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Short communication

Mechanism of interaction of the non-steroidal antiinflammatory drugs meloxicam and nimesulide with serum albumin

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Abstract

The mechanism of interaction of the non-steroidal antiinflammatory drugs meloxicam and nimesulide with human and bovine serum albumin has been studied using fluorescence spectroscopy. There was only one high affinity site on serum albumin for both the drugs with association constants of the order of 10^5. Negative enthalpy (ΔH^0) and positive entropy (ΔS^0) values in the case of both meloxicam and nimesulide showed that both hydrogen bonding and hydrophobic interactions play a role in the binding of these drugs. Binding studies in the presence of the hydrophobic probe 1-anilinonaphthalene-8-sulfonate (ANS) showed that the binding of meloxicam and nimesulide to serum albumin involves predominantly hydrophobic interactions. Stern–Volmer analysis of the quenching data showed that quenching is highly efficient and that the tryptophan residues in hydrophobic regions of the proteins are fully exposed to the drugs. Thus these drugs are bound to albumin by hydrophobic interactions as well as hydrogen bonding at a site, which is close to the tryptophan residues. An increase of the pH and ionic strength caused an increase in the concentration of free drug, although the effect was not very significant.

Keywords: Serum albumin; Meloxicam; Nimesulide; Fluorescence spectroscopy

1. Introduction

Reversible attachment to serum proteins significantly modulates the pharmacokinetics (volume of distribution, clearance and elimination half-life) and pharmacodynamics (biological activity and toxicology) of many drugs [1–3]. The free concentration of a drug in plasma is, therefore, a more reliable parameter for representing the intensity of pharmacological effect than the total plasma concentration [4,5]. The effect is especially significant for drugs which are highly protein bound, have a narrow therapeutic index and a small volume of distribution [6,7]. The nature of the forces involved in drug–protein interaction also plays a significant role in drug action because the dissociation of drug–protein complex can occur only when the driving force of dissociation is greater than the forces accounting for the binding. The forces of association must be strong enough to cause binding, yet weak enough so that minor modifications of the physical environment will provide sufficient driving force for dissociation [8]. Thus the nature and magnitude of drug–protein interaction significantly influences the biological activity of a drug [9–11]. Amongst plasma proteins, serum albumin, the most abundant protein in plasma, is undoubtedly the most important carrier for drugs and other small molecules and is considered as a model for studying drug–protein interaction in vitro [12].

Meloxicam and nimesulide are pharmacologically important, new generation, non-steroidal anti-inflammatory drugs (NSAIDs) with minimum adverse gastrointestinal and renal side effects associated with traditional NSAIDs [13–15]. Besides their primary functions as anti-inflammatory agents, they are also emerging as useful agents, in cancer treatment and Alzheimer's disease [16,17]. In various types of cancer, cox-2 is over-expressed. Meloxicam's ability to kill cancerous cells specifically by inhibiting cox-2 makes cox-2 inhibitors promising tools in the fight against cancer [18]. The cox-2 enzyme is also expressed in the brain and may be el-

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evated in patients with Alzheimer's disease. Nimesulide and other cox-2 inhibitors retard the progression of Alzheimer's disease [17]. They exhibit a high degree of binding to albumin, have a low apparent volume of distribution and a long plasma half-life [19]. Thus their binding characteristics are primary determinants of their pharmacokinetic properties. The effect of plasma protein binding on the diffusion of oxicams through the blood-brain barrier and the binding of nimesulide to blood components has been reported by some workers [19,20]. However, detailed studies on the molecular basis of their interaction with serum albumin have not been reported. Oravcova et al. [21,22] have recently reviewed the techniques used for studying drug–protein binding. Fluorescence spectroscopy has been the most widely used spectroscopic technique for monitoring drug binding to plasma albumin because of its sensitivity, accuracy, rapidity and ease of use. They have shown that the conventional approaches such as affinity and size-exclusion chromatography, equilibrium dialysis, ultrafiltration and ultracentrifugation, all suffer from lack of sensitivity or long analysis times or both and use of protein concentrations far in excess of the dissociation constant for the drug–protein complex. In the present work the interaction of meloxicam and nimesulide with human and bovine serum albumin has been studied under different environmental conditions using fluorescence spectroscopy. Results have been discussed in terms of the binding parameters and the nature of the forces involved in the interaction.

2. Experimental

2.1. Materials and methods

Pure meloxicam and nimesulide were obtained as gifts from Ms. Sun Pharmaceutical Industries Ltd., Mumbai, India and Panacea Biotec Ltd., Lalru, India, respectively. Human serum albumin (HSA), bovine serum albumin (BSA) and the fluorescent probe, 1-anilinonaphthalene-8-sulfonate (ANS), were purchased from Sigma Chemical Co., U.S.A. All other reagents were of analytical grade. The water used was double distilled in an all glass apparatus. Human and bovine serum albumin solutions were prepared based on molecular weights of 66,500 and 66,000, respectively. All experiments were carried out in 0.10 M phosphate buffer using fluorescence spectroscopic technique. A Perkin-Elmer fluorescence spectrophotometer (MPF 44B) equipped with a 150 W xenon lamp source was used.

2.2. Drug-serum albumin binding

For the determination of binding parameters, two millilitres of 10 μM albumin solution was taken in a quartz cell and increasing amounts of drug stock solution (250–300 μM) was added. The albumin concentration was kept fixed at 10 μM by adding the same volume of 20 μM albumin to the cell. Fluorescence spectra were recorded in the range 280–400 nm after excitation at 296 nm, in each case. Intrinsic fluorescence of protein was measured, drugs meloxicam and nimesulide did not have any fluorescence at the emission wavelength of proteins. The fluorescence data was corrected for inner filter effect using the equation [23]

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

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 the interaction was determined by the method of continuous variations [24,25]. The fluorescence change ($\Delta F = F_{\text{protein+drug}} - F_{\text{protein}}$) 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.

Thermodynamic parameters for drug–protein interactions were determined for both the drugs at pH 7.4 from the experiments conducted at three different temperatures 20, 27 and 37 °C. To study the effect of pH on binding parameters, the temperature was kept at 37 °C and the experiments were conducted at different pH values; 7.4, 8.8 and 10.0 using 0.05 M glycine–NaOH buffer. The effect of ionic strength was studied at 37 °C and pH 7.4 by using phosphate buffer containing 0.15 M NaCl as the electrolyte.

2.3. Drug-albumin interaction in the presence of the hydrophobic probe ANS

Experiments were also carried out in the presence of a hydrophobic probe, ANS. In the first set of experiments the interaction of drugs and ANS with albumin was studied under identical conditions. Albumin concentration was kept fixed at 10 μM and the ANS/drug concentration was varied from 1 to 14 μM. The fluorescence of albumin was recorded in the range 280–400 nm after excitation at 296 nm. In the second set of experiments the albumin–ANS interaction was studied in the presence and absence of drug. Increasing amounts of drug was added to an equimolar albumin–ANS mixture (10 μM each). The concentration of the albumin–ANS mixture was kept fixed at 10 μM each by adding the same volume of albumin–ANS mixture (20 μM each) to the cell. The fluorescence of ANS was recorded in the range 350–550 nm after excitation at 370 nm.

3. Results and discussion

3.1. Drug-serum albumin interaction

The structures of the drugs used in the present work meloxicam and nimesulide, are shown in Fig. 1. Perturba-

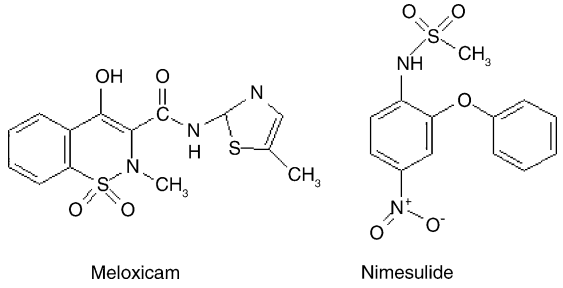

Fig. 1. Structures of meloxicam and nimesulide.

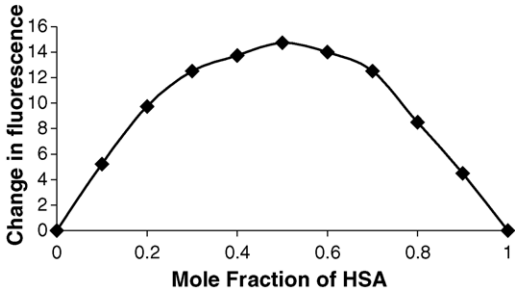

Fig. 2. Job's plot for nimesulide-HSA binding.

tion of the intrinsic protein fluorescence on drug binding was monitored and both drugs were found to quench the intrinsic fluorescence of serum albumin. However, there was no observable shift in the wavelength for maximum emission. The fractional occupancy of the total protein binding sites by drug was obtained from the ratio, $\theta = \Delta F/F_0$ [26,27], 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.

The number of binding sites on protein molecule (n) was estimated in each case by the continuous variation method [24,25]. As a representative example, the Job's plot for the nimesulide-HSA system at 37 °C and pH 7.4 is shown in Fig. 2. The maximum in the fluorescence change occurred at 0.5 mole fraction of drug in the case of both HSA and BSA, indicating thereby that the stoichiometry of the interaction is 1:1 in each case. Generally, spectroscopic methods are unable to identify more than one binding site. However, fluorescence quenching measurements are sensitive to perturbations in the tryptophan residue, which is the high affinity site for anionic drugs. Secondary binding at sites of lower affinity, remote from the fluorescent moiety are not detected.

If $[P_t]$ is the total protein concentration and n the number of binding sites, the total number of sites on protein is given by $n[P_t]$ and the concentration of bound sites on protein is given by $n\theta[P_t]$ [28], 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 using an iterative non-linear least squares regression program developed for this purpose.

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

The experimental data could be fitted into an equation for only one class of binding sites ($n = 1$) for both HSA and BSA. K and n values for both the drugs, are given in Table 1. The reported values are an average of three determinations with coefficient of variation less than 2% in each case. The association constants were lower in the case of HSA as compared to BSA. However, the order was 10^5 in each case suggesting strong drug–protein affinity for both the drugs.

3.2. Effect of temperature on protein binding of meloxicam and nimesulide

Thermodynamic parameters for the interaction of meloxicam and nimesulide with bovine and human serum albumin were determined from the binding studies carried out at three different temperatures. The binding parameters at different temperatures for meloxicam and nimesulide are given in Table 1.

It is seen that there was no significant change in the number of binding sites with increase in temperature indicating thereby increasing the 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 the drugs. Thermodynamic parameters, ΔG^0, ΔH^0 and ΔS^0 were calculated from the temperature-dependence of K, using Eqs. (3) and (4).

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

$$\log K = -\Delta H^0/2.303RT + \Delta S^0/2.303R \quad (4)$$

Table 1
Binding parameters for the interaction of meloxicam and nimesulide with bovine and human serum albumin at pH 7.4 and different temperatures

Temperature (K)	Meloxicam				Nimesulide			
	Association constant (K) $\times 10^{-5}$		Number of binding sites (n)		Association constant (K) $\times 10^{-5}$		Number of binding sites (n)	
	BSA	HSA	BSA	HSA	BSA	HSA	BSA	HSA
293.15	6.548	2.434	0.92	0.87	4.437	2.691	1.00	0.91
300.15	5.503	1.954	0.93	0.89	3.264	2.166	1.01	0.88
310.15	3.675	1.425	0.89	0.88	2.540	1.495	1.00	0.90

Table 2
Thermodynamic parameters for the interaction of meloxicam and nimesulide with bovine and human serum albumin

Thermodynamic parameter	Meloxicam		Nimesulide	
	BSA	HAS	BSA	HAS
ΔG^0 (kJ/mol)[a]	−33.049	−30.606	−32.096	−30.730
ΔH^0 (kJ/mol)	−25.602	−23.960	−24.079	−26.400
ΔS^0 (J/mol)	+24.282	+21.456	+25.785	+14.035

[a] ΔG^0 values have been calculated at 37 °C.

log 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. Values of various thermodynamic parameters for the interaction of meloxicam and nimesulide with human and bovine serum albumin are given in Table 2. It is seen that there is no significant difference in the sign and magnitude of thermodynamic parameters in the case of BSA and HSA. The positive entropy change occurs because the water molecules that are arranged in an orderly fashion around the ligand and protein acquire a more random configuration as a result of hydrophobic interactions. Negative ΔH^0 value cannot be attributed to electrostatic interactions since for electrostatic interactions, ΔH^0 is very small, almost zero [25,29]. Negative ΔH^0 and positive ΔS^0 values in the case of both meloxicam and nimesulide, therefore, showed that both hydrogen bond and hydrophobic interactions play a role in the binding of these drugs to HSA and BSA [30,31].

3.3. Binding studies in the presence of hydrophobic probe

In order to further understand the nature of interaction involved, binding was also studied in the presence of the hydrophobic probe ANS [32–34]. In the first set of experiments, the quenching of protein fluorescence by drugs and ANS was determined under identical conditions. Both drugs and ANS quench the fluorescence of albumin. ANS could quench about 46% of HSA fluorescence and about 72% of BSA fluorescence. The magnitude of quenching by drug was comparable to that of ANS in the case of meloxicam and slightly lower than that of ANS in the case of nimesulide in the case of both BSA and HSA (Fig. 3). The percentage quenching was 47 and 37% in the case of HSA and 68 and 60% in the case

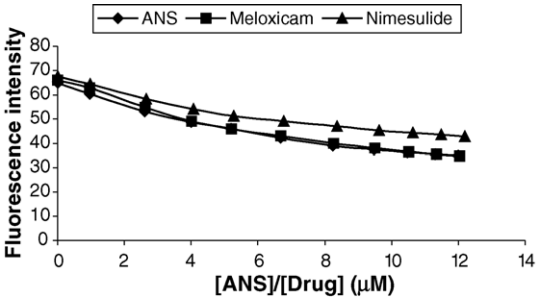

Fig. 3. Quenching of HSA fluorescence by ANS, meloxicam and nimesulide under identical conditions.

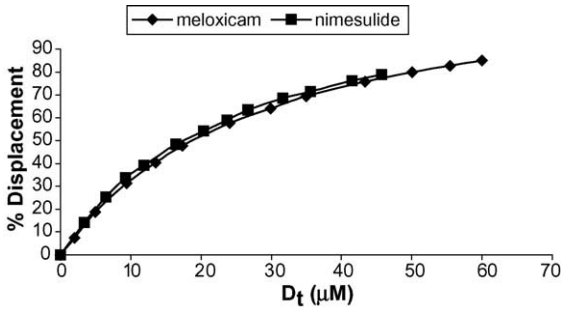

Fig. 4. Percentage displacement of hydrophobic probe, ANS from HSA by meloxicam and nimesulide.

of BSA for meloxicam and nimesulide, respectively, under identical conditions. It thus appears that the interaction is predominately hydrophobic and meloxicam and nimesulide share common site with ANS in albumin.

In another set of experiments ANS fluorescence was measured in albumin–ANS mixture in the absence and presence of increasing amounts of drug. It was found that in each case the presence of drug caused a significant decrease in the fluorescence of ANS, indicating thereby that the drugs displace ANS from its binding site. The effect was quantitatively studied by determining the percentage displacement of probe, D using the relationship, $D = (F_1 - F_2)/F_1 \times 100$, where F_1 and F_2 are the fluorescence intensities of ANS in the absence and presence of drug, respectively. The percentage displacement has been plotted against the drug concentration in Fig. 4. Maximum displacement of probe by drug, determined from the double reciprocal plot ($1/D$ versus 1/[drug]) (not shown), was found to be nearly 100% in the case of BSA and 76–79% in the case of HSA. Since ANS binds to albumin primarily by hydrophobic and to a lesser extent by ionic interactions [32,33], the present studies confirmed the earlier conclusion that the binding of meloxicam and nimesulide to serum albumin involves predominantly hydrophobic interactions.

3.4. Stern–Volmer analysis

Fluorescence quenching data at pH 7.4 and 37 °C was also analysed by the modified Stern–Volmer plot [35,36].

$$F_0/(F_0 - F) = 1/f_a + 1/(D_t f_a K_q) \quad (5)$$

where F_0 and F are the fluorescence intensities at 332 nm in the case of HSA and 344 nm in the case of BSA in the absence and presence of quencher (drug), respectively. K_q is the Stern–Volmer quenching constant and f_a is the fraction of fluorophore (protein) accessible to the quencher (drug). From a plot of $F_0/(F_0 - F)$ versus $1/D_T$ (not shown), f_a and K_q were determined. f_a was found to be close to unity indicating thereby that the tryptophan residues of HSA and BSA are fully accessible to the drugs. The Stern–Volmer quenching constant, K_q, was found to be 7.35×10^4 and $7.72 \times 10^4\ M^{-1}$, respectively in the case of meloxicam and nimesulide with HSA and 9.44×10^4 and $7.70 \times 10^4\ M^{-1}$,

Table 3
Effect of pH and presence of salt on the binding parameters of meloxicam and nimesulide with bovine serum albumin at 37 °C

pH/presence of salt	Meloxicam		Nimesulide	
	Association constant (K) × 10^{-5}	Number of binding sites (*n*)	Association constant (K) × 10^{-5}	Number of binding sites (*n*)
pH 7.4	3.675	0.89	2.540	1.00
pH 8.8	2.146	0.76	2.089	0.83
pH 10.0	2.331	0.80	2.431	0.86
0.15 M NaCl at pH 7.4	2.918	0.89	1.494	1.03

respectively in the case of interaction of meloxicam and nimesulide with BSA. 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 τ_0 value for tryptophan fluorescence in proteins is known to be of the order of 10^{-9} s [37], 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 drug. It can be shown that

$$k_q = 4\pi aDN_A \times 10^{-3} \quad (5')$$

where D is the sum of the diffusion coefficients of quencher and fluorophore, a the sum of molecular radii and N_A the Avogadro's number [38]. 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 the quenching is highly efficient, the tryptophan residues in hydrophobic regions of protein are highly exposed to drugs and thus the nature of interaction is mainly hydrophobic. Moreover, specific interactions such as hydrogen bonding increase the drug–protein encounter radius, '*a*' and make k_q larger. Thus the drugs are bound to albumin by hydrophobic interactions as well as hydrogen bond at a site which is close to the tryptophan residues.

3.5. Effect of pH

The binding of meloxicam and nimesulide with bovine serum albumin was also studied at three different pH values, 7.4, 8.8 and 10.0. Binding parameters are given in Table 3. These data showed a decrease in the association constants as well as the number of binding sites on increasing the pH from 7.4 to 8.8. The variation in the magnitude of binding parameters can either be due to the change in the degree of ionization of the protein or drug. Both meloxicam and nimesulide are almost fully ionized at pH 7.4 and 8.8. In the albumin molecule an increase in pH from 7.4 to 8.8 causes increased ionization of imidazole and amino groups, which results in an increase in the net negative charge on albumin. Since the drugs are also negatively charged, the repulsion between negatively charged species may be responsible for the decrease in the binding capacity. A small increase in the binding constant on increasing the pH from 8.8 to 10.0 can be attributed to the well known N-B transition in albumin in this pH range [11,39].

The concentration of free drug, $[D_f]$ at different pH values is given in Table 4 at different drug–protein ratios, ($[D_t]/[P_t]$). Thus the concentration of free drug in plasma is sensitive to the change in pH, although the effect is not very significant.

3.6. Effect of Ionic strength on binding

The interaction of meloxicam and nimesulide with bovine serum albumin was also studied in the presence of 0.15 M NaCl. Association constants and the number of binding sites for meloxicam and nimesulide in the presence and absence of 0.15 M NaCl are given in Table 3. In the case of both meloxicam and nimesulide the presence of salt decreased the association constants but the number of binding sites remained almost same. Since thermodynamic parameters for the binding of these drugs do not suggest electrostatic interactions, it appears that chloride ions displace the drugs from their binding sites and hence the binding constants are lowered in the presence of salt. A similar findings have also been reported by other workers [40,41]. It has been shown by

Table 4
Concentration of free drug at different drug:protein ratios for meloxicam–BSA and nimesulide–BSA systems

$[D_t]/[P_t]$	Concentration of free drug (D_f) (μM)							
	Meloxicam				Nimesulide			
	pH 7.4	pH 8.8	pH 10.0	In 0.15 M NaCl at pH 7.4	pH 7.4	pH 8.8	pH 10.0	In 0.15 M NaCl at pH 7.4
0.5	1.65	2.50	2.45	2.00	2.05	2.10	2.05	2.45
1.0	5.00	5.93	5.45	5.05	4.80	5.70	5.30	5.50
1.5	9.00	10.01	9.75	9.00	8.60	9.40	9.10	9.45
2.0	13.65	14.65	14.25	13.50	13.00	13.80	13.60	13.80
2.5	18.50	19.00	19.00	18.00	17.80	18.50	18.20	18.50
3.0	23.75	23.75	23.75	22.75	22.40	23.20	23.00	23.20

Wilting et al. [41] that chloride ions also affect N–B transition but at pH 7.4 a competition between drug and chloride ions is dominant.

$[D_f]$ values at different drug–protein ratios ($[D_t]/[P_t]$), in the presence and absence of salt are given in Table 4. It is seen that in the case of both meloxicam and nimesulide the presence of salt causes an increase in the concentration of free drug, although the effect is not very significant.

4. Conclusions

The nature of interaction of meloxicam and nimesulide with human and bovine serum albumin involve strong drug–protein interactions with only one high affinity site on the albumin molecule. The association constants were lower in the case of HSA as compared to BSA but the order was 10^5 in each case. Thermodynamic parameters as well as binding studies in the presence of a hydrophobic probe showed that the interaction is predominantly hydrophobic in nature. However, the role of hydrogen bonding cannot be ignored due to high negative enthalpy of binding. There was no significant difference in the sign and magnitude of thermodynamic parameters and hence the nature of interaction in the case of HSA and BSA. Stern–Volmer analysis of the fluorescence data showed that the tryptophan residues of HSA and BSA are fully accessible to the drugs. The high magnitude of the rate constant for quenching showed that the drugs are bound to albumin by hydrophobic interactions as well as hydrogen bonding at a site which is close to the tryptophan residues. Increase of pH and ionic strength caused a decrease in binding and an increase in the concentration of free drug but the effect was not very significant indicating thereby that electrostatic interactions are not involved.

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