INTERACTION OF NON-STEROIDAL ANTI-INFLAMMATORY DRUGS, ETORICOXIB AND PARECOXIB SODIUM, WITH HUMAN SERUM ALBUMIN STUDIED BY FLUORESCENCE SPECTROSCOPY

Neelam Seedher* and Sonu Bhatia

Department of Chemistry, Panjab University, Chandigarh, India

SUMMARY

The mechanism of interaction of the non-steroidal anti-inflammatory drugs, etoricoxib and parecoxib sodium, with human serum albumin (HSA) was studied using fluorescence spectroscopy. There was only one class of binding site with association constants of the order of 10^4. Thermodynamic parameters suggest that van der Waals and hydrogen bonding interactions in the case of etoricoxib, and electrostatic and hydrogen bonding interactions in the case of parecoxib sodium, are predominantly involved in the binding. Studies in the presence of the hydrophobic probe, 1-anilinonaphthalene-8-sulfonate (ANS), showed that hydrophobic interactions are not involved in the binding of these drugs to HSA. Displacement studies using the site-specific probe, dansylsarcosine piperidinium salt (DSS), showed that the drugs are bound at site II on the HSA molecule. However, etoricoxib and parecoxib sodium are bound at different regions within site II. Increase of pH and the presence of salt caused significant changes in the association constants and the concentration of free pharmacologically active drug. Stern-Volmer analysis of the binding data indicated that the tryptophan residues of albumin are not

* Author for correspondence:
Neelam Seedher
Department of Chemistry
Panjab University
Chandigarh, India
e-mail: nseedher@yahoo.com

fully accessible to anionic parecoxib sodium and a predominantly static quenching mechanism is operative in each case.

KEY WORDS

mechanism, interaction, human serum albumin, etoricoxib, parecoxib sodium, fluorescence spectroscopy

INTRODUCTION

Serum albumin, the most abundant protein in blood plasma, serves as a transport and depot protein for numerous endogenous and exogenous compounds due to its unique ligand-binding capabilities. Most of the drugs which enter the blood circulation bind to varying degrees with human serum albumin (HSA). The mechanism of binding of drugs to plasma proteins has important pharmacokinetic and pharmacodynamic implications since only the free drug is pharmacologically active /1,2/. The degree of protein binding affects the absorption, distribution, biotransformation and excretion of drugs. The effect is especially significant for highly protein-bound drugs, where only a small alteration in bound fraction can produce a profound change in the pharmacodynamically active free drug concentration /3,4/. A large number of reports have been published in recent years on various aspects of this problem /5,6/.

Etoricoxib and parecoxib sodium are non-steroidal anti-inflammatory drugs (NSAIDs) which are selective COX-2 inhibitors /7,8/. They are more than 90% bound, primarily to serum albumin which serves as a storehouse for the drug, and it is the unbound moiety that is pharmacologically active. We recently reported the molecular basis of the interaction of COX-2 inhibitors, meloxicam and nimesulide, with HSA /9/. There appears to be no such report on etoricoxib and parecoxib sodium. We report a study on the mechanism of interaction and detailed physicochemical characterization of the binding of etoricoxib and parecoxib sodium to HSA using fluorescence spectroscopy. The results are discussed in terms of the binding parameters under different environmental conditions, the thermodynamics of the binding process, the nature of forces involved in the interaction,

identification of the binding site, and the fluorescence quenching mechanism involved.

MATERIALS AND METHODS

Pure samples of etoricoxib and parecoxib sodium were obtained as a gift from Virdev Intermediates Pvt. Ltd., Surat, India. HSA and fluorescent probes, 1-anilinonaphthalene-8-sulfonate (ANS) and dansylsarcosine piperidinium salt (DSS), were purchased from Sigma Chemical Co., St. Louis, MO, USA. All other reagents were of analytical grade. Water used was double distilled in all glass apparatus. HSA solutions were prepared based on molecular weight of 66,500 Daltons. All experiments were carried out in 0.1 M phosphate buffer using fluorescence spectroscopy. A Perkin Elmer fluorescence spectrophotometer (MPF 44B) equipped with a 150 W xenon lamp source was used.

Drug-albumin binding

For the determination of binding parameters, 2 ml of 10 μM HSA solution was taken in a quartz cell and increasing amounts of drug stock solution (200 μM in the case of etoricoxib and 500 μM in the case of parecoxib sodium) were added. Due to the limited solubility of etoricoxib in phosphate buffer (pH 7.4), drug stock solution was prepared using 10% DMSO as co-solvent. The concentration of DMSO in the final mixture was not more than 2.5%. HSA concentration was kept fixed at 10 μM by adding the same volume of 20 μM HSA solution to the cell. The final drug concentration was in the range 5-50 μM in the case of etoricoxib and 5-100 μM in the case of parecoxib sodium. Fluorescence spectra were recorded in the range 300-400 nm after excitation at 296 nm in each case. Intrinsic fluorescence of protein was measured at 334 nm. The drugs did not have any fluorescence at the emission wavelength of protein. No correction for inner filter effect was applied in the case of parecoxib sodium since the drug-protein mixture had very low absorbance (less than 0.10) at the excitation and emission wavelengths. In the case of etoricoxib, the fluorescence data were corrected for inner filter effect /10/ using equation (1):

$$F_{corr} = F_{obs} \text{ antilog}(OD_{ex} + OD_{em})/2 \quad (1)$$

where F_{corr} and F_{obs} are the corrected and observed fluorescence intensity, and OD_{ex} and OD_{em} are the optical density of the sample at the excitation and emission wavelengths, respectively.

The stoichiometry of interaction was determined by the method of continuous variations /11,12/. The fluorescence change ($\Delta F = F_{protein+drug} - F_{protein}$) of a series of protein-drug mixtures was measured under such conditions that the total concentration of drug plus protein was held constant at 10 μM but the respective mole fraction of each was varied. ΔF was plotted against the mole fraction of drug (Job's plot) and the stoichiometry of binding was obtained from the maximum in the plot in each case.

Data analysis

Data were analysed as follows using Ward's method /13/. The fractional occupancy of the total protein binding sites by drug was obtained from the ratio, $\theta = \Delta F/\Delta F_{max}$ /14,15/, where $\Delta F = F_0 - F$. F_0 and F are the fluorescence intensities of serum albumin in the absence and presence of drug, respectively. ΔF_{max} values were obtained from the double reciprocal ($1/\Delta F$ versus $1/D_t$) plots.

If P_t is the total protein concentration and n is the number of binding sites, the total number of sites on protein is given by nP_t and the concentration of bound sites on protein is given by $n\theta P_t$ /13/, which is also equal to the concentration of the bound drug (D_b). D_f, the number of moles of free drug, was obtained from the difference, $D_t - D_b$, where D_t is the total drug added. The amount bound was expressed as moles of drug bound per mole protein, r (= D_b/P_t). The binding parameters were computed directly by fitting the experimental data (r and D_f values) to the following general equation (Scatchard equation) using an iterative non-linear least squares regression program developed for this purpose:

$$r = \sum_{i=1}^{i=m} n_i K_i D_f / 1 + K_i D_f \qquad (2)$$

Data are reported as means ± SEM (three measurements). The percentage of drug bound ($\beta = D_b/D_t \times 100$) was calculated from the association constants using the relationship, $\beta = \{[P_t]/([P_t] + (1/K_a) + [D_f])\} \times 100$ /16/.

Thermodynamic parameters for drug-protein interaction were determined for both drugs at pH 7.4, from the experiments conducted at three different temperatures, 27°C, 32°C and 37°C, using equations (3) and (4):

$$\Delta G^0 = -RT \ln K \quad (3)$$

$$\ln K = -\Delta H^0/RT + \Delta S^0/R \quad (4)$$

ln K versus 1/T plots were used to calculate the standard enthalpy change ΔH^0 and standard entropy change ΔS^0 for the binding process. To study the effect of ionic strength on binding parameters, the data at 37°C were also obtained in the presence of 0.15 M NaCl.

The percentage of free drug ($\alpha = D_f/D_t \times 100$) was calculated from the dissociation constants for drug-protein complex ($K_d = 1/K_a$) using the relationship, $(K_d + D_f)/\{[P_t] + K_d + [D_f]\} \times 100$ /16/.

Drug-HSA interaction in the presence of fluorescent probes

Hydrophobic probe, ANS

Experiments were also carried out in the presence of the hydrophobic probe, ANS. In the first set of experiments, interaction of drugs and ANS with HSA was studied under identical conditions. The HSA concentration was kept fixed at 10 μM, and ANS/drug concentration was varied from 1 to 11 μM. Fluorescence of HSA was recorded at 334 nm after excitation at 296 nm. In the second set of experiments, an increasing amount of drug (1-60 μM) was added to equimolar HSA-ANS mixture (10 μM each) and the fluorescence of ANS was recorded at 470 nm after excitation at 370 nm. The concentration of the HSA-ANS mixture was kept fixed at 10 μM each by adding the same volume of albumin-ANS/albumin-drug mixture (20 μM each) to the cell.

Site-selective probe

Fluorescence probe displacement experiments were carried out using the site II-selective probe, dansylsarcosine (DSS). The fluorescence of the probe was measured at 27°C in the probe-HSA mixture (1:1, 5 μM each) before and after the addition of drug (4-56 μM). DSS fluorescence was measured at 480 nm after excitation at 350 nm. The

probe to HSA ratio was kept at 1:1 in order to keep the non-specific binding of the probe to a minimum.

RESULTS AND DISCUSSION

Drug-serum albumin interaction

HSA is a single-chain protein containing 585 amino acids and has an isoelectric point of 4.7. The protein has three homologous domains (I-III), and each of these is comprised of two subdomains (A and B). It contains a single tryptophan residue at position 214 in subdomain IIA /17/. Intrinsic fluorescence of HSA is usually measured by selectively exciting the tryptophan residues at 296 nm. Etoricoxib, a pyridine diaryl-substituted COX-2 inhibitor, is a weakly basic drug with pK_a 3.68, and parecoxib sodium with an isoxazolyl central ring system is an anionic drug with pK_a 4.81 (Table 1). The structures of etoricoxib and parecoxib sodium are shown in Figure 1.

Perturbation of intrinsic protein fluorescence on drug binding was monitored. Both drugs were found to quench the intrinsic fluorescence of HSA. However, there was no observable shift in the wavelength for maximum emission. The stoichiometry of interaction, determined by the method of continuous variations /11,12/, was found to be 1:1 in the case of both etoricoxib and parecoxib sodium. As a representative example, the Job's plot for the etoricoxib-HSA system at 37°C and pH 7.4 is shown in Figure 2.

The experimental data could be fitted into an equation for only one class of binding sites (m = 1). The association constant (K) was of the order of 10^4 and the number of binding sites was close to one in the case of both drugs (Table 2). The values reported in the literature for other NSAIDs are also in the range 10^4-10^6 /12,18,19/. The percentage of drug bound at different drug:protein ratios is shown in Figure 3. At low drug:protein ratios, a significant fraction of the added drug was bound in each case. It may be mentioned that low drug:protein ratios are frequently encountered in the physiological system, since the serum albumin concentration in blood is very large (0.53-0.75 mM).

Some physico-chemical properties of the drugs are given in Table 1. pK_a values for the drugs show that etoricoxib (a basic drug) exists in predominantly unionised form and parecoxib sodium (an acidic drug) exists in predominantly ionised form at physiological pH.

TABLE 1

Some physico-chemical properties of etoricoxib and parecoxib sodium

	Etoricoxib	Parecoxib sodium
pK_a*	3.68	4.81
Partition coefficient (logD)***	2.22	0.82
Total polar surface area (TPSA)**	59.926	89.272
Molar volume*	276.4	–
H bond acceptors**	5	6
H bond donors**	0	1

* Calculated using software ACD/I-Lab.
** Calculated using software molinspiration.
*** Experimentally determined at pH 7.4 using shake flask method.

TABLE 2

Association constants for the interaction of etoricoxib and parecoxib sodium with human serum albumin at pH 7.4 at different temperatures

Temperature (°K)	**Association constant (K_a) x 10^{-4} M^{-1}**	
	Etoricoxib	**Parecoxib sodium**
293.15	–*	9.878 ± 0.125
300.15	4.783 ± 0.025	7.582 ± 0.052
305.15	3.748 ± 0.183	6.479 ± 0.100
310.15	3.079 ± 0.095	5.524 ± 0.101

* Data could not be obtained due to low solubility of etoricoxib at 293.15°K in phosphate buffer (pH 7.4).
Data are reported as means ± SEM (three measurements).

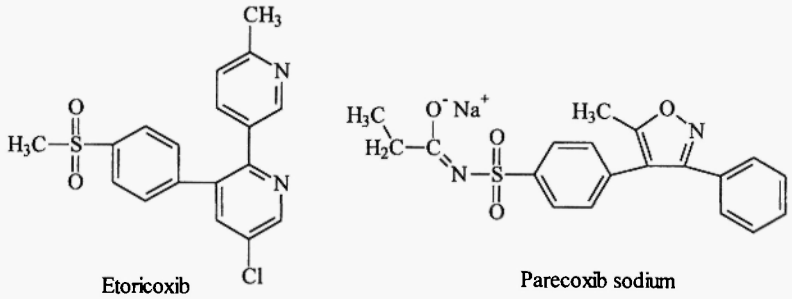

Fig. 1: Structures of etoricoxib and parecoxib sodium.

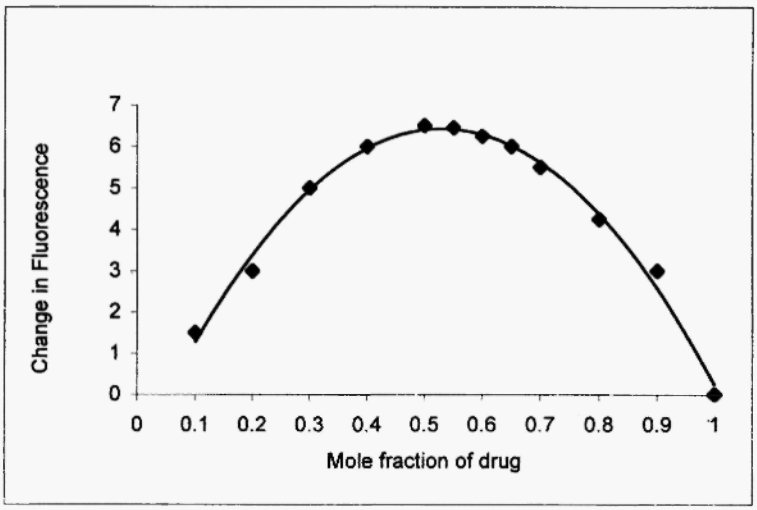

Fig. 2: Job's plot for etoricoxib-HSA system.

Higher log D values and lower total polar surface area (TPSA) values in the case of etoricoxib as compared to parecoxib sodium also show that etoricoxib is more hydrophobic than parecoxib sodium. However, the binding constant for etoricoxib was found to be smaller than that for parecoxib sodium, indicating that hydrophobic interactions may not be involved in the binding of these drugs to HSA. A further insight into the nature of the interaction involved could be obtained from the thermodynamic parameters for the binding equilibrium.

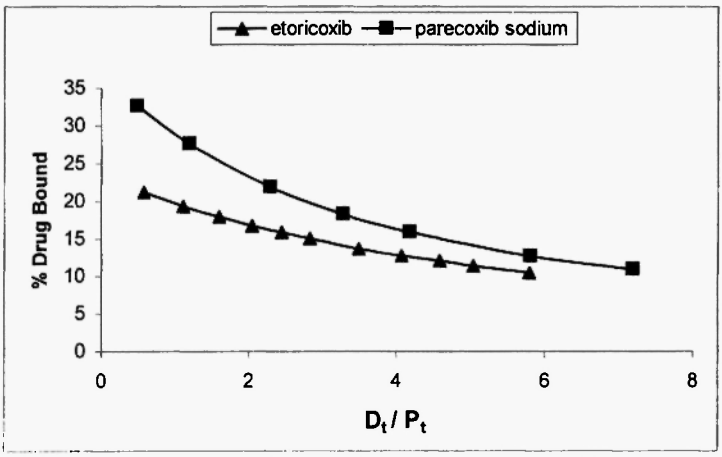

Fig. 3: Percentage of etoricoxib and parecoxib sodium bound to HSA at 37°C.

Effect of temperature

There was no change in the number of binding sites with increase in temperature, indicating that increase of temperature does not cause any major structural changes in the protein molecule. Association constants were found to decrease with increase in temperature in the case of both drugs. Thermodynamic parameters, ΔG^0, ΔH^0 and ΔS^0, for the interaction of etoricoxib and parecoxib sodium with HSA are given in Table 3. High negative ΔG^0 values show spontaneity of the binding process. The enthalpy change (ΔH^0) had high negative values in the case of both drugs, while the entropy change (ΔS^0) had a large negative value in the case of etoricoxib and a small positive value in the case of parecoxib sodium. Since etoricoxib is unionized at pH 7.4, the possibility of electrostatic interactions is ruled out /20/. High negative ΔH^0 and high negative ΔS^0 values in the case of etoricoxib suggest the involvement of van der Waals and hydrogen bonding interactions /21,22/. Parecoxib sodium, on the other hand, is anionic at pH 7.4. Although at pH 7.4 the net charge on the protein is negative, the HSA molecule has about 80 positively charged groups, and since the surface of the protein is greater than that of the drug, local electrostatic interactions can occur. For purely electrostatic interactions, ΔH^0 is very small /23/. High negative ΔH^0 values and small positive ΔS^0

TABLE 3

Thermodynamic parameters for the interaction of etoricoxib and parecoxib sodium with human serum albumin

Thermodynamic parameter	Etoricoxib	Parecoxib sodium
ΔG^0 (kJ/mol)*	-26.654	-28.162
ΔH^0 (kJ/mol)	-33.952	-25.749
ΔS^0 (J/mol)	-23.576	+7.743

* ΔG^0 values were calculated at 37°C.

values in the case of parecoxib sodium indicate the involvement of both electrostatic and hydrogen bonding interactions /24,25/. Small positive ΔS^0 values do not indicate hydrophobic interactions, since hydrophobic interactions are known to be endothermic /26/. It may be mentioned that both drugs are capable of hydrogen bond formation since they contain a large number of hydrogen bond acceptors (Table 1).

Binding studies in the presence of fluorescence probes

Hydrophobic probe, ANS

In order to further understand the nature of the interaction involved, studies were also carried out in the presence of the hydrophobic probe, ANS /27,28/. In the first set of experiments, the quenching of HSA fluorescence by drugs and ANS was determined under identical conditions. Both drugs and ANS quench the fluorescence of albumin. However, the extent of quenching by drugs was about half that of ANS in the case of both drugs (Fig. 4). The percentage quenching was about 45% in the case of ANS and about 20-22% in the case of parecoxib sodium and etoricoxib, under identical conditions. These results show that drugs and ANS do not share a common site in HSA.

In another set of experiments, ANS fluorescence was measured. When an increasing amount of drug (1-60 μM) was added to a fixed

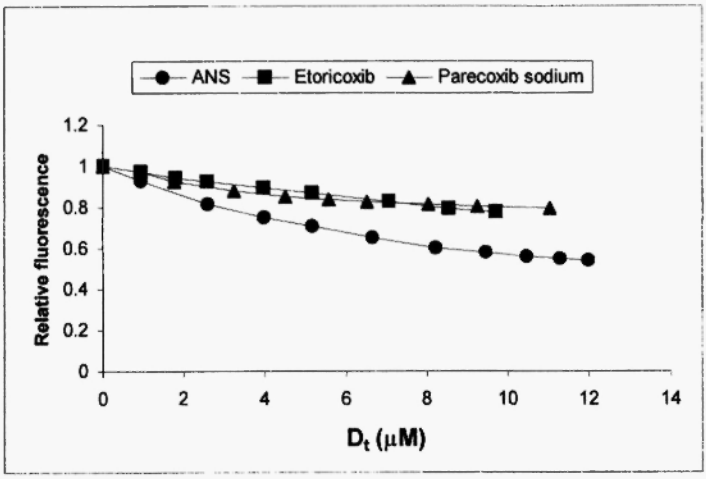

Fig. 4: Quenching of HSA fluorescence by ANS and drugs under identical conditions.

concentration of HSA-ANS mixture (10 μM each), there was no change in the fluorescence of ANS in the case of etoricoxib and a small decrease in fluorescence of ANS in the case of parecoxib sodium. Relative fluorescence intensity (F/F_0, where F and F_0 are the fluorescence intensity of ANS in the HSA-ANS system, in the presence and absence of drug) was plotted against the concentration of drug (Fig. 5). It is known that the hydrophobic probe, ANS, shows greatly increased fluorescence as a result of hydrophobic interaction with proteins and other macromolecules, due to the transfer of the probe from an aqueous to a non-polar environment /27,28/. The drug when added to the HSA-ANS system can compete with ANS for hydrophobic sites on the surface. In that case it would inhibit the binding of ANS, i.e., displace ANS from its binding site, and the fluorescence intensity should decrease. This happened in the case of parecoxib sodium. However, the percentage displacement was very small, only about 5%. In the case of etoricoxib, there was no displacement of ANS. Thus, from the studies carried out in the presence of the hydrophobic probe, ANS, it was concluded that hydrophobic interactions are not primarily involved in the binding of these drugs to HSA.

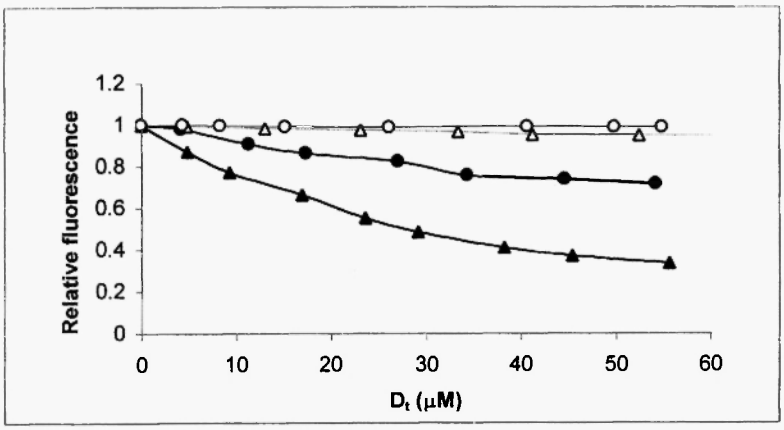

Fig. 5: Displacement of fluorescent probes from HSA by drugs. ○ = ANS-etoricoxib; ● = DSS-etoricoxib; Δ = ANS-parecoxib sodium; ▲ = DSS-parecoxib sodium.

Site-selective probe

In the case of both etoricoxib and parecoxib sodium, the addition of increasing amounts of drug to the HSA-DSS mixture resulted in decrease of DSS fluorescence, thus indicating that the drugs displace DSS from its binding site /29,30/. Relative fluorescence intensity (F/F_0, where F and F_0 are the fluorescence intensity of DSS in HSA-DSS system, in the presence and absence of drug) was plotted against the concentration of drug (Fig. 5). The percentage displacement was about 66% in the case of etoricoxib and only about 28% in the case of parecoxib sodium at the highest drug concentration used (~55 μM). High percentage displacement in the case of etoricoxib showed that etoricoxib is bound to site II in the HSA molecule. Only 28% displacement in the case of parecoxib sodium, however, showed that parecoxib sodium and DSS bind at different regions within site II. Maximum displacement was also calculated from the linear double reciprocal plot (1/displacement versus 1/drug concentration). Maximum displacement was nearly 100% in the case of etoricoxib and 65% in the case of parecoxib sodium.

Crystallographic analyses have assigned site II in HSA to subdomain IIIA, and among the individual amino acid residues in this

subdomain, ^{410}Arg and ^{411}Tyr are usually assumed to be important /20,31/. Most NSAIDs of arylpropionic acid type which possess a carboxylate at the end of an extended hydrophobic molecule (e.g. ketoprofen, ibuprofen and flufenamic acid) are bound to site II /32-34/. However, Watanabe *et al.* /35/ have shown that a carboxyl group is not obligatory for binding to site II, because diazepam, a basic drug that exists mainly in unionised form at neutral pH, also binds with high affinity to this site. It has been suggested that in site II, the guanidine moiety of ^{410}Arg, the phenolic oxygen and the aromatic ring of ^{411}Tyr are important for drug binding. The drugs possessing a carboxyl group probably interact electrostatically with the guanidino moiety, while the phenolic oxygen makes a hydrogen bond with the ligand. ^{410}Arg is, therefore, not important for ligands such as diazepam. Etoricoxib and parecoxib sodium also do not possess a carboxyl group and therefore ^{411}Tyr may be involved in binding within site II. This conclusion finds support from the thermodynamic parameters for the binding of these drugs which suggest predominantly hydrogen bonding interactions.

Stern-Volmer analysis

Stern-Volmer analysis is useful in the estimation of the accessibility of tryptophan residues in proteins to the drug (quencher) molecules. Fluorescence quenching data at pH 7.4 were also analysed by the Stern-Volmer law /36/:

$$F_0/F = 1 + K_q D_t \quad (4)$$

where F_0 and F are the steady state fluorescence intensities at 334 nm in the absence and presence of quencher (drug), respectively, and K_q is the Stern-Volmer quenching constant. F_0/F versus D_t plots (not shown) were linear for etoricoxib with intercept close to unity at all temperatures. Linearity of the plots showed that the tryptophan residues of HSA are fully accessible to the drug. In the case of parecoxib sodium, on the other hand, a simple Stern-Volmer plot showed a downward curvature at all temperatures (Fig. 6). Downward curvature indicates the presence of buried residues, that is, the tryptophan residues are not fully accessible to the drug /37/. This difference in the behaviour of the two drugs may be due to the fact that the anionic parecoxib sodium cannot effectively penetrate the

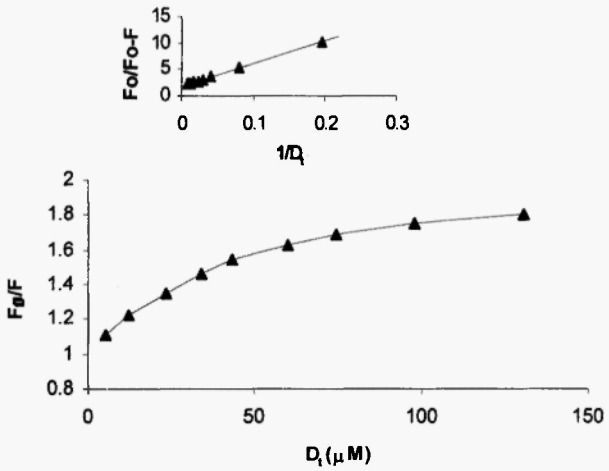

Fig. 6: Stern-Volmer plots for parecoxib sodium-HSA binding at 37°C.

hydrophobic interior of the drug binding site. The data in the case of parecoxib sodium were, therefore, analysed by the modified Stern-Volmer equation /38/:

$$F_0/ (F_0-F) = 1/f_a + 1/(D_t f_a K_q) \quad (6)$$

where f_a is the fraction of fluorophore (protein) accessible to the quencher (drug). The modified Stern-Volmer plots were linear with f_a values close to 0.5 at all temperatures (see inset to Fig. 6). K_q values for etoricoxib and parecoxib sodium at different temperatures are given in Table 4. Decrease in quenching constants with increase in temperature indicated that a predominantly static quenching mechanism is involved in the case of both drugs /39/.

For a bimolecular quenching process, $K_q = k_q \tau_0$, where τ_0 is the lifetime in the absence of quencher, and k_q is the rate constant for quenching. As the τ_0 value for tryptophan fluoresence in proteins is known to be of the order of 10^{-9} s /40/, the rate constant, k_q, would be of the order of 10^{13} $M^{-1}s^{-1}$. k_q depends on the probability of a collision between fluorophore and quencher and is a measure of the exposure of tryptophan residues to the drug. It can be shown that

$$k_q = 4\pi a D N_A \times 10^{-3} \quad (5)$$

TABLE 4

Stern-Volmer quenching constant for etoricoxib and parecoxib sodium at different temperatures

Temperature (°K)	Stern-Volmer quenching constant (K_q) x 10^4 M^{-1}	
	Etoricoxib	**Parecoxib sodium**
293.15	–	6.00
300.15	1.15	5.57
305.15	0.90	4.95
310.15	0.64	4.51

where D is the sum of the diffusion coefficients of quencher and fluorophore, a is the sum of molecular radii, and N_A is Avogadro's number /41/. The upper limit of k_q expected for a diffusion-controlled bimolecular process is 10^{10} $M^{-1}s^{-1}$. The high magnitude of k_q in the present study (10^{13} $M^{-1}s^{-1}$) shows that specific interactions, such as hydrogen bonding, increase the drug-protein encounter radius, a, and make k_q larger. Thus drugs are bound to albumin by hydrogen bonding at a site which is close to the tryptophan residues. The high magnitude of k_q in the present study (10^{13} $M^{-1}s^{-1}$) also shows that the quenching is not initiated by dynamic collision, but originates from the formation of a complex between the drug and the protein /42/. However, a collisional quenching mechanism is also involved, since the magnitude of the quenching constant is smaller than the association constant for the interaction.

Effect of pH and the presence of salt

Association constants for the binding and the number of binding sites at two different pH values, 7.4 and 8.0, determined at 37°C, are given in Table 5. With increase of pH, the binding constants increased in the case of etoricoxib and decreased in the case of parecoxib sodium, while the number of binding sites remained the same. Since pK_a values of etoricoxib (a basic drug) and parecoxib sodium (an acidic drug) are 3.68 and 4.81, respectively, the percentage ionization

TABLE 5

Effect of pH and presence of salt on the association constants of etoricoxib and parecoxib sodium with human serum albumin at 37°C

	Association constant (K_a) x 10^{-4} M^{-1}	
	Etoricoxib	**Parecoxib sodium**
pH 7.4	3.079 ± 0.095	5.524 ± 0.101
pH 8.0	5.880 ± 0.105	3.280 ± 0.095
pH 7.4 + 0.15 M NaCl	4.990 ± 0.098	1.550 ± 0.008

of the drugs will remain practically unchanged on increasing the pH from 7.4 to 8.0: etoricoxib will remain practically unionized, and parecoxib sodium will remain ionized in this pH range. In the protein molecule, the fraction of cationic surface decreases while the anionic and neutral surface increases with increase of pH. Etoricoxib being a basic drug, its binding constant increases as the fraction of anionic and neutral surface increases. On the other hand, binding of negatively charged parecoxib sodium decreases as the fraction of the cationic surface on the protein decreases. Percentage of free drug at different pH values is shown in Figures 7 and 8. With increase of pH, the free pharmacologically active drug decreases in the case of etoricoxib and increases in the case of parecoxib sodium.

The binding of etoricoxib and parecoxib sodium with HSA was also studied in the presence of 0.15 M NaCl. Binding parameters are given in Table 5. The presence of salt increased the association constants in the case of etoricoxib and decreased the association constants in the case of parecoxib sodium, but the number of binding sites remained almost the same. Decrease of binding constants in the case of parecoxib sodium again indicates the involvement of electrostatic interactions /19/. The increase in the binding constant of etoricoxib indicates that the presence of salt probably causes conformational changes in the albumin molecule. It has been shown by Wilting *et al.* /43/ that chloride ions also affect N-B transition. The percentage of free drug in the absence and presence of 0.15 M NaCl is

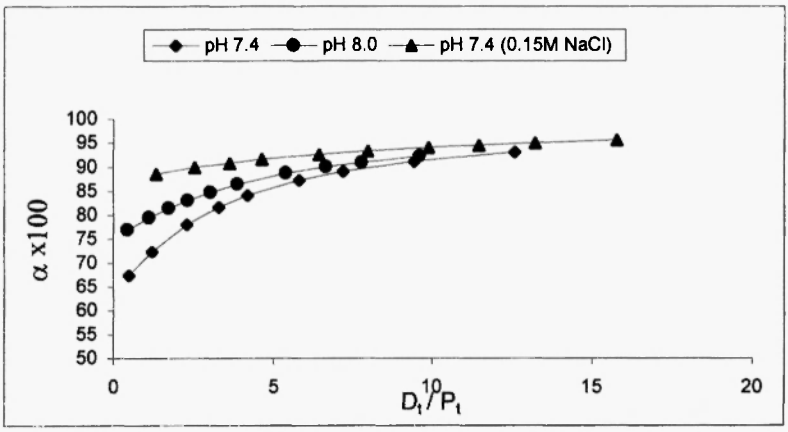

Fig. 7: Plot of percentage of free drug versus D_t/P_t for parecoxib sodium-HSA binding under different conditions.

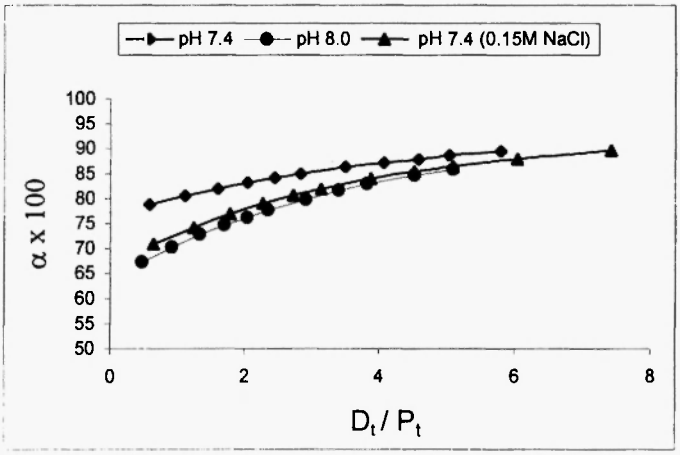

Fig. 8: Plot of percentage of free drug versus Dt/Pt for etoricoxib-HSA binding under different conditions.

shown in Figures 7 and 8 at different drug-protein (D_t/P_t) ratios. Thus in the presence of salt, the percentage of free drug decreased in the case of etoricoxib and increased in the case of parecoxib sodium. It is, therefore, recommended to adjust etoricoxib and parecoxib sodium dose in cases of diseases that induce pH or electrolytic imbalance.

CONCLUSIONS

The binding of the drugs, etoricoxib and parecoxib sodium, with HSA may be summarised thus: The drugs interact with HSA on a single site with association constants of the order of 10^4. Paracoxib sodium had a higher relative affinity for HSA compared to etoricoxib. Thermodynamic parameters for the binding showed that in the case of etoricoxib, hydrogen bonding and van der Waals interactions are predominantly involved. In the case of parecoxib sodium, on the other hand, electrostatic and hydrogen bonding interactions are involved. Binding studies in the presence of a hydrophobic probe (ANS) showed that hydrophobic interactions are not predominantly involved in the binding. Displacement of the site-specific probe, DSS, showed that site II is involved in binding. However, etoricoxib and parecoxib sodium are bound at different regions within site II. Stern-Volmer analysis of the fluorescence data showed that in the case of parecoxib sodium, the tryptophan residues of HSA are not fully accessible to the drug, and a predominantly static quenching mechanism is operative in each case. Increase of pH and the presence of salt significantly altered the percentage of free drug in plasma.

ACKNOWLEDGEMENTS

The authors are grateful to University Grants Commission, New Delhi, India for financial assistance, and to Virdev Intermediates Pvt. Ltd., Surat, India, for the gift samples of etoricoxib and parecoxib sodium.

REFERENCES

1. Bertucci C, Domenici E. Reversible and covalent binding of drugs to human serum albumin: methodological approaches and physiological relevance. Curr Med Chem 2002; 9: 1463-1481.
2. Kragh-Hansen U, Chuang VTG, Otagiri M. Practical aspects of the ligand-binding and enzymatic properties of human serum albumin. Biol Pharm Bull 2002; 25: 695-704.
3. Levy RH, Moreland TA. Rationale for monitoring free drug levels. Clin Pharmacokinet 1984; 9 (Suppl 1): 1-9.
4. Rowland M. Plasma protein binding and therapeutic drug monitoring. Ther Drug Monit 1980; 2: 29-37.

5. Xie M, Xu X, Wang Y. Interaction between hesperetin and human serum albumin revealed by spectroscopic methods. Biochim Biophys Acta 2005; 1724: 215-224.
6. Pang YH, Yang LL, Shuang SM, Dong C, Thompson M. Interaction of human serum albumin with bendroflumethiazide studied by fluorescence spectroscopy. J Photochem Photobiol 2005; 80: 139-144.
7. Needleman P, Isakson PC. The discovery and function of COX-2. J Rheumatol 1997; 24 (Suppl 49): 6-8.
8. Silas S, Clegg DO. Selective COX-2 inhibition. Bull Rheum Dis 1999; 40: 1-4.
9. Seedher N, Bhatia S. Mechanism of interaction of non-steriodal anti-inflammatory drugs meloxicam and nimesulide with serum albumin. J Pharm Biomed Anal 2005; 39: 257-262.
10. Oberfelder RW, Lee JC. Measurement of ligand-protein interaction by electrophoretic and spectroscopic techniques. Meth Enzymol 1985; 117: 381-388.
11. Job P. Formation and stability of inorganic complexes in solution. Ann Chim 1928; 9: 113-203.
12. Rahman MH, Maruyama T, Okada T, Yamasaki K, Otagiri M. Study of interaction of carprofen and its enantiomers with human serum albumin-I. Biochem Pharmacol 1993; 46: 1721-1731.
13. Ward LD. Measurement of ligand binding to proteins by fluorescence spectroscopy. Meth Enzymol 1985; 117: 400-414.
14. Weber G, Young LB. Fragmentation of bovine serum albumin by pepsin. I. The origin of the acid expansion of the albumin molecule. J Biol Chem 1964; 239: 1415-1423.
15. Maruyama T, Otagiri M, Schulman SG. Binding characteristics of coumarin anticoagulants to human-α_1-acid glycoprotein and human serum albumin. Int J Pharmaceut 1990; 59: 137-143.
16. Martin BK. Potential effect of the plasma proteins on drug distribution. Nature 1965; 207: 274-276.
17. He XM, Carter DC. Atomic structure and chemistry of human serum albumin. Nature 1992; 358: 209-215.
18. Russeva V, Mihailova D. Binding of phenylbutazone to human serum albumin. Arzneim-Forsch/Drug Res 1999; 49: 255-258.
19. Trivedi VD, Vorum H, Honore B, Qasim MA. Molecular basis of indomethacin-human serum albumin interaction. J Pharm Pharmacol 1999; 51: 591-600.
20. He W, Li Y, Xue C, Hu Z, Chen X, Sheng F. Effect of Chinese medicine alpinetin on the structure of human serum albumin. Bioorg Med Chem 2005; 13: 1837-1845.
21. Aki H, Yamamoto M. Thermodynamics of the binding of phenothiazines to human plasma, human serum albumin and alpha-acid glycoprotein. A calorimetric study. J Pharm Pharmacol 1989; 41: 674-679.
22. Seedher N, Singh B, Singh P. Mode of interaction of metronidazole with bovine serum albumin. Indian J Pharm Sci 1999; 61: 143-148.

23. Ross PD, Subramanian S. Thermodynamics of protein association reactions. Forces contributing to stability. Biochemistry 1981; 20: 3096-3102.
24. Fernandez GM, Lumbreras JM, Ordonez D. A thermodynamic approach to the binding mechanisms of cefotaxime to serum albumins. J Pharm Sci 1993; 82: 948-951.
25. Li Y, He W, Liu J, Sheng F, Hu Z, Chen X. Binding of the bioactive component jatrorrhizine to human serum albumin. Biochim Biophys Acta 2005; 1722: 15-21.
26. Barbosa S, Taboada P, Mosquera V. Analysis of the interactions between human serum albumin/amphiphilic penicillin in different aqueous media: an isothermal titration calorimetry and dynamic light scattering study. Chem Phys 2005; 310: 51-58.
27. Jun HW, Ruenitz PC. Interaction of tricyclic antipsychotic and antidepressant drugs with 1-anilino-8-naphthalenesulfonic acid. J Pharm Sci 1978; 67: 861-863.
28. Seedher N. In vitro study of the mechanism of interaction of trifluoperazine dihydrochloride with bovine serum albumin. Indian J Pharm Sci 1999; 62: 16-20.
29. Sudlow G, Birkett D, Wade D. The characterization of two specific drug binding sites on human serum albumin. Mol Pharmacol 1975; 11: 824-832.
30. Sudlow G, Birkett D, Wade D. Further characterization of specific drug binding sites on human serum albumin. Mol Pharmacol 1976; 12: 1052-1061.
31. Sugio S, Kashima A, Mochizuki S, Noda M, Kobayashi K. Crystal structure of human serum albumin at 2.5 Å resolution. Protein Eng 1999; 12: 439-446.
32. Chuang VT, Kuniyasu A, Nakayama H, Matsushita Y, Hirono S, Otagiri M. Helix 6 of subdomain III A of human serum albumin is the region primarily photolabeled by ketoprofen, an aryl propionic acid NSAID containing a benzophenone moiety. Biochim Biophys Acta 1999; 1434: 18-30.
33. Wanwimolruk S, Birkett DJ, Brooks PM. Structural requirements for drug binding to site II on human serum albumin. Mol Pharmacol 1983; 24: 458-463.
34. Otagiri M, Masuda K, Imai T, Imamura Y, Yamasaki M. Binding of pirprofen to human serum albumin studied by dialysis and spectroscopy techniques. Biochem Pharmacol 1989; 38: 1-7.
35. Watanabe H, Tanase S, Nakajou K, Maruyama T, Kragh-Hansen U, Otagiri M. Role of Arg-410 and Tyr-411 in human serum albumin for ligand binding and esterase-like activity. Biochem J 2000; 349: 813-819.
36. Eftink MR, Ghiron CA. Fluorescence quenching studies with proteins. Anal Biochem 1981; 114: 199-227.
37. Eftink MR, Ghiron CA. Fluorescence quenching of indole and model micelle systems. J Phys Chem 1976; 80: 486-493.
38. Lehrer SS. Solute perturbation of protein fluorescence. The quenching of tryptophan fluorescence of model compounds and of lysozyme by iodide ion. Biochemistry 1971; 10: 3254-3256.
39. Gonzalez-Jimenez J, Frutos G, Cayre I. Fluorescence quenching of human serum albumin by xanthines. Biochem Pharmacol 1992; 44: 824-826.

40. Lakowicz JR. Principles of Fluorescence Spectroscopy. New York: Plenum Press, 1983; 257-295.
41. Campbell ID, Dwek RA. Biological Spectroscopy. London: The Benjamin/ Cummings Publishing Company Inc., 1984; 108-109.
42. Cui FL, Fan J, Li W, Fan YC, Hu ZD. Fluorescence spectroscopic studies on 5-aminosalicylic acid and zinc 5-aminosalicylate - interaction with human serum albumin. J Pharm Biomed Anal 2004; 34: 189-197.
43. Wilting J, Van der Giesen WF, Janssen LHM. The effect of chloride on the binding of warfarin to albumin as a function of pH. Biochem Pharmacol 1981; 30: 1025-1031.