

Evaluation of Serum Protein Binding by Using *in Vitro* Pharmacological Activity for the Effective Pharmacokinetics Profiling in Drug Discovery

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The establishment of a new index for the profile of serum protein binding was analyzed theoretically. The *in vitro* pharmacological activity ratio of the inhibition constant in the absence of serum protein to that in its presence (activity ratio), which represents the extent of specific binding to serum protein, was suggested as the new index. To clarify the usefulness of the activity ratio, theoretical analysis by the activity ratio for 3% human serum albumin was examined in comparison with conventional methods of equilibrium dialysis. In-house very late antigen-4 antagonists were used as model compounds, whose pharmacokinetics were strongly influenced by serum protein binding. Although the theoretical and actual unbound fractions were similar, the latter tended to be slightly lower than the former. This small difference was considered to correspond to nonspecific binding. These results suggested that the specific and nonspecific binding could be discriminated by comparing the activity ratio data with those of conventional methods. Moreover, the activity ratio was suggested to be useful in profiling the influence of protein binding on pharmacokinetics. In conclusion, it was considered that the activity ratio could avoid the risk of misleading interpretation by nonspecific binding in pharmacokinetics/pharmacological activity. Moreover, the activity ratio was considered to be valuable as one of the useful parameters in pharmacokinetics profiling and as a tool of rational drug design for drug discovery.

Key words protein binding; pharmacological activity ratio; specific binding; nonspecific binding

The affinity of a drug to serum proteins has a major clinical significance on both pharmacokinetics and pharmacodynamics *in vivo*.^{1–3} It is well known that unbound drug to serum protein is excreted from the body by diverse elimination processes, while bound drug constantly serves to recruit unbound drug to the plasma concentration. Consequently, a high level of protein binding prolongs the duration of total drug concentration in blood, whereas protein binding reduces the unbound fraction (*fu*) and therefore the pharmacological activity of a drug. Thus, the degree of protein binding is a key factor in the delicate balance between the intended pharmacological activity and potential unintentional effects of the drug.

According to the recent advances in high-throughput screening technology,^{4–6} multifaceted approaches such as pharmacokinetics, toxicity and physicochemical properties are now becoming more important in the drug discovery process. Namely, the strategy to select a drug candidate shifts from a bias toward the pharmacological activity to a balance in the pleiotropic properties including ADME (absorption, distribution, metabolism, excretion), physicochemical parameter and so on. An early survey of the protein binding level is one of the significant evaluations, as well as other ADME parameters. In particular, the evaluation of the effect of protein binding on the pharmacological activity is essential information for drug design.

Many reports concerned with the assessment of protein binding and simplified approaches for the determination of protein binding are being considered. The conventional methods such as equilibrium dialysis,⁷ surface plasmon resonance (SPR),³ ultrafiltration,⁸ ultracentrifugation⁹ and so on were improved and/or developed as high-throughput screening technology.

In view of this, we need to pay attention to the difference of affinity to serum protein such as specific and nonspecific binding. 7-Hydroxystaurosporine was reported to be non-

specifically bound to rat α_1 -acid glycoprotein (rAGP),¹⁰ whereas its protein binding to human AGP (hAGP) was the sum of specific and nonspecific binding. The pharmacokinetics of 7-hydroxystaurosporine in humans have shown a very distinctive feature, *i.e.*, low clearance/distribution volume and a long half-life in contrast to the experimental animals. Therefore, Fuse *et al.* showed that the specific high affinity binding to hAGP is one of the reasons for unusual pharmacokinetics of 7-hydroxystaurosporine in clinical studies.¹⁰ Consequently, the difference of affinity, as well as the extent of bound was suggested to play a key role in drug pharmacokinetics and/or the pharmacological activity.

The concentration dependence of percent bound, *i.e.*, Scatchard plot analysis by equilibrium dialysis, has been studied in order to examine the difference of affinity. However, feasibility of the conventional methods relies either on a separation of bound and unbound drugs or on the physicochemical properties of drugs such as adsorption to membrane/the detection sensitivity. Therefore, tasks in the application of the conventional methods remain.

With regard to the effect of serum protein on the *in vitro* pharmacological activity, the activity assessment by the addition of whole/diluted serum is generally used as the modified evaluation. This modified evaluation was considered to be useful in simultaneously providing accurate information about the influence of protein binding on pharmacological activity, as well as the level of protein binding compared with conventional assay of protein binding. Furthermore, it was considered that this approach could avoid the misleading impact of nonspecific protein binding. However, there are few reports available that study the relation between the level of protein binding and *in vitro* pharmacological activity quantitatively, and the difference of affinity that is yet to be elucidated. Hence, we were interested in clarifying the details of the relationship between the pharmacological activity and protein binding. Concomitantly, getting usefulness and sim-

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ple surrogate markers of specific binding for the effective profiling approach was considered important in the early drug discovery stage.

This paper investigates the acquirement of a new index for the characteristics of protein binding, and to apply the index to pharmacokinetics analysis. As model compounds, in-house very late antigen-4 (VLA-4) antagonists were used because the pharmacokinetics of VLA-4 antagonists, *i.e.*, the interindividual variability/clearance was reported to be strongly influenced by protein binding.^{11,12} These antagonists were acidic compounds that have a carboxylic acid and three kinds of derivatives, in which its basic structure is shown in Chart 1.

At first, the relationship between specific binding to serum protein and the *in vitro* pharmacological activity was analyzed theoretically. The *in vitro* pharmacological activity ratio of the inhibition constant (K_i) in the absence of serum protein to that in its presence (activity ratio), which represents the extent of specific binding to human serum albumin (HSA), was studied as the new index. Next, to evaluate the usefulness of the activity ratio, theoretical analysis of the activity ratio for 3% HSA was examined in comparison with conventional methods of equilibrium dialysis. Moreover, the results were verified by SPR method. Finally, the application of the index to pharmacokinetics profiling was investigated.

Experimental

Materials Three kinds of derivative of in-house VLA-4 antagonists were synthesized by the Medicinal Chemistry Research Laboratory, Daiichi Sankyo (Tokyo, Japan). Human serum albumin (HSA), which is essentially fatty acid and globulin-free, was purchased from Sigma (St. Louis, MO, U.S.A.). All other chemicals used in this experiment were of analytical and reagent grade.

Theoretical Analysis The *in vitro* pharmacological activity of the inhibition constant (K_i) in the absence and presence of 3% HSA was measured by VLA-4/vascular cell adhesion molecule-1 (VCAM-1) binding assay reported previously.¹² Theoretical unbound fraction (*fu*) was calculated from the activity ratio based on the theoretical equation in the inhibition scheme (see Results and Discussion, Chart 2).

Partition Coefficient The *n*-octanol-pH 7.4 partition coefficient ($\log D_{7.4}$) of the test compound from an aqueous solution to an organic solution was determined as follows. After mixing a test compound aqueous solution (pH 7.4 phosphate buffer solution) with an organic solvent (*n*-octanol), the solutions were shaken for 30 min and then centrifuged for 10 min at 3000 rpm. The test compound concentration in the aqueous and the organic phases was measured by an LC/MS method. $\log D_{7.4}$ was calculated from the following equation.

$$\log D_{7.4} = \log \left[\frac{\text{(concentration of organic phase)}}{\text{(concentration of aqueous phase)}} \right]$$

Equilibrium Dialysis The measurement of protein binding using a rapid

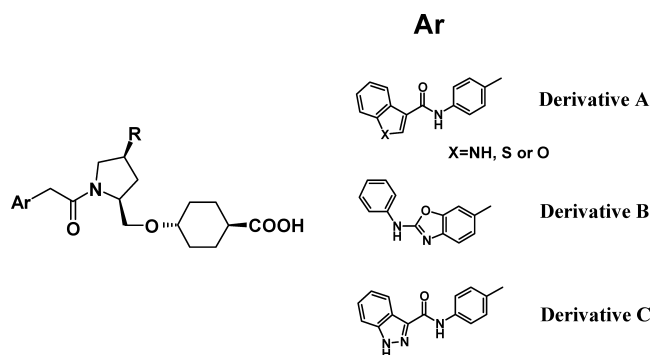


Chart 1. Basic Structure of VLA-4 Antagonist

equilibrium dialysis device (Linden Bioscience, Woburn, MA, U.S.A.) was carried out. The 3% HSA solution, including a test compound at a concentration of 10 μM was added to the membrane chamber, whereas pH 7.4 phosphate buffer solution was added to the buffer chamber. The sample solution was transferred from both sides after the equilibrium dialysis by incubation at 37 $^{\circ}\text{C}$ for more than 10 h. Both concentrations of compounds were measured by an LC/MS method. The apparent percent of unbound fraction was calculated using the following equation:

$$\% \text{ unbound} = (\text{buffer side/matrix side}) \times 100$$

$$\% \text{ bound} = 100 - \% \text{ unbound}$$

LC/MS Measurement Samples were injected onto a reverse phase column (Presto FT-C18 30 \times 4.6 mm i.d., Imtakt Japan). Mobile phase A consisted of 0.1% trifluoroacetic acid (TFA) solution, and mobile phase B consisted of acetonitrile containing 0.1% TFA. The gradient profile was 5% B for 0.2 min, increasing to 80% B at 2 min, 80% B for 0.5 min, then 5% B at 3.5 min and re-equilibrating after 1 min. The flow rate was 1.0 ml/min at 25 $^{\circ}\text{C}$.

SPR Measurement SPR measurement was carried out with a BIA-CORE 2000 (Biacore AB, Uppsala, Sweden). Sensor chip CM5, consisting of a carboxymethyl-modified dextran polymer linked to a gold-covered glass support, was used for analysis. HSA was immobilized to the sensor chip, using amine coupling. Unmodified dextran was used as a reference surface. The data obtained in the reference flow cell was subtracted from that obtained in the HSA flow cell. Dose-response curves were used for K_D determinations. To obtain an estimated K_D and response values at site saturation (R_{max}), response unit (RU) measured at different concentrations (C) of the compound was fitted to the equation:

$$RU = \frac{R_{\text{eq}}}{MW} = \frac{C \cdot R_{\text{max}}}{C + K_D} \quad (1)$$

where, R_{eq} and MW are response values at concentrations (C) of the compound and molecular weight, respectively. Unbound fraction was calculated from K_D . In this calculation, concentration of HSA and the compound used was 3% (0.45 mM) and 1 μM , respectively.

Application of the Activity Ratio to Pharmacokinetics. Index of Oral Clearance (CL_{po}) The CL_{po} is represented as follows:

$$CL_{\text{po}} = \frac{CL_{\text{tot}}}{F} = \frac{k_e \cdot V}{F} = k_e \cdot \frac{D_0 \cdot k_a}{C_0(k_a - k_e)}$$

CL_{tot} : total body clearance, k_e : elimination rate constant, k_a : absorption rate constant, V : distribution volume, D_0 : dose, F : bioavailability, C_0 : drug concentration at initial time

The plot of serum concentration of compound at 15 min (C_{15}) versus at 60 min (C_{60}) after oral administration to mice was in good agreement in this experiment (Fig. 1). The ratio of C_{15} and C_{60} was about 5 : 1. The maximum concentration time of this series of compounds was also reported to be within nearly 30 min.¹² These results suggested that the absorption process hardly influences serum concentration at or above 30 min after oral administration to mice. Moreover, these results indicated that the k_e value was nearly identical and k_a was quite higher than k_e . The estimated C_0 extrapolated from k_e and C_{60} was considered to be proportional to C_0 . C_{60} was thought to be a possible surrogate marker of C_0 . CL_{po} was thought to be nearly proportional to the inverse of C_{60} and was represented by the following simplified equation.

$$CL_{\text{po}} \propto \frac{1}{C_{60}}$$

Accordingly, the inverse of C_{60} was used as the surrogate index of CL_{po} in mice. The relationship between the activity ratio and the index of CL_{po} was analyzed. C_{15} and C_{60} after oral administration at 10 mg/kg to mice were determined by the reported bioassay method.¹² In this analysis, the test compounds of each derivative were selected based on high similarity of chemical structure.

Species Difference The *in vitro* pharmacological activity of the inhibition constant (K_i) in the absence and presence of 3% HSA and 10% mouse serum was measured by VLA-4/VCAM-1 binding assay reported previously.¹² The activity ratio for both species was used as the surrogate

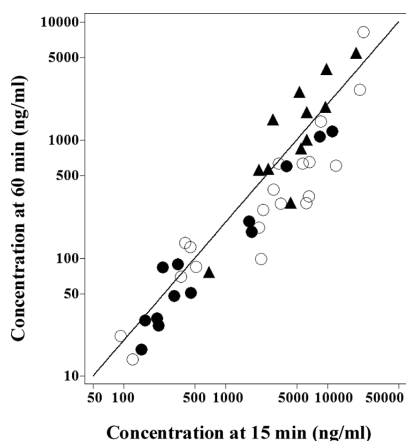


Fig. 1. Relationship between the Concentration of Compounds at 15 and 60 min after Oral Administration to Mice
 ○: Derivative A, ●: derivative B, ▲: derivative C.

index of specific protein binding. The relationship of the activity ratio for each species was analyzed for each derivative.

Results and Discussion

Theoretical Analysis Chart 2 shows the inhibition scheme in the presence of serum protein and the dissociation constant derived from the scheme. In this scheme, it was assumed that only free drug can bind to the receptor.

First, based on the inhibition scheme, *D* unbound to *R* (*D'*) can be written as follows:

$$\begin{aligned}
 [D'] &= [D] + [D \cdot P] + [D_2 \cdot P] + \dots + [D_n \cdot P] \\
 &= [D] \cdot \left(1 + \frac{[P]}{K_{p1}} + \frac{[D] \cdot [P]}{K_{p1} \cdot K_{p2}} + \dots + \frac{[D]^{n-1} \cdot [P]}{K_{p1} \cdot \dots \cdot K_{pn}} \right) \\
 &= [D] \cdot \left(1 + \sum_{n=1}^n \frac{[D]^{n-1} \cdot [P]}{\beta_n} \right)
 \end{aligned}$$

where, α is defined by the following equation.

$$\alpha = \sum_{n=1}^n \frac{[D]^{n-1} \cdot [P]}{\beta_n} = \frac{\text{bound fraction}}{\text{unbound fraction}}$$

where, α represents the extent of specific binding to serum protein, implying a stronger affinity compared with that of the receptor.

Similarly, *R* unbound to *D* (*R'*) can be written as follows:

$$[R'] = [R] \cdot \left(1 + \frac{[L]}{K_d} \right) = [R] \cdot (1 + \beta)$$

Since the initial amount of *[L]* is considerably higher than that of *[R]* in this experiment, *[L]* can be dealt as a constant. Accordingly, β is the constant in this experiment.

Next, in the presence of *P* and *L*, the conditional inhibition constant (*K_i'*) can be written as follows:

$$\begin{aligned}
 K_i' &= \frac{[D'] [R']}{[D \cdot R]} \\
 &= \frac{[D] \cdot (1 + \alpha) \cdot [R] \cdot (1 + \beta)}{[D \cdot R]} \\
 &= (1 + \alpha) \cdot (1 + \beta) \cdot K_i
 \end{aligned}$$

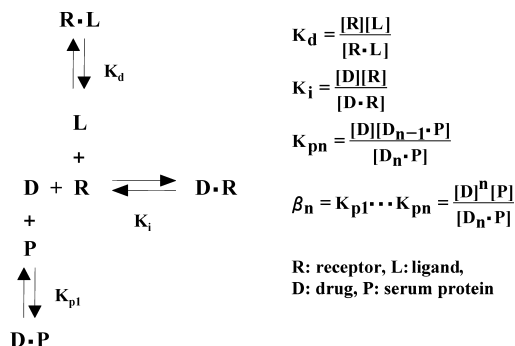


Chart 2. Inhibition Scheme in the Presence of Serum Protein and the Dissociation Constant

Consequently, the *in vitro* pharmacological activity ratio of *K_i* in the absence of serum protein to that in its presence (activity ratio) can be described as follows:

$$\frac{K_i}{K_i'} = \frac{1}{(1 + \alpha) \cdot (1 + \beta)}$$

The activity ratio depends on the degree of specific binding to serum protein. For instance, when the activity ratio is 0.1 and 0.01 under the experimental condition of 3% HSA, the unbound fraction corresponds to about 10% and 1%, respectively. In connection with the activity ratio, the *in vitro* pharmacological activity ratio of *IC₅₀* in the absence of serum protein to that (*IC₅₀'*) in its presence can be written as follows:

$$\frac{IC_{50}}{IC_{50}'} = \frac{1}{(1 + \alpha)}$$

This theoretical analysis was thought to be able to apply to serum proteins such as HSA.

Next, it is commonly assumed that the diluted serum instead of whole serum was used for the *in vitro* pharmacology assessment. Hence, the influence of serum protein content on the activity ratio was calculated by the different dissociation constant (*K_{p1}*) such as 1×10^{-4} , 1×10^{-5} and 1×10^{-6} . Figure 2 shows that the activity ratio decreased by the increase of the serum protein content using the three model compounds. However, the rate of reduction was nearly constant for each *K_{p1}*, and it was considered that there is no influence of serum protein content on the relative relation between activity ratio and protein binding. Therefore, it was determined that there was no relation to the usage of diluted/whole serum in case of relative comparison of the activity ratio. The extrapolation of the activity ratio of diluted to 100% whole serum was suggested as a possibility.

Comparison of Theoretical Analysis with Conventional Methods To clarify the usefulness of the activity ratio, which was the index of the extent of specific binding to HSA, the relationship between the unbound fraction (*fu*) obtained by the activity ratio and equilibrium dialysis by the conventional method was investigated. Compounds, whose partition coefficients at pH 7.4 (*log D_{7.4}*) as the index of lipophilicity, were largely different and were selected as test compounds. *Log D_{7.4}* plot versus *fu* measured by equilibrium dialysis is shown in Fig. 3a. A low/no correlation was found between

$\log D_{7,4}$ and f_u , thereby the major source of protein binding was assumed to be a specific interaction besides lipophilicity, a so-called nonspecific interaction.

Next, the relationship between the activity ratio and f_u was studied in these test compounds. The theoretical f_u calculated from the activity ratio and actual f_u measured by equilibrium dialysis were used as f_u , respectively. The relationship between the activity ratio and f_u are shown in Fig. 3b, and the data is also shown in Table 1. Theoretical and actual f_u were almost identical; however, the latter generally tended to be lower than the former. The differences among compounds (10, 11, 12), whose f_u were high, tended to be considerably larger.

f_u was verified by the SPR method. In this study, fatty acid and globulin-free HSA was used for the SPR assay. It was reported that the protein binding data obtained by the SPR method correlated with the data obtained by conventional methods including equilibrium dialysis.^{3,13} Dose-response curves of seven compounds bound to HSA are shown in Fig. 4. Compounds 1 and 2 displayed monophasic binding curves and approached saturation levels at low concentrations. Compounds 9 and 11 gave linear binding curves over a wide concentration range, and it was not possible to estimate saturation levels. K_D was calculated from Eq.1 (see Experimental)

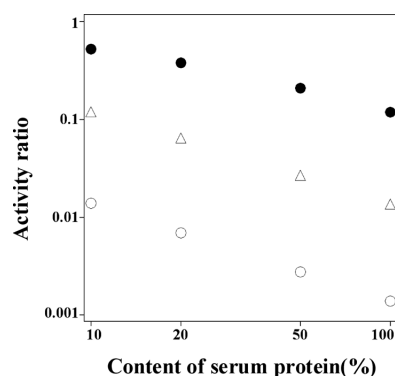


Fig. 2. Influence of Diluted Serum Protein on the Activity Ratio

The activity ratio was calculated for three model compounds which have dissociation constants (K_{pi}) of 1×10^{-4} , 1×10^{-5} and 1×10^{-6} . The calculated concentration of 100% serum protein and the compound was 0.6 mM and 1 μ M, respectively. \bullet : $K_{pi} = 1 \times 10^{-4}$, Δ : $K_{pi} = 1 \times 10^{-5}$, \circ : $K_{pi} = 1 \times 10^{-6}$.

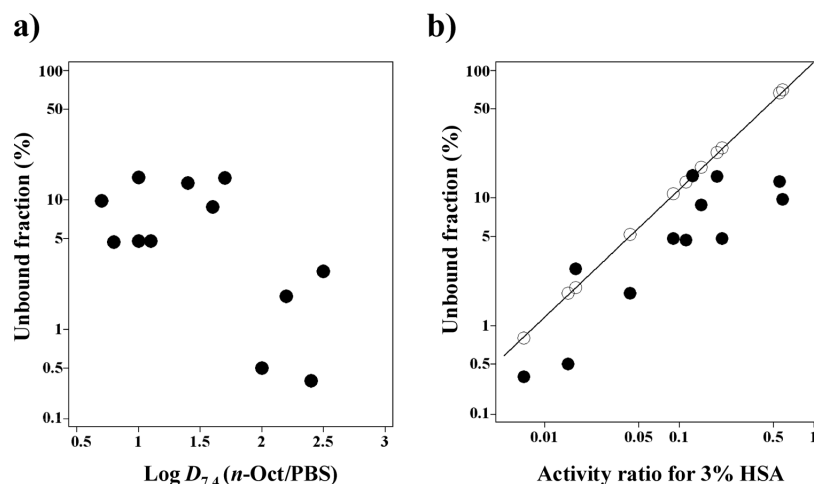


Fig. 3. Relationship between $\log D_{7,4}$ (a), the Activity Ratio for 3% HSA (b) and the Unbound Fraction

\circ : Activity ratio, \bullet : equilibrium dialysis, line: theoretical line.

by the dose-response curves.

Table 1 shows the results of protein binding obtained by different methods, including the SPR method. f_u obtained by different methods was almost identical among compounds (1–4, 7) whose f_u were lower. The validity of these methods was confirmed. On the other hand, the SPR data tended to be higher than the equilibrium dialysis data among compounds (9, 11) whose f_u were higher. The order of magnitude of the SPR response of 7, 9 and 11 was more similar to that of the activity ratio than the equilibrium dialysis. The strength of the specific interaction was thought to be strongly reflected in the SPR data when compared with the equilibrium dialysis data measured at an appropriate concentration. This affinity difference was not clearly elucidated because of the detection limit of the SPR method. However, the difference between the activity ratio and the equilibrium dialysis was considered to correspond to nonspecific binding.

The activity ratio was thus confirmed to be useful as a new index for the extent of specific binding to HSA. The difference of f_u in these methods was considered to correspond to nonspecific binding. These results suggested that the bound fraction obtained by the conventional method was the sum of specific and nonspecific binding, and that the two bindings could be discriminated by comparing the activity ratio data with those for conventional methods.

Application of the Activity Ratio to Pharmacokinetics.

Activity Ratio versus Oral Clearance (CL_{po}) The activity ratio was confirmed to be useful as the new index for the extent of specific binding to HSA as mentioned above. The above-mentioned characteristics of protein binding, such as specific and nonspecific binding strongly affected the pharmacokinetics of the drug. The activity ratio as a new index reflects the characteristics of protein binding, and was considered to be useful in assessing the correlation with pharmacokinetics of the drug. Regarding the application of the activity ratio to pharmacokinetics, the relationship between the activity ratio and CL_{po} in mice was investigated.

In the case of oral administration of hepatically eliminated drugs, the relationship between CL_{po} and intrinsic CL (CL_{int}) is generally represented as follows:

Table 1. Affinity for the Compound—Human Serum Albumin Interaction

Type of derivative	Compound	log $D_{7.4}$ (<i>n</i> -Oct/PBS)	Theoretical analysis data				Equilibrium dialysis data		SPR data	
			K_i (nM)	K'_i (nM)	Activity ratio	Calcd unbound fraction (%)	Unbound fraction (%)	K_D (M)	Calcd unbound fraction (%) ^{a)}	
B	1	2.4	1.80	264.0	0.007	0.8	0.4	2.69×10^{-6}	0.6	
A	2	2.0	1.60	106.0	0.015	1.8	0.5	3.05×10^{-6}	0.7	
A	3	2.5	3.36	208.4	0.017	2.0	2.8	6.87×10^{-6}	1.5	
A	4	2.2	4.82	118.6	0.043	5.2	1.8	2.30×10^{-5}	4.9	
A	5	1.1	1.62	18.0	0.090	10.8	4.8	NT ^{b)}		
A	6	0.8	0.73	6.5	0.112	13.4	4.7	NT		
A	7	1.0	3.45	28.0	0.125	15.0	14.9	9.27×10^{-5}	17.1	
A	8	1.6	0.32	2.2	0.145	17.4	8.8	NT		
A	9	1.7	2.75	14.5	0.190	22.8	14.8	$>1.24 \times 10^{-4}$	>21.6	
A	10	1.0	1.20	5.8	0.207	24.8	4.8	NT		
A	11	1.4	7.80	14.1	0.555	66.6	13.5	$>1.57 \times 10^{-4}$	>25.9	
A	12	0.7	3.80	6.5	0.585	70.2	9.8	NT		

a) Calculated from K_D under these conditions: [HSA]=0.45 mM, [Compound]=1 μ M. b) Not tested.

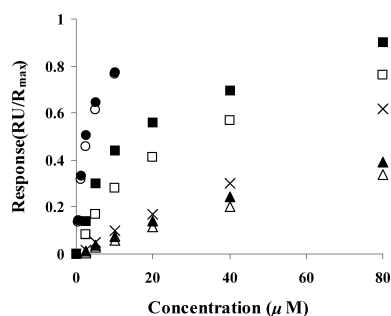


Fig. 4. Dose-Response Curves of Seven Compounds Bound to HSA

●: 1, ○: 2, ■: 3, □: 4, ×: 7, ▲: 9, △: 11.

$$CL_{po} = \frac{CL_{tot}}{F} = fu \cdot \frac{CL_{int}}{F}$$

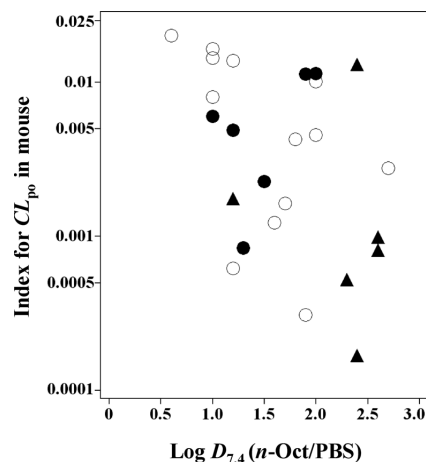
CL_{tot} : total body clearance, F : bioavailability

In this study, the relationship between CL_{po} and fu in mice was analyzed for VLA-4 antagonists, which were estimated as hepatically eliminated drugs. Additionally, the test compounds of each derivative were selected based on high similarity of chemical structure. The activity ratio for the diluted 10% mouse serum was used as the index for fu . As described in detail in the methods section, CL_{po} was thought to be nearly proportional to the inverse of serum concentration of the compound at 60 min (C_{60}) after oral administration in mice. Its relation can be written by the following simplified equation.

$$CL_{po} \propto \frac{1}{C_{60}}$$

Therefore, the inverse of C_{60} was used as the surrogate index of CL_{po} in mice. The plot of $\log D_{7.4}$ versus the index for CL_{po} is shown in Fig. 5. No correlation was found between $\log D_{7.4}$ and CL_{po} , thereby other factors except for lipophilicity, such as so-called nonspecific interaction was assumed to strongly participate in the extent of CL_{po} .

Next, the relationship between the activity ratio and CL_{po} was examined in these test compounds. The plots of the ac-

Fig. 5. Relationship between $\log D_{7.4}$ and the Index for CL_{po} in Mice

○: Derivative A, ●: derivative B, ▲: derivative C.

tivity ratio versus the index for CL_{po} were in good agreement as shown in Fig. 6. According to the reduction of the activity ratio, CL_{po} was lower. It was confirmed that CL_{po} was strongly influenced by specific binding. For each of the three groups, each plot of the activity ratio versus the index for CL_{po} was nearly linear with a slope of 1. Each group on the same regression line was thought to have similar CL_{po} . In view of this, the bioavailability of these compounds was considered to be quite similar because the oral absorption of each compound was quite high. Consequently, each group on the same regression line was thought to have similar CL_{int} . Moreover, the order of CL_{int} was derivative C > derivative B > derivative A. Derivative C indicated an advantage of potency in retaining the high concentration in mice when compared with other derivatives. The activity ratio was thus useful in profiling the influence of protein binding on pharmacokinetics.

Species Difference of Protein Binding Regarding the application of the activity ratio to pharmacokinetics, the species difference of protein binding was investigated. Figure 7 shows the relationship between the activity ratio for 3% HSA and 10% mouse serum. The extent of influence of each

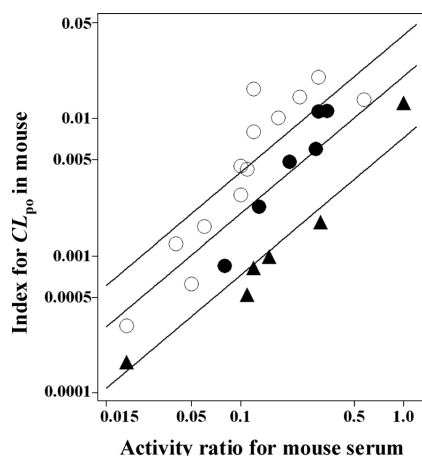


Fig. 6. Relationship between the Activity Ratio and the Index for CL_{po} in Mice

○: Derivative A, ●: derivative B, ▲: derivative C.

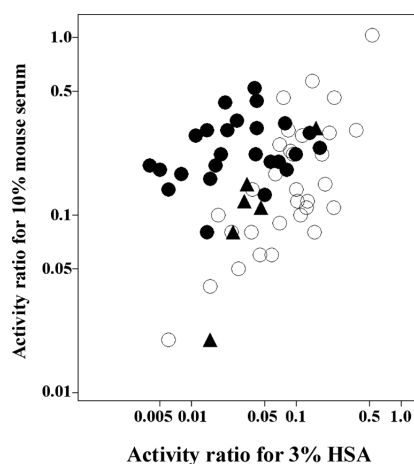


Fig. 7. Relationship between the Activity Ratio for 3% HSA and 10% Mouse Serum

○: Derivative A, ●: derivative B, ▲: derivative C.

serum protein was different in two derivatives, derivatives A and B. The affinities to mouse serum protein of both derivatives were almost identical, whereas, the affinities to HSA of derivative B tended to be higher than that of derivative A. As for derivative C, its species difference was not clear because the number of compounds was low when compared with other derivatives.

Next, the activity ratio of 3% HSA and 100% whole mouse serum as a modified evaluation *in vivo*, which was extrapolated by a factor of one-tenth of that for 10% diluted

mouse serum, was compared (not shown data). The differences in activity ratio among the two species of derivative B tended to be quite small. On the contrary, those of derivative A were comparatively larger. Namely, in comparison with the species difference of derivatives A and B, the former was larger than the latter. The activity ratio was thus useful in profiling species difference of protein binding.

In this study, we provided a new index which represents the degree of specific protein binding. The difference of affinity to serum protein, such as specific and nonspecific binding, was also easily separated by using the activity ratio and conventional method. Moreover, the application of the index to pharmacokinetics profiling was demonstrated.

In conclusion, it was considered that the activity ratio could avoid the risk of misleading interpretation by nonspecific binding in pharmacokinetics/pharmacological activity. Information on the activity ratio may be beneficial to elucidate the pharmacokinetics profile caused by protein binding or to predict pharmacokinetics. Furthermore, the activity ratio was valuable as one of the parameters in pharmacokinetics profiling and as a tool of rational drug design for drug discovery.

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