

Original Research Article

***In Vitro* analysis of the interaction between**

ketorolac tromethamine and bovine serum albumin using fluorescence spectroscopy

ABSTRACT

Objectives: To evaluate the *in vitro* mutual interaction of ketorolac tromethamine with bovine serum albumin (BSA) using fluorescence spectroscopy under different conditions.

Material and methods: Different concentrations of ketorolac were mixed with 20 μ M bovine serum albumin solution at pH 7.4 and stirred for 2 min at 298 K & 308 K temperatures. Finally different ketorolac-BSA complex at the excitation wavelength 280 nm & 293 nm was measured by the fluorescence spectrophotometer.

Results: It was observed that the fluorescence quenching of BSA by ketorolac is a result of dynamic quenching with probable involvement of tryptophan residue. Fluorescence quenching constants were determined using the Stern-Volmer equation. The thermodynamic parameters such as Gibb's free energy (ΔG), enthalpy change (ΔH), and entropy change (ΔS) at different temperatures were studied by using Van't Hoff equation. The values of ΔG , ΔH and ΔS at 298K were -33.25 KJ/mol, 17.093 KJ/mol and 168.94 J/mol for ketorolac. The binding process for ketorolac had been found to be spontaneous, exothermic and entropy driven as indicated by thermodynamic analysis and hydrophobic forces playing a major role in the ketorolac-BSA association.

Conclusions: The interaction of ketorolac with BSA was successfully explored using a fluorescence spectroscopic technique.

Key words: Ketorolac tromethamine, bovine serum albumin, fluorescence quenching, Thermodynamic parameters.

INTRODUCTION

Ketorolac tromethamine is a non-steroidal anti-inflammatory drug (NSAID) that exhibits analgesic activity. Ketorolac inhibits the synthesis of prostaglandins through inhibition of the cyclooxygenase enzyme system. Ketorolac tromethamine tablets are available in the market for the treatment of fever and inflammation or swelling and relieve pain. It is also used to treat pain, swelling and stiffness associated with arthritis.

Protein binding of a drug is a limiting factor for drug effect. The type and nature of protein binding depend on the physicochemical properties of the drug molecules, their concentration, pH of the medium and also on the concentration and number of available binding sites of the plasma proteins [1]. Serum albumins are the most abundant proteins in blood as it binds with the drug and forms a drug-protein complex which strongly influences drug distribution and the free fraction, which is available to the target [2]. Therefore the investigation of such molecules with respect to albumin binding is of imperative and fundamental importance. The interaction between drug molecule and protein may result in the formation of stable and reversible drug-protein complex having important effect on the distribution, metabolism and excretion of drug [3]. Bovine and human serum albumins display approximately 76% homology and 3D structure of BSA is believed to be similar to that of HSA. In this work BSA is selected as our protein model because of its medical importance, low cost, ready availability, and the results of all studies are consistent with the fact that bovine and human serum albumins are homologous proteins [4]. Spectral methods are powerful tool for the study of the reactivity of chemical and biological systems since it allows measurements of substance in low concentration under physiological conditions, and there are several studies of albumin induced by drugs or other bioactive small molecules using spectral methods [5]. In this work, we used fluorescence spectroscopic method to obtain information related to the binding mechanism of ketorolac to BSA such as participating residues, quenching rate constant, binding modes, binding constant and also the number of binding sites.

MATERIALS AND METHODS

Materials:

Ketorolac (KT) was obtained from Incepta Pharmaceuticals Ltd. Bangladesh. BSA (fatty acid free, fraction V, 96-98%) was purchased from Sigma Chemical CO, USA. All other chemicals were of analytical reagent grade and purchased from local supplier. Doubly distilled water was used throughout the work.

Apparatus:

Steady-state fluorescence measurements were carried out on a Hitachi F-7000 fluorescence spectrophotometer (Hitachi, Japan) equipped with 1.0 cm quartz cells. For different temperature a thermostat bath (Unitronic Orbital, P- Spectra, Spain) was used.

Spectroscopic measurement:

The fluorescence spectra of drug were performed at different temperatures (298° K and 308° K). The widths of both entrance and exit slit were set to 5 nm. Most of the fluorescence emission spectra in the range of 320- 460 nm were recorded at excitation wavelength of 280 nm and 293 nm, respectively, in the same experimental conditions. Ketorolac has no fluorescence in the range of 320- 460 nm. The mixture solution of BSA and ketorolac must be hatched at least 10 min before the spectrum measurements.

Principle of fluorescence quenching:

Fluorescence quenching is the decrease of the quantum yield of fluorescence from a fluorophore induced by a variety of molecular interaction with quencher molecule [4]. The fluorescence quenching data are usually analyzed by Stern- Volmer equation [5]:

$$F_0/F = 1 + K_{SV} [Q] \dots\dots\dots(1)$$

Where, F_0 and F are the fluorescence intensity of fluorophore in the absence and presence of quencher, respectively. $[Q]$ is the concentration of quencher and K_{SV} is Stern-Volmer quenching constant, which indicates the strength of the interaction. K_{SV} is the slope of the plot of F_0/F against $[Q]$ based on the fluorescence data at different temperatures.

RESULTS AND DISCUSSION

The interaction of Ketorolac with BSA:

There are three types of fluorophore in BSA, namely tryptophan residue, tyrosine residue and phenylalanine residue. When excited by appropriate wavelength of light all of them can emit fluorescence. In order to determine whether both tryptophan and tyrosine residues are involved in the interaction with ketorolac, the fluorescence of BSA excited at 280 nm and 293 nm in the presence of ketorolac is compared. When 280 nm excitation wave length is used, fluorescence of albumin comes from both tryptophan and tyrosine residues, whereas 293 nm wavelength only excites tryptophan residues [5].

The plots F/F_0 against $[Ketorolac]/[BSA]$ at excitation wavelength 280 nm and 293 nm are measured to determine the interaction of drug and BSA. Here, F_0 is the fluorescence intensity of BSA, F is the fluorescence intensity of BSA in presence of drug.

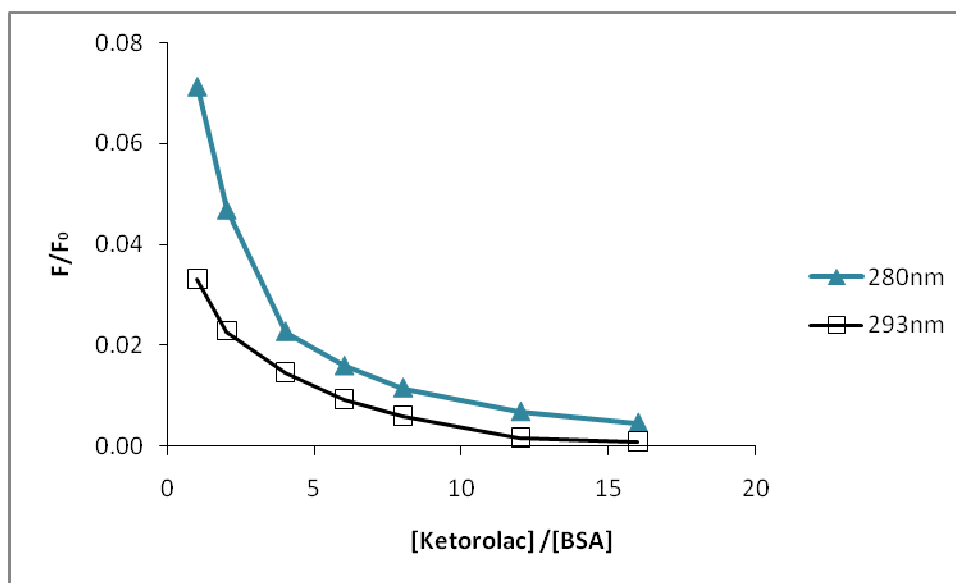


Fig. 1: Fluorescence titration curve of BSA in the presence of Ketorolac at $\lambda_{\text{max}} = 280 \text{ nm}$ and 293 nm

The plots F/F_0 against $[\text{Ketorolac}]/[\text{BSA}]$ were shown in the **figure 1**, which indicated that the fluorescence of BSA excited at 280 nm obviously differs from that excited at 293 nm in the presence of ketorolac. This significant difference between quenching of serum albumin fluorescence indicated that both tyrosine and tryptophan residues participated in the molecular interactions between BSA and ketorolac at excited state.

Effect of ketorolac on the fluorescence spectra of BSA:

At the excitation wavelength of 280 nm and 293 nm , the fluorescence spectra of BSA with varying concentration are shown in the **figure 2** and **figure 3** respectively. It showed the quenching of fluorescence of BSA in the presence of different concentration of ketorolac. This indicated strong interaction and energy transfer between ketorolac and BSA at 298K .

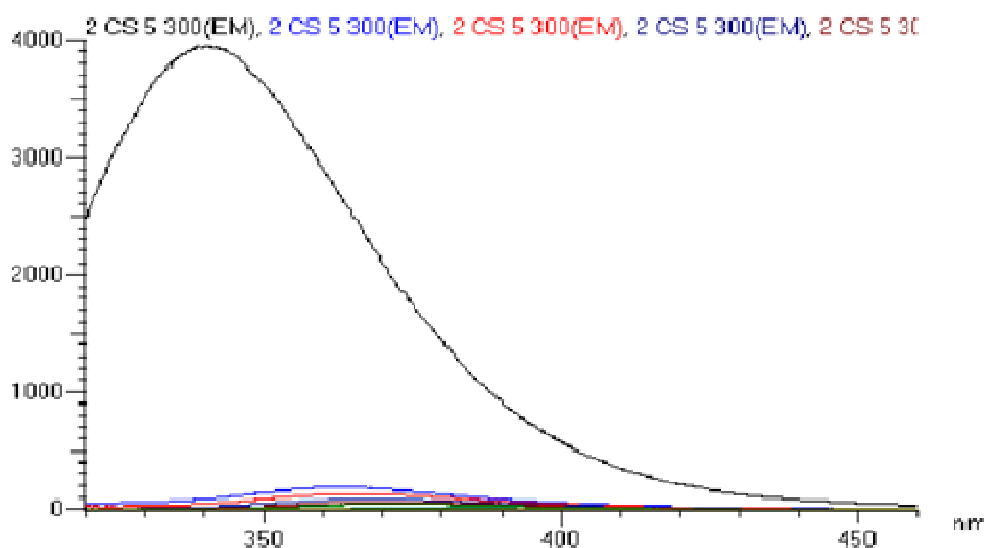


Fig. 2: Fluorescence emission spectrum of BSA in the presence of different concentrations of ketorolac ($\lambda_{\text{max}} = 280 \text{ nm}$, $T = 298\text{K}$)

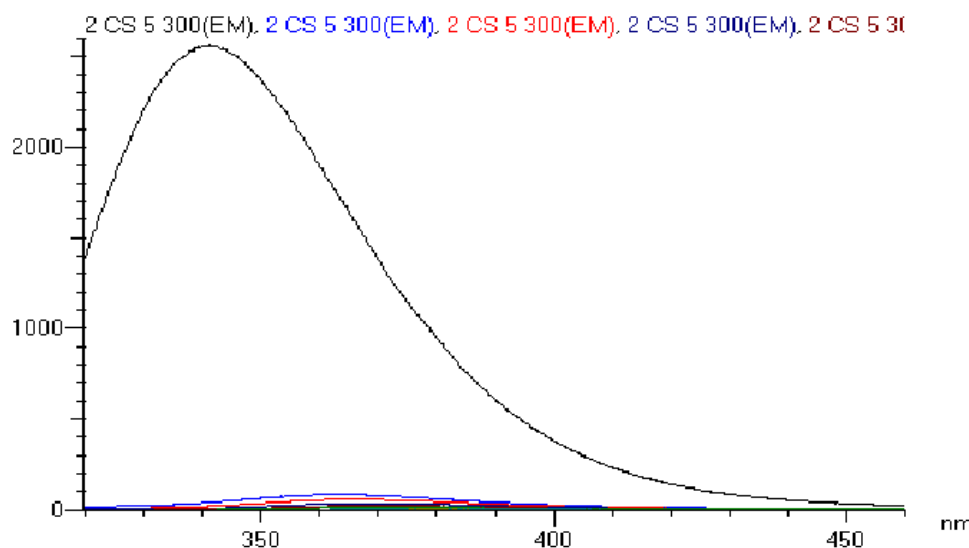


Fig. 3: Fluorescence emission spectrum of BSA in the presence of different concentrations of ketorolac ($\lambda_{\text{max}} = 293 \text{ nm}$, $T = 298\text{K}$)

Analysis of fluorescence quenching mechanism:

Fluorescence quenching is the decrease of the quantum yield of fluorescence from a fluorophore induced by a variety of molecular interaction with quencher molecule [4]. A variety of molecular interactions can result in quenching including excited state reactions, molecular rearrangements, ground state complex formation, and collisional quenching and so on. The different mechanisms of quenching are usually classified as either dynamic quenching or static quenching [5]. Static quenching refers to formation of complex between quencher and the fluorophore, while dynamic quenching refers to the collision of the quencher and fluorophore during the excitation [6].

The fluorescence quenching data are usually analyzed by Stern-Volmer equation [4]:

$$F_0/F = 1 + K_{SV}[Q]$$

K_{SV} was calculated from the slope of the plot of F_0/F versus $[K\text{etorolac}]$ based on the fluorescence data at different temperatures (298°K and 308°K).

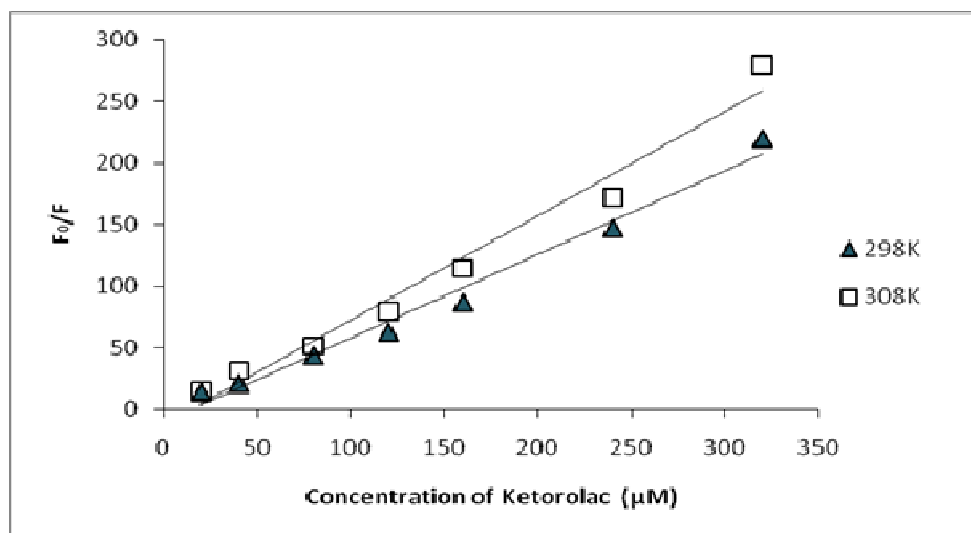


Fig. 4: The Stern-Volmer plots for the quenching of BSA by ketorolac at 298K and 308K.

Figure 4 displayed the Stern-Volmer plots of the quenching of BSA fluorescence by the quencher (Ketorolac) at different temperatures. The plot showed that within the experimental concentrations, the results were in good agreement with the Stern-Volmer equation. The plots were linear and Stern-Volmer quenching constants were obtained from the slopes at various temperatures and those were listed in **Table 1**. The Stern-Volmer quenching constant increases with increase in temperature for dynamic quenching while for the static quenching reverse effect is observed [7]. It was clear from Table 1 that the probable quenching mechanism for the ketorolac-BSA binding reaction was due to dynamic quenching.

Table 1: Stern-Volmer quenching constant (K_{SV}) of ketorolac-BSA system (R = Correlation co-efficient)

T (K)	K_{SV} (L mol ⁻¹)	R
298	6.77×10^5	0.984
308	8.47×10^5	0.977

Thermodynamic parameters and nature of the binding forces:

The interaction forces between quencher and fluorescence active molecule may include hydrophobic force, electrostatic interaction, van der Waals interaction, hydrogen bond, etc [8]. The thermodynamic parameters were calculated in order to elucidate the interaction between the ketorolac and BSA. The thermodynamic parameters can be determined from the Van't Hoff equation:

$$\ln K_a = -\left(\frac{\Delta H}{RT}\right) + \left(\frac{\Delta S}{R}\right)$$

130 Where,

131 ΔS = Entropy change

132 ΔH = Enthalpy change

133 K_a = analogous to the Stern-Volmer quenching constants K_{SV} at the corresponding temperature [2]

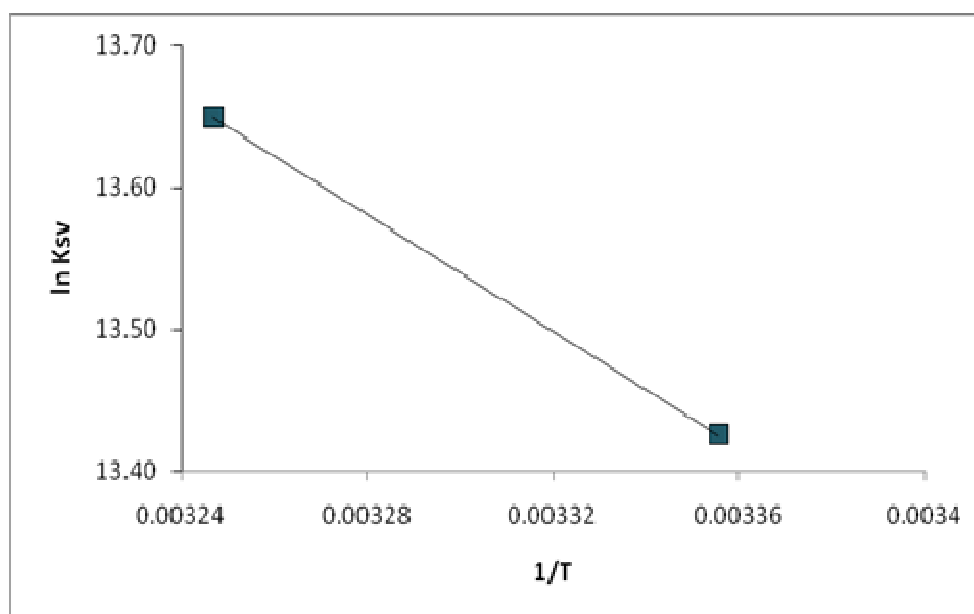
134 R = Gas constant.

135 The enthalpy change (ΔH) and the entropy change (ΔS) can be determined from the slope and intercept
136 of the curve of $\ln K_{SV}$ versus $1/T$, respectively.

137 The free energy, ΔG can be estimated from the following relationship:

138
$$\Delta G = \Delta H - T\Delta S$$

139 For reversible drug-protein binding, binding forces like hydrogen bonds, van der Waals forces,
140 hydrophobic forces and electrostatic forces are involved [9]. The Van't Hoff Plot for ketorolac-BSA system
141 was shown on **Figure 5**.



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143 **Figure 5:** The Van't Hoff Plot for ketorolac-BSA system; [BSA] = 20 μM

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Table 2: Thermodynamic parameters of ketorolac-BSA binding

T (K)	ΔH (KJ/mol)	ΔS (J/mol)	ΔG (KJ/mol)
298	17.093	168.94	-33.25

As shown in **Table 2**, the enthalpy change (ΔH) and the entropy change (ΔS) were positive, and the free energy change (ΔG) was negative. The negative value of ΔG indicated that binding of ketorolac to BSA was spontaneous. According to the rules summarized by Ross and Subramanian[10], for drug - protein interaction, the positive ΔS and ΔH values of interaction of ketorolac and BSA indicated that binding is mainly entropy driven and enthalpy is unfavorable for it, the hydrophobic forces playing a major role in the ketorolac-BSA system.

Binding constant and binding sites for ketorolac:

When small molecules bind independently to a set of equivalent sites on a macromolecule, the equilibrium between free and bound molecules is given by the following equation [11]:

$$\log \left\{ \frac{(F_0 - F)}{F} \right\} = \log K + n \log [Q]$$

Where, K is the binding constant to a site of albumin and n is the number of binding site for drug per albumin. The values of K and n were calculated from the values of intercept and slope of the plot of $\log \{(F_0 - F)/F\}$ versus $\log [Q]$, respectively shown in the **Figure 6**.

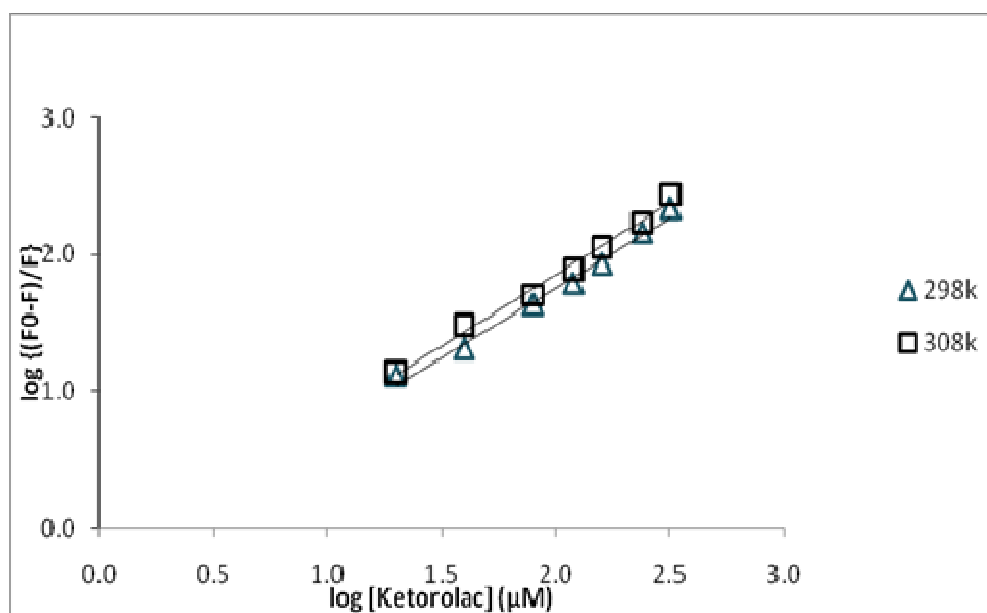


Fig. 6: Plot for binding constant and binding sites of ketorolac at 298K and 308K

Table 3: Binding constant and binding sites of ketorolac-BSA system

T (K)	$K (\mu\text{M}^{-1})$	n
298	0.522	1.020
308	0.603	1.038

Table 3 contained the values of K and n , at different temperatures, which were obtained from the intercept and slope of **Figure 6**. It was observed that the binding constant increases with the increase in temperature, resulting in increasing the collision of ketorolac-BSA system. The obtained values for n were found to be ≈ 1 indicate that only a single ketorolac molecule binds with BSA and temperature has almost no effects on bindings sites of drug on BSA.

CONCLUSION

In this paper, the interactions of ketorolac with BSA have been investigated by fluorescence spectroscopy. The experiment result indicates that both tryptophan and tyrosine residues participate in the interactions between ketorolac and BSA at 280 nm excitation wavelength. It was observed that the fluorescence quenching of BSA by ketorolac is a result of dynamic quenching. Fluorescence quenching constants were determined using the Stern-Volmer equation and van't Hoff equation to provide a measure of the thermodynamic parameters ΔG , ΔH , and ΔS at different temperatures. The binding process for ketorolac has been found spontaneous, exothermic and entropy driven as indicated by

thermodynamic analysis and hydrophobic forces playing a major role in the ketorolac-BSA association.

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