{-3}	9.14×10^{-4}
Diclofenac sodium 2-CH_2COOH, 2′,6′-Cl	1.89×10^{-4}	6.70×10^{-5}	2.60×10^{-3}	9.21×10^{-4}

a) pH 7.0 phosphate buffer containing 4% ethanol and 2% acetonitrile and at 25°.

The magnitude of the K^0_{IN} value indicates that the smaller the value, the stronger the binding of the inhibitor to the active site of HSA. Anthranilic acid did not inhibit the reaction of NPA with HSA up to its concentration of 4.36×10^{-4} M, while K^0_{IN} values for N-phenylanthranilic acid and diphenylamine were 3.54×10^{-5} M and 2.62×10^{-4} M, respectively. The values of K^0_{IN} for the inhibitors which have bulky N-phenyl groups such as flufenamic acid, mefenamic acid and meclofenamic acid are slightly smaller than that for N-phenylanthranilic acid. These may indicate that the bulkiness of the substituents on phenyl groups of N-arylanthranilic acids plays a role for the binding to the HSA active site. The difference of K^0_{IN} values between for diclofenac sodium and meclofenamic acid may be attributed to the methylene group between carboxyl and phenyl groups.

TABLE IV. Dissociation Constants for Bindings of Coumarin Derivatives and Prostaglandins to HSA

Drugs		K_{IN} (M)	K^0_{IN}*1	K_S from Eq. 4 (M)	$K_S{}^0$
Coumarin derivatives[a]	Dicumarol	1.44×10^{-4}	3.92×10^{-5}	3.32×10^{-3}	9.05×10^{-4}
	Warfarin	No inhibition		3.43×10^{-3}	9.35×10^{-4}
Prostaglandins[b]	$F_{2\alpha}$	4.77×10^{-3}	3.20×10^{-3}	1.47×10^{-3}	9.87×10^{-4}
	E_2	6.89×10^{-4}	4.62×10^{-4}	1.69×10^{-3}	1.13×10^{-3}

a) pH 7.0 phosphate buffer containing 1% pyridine and 2% acetonitrile and at 25°.
b) pH 7.0 phosphate buffer containing 2% acetonitrile and at 25°.
*1 calculated from equations; $K_{IN}=K^0_{IN}(1+[AN]_0/K_{AN}+[PY]_0/K_{PY})$ for coumarin derivatives
$K_{IN}=K^0_{IN}(1+[AN]_0/K_{AN})$ for prostaglandins.

Table IV lists the results for coumarin derivatives and prostaglandins. Although warfarin[15,16] was known to bind strongly to HSA, it showed interestingly no inhibition. This may mean that the esterase active site of HSA differs from the binding sites of this drug to HSA found in the literatures, which will be discussed in the next section. It has also been known that the interactions of HSA with long-chain fatty acids were very strong and HSA had three classes of binding sites for these acids.[17,18] The K_{IN}° values for prostaglandins in Table IV were, however, rather large, which was contrary to our expectation. This may be due to the complicated steric arrangement of the prostanoic acid structure compared with those of simple long-chain fatty acids.

Comparison of Esterase Active site to the Binding Sites on HSA

The protein bindings of small molecules have been studied by the several methods[19] such as equilibrium dialysis, gel filtration and spectrophotometry. All of the data obtained from the conventional static methods were analyzed using the following equation.

$$r = \sum_{i=1}^{m} \frac{n_i k_i [A]}{1 + k_i [A]} \tag{8}$$

Where r represents the moles of the bound small molecule per mole of the protein, [A] is the concentration of the nonbound (free) small molecule and m is the number of classes of independent sites, such that each class, i, has n_i sites with an intrinsic binding constant (association constant), k_i. If the value of n_i is unity, the reciprocal of the k_i value may be directly compared with the K_{IN}° value obtained from the kinetic method in this study. If the n_i value is not unity, the comparison of the reciprocal of the total association constant, $n_i k_i$, with the value of K_{IN}° should be made. Table V summarizes the literature values of $1/n_i k_i$ obtained from the various conventional static methods.

Table V. Literature Values of $1/n_i k_i$ obtained from Various Conventional Methods

Drugs		$1/n_i k_i$		Methods	References
		$1/n_1 k_1$	$1/n_2 k_2$		
N-Arylanthranilic acids	Flufenamic acid	7.94×10^{-7}	—	Fluorescence	3
	Mefenamic acid	1.26×10^{-6}	—	Fluorescence	3
	N-Phenylanthranilic acid	1.78×10^{-6}	—	Fluorescence	3
Coumarin Derivatives	Dicumarol	3.45×10^{-7}	5.56×10^{-6}	Circular dichroism	4, 20
		—	1.30×10^{-6}	Equilibrium dialysis	21
	Warfarin	1.19×10^{-5}	—	Equilibrium dialysis	15
		8.93×10^{-6}	—	Equilibrium dialysis	16

For N-arylanthranilic acids the values of $1/n_1 k_1$ determined by the fluorescence method[3] were about 20 to 30 times smaller than those of K_{IN}° in Table III. Chignell[3] measured the bindings of flufenamic acid to HSA also by the equilibrium dialysis and the circular dichroism methods. Although the association constants were not determined, he suggested that HSA had at least three binding sites with a very high affinity for flufenamic acid.[3] The literature values for coumarin derivatives[4,15,16,20,21] were also smaller than the K_{IN}° values determined

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by this kinetic method. Thus, the differences in the values between $1/n_i k_i$ and K^0_{IN} for N-aryl-anthranilic acids and coumarin derivatives may indicate that the binding sites on HSA observed from the conventional static methods differ from the esterase active site of HSA in this study.

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