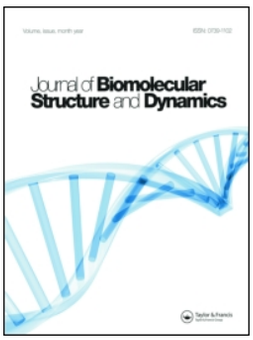

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LETTER TO THE EDITOR

Bio thermodynamic studies of diclofenac interaction with lysozyme under various conditions using diclofenac-selective membrane electrode and molecular docking

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Introduction

According to the fact that usually it is the unbounded drug that is pharmacologically active, the extent of drug–protein binding can significantly affect drug distribution and elimination. In some diseases, the blood albumin concentration decreases that known is as hypoalbuminemia (e.g. diabetes, hepatic, protein-losing enteropathy, nephrotic syndromes, etc.). In these cases, by decreasing serum albumin concentration, the free concentration of drugs (the active drug) increases at any given total drug concentration in the blood. The drug–protein binding causes the time-dependent release of drug and drug stability, and distribution can be controlled by protein binding. By decreasing free drug concentration in blood, the chemical potential for bonded drug decreases and so the protein releases the bonded drug for reducing chemical potential and reaching to new equilibrium. So drugs binding to proteins can increase drug life in blood circulatory system and suitable therapeutic drug concentration can be obtained. But by decreasing blood protein level, the drug metabolization, elimination, and local aggregation increases. This phenomenon may give rise to drug toxicity in patients who nonetheless have a total serum concentration of the drug within the therapeutic range. In addition to albumin, various blood constituents such as red blood cells and α-1-acid glycoprotein are capable of binding drugs. The concentration in plasma of α-1-acid glycoprotein, a protein that binds many basic drugs, increases with infectious, inflammatory, and malignant diseases, and after surgery (Kwong, 1985; Piafsky, Buda, & MacDonald, 1980; Piafsky & Mpamugo, 1981; Soldin, 1999; Soldin & Soldin, 2002). Drug–protein binding studies can be useful in construction and application of drug–protein complexes in therapeutic uses and drug concentration and distribution controlling. Pharmacokinetic behavior, pharmacological response, delivery rate, biosafety, toxicity, therapeutic dosage, and efficacy and drugs design are dependent on the nature, mechanism, biomolecular forces, and the magnitude of protein–ligand interactions. The model proteins have a great role in these studies and have improved the scientific understandings of these biochemical phenomena.

Lysozyme is a small monomeric low molecular weight (14,307 Da) globular protein that was discovered by Fleming in 1922. Lysozyme has emerged as a model protein for various investigations on protein structure and function. This enzyme is a single-chain polypeptide that contains a-helix, b-sheet turns, and disordered structural elements. The primary structure of lysozyme consists of 129 amino acid cross-linked with four disulfide bridges residues including six tryptophan and three tyrosine residues. The enzyme isoelectric point (pI) is 11.35. Lysozyme is often used for lysing bacterial cells by hydrolyzing the peptidoglycan present in the cell walls. Gram-positive bacterial cells are quite susceptible to this hydrolysis as their cell walls have a high proportion of peptidoglycan. Gram-negative bacteria are less susceptible due to the presence of an outer membrane and a lower proportion of peptidoglycan (Bordbar, Hosseinzadeh & Norozi, 2007; Kuehner et al., 1999; Lu, Su, & Howlin, 1999; Sigma-Aldrich, 2013; Strang, 1984).

Diclofenac is a nonsteroidal anti-inflammatory drug (NSAID) that known as diclofenac in generic name (dye KLOE fen ak), also the common brand names that used for diclofenac are; Cambia, Cataflam, Voltaren-XR, Zipsor, Zorvolex, Voltaren, Dyloject (Ensafi, Izadi, & Karimi-Maleh, 2013). The name "diclofenac" is derived from its chemical name; 2-(26-dichloranilino) phenyl acetic acid. Diclofenac works by reducing substances in

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the body that cause pain and inflammation and used to treat mild to moderate pain, or signs and symptoms of osteoarthritis or rheumatoid arthritis. Diclofenac can interact and bind extensively to various proteins as well as plasma proteins (Hosseinzadeh & Gheshlagi, 2009; Hosseinzadeh, Maleki, & Matin, 2007; Maleki, Matin, Hosseinzadeh, & Jouyban, 2007).

In the current work, interactions of diclofenac with lysozyme were considered using diclofenac-selective membrane electrode as diclofenac detector in solution and also molecular docking methods. Obtained data play an important role in understanding drug transport, distribution, illumination, and interaction using simple, fast, and accurate potentiometric method by diclofenac-selective membrane electrode.

Materials and methods

Materials

Lysozyme obtained from Sigma Chemical Co., was used as received. All of the calculations were made assuming 14,000 Da as lysozyme molecular weight. Dioctyl phetalate (DOP) and poly vinylchloride PVC (high relative molecular weight) were supplied by Fluka. All of the used salts were purchased from Merck chemical company that were at analytical grade purity and used without further purification. Standard solutions and buffers were prepared freshly using deionized water. HPLC grade tetrahydrofuran (THF), as PVC solvent, was obtained from Merck. Pure sodium diclofenac powder was a gift from Drug Applied Research Center (Tabriz, Iran). Potentiometric measurements were made at 25°C using Metrohm 744 (Switzerland) instrument. Ag/AgCl reference electrodes were purchased from Azar Electrode (Urmia, Iran).

Methods

The diclofenac-selective membrane electrode was constructed based on method that was reported previously (Hosseinzadeh, Maleki, & Matin, 2007). The method for constructing the electrode was described briefly here; the membrane was fabricated by dissolving certain amounts of powdered PVC, plasticizer (DOP), and diclofenac-silver ion-pair complex in 5 mL of THF. The resulting mixture was transferred into a glass dish. The THF was evaporated slowly for obtaining concentrated mixture. Pyrex tubes (3–5 mm o.d.) were dipped into the mixture for constructing the membrane of about .5-mm thickness. The produced membranes were kept at room temperature and in the dust-free place for about 4 h for THF evaporation and final membrane structure obtaining. A .1 micromole solution of diclofenac was used for membrane conditioning and also as internal reference solution. The electrode conditioning was done by considering for 24 h. The produced membrane electrode was used for measurement of the free concentration of diclofenac ions in experimental solutions. The following electrochemical assembly was used for potentiometric measurements:

Ag/AgCl | internal solution (1.0×10^{-4} M) | PVC membrane |test solution | AgCl/Ag

The diclofenac-selective membrane electrode was used for measurement of the free concentration of diclofenac in equilibrium with lysozyme–diclofenac complexes under various conditions. In all experiments, sample solution was continuously stirred using a magnetic stirrer.

Molecular modeling

Molecular docking was carried out using AutoDock 4.2 and AutoDock Tools version 1.5.6 with standard parameters (Hosseinzadeh, Khorsandi, Sheikh-Hasani, & Khatibi, 2016; Morris et al., 2009). Crystal structure of lysozyme was obtained from Protein Data Bank (pdb code 2LYZ) (Sanner, 1999) and ligand structure was constructed and energy minimized using PRODRG online server (Hosseinzadeh et al., 2016; Schüttelkopf & van Aalten, 2004). Schematic diagram of protein–ligand interactions was generated using LigPlot + version 1.4.5 (Figure 6) (Wallace, Laskowski, & Thornton, 1995).

Results and discussion

The plot of potentials (Emf) vs. logarithm of added concentration of diclofenac in the presence and absence of lysozyme is presented in Figure 1 and specified experimental conditions are mentioned in figure legend. It is obvious that in the absence of lysozyme, Emf is directly proportional to logarithm of diclofenac

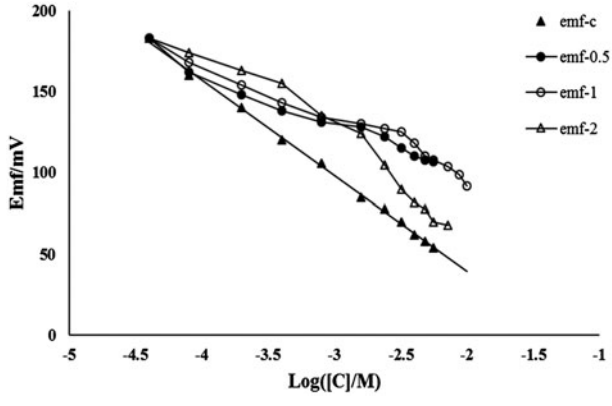

Figure 1. The plot of Emf vs. log[Diclofenac], at 25°C and in absence or at various concentrations of lysozyme.

concentration with Nernstian slope. According to the Figure 1, in the presence of lysozyme, the curves show two distinct sections as following: 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 lysozyme. Hence, it can be concluded that there is no measurable interaction between lysozyme and diclofenac at low concentrations. The second region begins with a distinct break in plot. This deviation from linearity is due to the interaction of diclofenac with lysozyme. By considering this fact that Emf decreasing in the presence of lysozyme is due to drug–protein binding so the amount of bounded drug to lysozyme can be estimated. These plots can be analyzed in order to obtain the average number of bound diclofenac ions per lysozyme molecule, ν, at various free concentrations of diclofenac using previously reported method (Bordbar & Hosseinzadeh, 2006; Bordbar, Sohrabi, & Gharibi, 2004; Hosseinzadeh, Maleki et al., 2007). Figure 2 shows the binding isotherms in interaction of diclofenac with lysozyme, at specified experimental conditions. The similar plots were obtained under other different conditions. All plots are concave that is the characteristic of cooperative binding. The Scatchard method is common procedure for calculating the affinity constant of a ligand binding to protein. The Scatchard plots were constructed from Scatchard equation. For instance, the corresponding Scatchard plot related to binding isotherms in Figure 2 is presented in Figure 3. These are not coincident with usual shapes of Scatchard plots and can represent the existence of more than one binding set in diclofenac interaction with lysozyme. The plot of binding capacity, θ, vs. $\log[\text{Diclofenac}]_{\text{free}}$ is 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 lysozyme and related to the

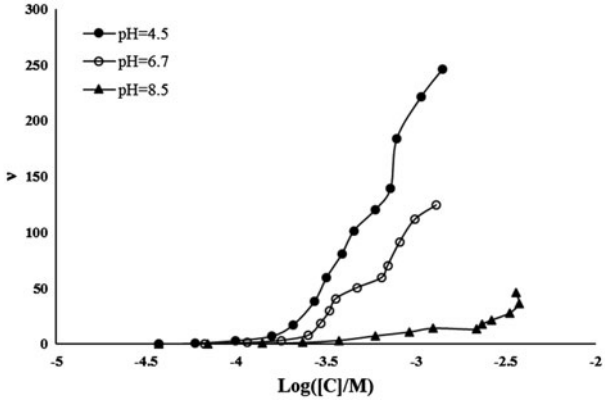

Figure 2a. Binding isotherm of interaction of diclofenac with lysozyme at various pHs.

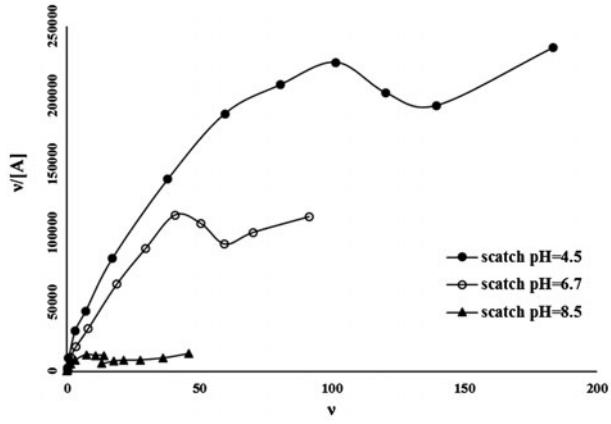

Figure 2b. Schatchard plots correspondence to binding isotherm of interaction of diclofenac with lysozyme at various pHs.

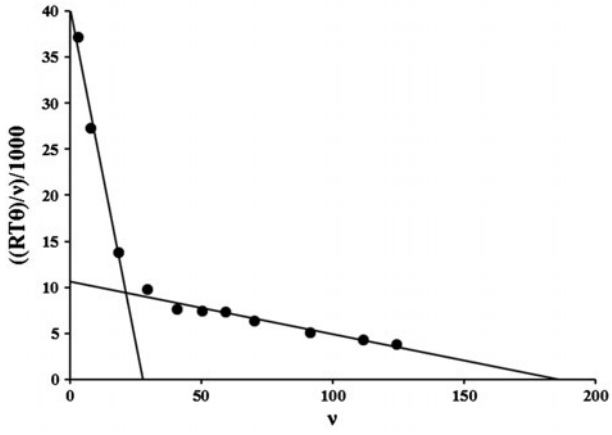

Figure 3a. The sample plot of RTθ/ν vs. ν for interaction of diclofenac with lysozyme at 25°C, pH 6.7 and 1 mg/mL of Lysozyme.

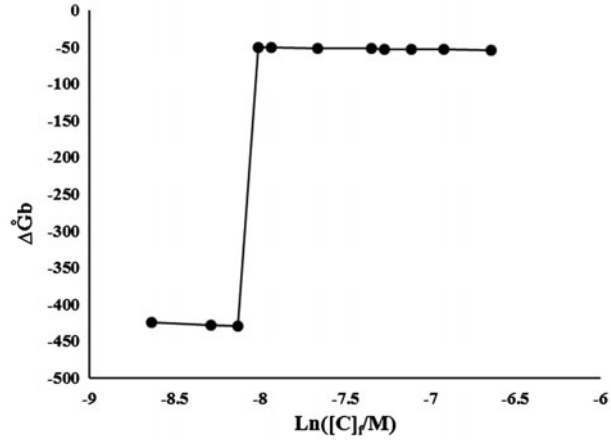

Figure 3b. The variation of intrinsic Gibbs free energy of binding per mole of diclofenac, as a function of log[Diclofenac] 25°C, pH 6.7, and 1 mg/mL of lysozyme.

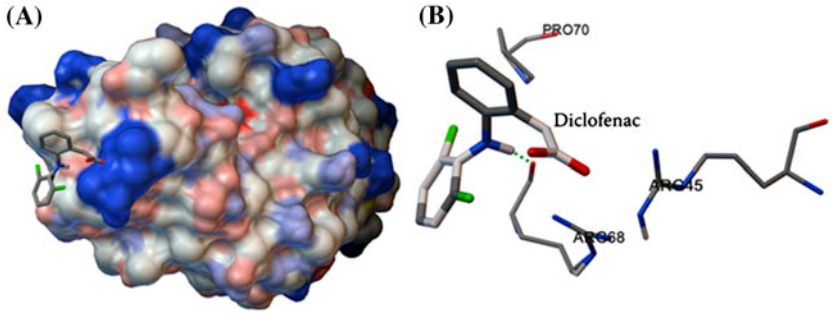

Figure 4. (A) Schematic diagram of lysozyme–diclofenac interactions. (B) Docking schematic surface interactions between diclofenac and lysozyme.

log[Diclofenac]$_{free}$ according to the binding capacity concept equation. This equation can provide a measure of steepness of the binding isotherm and hence can be a measure of cooperativity as expected by the Hill coefficient, n_H so this concept is directly related to the type and the extent of cooperativity (Bordbar et al., 2004; Hosseinzadeh, Bordbar, Matina, & Maleki, 2007; Hosseinzadeh, Maleki et al., 2007).

The corresponding plot of [RTθ/υ] vs. υ, for obtained binding isotherms in Figure 2, is shown in Figure 4. Similar plots obtained for other experimental conditions. All of these curves can be divided into two separated linear regions. The values of x-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. According to obtained results, it is clear that there are two binding set for diclofenac binding to lysozyme. g_1 and g_2 represent total binding sites in the first and second binding sets, respectively. According to the difference between binding affinity in interaction of diclofenac with lysozyme sites on the first and second binding sets, the energetics of interaction differ at these sets. All of the binding parameters of Hill equation are estimated and listed in Table 1. According to the results, the Hill coefficient (n_H) differs for each set but the both sets show positive cooperativity ($n_H > 1$) in binding of diclofenac to lysozyme. By increasing ionic strength, the electrostatic interactions decrease, so it can be concluded that the second binding set contains both electrostatic and hydrophobic interactions. In second binding sets, the hydrophobic forces have major contribution in binding forces. The intrinsic Gibbs free energy of binding per mole of diclofenac for the first and the second binding sets can be obtained using related equations (Hosseinzadeh et al., 2016). Figure 3b represents the variation of Gibbs free energy of binding sets vs. Ln[Diclofenac]$_{free}$ for interaction of diclofenac with lysozyme at figure legend specified experimental conditions. The big jump, which has been observed in plots at

Table 1. Hill equation parameters for diclofenac binding to lysozyme.

	g_1	g_2	n_1	n_2	Ln K_1	Ln K_2
.5 mg/mL	29	385	4.3	1.85	35.26	12.63
1 mg/mL	28	157	5	1.97	41.22	14.32
2 mg/mL	2	24	5.64	1	52.98	5.4
pH 4.5 (1 mg/mL)	60	261	4.72	2.6	39.21	19.49
pH 6.7 (1 mg/mL)	28	157	5	1.97	41.22	14.32
pH 8.5 (1 mg/mL)	2	116	2.98	2.09	25.28	10.83
IS = .01	22	112	2.79	1.33	22.43	9.91
IS = .1	28	168	3.33	1	26.49	5.98
IS = .5	6	107	2.05	2.26	15.99	14.34

Figure 3b, occurred after the occupation of the first binding set. This is due to the large difference in binding affinity of two binding sets on the protein. The binding forces in second binding set are predominantly weak hydrophobic interactions, while a combination of strong electrostatic and hydrophobic forces are accounted in the first binding set. According to the isoelectric point of lysozyme, at pHs below 10, the net charge on the surface of enzyme is positive charge and so the electrostatic attractive ionic interactions have great role in first binding sets in all experiments. The effect of ionic strength on binding forces is in agreements with this statement and in agreement with results of various pHs' effect on binding of diclofenac to lysozyme. According to molecular docking results, diclofenac molecule showed a high affinity to the surface of lysozyme. This was mostly because of the electrostatic interactions between polar parts of drug and surface of the protein. As it can be seen from Figure 4(A), a good shape and charge complementarity occurred between drug and protein surface which can be seen with more details in Figure 4(B). According to this figure, electrostatic and hydrogen bonding are dominant factors in diclofenac–lysozyme interactions. The protein aggregation also can affect the binding of diclofenac to lysozyme. According to the obtained results at higher concentration of lysozyme,

the protein shows higher potential for aggregation in the presence of diclofenac that it is due to the occupation of surface positive charges on the lysozyme with diclofenac anions that increase in hydrophobicity of complex and consequently increased the aggregation potential at higher concentration of protein.

Conclusion

Binding capacity concept was used for binding data analysis for diclofenac interaction with lysozyme and was shown there are at least two binding sets for diclofenac on lysozyme enzyme. The results demonstrate a combination of electrostatic and hydrophobic forces existing in the binding of diclofenac to lysozyme. The results show positive cooperativity in both binding sets in all of the studied conditions. The positive cooperativity at first binding set demonstrated that both electrostatic and hydrophobic interactions contribute in this set, but electrostatic interaction is the major interaction in this set. Also, the positive cooperativity in the second set refers to majority of hydrophobic forces in the second set. The binding strength of the first and second sets decreased by an increase in lysozyme. The results show that binding strength was strongly affected by variation of protein concentration and indicated that at high concentration of lysozyme, aggregation of protein has occurred. At pHs below 10, the net charge on the surface of enzyme is positive and so the electrostatic interactions have a great role in first binding sets. The hydrophobic forces are greater in second set. These results are in good agreement with effect of ionic strength in diclofenac binding to lysozyme.

Disclosure statement

No potential conflict of interest was reported by the authors.

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