Arch. Pharm. Res. 6(1), 55~62(1983)

Drug-Biomacromolecule Interaction IV

Binding of Six Cephalosporins to Bovine Serum Albumin

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(Received 18 May 1983)

Abstract □ Binding of six cephalosporins (cefotaxime, cefuroxime, cefazoline, cephalothin, cephaloridine, cephacetrile) to bovine serum albumin was studied. Fluorescence probe technique and difference spectrophotometry were employed to evaluate the nature and degree of association of cephalosporin-albumin complex. 1-Anilinonaphthalene-8-sulfonate (ANS) was used as the fluorescence probe. 2-(4′-hydroxybenzeneazo) benzoic acid(HBAB) was employed as the UV spectrophotometric probe. Competitive bindings between cephalosporins and probes were observed. The number of binding sites of bovine serum albumin for each cephalosporin is 2. Among six cephaloporins, cefotaxime has the highest binding constant followed by cefazoline, cefuroxime, cephalothin, cephaloridine and cephacetrile.

Keywords □ Cefotaxime, Cefazoline, Cefuroxime, Cephalothin, Cephaloridine, Cephacetrile, Bovine serum albumin, 1-Anilinonaphthalene-8-sulfonate (ANS), 2-(4′-Hydroxybenzeneazo) benzoic acid (HBAB), Fluorescence probe technique, Difference spectra.

Binding of drug to plasma protein is generally considered as an important factor in the transport, distribution and elimination of drug.[1] Higher molecular weight of plasma proteins prevents passage across the capillary walls and assures longer retention time in the vascular space.[2] The unbound drug molecules are presumed to be available for transport to the receptor site and for distribution into the extravascular space and/or for certain modes of elimination. Hence, binding to plasma protein may have a marked effect on the apparent volume of distribution and pharmacologic effect of drug.[3] The most important contribution to drug binding is made by albumin which includes about one half of total plasma proteins.[4]

The scope of present work is a quantitative study of the interaction of six available cephalosporins with bovine serum albumin. Fluorescence probe technique and ultraviolet difference spectroscopy were applied to evaluate the stoichiometry and strength of the binding.

EXPERIMENTAL METHODS

Materials

Cephaloridine, sodium cephalothin and sodium cefuroxime were obtained from Chong Kun Dang Co.. Sodium cephacetrile and sodium cefotaxime were supplied by Han Dok Remedia Ind. Co.. Sodium cefazoline was provided by Yu Han Co.. All cephalosporins were used without further purification. Bovine serum albumin(BSA), Fraction V was purchased from Sigma Co. and its molecular weight was assumed to be 69,000. The concentration of albumin solution was determined from the absorbance of the peak at 280 nm. The molar concentration was calculated on the basis of $E^{1\%}_{1cm}=6.67$. The fluorescence probe, 1-anilinonaphthalene-8-sulfonate (ANS),

was purchased from Sigma Co.. The spectrophotometric probe, 2-(4′-hydroxybenzeneazo) benzoic acid(HBAB), was purchased from ICN Pharmaceuticals Inc.. All other chemicals used were of analytical reagent grade. The water used was double distilled from glass.

Instrument

All fluorescence measurements were made with Baird-Automic Spectrophotometer Model FC100 equipped with 150 watts xenon lamp. The relative fluorescence intensities were recorded directly from the recorder in fluorometer. The entrance slit for the excitation light and the exit slit for the fluorescence emission were 32 nm, respectively.

The ultraviolet difference spectra were measured on the model SP1750 Pye Unicam Spectrophotometer. The temperature of all measurements was maintained at 20°C using a circulator(Techne Co.).

Fluorescence Measurement

The binding of the probe, ANS, to BSA was determined by measuring the fluorescence intensity as a function of ANS concentration according to the method described in previous papers.[4-7] The fluorescence measurement was made at 480 nm using an excitation wavelength of 375 nm which is isosbestic for the ANS-BSA binding. BSA was dissolved in 0.05 M phosphate buffer at pH 7.4. Stock solution of ANS was prepared using methanol at a concentration of 1×10^{-3}M. Successive aliquots of 2 μl of probe solution were added to each 5 ml of albumin solutions. Titrations with ANS were carried out at both low(7.25×10^{-7}M) and high (7.25×10^{-6}M) albumin concentrations. The titration of BSA solution of low concentration was repeated in the presence of each cephalosporin, individually. To minimize the photodecomposition of ANS, samples were exposed to the light only for the short measuring period. Blank titration in the buffer was made to correct the fluorescence of free probe in the absence of BSA. The inner filter effect was not observed by keeping absorbance low at the excitation wavelength. Methanol did not affect the binding of the probe in the concentration range used.

Difference Spectra Measurement

The binding of the spectrophotometric probe, HBAB, to BSA was determined by measuring the difference spectra as a function of HBAB concentration according to the method described in previous paper.[8] The absorbance difference titrations were carried out at 20°C controlled by circulator. Tandem cells were used to correct for solvent absorbance reflectance. The absorbance difference was measured at 484 nm.

The HBAB was dissolved in methanol at a concentration of 1×10^{-2}M. The BSA was dissolved in 0.05 M phosphate buffer, pH 7.4. Titrations with HBAB solution were performed at low(2.9×10^{-5}M) and high(1.5×10^{-4}M) BSA concentrations, respectively. Two, 2 ml of BSA solution were pipetted into two compartments of tandem cells and two, 2 ml of buffer solution were pipetted into two remaining compartments of the cells. These solutions were placed in the reference and sample beams as shown in Figure

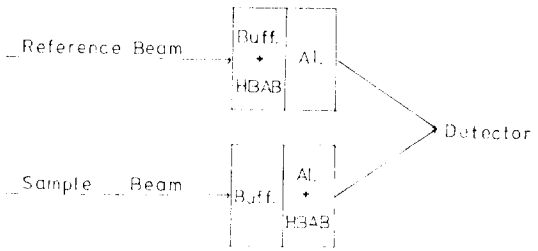

Fig. 1: Tandem cell arrangement for difference spectrophotometry.
Al.: albumin solution, Buff.: 0.05M phosphate buffer at pH 7.4

1. After drawing a base line, buffer solution in the reference beam and BSA solution in the sample beam were titrated simultaneously with successive additions of 5 μl of the HBAB solution and absorbance differences were measured in each time. A repetitive titration of BSA solution of low concentration was carried out in the presence of 1×10^{-3}M of each cephalosporin, individually.

Data treatment

Enhancement of the fluorescence or difference absorbance of the probe upon addition to BSA solution at two concentrations, and the subsequent decreases of fluorescence or difference absorbance in the presence of the binding cephalosporins were used to calculate the binding parameters for the probe and drugs. The fraction of probe bound, X, was calculated using the following equation[9].

$$X=\frac{I_p-I_0}{I_b-I_0}$$

where I_p and I_0 are the fluorescence intensities or difference absorbances of a given concentration of probe in solutions of low BSA concentration and in solutions without BSA, respectively; I_b is the fluorescence intensity or difference absorbance of the same concentration of probe fully bound to BSA for high concentration. After the value, X, was found for each point along the titration curve, the Scatchard equation was applied to determine the binding parameters of the protein-probe interaction according to the method described in previous reports.[7,8]

$$\frac{V}{A}=nKa-VKa$$

where V is the number of moles of bound probe per mole of BSA, A is the concentration of free probe, n is the number of binding sites on the BSA molecule, and Ka is the binding constant of the probe to BSA. The competitive binding of cephalosporins was studied using ANS as the fluorescence probe and HBAB as the spectrophotometric probe. The binding of probe to BSA was determined in the presence and absence of competitor by varying the concentration of probe at fixed concentration of BSA and cephalosporin. The binding constants of cephalosporins were calculated by using the equation derived by Klotz et al.[10]

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

where,

Kb = association constant for competitive drug
Ka = association constant for probe
(A) = concentration of free probe
(PA) = concentration of bound probe
n = number of binding sites
(Po) = total concentration of protein
(Bt) = total concention of competitive drug

RESULTS AND DISCUSSION

The fluorescence intensity of ANS in pH 7.4 phosphate buffer is not significant, but the fluorescence intensity of ANS in the presence of BSA is greatly enhanced.[7] The fluorometric titration curves of BSA are shown in Figure 2. The relative fluorescence intensities were obtained directly from fluorometer reading at uncorrected excitation wavelength 375 nm and emission wavelength 480 nm for ANS albumin complex.

To calculate the fraction of free and bound ANS concentration, binding data of ANS at a high BSA concentration which provides excess binding sites are required. The enhancement in fluorescence intensity was approximately linear at high concentration of BSA, 7.25×10^{-6}M, suggesting a proportional increase in

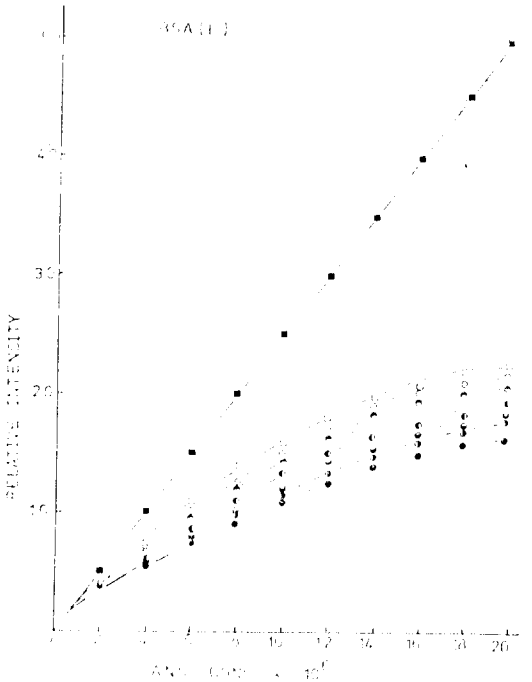

Fig. 2: Fluorescence titration curve of BSA with ANS at high (■) and low (□) albumin concentration. Curve ●, ◑, ◒, ◐, ◓, and ○ are the titration curves of low albumin concentration with ANS in the presence of 5×10^{-4}M cefotaxime, cefazoline, cefuroxime, cephalothin, cephaloridine and cephacetrile, respectively.

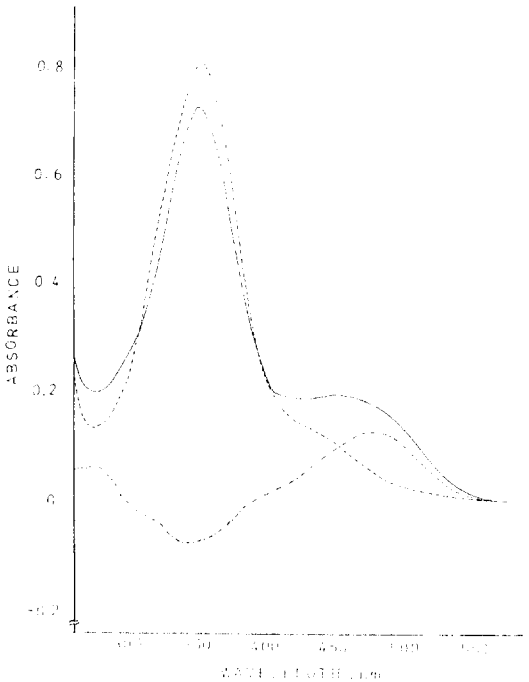

Fig. 3: Spectral change of HBAB bound to BSA.
----, $1\times10,^{-4}$M HBAB
——, 1×10^{-4}M HBAB and 2.89×10^{-5}M BSA
—·—, difference spectrum of HBAB and BSA

the bound form of ANS in the system. The curve of low concentration of BSA, 7.25×10^{-7}M, is shown that the ANS is only partially bound. The fluorescence intensities of ANS-BSA complex are reduced by the addition of cephalosporins. A decrease in fluorescence intensity of ANS-BSA in the presence of cephalosporins is the indication of the competition between ANS and drug for the same binding sites on BSA.

Figure 3 shows the UV spectra of HBAB in the absence and presence of BSA in pH 7.4 phosphate buffer. The difference spectra were characterized by two positive peaks at 484 nm and 262 nm, and one negative peak at 345 nm in the range of 250-600 nm. The difference spectra were measured at 484 nm for BSA-HBAB system.

The difference absorbance titration curves are shown in Figure 4. The difference absorbance is linearly increased with the addition of HBAB at high concentration of BSA, 1.5×10^{-4}M. This result indicates that all HBAB added is fully bound to BSA under this experimental condition. The curve of low concentration of BSA, 2.9×10^{-5}M, is shown that the binding sites of BSA are gradually saturated with HBAB. The difference absorbances of HBAB-BSA complex are

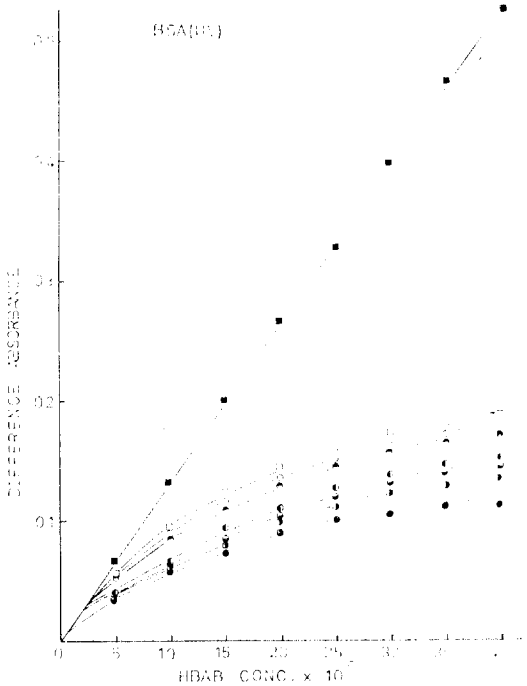

Fig. 4: Difference absorbance titration curve of BSA with HBAB at high (■) and low (□) albumin concentration. Curve ●, ◑, ◒, ◐, ◓, and ○ are the titration curves of low albumin concentration with HBAB in the presence of 1×10^{-3}M cefotaxime, cefazoline, cefuroxime, cephalothin, cephaloridine and cephacetrile, respectively.

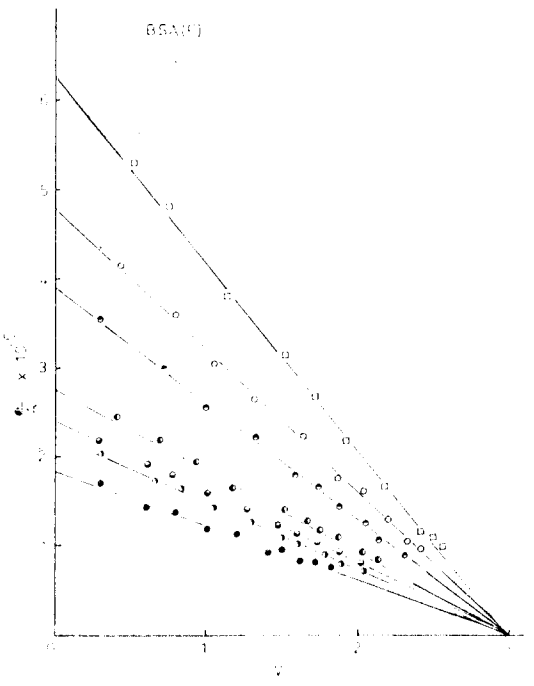

Fig. 5: Scatchard plots for the binding of ANS to BSA □, in the absence of drug ●, ◑, ◒, ◐, ◓, ○, in the presence of 5×10^{-4}M cefotaxime, cefazoline, cefuroxime, cephalothin cephaloridine and cephacetrile, respectively.

reduced by the addition of cephalosporins. A decrease in difference absorbance of HBAB-BSA complex in the presence of cephalosporins shows competition between HBAB and drug for the same binding sites on the BSA.

Figure 5 shows the Scatchard plots of the ANS-BSA complex in the absence and presence of fixed amounts of each of the six competitive cephalosporins. Figure 6 shows the Scatchard plots of the HBAB-BSA complex in the absence and presence of fixed amounts of each of the six competitive cephalosporins. If the competition is reversiblee, the total number of binding sites will remain constant and competition will result in a reduced affinity of the drug for BSA. If, however, competition is not reversible, the affinity of the drug will remain the same, but the total number of binding sites will be greatly reduced.[11] In Figure 5 and 6, the plots in the absence and presence of cephalosporins intercept at the same point on the abscissa, indicating the competition between the probes and cephalosporins at the same binding sites or adjacent hydrophobic sites.

Based on the plots, the BSA molecule appeared to have three primary binding sites for ANS and HBAB under the described experimental conditions. The binding constants were found to be $2.08\times10^{6}M^{-1}$ for ANS with BSA and $3.49\times10^{4}M^{-1}$ for HBAB with BSA, respe-

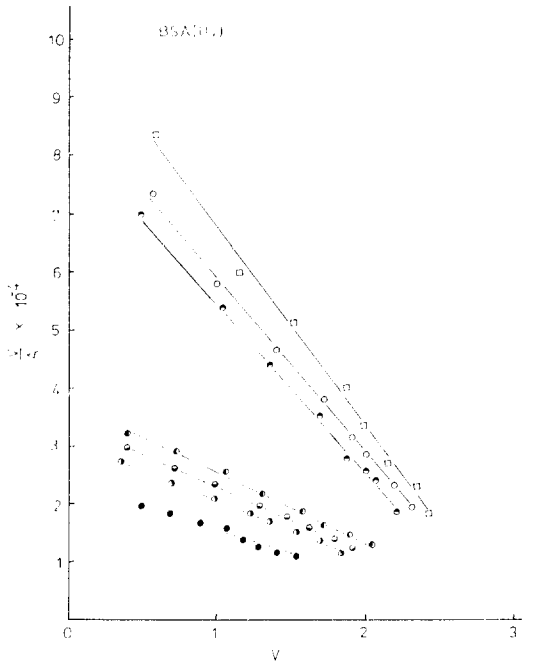

Fig. 6: Scatchard plots for the binding of HBAB to BSA □, in the absence of drug ●, ◑, ◒, ◐, ◓, ○, in the presence of 1×10^{-3}M cefotaxime, cefazoline, cefuroxime cephalothin, cephaloridine and cephacetrile, respectively.

ctively. The binding constants for cephalosporins with BSA by fluorescence probe technique and difference spectra were calculated according to the Klotz equation. The resulting parameters obtained are summerized in Table I.

As with the other properties of cephalosporins, serum albumin binding is dependent upon the interaction between the 7-acyl group and the 3-methyl substituent.[12] The degree of cephalosporins binding to serum albumin probably depends on the overall polarity of the molecule. The stability of a drug-protein complex is expressed by its binding constant, which is also important for the pharmacokinetic behavior of the drug.[13] Based on the average binding constants of cephalosporins, cefazoline and cefuroxime can be classified as highly bound, with

Table I: Binding parameters of cephalosporins to bovine serum albumin.

			n	Method
		8.98	3	F
			3	U.V.
			3	F
		2.70	3	U.V.
		4.03	3	F
		2.19	3	U.V.
		4.00	3	F
		2.01	3	U.V.
		5.30	3	F
		1.00	3	U.V.
		7.19	3	F
		0.73	3	U.V.

n: number of binding sites, K^{obs}; observed binding constant, F.: fluorescence probe technique, U.V.: UV difference spectra method.

affinities higher than $7\times10^{3}M^{-1}$, cephalothin, cephaloridine can be classified as moderately bound, with affinities between $7\times10^{3}M^{-3}$ and $4\times10^{3}M^{-1}$, cephacetrile is weakly bound, with affinities below $4\times10^{3}M^{-1}$.

ACKNOWLEDGEMENT

This work was partly supported by the research grant from the Korea Science & Engineering Foundation in 1980-81.

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