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Effects of Drug Binding on the Esterase-Like Activity of Human Serum Albumin. IV.[1] Application of an Analog Computer to Determination of the Multiple Dissociation Constants

YUKIHISA KURONO* and KEN IKEDA

Faculty of Pharmaceutical Sciences, Nagoya City University, Tanabe-dori, Mizuho-ku, Nagoya, 467, Japan

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The inhibition parameters of various drugs (inhibitor, I) for the reaction of *p*-nitrophenyl acetate (substrate, S) with human serum albumin (HSA) could not be directly determined, because HSA has two esterase-like active sites for S (*i.e.* R and T sites) and three drug binding sites (*i.e.* R, T and U sites) [Ozeki *et al.*, *Chem. Pharm. Bull.*, **28**, 525 (1980)]. In the present study, the dissociation constants between I and the individual binding sites on HSA, $K_{I,R}$, $K_{I,T}$ and $K_{I,U}$, were determined by analog computer simulation of the inhibition. Clofibric acid, ibuprofen and dansylsarcosine were found to bind solely to the R site, and their $K_{I,R}$ values were determined. Flufenamic acid, ethacrynic acid and salicylic acid bind primarily to the R site and then to the T site, and their $K_{I,R}$ and $K_{I,T}$ values were estimated. The $K_{I,U}$ and $K_{I,R}$ values for phenylbutazone and sulfinpyrazone were also determined, and their $K_{I,U}$ values were smaller than their $K_{I,R}$ values. It is likely that warfarin binds primarily to two binding sites unconcerned with the esterase-like activities and then to the R site of HSA, and these three dissociation constants were estimated. Since this kinetic method distinguishes the drug binding sites on HSA and gives the dissociation constants for the individual sites, it is very useful for studies on drug interaction with HSA.

Keywords——human serum albumin; protein binding; esterase-like activity; enzyme kinetics; analog computer simulation; competitive inhibition; distinction and identification of drug binding sites; *p*-nitrophenyl acetate; anionic drugs

The distinction and identification of drug binding sites on human serum albumin (HSA), and the estimation of the binding affinities are important from the standpoint of drug displacement *in vivo*. In the previous studies,[1,2] the inhibition type of the reaction of *p*-nitrophenyl acetate (substrate, S) with HSA caused by Sudlow's site I and site II drugs (inhibitor, I)[3] indicated that HSA has two esterase-like active sites, *i.e.* the primarily reactive site (R site) and the secondarily reactive site (T site). Furthermore, it was found that there is a third site (U site) which is unconcerned with the esterase-like activities and is the drug binding site. For convenience, the relationship between the esterase-like active sites and the drug binding sites on HSA is shown schematically in Fig. 1. The R site was found to correspond to Sudlow's site II[3] and to be located close to Tyr-411 of the HSA amino acid sequence.[4] The U site was identical with Sudlow's site I and close to Trp-214 of the HSA sequence. Out of the many parameters shown in Fig. 1, the kinetic parameters ($K_{S,R}$, $K_{S,T}$, $k_{2,R}$, $k_{2,T}$ and k_0) for the reactions of S with HSA were determined in the previous study.[1] However, it was difficult to determine the dissociation constants ($K_{I,R}$, $K_{I,T}$ and $K_{I,U}$) between I and the individual binding sites by the conventional analytical method, because the system related to the inhibition was very complicated as shown in Fig. 1.[2]

In this paper, we report the application of an analog computer to determine the dissociation constants between I and the binding sites on HSA. The dissociation constants for the following drugs[2] were successfully estimated: clofibric acid, ibuprofen and dansylsarcosine as R type inhibitors; flufenamic acid, ethacrynic acid and salicylic acid as R–T type inhibitors; phenylbutazone, sulfinpyrazone and warfarin as U–R type inhibitors. In this study as well as the previous ones,[1,2] it is assumed that the reactive sites and the binding sites are inde-

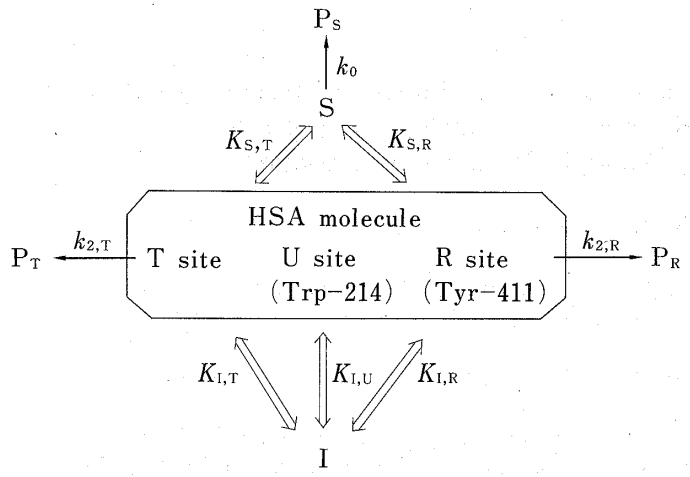

Fig. 1. The Relationship between the Esterase-Like Active Sites and the Drug Binding Sites on HSA

$K_{S,R}$ and $K_{S,T}$ are the dissociation constants between S and the esterase-like active sites (R and T sites), respectively. $K_{I,R}$, $K_{I,U}$ and $K_{I,T}$ are the dissociation constants between I and the corresponding binding sites (R, U and T sites). $k_{2,R}$, $k_{2,T}$ and k_0 show the first-order rate constants of the complexes between S and the reactive sites, and of the uncatalyzed reaction of S, respectively. P_R, P_T and P_S represent respectively the products from the reactions characterized by $k_{2,R}$, $k_{2,T}$ and k_0, all of which are *p*-nitrophenol.

pendent of each other, that is, one HSA molecule acts on S and I just as if different molecules were involved with respect to the sites (see Charts 1, 2 and 3).

Experimental

Apparatus——The analog computer used was a Hitachi ALS-20M machine (Hitachi-Denshi Ltd., Tokyo).

Equations for Simulation of the Inhibitions and Analog Circuit Diagrams——1. R Type Inhibition: The basic kinetic parameters for the reactions of S with HSA shown in Fig. 1, which were determined in the previous study,[1] are listed in Table I. The reaction scheme for the R type inhibition can be represented as in Chart 1.[1,2] In this chart the uncatalyzed reaction (k_0) of S is neglected, since k_0 is very small compared with $k_{2,R}$ and $k_{2,T}$ (see Table I). HSA_R and HSA_T are the R and T sites on HSA, respectively. $S \cdot HSA_R$, $S \cdot HSA_T$ and $I \cdot HSA_R$ represent the complexes between S or I and the corresponding sites on HSA. Acetyl-HSA_R and acetyl-HSA_T show the R and T sites acetylated with S, respectively.[1]

Table I. Kinetic Parameters for the Reactions of *p*-Nitrophenyl Acetate (S) with HSA[a]

Parameter	Value
$k_{2,R}$	3.14×10^{-2} (s^{-1})
$k_{2,T}$	9.07×10^{-3} (s^{-1})
k_0	1.67×10^{-5} (s^{-1})
$K_{S,R}$	1.33×10^{-4} (M)
$K_{S,T}$	3.09×10^{-4} (M)

a) These parameters were obtained previously.[1]

$$S + HSA_R \underset{}{\overset{K_{S,R}}{\rightleftharpoons}} S \cdot HSA_R \xrightarrow{k_{2,R}} P_R + \text{acetyl-}HSA_R$$

$$I + HSA_R \overset{K_{I,R}}{\rightleftharpoons} I \cdot HSA_R$$

$$S + HSA_T \overset{K_{S,T}}{\rightleftharpoons} S \cdot HSA_T \xrightarrow{k_{2,T}} P_T + \text{acetyl-}HSA_T$$

Chart 1

The following conservation and rate equations are derived according to Chart 1. The conservation equations are as follows:

$$[S]_0 = [S] + [S][HSA_R]/K_{S,R} + [P_R] + [S][HSA_T]/K_{S,T} + [P_T] \quad (1)$$

$$[HSA_R]_0 = [HSA_R] + [S][HSA_R]/K_{S,R} + [P_R] + [I][HSA_R]/K_{I,R} \quad (2)$$

$$[I]_0 = [I] + [I][HSA_R]/K_{I,R} \quad (3)$$

$$[HSA_T]_0 = [HSA_T] + [S][HSA_T]/K_{S,T} + [P_T] \quad (4)$$

$$[P] = [P_R] + [P_T] \quad (5)$$

where subscript 0 represents the initial concentration of the individual species. [P] is the total concentration of the products formed from the reactions characterized by $k_{2,R}$ and $k_{2,T}$. The rate equations for the product formations are as follows:

$$d[P_R]/dt = k_{2,R}[S][HSA_R]/K_{S,R} \quad (6)$$

$$d[P_T]/dt = k_{2,T}[S][HSA_T]/K_{S,T} \quad (7)$$

After amplitude and time scaling for equations (1)—(7), the analog circuit diagram shown in Fig. 2 was made. The details of the amplitude and time scaling, and of the programing are described in Appendix 1.

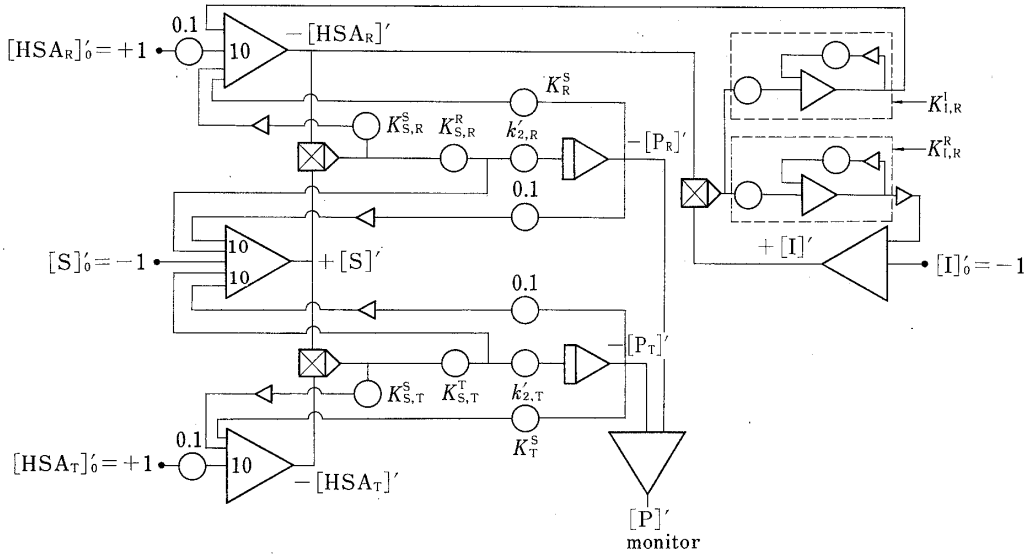

Fig. 2. Analog Circuit Diagram for the R Type Inhibition

$$S + HSA_R \underset{}{\overset{K_{S,R}}{\rightleftharpoons}} S\cdot HSA_R \xrightarrow{k_{2,R}} P_R + \text{acetyl-}HSA_R$$

$$I + HSA_R \overset{K_{I,R}}{\rightleftharpoons} I\cdot HSA_R$$

$$S + HSA_T \overset{K_{S,T}}{\rightleftharpoons} S\cdot HSA_T \xrightarrow{k_{2,T}} P_T + \text{acetyl-}HSA_T$$

$$I + HSA_T \overset{K_{I,T}}{\rightleftharpoons} I\cdot HSA_T$$

Chart 2

2. R–T Type Inhibition: Chart 2 shows the reactions for the R–T type inhibition.[2] The reaction characterized by k_0 shown in Fig. 1 is neglected in this chart, since in the presence of I up to the ratio $[I]_0/[HSA]_0$ of 10, the contribution of the reaction (k_0) to the inhibition curves was practically negligible.

The following equations (8) and (9) instead of equations (3) and (4), respectively, are required for the simulation of the R–T type inhibition.

$$[I]_0 = [I]+[I][HSA_R]/K_{I,R} + [I][HSA_T]/K_{I,T} \quad (8)$$

$$[HSA_T]_0 = [HSA_T] + [S][HSA_T]/K_{S,T} + [P_T] + [I][HSA_T]/K_{I,T} \quad (9)$$

Equations (1), (2), (5), (6) and (7) for the R type inhibition are also applicable to the R–T type inhibition. Figure 3 shows the analog circuit diagram for the R–T type inhibition.

3. U–R Type Inhibition: The reactions for the U–R type inhibition are shown in Chart 3.[2] In this chart, HSA_U and $I\cdot HSA_U$ are the U site of HSA and the complex between I and HSA_U, respectively. The following equation (10) instead of equation (3) is used for the simulation of the U–R type inhibition.

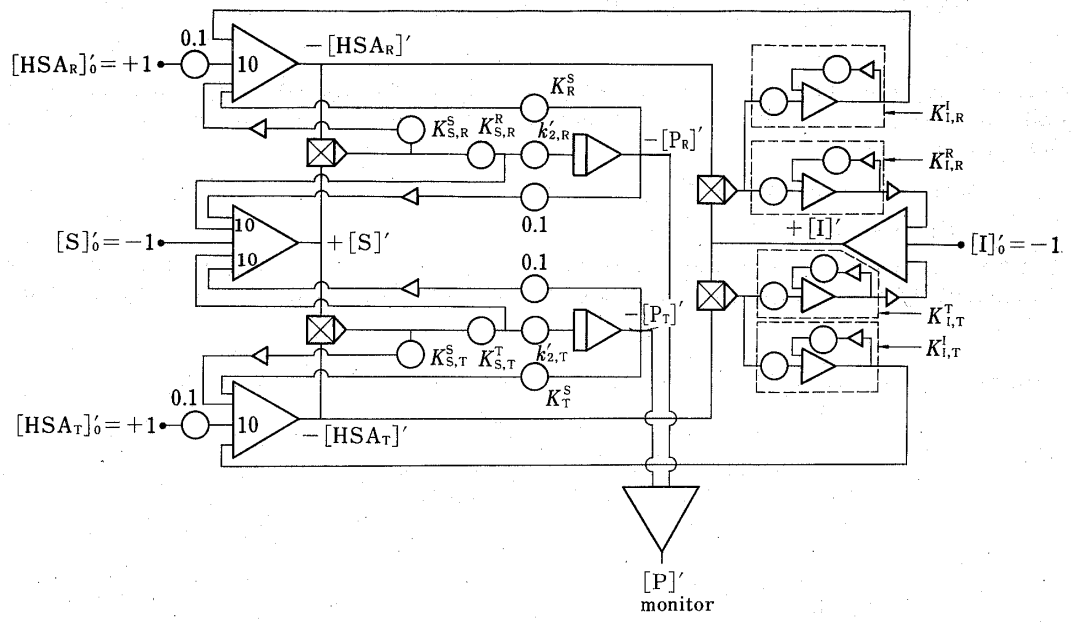

Fig. 3. Analog Circuit Diagram for the R–T Type Inhibition

$$\mathrm{S} + \mathrm{HSA_R} \underset{}{\overset{K_{S,R}}{\rightleftharpoons}} \mathrm{S{\cdot}HSA_R} \xrightarrow{k_{2,R}} \mathrm{P_R} + \text{acetyl-HSA}_R$$

$$\mathrm{I} + \mathrm{HSA_R} \overset{K_{I,R}}{\rightleftharpoons} \mathrm{I{\cdot}HSA_R}$$

$$\mathrm{I} + \mathrm{HSA_U} \overset{K_{I,U}}{\rightleftharpoons} \mathrm{I{\cdot}HSA_U}$$

$$\mathrm{S} + \mathrm{HSA_T} \overset{K_{S,T}}{\rightleftharpoons} \mathrm{S{\cdot}HSA_T} \xrightarrow{k_{2,T}} \mathrm{P_T} + \text{acetyl-HSA}_T$$

Chart 3

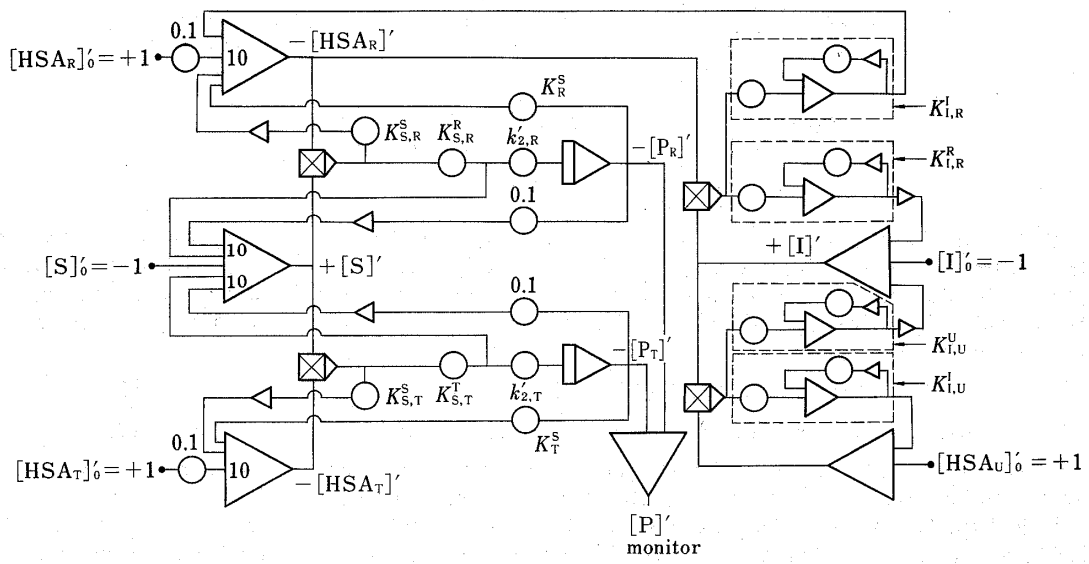

Fig. 4. Analog Circuit Diagram for the U–R Type Inhibition

$$[I]_0 = [I] + [I][HSA_R]/K_{I,R} + [I][HSA_U]/K_{I,U} \quad (10)$$

In addition to equations (1), (2), (4)—(7) and (10), the following equation (11) is necessary for the simulation.

$$[HSA_U]_0 = [HSA_U] + [I][HSA_U]/K_{I,U} \quad (11)$$

Figure 4 shows the analog circuit diagram for the U–R type inhibition.

Determination of the Dissociation Constants——Both in the presence and absence of I, the concentrations of S and HSA for the simulation were fixed at 1×10^{-5} M and 5×10^{-5} M, respectively.[1,2] Then, pseudo first-order analyses could be applied for the product formations simulated by means of the analog computer using the kinetic parameters in Table I. The pseudo first-order rate constants (k_{obs})[5] were calculated from the half-lives ($t_{1/2}$) by employing the equation $k_{obs}=0.693/(t_{1/2})$.

The product formations with at least ten different concentrations of I were simulated, using a chosen dissociation constant between I and the binding site on HSA. Then the inhibition curve was drawn by plotting k_{obs}/k^0_{obs} *versus* $[I]_0/[HSA]_0$,[2] where k^0_{obs} is the rate constant in the absence of I. The theoretical curve thus obtained is characterized by the dissociation constant used. The inhibition curve giving the best fit to the experimental results was sought by varying the dissociation constants. Finally, the constant giving the best fit was chosen as the dissociation constant to be determined for each drug.

Results and Discussion

R Type Inhibitors

Figure 5 shows the R type inhibition curves at different $K_{I,R}$ values. The smaller the values of $K_{I,R}$, the steeper the inhibition curves, as expected. The closed circles in Fig. 5 are experimental results for clofibric acid obtained in the previous study.[1,2] The value of $K_{I,R}$ for clofibric acid, therefore, can be estimated as about 5×10^{-6} M.

Since the binding affinity of a drug to HSA given in the literature is generally expressed as the binding constant (the association constant), the reciprocal values ($1/K_{I,R}$) of the dissociation constants obtained in this study are used for comparison. Table II lists the reciprocals of $K_{I,R}$ for the three R type inhibitors along with the literature binding parameters. In this table, n and K are the number of the binding sites and the binding constant, respectively. Subscripts 1 and 2 represent the high and low affinity binding sites, respectively. Differences by a factor of about 2 between $1/K_{I,R}$ and the literature binding constants (K_1) are observed for each drug. However, these variations may be allowable, since it is known that binding parameters often vary with slight differences of experimental conditions such as buffer components, ionic strength, pH and source of HSA, and with the method used to obtain the pa-

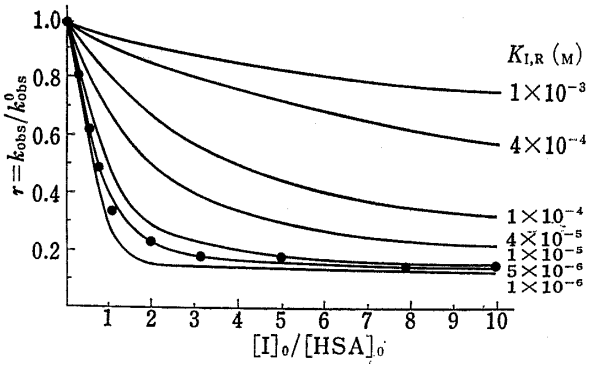

Fig. 5. R Type Inhibition Curves at Different $K_{I,R}$ Values

●, experimental results for clofibric acid.
$[HSA]_0=[HSA_R]_0=[HSA_T]_0=5\times10^{-5}$M, $[S]_0=1\times10^{-5}$M.

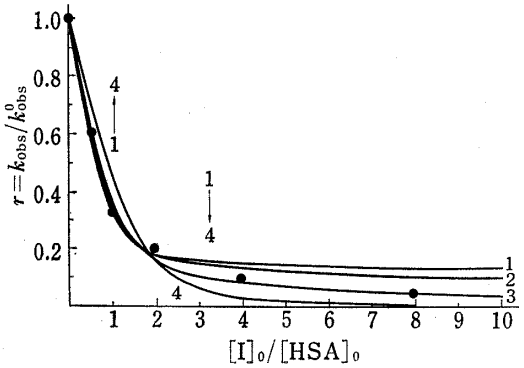

Fig. 6. R–T Type Inhibition Curves at Different $K_{I,T}$ Values and Constant $K_{I,R}$ Value (2×10^{-6} M)

●, experimental results for flufenamic acid.
$[HSA]_0=[HSA_R]_0=[HSA_T]_0=5\times10^{-5}$ M,
$[S]_0=1\times10^{-5}$ M.
$K_{I,T}$ (M): 1; ∞, 3; 1×10^{-4},
2; 1×10^{-3}, 4; 1×10^{-5}.

Table II. Reciprocals of the Dissociation Constants obtained for the R Type Inhibitors and Binding Parameters from the Literature

Inhibitor	$1/K_{I,R}$ (M^{-1})	Literature binding parameters				Reference
		n_1	K_1 (M^{-1})	n_2	K_2 (M^{-1})	
Clofibric acid	2×10^5	0.861	1.73×10^5	5.86	1.57×10^3	1
		1	1.3×10^5	16	1.5×10^3	6
Ibuprofen	2×10^5	1	1×10^5	—a)	—a)	7
Dansylsarcosine	3×10^5	1	1.3×10^5	—a)	—a)	8

a) Accurate binding parameters could not be found in the literature.

rameters. Therefore, this kinetic method can be applied to the determination of the dissociation constants between drugs and HSA.

R-T Type Inhibitors

In the case of the R–T type inhibition, there are two unknown parameters, that is, $K_{I,R}$ and $K_{I,T}$. Hence, it is necessary for examination of the features of the inhibition curve that one parameter is fixed at a constant value and the other is varied. Figure 6 shows the theoretical curves for the R–T type inhibition at different $K_{I,T}$ values and constant $K_{I,R}$ value. The $K_{I,R}$ value of 2×10^{-6} M was chosen from the trial-and-error calculations. The solid circles are the experimental results for flufenamic acid.[2] The $K_{I,R}$ and $K_{I,T}$ values for flufenamic acid are, therefore, 2×10^{-6} M and 1×10^{-4} M, respectively.

Table III. Reciprocals of the Dissociation Constants obtained for the R–T Type Inhibitors and Binding Parameters from the Literature

Inhibitor	$1/K_{I,R}$ (M^{-1})	$1/K_{I,T}$ (M^{-1})	Literature binding parameters				Reference
			n_1	K_1 (M^{-1})	n_2	K_2 (M^{-1})	
Flufenamic acid	5×10^5	1×10^4	3	$>1.5\times10^6$	8	$<7\times10^2$	9
Ethacrynic acid	1×10^6	2×10^4	—a)	—a)	—a)	—a)	
Salicylic acid	3×10^4	1×10^4	1.7	2.5×10^4	15	1.0×10^2	10
			2.2	2.8×10^4	—a)	—a)	11

a) Accurate binding parameters could not be found in the literature.

Table III contains the reciprocals of the dissociation constants for the R–T type inhibitors and the literature binding parameters. From the literature binding parameters for flufenamic acid and salicylic acid, it is difficult to deduce which sites of HSA give the binding constants (K_1) because of the multiple n_1 values. In contrast, this kinetic analysis can distinguish the drug binding sites (R and T sites) and give the dissociation constants for the individual sites ($K_{I,R}$ and $K_{I,T}$). For example, the two high affinity binding sites (n_1 values) for salicylic acid appear to be the R and T sites, since the values of $1/K_{I,R}$ and $1/K_{I,T}$ are near the K_1 values listed in Table III. Hence, the kinetic method applying an analog computer is of great value in studies on the interaction of drugs with HSA.

U-R Type Inhibitors

Figure 7 shows the U–R type inhibition curves at different $K_{I,R}$ values and constant $K_{I,U}$ value (1×10^{-6} M). When the $K_{I,R}$ values are larger than the $K_{I,U}$ value, shoulders are observed at low ratios of $[I]_0/[HSA]_0$. This indicates that the U–R type inhibition is observed only when the binding affinity of I to the U site of HSA is higher than that to the R site of HSA. The closed circles in Fig. 7 are the experimental results for phenylbutazone.[2] There-

fore, the $K_{I,U}$ and $K_{I,R}$ values for phenylbutazone can be estimated as 1×10^{-6} M and 3×10^{-5} M, respectively.

In the previous study,[2] warfarin hardly inhibited the reaction of S with HSA up to the warfarin-to-HSA ratio of about 2. Hence, besides the U site the existence of another binding site was presumed in order to fit the theoretical curve to the experimental results for warfarin. The binding site for warfarin is tentatively named the V site in this study. The V site on HSA as well as the U site is unconcerned with the esterase-like activities of HSA. Unfortunately, since the V site was not distinguished from the U site by this kinetic method, the dissociation constant ($K_{I,V}$) between warfarin and the V site was assumed, for simplicity of the simulation, to be the same as that ($K_{I,U}$) for the U site. Then, twice the concentration (1×10^{-4} M) of the U site instead of the concentration (5×10^{-5} M) of the V site was employed for the simulation. Furthermore, the dissociation constant between warfarin and the binding site (HSA_{2U}, 1×10^{-4} M) was expressed as $K_{I,2U}$ to distinguish it from the original $K_{I,U}$. Figure 8 shows the U–R type inhibition curves thus obtained. The curve at $K_{I,2U}=2\times10^{-6}$ M and $K_{I,R}=2\times10^{-4}$ M is well fitted to the experimental results for warfarin (closed circles). The $K_{I,2U}$ value of 2×10^{-6} M in this case implies that the dissociation constants for both U and V sites are 2×10^{-6} M, that is, $K_{I,U}=2\times10^{-6}$ M and $K_{I,V}=2\times10^{-6}$ M (see Appendix 2).

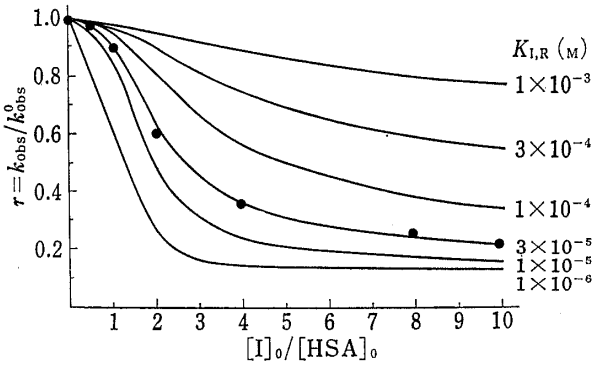

Fig. 7. U–R Type Inhibition Curves at Different $K_{I,R}$ Values and Constant $K_{I,U}$ Value (1×10^{-6} M)

●, experimental results for phenylbutazone.
$[HSA]_0=[HSA_R]_0=[HSA_T]_0=[HSA_U]_0=5\times10^{-5}$ M,
$[S]_0=1\times10^{-5}$ M.

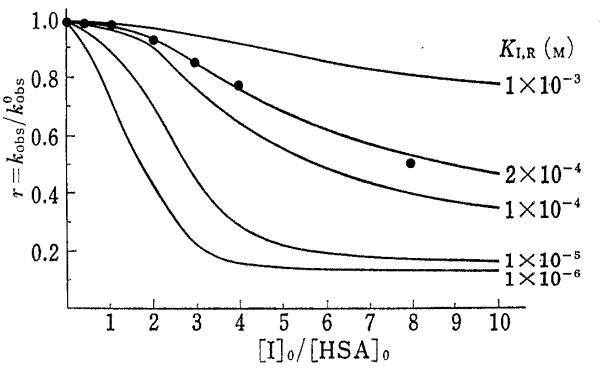

Fig. 8. U–R Type Inhibition Curves at Different $K_{I,R}$ Values and Constant $K_{I,2U}$ Value (2×10^{-6} M)

●, experimental results for warfarin.
$[HSA]_0=[HSA_R]_0=[HSA_T]_0=5\times10^{-5}$ M,
$[HSA_{2U}]_0=1\times10^{-4}$ M, $[S]_0=1\times10^{-5}$ M.

TABLE IV. Reciprocals of the Dissociation Constants obtained for the U–R Type Inhibitors and Binding Parameters from the Literature

Inhibitor	$1/K_{I,U}$ (M^{-1})	$1/K_{I,R}$ (M^{-1})	Literature binding parameters				Reference
			n_1	K_1 (M^{-1})	n_2	K_2 (M^{-1})	
Phenylbutazone	1×10^6	3×10^4	1	7×10^5	1	1×10^5	12
Sulfinpyrazone[b]	1×10^6	3×10^4	—[a]	—[a]	—[a]	—[a]	
Warfarin[c]	5×10^5	5×10^3	1	1.4×10^6	2	1.8×10^4	12
			1.46	2.41×10^5	2.42	5.6×10^3	13

a) Accurate binding parameters could not be found in the literature.
b) The slight binding of sulfinpyrazone to the T site of HSA[2] was neglected.
c) The reciprocal value of $K_{I,V}$ is also 5.0×10^5 M^{-1} (see the text).

Table IV lists the reciprocals of the dissociation constants for the U–R type inhibitors and the literature binding parameters. Since the binding parameters for phenylbutazone and warfarin found in the literature vary widely, we arbitrarily chose binding parameters near the values obtained in this study. The high (n_1) and low (n_2) affinity sites for phenyl-

butazone appear to be the U and R sites, respectively. The slight variations between the binding parameters (K_1 and K_2) and the reciprocals of the dissociation constants ($1/K_{I,U}$ and $1/K_{I,R}$) probably arise from the different experimental conditions (*e.g.* temperatures of 4°C and 25°C). Therefore again, the kinetic method seems to distinguish and identify the drug binding sites on HSA and to give their dissociation constants. However, for the U–R type inhibitions, this kinetic method sometimes has the disadvantage that multiple binding sites unconcerned with the esterase-like activities are not distinguished by this method alone, as in the case of warfarin.

Appendixes

1. Amplitude and Time Scaling for the R Type Inhibition

Calculations with an analog computer generally require amplitude and time scaling.[14] Here, as an example, the scaling in the case of the R type inhibition is interpreted in detail. The individual variables and initial concentrations in equations (1)—(7) are normalized by dividing by their likely maximum values. The new normalized variables and initial conditions expressed by the prime (′) are as follows:

$$[S]_0' = [S]_0/[S]_0 = 1,\ [S]' = [S]/[S]_0,\ [P_R]' = [P_R]/[S]_0,\ [P_T]' = [P_T]/[S]_0,\ [P]' = [P]/[S]^0,\ [HSA_R]_0' = [HSA_R]_0/[HSA_R]_0 = 1,\ [HSA_R]' = [HSA_R]/[HSA_R]_0,\ [HSA_T]_0' = [HSA_T]_0/[HSA_T]_0 = 1,\ [HSA_T]' = [HSA_T]/[HSA_T]_0,\ [I]_0' = [I]_0/[I]_0 = 1 \text{ and } [I]' = [I]/[I]_0.$$

When the new variables are used, equations (1)—(7) become equations (12)—(18), respectively.

$$[S]_0' = [S]' + K^R_{S,R}[S]'[HSA_R]' + [P_R]' + K^T_{S,T}[S]'[HSA_T]' + [P_T]' \tag{12}$$

where $K^R_{S,R} = [HSA_R]_0/K_{S,R} = 5\times10^{-5}/1.33\times10^{-4} = 0.376$ and $K^T_{S,T} = [HSA_T]_0/K_{S,T} = 5\times10^{-5}/3.09\times10^{-4} = 0.162$ (see Table I for the $K_{S,R}$ and $K_{S,T}$ values).

$$[HSA_R]_0' = [HSA_R]' + K^S_{S,R}[S]'[HSA_R]' + K^S_R[P_R]' + K^I_{I,R}[I]'[HSA_R]' \tag{13}$$

where $K^S_{S,R} = [S]_0/K_{S,R} = 1\times10^{-5}/1.33\times10^{-4} = 0.075$, $K^S_R = [S]_0/[HSA_R]_0 = 0.2$ and $K^I_{I,R} = [I]_0/K_{I,R}$.

$$[I]_0' = [I]' + K^R_{I,R}[I]'[HSA_R]' \tag{14}$$

where $K^R_{I,R} = [HSA_R]_0/K_{I,R}$.

$$[HSA_T]_0' = [HSA_T]' + K^S_{S,T}[S]'[HSA_T]' + K^S_T[P_T]' \tag{15}$$

where $K^S_{S,T} = [S]_0/K_{S,T} = 0.032$ and $K^S_T = [S]_0/[HSA_T]_0 = 0.2$.

$$[P]' = [P_R]' + [P_T]' \tag{16}$$

$$d[P_R]'/dt = k_{2,R}K^R_{S,R}[S]'[HSA_R]' \tag{17}$$

where $K^R_{S,R} = [HSA_R]_0/K_{S,R} = 0.376$.

$$d[P_T]'/dt = k_{2,T}K^T_{S,T}[S]'[HSA_T]' \tag{18}$$

where $K^T_{S,T} = [HSA_T]_0/K_{S,T} = 0.162$.

When the $k_{2,R}$ and $k_{2,T}$ values in Table I were applied to equations (17) and (18), respectively, calculations by the computer was too slow to follow conveniently on the recorder. Thus, time scaling was necessary for this system.

When the computer time and real time are represented respectively by τ and t, $\tau = \beta t$ where β is the time scaling factor. Then, equations (17) and (18) become equations (19) and (20), respectively.

$$d[P_R]'/d\tau = k'_{2,R}K^R_{S,R}[S]'[HSA_R]' \tag{19}$$

where $k'_{2,R} = k_{2,R}/\beta$.

$$d[P_T]'/d\tau = k'_{2,T}K^T_{S,T}[S]'[HSA_T]' \quad (20)$$

where $k'_{2,T}=k_{2,T}/\beta$.

If $\beta=1/60$, then $k'_{2,R}=1.88$ and $k'_{2,T}=0.544$. The calculation time on the computer using this factor is suitable to follow on the recorder. The time scaling factor (1/60) means that one second of the computer time corresponds to one minute of real time.

The analog circuit diagram for the R type inhibition shown in Figure 2 was drawn on the basis of equations (12)—(16), (19) and (20). For easy understanding of the diagram, the notations (*e.g.* $K^R_{S,R}$) for the potentiometers are employed instead of using the concrete numbers (*e.g.* 0.376).

2. Calculation of the Bound Warfarin Concentration

The following calculations (case 1 and case 2) of the bound warfarin concentrations may confirm the inference of the $K_{I,U}$ and $K_{I,V}$ values from the $K_{I,2U}$ value in the text.

case 1. If $[HSA_{2U}]_0=1\times10^{-4}$ M, $[I]_0=1\times10^{-4}$ M and $K_{I,2U}=2\times10^{-6}$ M, then

$$K_{I,2U} = [I][HSA_{2U}]/[I\cdot HSA_{2U}]$$
$$= (1\times10^{-4} - [I\cdot HSA_{2U}])^2/[I\cdot HSA_{2U}] = 2\times10^{-6} \quad (21)$$

From equation (21), the bound warfarin concentration ($[I\cdot HSA_{2U}]$) can be calculated as 8.68×10^{-5} M.

case 2. If $[HSA_U]_0=5\times10^{-5}$ M, $[HSA_V]_0=5\times10^{-5}$ M, $[I]_0=1\times10^{-4}$ M, $K_{I,U}=2\times10^{-6}$ M and $K_{I,V}=2\times10^{-6}$ M, then

$$K_{I,U} = [I][HSA_U]/[I\cdot HSA_U] = 2\times10^{-6}$$
$$= (1\times10^{-4} - [I\cdot HSA_U] - [I\cdot HSA_V])(5\times10^{-5} - [I\cdot HSA_U])/[I\cdot HSA_U] \quad (22)$$

Similarly,

$$K_{I,V} = [I][HSA_V]/[I\cdot HSA_V] = 2\times10^{-6}$$
$$= (1\times10^{-4} - [I\cdot HSA_U] - [I\cdot HSA_V])(5\times10^{-5} - [I\cdot HSA_V])/[I\cdot HSA_V] \quad (23)$$

Since $[I\cdot HSA_V]$ has to be equal to $[I\cdot HSA_U]$, equation (22) becomes equation (24).

$$K_{I,U} = (1\times10^{-4} - 2[I\cdot HSA_U])(5\times10^{-5} - [I\cdot HSA_U])/[I\cdot HSA_U] = 2\times10^{-6} \quad (24)$$

From equation (24), $[I\cdot HSA_U]$ can be calculated as 4.34×10^{-5} M. Thus, the warfarin concentration bound to the U and V sites ($[I\cdot HSA_U]+[I\cdot HSA_V]=2[I\cdot HSA_U]$) is 8.68×10^{-5} M.

The agreement of the bound warfarin concentrations between case 1 and case 2 suggests the propriety of the inference.

References and Notes

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5) The expression of k_{obs} using the individual parameters for the R type inhibition is presented as an example. According to Chart 1, the k_{obs} value can be represented as follows:[1)]

$$k_{obs} = \frac{\left\{k_{2,R}K_{S,T} + k_{2,T}K_{S,R}\left(1+\frac{[I]}{K_{I,R}}\right)\right\}[HSA]_0}{K_{S,T}K_{S,R}\left(1+\frac{[I]}{K_{I,R}}\right) + \left\{K_{S,T} + K_{S,R}\left(1+\frac{[I]}{K_{I,R}}\right)\right\}[HSA]_0}$$

where $[HSA]_0$ is constant because of its excess over the initial concentration of the substrate, and [I] which can be determined from $[I]_0$ added, $[HSA]_0$ and $K_{I,R}$ is also constant through the reaction.
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