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Binding of diclofenac sodium with bovine serum albumin at different temperatures, *p*H and ionic strengths

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The study was designed to examine the binding of diclofenac sodium with bovine serum albumin (BSA) at different temperatures (20°, 30° and 40°C), *p*H (6.4,7.4 and 8.4) and ionic strengths (μ = 0.1, 0.2 and 0.3) by means of equilibrium dialysis method. The concentration of diclofenac sodium was maintained at wider range from 15 to 900 μmole/l and BSA concentration was maintained at 61.5 μmole/l. The data obtained were interpreted by nonlinear regression method using Graphpad prism software. The analysis showed that the interaction between diclofenac sodium with BSA results in two-site saturable binding. A decrease in association constant was observed with increasing temperature. The average standard free energy change (ΔG°) value was –7.07 (site I) and –4.2 (site II) Kcal/mol. The standard enthalpy change (ΔH°) and the standard entropy change (ΔS°) were–7.8 Kcal/mole, –2.35 cal/mole (site I) and –7.4 Kcal/mole, –10.5 cal/mole (site II), respectively. The negative enthalpy change suggested the binding between diclofenac sodium and the binding sites of BSA were spontaneous and exothermic. The negative value of ΔH° and ΔS° indicated hydrogen bonding and van der Waal's force was the major mechanism for diclofenac sodium and BSA interaction. Increase in *p*H and ionic strength also caused decrease in association constant of diclofenac sodium and BSA binding.

Keywords: Albumin, Bovine serum, Diclofenac sodium, Ionic strength

A drug in plasma binds, much or less, to plasma proteins such as albumin[1], α_1-acid glycoprotein[2-4] and in a rare case with immunoglobulins[5] and quickly establishes binding equilibrium. The plasma protein binding of drugs has been shown to have significant effects on numerous aspects of pharmaco-kinetics (such as hepatic metabolism rate, renal excretion, biomembrane permeation rate and steady state distribution volume)[6,7] and pharmacodynamics[8]. If the drug is highly protein bound then displacement of drug from the binding site due to pathological states like impaired renal or liver function[9], presence of other competitive drugs for same site[10] or age[11] can increase or decrease the free and active form of drug serum concentration. It has been reported that diclofenac, 2-arylacetic acid, a non-steroidal anti-inflammatory drug binds to human plasma protein[12]. Existence of two classes of binding sites has been reported on human serum albumin for diclofenac sodium[13]. The present work was designed to perform an exhaustive study to evaluate the effect of concentration, temperature, *p*H and ionic strength on diclofenac sodium and bovine serum albumin (BSA) interaction, as studies related to the influences of these physicochemical properties on binding interaction are meagre. A probable mechanism of diclofenac sodium and BSA interaction was also postulated by determining different thermodynamic parameters.

Materials and Methods

Diclofenac sodium was obtained from Bengal Chemicals and Pharmaceutical Works Ltd., Kolkata, as a generous gift. BSA was obtained from Merck (I) Ltd. and dialysis tubing was obtained from Sigma Chemical Company, St.Louis, USA. All other chemicals and reagents used were of analytical and UV grade.

Determination of in vitro BSA-drug binding— Extent of protein binding of drug can be determined by equilibrium dialysis method[14-15]ultrafiltration[16], Capillary electrophoresis[17], high performance frontal

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analysis[18], bioassay[19] and chromatographic techniques[20], but equilibrium dialysis and ultra filtration are the most routinely used methods. Both of these methods have been proven to be experimentally sound and to yield adequate protein binding data[21]. The equilibrium dialysis was chosen for this study because of its ability to reasonably approximate physiological conditions and the per cent bound was determined indirectly by measuring the unbound fraction spectrophotometrically at 272 nm using UV-VIS spectrophotometer (Spectronic, U.K.). BSA solutions (0.4%w/v) were prepared using phosphate buffer and 0.1*M* of sodium chloride was added to adjust the required ionic strength (μ) based on the following equation,

$$\mu = \frac{1}{2}\sum_{1}^{j} c_i z_i^2 \quad \ldots (1)$$

where *c* is the concentration of any ionic species and z is its valence.

Study of diclofenac sodium and BSA binding at different temperatures—Dialysis tubes similar to those described in literature[22] were treated according to manufacturers' instruction and then it was soaked for 24 hr in the phosphate buffer solvent prior to use. A dialysis bag was prepared from the tube and 5 ml of BSA solution (0.4%w/v) containing 2ml of diclofenac sodium solutions in buffer (*p*H 7.4), ionic strength 0.1 of various concentrations in the range from 15 μ*M*/l to 900 μ*M*/l were lowered in 30 ml of dialyzing solutions. The dialysis procedure was performed for 24 hr with continuous stirring at very slow speed (6-10 rpm) at 20°C. A control containing drug in buffer solution was run simultaneously to determine the loss due to membrane binding of drugs in each case. In no case it was observed that not more than 3% of drug bound with the membrane and this correction factor was used to calculate the drug protein binding. Another control without drugs was also run to determine the probable albumin transfer across the membrane. Measuring the UV absorbance of the solvent at 278 nm checked albumin leakage. The limit of detection of this method was 0.02% of albumin. In all determinations the BSA concentration used was 0.4%(w/v) i.e. 61.5 μ*M*/l. The bound drug concentration was usually determined indirectly by measuring the concentration of unbound fraction in the drug or drug-protein complex. In the present work, and free drug concentration was measured spectrophotometrically at 272 nm after 24 hr of incubation. The same dialysis procedure was also performed at 30° and 40°C but at *p*H 7.4 and ionic strength 0.1. Three different temperatures were selected to produce the vant Hoff plot and higher temperatures were not selected as it might cause denaturation of protein and loss of solvent due to evaporation.

Study of diclofenac sodium and BSA binding at different pH—The equilibrium dialysis study of different concentrated solutions of diclofenac sodium and BSA were also performed using phosphate buffer (*p*H 6.4) and borate buffer (*p*H8.4) but at 30°C and ionic strength 0.1. The selection of buffer solution of *p*H 7.4 was based on physiological *p*H. The other two solutions were selected to observe the effect at lower and higher *p*H. Other conditions maintained as above. All controls were also run following the same conditions. The free fraction of drug was measured similarly by spectrophotometer at 272 nm.

Study of diclofenac sodium and BSA binding at different ionic strengths—Phosphate buffer solution (*p*H 7.4) was prepared and sodium chloride was added according to equation 1 to prepare buffer solution of ionic strength 0.2 and 0.3. The equilibrium dialysis study of different concentrated solutions of diclofenac sodium and BSA were done using the solvents and the free fraction of diclofenac sodium was measured to generate binding data. BSA concentration was maintained at 61.5 μ*M*/l. Higher concentration of salt was maintained to combat the donan effect. All controls were also run as described above.

Protein binding expressed in percentage was calculated from the following formula,

$$\%\ \text{Bound} + 100 \times \left(1 - \frac{\text{conc. of free drug in test sample}}{\text{conc. of free drug in control sample}}\right) \quad \ldots (2)$$

Results and Discussion

The binding of diclofenac sodium to BSA appeared to be a saturable process. The bound fraction decreased with increasing drug concentrations. The equilibrium dialysis study of solutions containing different concentrations of diclofenac sodium was performed in the absence and in the presence of BSA and the bound drug fraction was determined using equation 2. The most accepted way to treat the binding data using saturation binding according to the

following equations for both one-site and two-site bindings.

$$[D_b] = \frac{B_{max}.[D_f]}{K_d + [D_f]} \text{(for one-site binding)} \quad \ldots (3)$$

$$[D_b] = \frac{B_{max1}[D_f]}{K_{d1} + [D_f]} + \frac{B_{max2}[D_f]}{K_{d2} + [D_f]} \text{(for two-site binding)} \quad \ldots (4)$$

where $[D_b]$ is the concentration of drug bound, $[D_f]$ is the concentration of free fraction, B_{max} is the protein concentration and K_d is the dissociation constant for drug protein interaction.

The binding data obtained at different temperatures (20°, 30° and 40°C) and *p*H (6.4, 7.4 and 8.4) conditions were analyzed by nonlinear regression method using GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego, California USA, for both one- and two-site binding. Performing F-test, we obtained that the two-site binding was the best-fit model for diclofenac sodium and BSA interaction. The best-fit model equations are given below

At 20°C, *p*H7.4 and ionicstrength 0.1,

$$D_b = \frac{13.25*10^{-5} D_f}{4.973*10^{-6} + D_f} + \frac{6.743*10^{-4} D_f}{6.28*10^{-4} + D_f} \quad \ldots (5)$$

[Comparison of Fits for both one-site and two-site bindings DFn, DFd 2, 7, F143.4; *P*<0.0001]

At 30°C, *p*H 7.4 and ionicstrength 0.1,

$$D_b = \frac{15.24*10^{-5} D_f}{9.16*10^{-6} + D_f} + \frac{6.3*10^{-4} D_f}{9.63*10^{-4} + D_f} \quad \ldots (6)$$

[Comparison of Fits for both one-site and two-site bindings: DFn, DFd 2, 7 F 43.7; *P*<0.0001]
At 40°C, *p*H 7.4 and ionicstrength 0.1,

$$D_b = \frac{12.7*10^{-5} D_f}{10.47*10^{-6} + D_f} + \frac{6.9*10^{-4} D_f}{11.4*10^{-4} + D_f} \quad \ldots (7)$$

[Comparison of Fits for both one-site and two-site bindings: DFn, DFd 2, 7 F 77.75; *P*<0.0001]

At *p*H 6.4, constant temperature 30°C, and ionic-strength 0.1,

$$D_b = \frac{12.2*10^{-5} D_f}{5.55*10^{-6} + D_f} + \frac{7.9*10^{-4} D_f}{8.3*10^{-4} + D_f} \quad \ldots (8)$$

[Comparison of Fits for both one-site and two-site bindings: DFn, DFd 2, 7 F 102.9; *P*<0.0001]

At *p*H 8.4, constant temperature 30°C, and ionic-strength 0.1,

$$D_b = \frac{12.78*10^{-5} D_f}{11.05*10^{-6} + D_f} + \frac{6.3*10^{-4} D_f}{10.2*10^{-4} + D_f} \quad \ldots (9)$$

[Comparison of Fits for both one-site and two-site binding: DFn, DFd 2, 7 F 102.9; *P*<0.0001]
where $[D_b]$ is the concentration of drug bound, $[D_f]$ is the concentration of free fraction, B_{max} is the protein concentration and K_d is the dissociation constant for drug protein interaction.

The binding data for two sites have been depicted in Fig. 1a at different temperatures and Fig. 1b at different *p*H. The analyzed value of $K_{ass}(1/K_d)$ and B_{max} for two site binding have been given in Table 1.

To determine the effect of salt concentration on the diclofenac and BSA binding the equilibrium study was performed at different ionic strength (μ=0.2 & 0.3) but at 20ºC and *p*H 7.4. From the previous study, it has been observed that the intrinsic binding affinity for site II binding of BSA and diclofenac sodium is very small as compared to former one. Hence, to study the effect of salt concentration on the interaction of diclofenac and BSA, the concentration of diclofenac sodium was taken at lower side to form interaction at site I only. Data was analyzed by nonlinear regression method using graph pad prism software and has been shown in Fig. 1c. The analyzed value of $K_{ass}(1/K_d)$ at ionic strength 0.2, 0.3 for first site binding were obtained as 1.45×10^5 M^{-1}, 1.38×10^5 M^{-1} and the value of B_{max} for the same were 14.8×10^{-5} M/l, 14.3×10^{-5} M/l, respectively.

Table 1—Diclofenac sodium binding with BSA at different conditions (two-site binding results)

Different temp./*p*H	K_{ass1}	B_{max1}	K_{ass2}	B_{max2}
		*Temp. (°C)		
20	2.004×10^5	13.26×10^{-5}	1.59×10^3	67.53×10^{-5}
30	1.09×10^5	15.1×10^{-5}	1.04×10^3	62.8×10^{-5}
40	0.880×10^5	13.24×10^{-5}	0.727×10^3	76.58×10^{-5}
		**pH		
6.4	1.80×10^5	12.8×10^{-5}	1.2×10^3	76.61×10^{-5}
8.4	0.905×10^5	12.84×10^{-5}	0.98×10^3	63.2×10^{-5}

*pH 7.4 and ionic strength=0.1; **30°C, and ionic strength=0.1

Thermodynamic analysis and mechanism of interaction—The data obtained at different temperatures were treated thermodynamically to calculate the standard free energy (ΔG°) of drug-protein binding and the standard enthalpy change (ΔH°) using the following equations,

$$\Delta G^\circ = -2.303RT \log K_{ass} \quad \ldots (10)$$

The standard enthalpy change ΔH° can also be obtained from the following relationship

$$\text{Log } K_{ass} = -\Delta H^o/2.303RT + \text{constant} \quad \ldots (11)$$

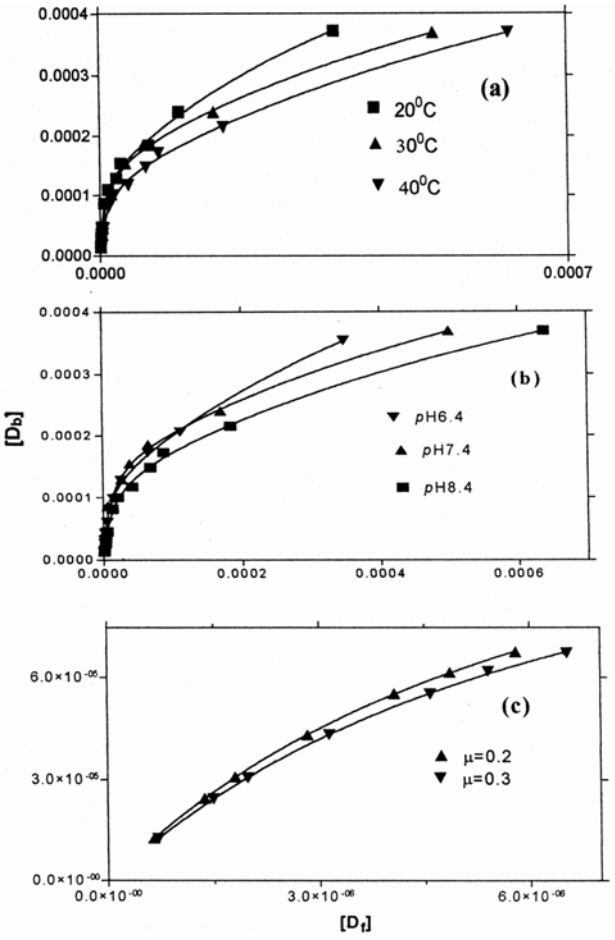

Fig 1—Diclofenac sodium-BSA interaction at – (a) different temperatures; (b) different *p*H; and (c) different ionic strengths

The constant term in equation (6) was actually equal to ΔS°/2.303RT [where ΔS° represents the standard entropy change].

Assuming that there is no significant temperature dependence of enthalpy change within the temperature range in which the interaction was carried out, it was possible to estimate the standard enthalpy change (ΔH°) from equation 6 by plotting log K vs 1/T(Van't Hoff equation) and the value of ΔS° computed from the following equation,

$$\Delta G^\circ = \Delta H^\circ - T.\ \Delta S^\circ \quad \ldots (12)$$

The value of thermodynamic parameters is presented in Table 2.

Decrease in association constant of BSA-diclofenac sodium interaction with increasing temperature clearly explained that it was an exothermic reaction and the negative sign for ΔG° meant that the binding process was spontaneous. The high negative ΔH^o value clearly explains that no electrostatic interactions between drug and protein molecules took place[23, 24]. The *p*H at which the interaction was carried out both the drug and BSA were in anionic form so it was again assumed that electrostatic interactions were not present. Small negative ΔS° value suggested that hydrophobic interaction was not predominated, because hydrophobic interactions result in positive entropy change[25,26]. Both enthalpy and entropy changes of the binding process were also negative which indicated some kind of donor accepter relationship between diclofenac sodium and binding sites of BSA[27, 28]. So, hydrogen bonding and van der Waal's forces are supposed to make major contribution to the binding of diclofenac sodium and BSA. The driving force of the binding sites (1st type) has been explained by 93% contribution of enthalpy change to free energy change. The results of diclofenac sodium and BSA binding at different *p*H suggested that intrinsic association constant was decreasing with increasing *p*H. It could also be concluded that binding was more predominated in unionized condition at lower *p*H since the pK_a of diclofenac was 4. Hence, it also indicated van der Waal's force and hydrogen bonding

Table 2—Thermodynamic parameters obtained from diclofenac sodium and BSA binding at different temperatures

Temperature (°C)	log K_{ass1}	log K_{ass2}	ΔG°_1 (Kcal/mole)	ΔG°_2 (Kcal/mole)	ΔH°_1 (Kcal/mole)	ΔH°_2 (Kcal/mole)	ΔS°_1 (cal/mole)	ΔS°_2 (cal/mole)
20°C	5.31	3.2	7.13	4.31	-7.8	-7.4	-2.35	-10.5
30°C	5.04	3.02	6.99	4.19				
40°C	4.94	2.86	7.09	4.10				

might play the major role in diclofenac sodium and BSA binding[29,30]. Increasing salt concentration also caused decrease in intrinsic binding affinity between diclofenac sodium and BSA. This suggested that hydrogen bonding played a role in diclofenac sodium and BSA binding[31].

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