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Drug-Biomacromolecule Interaction XII: Comparative binding study of sulfaethidole to bovine serum albumin by equilibrium dialysis, fluorescence probe technique, uv difference spectrophotometry and circular dichroism

Chong-Kook Kim[§], Yang-Sook Chun and Woon-Lyong Lah

College of Pharmacy, Seoul National University, Seoul 151-742, Korea

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Abstract□Binding of sulfaethidole to bovine serum albumin was studied by equilibrium dialysis, fluorescence probe technique, uv difference spectrophotometry and circular dichroism. Equilibrium dialysis method enabled us to estimate the total number of drug binding sites of albumin molecule. For sulfaethidole, albumin had 6 primary and 40 secondary binding sites. The primary and secondary binding constants were 0.9×10^5 M^{-1} and 0.2×10^6 M^{-1}, respectively. 1-Anilino-8-naphthalenesulfonate (ANS) and 2-(4'-hydroxylbenzeneazo)-benzoic acid (HBAB) were used as the fluorescence probe and the uv spectrophotometric probe, respectively. In fluorescence probe technique, results indicated that the number of higher affinity drug binding site of albumin was 1 and the number of lower affinity drug binding sites of albumin was 3, and the primary and secondary drug binding constants for bovine serum albumin were 2.15×10^5 M^{-1} and 1.04×10^5 M^{-1}, respectively. In uv difference spectrophotometry, binding sites were 3 and binding constant was 1.88×10^5 M^{-1}. The above results suggest that several different methods should be used in ompensation for insufficient information about drug binding to albumin molecule given by only one method.

Key words□Sulfaethidole, bovine serum albumin, protein binding, equilibrium dialysis, fluorescence probe technique, uv difference spectrophotometry, circular dichroism.

Binding of drugs to serum albumin is a well known and much studied phenomenon. Drug binding to various blood and tissue proteins can influence therapeutic, pharmacokinetic and toxicologic actions of drugs.[1] Since only free fraction of drug molecules is available for transport to the receptor site and contributes to pharmacological activity, protein binding has a significant effect on diffusion rate and apparent volume of distribution of drugs.[2,3]

Binding of small molecules can also influence chemical reactivity of macromolecules. This phenomenon is mainly due to long range electrostatic force, shorter range specific interactions such as hydrogen bonds, hydrophobic bonds, and proton dispersion forces. Primary drug-protein complexes are often stabilized by charge transfer forces. These forces, however, should not be used to estimate the overall complex stability, because the drug-protein binding is mainly due to van der Waal's forces. The stability of a drug-protein complex is expressed by its association constant, which is also important for the pharmacokinetic behavior of the drug.[4]

A vast array of physicochemical methods have been used to study ligand binding of protein.[5] Equilibrium dialysis is most common tool for drug-protein binding study. In recent years, spectroscopic techniques, including ultraviolet and visible absorption spectroscopy, fluorescence spectroscopy, optical rotatory dispersion and circular dichroism, nuclear magnetic resonance and electron spin resonance have played an important role in the studies of drug interactions with macromolecules.[6-8]

One important advantage that spectroscopic techniques have over the more classical techniques, such as equilibrium dialysis, is that they can

[§]To whom inquires should be directed

measure the amount of bound and free drug in a given system, and can also yield additional information on the nature of the drug-macromolecule interaction.[9,10] The spectrophotometric probe technique is a simple, rapid and reproducible method which yields information concerning the nature of binding, specific binding sites of macromolecules and spectral properties of the probe used.[11,14]

A fluorescence probe[12,13] is defined as a compound that undergoes changes in one or more of its fluorescence properties, when it is bound to certain proteins. ANS has been used as a fluorescence probe to study drug-protein binding at the hydrophobic sites of proteins.[12] Competition with a drug for the same binding sites reduces the fluorescence intensity of the probe-protein complex. In uv difference spectrophotometry, the usefulness of a dye probe is evident from the fact that the absorption spectrum of the dye changes as a result of drug binding to protein. HBAB is a dye that interacts with serum albumins to form characteristic spectrophotometric peaks.[10] The intensities of these peaks can be related to the extent of binding of the dye to serum albumin, therefore, this method can be used as a convenient means for studying displacement reactions.

When optically inactive compounds bind to optically active compounds such as proteins, new circular dichroism bands are induced in the absorption bands of optically inactive compounds.[19] The binding sites on the protein responsible for the Cotton effects are known to contain a hydrophobic region and a hydrophilic region involving a cationic center or a center capable of forming a hydrogen bond. Since the Cotton effects can give information about conformational change of protein when drug attaches to protein, circular dichroism method is applicable to the study of drug-protein interaction.

In this study, equilibrium dialysis, fluorescence probe technique and uv difference spectrophotometry were applied to determine the binding parameters of sulfaethidole to bovine serum albumin (BSA), because each of these methods provides only limited information on the nature involved in the formation of the BSA-sulfaethidole complex.

EXPERIMENTAL

Materials

Bovine serum albumin (BSA), Fraction V, was purchased from Sigma Co., and its molecular weight was assumed to be 69,000. The concentrations of albumin solutions were determined by uv at 280 nm. The molar concentration was calculated on the basis of $E^{1cm}_{1\%} = 6.67$.

N'-(5-ethyl-1,3,4-thiadiazol-2-yl)-sulfanilamide (sulfaethidole), 1-anilinonaphthalene-8-sulfonate (ANS) and 2-(4'-hydroxybenzeneazo) benzoic acid (HBAB) were purchased from E. Merk (Darmstadt, Germany), Sigma Co., and ICN pharmaceutical Inc., respectively. All solvents used were of spectral grade.

Equilibrium dialysis

Solutions of BSA and sulfaethidole were prepared in 0.054 M sod. phosphate buffer(pH 7.4). For equilibrium dialysis, a multicompartment dialysis system consisting of two plastic blocks with five wells was employed. Semipermeable membrane (Visking tube) sufficiently swollen and rinsed with distilled water was boiled for 15 min in a 10 % acetic acid solution, followed by repeated rinsing with distilled water. The treated dialysis membrane was cut into a single sheet and placed between the plastic blocks, which were then clamped together with bolts and screws. The concentrations of drug were applied over the range of 2.85×10^{-5} M and 1×10^{-4}M, and BSA concentration was fixed at 2.89×10^{-5}M. BSA-drug solutions were added to one side of the membrane, and phosphate buffer (pH 7.4) solutions were added to the other side. The sampling ports were then sealed, and the unit was gently agitated overnight in a water bath at 20 °C. This procedure was allowed to be at equilibrium. Then, the bound fraction of sulfaethidole to BSA was determined by measuring the free sulfaethidole concentration in the phosphate buffer compartment with a spectrophotometer (LKB Ultraspec 4050) at 280 nm. Membrane effects were controlled by carrying out the ligand blank test only with phosphate buffer in one of five wells.

Fluorescence probe technique

The binding of the probe (ANS) to BSA was determined by measuring the increase in fluorescence observed as ANS was added to the BSA solution. The fluorescence intensity was measured with fluorescence spectrophotometer (Bario-Automic Spectrophotometer Model FC 100) with excitation at 375 nm and emission at 480 nm. Solutions of albumin were prepared in phosphate buffer (pH 7.4) and solution of ANS was prepared to a concentration of 1×10^{-3} M in absolute methanol. Titrations with ANS were carried out at both lower (7.25×10^{-7} M) and higher (7.25×10^{-6} M) BSA concentration. Successive 2 μl aliquots of ANS

solution were added to 5 m*l* of BSA solutions. Titrations of BSA solution of low concentration were repeated in the presence of 1.4×10^{-5} M sulfaethidole. Sulfaethidole was added to the BSA solution prior to titration. Blank titration in the buffer was carried out to correct the fluorescence of free ANS in the absence of BSA.

UV difference spectrophotometry

The binding of the probe (HBAB) to BSA was determined by measuring the increase in uv difference absorbance following the titration of the BSA solution with HBAB in the procedure as previously described.[14,15] The HBAB solution was prepared to a concentration of 1×10^{-2} M in absolute methanol. The BSA solutions were prepared in 0.054 M phosphate buffer(pH 7.4). The lower concentration of BSA solution was 2.9×10^{-5} M and the concentration of sulfaethidole was 1.0×10^{-4} M. Tandem cells were arranged in the procedure as previously described.[14] Aliquots of the BSA solutions (1 m*l*) were pipetted into two compartments of tandem cells and then, two aliquots of buffer solutions (1 m*l*) were pipetted into two compartments of the cells. These solutions were placed in the reference and sample beams so that a buffer and a BSA solution compartments might be in tandem in each beam. After drawing a base line, the contents of buffer solution compartment in the reference beam and the BSA solution compartment in the sample beam were titrated with successive addition of 5 μl of HBAB solution and then uv difference absorbance were measured in each time with a double beam spectrophotometer (Pye Unicam RF-150) equipped with tandem cell holder at 484 nm for HBAB-BSA system. Titrations of BSA solution of low concentration were repeated in the presence of 1×10^{-4} M sulfaethidole. Sulfaethidole was added to the BSA solution in sample beam prior to titration.

RESULTS AND DISCUSSION

Equilibrium dialysis method enables us to estimate the total number of drug binding sites. In equilibrium dialysis, the amount of bound drug to m classes of independent sites in a non-cooperative process per mole of albumin(V) is given by

$$V = \sum_{i=1}^{m} \frac{n_i K_i A}{1 + K_i A} \quad (1)$$

where each class, i, has n_i sites having intrinsic binding constant, K_i, and A is the concentration of unbound drug. In the case of two classes of binding sites, equation (1) reduced to

$$V = \frac{n_1 K_1 A}{1 + K_1 A} + \frac{n_2 K_2 A}{1 + K_2 A} \quad (2)$$

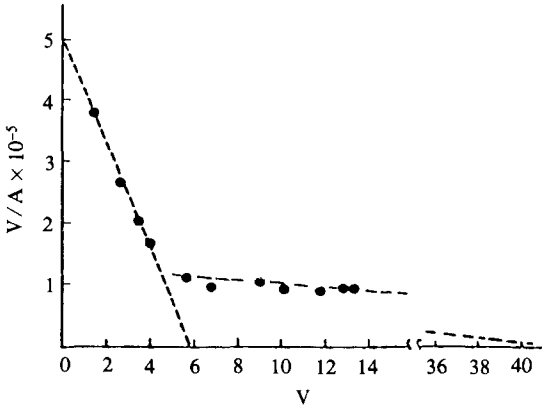

Fig. 1. Scatchard plot of sulfaethidol binding to BSA.

The curvature shown in a Scatchard plot (Fig. 1) was indicative of more than one class of binding site. For sulfaethidole, BSA has 6 primary and 40 nonspecific secondary binding sites, and 0.9×10^5 M^{-1} primary and 0.2×10^5 M^{-1} secondary binding constants.

The fluorescence titration curves of BSA with ANS and uv difference absorbance titration curves of BSA with HBAB in the presence or absence of sulfaethidole are shown in Fig. 2 and 3, respectively. The fluorescence or uv difference absorbance of the probe-BSA complex at higher and lower BSA concentrations, and the subsequent decrease of fluorescence or uv difference absorbance in the presence of sulfaethidole were used to calculate the bound and unbound fraction of probes and to calculate the binding parameters for probes and drug. Bound fraction of ANS, X, was calculated using the following equation.[21]

$$X = \frac{I_p - I_o}{I_b - I_o} \quad (3)$$

where, I_p and I_o are the fluorescence intensities of a given concentration of ANS in solutions of low BSA concentration and in solutions without BSA, respectively, and I_b is the fluorescence intensity of the same concentration of ANS fully bound to a high concentration of BSA. The fluorescence intensity of ANS-BSA complex was reduced by the addition of sulfaethidole. Fig. 2 shows the effect of sulfaethidole on the fluorescence emission spectra of the ANS-BSA complex, and the addition of sul-

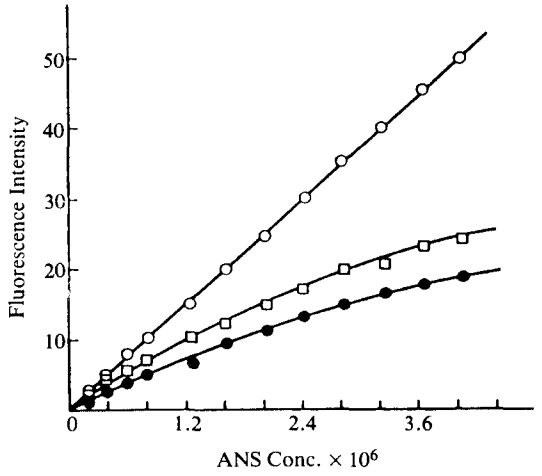

Fig. 2. Fluorescence titration curves of BSA with ANS at high(○) and low(□) concentration of BSA. Curve (●) is the titration curve of low BSA concentration with ANS in the presence of sulfaethidole.

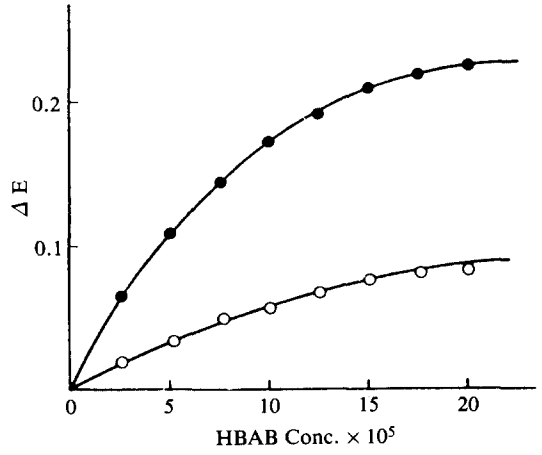

Fig. 3. Difference absorbance titration curves of BSA (2.90×10^{-5}M) solution with HBAB in the absence(•) and in the presence(○) of 1.0×10^{-4}M sulfaethidole, respectively.

faethidole resulted in quenching the fluorescence intensity. The bound fraction of HBAB, X, was calculated by using the following equation.

$$X=\frac{El}{Eh} \tag{4}$$

where El and Eh refer to the uv difference absorbances of HBAB in solutions at lower and higher

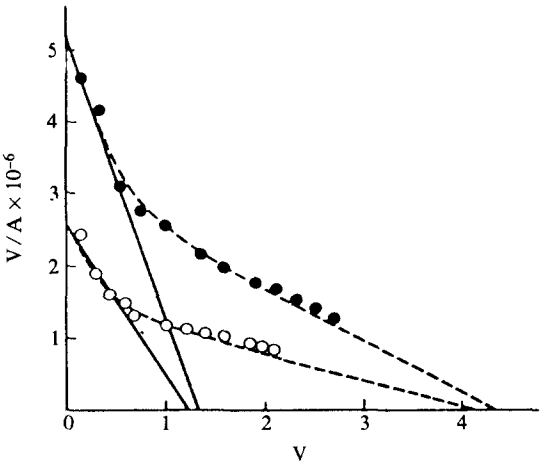

Fig. 4. Scatchard plots of ANS binding to BSA in the absence(●) and in the presence (○) of 1.4×10^{-5}M sulfaethidole, respectively.

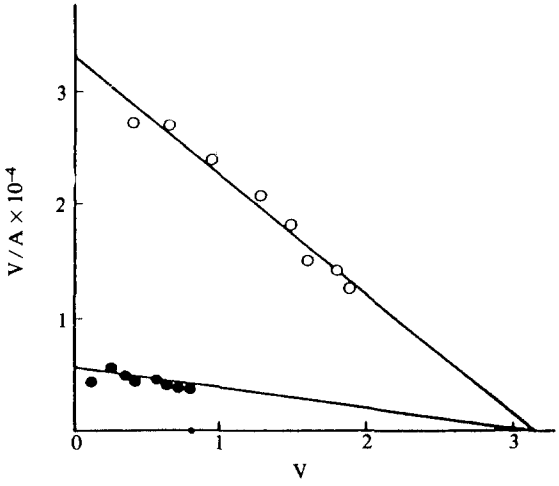

Fig. 5. Scatchard plots of HBAB to BSA in the absence (○) and in the presence (●) of 1.4×10^{-5}M sulfaethidole, respectively.

BSA concentrations, respectively. To determine the value of Eh for given concentration of HBAB, uv difference absorbance titrations were carried out at various BSA concentrations, and the values of Eh were obtained from extrapolation to the intercepts of plots of 1/E versus 1/(P), where E and (P) represent uv difference absorbance and the total concentration of BSA, respectively.[14] After values for the bound fraction of probe were found from the titration curve, the data obtained from flu-

orescence probe technique and uv difference spectrophotometry were treated according to the Scatchard equation[16]

$$V/A = nKa - VKa \quad (5)$$

where V is the number of moles of bound probe per mole of albumin; A, the concentration of free probe; n, the number of binding sites on BSA; Ka, the intrinsic binding constant of the complex.

The Scatchard plots in equilibrium dialysis, fluorometric titration, and uv difference spectrophotometry are shown in Fig. 1, 4, and 5, respectively. Least square analysis was employed for fitting the experimental data. The common intercept indicated that drug binding occurred at the same sites on BSA where the probe was bound. The Scatchard plots were linear in Fig. 5 but curved in Fig. 1 and 4. These curvatures are generally indicative of more than one class of sites.[16] In these cases, simple extrapolation or evaluation of the slope from the linear portion of the curve may give inaccurate estimates of the binding constants for the various classes. These aspects have been overlooked by many authors, but linear extrapolaton is probably still the most popular means to determine binding constants.[17]

Fig. 4 shows that the number of binding sites for ANS to BSA increased from one to four as raising the molar ratios of ANS to BSA and the number of the higher affinity binding site seemed to be close to one. The Scatchard plot of the sulfaethidole-BSA binding shows that sulfaethidole and ANS has the same binding sites. Fig. 5 shows that the number of binding sites for HBAB to BSA are three. And the Scatchard plot of the sulfaethidole-BSA binding shows that sulfaethidole and HBAB has the same binding sites. The binding constants of sulfaethidole to BSA determined by uv difference spectra or fluorescence probe technique were calculated according to the following Klotz equation.[18]

$$Kb = \frac{n(Pt)Ka(A) - Ka(A)(PA) - (PA)}{(Bt)Ka(A) - n(Pt)Ka(A) + Ka(A)(PA) + (PA)} \times \frac{Ka(A)}{(PA)}$$

where Ka and Kb are the binding constants for probe and sulfaethidole, respectively, PA is the concentration of probe bound, Pt and Bt are the total concentrations of the protein and sulfaethidole, respectively, A is the concentration of the free probe, and n is the number of binding sites. In equilibrium dialysis, Fig. 1 indicates that the number of binding sites for sulfaethidole increase from 6 to 40 as the molar ratios of sulfaethidole to BSA increase, and the numbers of higher and lower affinity binding sites are 6 and 40, respectively. In the fluorophotometric probe technique, the numbers of higher and lower affinity binding sites of the sulfaethidole on BSA are 1 and 3, respectively. The primary and secondary binding constants of sulfaethidole to BSA are 2.15×10^5 M^{-1} and 1.04×10^5 $M,^{-1}$ respectively. In uv difference spectrophotometry, binding sites are 3 and binding constant is 1.88×10^5 M^{-1}. In a preceding paper, binding of sulfaethidole to BSA was studied by circular dichroism. It was found that one primary binding site on BSA was capable of inducing optical activity in the presence of sulfaethidole and the primary binding constant was 5.4×10^5 M^{-1}.[19] Binding parameters obtained from four different methods were summarized in Table I.

Table I. Binding parameters of sulfaethidole to bovine serum albumin

Binding parameter Method	No. of Binding Sites(n)	Binding Constant(K)
UV	3	1.88×10^5 M^{-1}
F	n_1 : 1	K_1: 2.25×10^5 M^{-1}
	n_2 : 3	K_2: 1.04×10^5 M^{-1}
EQ	n_1 : 6	K_1: 0.9×10^5 M^{-1}
	n_2 : 40	K_2: 0.2×10^5 M^{-1}
CD(a)	1	5.4×10^5 M^{-1}

n_1 : No. of primary binding sites
n_2 : No. of secondary binding sites
K_1: Primary binding constant
K_2: Secondary binding constant
a : Data from ref. 20.

CONCLUSION

Although four different methods have been used to study sulfaethidole-BSA binding, the techniques commonly used for binding studies have generally yielded somewhat limited information on the nature involved in the formation of the complex. It means that different detection mechanism of each method, and different concentration range of drug and BSA solution result in the different binding parameters as shown in Table I. Therefore, in the drug-macromolecule interaction studies, it is suggested that several different methods should be used in compensation for insuficient information given by only one method.

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