Evaluation of Hydrophobic Interaction between Acidic Drugs and Bovine Serum Albumin by Reversed-Phase High-Performance Liquid Chromatography

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The contribution of hydrophobic interaction to the protein binding of acidic drugs has been evaluated in terms of a new hydrophobic index (r -value), defined as the slope of the log-log plots of capacity factor vs. reciprocal of methanol concentration in an aqueous binary mobile phase, measured by the reversed-phase high-performance liquid chromatography. The logarithms of the binding constants ($\log K_1$) of the selected acidic drugs and the related aromatic carboxylic acids indicated linear relationship with their r -values, suggesting that the effect of hydrophobicity on protein binding can be explained similarly to that on the retention onto the reversed-phase stationary ligand.

Keywords HPLC; hydrophobic index; bovine serum albumin; drug-protein binding; protein binding; hydrophobic interaction

Introduction

The protein binding of a drug exerts significant effect on its pharmacological and pharmacokinetic properties,¹⁻⁵⁾ which is generally recognized as a combined effect of the specific and the non-specific interactions between drug molecule and the binding sites of protein. The binding force involves not only electrostatic interaction and hydrogen bonding but also hydrophobic interaction. Many drugs are known to have specific binding sites on an albumin molecule,⁶⁻¹⁰⁾ the specificity being a function of such various modes of interactions.

Hansch *et al.*¹¹⁻¹⁴⁾ showed that the non-specific binding is solely a function of the hydrophobicity of a solute as expressed by

$$\log(1/C) = a \cdot \log P + b \quad (1)$$

where C is the molar concentration of a solute necessary to form a 1:1 complex with albumin, representing the bindability, and P is the 1-octanol/water partition coefficient of a solute widely used as a hydrophobic index. Hydrophobicity of a drug is also expected to play an important role in the specific albumin binding.

The coefficient a in Eq. 1 usually takes a value between 0.5 and 0.7.¹³⁾ This value is smaller than the coefficient (usually almost unity) for the correlation between $\log P$ and anesthetic¹⁵⁾ or hemolytic activities^{16,17)} of drugs where C in Eq. 1 presents the molar concentration of a drug causing the standard biological responses. These activities depend obviously on the partition to the biomembranes. This fact suggests that the hydrophobicity of a drug makes a smaller contribution to the protein binding than to the partition. It can be considered, therefore, that a drug binds with protein at its surface but not inside of the protein structure. Thus, it becomes necessary to evaluate the hydrophobic surface adsorbability of a drug to comprehend the protein binding. The partition coefficient ($\log P$), however, does not necessarily reflect the surface hydrophobicity of a drug. For instance, the partition of a certain compound bearing strongly polar functional groups is much smaller than that expected from its hydrophobic adsorbability.¹⁸⁾

In the previous papers, the authors evaluated the solute hydrophobicity in terms of the r -value measured by reversed-phase high-performance liquid chromatography (RP-HPLC) with methanol (MeOH)/water binary mobile phase.¹⁹⁻²¹⁾ The r -value, defined as a slope of the following equation,

$$\log k' = r \cdot \log(1/[MeOH]) + q \quad (2)$$

is a hydrophobic index reflecting the adsorbability of a solute to the non-polar surface of the stationary ligand. It allows not only to evaluate the solute hydrophobicity but also to consider the molecular posture of the adsorbed solute and the surface properties of the stationary ligand.^{19,21)}

The present paper aims to correlate the r -value with the specific albumin binding of drugs. Several acidic drugs and the related aromatic carboxylic acids are selected as the model compounds bound specifically with serum albumin.

Experimental

Materials The clinically available acidic drugs used in this study were indomethacin (Nacalai Tesque Inc., Kyoto, Japan), ibuprofen (Kaken Seiyaku Co., Tokyo, Japan), ethacrynic acid (Sigma, St. Louis, U.S.A.), ketoprofen (Wako Pure Chemical Industries, Osaka, Japan), naproxen (Sigma) and salicylic acid (Nacalai Tesque). The structurally related aromatic carboxylic acids, biphenyl-4-carboxylic acid, naphthalene-2-carboxylic acid, 4-ethylbenzoic acids, naphthalene-1-carboxylic acid, 4-chlorobenzoic acid and 4-toluic acid were also used as supplied from Tokyo-Kasei Kogyo Co., Tokyo, Japan) and Nacalai Tesque. The chemical structures of these compounds are shown in Fig. 1. Bovine serum albumin (BSA) obtained from Sigma was fatty acid free.

Measurement of r -Value The retention time of a drug was measured on a Model LC-5A liquid chromatograph system (Shimadzu, Kyoto, Japan) equipped with a Model SPD-2A variable wave-length UV-detector (Shimadzu). The stationary phase used was a Nucleosil 5-C18 ODS silica (Macherey-Nagel, Duren, FRG) packed in a 15 cm $\times$ 4.6 mm i.d. stainless steel tubing. The column was kept at 40°C in a thermostatic oven. The mobile phases were the binary mixtures of methanol and 10 mM KCl-HCl buffer solution (pH=2.2). The methanol concentration was varied from 30% to 80% at an interval of 5%. The flow rate was fixed at 0.7 ml/min. The capacity factor (k') was calculated as

$$k' = (t_R - t_0)/t_0 \quad (3)$$

where t_R is the solute retention time, and t_0 is the retention time of nitrate ion. From the k' values obtained, $\log k'$ was plotted against $\log(1/[MeOH])$, and the slope of the linear part of the plots was calculated by the least squares method.¹⁹⁾ In most cases, the linearity was observed between 45% and 65% of methanol concentration. The resulting r -values of the drugs are given in Table I.

Measurement of Free Drug Concentration The binding of a drug with BSA was determined as follows. A 3 ml portion of 10 or 50 μ M BSA solution in the phosphate buffer (pH 7.4, ionic strength 0.17) was mixed with a 3 ml portion of the same phosphate buffer solution of a drug, and the mixture was incubated at 37°C for 20 min. The drug concentration was varied to make the binding ratio, C_b/C_p (bound drug concentration/BSA concentration), to be in a range of 0.2 to 2.0. A 1.5 ml portion of the mixed solution was subjected to the ultrafiltration using Molcut II (UFPI LGC, Millipore Co.) and a 0.1 ml portion of the filtrate was collected. For the drugs adsorbable to the filtration membrane, the first

Fig. 1. Structures of the Acidic Drugs and the Related Aromatic Carboxylic Acids Used in This Study

The symbols, see Table I.

TABLE I. *r*-Values and BSA Binding Parameters Estimated by the Two-constant Equation and by the Klotz Equation

Solutes	<i>r</i>	<i>N</i>	Two-constant ^{a)} <i>K</i> ₁ [M ⁻¹] (S.D.)	Klotz ^{b)} <i>K</i> ₁ [M ⁻¹] (S.D.)
A. Indomethacin	6.76	10	321300 (10000)	c)
B. Ibuprofen	6.42	15	1242000 (135000)	c)
C. Ethacrynic acid	5.60	8	629200 (46000)	710500 (141700)
D. Ketoprofen	5.16	7	480400 (8600)	c)
E. Naproxen	5.02	11	340100 (66500)	c)
F. Salicylic acid	2.63	10	96600 (8800)	87500 (22300)
G. Biphenyl-4-carboxylic acid	5.33	10	640300 (77900)	711700 (236100)
H. Naphthalene-2-carboxylic acid	4.36	12	482400 (63500)	c)
I. 4-Ethylbenzoic acid	4.03	14	238700 (29100)	262000 (38800)
J. Naphthalene-1-carboxylic acid	4.02	16	136400 (8300)	124800 (11100)
K. 4-Chlorobenzoic acid	3.56	6	138400 (22300)	c)
L. 4-Toluic acid	3.28	7	129800 (3900)	121500 (3600)

r: hydrophobic index estimated by Eq. 2. *N*: Number of data for binding analysis. S.D.: standard deviation. a) Eq. 4. b) Eq. 5. c) Pertinent values, not obtained.

0.1 ml portion of the filtrate was discarded and the following 0.1 ml portion was collected. The drug concentration in the filtrate was determined by RP-HPLC under the following condition: Column, Capcell-pak

C18 (Shiseido, Tokyo, Japan) packed in a 15 cm × 4.6 mm i.d. stainless tubing; mobile phase, acetonitrile/phosphate buffer solution (pH 4); detection, UV-210 nm. The mobile phase composition and the flow rate were selected appropriately.

Estimation of Binding Parameters The two-constant equation given by Larsen *et al.*²²⁾ was adopted to estimate the binding parameters.

$$R = a \cdot \ln(b \cdot C_f + 1) \quad (4)$$

where *R* is the binding ratio (*C_b*/*C_p*), *C_f* is the free drug concentration, and *a* and *b* are the coefficients. This empirical equation has advantages over the Klotz (Adair) equation (Eq. 5)²³⁾ derived based on the stepwise equilibrium and Scatchard equation²⁴⁾ based on Langmuir type adsorption, because it involves a least number of the coefficients to be determined and does not show a plateau due to saturation.²²⁾ Klotz equation is

$$R = \frac{K_1 \cdot C_f + 2 \cdot K_1 \cdot K_2 \cdot C_f^2 + \dots + n \cdot K_1 \cdot K_2 \dots K_n \cdot C_f^n}{1 + K_1 \cdot C_f + K_1 \cdot K_2 \cdot C_f^2 + \dots + K_1 \cdot K_2 \dots K_n \cdot C_f^n} \quad (5)$$

where *K*₁, *K*₂, ... and *K_n* are the first, the second, ... and the *n*-th binding constants. The coefficients *a* and *b* in Eq. 4 are correlated with *K*₁ and *K*₂²²⁾ as

$$a = \frac{K_1}{2(K_1 - 2 \cdot K_2)} \quad (6)$$

$$b = 2(K_1 - 2 \cdot K_2) \quad (7)$$

The pertinent values of *a* and *b* were estimated by the non-linear least squares fittings of Eq. 4 to the data obtained by the binding experiment mentioned above. From the results, the binding constants were calculated according to Eqs. 6 and 7.

Results and Discussion

The binding constants (*K*₁) for 12 acidic drugs, which reflect dominantly the binding to the primary specific sites on an albumin molecule, are listed in Table I. The values of the standard deviation given in parentheses indicate good fitness to the two-constant equation (Eq. 4). However, the

Fig. 2. Plots of $\log K_1$ vs. r -Value

The conditions for the measurements of r -values, see text. The symbols, see Table I.

fit with the Klotz equation (Eq. 5) did not give the pertinent values for some drugs (see Table I) which exhibited scattered or a small number of data points. In contrast, for the drugs which fit the Klotz equation, the results indicated good agreement with those obtained by the two-constant equation. This confirmed adequacy of the fit.

Figure 2 depicts the relationship between $\log K_1$ and r -value given in Table I. All the data points corresponding to the drugs and the related aromatic carboxylic acids except for indomethacin are almost linear. This fact suggests that among the various natures of drugs responsible for the specific binding to proteins, the hydrophobicity, represented by r -value, plays a predominant role in the prediction of protein binding ability of structurally related compounds (e.g. aromatic carboxylic acids in this study). The least squares analysis of the 11 data points (excluding indomethacin) in Fig. 2 gave a regression line

$$\log K_1 = 0.304 \cdot r + 4.121 \quad (8)$$

with the correlation coefficient 0.950.

Among the drugs used in this study, ibuprofen and ethacrynic acid are known to share the same primary binding site on albumin.^{6,7)} These compounds have electrostatic interaction with the basic functional group at the binding site and hydrophobic interaction with the surrounding non-polar moiety. It is interesting to note that the constant K_1 , mainly reflecting the affinity to the primary site, is closely related with the hydrophobic adsorbability to the ODS ligand on the surface of the stationary phase of RP-HPLC. This fact suggests that the hydrophobicity of a drug makes a similar contribution to both the protein binding and the non-polar adsorption, and that these drugs show almost the same contribution of the non-hydrophobic interactions, such as electrostatic interaction and hydrogen bonding, to the protein binding. Therefore, it can be speculated that the related aromatic acids in Table I may also have the same primary binding site on albumin or a different binding site bearing similar specificity.

Fig. 3. Plots of $\log K_1$ vs. $\log k'$ Obtained by Using 60% Methanolic Mobile Phase

The symbols, see Table I.

On the other hand, the point due to indomethacin in Fig. 2 is widely deviated downward from the straight line, showing weaker bindability than that expected from its hydrophobicity. That is, the hydrophobicity of indomethacin exerts a lower effect on the binding than that of other acidic drugs. This may imply that the primary binding site of indomethacin is different from those of others. Such a low affinity of indomethacin may be reasoned by the structural factors; the posture of indomethacin molecule bound to albumin will be restricted by the directional polar interactions and/or the dimension of hydrophobic circumstance around of the binding site, while the hydrophobic adsorption to the non-polar stationary ligand in RP-HPLC will occur to expose maximum surface area to contact with the ligand. Since indomethacin molecule has wider hydrophobic surface area than others as depicted in Fig. 1, this excess hydrophobicity may vainly contribute to the protein binding. This is why indomethacin gave the point deviating downward from the line in Fig. 2. This speculation agrees with the idea that the hydrophobic interaction at the binding site takes place on a limited area of "hydrophobic patch"²⁵⁾ scattered on an albumin molecule.

When $\log K_1$ is plotted against $\log k'$ (capacity factor) instead of r -value, we obtain also a linear relationship. Figure 3 shows the result obtained from k' values of the acidic drugs and the related aromatic carboxylic acids in Table I, measured by using the binary mobile phase containing 60% methanol.

$$\log K_1 = 1.283 \cdot \log k'(55\%) + 4.232 \quad (9)$$

$$\log K_1 = 1.513 \cdot \log k'(60\%) + 4.272 \quad (10)$$

$$\log K_1 = 1.806 \cdot \log k'(65\%) + 4.304 \quad (11)$$

$$\log K_1 = 2.212 \cdot \log k'(70\%) + 4.330 \quad (12)$$

Equation 10 is the regression equation for the line in Fig. 3. Equations 9, 11 and 12 are those obtained with use of

the mobile phases containing 55%, 65% and 70% methanol, respectively. The correlation coefficients of Eqs. 9 to 12 are 0.932, 0.921, 0.906 and 0.889, respectively. As readily found from these results, the lower the methanol concentration of the mobile phase, the better the correlation, but all the values of correlation coefficients are lower than that (0.950) for the $\log K_1$ vs. r equation (Eq. 8). Therefore, the r -value is a better index than the capacity factor to evaluate contribution of the hydrophobicity to the protein binding. The low methanol concentration in the mobile phase gives rise to high contribution of the hydrophobicity to the non-polar adsorption, and hence results in relatively small effects of the non-hydrophobic interaction on k' value. Thus, the value of the correlation coefficient was increased with decreasing concentration of methanol. However, the excessive decrease causes the retention time of a hydrophobic drug to be impractically prolonged. For instance, the retention times of indomethacin and ibuprofen exceeded 1 h when 50% methanol was used as a mobile phase. The r -value is a convenient index of hydrophobicity, since it is easy to measure and is scarcely affected by non-hydrophobic forces.

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