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Drug-Biomacromolecule Interaction VII

Binding of Six Cephalosporins to Human Serum Albumin Using Fluorescence Probe Technique and Difference Spectra

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Abstract □ Binding of six cephalosporins (cefotaxime, cefuroxime, cefazoline, cephalothin, cephaloridine, cephacetrile) to human 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 was used as the fluorescence probe, and 2-(4′-hydroxybenzeneazo) benzoic acid as the UV spectrophotometric probe. Competitive bindings between cephalosporins and probe were observed. For the binding of cephalosporins to human serum albumin, three binding sites were identified by fluorescence probe technique but four binding sites by UV spectrophotometry. The binding constants of cephalosporins to human serum albumin measured by fluorescence probe technique are higher than those measured by UV spectrophotometry.

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

Before testing a drug in man, animal pharmacokinetic data are usually required. The binding of drugs to plasma proteins may play an important role in the pharmacokinetic consequences and pharmacologic results of drugs.[1] Measurements of binding to human plasma and plasma of animals in which pharmacologic action and toxicty were studied should be an integral part of preclinical investigations. The most important contribution to drug-plasma protein binding is made by serum albumin which comprises about one half to total plasma protein.[2] Binding of drugs to serum albumin in various species is not predictable. Since the structure of the albumins (not only amino acid composition) are unknown, extrapolations from one species to another are not possible.

Marre et al tested for pharmacokinetic properties of eleven cephalosporins in rat.[3] Kim et al determined the binding affinity of six cephalosporins to bovine serum albumin.[4] This work is a quantitative study of the interaction of cefotaxime, cefuroxime, cefazoline, cephalothin, cephaloridine and cephacetrile with human serum albumin using spectroscopic methods.

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.. The fluorescence probe, 1-anilinonaphthalene-8- sulfonate (ANS), was purchased from Sigma Co.. The spectropothometric probe, 2-(4′-hydroxybenzeneazo) benzoic acid (HB-

AB), was purchased from ICN Pharmaceutical Inc.. Human serum albumin(HSA), Fraction V was purchased from Sigma Co.. The molar concentration of HSA was calculated on the basis of $E_{1\%}^{1cm}=6.67$. All other chemicals were of analytical reagent grade. The water used was double distilled from the glass.

Fluorescence Measurement

The fluorescence intensity of HSA-ANS complex as a function of ANS concentration was measured in the absence and in the presence of each cephalosporins. All measuring procedures were exactly identical with those described in the previous paper.[4] All fluorescence measurements were made with Baird-Automic Spectrophotometer Model FC100.

Difference Absorbance Measurement

The binding of spectrophotometric probe, HBAB, to HSA was determined by measuring the difference absorbance as a function of HBAB concentration according to the procedure described in the previous paper.[5] All UV difference absorbances were measured on the model SP1750 Pye Unicam Spectrophotometer.

Data Treatment

The fraction of probe bound was calculated using the method described in the previous reports.[4,5] The Scatchard equation was applied to determine the binding parameters of the HSA-probe interaction.

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

where V is the number of moles of bound probe per mole of HSA, A is the concentration of

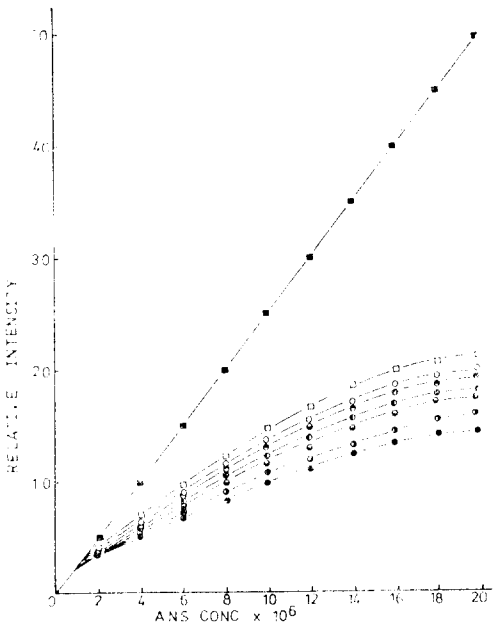


Fig. 1: Relative fluorescence intensity of HSA as a funtion of ANS concentration at high (■) albumin concentration, at low (□) albumin concentration alone, in the presence of 5×10^{-4} M cefotaxime (●), cefazoline (◑), cefuroxime (◒), cephalothin (◐), cephaloridine (◓), and cephacetrile (○).

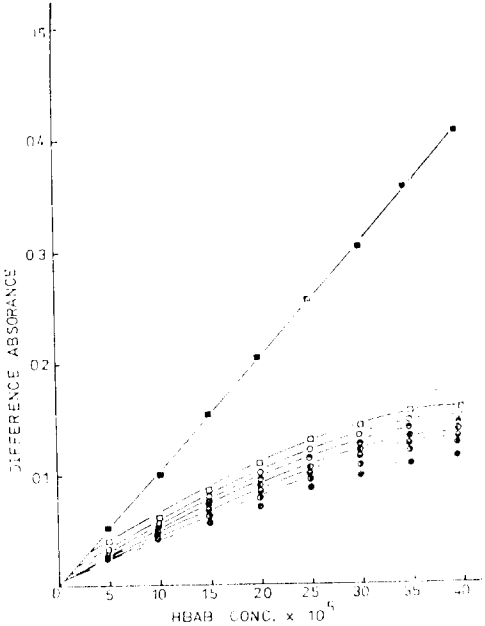


Fig. 2: Difference Absorbance of HSA with HBAB at high (■) albumin concentration, at low (□) albumin concentration alone, in the presence of 5×10^{-4}M cefotaxime (●), cefazoline (◑), cefuroxime (◒), cephalothin (◐), cephaloridine (◓) and cephacetrile (○).

free probe, n is the number of binding sites on the HSA molecule, and Ka is the binding constant of probe to HSA. The binding constants of cephalosporins were calculated by using the equation derived by Klotz et al.[6]

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 HSA is greatly enhanced.[7,8] The relative fluorescence intensities of ANS-HSA complex in Figure 1 were obtained directly from the fluorimeter reading at uncorrected excitation wavelength 375 nm and emission wavelength 480 nm. The fluorescence intensity linearly enhanced with the addition of ANS at high concentration of HSA, 7.25×10^{-6}M, suggesting a proportional increase in the bound form of ANS under this experimental conditions. ANS is only partially bound at low concentration of HSA, 7.25×10^{-7}M. The decrease in fluorescence intensity of ANS-HSA complex in the presence of cephalosporins is the indication of the competition between ANS and drug for the same binding sites on the HSA.

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 as the spectrum from the previous report.[5] The difference absorbances in Figure 2 were measured at 484 nm for HBAB-HSA complex. The difference absorbance linearly increased with the addition of HBAB at high concentration of HSA, 1.5×10^{-4}M. This result

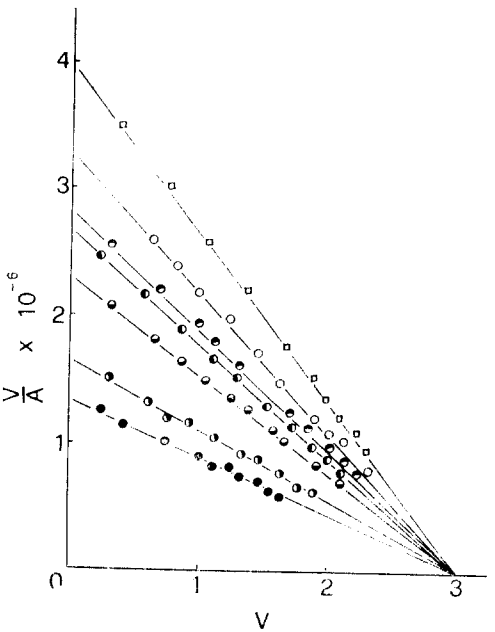


Fig. 3: Scatchard plots for the binding of ANS to HSA in the absence of drug (□), in the presence of 5×10^{-4}M cefotaxime (●), cefazoline (◑), cefuroxime (◒), cephalothin (◐), cephaloridine (◓) and cephacetrile (○), respectively.

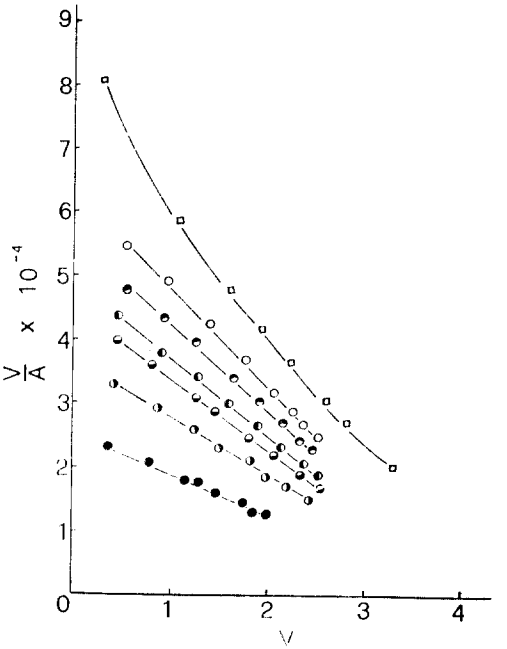


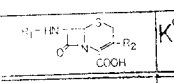
Fig. 4: Scatchard plots for the binding of HBAB to HSA in the absence of drug (□), in the presence of 1×10^{-3}M cefotaxime (●), cefazoline (◑), cefuroxime (◒), cephalothin (◐), cephaloridine (◓) and cephacetrile (○), respectively.

indicated the all HBAB added is fully bound to HSA under current experimental conditions. The curve of low concentration of HSA, 2.9×10^{-5}M, shows that the binding sites of HSA are gradually saturated with HBAB. The decrease in the difference absorbance of HBAB-HSA complex in the presence of cephalosporins shows competition between HBAB and drug for the same binding sites on the HSA.

Figure 3 shows the Scatchard plots of the ANS-HSA complex in the absence and presence of fixed amounts of each competitive cephalosporin using fluorescence probe technique. Figure 4 shows the Scatchard plots of the HBAB-HSA complex in the absence and presence of fixed amounts of each competitive cephalosporin using difference spectra. In both figures, the plots in the absence and in the presence of cephalosporins intercepted at the same point on the abscissa, indicating the competition between the probes and cephalosporins at the same binding sites or adjacent hydrophobic sites.

The Scatchard plots for the HBAB-HSA deviate a little from the linearity. Such deviation might be considered due to the heterogeneity of the albumin with respect to its binding properties. Secondary binding sites might be involved in drug-HSA binding at high concentration of HSA. Based on Figure 3 and Figure 4, four binding sites are identified by UV study but three binding sites by fluorescence probe technique. The binding constants obtained by using the UV difference spectra were lower than those obtained from fluorescence probe technique. It is not easy to explain this differences. However, the difference of binding properties measured by two different methods might be explained in several ways. Under the current experimental conditions, difference in number of binding sites might be due to the presence of nonfluorescent

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

Compounds		$K^{obs}\times10^{3}$ M^{-1}	n	Met.
Cefotaxime		6.60	3	F
		1.28	4	UV
Cefazoline		6.36	3	F
		1.21	4	UV
Cefuroxime		4.37	3	F
		1.15	4	UV
Cephalothin		3.30	3	F
		1.09	4	UV
Cephaloridine		2.86	3	F
		0.50	4	UV
Cephacetrile		2.61	3	F
		0.24	4	UV

binding site which was detected by UV measurement or sharing of one drug molecule by more than one HSA molecule in the binding at low concentration. Also, conformational difference of HSA resulting from the concentration difference between the two methods could be considered. Lower binding constant measured by difference spectrophotometry might be due to contribution of secondary binding site because of concentration difference and conformational change of HSA. The resulting binding parameters are summarized in Table I.

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