Arch. Pharm. Res. 4(2), 99~107 (1981)

Drug-Biomacromolecule Interaction I

Physico-Pharmaceutical Characteristics of Bovine Serum Albumin Binding of Ibuprofenlysine

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(Receive 30 November 1981)

Abstract To investigate the protein binding characteristics of ibuprofenlysine, the effects of drug conentration, pH, ionic strength and protein concentration on the binding of drug to protein were studied by fluorescence probe method. The conformational change of protein was investigated by circular dichroism(CD) measurement. As the concentration of drug increases, the association constant decreases. These may be due to complex formation of the probe and drug ,or the interaction of the protein-probe complex and drug. The association constant for ibuprofenlysine increased with increasing protein concentration. These findings suggest a sharing of one ibuprofenlysine molecule by more than one protein molecule in the binding. The binding between ibuprofenlysine and protein was dependent on pH and ionic strength. It seems that both hydrophobic binding and some electrostatic forces are involved in the binding of ibuprofenlysine to protein.

Key words drug interactions; ibuprofenlysine; bovine serum albumin; binding and displacement; ANS; drug concentration, protein concentration, pH, ionic strength dependency; fluorescence titration; conformational change; CD measurements

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

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Ibuprofen, [(RS)-2-(4-isobutylphenyl)-propionic acid], reported in 1967, is widely used in the treatment of rheumatoid arthritis and osteoarthroses[1]. Masumoto *et al*[2] indicated that ibuprofen was effective in inhibiting adjuvant-induced arthritis in rats. The inhibiting effect was 3 times that of phenylbutazone and 5-10 times that of aspirin.

(+)-Ibuprofen was highly active, whereas (−)-ibuprofen had very little activity, in inhibiting a bovine seminal vesicle microsomal preparation of prostaglandin synthetase, *in vitro*[4].

Mills *et al*[10] have reported that ibuprofen at a concentration of 20 μg/ml was 99% bound in whole human plasma. Studies in rats showed that ibuprofen can displace warfarin from serum protein binding sites and that this displacement causes an increase in the total body clearance of warfarin and in the anticoagulant effect produced by a given concentration of total(free and bound)drug[3].

Although ibuprofen is widely used as anti-inflammatory-antirheumatic agents, this drug has poor solubility in water. The solubility of ibuprofen is increased markedly by the addition of lysine to the carboxyl group of ibuprofen[5]. In order to investigate the nature

of the binding of ibuprofenlysine, the effects of drug concentration and bovine serum albumin concentration, pH and ionic strength on the binding of drug were studied by fluorescence, using 1-anilinonaphthalene-8-sulfonate as a probe.

Conformational change of bovine serum albumin under the experimental condition was measured by circular dichroism spectra.

EXPERIMENTAL

Materials

Bovine serum albumin(BSA), Fraction V (Sigma Co.), was used in this study, and its molecular weight was assumed to be 69000. The concentrations of albumin solutions were 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 fluorescent probe, 1-anilinonaphthalene-8-sulfonate (ANS), was purchased from Sigma Co. Ibuprofenlysine used was purchased from Il-Yang Pharm. Co. All other chemicals used were of analytical reagent grade. The water used was double distilled from glass.

Apparatus

All fluorescence measurements were made with Baird-Automic Spectrophotometer Model FC 100 equipped with 150 watts Xenon lamp and spectra were recorded on Bryans Model 2500 X-Y recorder. The entrance slit for the excitation light and the exit slit for the fluorescence emission were 32 nm, respectively.

Circular dichroism spectra were obtained with a JASCO Model J-20C spectropolarimeter.

Methods

1)Fluorescence Titration

The binding of the probe to BSA was determined by measuring the increase in fluorescence following the titration of the protein solution with the probe as described by Kim et al.[6]

The excitation and emission wavelengths were 375 nm and 470 nm, respectively.

BSA solutions were prepared in 0.05 M phosphate buffer. The probe, ANS, was dissolved in methanol at a concentration of 1×10^{-3} M. To minimize the photodecomposition of probes, samples were exposed to the light only for the short measurement period. Three milliliters of BSA solution of low concentration was titrated with successive addition of 1 μl of ANS solution. Titration was carried out manually at 23°C with microsyringe. Methanol did not affect the binding of the probe in the concentration range used.

A separate titration was performed for BSA at the same concentration but containing ibuprofenlysine. BSA solution of high concentration in which all the ANS was bound was also titrated. Blank titration in the buffer was made to correct the fluorescence of free probe in the absence of protein. The inner filter effect was not observed by keeping absorbance low at the excitation wavelength (less than 0.04).[7]

2) CD measurement

Conformational change of drug bound protein was detected by CD measurement as

follows.

Three milliliters of BSA solution(8.7×10^{-7} M) in 0.05 M phosphate buffer were titrated with 10 μl of 1×10^{-4} M of ibuprofenlysine.

All solutions were scanned from 250 to 200 nm in 10 mm cells. Spectropolarimeter was adjusted as follows: N_2 gas flow rate was 2-3 kg/cm^2 min. Chart speed was 2 cm/min. Time constant was 4. Wavelength expansion was 10 nm/cm^2. Scale was 20 mdeg/cm.

Data Treatment

Enhancement of the fluorescence of the probe upon addition to BSA at two concentrations and the subsequent decrease of fluorescence in the presence of the binding drug, ibuprofenlysine, were used to calculate the binding parameters for the probe and drug. The fraction of probe bound, X, was calculated using the following equation[8]:

$$X=\frac{F_p-F_0}{F_b-F_0} \qquad \cdots\cdots\cdots(1)$$

where F_p and F_0 and the fluorescence intensities of a given concentration of probe in solutions of low protein concentration and in solutions without protein, respectively; and F_b is the fluorescence intensity of the same concentration of probe fully bound to a high concentration of protein. Such treatment yields true values of X, provided the fluorescence intensity of the bound species, protein-probe, is a linear function of the concentration. After the value X is found for each point along the titration curve, the Scatchard equation may be applied to determine the binding parameters of the protein-probe interaction:

$$\frac{V}{A}=nKa-VKa \qquad (2)$$

where V is the number of moles of bound probe per mole of protein, A is the concentration of free probe, n is the number of binding sites on the protein molecule, and Ka is the association constant of the probe to the protein.[8]

The competitive binding of ibuprofenlysine was studied using ANS as the indicating probe. The binding of probe to BSA was determined in the presence and absence of competitor by varying the concentration of probe at constant BSA and ibuprofenlysine concentrations. The association constant of ibuprofenlysine was calculated by using the equation of Klotz et al.[9]:

$$K_b=\frac{n[P_o]K_a[A]-K_a[A][PA]-[PA]}{[B_t]K_a[A]-[P_o]K_a[A]+K_a[A][PA]+[PA]}\times\frac{Ka[A]}{[PA]} \qquad \cdots\cdots\cdots(3)$$

where

K_b; association constant for competitive drug

K_a; association constant for probe

[A]; concentration of free probe

[PA]; concentration of bound probe

n; number of binding sites

$[P_o]$; total concentration of protein

$[B_t]$; total concentration of competitive drug

The observed ellipticity (θ_{obs}) in degrees could be calculated from CD spectra. θ_{obs} was converted to mean residue ellipticity $[\theta]$ in degrees$\cdot cm^2\cdot dmole^{-1}$ by means of the following equation[11]:

$$[\theta]=\frac{\theta_{obs}\cdot M}{c\cdot l\cdot 10} \qquad \cdots\cdots\cdots(4)$$

where M is the mean residue weight of 114, c is the concentration of protein in g/ml and

l is the path-length in cm. The α-Helix contents were calculated by the method at Greenfield and Fasman[12].

$$\alpha\text{-Helix} = \frac{-[\theta]-4000}{33000-4000} \quad \cdots\cdots\cdots(5)$$

RESULTS AND DISCUSSION

Effect of Drug Concentration

Using ANS of which fluorescence intensity is thought to be induced by binding to the hydrophobic region of BSA, the contribution of the hydrophobic force for the binding of ibuprofenlysine to BSA was examined. The binding of ANS to BSA was studied in the presence of a constant total concentration of ibuprofenlysine where four levels of ibuprofenlysine were used.

Fig. 1 shows the fluorescence emission

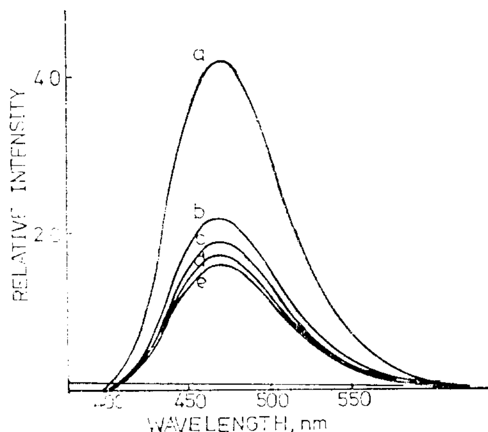

Figure 1: Fluorescence emission spectra for the probe-protein complex in the presence and absence of drug.
a, in the absence of drug
b, c, d, e, in the presence of 1×10^{-4}M, 2×10^{-4}M, 3×10^{-4}M, 4×10^{-4}M of Ibuprofenlysine, respectively.

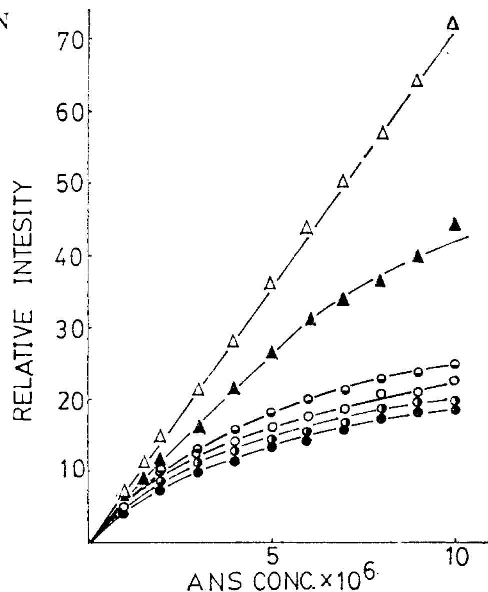

Figure 2: Fluorescence titration curves of BSA with the probe at high (△) and low (▲) protein concentration. Curve ◒,○,◑, and ●are the titration curves of low protein concentration with the probe in the presence of 1×10^{-4}M, 2×10^{-4}M, 3×10^{-4}M, and 4×10^{-4}M Ibuprofenlysine, respectively.

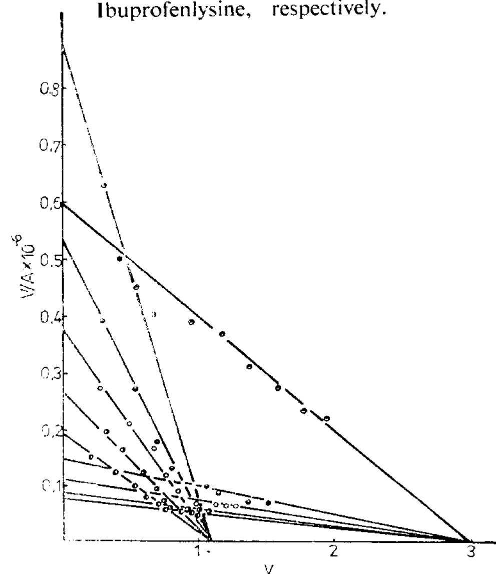

Figure 3: Scatchard plots of the binding of probe to BSA.
◓, in the absence of drug
◒,○,◑,◐, in the presence of 1×10^{-4}M, 2×10^{-4}M, 3×10^{-4}M, and 4×10^{-4}M of ibuprofenlysine, respectively.

Table I: Effect of concentration of Ibuprofenlysine on the binding of Ibuprofenlysine to BSA.

Concentration of drug (M)	Association constant (M^{-1})	
	K_{b_1}	K_{b_2}
1×10^{-4}	1.80×10^4	5.18×10^4
2×10^{-4}	1.54×10^4	4.00×10^4
3×10^{-4}	1.48×10^4	3.00×10^4
4×10^{-4}	1.45×10^4	2.47×10^4

spectra of ANS-BSA in the absence and presence of drug. The fluorescence intensity of the probe itself in pH 7.4 phosphate buffer was not significant, but when ANS was added to protein, the fluorescence intensity was greatly enhanced. A decrease in fluorescence of ANS-BSA complex in the presence of ibuprofenlysine is indication of the competition between ANS and drug for the same binding sites on the protein.

The fluorometric titration curves of ANS-BSA are shown in Fig. 2, respectively. As the concentration of ibuprofenlysine increases, fluorescence intensity decreases.

The results represented as Scatchard plots are given in Fig. 3. The data are consistent with competition between ibuprofenlysine and ANS for the same sites on the protein. Thus, the mechanism of inhibition of ANS-BSA complex formation by ibuprofenlysine may involve competition for the same binding sites. This also suggests that the polarity in the vicinity of the binding sites for ANS may be decreased in the presence of ibuprofenlysine. Under the experimental conditions, the number of binding sites for the probe appears to increase from one to three as the molar ratio of probe to protein increases. As the con centration of ibuprofenlysine increases, the displacement of ANS from its binding sites also increases.

The results obtained from Table I give same indication of a decrease in K_b with increasing drug concentrations. Variation of the binding constants by different concentrations may be due to complex formation of the probe and drug, or the interaction of the ANS-BSA complex and drug. the intermolecular forces between ANS and ibuprofenlysine involve electrostatic and localized charge transfer interactions[8].

Generally, in the short wavelength region, 200–250 nm, the CD changes are mainly due to the backbone structure that is α-helix, β-structure, and random coil of the polypeptide chain of the protein molecule[13]. The effects of ibuprofenlysine are seen in Fig. 4. The spectra were analyzed according to the

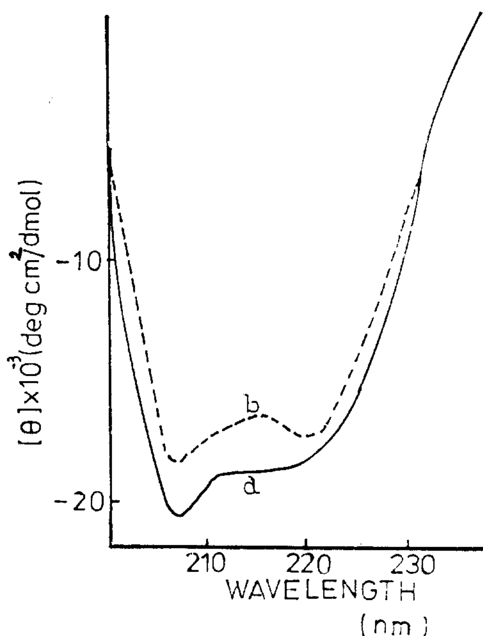

Figure 4: CD spectra of BSA in pH 7.4 phosphate buffer.
a, in the absence of ibuprofenlysine.
b, in the presence of ibuprofenlysine.

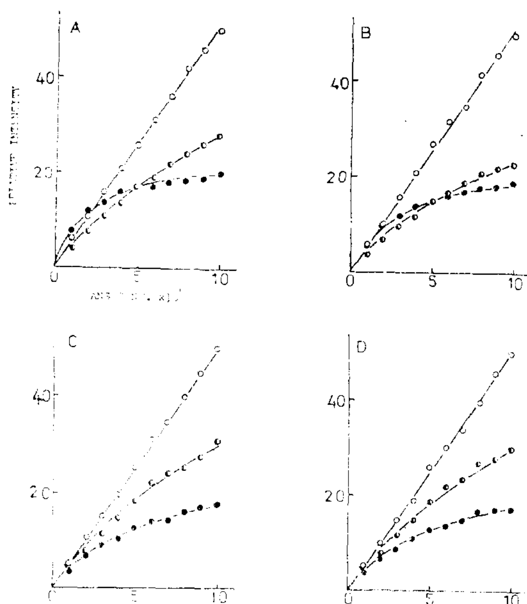

Figure 5: Fluorescent titration curves of BSA with ANS at pH 6.5 (A), 7.0 (B), 7.4 (C), and 8.0 (D).
○, at high protein concentration.
◐, at low protein concentration.
●, at low protein concentration in the presence of drug.

procedure of Greenfild and Fasman[12]. In the present experimental conditions of drug-BSA binding study, the α-helical content of BSA is reduced from 54.7% to 53%.

Effects of pH and Ionic Strength

Fig. 5 shows the fluorescent titration curves of BSA-ANS at various pH values and at 0.2 ionic stength. In acidic and neutral region, unexpected data were obtained. At lower molar ratios of ANS to protein, fluorescence intensity of BSA-ANS in the presence of drug was higher than that of BSA-ANS in the absence of drug. At pH 7.4, the primary and secondary association constant for ibuprofenlysine to BSA were $1.80\times10^4M^{-1}$ and $5.18\times10^4 M^{-1}$, and at pH 8.0 the primary and secondary binding constant $2.20\times10^4M^{-1}$ and $5.04\times10^4 M^{-1}$. The results indicate that binding affinity of ibuprofenlysine is nearly constant in alkaline region, but increases markedly in neutral and acidic region. However, the change of pH did not cause the change in the CD spectra of BSA-ibuprofenlysine under described experimental conditions. Thus, it is considered that this increase is mainly due to the increase of cationic charge of BSA in acidic region[14]. This suggests that, although hydrophobic binding has some role, the ionic interaction cannot be ignored. It is well known that acidic compounds are non-specifically bound to serum albumin, chiefly by the electrostatic forces between acidic groups of the compounds and cationic groups of albumin.

In order to study the effect of ionic strength, phosphate buffer solutions varying ionic strength were prepared by adding sodium chloride. The influence of the ionic strength of the medium at pH 7.4 on the binding of ibuprofenlysine to protein is shown in Fig. 6. It demonstrated that fluorescence intensity of BSA-ANS in the absence of ibuprofenlysine decreased with increasing ionic strength.

Table II shows that the ionic strength influences CD spectra of BSA. α-Helical content (%) of BSA was changed from 54.7 to 50.8 as ionic stength increased from 0.2 to 0.8. That of BSA in presence of ibuprofenlysine was reduced from 53 to 48.7. It is generally accepted that at low ionic strength most protein forms heavy complexes by aggregation and at high ionic strength

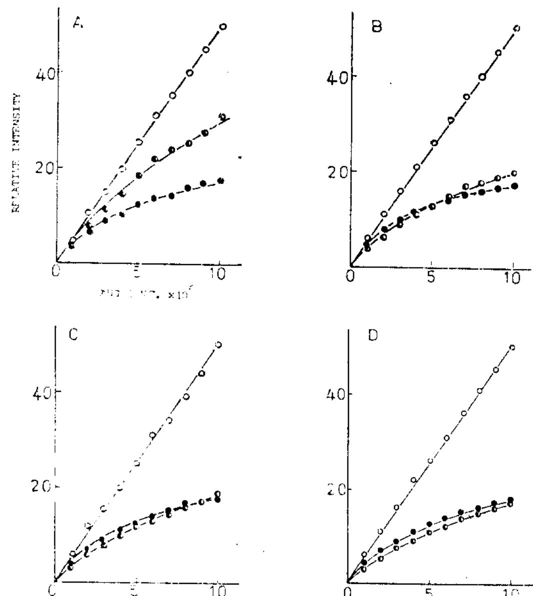

Figure 6: Fluorescent titration curves of BSA with ANS at ionic strength 0.2 (A), 0.4 (B), 0.6 (C), 0.8 (D).
○, at high protein concentration.
◐, at low protein concentration.
●, at low protein concentration in the presence of drug.

Table II: Percentage of α-helical content of BSA in absence and presence of ibuprofenlysine at various ionic strength.

Ionic strength	α-Helix content (%)	
	BSA	BSA containing Ibuprofenlysine
0.2	54.7	53
0.4	53	51
0.6	52.1	50.7
0.8	50.8	48.7

dissociates to subunits[16].

The effect of salt concentration on the interaction between ibuprofenlysine and BSA may be explained in terms of: (a) changes in ionic atmosphere of the associating protein molecule, (b) competitive binding by the chloride ions, (c) a salting out effect[17].

Taking the pH dependency, and the effect of ionic strength into consideration, it seems reasonable to say that drugs have mainly hydrophobic properties, but large hydroph-

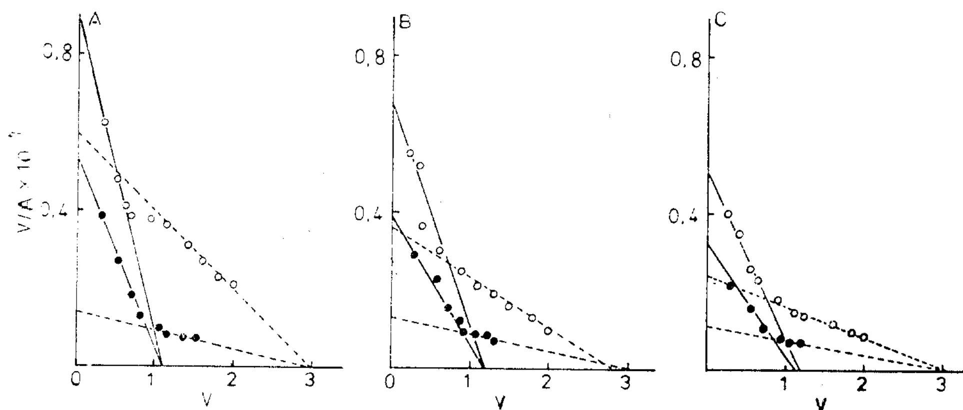

Figure 7: Effect of BSA concentration on the Scatchard plots for ANS-BSA in the presence (●) and absence (○) of drug.
A, at BSA concentration of 8.7×10^{-7}M.
B, at BSA concentration of 7.25×10^{-7}M.
C, at BSA concentration of 5.8×10^{-7}M.

Table III: Effect of BSA concentration on the association constant and n value of ibuprofenlysine binding to BSA.

Binding parameter	Concentration of BSA (M)		
	8.7×10^{-7}	7.25×10^{-7}	5.8×10^{-7}
n_1	1.1	1.2	1.1
n_2	3.0	3.0	3.1
K_{b_1} (M^{-1})	1.80×10^4	1.58×10^4	1.22×10^4
K_{b_2} (M^{-1})	5.80×10^4	3.36×10^4	0.88×10^4

obic areas are often combined with more hydrophilic groups, which can participate in hydrogen bonds, dipole-dipole interactions, and electrostatic interactions[18].

Effect of BSA Concentration

The Scatchard plots for binding of probe at a fixed concentration of 1×10^{-4}M of ibuprofenlysine to various dilutions of BSA are shown in Fig. 7. As BSA concentration decreases, association constant of ANS binding to BSA decreases. Primary constant decreases from $8.05\times10^6M^{-1}$ to $4.14\times10^6M^{-1}$ and secondary constant from 1.98×10^6 to $0.78\times10^6M^{-1}$.

As shown in Table III, association constant of ibuprofenlysine to BSA decreased dramatically with protein dilution while the number of binding sites were not greatly changed. It is difficult to explain the observed differences. However, the BSA concentration-dependent differences in association constant can be explained in two ways. There is evidence that phosphate buffer may compete with drug molecule for binding site[19]. Also it may suggest a sharing of one ibuprofenlysine molecule by more than one protein molecule in the binding[20]. This observation is further substantiated by Fig. 7. The ratio of bound drug to free drug concentrations at high protein concentration is larger than that at low protein concentration.

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