약제학회지(1993), 제23권 제3호
J. Kor. Pharm. Sci., Vol. 23, No. 3, 119-125 (1993)

The Effect of Physicochemical Properties of Salicylate Analogs on Binding to Bovine Serum Albumin

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(Received July 29, 1993)

살리실산 유사체류의 물성이 우혈청 알부민 결합에 미치는 영향

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영남대학교 약학대학

(1993년 7월 29일 접수)

The protein binding of salicylate analogs has been investigated by equilibrium dialysis. A series of binding experiments were performed in order to elucidate the effects of physicochemical properties of salicylate analogs on the binding with bovine serum albumin. Attempts to correlate affinity constants with capacity factor, steric factor and Hammett σ values suggested hydrophobic forces to be involved in the binding of salicylate analogs. Steric factor contributes to binding process partly, whereas electronic interaction appears to be insignificant.

Keywords—Protein binding, Salicylate analogs, Equilibrium dialysis, Capacity factor, Steric factor, Electronic factor

It has been well recognized that the interaction of drugs with various blood or tissue proteins can profoundly influence the therapeutic, pharmacodynamic and toxicological actions of drugs.[1,2] Most drugs bind or complex with proteins by a reversible process in which weaker chemical bonds such as hydrogen bonds or van der Waals forces are involved. The amino acids, components of the protein chain, have hydroxyl, carboxyl, or other sites available for reversible drug interactions.

The protein-drug complex acts as a transport system to carry the drug to the sites of action; this transport is extremely important for drugs that exhibit low solubility in the water portion of the plasma. Protein binding slows the disappearance of free drug from the plasma into tissues by decreasing the concentration gradient. It also serves as a depot system for free drug to replace that removed by various distribution and elimination processes.[3-5] It is generally accepted that only unbound drug is pharmacologically active and capable of diffusing across biological membranes. Thus, the extent of binding may markedly exert profound effects on drug distribution between the blood and extravascular fluids, interactions with other drugs and may also affect both hepatic and renal clearance.[6] Drug protein binding is an important determinant of modified drug pharmacokinetics in the disease state. For example, in patients with heatic diseases, the binding of many acidic drugs to plasma proteins is frequently decreased. The decreased plasma protein binding of drug has been related to either the reduction of concentrations of plasma proteins such as albumin and lipoproteins or the binding competition between acidic drugs and endogenous substances such as bilirubin and free fatty acids on binding sites of those plasma proteins.[2]

Free drug, not total drug, is inherently the most reliable indices of the intensity of drug action and can be used as a guide to optimal drug dosage.[7] In this context, clinical evidence also indicates that the concentration of free drug in plasma correlates better with biological activity than the total drug concentration.[8] For example, serum unbound cortisol is thought to closely reflect the physiologically active hormone level. In situations where binding proteins are abnormal, measurement of total cortisol will not necessarily correlate with physiological activity. For this reason, there has long been interest in measurement of free cortisol in serum using a variety of techinques.[9]

It is generally accepted that albumin is the principal protein in the body responsible for the nonspecific binding of weakly acidic drugs.[3] Studies on binding to isolated plasma proteins, specifically to albumin, may provide information as to the quantitative binding characteristics, *i.e.*, the number and type of binding sites and association binding constants.

This study was undertaken to assess the effect of physicochemical properties of salicylate analogs on interaction with bovine serum albumin. Drug protein binding provides clinically valuable information on appropriate therapeutic uses of the drug and predictions of possible drug interactions.

EXPERIMENTAL

Materials

Salicylate analogs such as 3-ethylsalicylic acid(3-EtSA), 3-isopropylsalicylic acid(3-ProSA), 3-tert-butylsalicylic acid(3-BuSA), 5-ethylsalicylic acid(5-EtSA), 5-isopropylsalicylic acid(5-ProSA) and 3,5-dimethylsalicylic acid(DMSA), 5-tert-butylsalicylic acid(5-BuSA) were obtained from previous work.[10] Methyl alcochol and ethyl alcohol were HPLC grade and were used as supplied by Fisher Scientific Co. (Fair Lawn, NJ). Sodium monophosphate, sodium diphosphate, sodium hydroxide and potassium chloride, all reagent grade, were also from Fisher. Bovine albumin, No. A 4378, crystallized and lyophilized, was obtained from Sigma Chemical (St. Louis, MO). The molecular weights was assumed to be 66,500. All other chemicals were also of analytical reagent grade and all solutions were prepared in deionized and distilled water. All protein and drug solutions were prepared in 0.1 M sodium phosphate buffer (pH 7.4).

Binding Experiments

The binding isotherms of the salicylate analogs were obtained by equilibrium dialysis performed at 20℃. For equilibrium dialysis, solutions of bovine albumin in 0.2 M phosphate buffer pH 7.4, were pipetted into cellophane bags (Visking, 18 mm diamter). After closure they were placed in appropriate volume of salicylate analogs solution with various quantities, prepared in the same buffer. This system was carefully closed with Parafilm (American Can Co., Greenwich, CT), placed in a temperature-controlled water bath, and shaken gently for 14-18 hr. Salicylate analogs were not bound measurably to chamber and membrane at free concentrations below 150 μM. Equilibrium dialysis experiments accordingly were confined to concentrations below 150 M. Other potential sources of errors, such as leakage of albumin through the membrane, albumin decay, osmosis of water, UV-absorbing impurities from the membrane, unequal distribution of free ligand, or hydrogen ions due to the Donnan effect, were found negligible under our experimental conditions. Equilibrium concentrations of unbound salicylate analogs in the outer media were determined, after suitable dilution with phosphate buffer, by spectrophotometry at a light absorption maximum. Lineraity of extinction with varying concentations was verified for all salicylate analogs. Control experiments with salicylate analogs-containing, but albumin-free, solutins showed that the dialysis membrane was fully permeable for all salicylate analogs and that equilibria were established within the periods of time employed.

The amount of protein was measured by the method of Bradford.[11]

Capacity Factor Determinations

One of the parameters which represent the lipophilicity of drug molecules is capacity factor (CF). High performance liquid chromatography using reverse-phase columns has proven to be a

valuable tool for estimating partition coefficients from retention data.[12] HPLC was performed on Beckman Ultrasphere ODS column (5 μm, 4.6 mm×250 mm) for capacity factor determinations. Samples (20 μl) containing about 20 μg/ml of salicylate analogs were injected using model 210A sample injection valve. Mobile phase was 50% MeOH in potassium phosphate buffer (0.05 M, pH 7.4) with a flow rate of 1.2 ml/min. Beckman 114M solvent delivery module (pump) and model 421A system controller were used to control the flow rate. Wavelengths were monitored at 230 and 296 nm using the Hewlett Packard 8451A diode array spectrophotometer. The program LCQUANT employing the HP 9121D Disc Memory was run for each sample. Capacity factor, k', was then determined for each sample as below;

$$k' = (t_R - t_0)/t_0 \quad (1)$$

t_R: retention time for salicylate analog

t_0: retention time for unretained compound

Binding Parameter Determinations

The characteristics of the binding parameters were quantitatively examined by the use of nonlinear regression analysis.

In order to find the best possible solution, the data of binding experiments to albumin were subjected to curve fitting based on Scatchard's equation,[13] formulated for one high-affinity site and several weaker, independent, and equal sites,

$$r = ck_1/(1+ck_1) + n_2ck_2/(1+ck_2) \quad (2)$$

where r is the average number of bound salicylate analogs per molecule of albumin, c is the free salicylate concentration, k_1 and k_2 are the site-binding constants for the high affinity site and the weak sites, respectively, and n_2 is the number of weak sites (number of binding sites per mole of protein).

Structure-Activity Relationship Study

In an attempt to elucidate the binding mode of salicylate to albumins, quantitative relationships between primary affinity constants of salicylate analogs and their physicochemical parameters were investigated.

The physicochemical model for albumin binding in this investigation assumes that albumin binding would be governed by electronic parameter, steric parameter, and hydrophobic parameter.[14] The capacity factor reflects lipophilic character of the salicylate analogs. E_s is Taft's steric effect[15] and is the substituent electronic effect of Hammett.[16]

It is the primary binding constant that is of major clinical importance and is the one of interest in this study.

Correlation and regression equations were calculated by linear least squares regression analysis on a PC-386 personal computer employing the SAS program. The improvement and reliability of regression analysis were judged by F and t test. The relative importance of these three parameters was evaluated, which might provide information on the albumin-drug binding forces. A general equation[17] for the multiparameter approach to structure-activity relationships can be written as follows;

$$\log k_1 = a(\log CF) + b(\Sigma\sigma) + c(E_s) + d \quad (3)$$

where binding affinity of salicylate analog with albumin is correlated with the change in CF, $\Sigma\sigma$ and E_s caused by structural modifications within a class.

RESULTS AND DISCUSSION

With a series of salicylate analogs, it is of interest to evaluate the importance of hydrophobic forces in the binding process. Capacity factor can be used to serve as a measure of relative hydrophobicity of salicylate analogs.[12] Therefore, we determined capacity factors for the salicylate analogs using HPLC and listed in Table I.

Drug binding is predictable from the laws of mass action and characterized mainly by two parameters: the affinity constant (K_i) of the drug for albumin, and the number of sites (n_i) on albumin to which it can bind. These parameters as well as the binding forces and the location of the sites dictate the significance of the plasma protein

Table I—*Capacity Factors of Salicylate Analogs*

Salicylate analog	Retention time	Capacity factor[a]
SA	2.8	0.077
3-MeSA	3.0	0.154
3-EtSA	3.5	0.346
3-ProSA	4.2	0.615
3-BuSA	6.8	1.615
5-MeSA	3.1	0.192
5-EtSA	3.7	0.423
5-ProSA	4.4	0.692
5-BuSA	6.9	1.654
DMSA	3.4	0.308

t_0=2.6 min.
[a]Calculated by equation 1.

Table II—*Binding Characteristics for Salicylate Analogs*

	Site I		Site II	
	n_1	$k_1(\times 10^{-5})$	n_2	$k_2(\times 10^{-3})$
SA	1	1,371	4.34	4.10
3-MeSA	1	6.095	5.10	7.93
3-EtSA	1	6.887	4.71	8.35
3-ProSA	1	6.950	4.23	8.93
3-BuSA	1	8.375	3.95	8.51
5-MeSA	1	4,365	4.50	7.39
5-EtSA	1	7,834	4.23	8.64
5-ProSA	1	9,226	4.61	9.42
5-BuSA	1	7.962	5.12	9.20
DMSA	1	5.702	4.15	6.87

Results are the means of 4 determinations. k_i is expressed in M^{-1}.
The data have been obtained from equation 2. n_1 and n_2 are the number of binding sites in the first and second binding class, respectively, and k_1 and k_2 are the corresponding Scatchard affinity constants.

binding for distribution and pharmacological activity. The binding characteristics of the salicylate analogs, as obtained from equilibrium dialysis experiments, are summarized in Table II, which clearly show the presence of at least two different binding regions for high affinity drug binding and low affinity binding.

Drug binding of most drugs to serum albumin is quantitatively the most important and often responsible for the entire drug binding in plasma.

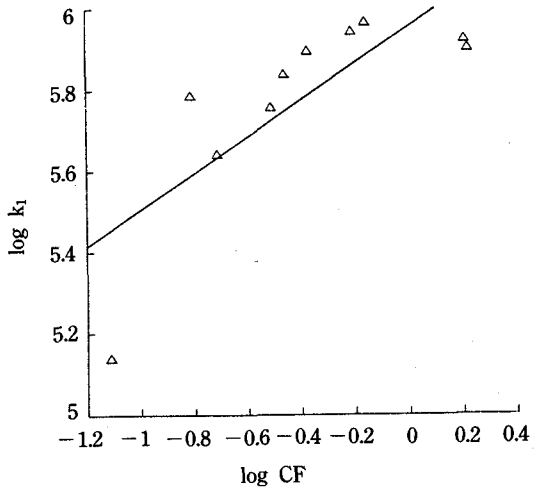

Figure 1—Relationship between log (affinity constant) and log (capacity factor).

Many highly albumin-bound drugs show poor solublity in water, and for such drugs hydrophobic binding to hydrophobic sites on albumin is often important.[18] Both ionic and hydrophobic bonds determine binding affinity. Highly bound drugs often have an ionizable acidic group at physiological pH and are hydrophobic and at least double-ring molecules.[4] Hydrogen-bonding and dipolar effects are probably also of importance for drug binding.[19]

Fig. 1 shows the correlation between physicochemical parameters of salicylate analogs and their primary binding affinity to bovine serum albumin, in which is seen a general increase of log k_1 as log CF increases. This finding suggests the involvement of hydrophobic forces in binding processes. Helmer *et al.*[20] also found a good correlation when plotting binding parameters of a great number of different organic ligands as a function of their octanol-water partition coefficients. However, because the correlations between log k_1 and log CF are relatively poor as shown in Eq. 4, other binding forces must exist between the salicylate analogs and albumin, probably a steric and electostatic nature.

$$\log k_1 = 0.440(\log CF)(\pm 0.2873) + 5.941(\pm 0.1623)$$
$$r^2 = 0.6840 \quad SD = 0.15982 \qquad (4)$$

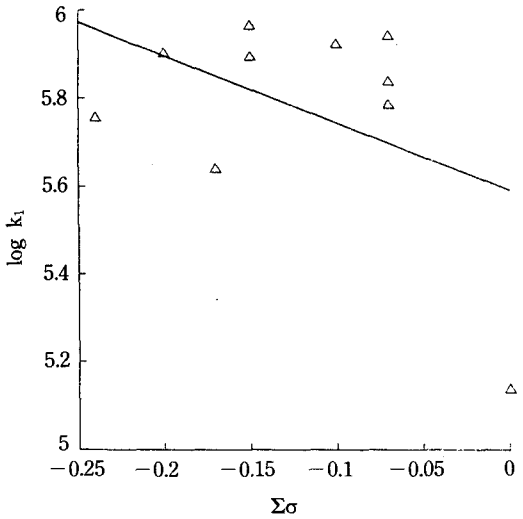

Figure 2—Relationship between log (affinity constant) and electronic factor.

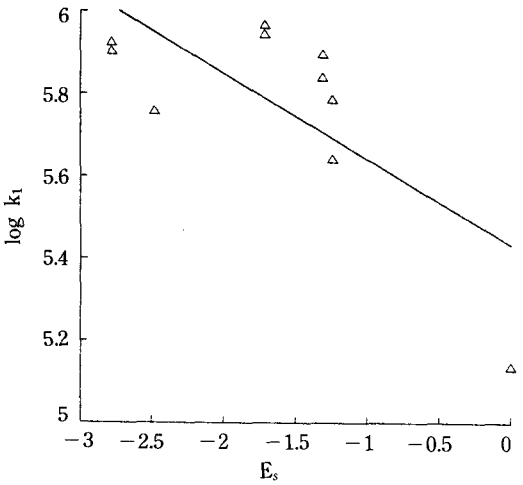

Figure 3—Relationship between log (affinity constant) and steric factor.

Fig. 3 shows the correlation of the binding constants of salicylate analogs with the Hammett sigma constants. Eq. 5 was derived using only $\Sigma\sigma$ as the independent variable, where r^2 is the square of correlation coefficient, SD is the standard deviation of estimate and values in parentheses are the 95 % confidence intervals.

The R-square of 0.2403 suggests an insignificant influence of electrostatic forces on the binding process.

Table III—*Physicochemical Data for Salicylate Analogs*

Salicylate analog	log k_1	log CF	E_s[a]	$\Sigma\sigma$[b]
SA	5.137	−1.114	0.00	0.00
3-MeSA	5.785	−0.813	−1.24	−0.07
3-EtSA	5.838	−0.461	−1.31	−0.07
3-ProSA	5.842	−0.211	−1.71	−0.07
3-BuSA	5.923	0.208	−2.78	−0.10
5-MeSA	5.640	−0.716	−1.24	−0.17
5-EtSA	5.894	−0.374	−1.31	−0.15
5-ProSA	5.965	−0.160	−1.71	−0.15
5-BuSA	5.901	0.219	−2.78	−0.20
DMSA	5.756	−0.512	−2.48	−0.24

[a]Adapted from ref. 15.
[b]Adapted from ref. 23 and calculated with respect to the carboxyl group.

$$\log k_1 = -1.627(\Sigma\sigma)(\pm 2.3586) + 5.570(\pm 0.3304)$$
$$r^2 = 0.2403 \quad SD = 0.22259 \qquad (5)$$

Eq. 6 derived only employing Es showed a better correlation than Eq. 10, even though neither Eq. 6 nor Eq. 5 is sufficient to explain binding process. As shown in Fig. 3, steric factor might have some role in protein binding under our experimental condition.

$$\log k_1 = -0.207(E_s)(\pm 0.1569) + 5.426(\pm 0.2892)$$
$$r^2 = 0.5355 \quad SD = 0.17405 \qquad (6)$$

Tanford *et al.*[21] proposed that binding sites were hydrophobic patches on the protein molecules; this implicates that these sites have some steric limitations. In fact, steric effects were important in some cases of protein binding. The molecular size of the coumarins was shown to exert influneces on the interaction of coumarins with 1-acid glycoprotein.[22]

Attempts were made to combine any two factors in a single equation to find better correlations. However no significant improvement in correlation could be found as shown below;

$$\log k_1 = 0.324(\log CF)(\pm 0.5990) - 0.068(E_s)$$
$$(\pm 0.3002) + 5.784(\pm 0.7205)$$
$$r^2 = 0.6236 \quad SD = 0.1675 \qquad (7)$$

$$\log k_1 = 0.393(\log CF)(\pm 0.3315) - 0.666(\Sigma\sigma)$$

$$(\pm 1.9524)+5.841(\pm 0.3384)$$
$$r^2=0.6417 \quad SD=0.16341 \qquad (8)$$

$$\log k_6=-0.015(\Sigma\sigma)(\pm 2.7120)-0.206(E_s)$$
$$(\pm 0.2308)+5.425(\pm 0.3261)$$
$$r^2=0.5355 \quad SD=0.18606 \qquad (9)$$

As expected from Eq. 5, electronic effects were found to be clearly unimportant since no significant improvement was obtained by addition of this term to Eq 4. Addition of electronic factor to Eq. 4 (Eq. 8) resulted in a slightly better correlation than that of steric factor (Eq. 7) did. This, however, was not statistically important.

The equation employing the three parameters, CF, E_s and $\Sigma\sigma$, did not give a significant improvement, with r^2 values less than 0.6840.

$$\log k_1=0.389(\log CF)(\pm 0.7138)-0.656(\Sigma\sigma)$$
$$(\pm 2.9097)-0.002(E_s)(\pm 0.4365)+5.837$$
$$(\pm 0.8205)$$
$$r^2=0.6417 \quad SD=0.1765 \qquad (10)$$

Comparing the statistics for equations 4-10, it may be concluded that capacity factor plays a key role in the albumin-salicylate analog binding. Steric factor may also contribute partly and electrostatic interactions appear to be insignificant. Further studies with expanded series of salicylate analogs are necessary to elucidate the exact role of each factor.

CONCLUSION

Hydrophobicity of salicylate analogs appears to be the most important factor, whereas steric factor seems to be of secondary importance and electronic factor is of little importance in salicylate analog binding to bovine serum albumin.

ACKNOWLEDGEMENT

This work was supported by a grant for professor from Yeungnam University.

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