ABSTRACT

**Aims:** This paper describes the *In vitro* study of protein binding by sildenafil citrate (SC) in presence of bisoprolol fumarate (BF) and metformin hydrochloride (MH).

**Study Design:** Study was designed to assess *In vitro* of quenching of bovine serum albumin (BSA) by sildenafil citrate (SC) in presence of bisoprolol fumarate (BF) and metformin hydrochloride (MH) by fluorescence spectrophotometry.

**Place and Duration of Study:** Drug Analysis and Research Laboratory, Centre for Advanced Research in Sciences, University of Dhaka, Dhaka-1000, Bangladesh between December 2013 and March 2014.

**Methodology:** In the present work, the *In vitro* study of quenching of BSA by SC in presence of BF...
and MH have been studied by fluorescence emission spectroscopy under different conditions. At first, the BSA solution (20 µM) was prepared in phosphate buffer (pH =7.4) in eight test tubes and different amounts of sildenafil citrate was added to each BSA solution to obtain the final concentrations as 0, 20, 40, 80, 120, 160, 240 and 320 × 10⁻⁶ molL⁻¹, respectively. Then the fluorescence emission spectra of BSA-SC system were recorded for eight test tubes at two excitation wavelengths of BSA (λEx max = 280 nm and λEx max = 293 nm) at 298 K and 308 K. Similarly, the fluorescence emission spectra of BSA-(SC+BF), BSA-(SC+MH) and BSA-(SC+BF+MH) systems were recorded at 280 nm and 293 nm at 298 K and 308 K. Quenching constants were determined using the Stern-Volmer equation to provide a measure of the strength of quenching of BSA by SC in presence of BF and MH in all the systems.

**Results:** The quenching of BSA by SC was increased in presence of BF and MH but remained close in presence of both BF and MH. Quenching constants were larger for the BSA-(SC+BF) system and ranked in the order as BSA-(SC+BF) > BSA-(SC+MH) > BSA-(SC+BF+MH) > BSA-SC at 280 nm at two different temperatures, respectively. But quenching at the excitation wavelength of 293 nm was ranked in order as BSA-(SC+BF) > BSA-(SC+MH) > BSA-(SC+BF+MH) > BSA-SC at 298 K and 308 K, respectively.

**Conclusion:** It was found that BSA quenched by SC in presence of BF and MH, which indicated that the effectiveness of SC might be predominately influenced by these drugs.

**Keywords:** Protein binding; quenching; Bovine Serum Albumin (BSA); sildenafil citrate; bisoprolol fumarate; metformin hydrochloride; fluorescence emission spectroscopy.

1. **INTRODUCTION**

Sildenafil citrate (SC) (Fig. 1A) is a drug used to treat erectile dysfunction and pulmonary arterial hypertension (PAH). It acts by inhibiting cGMP-specific phosphodiesterase type 5 (PDE5), an enzyme that promotes degradation of cGMP, which regulates blood flow in smooth muscle [1,2]. Sildenafil has no direct relaxant effect on isolated human corpus cavernosum, but enhances the effect of nitric oxide (NO) by inhibiting phosphodiesterase type 5 (PDE5), which is responsible for degradation of cGMP in the corpus cavernosum.

Bisoprolol fumarate (BF) (Fig. 1B) is a highly selective β1-adrenoreceptor antagonist drugs used for the treatment of coronary disease and hypertension. It is responsible for the therapeutic effect of reducing blood pressure and most of its beta-blocking activity [3,4].

Metformin hydrochloride (MH) (Fig. 1C) is an oral antidiabetic drug used for the treatment of type 2 diabetes, in particular, in overweight and obese people and those with normal kidney function [5,6]. It is also used in the treatment of polycystic ovary syndrome, and has been investigated for other diseases where insulin resistance may be an important factor. Metformin works by suppressing glucose production by the liver. It helps reduce LDL cholesterol and triglyceride levels, and is not associated with weight gain.

Serum albums are the most abundant soluble protein in mammalian blood plasma and they have many important physiological functions. The most important physiological feature of serum albumin serving as depot protein and binding of numerous ligands, such as fatty acids, drugs, and metal ions, in the bloodstream to their target organs [7,8]. Therefore, investigation of such molecules with respect to serum albumin binding is of imperative and fundamental importance. Moreover, serum albumin is considered as a model to study the drug-protein interaction In vitro [9]. Bovine serum albumin (BSA) is an extensively studied ideal protein model of albumin group since it displays unusual ligand-binding properties and structural homology (80%) with human serum albumin (HSA) [10]. BSA is an ideal protein of a single polypeptide chain of 583 amino acid residues and three structurally homologous domains (I-III) which are divided into nine loops (L1-L9) by 17 disulfide bonds, and each domain is further divided into two sub-domains (A and B) [11]. It is a convenient protein for intrinsic fluorescence measurement due to the presence of two intrinsic tryptophan (Trp) residues which is highly sensitive to its local environment, and can be used to observe changes in the fluorescence emission spectra due to protein conformational changes, binding to substrates, and denaturation [12]. There also numerous tyrosine residues of BSA depending on the excitation wavelength selected which have minor contribution for intrinsic fluorescence.
Trp-212, located within the hydrophobic binding pocket of sub-domain IIA (site-I), and Trp-134, located on the surface of sub-domain IB (site-II) [13-15]. The binding sites of BSA for endogenous and exogenous ligands may be in these domains, and some ligands specifically bind to the different domains of serum albumin [16]. However, BSA plays an important role in binding of numerous drugs in the bloodstream to their target organs for understanding the pharmacokinetics and pharmacodynamics properties of drug candidates.

Drugs can be bound at molecular level to proteins are acted as carriers which leads to the interpretation of the metabolism, distribution, free concentration, efficacy and transporting process of drugs [17]. Moreover, investigation of drug protein binding provides the information of structural features determining the therapeutic effect of drugs helps to understand the drug toxicity and playing a key role in the researching pharmacology, pharmacodynamics and biochemistry [18]. Therefore, drug-protein binding has become an important research field in life sciences, chemistry and clinical medicine [18,19]. Consequently, some popular techniques have been used to investigate the interaction between drugs and BSA. Fluorescence quenching is one of the powerful techniques to study molecular interactions which changes local environment of fluorophore and helps to predict the binding phenomenon of drugs to BSA [18]. However, the In vitro mechanism of interactions of SC with BSA in presence of BF and MH has not been explored. So it is significant to study the interaction between SC and BSA in presence of BF and MH by fluorescence quenching.

In the present study, In vitro quenching of BSA by SC in presence of BF and MH have been studied by fluorescence emission spectroscopy under different conditions. At first, quenching of BSA by SC for BSA-SC system were measured at two excitation wavelengths of BSA (280 nm and 293 nm) at two different temperatures (298 K and 308 K). In order to observe the effect of other drugs on fluorescence quenching of BSA by SC quenching for BSA-(SC+BF) and BSA-(SC+MH) systems were measured in presence of BF and MH, respectively at same conditions. Finally, combined effect of drugs in fluorescence quenching of BSA by SC in presence of both BF and MH in BSA-(SC+BF+MH) system were observed at mentioned conditions. However, to our best knowledge, the interactions of SC with BSA in presence of BF and MH have not been reported. The interactions of drug-serum albumin also depend on surrounding circumstances such as pH, temperature, concentrations of BSA and drug, and the presence of other endogenous and exogenous compounds. Therefore, it is necessary to understand the level of binding of SC for the BSA in the presence of BF and MH which would be important to concomitant use of these drugs.

2. MATERIALS AND METHODS

2.1 Reagent and Materials

All chemicals and reagent were analytical grade and doubly distilled water was used throughout the study. BSA (fatty acid free, fraction V, 96-98%) was purchased from Sigma Chemical Co., USA and was used without further purification. Sildenafil citrate (99.4%) was obtained from the Drug International Ltd., Bangladesh. Metformin hydrochloride (99.5%) and bisoprolol fumarate (99.5%) were obtained from ACI Pharmaceuticals Ltd., Bangladesh. The solutions of drug and BSA were prepared in phosphate buffer of pH 7.4 containing 0.01 M Na_2HPO_4 (Active Fine Chemicals Ltd., Bangladesh) and 0.02 M KH_2PO_4 (Active Fine Chemicals Ltd., Bangladesh). Phosphate buffer of pH 7.4 was prepared by the method as described by Perrin & Boyd Dempsey (1974).
2.2 Apparatus

All fluorescence spectra were recorded on F-7000 spectrophotometer (Hitachi, Japan) equipped with 1.0 cm quartz cell. For different temperature a thermostat bath (Unitronic Orbital, P- Spectra, Spain) was used.

2.3 Sample Preparation

A 5.0 mL of 20 × 10^{-6} molL^{-1} BSA solution, previously prepared in phosphate buffer of pH 7.4 was taken in each of the eight test tubes. Sildenafil citrate was added in different volumes to seven out of eight test tubes to have the following concentrations: (20, 40, 80, 120, 160, 240 and 320) × 10^{-6} molL^{-1}, respectively. The ratio of SC and BSA ([SC]/[BSA]) in BSA-SC system of seven test tubes were 1, 2, 4, 6, 8, 12 and 16, respectively. The mixture solution of BSA and SC must be hatched at least 5 min before the spectrum measurements. Similarly, BF (20 × 10^{-6} molL^{-1}) and MH (20 × 10^{-6} molL^{-1}) were added to BSA+SC solution to prepare other three systems of BSA-(SC+BF), BSA-(SC+MH) and BSA-(SC+BF+MH). The ratio of SC and BSA ([SC]/[BSA]) in these systems were also 1, 2, 4, 6, 8, 12 and 16, respectively. These mixture of solutions in the previous systems also hatched at least 5 min before the spectrum measurements.

2.4 Spectroscopic Measurement

The fluorescence emission spectra for all the four systems were recorded at the two excitation wavelengths of BSA (280 nm and 293 nm) at two different temperatures (298 K and 308 K). The widths of both entrance and exit slit were set to 5 nm. These emission spectra for all the four systems were recorded in the range of 320-460 nm for BSA at same experimental conditions since there were no emission spectra of SC, BF and MH in this range.

3. RESULTS AND DISCUSSION

3.1 Fluorescence Quenching Analysis

The decrease of the quantum yield of fluorescence from a fluorophore induced by a variety of molecular interaction with quencher molecule is known as fluorescence quenching [20]. Quenching can occur due to non-molecular mechanisms, e.g. attenuation of the incident light by the fluorophore itself or by other absorbing species. Other mechanisms of quenching include a variety of competing processes including excited-state reactions, molecular rearrangements, ground-state complex formation, collisional quenching and so on, which induce nonradiative relaxation of excited electrons to the ground state without light emission. Formation of complex between quencher and the fluorophore refers to static quenching. On the other hand collision of the quencher and fluorophore during the excitation refers to dynamic quenching [21]. The fluorescence quenching data are usually analyzed by Stern-Volmer equation [22].

\[
\frac{F_0}{F} = 1 + K_{sv} [Q]
\]

Where, \(F_0\) and \(F\) are the fluorescence intensities in the absence and presence of quencher, \([Q]\) is the quencher concentration and \(K_{sv}\) is the Stern-Volmer quenching constant which indicates the strength of interaction between albumin protein and quencher molecule. Hence, this equation was applied to determine \(K_{sv}\) by linear regression of a plot of \(\frac{F_0}{F}\) against \([Q]\). The static quenching distinguished from dynamic quenching by their differing dependence of temperature [12]. Dynamic quenching depends upon diffusion: higher temperatures result in larger diffusion coefficients. As a result, the Stern-Volmer quenching constants (\(K_{sv}\)) are expected to increase with increasing temperature. In contrast, increased temperature is likely to result in decreasing stability of complexes, and thus lower value of static quenching constants [22].

3.1.1 Effect of SC on the fluorescence spectra of BSA

The fluorescence emission spectra of BSA with varying concentration of SC were measured at two excitation wavelength of BSA (280 nm and 293 nm) at two different temperatures (298 K and 308 K) are shown in Fig. 2. The fluorescence spectra of BSA with varying concentrations of SC are shown in Fig. 2. The fluorescence of BSA regularly decreased with the increasing concentration of SC, indicating that there was a strong interaction and energy transfer between SC and BSA at the both excitation wavelengths of BSA (\(\lambda_{Ex_{max}}\) of BSA = 280 nm and 293 nm). As a result, there were quenching of intrinsic fluorescence of BSA but no significant shift of the emission maximum wavelength was observed. The pattern of quenching of BSA fluorescence by SC in BSA-
SC system can be determined by measuring the value of Stern-Volmer quenching constant (Ksv) at the excitation wavelength of BSA (280 nm and 293 nm) at two different temperatures (298 K and 308 K). Ksv was calculated from the slope of the plot of F/Fo versus concentration of SC based on the fluorescence data (Fig. 3) at the above conditions.

Fig. 3 displays the Stern-Volmer plots of the quenching of BSA fluorescence by SC at two excitation wavelength of BSA (280 nm and 293 nm) at two different temperatures. The plots showed that within the experimental concentrations, the results were good agreement with the Stern-Volmer equation. The plots were linear and Stern-Volmer quenching constants were obtained from the slopes at two different temperatures; these are listed in Table 1. The Stern-Volmer quenching constant decreases with increasing temperature for static quenching while for dynamic quenching the reverse effect is observed [23]. It has been seen from the Table 1 that the Ksv decreased by increasing temperature at 280 nm but increased by increasing temperature at 293 nm. So it was observed that the probable BSA fluorescence quenching by SC at 280 nm was static quenching (complex formation) but dynamic (collision) at 293 nm.

| Temperature (K) | Ksv ($\times 10^{-3}$ Lmol$^{-1}$) at 280 nm | Ksv ($\times 10^{-3}$ Lmol$^{-1}$) at 293 nm |
|----------------|------------------------------------------|------------------------------------------|
| 298            | 10.2                                     | 9.7                                      |
| 308            | 9.5                                      | 11.0                                     |

Table 1. The Stern-Volmer quenching constant (Ksv) for BSA-SC system at 280 nm and 293 nm at two different temperatures (298 K and 308 K)

To determine the effect of BF on fluorescence quenching of BSA by SC, the fluorescence emission spectra of BSA with varying concentration of SC in presence of BF were measured at two excitation wavelengths of BSA (280 nm and 293 nm) at two different temperatures (298 K and 308 K) as shown in Fig. 4.

Fig. 4 indicates that in presence of BF, there was a strong interaction and energy transfer between SC and BSA in both excitation wavelength of BSA ($\lambda_{\text{Ex}}$ of BSA = 280 nm and 293 nm) at two different temperatures (298 K and 308 K). As a result, the fluorescence of BSA regularly decreased with the increasing concentration of SC in presence of BF indicating that drugs interacted with BSA and quenched its intrinsic fluorescence. Quenching of BSA fluorescence by SC in presence of BF was determined by measuring the value of Stern-Volmer quenching constant (Ksv) from the slope of the plot of F/Fo versus concentration of SC based on the fluorescence data (Fig. 5) at previously mentioned conditions.

Table 2 contains the values of Ksv at different temperatures, which obtained from the slopes of the Stern-Volmer plots (Fig. 5) for quenching of BSA by SC in presence of BF at two excitation wavelengths (280 nm and 293 nm). It was observed that Stern-Volmer quenching constant (Ksv) decreased with increasing temperature at 280 nm but increased with increasing temperature at 293 nm. So it was clear from the Table 2 that the probable BSA fluorescence quenching by SC in presence of BF was static (complex formation between BSA and drugs) at 280 nm but dynamic (collision of drugs and BSA) at 293 nm. It has been seen that Ksv increased in BSA-(SC+BF) system than BSA-SC system at both excitation wavelengths at both temperatures. It indicated that the fluorescence quenching (static and dynamic) of BSA by SC was increased in presence of BF.

| Temperature (K) | Ksv ($\times 10^{-3}$ Lmol$^{-1}$) at 280 nm | Ksv ($\times 10^{-3}$ Lmol$^{-1}$) at 293 nm |
|----------------|------------------------------------------|------------------------------------------|
| 298            | 12.5                                     | 13.3                                     |
| 308            | 11.8                                     | 15.1                                     |

Table 2. The Stern-Volmer quenching constant (Ksv) for BSA-(SC+BF) system at 280 nm and 293 nm at two different temperature (298 K and 308 K)

3.1.2 Effect of SC on the fluorescence emission spectra of BSA in presence of BF

3.1.3 Effect of SC on the fluorescence emission spectra of BSA in presence of MH

To determine the effect of MH on fluorescence quenching of BSA by SC, the fluorescence emission spectra of BSA with varying concentration of SC in presence of MH were measured at two excitation wavelengths of BSA (280 nm and 293 nm) at two different temperatures (298 K and 308 K) as shown in Fig. 6.
Fig. 6 indicates that there was a strong interaction and energy transfer between drugs and BSA in both excitation wavelengths of BSA ($\lambda_{\text{Ex, max}}$ of BSA = 280 nm and 293 nm) at two different temperatures (298 K and 308 K). As a result, the fluorescence of BSA regularly decreased with the increasing concentration of SC in presence of MH indicating that drugs interacted with BSA and quenched its intrinsic fluorescence. Quenching of BSA fluorescence by SC in presence of constant concentration of BF and MH was determined by measuring the value of Stern-Volmer quenching constant ($K_{sv}$) from the slope of the plot of $F/F_0$ versus concentration of SC based on the fluorescence data (Fig. 7) at two different temperatures.

Table 3 contains the values of $K_{sv}$ at different temperatures, which obtained from the slopes of the Stern-Volmer plots (Fig. 7) for quenching of BSA by SC in presence of BF at two excitation wavelengths (280 nm and 293 nm). It was observed that Stern-Volmer quenching constant ($K_{sv}$) decreased with increasing temperature at 280 nm but increased with increasing temperature at 293 nm. So it was clear from the Table 3 that the probable BSA fluorescence quenching by SC in presence of MH was static (complex formation between BSA and drugs) at 280 nm but dynamic (collision of drugs and BSA) at 293 nm. It has been seen that $K_{sv}$ increased in BSA-(SC+BF) system than BSA-SC system but decreased from BSA-(SC+MH) system at both excitation wavelengths at both the temperatures. It indicated that fluorescence quenching of BSA by SC in BSA-(SC+MH) system was strong than the quenching in BSA-SC system but weak from BSA-(SC+BF) system at mentioned conditions. So, MH was also less effective for fluorescence quenching of BSA by SC than BF at 280 nm and 293 nm at 298 K and 308 K.

### 3.1.4 Effect of SC on the fluorescence emission spectra of BSA in presence of (BF + MH)

In order to confirm the combined effect of BF and MH on fluorescence quenching of BSA by SC, the fluorescence emission spectra of BSA with varying concentration of SC in presence of both BF and MH were measured at two excitation wavelengths of BSA (280 nm and 293 nm) at two different temperatures (298 K and 308 K) as shown in Fig. 8.

Fig. 8 shows that the fluorescence of BSA regularly decreased with the increasing concentration of SC in presence of both BF and MH indicating that drugs interacted with BSA and quenched its intrinsic fluorescence. Quenching of BSA fluorescence by SC in presence of constant concentration of BF and MH was determined by measuring the value of Stern-Volmer quenching constant ($K_{sv}$) from the slope of the plot of $F/F_0$ versus concentration of SC based on the fluorescence data (Fig. 9) at two different temperatures.

**Table 3. The Stern-Volmer quenching constant ($K_{sv}$) for BSA-(SC+MH) system at 280 nm and 293 nm at two different temperatures (298 K and 308 K)**

| Temperature (K) | $K_{sv}$ ($\times 10^3$ Lmol$^{-1}$) at 280 nm | $K_{sv}$ ($\times 10^3$ Lmol$^{-1}$) at 293 nm |
|----------------|-----------------------------------------------|-----------------------------------------------|
| 298            | 11.4                                          | 11.8                                          |
| 308            | 11.1                                          | 13.7                                          |

Table 4 shows that Stern-Volmer quenching constant ($K_{sv}$) decrease with increasing temperature at both excitation wavelengths of BSA. So, it was clear from the Table 4 that there was complex formation between drugs and BSA at both excitation wavelengths. As a result, static quenching was observed in BSA-(SC+BF+MH) system at both excitation wavelengths of BSA. It was also observed that the value of $K_{sv}$ in BSA-(SC+BF+MH) system was close the value of BSA-SC system but decreased from BSA-(SC+BF) and BSA-(SC+MH) systems at 280 nm and 293 nm. So, it might be expected that the quenching of BSA by SC in BSA-(SC+BF+MH) system at 280 nm was decreased due to interaction between drugs.

**Table 4. The Stern-Volmer quenching constant ($K_{sv}$) for BSA-(SC+BF+MH) system at 280 nm and 293 nm at two different temperatures (298 K and 308 K)**

| Temperature (K) | $K_{sv}$ ($\times 10^3$ Lmol$^{-1}$) at 280 nm | $K_{sv}$ ($\times 10^3$ Lmol$^{-1}$) at 293 nm |
|----------------|-----------------------------------------------|-----------------------------------------------|
| 298            | 10.5                                          | 11.2                                          |
| 308            | 9.32                                           | 10.8                                          |

### 3.2 Binding Constant and Binding Sites for BSA-SC System

When small molecule binds independently to a set of equivalent sites on a macromolecule, the equilibrium between free and bound molecule is given by the following equation [24].
log \left\{\frac{(F_0 - F)}{F}\right\} = \log K + n \log [Q]

Where,

K = Binding constant to site of albumin
n = Number of binding sites for drug per albumin

The values of K and n are calculated from the values of intercept and slope of the plot of log \left\{\frac{(F_0 - F)}{F}\right\} versus log [Q].

Table 5 contains the values of binding constant (K_b) at 280 nm at two different temperatures, which were obtained from the intercept of Fig. 10. It was observed that the binding constant decreased with the increase in temperature in all the systems, resulting in the reduction of stability of the complex. The value of binding number (n) for binding of BSA with SC in presence of BF and MH were found to be 1.064 (≈ 1) which indicated that one mole SC bound with 1 mole of BSA. It was also observed that value of binding constants in four systems were ranked in order as BSA-(SC+BF) > BSA-(SC+ MH) > BSA-SC > BSA-(SC+BF+MH). It was found that binding of BSA with SC increased in presence only of BF and MH but decreased when both BF and MH present in BSA and SC binding sites. So it has been found that SC effected BSA weakly when both BF and MH present in the medium.

3.3 Thermodynamic Parameters and Nature of Binding Forces

There are many interaction forces (e.g. hydrophobic force, electrostatic interactions, Vander Waals interactions, hydrogen bonds, etc.) between quencher and fluorescence active molecule [15].

The thermodynamic parameters are calculated in order to elucidate the interaction between the drug and BSA, which can be determined from the Van’t Hoff equation:

\[ \ln K_a = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \]

Where, \( \Delta S = \) Entropy change, \( \Delta H = \) Enthalpy change, \( R = \) Universal gas constant
And \( K_a = \) Analogous to the Stren – Volmer quenching constants \( K_{sv} \) at the corresponding temperature [25].

The enthalpy change (\( \Delta H \)) and the entropy change (\( \Delta S \)) can be determined from the slope and intercept of the fitted curve of \( \ln K_{sv} \) against \( 1/T \) respectively. The free energy \( \Delta G \) can be estimated from the following relationship:

\[ \Delta G = \Delta H - T\Delta S \]

Tables 6 and 7 show that \( \Delta S \) was a positive value, and \( \Delta H \) was a small negative value. The negative value of \( \Delta H \) revealed that the formation of BSA-SC complex in both BSA-SC and BSA-(SC+BF) system was an exothermic process. Moreover, the negative sign for \( \Delta G \) indicated the spontaneity of the binding process of SC with BSA. According to the views of Ross and Subramanian [26], the model of interaction between drug and biomolecule can be summarized as follows: (1) the positive \( \Delta S \) value is frequently regarded as the evidence for a hydrophobic interaction [27] because the water molecules arranged in an orderly fashion around the drug and protein established a more random configuration; (2) the negative value of \( \Delta H \) can be obtained whenever there was a possibility of hydrogen bonding [28,29].

Table 5. Binding constant and binding sites for four systems at 280 nm at two different temperatures (298 K and 308 K)

| Temperature (K) | \( K_b \left(10^{-3} \times \text{Lmol}^{-1}\right) \) for BSA-SC system | \( K_b \left(10^{-3} \times \text{Lmol}^{-1}\right) \) for BSA-(SC+BF) System | \( K_b \left(10^{-3} \times \text{Lmol}^{-1}\right) \) for BSA-(SC+MH) system | \( K_b \left(10^{-3} \times \text{Lmol}^{-1}\right) \) for BSA-(SC+BF+MH) system |
|----------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| 298            | 14.32                                           | 30.38                                           | 20.09                                            | 9.30                                            |
| 308            | 12.37                                           | 25.19                                           | 13.60                                            | 2.33                                            |
Fig. 2. Fluorescence emission spectra for quenching of BSA by SC in BSA-SC system
(A) At the excitation of 280 nm at 298 K (B) At the excitation of 280 nm at 308 K (C) At the excitation of 293 nm at 298 K and (D) At the excitation of 293 nm at 308 K

Fig. 3. The stern-volmer plots for quenching of BSA by SC in BSA-SC system
(a) At 280 nm at two different temperatures (298 K and 308 K) and (b) At 293 nm at two different temperatures (298 K and 308 K)
Fig. 4. Fluorescence emission spectra for quenching of BSA by SC in presence of BF in BSA-(SC+BF) system
(A) At the excitation of 280 nm at 298K (B) At the excitation of 280 nm at 308K (C) At the excitation of 293 nm at 298K and (D) At the excitation of 293 nm at 308K

Fig. 5. The Stern-Volmer plots for quenching of BSA by SC in presence of BF in BSA-(SC+BF) system
(A) At 280 nm at two different temperatures (298 K and 308 K) and (B) at 293 nm at two different temperatures (298 K and 308 K)
Fig. 6. Fluorescence emission spectra for quenching of BSA by SC in presence of BF in BSA-(SC+MH) system
(A) At the excitation of 280 nm at 298 K (B) At the excitation of 280 nm at 308 K (C) At the excitation of 293 nm at 298 K and (D) At the excitation of 293 nm at 308 K

Fig. 7. The Stern-Volmer plots for quenching of BSA by SC in presence of MH in BSA-(SC+MH) system
(a) At 280 nm at two different temperatures (298 K and 308 K) and (b) At 293 nm at two different temperatures (298 K and 308 K)
Fig. 8. Fluorescence emission spectra for quenching of BSA by SC in presence of BF in BSA-(SC+BF+MH) system
(A) At the excitation of 280 nm at 298 K (B) At the excitation of 280 nm at 308 K (C) At the excitation of 293 nm at 298 K and (D) At the excitation of 293 nm at 308 K

Fig. 9. The Stern-Volmer plots for quenching of BSA by SC in presence of MH in BSA-(SC+BF+MH) system
(a) At 280 nm at two different temperatures (298 K and 308 K) and (b) At 293 nm at two different temperatures (298 K and 308 K)
Fig. 10. Plot for binding constant and binding sites for four systems at 280 nm at two different temperatures (298 K and 308 K)
(a) BSA-SC system at 280 nm at two different temperatures (b) BSA-(SC+BF) at 280 nm at two different temperatures (c) BSA-(SC+MH) at 280 nm at two different temperatures (d) BSA–(SC+BF+MH) at 280 nm at two different temperatures

Fig. 11. The Van’t Hoff plot for BSA-SC system at 280 nm at two different temperatures (298K and 308K)
Table 6. Thermodynamic parameters for BSA-SC system at 280 nm at two different temperatures (298 K and 308 K)

| Temperature (K) | ΔH (KJ/mol) | ΔS (J/mol) | ΔG (KJ/mol) |
|----------------|-------------|------------|-------------|
| 298            | -5.89       | 57.01      | -22.87      |
| 308            | -23.44      |            | -23.44      |

Table 7. Thermodynamic parameters for BSA-(SC+BF) system at 280 nm at two different temperatures (298 K and 308 K)

| T (K) | ΔH (KJ/mol) | ΔS (J/mol) | ΔG (KJ/mol) |
|-------|-------------|------------|-------------|
| 298   | -8.30       | 50.48      | -23.34      |
| 308   | -23.85      |            | -23.85      |

4. CONCLUSION

Quenching of BSA by SC in presence of BF and MH were successfully investigated. BSA quenched more by SC when BF and MH were present solely. But quenching of BSA by SC in the presence of both BF and MH did not increase as did as the single drug (only BF or MH). Thus both hydrogen bonding and hydrophobic interactions were present in the SC-BSA binding in both BSA-SC and BSA-(SC+BF) system at 280 nm at both temperatures. It was also observed that in BSA-(SC+BF) system, value of ΔH and ΔS increased and value of ΔS decreased which indicated that in presence of bisoprolol fumarate, the binding of SC and BSA increased in BSA-(SC+BF) system very spontaneously than did in BSA-SC system.

Therefore, it was found that BSA quenched by SC in presence of BF and MH, this indicated that the effectiveness of SC might be predominately influenced by these drugs.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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