Binding of sulfamerazine and sulfamethazine to bovine serum albumin and nitrogen purine base adenine: a comparative study

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ABSTRACT. Quenching of bovine serum albumin (BSA) and DNA base (adenine) by sulfamerazine (SM) and sulfamethazine (SMT) was studied using UV-visible, fluorescence cyclic voltammetry and molecular docking methods. A strong fluorescence quenching reaction of SM and SMT to BSA/adenine was observed and the quenching mechanism was suggested as static. Both drugs can bind to BSA and adenine with stoichiometric ratio of 1:1 and the protein - drug complexes are stabilized mainly by hydrogen bonds and van der Waals interaction. Compared to SM, SMT contributes substantially higher binding efficiency with BSA/adenine. With addition of drug solution to the adenine/BSA, the oxidation and the reduction peaks shifted towards high and low potentials, respectively. \( R_0, J, r \) and \( E \) values in the BSA-drugs are higher than that of adenine – drug molecules suggest that binding of the sulfa drugs with BSA is higher than adenine – drug molecules. Docking method specify that bioactive site of sulfa drugs moiety, the aniline group is interacted with the BSA molecules.

1. INTRODUCTION

Serum albumin is the most abundant protein in blood plasma (\(~ 60\%)\), which has many physiological functions, such as maintaining the pH of blood, osmotic pressure and scavenging free radicals as an antioxidant [1]. It is an attractive macromolecular carrier, the lack of toxicity and immunogenicity make it an ideal candidate for drug delivery. In addition, serum albumin is the most multifunctional transport protein and plays an important role in the transport and deposition of a variety of endogenous and exogenous substances in blood [1]. Bovine serum albumin (BSA) has been one of the most widely studied of this group of proteins, not only because of its medical importance, low cost, ready availability, abundance, ease of purification, unusual ligand-binding properties and it is widely accepted in the pharmaceutical industry [2–3], but also because of its structural homology with human serum albumin (HSA) [4–6]. The studies on this aspect can provide information of the structural features that determine the therapeutic effectivity of drugs, and have become an interesting research field in life science, chemistry, biochemistry and clinical medicine.

Besides DNA and RNA, adenine is also an important part of adenosine triphosphate, or ATP. Adenosine triphosphate is the nitrogenous base adenine bonded to a five carbon sugar. This molecule is important because it has the ability to phosphorylate, or add a phosphate group to, other molecules. This transfer of a phosphate group allows energy to be released. It is this energy which is used by cells in living organisms. This is why the molecules ATP, and its nitrogenous base adenine, are so important.

Sulfur drugs, which are also known as sulfonamide drugs, are a family of synthetic drugs used pharmacologically as antimicrobial agents to treat human and animal infections [7–8]. Their structures are featured by the existence of sulfanilamide group and distinct six- or five-member heterocyclic rings. Sulfonamides are not readily biodegradable and have various serious side effects that can lead to diseases in humans, such as disorders in the central nervous system, which in turn necessitates understanding of their pharmacodynamics and pharmacokinetics in humans and animals [9]. Sulfamerazine (4-amino-N-(4-methyl pyrimidin-2-yl) benzenesulfonamide) and
sulfamethazine, (4-amino-N-(4,6-dimethyl pyrimidin-2-yl) benzenesulfonamide), structure is shown in Fig. 1, is a member of the sulfonamides family widely used in veterinary medicine for treating various diseases, including nonlactating dairy cattle, poultry, and others [10,11]. However, recent studies have demonstrated that SM and SMT may exist at unhealthy concentration level in various kinds of food, which may cause side effects on human health [12–14].

![Chemical structures of sulfamerazine (SM) and sulfamethazine (SMT)](image)

**Fig. 1** Chemical structures of sulfamerazine (SM) and sulfamethazine (SMT)

Knowledge of interaction mechanisms between these two antimicrobial drugs and serum albumin is very important for us to understand the pharmacokinetics and pharmacodynamics of them. First, the drug–serum albumin interaction plays a dominant role in the bioavailability of drugs because the bound fraction of drugs is a depot, whereas the free fraction of drugs shows pharmacological effects [15]. Second, drug distribution is mainly controlled by serum albumin, because most drugs circulate in plasma and reach the target tissues by binding to serum albumin [16]. If a drug is metabolized and excreted from the body too fast because of low protein binding, the drug won’t be able to provide its therapeutic effect. Alternatively, if a drug has high protein binding and is metabolized and excreted too slowly, it may increase the drugs half life in vivo and lead to undesired side effects [17]. Furthermore, very high affinity binding of a drug to serum albumin may prevent the drug from reaching the target at all, resulting in insufficient tissue distribution and efficacy. Third, the competition between two drugs for the binding sites on serum albumin may result in a decrease in binding and hence an increase of the concentration of the free biologically active fraction of one or both of the drugs. Co-administration of two drugs increases the free concentration of the drug with the lower affinity to serum albumin [18]. In addition, these hydrophobic binding pockets enable serum albumin to increase the apparent solubility of the hydrophobic drugs in plasma and modulate their delivery to the cells in vivo [19]. In a word, the absorption, distribution, metabolism, and excretion properties of a drug can be significantly affected as a result of its binding to serum albumin.

Thus, providing insights on the interaction between such drugs and bio-macromolecules, including proteins, is a necessity. This approach originally developed to illuminate the structure and the excited state behavior of organic molecules and drugs included in cyclodextrin macrocycles through experimental and theoretical [20–26] explorations. In the view of increasing attention directed toward the importance of investigating interaction between proteins and drugs, we present in this work utilizing UV-visible and fluorescence spectroscopy, cyclic voltammetry and molecular docking studies for investigating the interaction between SM/SMT with BSA/adenine. In particular, our investigation is focused on fluorescence quenching, determination of binding constant and binding sites of the SM/SMT – BSA/adenine system, energy transfer and binding distance between BSA/adenine and drugs, thermodynamic free energy and binding modes and conformation changes of BSA/adenine upon binding to drugs.

2. EXPERIMENTAL

2.1. Materials

SM, SMT, adenine and BSA were obtained from Sigma-Aldrich chemical company, USA and used without further purification. All other reagents were of analytical grade. The purity of the compound was checked by similar fluorescence spectra when excited with different wavelengths. Triply distilled water was used for the preparation of aqueous solutions. BSA solutions were
prepared in $2 \times 10^{-5}$ M Tris HCl buffered at pH ~7.4. 0.2 ml of sulfa drug in methanol solution was used for all binding experiments. The concentration of the sulfa drug solutions was varied from $1 \times 10^{-3}$ to $1 \times 10^{-5}$ M. All solutions were stored in a refrigerator at 4 °C in the dark.

2.2. Apparatus
Absorption spectral measurements were carried out with a UV-visible spectrophotometer (model-UV-2600 Shimadzu, Japan) and fluorescence measurements were performed on a spectrofluorophotometer (model-RF-5301PC, Shimadzu, Japan) equipped with 1.0 cm quartz cells. The excitation wavelength for all the sulfa drugs is 280 nm. Cyclic voltammetry measurements were performed through an electrochemical workstation (model-CHI 620D, CH Instruments, USA) with a three electrode system: surface area 0.1963 cm$^2$ platinum disc as working electrode, saturated silver electrode as reference electrode and a platinum foil as counter electrode. Prior to use, the working electrode was polished with 0.05 µm alumina and thoroughly washed in an ultrasonic bath for 5 min. Before experiments, the solution within a single-compartment cell was deaerated by purging with pure N$_2$ gas for 5 min. The pH values in the range 2.5-11.5 were measured on Elico pH meter model LI-120.

2.3. Molecular docking
Molecular docking calculations were carried out using online docking Server (http://www.dockingserver.com) [27]. The MMFF94 force field was used for energy minimization of ligand molecule (SM and SMT) using Docking Server [28]. Gasteiger partial charges were added to the ligand atoms. Non-polar hydrogen atoms were merged, and rotatable bonds were defined. Docking calculations were carried out on 4F5S protein model. Essential hydrogen atoms, Kollman united atom type charges, and solvation parameters were added with the aid of Auto Dock tools [29]. Affinity (grid) maps of 20×20×20 Å grid points and 0.375 Å spacing were generated using the Auto grid program [29]. Auto Dock parameter set and distance dependent dielectric functions were used in the calculation of the van der Waals and the electrostatic terms, respectively. Docking simulations were performed using the Lamarckian genetic algorithm (LGA) and the Solis & Wets local search method. Initial position, orientation, and torsions of the ligand molecules were set randomly. Each docking experiment was derived from 10 different runs that were set to terminate after a maximum of 250000 energy evaluations. The population size was set to 150. During the search, a translational step of 0.2 Å, and quaternion and torsion steps of 5 were applied.

3. RESULTS AND DISCUSSION
3.1. Absorption spectroscopic studies
Absorption and fluorescence spectra of BSA and adenine ($2 \times 10^{-5}$ M) were recorded in the absence and presence of different SM and SMT concentrations (pH~ 7.4) are shown in Table 1, Fig. S1(Supplementary data) and Fig. 2 respectively. In water, the absorption maxima of both SM and SMT drugs appears at 268 and 243 nm with high molar absorption coefficient values ($10^4$ cm$^{-1}$), whereas the absorption spectrum of BSA and adenine appears at ~278, 210 nm and 260 nm respectively. Both sulfa drugs exhibit similar absorption maxima; this is because tautomeric structure is present in both drug molecules [20-25]. With an increasing the concentration of SM and SMT, the absorption maximum of both sulfa drugs was completely lost whereas BSA and adenine absorbance was decreased at the same wavelength; i.e., in varying concentration of sulfa drugs, no remarkable spectral shift was noticed in BSA and adenine except a decrease in the intensity. Inset Fig. S1 indicate that absorbance of BSA and adenine was gradually decreased with increase the SM and SMT concentrations. In addition to this an obvious isosbestic point was observed, which designates a formation of well-defined 1:1 interaction formed between the drugs into BSA and adenine. The above results indicate that both sulfa drugs are interacted with BSA and adenine which agreed with an earlier rep
The variation in absorbance indicates the change in polarity around the tryptophan residue and the change in peptide strand of BSA molecules and hence the change in hydrophobicity [31]. These above observations signify that with the addition of SM and SMT, the peptide strands of BSA molecules were extended more and hydrophobicity was decreased [32] and this perspective was also validated by the following fluorescence spectroscopy studies and its quenching mechanism.

### Table 1. Absorption and fluorescence maxima of BSA and adenine with different concentrations of SM and SMT.

| Concentration of drugs (M) \(10^{-5}\) | BSA | Adenine |
|----------------------------------------|-----|---------|
|                                        | SM  | SMT     | SM  | SMT |
| (without drug)                         | \(\lambda_{abs}\) | \(\log\varepsilon\) | \(\lambda_{flu}\) | \(\lambda_{abs}\) | \(\log\varepsilon\) | \(\lambda_{flu}\) | \(\lambda_{abs}\) | \(\log\varepsilon\) | \(\lambda_{flu}\) |
| 0                                     | 278 | 4.57    | 338 | 278 | 4.55    | 338 | 260 | 4.32    | 288 | 4.35    | 288 |
| 1                                     | 278 | 4.41    | 338 | 278 | 4.53    | 338 | 260 | 4.28    | 288 | 4.33    | 288 |
| 3                                     | 278 | 4.40    | 338 | 277 | 4.46    | 338 | 260 | 4.24    | 288 | 4.31    | 288 |
| 5                                     | 279 | 4.38    | 338 | 277 | 4.44    | 339 | 260 | 4.20    | 288 | 4.29    | 288 |
| 7                                     | 279 | 4.38    | 338 | 277 | 4.43    | 339 | 260 | 4.18    | 288 | 4.26    | 288 |
| 9                                     | 279 | 4.35    | 338 | 277 | 4.42    | 339 | 260 | 4.17    | 288 | 4.25    | 288 |
| 10                                    | 279 | 4.34    | 339 | 277 | 4.41    | 340 | 260 | 4.17    | 288 | 4.21    | 288 |

| Excitation wavelength (nm)           | -   | 280    | -   | 280    | 270    | 270 |
|--------------------------------------|-----|--------|-----|--------|--------|-----|
| \(R_0(10^{-9})\)                     | 1.18| 1.20   | 1.08| 1.10   |
| \(J(10^{-14})\)                      | 1.55| 1.78   | 0.89| 0.77   |
| \(r(10^{-9})\)                       | 1.21| 1.25   | 0.94| 0.97   |
| \(E\)                                | 0.62| 0.66   | 0.48| 0.49   |

The variation in absorbance indicates the change in polarity around the tryptophan residue and the change in peptide strand of BSA molecules and hence the change in hydrophobicity [31]. These above observations signify that with the addition of SM and SMT, the peptide strands of BSA molecules were extended more and hydrophobicity was decreased [32] and this perspective was also validated by the following fluorescence spectroscopy studies and its quenching mechanism.

### 3.2. Fluorescence spectra

Table 1, Figs. 2 displays the fluorescence spectra and the corresponding data of BSA and adenine in aqueous solution as a function of SM and SMT concentrations. The effects of BSA and adenine on the emission spectra of the drug molecule are more pronounced than the corresponding effect on the absorption spectra with respect to the concentration of SM and SMT. BSA and adenine concentration was kept at \(2 \times 10^{-5}\) mol L\(^{-1}\). The excitation and emission slit widths were fixed at 5 nm. The excitation wavelength was set at 280 nm (excitation of the Trp and Tyr), and the emission spectra were recorded at 290 –500 nm. SM and SMT emit two emission maxima at 348 and 434 nm respectively with a shoulder at 372 nm when excited at 310 nm (not shown in the figure). It has
been shown in our earlier studies [9,10] that sulfonamide derivatives undergo normal as well as highly Stokes shifted fluorescence. In isolated adenine, multiple emission maxima appear at 430, 330 and 288 nm where as single emission was observed (338 nm) in the BSA molecule. When the sulfa drugs were added into the BSA or adenine solution, the emission maxima of both sulfa drugs are completely quenched. However, the fluorescence intensity of BSA and adenine dropped regularly with increasing the concentration of SM or SMT.

In the absence and presence of SM and SMT, the fluorescence spectra of adenine consists of three distinct bands, the maxima at around 290 nm (F₁) 330 nm (F₂) 430 nm (F₃) with SM and SMT. The intensity of the pure adenine bands is in the order F₁ > F₂ > F₃; however with the addition of the drugs the intensity of the bands is in the order F₂ > F₁ > F₃ The yields of the bands are in conformity with some other findings for similar other compounds. Fig 2 shows that as the drugs concentration increases in the adenine solution F₁, F₂ and F₃ band intensity gradually decreases at the same wavelength. Fluorescence quenching refers to any process that decreases the fluorescence intensity of a fluorophore. Therefore, SM or SMT acted as a quencher and quenched the fluorescence of BSA. The results demonstrated that SM or SMT bound with BSA and adenine formed a non-fluorescent complex. The binding parameters were then obtained according to the following equation:

$$\log \left[ \frac{(F₀ - F)}{F} \right] = \log K_a + n \log [Q]$$  \hspace{1cm} (1)

where $n$ is the number of binding sites, $K_a$ is the binding constant or the apparent association constant for drug– protein interaction. Values of $n$ and $K_a$ can thereby be determined from the intercept and slope by plotting $\log \left( \frac{(F₀ - F)}{F} \right)$ versus $\log [Q]$ which is shown in Fig. 3. The data in Table 2 show that the value of $n$ is approximately equal to 1, indicating that there is one class of binding sites for SM/SMT in BSA/adenine.
Fig. 2 Fluorescence spectra of BSA and adenine with different concentrations of SM and SMT (x $10^{-3}$ M): 1) 0, 2) 1, 3) 3, 4) 5, 5) 7, 6) 9 and 7) 10; Inset fig.: fluorescence intensity ($I_F$) vs. [drug].
Table 2 Stern-Volmer quenching constant ($K_{sv}$), modified Stern-Volmer association constant $K_a$ and bimolecular quenching rate constant ($K_q$) of the adenine and BSA with SM and SMT.

| Method                  | Drugs | $K_{sv}$ (M$^{-1}$) | $K_q$ (M$^3$s$^{-1}$) | $R^a$ | SD  |
|-------------------------|-------|--------------------|-----------------------|-------|-----|
| S-V quenching           | BSA   | SM  1.92 × 10$^5$  | 1.92 × 10$^{12}$     | 0.999 | 0.592 |
|                         |       | SMT  1.88 × 10$^5$  | 1.88 × 10$^{12}$     | 0.999 | 0.761 |
|                         | Adenine | SM     | 3.93 × 10$^4$      | 3.93 × 10$^{14}$ | 0.990 | 0.492 |
|                         |       | SMT  4.21 × 10$^4$  | 4.21 × 10$^{14}$     | 0.991 | 0.668 |
| Modified S-V quenching  | BSA   | SM    | 1.37 × 10$^5$      | -20.93  | 0.988 | 0.999 |
|                         |       | SMT  1.32 × 10$^5$  | -20.98    | 0.954 | 0.998 |
|                         | Adenine | SM     | 2.01 × 10$^4$      | -19.02  | 0.789 | 0.996 |
|                         |       | SMT  2.11 × 10$^4$  | -19.15    | 0.921 | 0.999 |

$R^a$ - linear correlation coefficient, SD – Standard deviation, n – number of binding site

The value of the binding constants are 1.37 × 10$^4$ M$^{-1}$ (R = 0.999) and 1.32 × 10$^4$ M$^{-1}$ (R = 0.998) for BSA and 2.01 × 10$^4$ M$^{-1}$ (R = 0.996) and 2.11 × 10$^4$ M$^{-1}$ (R = 0.999) for adenine. Thermodynamic parameters of the binding reaction provide the majority of the evidence to confirm the binding force. Hence, the free energy change ($\Delta G$) can be derived from equation:

$$\Delta G = -RT \ln K$$

(2)

where, $K$ is the binding constant at a corresponding temperature; $R$ is the gas constant; and $T$ is the absolute temperature. The negative value of $\Delta G$ indicated the spontaneity of the binding between SM and SMT with BSA/adenine.

3.3. Quenching mechanism

In order to understand quantitatively the magnitude of the SM and SMT to quench the emission intensity of BSA/adenine, the linear Stern–Volmer equation is also employed:

$$F_0/F = 1 + K_{sv}Q$$

(3)

where $F_0$ and $F$ represent the fluorescence intensities in the absence and presence of the quencher, respectively. $Q$ is the concentration of the quencher. $K_{sv}$ is the linear Stern–Volmer quenching constant. $K_q$ is the bimolecular quenching rate constant, and $\tau_0$ is the average lifetime of the fluorophore in the absence of the quencher, which is 10$^{-8}$ s. Figs. 3, S2 shows the quenching plots of $F_0/F$ versus [Q]. The Stern Volmer quenching constant $K_{sv}$ and $K_q$ values obtained from the equation (1) are 1.92 × 10$^7$ M$^{-1}$ and 1.92 × 10$^{12}$ M$^{-1}$ s$^{-1}$ (R = 0.999 for SM with BSA) and 1.88 × 10$^5$ M$^{-1}$ and 1.88 × 10$^{12}$ M$^{-1}$ s$^{-1}$ (R = 0.999 for SMT with BSA). Obviously, the rate constants $K_q$ of BSA/adenine quenching initiated by the both drugs are higher than 2.0 × 10$^{10}$ M$^{-1}$ s$^{-1}$, which is the maximum collision quenching constant of various kinds of quenchers to bio-macromolecules [33], indicating that the above mentioned quenching is not initiated by dynamic collision. This standpoint was furthermore legitimizing by the following theory [Forster non-radiative energy transfer (FRET)].
3.4. Energy Transfer theory

The energy transfer effect is related not only to the distance between the acceptor and the donor, but also to the critical energy transfer distance $R_0$, that is

$$E = \frac{R_0}{R_0^6 + r_6}$$  \hspace{1cm} (4)

where $r$ is the distance between the acceptor and the donor and $R_0$ is the critical distance when the transfer efficiency is 50%, which can be calculated by

$$R_0^6 = 8.79 \times 10^{-25} \cdot K^2 \cdot n^{-4} \cdot \varphi \cdot J$$  \hspace{1cm} (5)

where $k^2$ is the spatial orientation factor between the emission dipole of the donor and the absorption dipole of the acceptor. The dipole orientation factor, $k^2$ is the least certain parameter in the calculation of the critical transfer distance ($R_0$). Although theoretically $k^2$ can range from 0 to 4, the extreme values require very rigid orientations. If both donor and acceptor are tumbling rapidly and free to assume any orientation, then $k^2$ equals 2/3 [34]. If only the donor is free to rotate, then $k^2$ can vary from 1/3 to 4/3 [35,36], $N$ is the refractive index of the medium, the fluorescence quantum yield of the donor and $J$ is the overlap integral of the fluorescence emission spectrum of the donor and the absorption spectrum of the acceptor, given by

$$J = \frac{\int_{-\infty}^{\infty} F(\lambda) \varepsilon(\lambda) \lambda^4 d\lambda}{\int_{0}^{\infty} F(\lambda) d\lambda}$$  \hspace{1cm} (6)

where $F(\lambda)$ is the fluorescence intensity of the fluorescent donor in wavelength $\lambda$ and dimensionless, $\varepsilon(\lambda)$ is the molar absorption coefficient of the acceptor in wavelength $\lambda$ and unit is cm$^{-1}$ mol$^{-1}$ L. The energy transfer efficiency is frequency calculated from the relative fluorescence yield in the presence ($F$) and absence of acceptor ($F_0$):

$$E = 1 - \frac{F}{F_0} = R_0 / (R_0^6 + r_6)$$  \hspace{1cm} (7)
On the basis of equations (4) – (7), the following parameters are obtained for BSA – SM and BSA – SMT respectively: \( J = 1.55 \) and \( 1.78 \times 10^{-14} \) cm\(^3\) L mol\(^{-1}\), \( R_0 = 1.18 \) and 1.20 nm, \( E = 0.62 \) and 0.66, \( r = 1.21 \) and 1.25 nm (Table 1). The binding distance between the drugs (SM and SMT) and tryptophan residue of BSA is 1.21 and 1.25 nm, which is smaller than 7 nm. According to conditions of Foster’s non-radioactive energy transfer theory the static quenching interaction between BSA and SM or SMT could be confirmed.

3.5. Cyclic voltammetry studies

Figure 4 and Table 3 shows the cyclic voltammograms of BSA and adenine in the absence and presence of different amounts of SM and SMT. Curve 1 was the redox curve of SM and SMT drugs with the peak potential at, SM \( \sim E_{pc} \) – 0.163, -0.713V and \( E_{pa} \) – 1.057 V, SMT \( \sim E_{pc} \) – 0.178, -0.610, -1.1835 and \( E_{pa} \) – 1.057 V respectively (vs. SCE). From curve 2, it can be seen that in the absence of drugs, BSA and adenine displays a oxidation waves corresponding to amino acids. On the addition of BSA/adenine, the reductive peak current of both drugs increased greatly without the shift of peak potential and no new reductive peaks appeared (curve 3 to 5).

![Cyclic voltammograms of BSA and adenine with different concentrations of SM and SMT](image)

**Fig. 4.** Cyclic voltammograms of BSA and adenine with different concentrations of SM and SMT \((x \times 10^{-4} \text{ M})\): 1) 0, 2),3, 3) 7 and 4) 10.

The oxidation peak shifted towards higher potentials and an increasing oxidation current was observed. When more drug added to the BSA/adenine, the greater the peak current \( (I_{pa}) \) increased.

With the addition of BSA/adenine to the SM and SMT drugs, the voltammetric oxidation peak currents increased and negatively shifted apparently, indicating that there exist interactions between the drugs and BSA / adenine. The drop of the voltammetric reduction current in the presence of BSA/adenine may be attributed to slow diffusion of the BSA/adenine binding with SM/SMT. The current charge (whether increase or decrease) is due to interaction of sulfa drugs with adenine or BSA. The results also indicated that there were binding reaction happened in the reaction solution and the electrode process was also irreversible.
Table 3 CV for BSA/adenine with SM and SMT (scan rate, 100 mV s\(^{-1}\), concentration of adenine and BSA 2×10\(^{-6}\) M; drugs concentration: 0, 3, 7 and 10 ×10\(^{-3}\) M).

| Drug-BSA or adenine | Drug concentration (× 10\(^{-3}\)) | \(E_{pa}\) | \(I_{pa}\) | \(E_{pe}\) | \(I_{pc}\) | \(E_{pa} - E_{pc}\)/2 | \(I_{pa}/I_{pc}\) |
|---------------------|-----------------------------------|--------|--------|--------|--------|----------------|------------------|
| SM only             | 2 × 10\(^{-3}\)                  | 1057   | 0.643  | 1638   | -0.584 | -818          | -1.101           |
|                     |                                   |        |        |        |        | -713          | -0.654           |
| SMT only            | 2 × 10\(^{-3}\)                  | 1042   | 0.901  | 1782   | -1.009 | 1412          | -0.892           |
|                     |                                   |        |        |        |        | -1183         | -0.997           |
|                     |                                   |        |        |        |        | -1042         | -0.698           |
| BSA only            | 2 × 10\(^{-6}\)                  | 973    | 0.524  | -      | -      | 487           | 0                |
| Adenine only        | 2 × 10\(^{-6}\)                  | 987    | 0.613  | -      | -      | 678           | 0                |
| BSA - SM            | 3 × 10\(^{-8}\)                  | 1080   | 2.524  | -724   | -1.096 | 1033          | -0.434           |
|                     | 7 × 10\(^{-8}\)                  | 1158   | 2.714  | -698   | -1.387 | 928           | -0.511           |
|                     | 10 × 10\(^{-8}\)                 | 1343   | 2.739  | -684   | -1.502 | 882           | -0.548           |
| BSA - SMT           | 3 × 10\(^{-8}\)                  | 1073   | 1.463  | -796   | -1.056 | 1024          | -0.721           |
|                     | 7 × 10\(^{-8}\)                  | 1106   | 1.881  | -724   | -1.094 | 915           | -0.581           |
|                     | 10 × 10\(^{-8}\)                 | 1253   | 2.248  | -710   | -1.372 | 891           | -0.610           |
| Adenine - SM        | 3 × 10\(^{-8}\)                  | 1083   | 0.621  | -446   | -0.970 | 840           | -1.562           |
|                     | 7 × 10\(^{-8}\)                  | 1171   | 0.708  | -451   | -1.181 | 811           | -1.668           |
|                     | 10 × 10\(^{-8}\)                 | 1235   | 1.033  | -463   | -1.308 | 773           | -1.266           |
| Adenine - SMT       | 3 × 10\(^{-8}\)                  | 995    | 0.890  | -714   | -1.108 | 646           | -1.244           |
|                     | 7 × 10\(^{-8}\)                  | 1058   | 1.186  | -744   | -1.342 | 529           | -1.136           |
|                     | 10 × 10\(^{-8}\)                 | 1293   | 1.221  | -782   | -1.388 | 869           | -1.065           |

The reductive reaction of sulfa drug-BSA/adenine interaction solution had the characteristics of the strong adsorption and emission behaviour and the irreversible electrode process. So Laviron’s equation [37,38] may be used to evaluate the kinetic constants of electrode reaction in the absence and presence of protein.

\[
E_p = E^0 + \frac{RT}{\alpha n F} \left[ \ln \left( \frac{RT k_s}{\alpha n F} \right) - \ln v \right]
\]

Where \(\alpha\) is the electron transfer coefficient, \(k_s\) the standard rate constant of the surface reaction, \(v\) the scan rate, \(E^0\) the formal potential and \(n\) the electron transfer number.

According to above equation, parameters of the SM and SMT – BSA/adenine reaction were calculated with the same method and the results were also got from Fig. S3a and S3b, if the \(E^0\) is known, plot of \(E_p\) vs. \(v\) is well-defined straight line (Figs. S3) and the \(\alpha n\) value can be calculated from the slope and \(k_s\) from the intercept. \(E^0\) value can be deduced from the intercept of \(E_p\) vs. \(v\) plot on the ordinate by extrapolating the line to \(v = 0\), when \(v\) was approached to zero, then \(E_p\) was approached to \(E^0\).

The plot of \(E_p\) vs. \(\ln v\) was shown in Figs. S3 (c and d), which was non linear line. From the slope, the \(\alpha n\) values of SM and SMT can be determined, and from the intercepts, the \(k_s\) values can be calculated.

### 3.6. Measurement of stoichiometry of complex

According with our earlier report [30] the composition and the equilibrium constant can be calculated based on the changes of peak current.

\[
\log \left[ \frac{\Delta I}{\Delta I_{\max}} \right] = \log \beta_k + \log [\text{Drug}]
\]

From Eq. (9), the relation of \(\log [\Delta I/ (\Delta I_{\max}\Delta I)]\) with \(\log [\text{Drug}]\) was calculated and plotted in Fig. 5. From the intercept and slope \(m\) and \(\beta_k\) were deduced, which indicated that SM and SMT binding to BSA/adenine formed the complex of BSA/adenine – drugs. Further the plot of \(\log [\text{drug}]\) vs. \(\log [\Delta I/(\Delta I_{\max} - \Delta I)]\) was linear (Fig 5) indicates that both sulfa drugs formed a single complex with protein.
3.7. Molecular docking studies

BSA and the drugs were further ascertained using the molecular docking technique as described below. In this study, the molecular docking server was used to realize the binding mode of SM and SMT at the active site of BSA. The binding site were obtained as Tyr150, Glu153, Ser192, Lys195, Glu196, Ser234, Leu238, His242, Arg257, Ile264, Ile290, Ala291 and Glu292. The estimation free energy of binding for the binding of SM and SMT to BSA were -5.81 and -7.63 kcal/mol respectively (Table 4).

The docking results showed that SM and SMT bind within the binding pocket of subdomain IIA (Figs. 6a and 6b). The binding site of the BSA was studied to understand the nature of the residues defining the site.

**Table 4.** Estimated free energy, Inhibition constant, Electrostatic energy and Total intermolecular energy of the BSA with SM and SMT.

| Drug | Est. Free Energy kcal/mol | Est. Inhibition Constant, Ki µM | vdW + H-bond + desolv Energy kcal/mol | Electrostatic Energy kcal/mol | Total Intermolecular Energy kcal/mol | Frequency | Intert. Surface |
|------|---------------------------|--------------------------------|--------------------------------------|-----------------------------|----------------------------------|----------|----------------|
| SM   | -5.81                     | 55.35                          | -6.40                                | -0.22                       | -6.62                            | 70%      | 671.19         |
| SMT  | -7.63                     | 2.55                           | -8.59                                | +0.07                       | -8.52                            | 90%      | 577.11         |
The distance between the SM/SMT and the TRP residue was 2.53 and 3.04 Å that explain the fluorescence quenching of BSA emission in the presence of SM and SMT. Hydrogen bonding plots and its two-dimensional schematic representation was used to explore the hydrogen bonds interactions between BSA with SM and SMT as shown in Fig. 7.

A hydrogen bonding interaction is observed between the hydroxy (OH) group of Thr190/Thr578 in BSA and bioactive group of sulfa drugs, with a distance of SM ~ 2.53 Å/ SMT ~ 3.04 Å.

**Fig. 6** The best binding mode between BSA with (a) SM and (b) SMT, important residues of BSA are represented using lines and the ligand structure is represented using a “Ball and Stick” format. Colour of the atoms: skeleton structure – BSA, blue- nitrogen, red –oxygen, yellow –sulphur, green- carbon.
Fig. 7. Hydrogen bonding plots between sulfa drugs (c) SM and (d) SMT with BSA. The BSA residues are represented using black dots and the hydrogen bonding interactions are represented using red dots. Two-dimensional schematic representation of hydrogen bonding interactions of BSA with (a) SM and (b) SMT. Hydrogen bond depicted in dashed line. Colour of the atoms: blue – nitrogen, red – oxygen, yellow – sulphur, black – carbon.
4. CONCLUSIONS

The interaction between BSA and adenine with SM and SMT drugs has been investigated by using different biophysical techniques. BSA fluorescence can be statically quenched by both drugs, which implies that SM and SMT can bind to BSA and adenine to form like protein - drug complex. Both drugs can bind to BSA and adenine with stoichiometric ratio of 1:1 and the protein - drug complexes are stabilized mainly by hydrogen bonds and van der Waals interaction. Compared to SM, SMT contributes substantially higher binding efficiency with BSA/adenine. With addition of drug solution to the adenine/BSA, the oxidation and the reduction peaks shifted towards high and low potentials, respectively. Molecular docking studies shows, BSA was observed to be similar in shape, in the presence and absence of the drugs, which meant that the second structure of BSA changed after binding of the drugs.

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Fig. S1. Absorbance spectra of BSA and adenine with different concentrations of SM and SMT (x 10^{-5} M): 1) 0, 2) 1, 3) 3, 4) 5, 5) 7, 6) 9 and 7) 10; Inset figure: Absorbance vs. [drug].
Fig. S2. Stern-Volmer and modified Stern Volmer plots for quenching of adenine with SM and SMT at 300 K; $C_{\text{adenine}} = 2.0 \times 10^{-5}$ M; pH 7.4, $\lambda_{\text{ex}} = 270$ nm, $\lambda_{\text{em}} = 270–500$ nm.

Fig.S3. Dependence of the peak potential $E_p$ on the potential scan rate ($\nu$) of SM/SMT with BSA and adenine. Semilogarithmic dependence of the peak potential $E_p$ on potential scan rate (ln $\nu$) of SM/SMT with BSA and adenine.
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