Effect of Albumin Conformation on the Binding of Ciprofloxacin to Human Serum Albumin: A Novel Approach Directly Assigning Binding Site

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Human serum albumin (HSA) is known to exist as N (pH ∼7), B (pH ∼9), and F (pH ∼3.5) isomeric forms and an equilibrium intermediate state (I) accumulate in the urea induced unfolding pathway of HSA around 4.8−5.2 M urea concentrations. These states displayed characteristic structure and functions. To elucidate the ciprofloxacin (CFX) binding behavior of HSA, the binding of ciprofloxacin with these conformational states of human serum albumin (HSA) has been investigated by fluorescence spectroscopy. The binding constant (K) for N, B, F, and I conformation of HSA were 6.92×10^5, 3.87×10^5, 4.06×10^5, and 2.7×10^5 M^{-1} and the number of binding sites (*n*) were 1.26,1.21, 1.16, and 1.19, respectively. The standard free energy changes ($\Delta G^0_{binding}$) of interaction were found to be −33.3 (N isomer), −31.8 (B isomer), −32 (F isomer), and −30.0 kJ mol^{-1} respectively. By using unfolding pathway of HSA, domain II of HSA has been assigned to possess binding site of ciprofloxacin. Plausible correlation between stability of CFX-N and CFX-B complexes and drug distribution have been discussed. At plasma concentration of HSA fraction of free CFX, which contributes potential to its rate of transport across cell membrane, was found to be ∼80% more for B isomers compared to N isomers of HSA. The conformational changes in two physiologically important isomers of HSA (N and B isomers) upon ciprofloxacin binding were evaluated by measuring far, near-UV CD, and fluorescence properties of the CFX-HSA complex.

1. Introduction

Serum albumin being the major ligand binding and transport protein of circulatory system is considered as a model for studying drug−protein interaction in in vitro.[1] It consists of three homologous domains. Each domain consists of two subdomains A and B.[2] The principal sites of ligand binding to HSA are located in hydrophobic cavities in subdomains IIA and IIIB.[2] HSA is known to undergo different pH dependent conformational transitios: the N−F transition between pH 5.0 and 3.5, the F−E transition between pH 3.5 and 1.2, and the N−B transition between pH 7.0 and 9.0.[3−5] The N−F isomerization involves unfolding and separation of domain III from rest of the molecule without significantly affecting rest of the molecule.[6−7] Moreover, urea induced unfolding of HSA showed a two-step three state transition with accumulation of an intermediate (I) around 4.8−5.2 M urea concentration.[8−9] The I state is characterized by unfolding of domain III together with partial but significant loss of native conformation of domain I. The domain II of HSA remains unaffected in intermediate state. That the N−B transition has physiological significance is suggested by the fact that, under increased Ca^{2+} ions concentration in blood plasma, the B isomer predominates.[10] Moreover, it is believed that the transport function of albumin is controlled through this transition or akin to it.[11−14]

In view of this, we planed to carry out the detailed investigations on the interaction of ciprofloxacin with N, B, F, and I states of HSA using fluorescence and circular dichroism techniques. Ciprofloxacin is a flouroquinolone (Figure 1) with

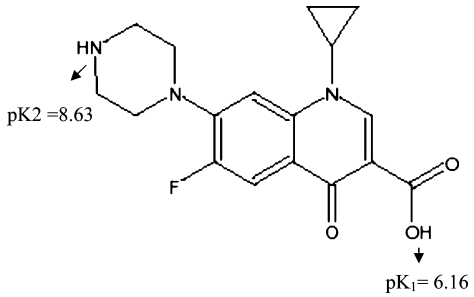

Figure 1. Chemical structure of ciprofloxacin.

a broad-spectrum activity against both gram-negative and gram-positive organisms.[15] The ciprofloxacin is used world over for the treatment of urinary tract infections, lower respiratory tract infections, nosocomial pneumonia, skin and skin structure infections, intra abdominal infections, bone and joint infections, and chronic bacterial prostitites.[16] Although blood pH is generally stable, there is a pH difference among blood, cerebral blood flow, and intracellular and extracellular environments where the ligand−HSA interaction occurred. The pH variation may affect drug−HSA interaction.[17−18] The binding data of physiological isomers of HSA (N and B) will prove helpful in providing basic information on the pharmacological actions, biotransformation, biodistribution, etc. of drugs, because it has been shown that the distribution and free concentration of various drugs can be significantly altered as a result of their binding to HSA. Moreover, binding phenomena of N, B, F, and I conformations will help to locate the ciprofloxacin binding site on the HSA domains as these isomers possess different domain status. Thus, this is the first study of its kind, using the unfolding pathway of HSA to locate the binding site of a drug.

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2. Experimental Section

2.1. Materials. Human serum albumin (essentially free of fatty acid and globulin) and urea were from Sigma (lot no. 90K7604). Ciprofloxacin was a product Ranbaxy Laboratories Ltd., India. All of the other reagents were of analytical grade. HSA was freed from dimers and higher-mers by passing through Sephacryl-S100 (HR) (76 × 1.8 cm) gel filtration column.

2.2. Preparation of Different Isomers of HSA. The N, B, F, and I conformational states of human serum albumin were produced by mixing 20 μL of HSA monomer stock solution (250 μM) with 980 μL of pH 7.0 (60 mM sodium phosphate), pH 9.0 (10 mM glycine·NaOH), and pH 3.5 (10 mM acetic acid/sodium acetate) buffers and desired volume of stock urea solution (10 M). The protein concentration was detemined spectrophotometrically using $E^{1\%}_{1cm}$ of 5.3[19] at 279 nm on a Hitachi spectrophotometer, model U-1500.

2.3. Circular Dichroism (CD) Measurements. CD measurements were carried out with a Jasco spectropolarimeter, model J-720 equipped with a microcomputer. The instrument was calibrated with D-10-camphorsulfonic acid. All of the CD measurements were made at 25 °C with a thermostatically controlled cell holder attached to Neslab's RTE − 110 water bath with an accuracy of ±0.1 °C. Spectra were collected with scan speed of 20 nm/min and response time of 1 s. Each spectrum was the average of four scans. Far and near-UV CD spectra were taken at protein concentrations of 5 and 20 μM with 0.1 and 1.0 cm path length cells, respectively. The results were expressed as MRE (Mean Residue Ellipticity) in deg·cm^2·dmol^{-1} which is defined as

$$\mathrm{MRE} = \theta_{obs}/(10nlC_p) \quad (1)$$

where θ_{obs} is the CD in milli-degree, n is the number of amino acid residues (585), l is the path length of the cell, and C_p is mole fraction. Helical content was calculated from the MRE values at 222 nm using the following equation as described by Chen et al.[20]

$$\%\ \alpha\text{-helix} = (\mathrm{MRE}_{222nm} - 2340/30300) \times 100 \quad (2)$$

2.4. Fluorescence Measurements. Fluorescence measurements were performed on a Shimadzu spectrofluorimeter, model RF-540, equipped with a data recorder DR-3. The fluorescence spectra were measured at 25 ± 0.1 °C with a 1 cm path length cell. The excitation and emission slits were set at 5 and 10 nm, respectively. Intrinsic fluorescence was measured by exciting the protein solution at 280 or 295 nm and emission spectra was recorded in the range of 300−500 nm.

2.5. Ciprofloxacin Binding. Ciprofloxacin solution was prepared by dissolving 5 mg of solid ciprofloxacin in distilled water and immediately diluting it to the desired volume of respective buffers of different pH. Binding of ciprofloxacin to different conformational forms of HSA was studied by fluorescence quench titration method using intrinsic fluorescence of HSA as a probe. To a fixed volume (3.0 mL) of protein solution was added an increasing volume (1−10 μL) of ciprofloxacin. Fluorescence was measured after 30 min at 340 nm after exciting the sample at 280 nm.

2.6. Denaturation Experiments. Stability studies of different isomers of HSA upon ciprofloxacin binding were carried out by urea-induced unfolding of HSA in the absence and presence of ciprofloxacin (ciprofloxacin/HSA= 2/1 molar ratio). Stock protein solutions (for N, B, and F isomers) were prepared by exhaustive dialysis of HSA monomer against respective buffers. To a 0.5 mL stock protein solution (N, B, and F isomer) were first added different volumes of the desired buffer, followed by the addition of stock urea solutions prepared in their respective buffer (10 M) to get a desired concentration of denaturant. The final solution mixture (3.0 mL) was incubated for 10−12 h at room temperature before optical measurements.

3. Results and Discussion

3.1. Characterization of Different Conformational States of HSA. To confirm whether the HSA conformation at pH 7.0. 9.0, and 3.5 and in the presence of 5.0 M urea (pH 7.0) represented the N, B, and F isomers and the I state, respectively, as reported previously,[3,9] we compared the far-UV, near-UV CD spectra, and various fluorescence properties of the different conformational states of HSA. Figure 2a shows the far-UV CD spectra of various states of HSA. The spectrum of HSA at pH 7.0 has two minima, one at 208 and the other at 222 nm, characteristic of α-helical structure. The N, B, F, and I conformational states of HSA contained around 58.3±0.8%, 53.9±0.3%, 46.3±0.7%, and 42±1.0% α- helical structure (Table 1) as determined by the method of Chen et al.,[20] which is in agreement with the values reported by other investigators.[3,8,9,21] Figure 2b shows the near-UV CD spectra in the 250−300 nm ranges for above transitions of HSA. The CD spectra in the near UV range provide information on fluctuation of the tertiary structure of the protein. The near UV CD spectrum for native state (pH 7.0) showed two minima at 262 and 268 nm and shoulders at 275 nm and 290 nm, characteristic of disulfide and aromatic chromophore.[22] By increasing the pH of the HSA

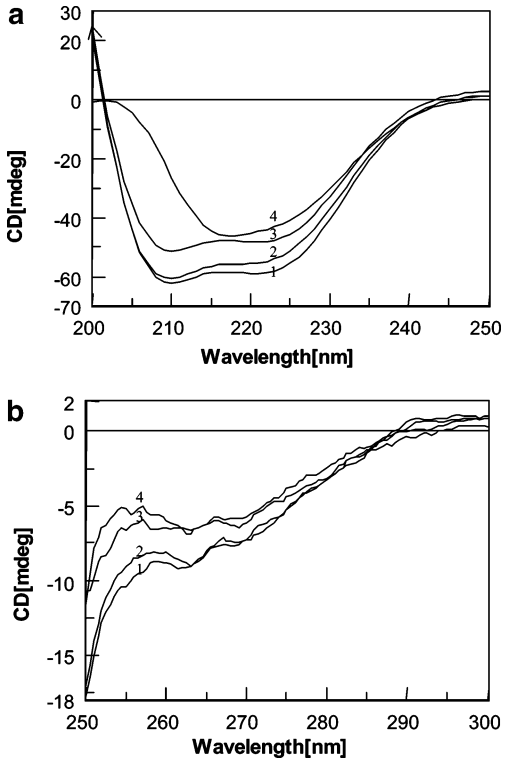

Figure 2. Far-UV CD (a) and near-UV CD spectra (b) of N (pH 7.0; curve 1), B (pH 9.0; curve 2), F (pH 3.5; curve 3), and I (5.0 M urea; curve 4) conformations of HSA.

Table 1. Summary of Structural Properties of Different States of HSA

variable	N isomer (pH 7)	B isomer (pH 9)	F isomer (pH 3.5)	I state (5.0 M urea)
MRE at 222 nm[a]	−20000	−18700	−16376	−15077
MRE at 262 nm	−166	−166	−155	−148
% α-helix[b] (ext. 295 nm.)	58.3	53.9	46.3	42.0
λ_{max}	344	340	334	344
FI at 344 nm (exct. 280 nm)	100	94.6	107.5	98.8
λ_{max}	340	335	334	340
FI at 340 nm	100	94.7	113.4	65

[a] MRE deg·cm^2·dmol^{-1}. [b] The mean value of 3 individual experiments with standard deviation ± 0.3 to ±1.0%.

solution from 7.0 to 9.0, we observed a small increase in the MRE at 268 and 262 nm (Table 1) and a small reduction in the CD signal in the region between 300 and 280 nm, denoting perturbations around disulfide and tryptophan residue.[23] Since during the N−B transition domain I undergoes tertiary structure isomerization, the observed changes in the tertiary structure might be ascribed to alteration of native conformation of domain I. At pH 3.5, there is an increase in the MRE between 280 and 250 nm and a decrease between 300 and 280 nm, denoting loss of tertiary structure, in agreement with the changes in the secondary structure and tryptophanyl fluorescence (Table 1). In view of previous results showing unfolding of domain III during N−F transition, the loss of the secondary and tertiary structures may be attributed to unfolding of domain III. In the presence of 5.0 M urea, the near-UV CD spectra of HSA also shows significant alteration in the tertiary structure. These changes in the tertiary structure may be due to the unfolding of domain III and the partial loss of the native conformation of domain I as described previously.[9]

As shown in Table 1, increasing the pH of HSA solution from 7.0 to 9.0 caused a blue shift of 4 nm and a slight decrease of FI at 340 nm, indicating internalization of tryptophan in the nonpolar environment. The slight quenching of tryptophanyl fluorescence might be due to deprotonation of lys-199 and -195 which occurs at distances of 3.7 and 7.4 Å to trp-214 in the crystal structure of HSA.[2] The wavelength maximum of fluorescence of HSA shifted from 340 nm at pH 7.0 to 334 nm at pH 3.5, which was reported previously for the N−F transition of HSA.[3] Since HSA contains only one tryptophan in domain II, the absence of any significant change in tryptophanyl fluorescence of HSA in 5.0 M urea indicated that unfolding of domain II did not occur in the N−I transition. This has also been reported earlier suggesting noninvolvement of domain II in the N−I transition by the other probe.[24] The loss of secondary and tertiary structures may be attributed to the unfolding of domains III and/or I. In conclusion, we were able to demonstrate that HSA at pH 7.0, 9.0, 3.5 and in the presence of 5.0 M urea represents the N, B, F, I conformational states, respectively. These states were further used for the ciprofloxacin binding study.

3.2. Ciprofloxacin-Induced Quenching Mechanism of HSA Fluorescence. Fluorescence quenching of protein could be used to derive much drug protein binding information.[25−28] The quenching of fluorescence is known to occur mainly by a collisional process (dynamic quenching) and/or formation of a complex between quencher and fluorophore (static quenching). Here we are interested in knowing whether CFX forms a complex with HSA and upon which the quenching mechanism acts. Ciprofloxacin-induced fluorescence quenching of HSA isomers was monitored at 340 nm after exciting the protein sample at 280 nm. The fluorescence quenching data of different forms of HSA (N, B, and F isomers and intermediate state) induced by ciprofloxacin were presented as a Stern−Volmer plot as F_0/F versus ciprofloxacin concentrations (Figure 3). It is apparent from the figure that equilibration of all preparation of HSA with ciprofloxacin caused concentration dependent quenching of HSA fluorescence which is suggestive of CFX binding to HSA isomers. The fluorescence quenching data were analyzed according to the Stern−Volmer equation[29]

$$F_0/F = 1 + k_{SV}[Q] \qquad (3)$$

In eq 3, F_0 and F are the fluorescence intensity in the absence and presence of the quencher, Q is the quencher concentration, and k_{SV} is the Stern−Volmer quenching constant. k_{SV} was

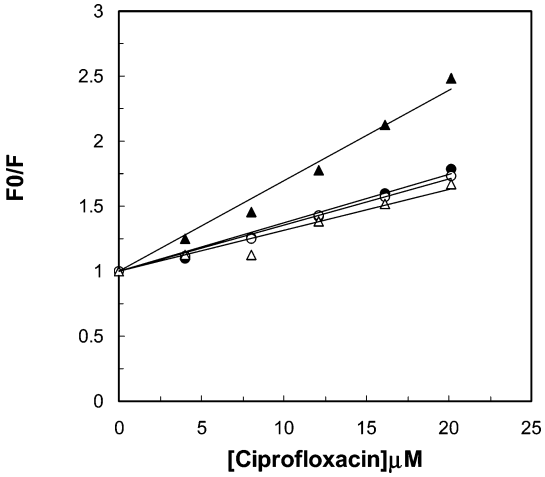

Figure 3. Stern−Volmer plots of ciprofloxacin quenching for N (●), B (○), F (▲), and I (△) conformations of HSA. Each data point was the mean of 4 independent observations ±S. D. ranging 0.06−0.11%.

Table 2. Quenching Constants and Ciprofloxacin Binding Parameters to Different Conformational States of HSA

HSA states	K_{sv}[a] ($\times 10^4$ L·M^{-1})	K ($\times 10^5$ M^{-1})	n	$\Delta G^0_{binding}$ (kJ M^{-1})
native	3.71	6.92	1.26	−33.3
basic	3.54	3.87	1.21	−31.8
fast moving	6.95	4.06	1.16	−32
urea induced intermediate	3.15	2.70	1.19	−30.9

[a] The mean value of 4 individual experiments with standard deviation ± 0.06 to ±0.11%.

obtained from the slope of the plot F_0/F versus [Q] (Figure 3), which followed the straight-line equation $Y = mx + c$. The k_{SV} for ciprofloxacin and various preparations of HSA were found to be of the order of 10^4 L mol^{-1} (Table 2).

3.3. Binding Constant (*K*), Binding Capacity (*n*), and Binding Energy ($\Delta G^0_{binding}$). Ciprofloxaci-induced fluorescence quenching data of different forms of HSA were analyzed to obtained various binding parameters. The binding constant (K) and binding affinity were calculated using equation[30]

$$\log[(F_0 - F)/F] = \log K + n \log[Q] \qquad (4)$$

where F_0 and F are the fluorescence intensity without and with the drug, respectively.

A plot of $\log[(F_0 - F)/F]$ versus log[Q] gave a straight line (Figure 4) using least-squares analysis whose slope was equal to n (binding affinity) and the intercept on Y axis to log K (K= binding constant). The binding constant (K) thus obtained was used to calculate the standard free energy change ΔG^0 of the drug binding to HSA from the relationship[31]

$$\Delta G^0_{binding} = -2.303RT \log K \qquad (5)$$

The values of binding constant K and binding capacity n thus determined were listed in Table 2. The value of the CFX−HSA complex (N isomer) binding constant K (6.92×10^5 M^{-1}) and binding capacity n (1.26) were similar to the binding constant reported for the interaction of gatifloxacin a methoxy fluoroquinolone derivative of ciprofloxacin with bovine serum albumin.[32]

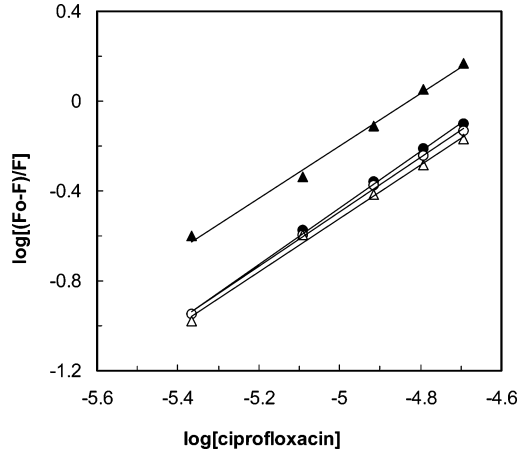

Figure 4. log($F_0/F - 1$) versus log[Q] plots N (●), B (○), F (▲), and I (△) conformations of HSA for binding constant and binding sites.

3.4. pH Dependence of *K* Ascribed to Conformational Changes of HSA. Whether the origin of the pH dependence of binding constant *K* resides in the ionic state of CFX or the protein molecule remains an interesting point. The extent of ionization of ionizing groups (−COOH, pK_1 = 6.16 and −NH, pK_2 = 8.63) of CFX (Figure 1) were determined according to Henderson−Hasselbatch equation

$$\mathrm{pH} = \mathrm{p}K + \log(\alpha/1 - \alpha)$$

where α is the extent of protonation. Using this equation, the following equations were derived which describe the extent of ionization of ionizing groups of CFX at pH 7, 9, and 3.5.

pH 7.0	pH = p*K*1 +0.84	∼90% $-COO^-$
	pH = p*K*2−1.6	∼100% $-NH_2^+$
pH 9.0	pH = p*K*1 +2.68	100% COO^-
	pH = p*K*2 +0.35	25% NH_2^+
pH 3.5	pH = p*K*1−2.68	100% COOH
	pH = p*K*1−5.13	∼100% $-NH_2^+$

Based on these, the ionic state equations of CFX at different pH values were determined, and it can be noted that CFX has almost equal (slightly positive) positive and negative charge at pH 7.0, negatively charged at pH 9.0, and positively charge at pH 3.5. The decrease of *K* in the both high and low pH range (Table 2) indicated that the origin of the pH dependence of *K* resided mainly in the protein molecule. This is obvious because of N−B and N−F conformational changes of HSA at pH 9 and 3.5, respectively.

3.5. Effect of N−B Transition on Ciprofloxacin Binding: Correlation with Drug Distribution. The results presented in Table 2 indicated a 44% decrease in the association constant *K* when HSA underwent N-to-B conformational changes. On the other hand number of binding sites *n* remained unaffected. These indicated decreased stability of B−CFX complex ($K = 3.87 \times 10^5\ M^{-1}$) compared to N−CFX complex ($K = 6.92 \times 10^5\ M^{-1}$) on the single class of CFX binding site. Since under increased Ca^{2+} concentration in the blood plasma, the B isomer predominates, it is suggested that N−B conformational changes have physiological significance.[2,10] The decrease in the binding constant of the B isomer may modify its distribution in the body and therefore may affect both the dose response relationship and the rate of drug elimination.[5,33−35] Because both the dose response relationship and the rate of drug elimination depend on the fraction of free drug (α_F) in the body, the fraction of total drug present in free form depend on *K*, for a given drug concentration and single class of binding sites according to the following equation:[36]

$$\alpha_F = (K_{dis} + [D_f])/([P_t] + K_{dis} + [D_f]) \quad (6)$$

The differences in drug distribution arising from differences in *K* may becomes significant at low concentration of drug, i.e., when $[D_f] \rightarrow 0$, α becomes proportional to K_{dis}

$$\alpha_F = K_{dis}/([P_t] + K_{dis}) \quad (7)$$

At a plasma concentration of HSA (6.7×10^{-4} M), the fraction of free CFX was calculated according to eq 7 and was found to be ∼80% more for the B isomer compared to the N isomer of HSA.

3.6. Ciprofloxacin Binding Site Located on Domain II of HSA. The acid- and urea-induced unfolding pathway of HSA has been studied by a number of workers.[6−9,37−38] The consensus exists today that between pH 7.0 and 3.5 and 4.8−5.2 M urea, human serum albumin undergoes N−F and N−I transitions, respectively. The F isomer, which predominates at pH 3.5, is characterized by unfolding and separation of domain III, and the I state is characterize by unfolding of domain III and partial but significant loss of the native conformation of domain I. Domain II is known to be unaffected by either N−F or N−I transitions. No effect in the number of binding sites (1.2) of these conformational states compared to native (1.2) (Table 3) indicated that the binding site for CFX may be located in domain II. The decreased binding constant (Table 3) can be understood to be the result of the loss of complex inter- and intradomain interactions that stabilize the albumin structure. A domain in the presence of other unfolded domains cannot be expected to fully reflect its behavior compared to native protein as they are connected through various interdomain forces such as salt bridges, hydrophobic interactions, hydrogen bonding, and natural boundaries between domains.[2]

3.7. Effect of CFX Binding on the Conformation of HSA. *Far-UV CD.* The changes in the secondary structures of N and B isomers of HSA were monitored by far-UV CD in the range 250−200 nm. Figure 5, panels a and b, shows the far-UV CD spectra of N and B isomers of HSA in the absence and presence of different drug-to-protein molar ratios. The increases in the magnitude of MRE at 222 and 208 nm were indicative of stabilization of helical structures of both N and B isomers when the CFX/HSA molar ratio is more than 1, and this stabilizing effect of CFX was slightly more (6.8%) in the case of the B isomeric form than for the N isomer (2.9%) of HSA. HSA at pH 7.0 did not show any significant change in the helical structure at a drug/HSA molar 1:1 ratio, whereas at pH 9.0, the B isomer showed a significant change at 208 nm indicating alteration of helical structure of the protein.

Near UV CD. Figure 6, panels a and b, shows the near-UV CD spectra of N and B isomers of HSA in the presence of different drug-to-protein molar ratios. The spectra for N and B isomers were also included for comparison. As shown in the figures, addition of the drug has changed the shape of the spectra of both isomers indicating alterations in the tertiary structure of HSA upon binding to the drug. The ellipticity 268/262 nm ratios of the N isomer in the absence and presence of CFX were found to be 0.88 and 1.07, respectively. The increase in the ellipticity 268/262 nm ratio is due to the appearance of a more prominent minimum at 268 nm and a flatting of the minimum at 262 nm upon CFX binding (Figure 6a). In the case of the B isomer, loss of the minimum at 262 nm was also accompanied

Table 3. Effects of Ciprofloxacin Binding on Conformation of N and B Isomers of HSA

						fluorescence properties Λ emission maxima	
HSA isomers	CFX/HSA molar ratio	MRE_{222nm}[a]	% helices[b]	minimum 262 nm	minimum 268 nm	ext.280 nm	ext. 295 nm
N isomer (pH7.0)	0:1	−20000	58.3	present	present	340	344
	1:1	−19794.8	57.6			342	344
	2:1	−20581.2	60.2	absent, signal decreased		350	350
	3:1	−20615.3	60.3			350	350
B isomer (pH 9.0)	0:1	−18700	53.9	present	present	335	340
	1:1	−18376	52.9			343	344
	2:1	−19982.9	58.2	absent signal increased		347	348
	3:1	−20478.6	59.8			348	348

[a] MRE deg·cm^2·dmol^{-1}. [b] Average values for three independent observations and the SD was 0.3−0.7%.

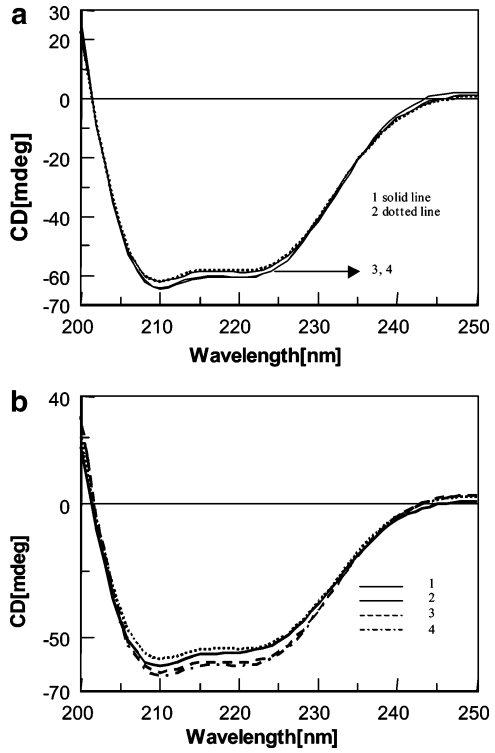

Figure 5. Far-UV CD spectra of N isomer (a) and B isomer (b) of HSA in the absence (curve 1) and presence of CFX; CFX/HSA molar ratio 1:1 (curve 2), 2:1 (curve 3), and 3:1 (curve 4).

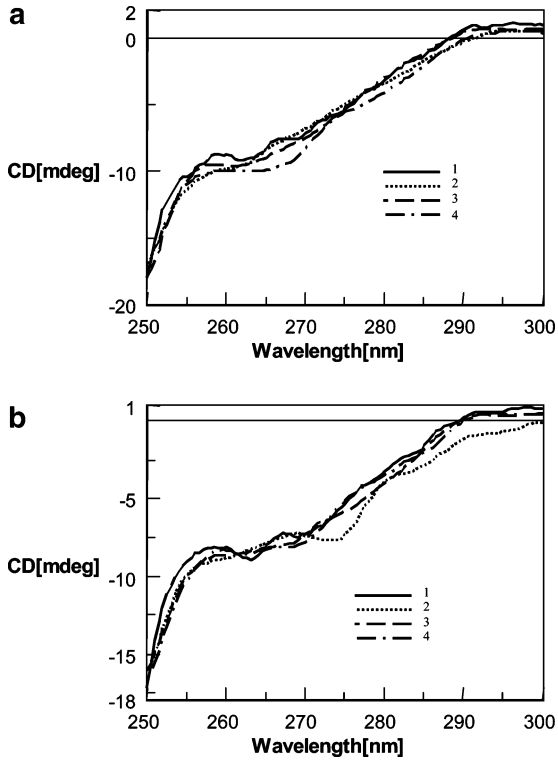

Figure 6. Near-UV CD spectra of N isomer (a) and B (b) isomer of HSA in the absence (curve 1) and presence of CFX; CFX/HSA molar ratio 1:1 (curve 2), 2:1 (curve 3), and 3:1 (curve 4).

by a small loss of the CD signal around this wavelength, which might indicate perturbation around disulfide bridges.[23,39]

Fluorescence Measurements. Figure 7, panels a and b, shows the fluorescence spectra of CFX and the N isomer of HSA in the absence and presence of CFX/HSA molar ratios of 2:1 and 0:1 in the 300−500 nm range after exciting the protein at 280 and 295 nm, respectively. HSA and CFX showed strong maximum fluorescence at 340 and 420 nm, respectively, when the excitation wavelength was 280 nm. When the excitation wavelength was increased to 295 nm where only tryptophan has fluorescence, the HSA fluorescence maximum was red shifted by 5 nm. This indicated the partially buried nature of lone tryptophan of HSA as reported previously.[2] The fluorescence spectra of the HSA−CFX complex have distinct quenching with red shifts of 10 and 5 nm when protein was excited at 280 and 295 nm. This indicated that conformational changes of HSA after binding with CFX have brought lone tryptophan to a more hydrophilic environment. The obvious changes; a blue shift and small quenching in the fluorescence spectra of CFX and HSA mixture further provided evidence for complex formation. But the increased fluorescence spectra of CFX and HSA mixture in the region of CFX fluorescence (∼420 nm) was due to cumulative effect of fluorescence of HSA and CFX at 420 nm after excitation at 295 nm. This suggested that CFX does not directly complex with tryptophan. Similar effects were observed also on the B isomeric form of HSA (Figure 8, panels a and b). As shown in Table 3, it appears that binding of ciprofloxacin has a more pronouced effect on the conformation of B isomer compared to N isomer of human serum albumin.

3.8. Structural Stability. The effect of ciprofloxacin binding on the stability of different isomers of HSA (N, B, and F isomers) was investigated by urea-induced unfolding of the proteins as monitored by measurements of fluorescence intensity at 340 nm after exciting the protein at 280 nm. Figure 9a−c, shows the normalized transition curves of different isomers of HSA both in the absence and presence of ciprofloxacin. In the absence of the drug, urea-induced unfolding of N and B isomers of HSA followed a two-step three state transition with accumulation of equilibrium intermediate states (I state) around 4.8−5.2 M and 3.0−3.5 M urea concentrations, respectively.

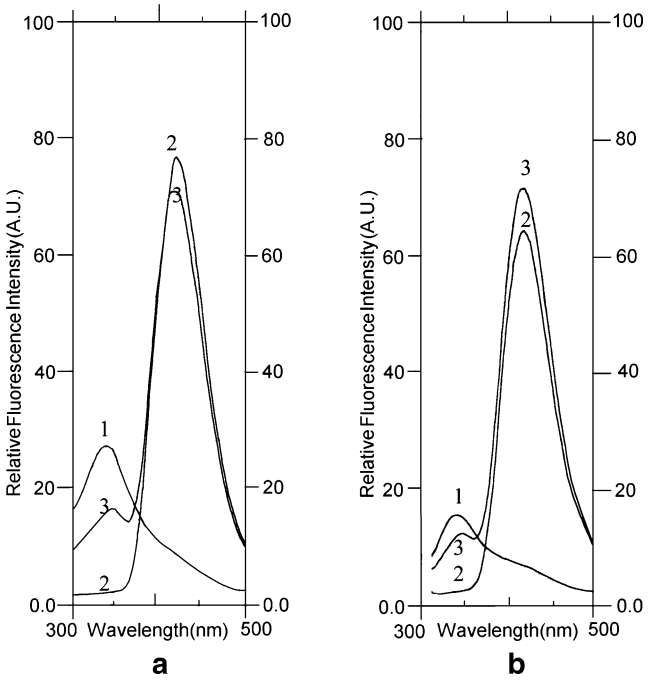

Figure 7. Fluorescence spectra of N form of HSA (curve 1), CFX (curve 2), and HSA−CFX complex at CFX/HSA molar ratio 2:1 (curve 3) monitored by exciting the protein at 280 nm (a) and 295 nm (b).

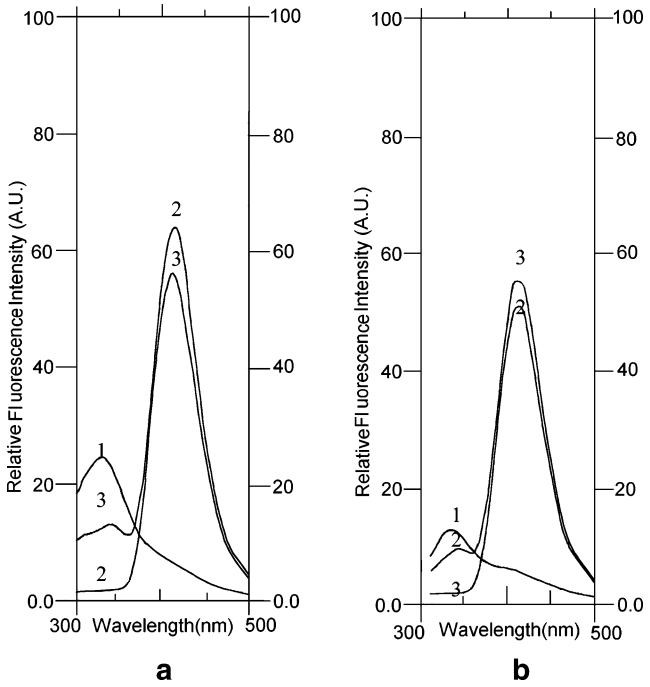

Figure 8. Fluorescence spectra of B form of HSA (curve 1), CFX (curve 2), and HSA−CFX complex at CFX/HSA molar ratio 2:1 (curve 3) monitored by exciting the protein at 280 nm (a) and 295 nm (b).

On the other hand, urea-induced unfolding of the F isomer of HSA in the absence of the drug did not show accumulation of intermediate states. These results are in agreement with our earlier observation made HSA.[9,40] N−I, B−I, and N−F transitions have been characterized by unfolding of domains III and I,[3,9,40] whereas I−U and F−U transitions have been characterized mainly due to the unfolding of domain II.[9,40] When the urea denaturation of different isomers of HSA were studied in the presence of ciprofloxacin (CFX/HSA = 2/1 molar ratio), the second transition which corresponded to the transformation of

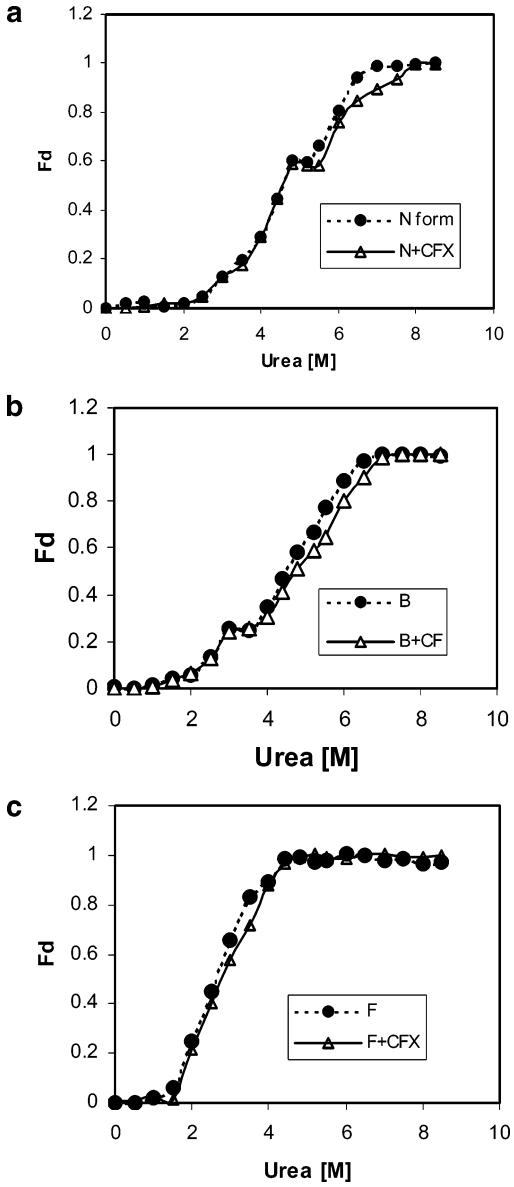

Figure 9. Normalized transition curves for urea induced unfolding of N (a), B (b), and F (c) isomers of HSA in the absence and presence of ciprofloxacin (drug/protein molar ratio of 2:1) as followed by intrinsic fluorescence measurements at 340 nm after exciting the proteins at 280 nm.

I state to U state shifted toward higher urea concentration by about 0.5 M, whereas the onset of the first transitions i.e., N−I and B−I, was not significantly affected. In other words, the drug was found to stabilize I−U and F−U transitions which corresponded to the unfolding of domain II. Therefore, these results again provide evidence that ciprofloxacin binding site is located on domain II.

Conclusion

The results indicate that the binding constant of ciprofloxacin to the physiologically important isomers (N and B) is significantly different. In view of the predominance of the B isomer in blood plasma under increased Ca^{2+} concentration, our report may prove significant in providing a useful guide to those effects of drug behavior, which are related to its distribution. By using different conformational states of HSA, we have concluded that ciprofloxacin interacts with domain II of HSA. It is a novel

approach that can be used to locate the binding site of a ligand to HSA directly.

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