Short communication

Interaction of Diclofenac with Bovine Serum Albumin Investigated by Diclofenac-Selective Electrode

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Abstract

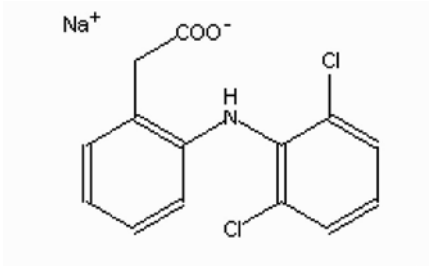
The binding of sodium diclofenac (SD) with bovine serum albumin (BSA) has been extensively studied at various concentration of BSA, using diclofenac-selective membrane electrodes constructed in our laboratory. The accurate binding isotherms have been obtained and analyzed in terms of binding capacity concept. The results represent two binding set system for all of studied conditions. The values of Hill equation parameters have been estimated and used for calculation of intrinsic Gibbs free energy of binding.

Keywords: Diclofenac sodium, bovine serum albumin, binding isotherm, binding capacity, diclofenac-selective electrode, Gibbs free energy.

1. Introduction

It is important to study the interactions of small ions, and molecules, with proteins in order to understand the nature of transportation and distribution of these species in biological systems because such interactions play a key role in transportation and distribution processes. Serum albumins are the major soluble protein constituents of the circulatory system; they have many physiological functions. The most outstanding property of albumins is that they serve as depot proteins and the transport proteins for numerous endogenous and exogenous compounds. The exogenous substances bonds with a high affinity to proteins are drugs. This interaction between protein and drug molecules results in a stable protein-drug complex formed. However the metal ions at trace concentration levels, which are stored in blood plasma, may affect the binding reactions of protein-drug complexes. Thus, it is necessary to investigate protein-drug interactions both in the presence and absence of metal ions. The binding data of serum albumin differ from species to species. BSA is well-suited to these initial studies because of their high affinity toward various ligands, since it has been extensively characterized.[1] BSA is a globular protein and has a compact ellipsoid structure. The stability of this structure originates mainly from hydrophobic interactions.[2–4] Study of binding phenomena will also help to explain the relationship between the structures and functions of proteins.

Sodium diclofenac (SD), or sodium 2-[(2,6-dichlorophenyl) amino]phenyl acetate (Scheme 1), is a nonsteroidal anti-inflammatory drug (NSAID) that is widely used as a therapeutic agent against rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, and acute gouty arthritis. Diclofenac binds extensively to plasma proteins.



Scheme 1. Structural formula of sodium diclofenac.

Drug interactions at protein binding level, in most cases significantly will affect the apparent distribution volume of the drugs and also affect the elimination rate of drugs. Therefore, the studies on this aspect can provide information of the structural features that determine the therapeutic effectiveness of drug, and have been an interesting research field in life sciences, chemistry and clinical medicine.[5–7]

The binding data for ionic ligand-protein interaction can be measured experimentally, using equilibrium dialysis and potentiometric techniques. Binding data analysis can reveal some important features of binding mechanism. However, different models of analysis that depends on various features of the binding process are required for this purpose. A number of methods for graphical and computer assisted analysis of the binding data for ligand (particularly surfactants)-protein interaction have been emplo-

yed. One of the most common presentations of such data is the Scatchard plot. However, there is not a general and comprehensive method for characterization of binding sets in ligand-protein system. One of the most popular concepts which introduced recently is the binding capacity concept (θ). It is the homotropic second derivative of the binding potential with respect to the chemical potential of the ligand and provides a measure for the steepness of the binding isotherm.[8,9,19]

The present study focused on interactions of diclofenac with Bovine serum albumin that play an important role in understanding drug transport, distribution and interaction, using diclofenac selective membrane electrode.

2. Experimental

2.1. Materials

BSA (Bovine Serum Albumin) obtained from Sigma Chemical Co., was used as received. Calculations were made assuming a molecular weight of 68000 D for BSA. Reagent grade dioctylphetalate (DOP) and high relative molecular weight PVC were purchased from Fluka and used as received. Tetrahydrofuran (THF) and all the salts (from Merck) were of the highest purity and used without further purification. Standard solutions and buffers were prepared using doubly distilled water. Pure sodium diclofenac powder was a gift from Drug Applied Research Center. (Tabriz, Iran). All potentiometric and pH measurements were made at 25 °C using Metrohm 744 (Switzerland). Ag/AgCl reference electrodes were purchased from Azar Electrode Co. (Urmia, Iran). All solutions were prepared with double distilled water.

2.2. Methods

Electrode preparation: The diclofenac-selective electrode which was used in this investigation has been constructed previously in our research laboratory and characterized extensively.[10] Our electrode shows some advantages in comparison with the other diclofenac-selective electrodes, reported in literatures due to its cheap and simple preparation method, long life time, short response time and etc.[10–16] The master membrane was fabricated by dissolving 42.0 mg of powdered PVC, 103.5 mg of plasticizer DOP and 4.5 mg of diclofenac-silver ion-pair complex in 5 mL of THF. The resulting mixture was transferred into a glass dish of 2 cm diameter. The THF was evaporated slowly at room temperature, until an oily concentrated mixture was obtained. A Pyrex tube (3–5 mm o.d.) was dipped into the mixture for about 10 s so that a membrane of about 0.5 mm thickness was formed. The tube was then pulled out from the mixture and kept at room temperature for about 4 h. A 1.0×10^{-4} mol L^{-1} of diclofenac solution was used as internal reference solution. The electrode was finally conditioned by soaking in 1.0×10^{-4} mol L^{-1} diclofenac solution for 24 h.

Emf measurements: The diclofenac ion-selective electrode (ISE) was used for measurement of the free concentration of diclofenac ions, $[D]_f$, in equilibrium with BSA- diclofenac complexes at various conditions. The following assembly was used for emf measurements:

Ag | AgCl | 3 M KCl ¦ Internal Solution
(1.0×10^{-4} M SD) | PVC membrane |
test solution ¦ 3 M KCl | AgCl | Ag

The potentials were measured relative to a Ag/AgCl reference electrode. In all experiments sample solution was continuously stirred using a magnetic stirrer.

The activity coefficient for an ionic species measures the deviation from ideal behavior resulting mainly from interionic interaction of an electrostatic nature. As at low ionic strength, the mean activity coefficient of different ions irrespective of charge and shape leads to unity. Because of the above discussions it is reasonable to assume that:

$$\gamma_{Cl^-} = \gamma_{dic^-} \approx 1.$$

Also by considering this fact that the electrode response depends on both diclofenac and co-ion (Cl^-) concentration, so at low and constant concentration of co-ion (ionic strength adjusted and kept constant using 1.0×10^{-4} mol L^{-1} KCl solution at all solutions), the electrode response varied only by variation at diclofenac concentration.[17,18] Least mean squares method was used for determination of the slope and intercept for each set of data.

3. Results and Discussion

Figure 1 shows the plot of emf versus logarithm of total diclofenac concentration, $\log[D]_t$, in the absence and presence of BSA at specified experimental conditions.

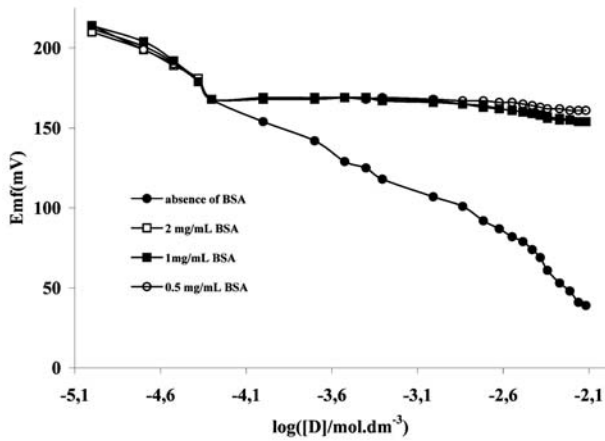


Figure 1. The variation of Emf vs. $\log[D]_t$, at 25 °C and pH = 6.9 in the absence and presence of BSA.

It is obvious that in the absence of BSA, emf is directly proportional to log[D]$_t$, with Nernstian slope (slope = 57–60 mV and R = 0.98) at 10^{-6} mol L^{-1} to 10^{-2} mol L^{-1} concentration interval of diclofenac.

But in the presence of BSA, the curves show two distinct regions as following (see Figure 1):

I) The first region, which is at very low concentrations of diclofenac, shows Nernstian slope, which is approximately equal to the corresponding value in the absence of BSA. Hence, it can be concluded that there is no measurable interaction between BSA and diclofenac at low concentrations.

II) The second region begins with a distinct break. This deviation from linearity is due to the interaction of diclofenac with BSA. The break point is known as critical aggregation concentration (CAC).

By considering this fact that emf is reduced in the presence of BSA, the amount of diclofenac bound to BSA can be calculated.

The average number of diclofenac molecules bound to each BSA molecule has been calculated as:

$$\nu = \frac{[D]_t - [D]_f}{C_p}, \qquad (1)$$

where C_p is the total concentration of BSA, $[D]_t$ and $[D]_f$ are total concentration of diclofenac ion was added to the potentiometric cell, and free concentration of diclofenac, respectively.

Figure 2 shows the binding isotherms (a semi logarithmic plot of the average number of diclofenac molecules bound to BSA molecule, ν, against the logarithm of the free diclofenac concentration, [D]$_f$) for interaction of diclofenac with BSA at the specified conditions. All plots are concave, characteristic of cooperative binding.

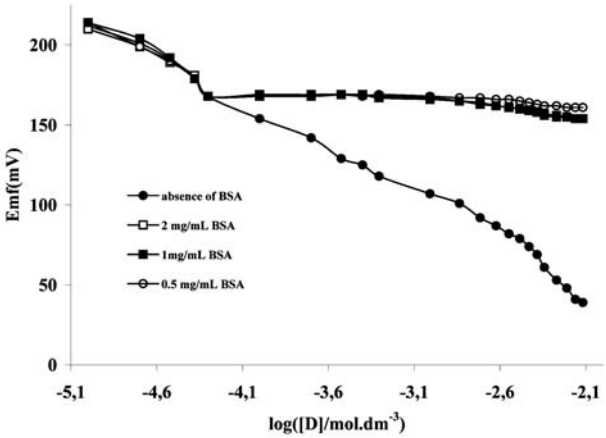


Figure 2, Binding isotherms of diclofenac interaction with BSA at 25 °C, pH = 6.9 and various concentration of BSA. (●) 0.5, (○) 1, and (■) 2 mg mL^{-1}.

The plot of binding capacity, θ, versus log[D]$_f$ can be a very useful approach for analyzing the binding isotherms; θ is derivative of the binding isotherms representing the change in the number of mole of diclofenac bond per mole of protein (ν), and related to the log[D]$_f$ according to the following equation:

$$\theta = \frac{\partial \nu}{\partial \mu_D} = \frac{\partial \nu}{RT\partial \ln[D]_f} = \frac{\partial \nu}{2.303RT\partial \log[D]_f} = \Sigma\theta_i, \qquad (2)$$

where μ_D is chemical potential of the diclofenac; the value of θ at any ν can be determined by calculating the slope of the binding isotherms. Where θ_i, is the binding capacity of i^{th} binding set, it provides a measure of steepness of the binding isotherm and hence can be a measure of cooperativity as expected by the Hill coefficient, n_{Hi}. In order to extract a relationship between, θ_i and n_{Hi}, the following usual definition of n_{Hi} can be used:

$$n_{Hi} = d\ln\left(\frac{y_i}{1-y_i}\right) \Big/ d\ln[CPC]_f = \left(\frac{1}{y_i(1-y_i)}\right)\left(\frac{dy_i}{d\ln[CPC]_f}\right), \qquad (3)$$

where y_i is the fractional saturation of ith binding set by CPC which is equal to ν_i/g_i.

From the definition of binding capacity, Eq.2, the following linear equation can be written:

$$\frac{RT\theta_i}{\nu_i} = n_{Hi} - n_{Hi}\left(\frac{\nu_i}{g_i}\right), \qquad (4)$$

It suggests that the plot of $RT\theta_i/\nu_i$ versus ν_i should be linear, where the slope, y and x-intercepts are $(-n_{Hi}/g_i)$, n_{Hi} and g_i, respectively. However; the total binding capacity (θ) can be determined from binding data is not θ_i, on the other hands, we can only calculate the total binding capacity (θ) from the experimental data, because θ is the summation of θ_i. By the way; for a system with relative high difference in binding affinity of sets, every set behave independently and so in which it can be assume that the i^{th} binding set has not been occupied until the full occupation of $(i-1)^{th}$ binding set has been occurred, for such system the curve of $(RT\theta/\nu)$ vs. ν can be divided to N-consecutive linear parts, corresponding to N binding sets. For evaluation of this assumption, the plots of $(RT\theta/\nu)$ vs. ν have been constructed for binding of diclofenac to BSA at various experimental conditions (Figures 3–5).

All of these curves can be divided to two separated linear regions. Intercept of first and second parts should be equal to g_1 and $g_1 + g_2$, respectively. The corresponding Hill plots for first and second binding sets can be constructed from estimated values of g_1 and g_2, respectively.[19,20] the collective estimated binding parameters of Hill equation are listed in Table 1. n_{Hi} is the Hill coefficient , Eq.3, (amount of the Hill coefficient is always po-

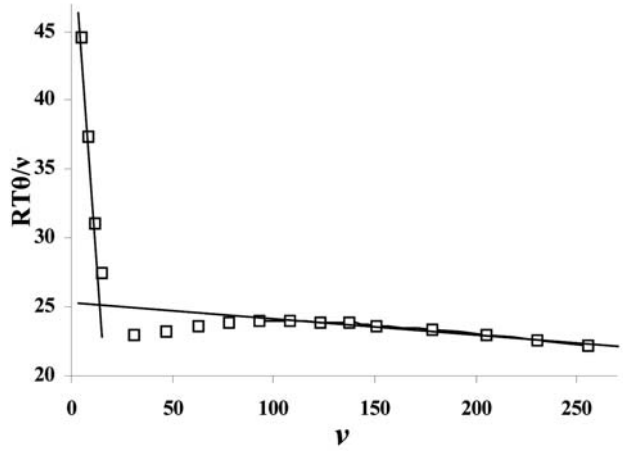


Figure 3. The plots of RTθ/ν versus ν for interaction of diclofenac with BSA at 25 °C, pH = 6.9 and 0.5 mg mL^{-1} of BSA.

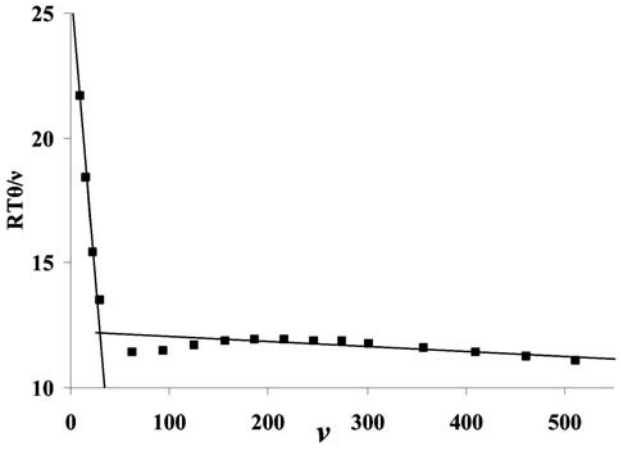


Figure 4, The plots of RTθ/ν versus ν for interaction of diclofenac with BSA at 25 °C, pH = 6.9 and 1mg mL^{-1} of BSA.

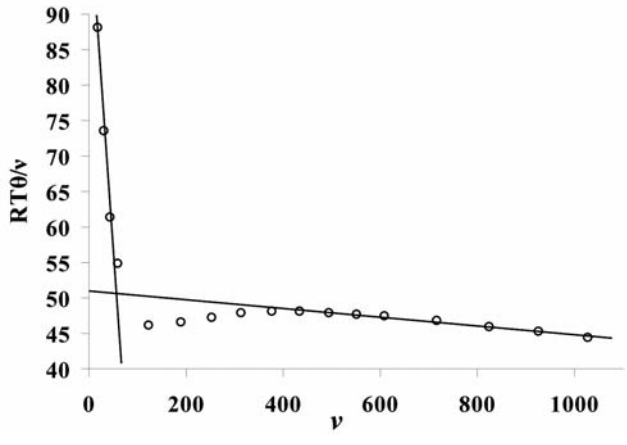


Figure 5. The plots of RTθ/ν versus ν for interaction of diclofenac with BSA at 25 °C, pH = 6.9 and 2 mg mL^{-1} of BSA.

sitive $n_H > 0$) , if $n_H < 1$, the systems by negative cooperativity affect binding and if $n_H > 1$ it indicated the systems by positive cooperativity in binding process. K_H is the binding constant, from the related amount for K_H in Table 1, it can be seen that binding strength at second binding set has been decreased by increasing of protein concentration but the binding strength in the first binding set remained approximately constant (see Table 1). Because in the first binding set, interaction driven forces are both electrostatic and hydrophobic interactions and at the second binding set, hydrophobic interactions have the major role in diclofenac-BSA interaction (the amount of K_H for the first set is greater than the K_H amount for the second set in all BSA concentrations). Also it can be concluded that, due to protein self-aggregation the binding affinity of second set decreased by increasing of protein concentration (protein self-aggregation affected the hydrophobic interactions in the second binding set and decreased interactions affinity on this set). Decrease in the g value (the total binding centers in each binding set) for the both sets, by increasing of BSA concentration shows self-aggregation of protein at high concentrations. Results were summarized in Table 1.

Table 1. Values of Hill parameters for interaction of diclofenac with BSA at 25 °C.

pH	[BSA] (mg mL^{-1})	g_1	g_2	lnK_{H1} (mol L^{-1})$^{-1}$	$lnKH_2$ (mol L^{-1})$^{-1}$	nH_1	nH_2
6.9	0.5	118	1282	9.55	8.46	4.16	6.1
6.9	1	58	762	9.69	5.75	4.21	4.57
6.9	2	30	545	9.42	3.78	4.11	3.52

The intrinsic Gibbs free energy of binding per mol of diclofenac for the first, $\Delta G^{\circ(1)}_{b,\nu}$, and the second, $\Delta G^{\circ(2)}_{b,\nu}$, binding sets can be obtained by the following equations.[20]

$$\Delta G^{\circ(1)}_{b,\nu} = -RTn_{H1}\ln K_{H1} + RT(1-n_{H1})\ln[D]_f, \quad (5)$$

$$\Delta G^{\circ(2)}_{b,\nu} = -RTn_{H2}\ln K_{H2} + RT(1-n_{H2})\ln[D]_f, \quad (6)$$

Figure 6, shows the variation of $\Delta G^{\circ(i)}_{b,\nu}$ versus $\ln[D]_f$ for interaction of diclofenac with various concentrations of BSA. The big jump, which has been observed in all of the curves in Figure 6 is occurred after the occupation of the first binding set. This is due to the large difference in binding affinity of two sets (the binding forces in second binding set are predominantly weak hydrophobic interactions while a combination of strong electrostatic and hydrophobic attractive forces are account in the first binding set).

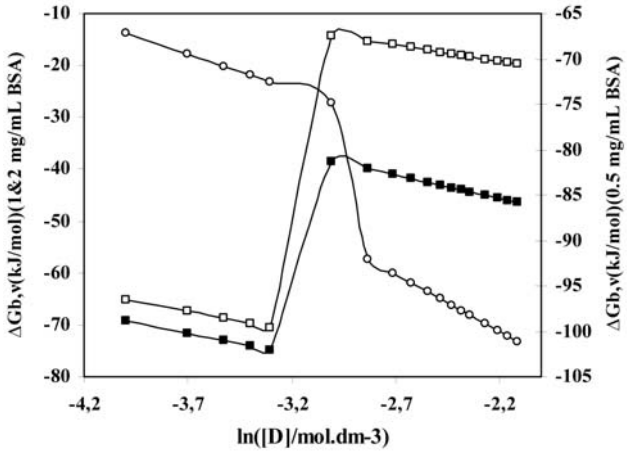


Figure 6. The variation of intrinsic Gibbs free energy of binding per mole of diclofenac, $\Delta G_{b,v}$,as a function of $\log[D]_f$ for binding of diclofenac to BSA at 25 °C, pH = 6.9 and various concentration of BSA: (○) 0.5 mg mL^{-1} (■) 1 mg mL^{-1} and (□) 2 mg mL^{-1}.

4. Conclusions

The process shows positive cooperativity in both binding sets ($n_{Hi} > 1$) for all of the studied conditions. The positive cooperativity in first binding set represents that both electrostatic and hydrophobic interactions contribute in this set. The positive cooperativity in the second binding set refers to essentially hydrophobic nature of interaction in this set.

The $\Delta G^{\circ}_{b,v}$ curves represents the relative variation of intrinsic Gibbs free energy of binding. The slope of the lines in these curves relates to Hill coefficient. Figure 6 shows that the binding strength of the first and second binding sets decreased by increasing of BSA concentration from 0.5 mg mL^{-1} to 2 mg mL^{-1}. Also comparison between binding strength in second binding set shows that binding strength, strongly affected by variation of protein concentration and indicated that at high concentration of BSA aggregation of protein have been occurred.

The big jump which has been observed in all of the curves in Figure 6 is occurred after the occupation of the first binding set. This is due to the large difference in binding affinity of two sets.

5. Acknowledgement

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6. References

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Povzetek

Vezavo natrijevega diklofenaka na goveji serumski albumin (BSA) smo raziskali za različne koncentracije BSA z uporabo diklofenak selektivne membranske elektrode lastne konstrukcije. Dobili smo izoterme vezanja in jih analizirali glede na kapaciteto vezanja. Rezultati kažejo, da gre za sistem z dvema vrstama vezavnih mest za vse preučevane pogoje. Ocenili smo vrednosti parametrov Hillove enačbe in jih uporabili za izračun Gibsove proste energije vezanja.