Exploration of binding interaction between doxycycline and bovine serum albumin by spectroscopic and voltammetric methods

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ABSTRACT

The interaction of bovine serum albumin (BSA) with the doxycycline (DOX) was investigated by different spectroscopic and voltammetric techniques under experimentally optimised physiological conditions. The alteration of functional properties of BSA when quenched by DOX was illustrated by the continuous decrease of the fluorescence intensity of BSA upon addition of DOX. The mechanism of BSA-DOX system was dynamic quenching process and was confirmed by lifetime measurements. The number of binding sites, binding constants and other binding characteristics were computed. The binding constants (K) at three different temperatures were calculated and thermodynamic parameters were determined. The positive values of ΔH^0 and ΔS^0 suggested the hydrophobic interactions play the major roles in the binding reaction. The conformational change of BSA after binding with DOX was demonstrated by the UV-visible spectrum, synchronous, 3D florescence spectra, FTIR and differential pulse voltammetric results. The effects of some common metal ions and site probes on binding of BSA– DOX were also investigated.

Keywords: Interaction, quenching, binding, spectra, bovine serum albumin.

1. Introduction

Protein plays a significant role in the living organisms by performing various biological activities. Serum albumin has been employed as a model for studying drug–protein interaction in vitro. Many drugs and other bioactive small molecules bind reversibly to albumin and other serum components so that they function as carriers [1]. This type of interaction can also influence the drug stability and toxicity during the chemotherapeutic process [2]. They play a significant role in the transport and deposition of endogenous and exogenous functional groups in blood as serum albumins often increase the apparent solubility of hydrophobic drugs in plasma and modulate their delivery to cells in vivo and in vitro. They also play an important role in storage and transport of energy [3]. The binding ability of drug-protein in blood stream may have a significant impact on distribution, free concentration and metabolism of drug. Because of the medicinal importance, low cost, ready availability and unusual ligand binding properties [4], bovine serum

albumin (BSA) (Figure 1A) is used as model protein. BSA and human serum albumin (HSA) display approximately 76% sequence homology [5]. BSA consists of 582 amino acids with 2 tryptophan moiety located at position 134 (located on the surface of domain I) and 214 (located within the hydrophobic pocket of domain II). Tryptophan residues are the main intrinsic fluorophores that are extremely sensitive to their microenvironment. The knowledge on the mechanism of interaction between the drug and plasma protein is of crucial importance to understand the pharmacodynamics and pharmacokinetics of a drug [6].

Doxycycline ((4S, 4aR, 5S, 5aR, 6R, 12aS)-4-(dimethylamino)-3,5,10,12,12a-pentahydroxy-6-methyl-1, 11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide) (Figure 1B) an antibiotic and anti-inflammatory drug was used in the treatment of infections caused by bacteria and protozoa, lung diseases associated with an influx of monocytes such as panbronchiolitis, asthma, cystic fibrosis, and bronchitis [7] . It is also useful for the treatment of malaria when used with quinine [8]. Doxycycline (DOX) can be used either by mouth or intravenously. Side effects of doxycycline are allergic reaction like hives; difficulty in breathing; swelling of face, lips, tongue, or throat, etc. Drug interactions at protein binding level will in most cases significantly affect the apparent distribution volume of the drugs and also affect the elimination rate of drugs. Therefore, the studies on this aspect can provide information on the structural features that determine the therapeutic effectiveness of drug and have been an interesting field of research in life sciences, chemistry and clinical medicine [9].

The literature survey revealed that attempts have not been made so far to investigate the binding mechanism of BSA with DOX by spectroscopic and cyclic voltammetric techniques. The aim of this study is to understand the interaction mechanism of BSA with DOX by investigating the binding parameters such as binding constants, number of binding sites, thermodynamic parameters, effect of some common metal ions, site probes and the conformational change of BSA with DOX by using different spectroscopic and voltammetric techniques. The study is expected to provide important insight into the interactions of protein, BSA with DOX under physiological conditions.

2. Experimental

2.1. Reagents and Chemicals

Bovine serum albumin (BSA) and doxycycline were purchased from Sigma Aldrich and used as such. Site probes warfarin, ibuprofen and digitoxin purchased from Sigma Chemical Company were initially dissolved in 5% methanol-water and then diluted with distilled water. The solutions of BSA, site probes and metal ions were prepared in 0.1M phosphate buffer (0.1 M NaH_2PO_4 and 0.1M Na_2HPO_4) of physiological pH 7.4. BSA solution was prepared based on the molecular weight 65,000 and DOX solution prepared in Millipore water. All other materials were of analytical reagent grade and Millipore water was used throughout the experiment.

2.2. Instrumentation

Fluorescence spectra were recorded using a RF-5301 PC Hitachi Spectrofluorometer Model F-2000 (Tokyo, Japan) with a 150 W Xenon lamp, a 1 cm quartz cell and thermostatic cuvette holder. The excitation and emission bandwidths were both 5 nm and the scan rate was 1200 nm/sec. The temperature of the sample was maintained by recycling water throughout the experiment. The absorption spectra were recorded on a double beam CARY 50-BIO UV–visible spectrophotometer (Victoria, Australia) with a scan rate of 600nm/min. The FT-IR measurements were made at room temperature on a Nicolet 5700 USA FT-IR spectrometer equipped with a germanium attenuated total reflection (ATR) accessory, a DTGS KBr detector and a KBr beam splitter. The pH measurements were performed with an Elico LI120 pH meter (Elico Ltd., India).Fluorescence life-time measurements were carried out in an ISS' Chronos BH fluorescence life time spectrometer. Electrochemical measurements were carried out on a CHI 630D electrochemical analyzer (CH Instruments Inc., USA).

2.3. Procedures

2.3.1. Fluorescence quenching of BSA–DOX interaction study

A stock solution of 250μM BSA and DOX are prepared in phosphate buffer solution (pH = 7.4). An appropriate concentration of the BSA solution (5μM from 250μM stock), DOX solution (5 μM from 250 μM stock) were transferred into a 5mL conical flask containing 2mL of phosphate buffer solution (pH 7.4) and then were shaken. On the basis of preliminary experiments, BSA concentration was fixed at 5μM and drug concentration was varied from 5μM to 45μM. Fluorescence spectra were recorded at three different temperatures (288, 298 and 308 K) in the range 290–550 nm upon excitation at wavelength of 296 nm in each case.

2.3.2. Fluorescence life time measurement

The fluorescence lifetime measurements of BSA in the presence and absence of DOX were recorded by fixing 296 nm as the excitation wavelength and 343 nm as the emission wavelength. The BSA concentration was fixed at 5μM while the drug concentration was varied from 5μM to 30μM in the presence of phosphate buffer at room temperature

2.3.3. UV measurements

The UV measurements of BSA in the presence and absence of DOX was made in the range of 200–400 nm. BSA concentration was fixed at 5μM while the drug concentration was varied from 5μM to 45μM in the presence of phosphate buffer as a solvent. DOX does not show any absorption at this wavelength.

2.3.4. Synchronous fluorescence measurements

The synchronous fluorescence spectral measurements of DOX–BSA were recorded at different scanning intervals of $\Delta\lambda$ ($\Delta\lambda=\lambda_{ex}-\lambda_{em}$). The spectrum behavior of tyrosine and tryptophan residues of BSA was observed when $\Delta\lambda$ = 15 nm and $\Delta\lambda$ = 60 nm, the spectra were recorded in the range of 200–500 nm.

2.3.5. 3D fluorescence spectra

The three-dimensional fluorescence spectra (3D) of BSA were recorded with and without DOX. 5µM of protein solution was transferred to a quartz cell, diluted to 2.0mL with phosphate buffer and mixed well. To this, 5µM of DOX was added and the 3D fluorescence spectra were recorded by scanning excitation wavelength in the range of 200–500nm and emission wavelength from 200 to 600nm at an interval of 10nm. The scanning parameters were the same as the fluorescence quenching experiments.

2.3.6. FT-IR measurements

The FT-IR spectra of BSA in presence and absence of DOX at 298 K were recorded in the range of 1400–1800 cm^{-1}. The concentrations of BSA and DOX were 5 µM in presence of phosphate buffer.

2.3.7. Voltammetric measurements

The voltammetric measurements were carried out in a 10 mL single compartment three-electrode glass cell with Ag/AgCl as a reference electrode, a platinum wire as counter electrode and a glassy carbon electrode as working electrode. The differential pulse voltammograms of BSA-DOX system were recorded in the potential range of 0.5 V to 1.0 V, the concentration of BSA was fixed at 5µM with increasing concentration of DOX from 5µM to 45µM. Also the voltamogrammas of BSA-DOX system with increasing concentration of BSA from 5µM to 45µM were recorded in presence of DOX (5µM) in phosphate buffer solution (pH 7.4) at room temperature.

The parameters of DPV were initial potential: 0.5 V; final potential: 1.0 V; increases potential: 0.004V; amplitude: 0.05V; pulse width: 0.06s; sample width: 0.02s; pulse period 0.5s; with time: 2s; and sensitivity: 10^{-7} A/V.

2.3.8. Competitive binding studies

The competitive binding studies were performed using different site probes viz., warfarin, ibuprofen and digitoxin for site I, II and III, respectively [10] by keeping the concentration of BSA and the probe constant (5µM each). The fluorescence quenching titration was used as before to determine the binding constant of BSA - DOX in presence of above site probes.

2.3.9. Effects of some common metal ions

The effects of some common metal ions viz., Co^{2+} ($CoCl_2$), Cu^{2+} ($CuCl_2$), Ni^{2+} ($NiCl_2$), Ca^{2+} ($CaCl_2$) and Zn^{2+} ($ZnCl_2$) were investigated on DOX-BSA interactions. The fluorescence spectra of BSA- DOX system were recorded in the range of 290nm to 550nm upon the excitation at 296 nm. The overall concentration of BSA and that of the common metal ions was fixed at 5µM.

3. Results and discussion

3.1. Fluorescence quenching of BSA by doxycycline

Fluorescence methods have been widely used to investigate the interaction between ligands and proteins and can give information about the quenching mechanism, binding constants and binding sites. We utilized the fluorescence technique to study the interaction between BSA and DOX. The fluorescence spectra of BSA at various concentrations of DOX were shown in Figure. 2. The fluorescence intensity of BSA

decreased regularly with an increasing concentration of DOX, which indicated that DOX can bind to BSA. The fluorescence intensity of system decreased tardily in each temperature curve which indicates the beginning of saturation of the BSA binding site. Furthermore, the maximum wavelength of BSA shifted from 343nm to 333 nm after the addition of DOX, so a blue shift of the maximum emission wavelength was observed and it could be deduced that the fluorophore of protein was placed in a more hydrophobic environment after the addition of DOX. It was probably owing to the loss of the compact structure of hydrophobic subdomain IIA where tryptophan is placed [11]. The different mechanisms of fluorescence quenching are usually classified as dynamic quenching or static quenching [12]. Dynamic quenching results from interaction through collision between the fluorophore and the quencher whereas static quenching is caused by formation of a non-fluorescent ground state fluorophore–quencher complex. Dependence of fluorescence quenching constants on different temperatures and fluorescence lifetime measurements can helpful in distinguish the dynamic and static quenching [13]. The quenching constants decreased with increasing temperature for static quenching, but the reverse effect is observed in the case of dynamic quenching [14]. In both the cases, molecular contact is required between the flurophore and the quencher for fluorescence quenching [15]. To confirm the possible quenching mechanism, the fluorescent quenching data were subjected to well known Stern–Volmer [16] equation (1).

$$\frac{F_0}{F} = 1 + K_{SV}[Q] = 1 + k_q \tau_0 [Q] \qquad (1)$$

where F_0 and F are the fluorescence intensities of BSA in the absence and presence of the quencher, K_{SV} is the Stern–Volmer quenching constant, [Q] is the concentration of the quencher, k_q is the quenching rate constant of the biomolecule and τ_0 is the average lifetime of the biomolecule without quencher [17]. The Stern–Volmer plots of interactions carried out at different temperatures (288, 298 and 308 K) were shown in figure SI Figure. 1. The Stern–Volmer quenching constant, K_{SV} and the correlation coefficient of each curve were calculated from the slope of the regression curves. The values of K_{SV} for BSA- DOX system at different temperatures were given in Table 1. Increase in K_{SV} values with increase in temperature reveals the dynamic quenching mechanism between BSA and DOX [18]. The quenching rate constant can be calculated by using the following equation (2)

$$k_q = K_{SV}/\tau_0 \qquad (2)$$

The fluorescence lifetime of the biopolymer is 10^{-8} s [19]. The quenching rate constant at 298 K was calculated to be 6.3 $\times 10^{13}$ $Lmol^{-1}s^{-1}$ and the values at different temperatures are listed in Table 1. The quenching rate constant values increased with increasing temperature which supports the dynamic quenching interaction between BSA and DOX. However, the maximum scatter collision quenching constant, k'_q of various quenchers with the biomolecule is 2×10^{10} $Lmol^{-1}s^{-1}$. Hence the quenching rate constant BSA−DOX system is greater than quenching constant of the biomolecule.

3.2. Fluorescence life time measurement studies

Time resolved fluorescence life time measurement is an ideal nanoscale probe detection method. Here the emission of a fluorophore can be highly influenced by its environment or the presence of other interacting molecules [20]. The steady state emission spectrum life time decay of the excited state to the ground state is in homogeneous environment and quenching process. For these measurements the sample is exposed to a pulse of light, where the pulse width is typically shorter than the decay time of the molecules. Lifetime based measurements are rich in information and provide unique insights into the systems under investigation [16]. Time-resolved fluorescence lifetime measurements were carried out for BSA in the absence and presence of DOX (Figure 3). Time resolved fluorescence spectroscopy was used to determine the decay times, thus enabling the differentiation between the presence of static and dynamic quenching. The dynamic quenching constant was determined by lifetime measurements by using the equation (3).

$$\frac{\tau_0}{\tau} = 1 + K_D[Q] \qquad (3)$$

where τ_0 and τ are the fluorescence lifetimes of BSA in the absence and presence of DOX respectively and K_D is the dynamic quenching constant. The fluorescence lifetimes (τ), average life-time ($<\tau>$), intensity fraction (f), Chi- square (χ^2) and their relative amplitudes (α) are listed in SI Table 1. From the plot of τ_0/τ versus [Q] the K_D value was found to be 2.51×10^3 M^{-1}. The value of static quenching constant, K_S, was calculated [16] by using the equation (4).

$$\frac{\left[\frac{F_0-F}{F}\right]}{[Q]} = (K_S + K_D) + K_S K_D[Q] \qquad (4)$$

From the plot of $\{(F_0 - F)/F\}/[Q]$ versus [Q] and with the value of known K_D, the value of K_S was found to be 0.96×10^3 M^{-1}. It reveals that the value K_D was greater than that of K_S which suggests that quenching mechanism of BSA−DOX was predominantly dynamic quenching than that of static quenching.

3.3. Binding parameters

When small molecules bind independently to a set of equivalent sites on a macromolecule [21], the binding constant (K) and the number of binding sites (n) can be obtained from the equation (5).

$$\log\frac{(F_0-F)}{F} = \log K + n \log[Q] \qquad (5)$$

where F_0 and F are the fluorescence intensities of protein in the absence and presence of drug, respectively and [Q] is the concentration of drug. The values of K and n are obtained from the intercept and slope of the plot of log $(F_0-F)/F$ versus log [Q] (Figure 4) and are shown in Table 1. The value of K increases with increase in temperature which also supports the dynamic quenching mechanism. The value of n is helpful to know the number of binding sites. In the present study, the number of binding sites n obtained was approximately equal to 2, indicating that there were two binding sites in BSA for DOX during their interaction, one of which is a strong binding site and the other is a weak one. This may be due to the DOX involved other sites with higher binding affinity and selectivity. Although in most of the studies on binding of drug–protein interactions, the value of n obtained was unity, however, there are also a few reports [14,22]

where the value of n was more than unity. The value of K is of the order of 10^8, indicating that a strong interaction exists between BSA and DOX. Even if a low concentration of DOX is present in organs, DOX can interact with BSA easily.

3.4. Determination of the binding forces

Thermodynamic measurements can help to determine the major binding forces between drug and BSA. There are four types of interactions between small molecule, ligands and biological macromolecules: hydrophobic forces, hydrogen bonds, Vander Waals' interactions and electrostatic forces, so on [23]. Thermodynamic parameters are important for confirming the non covalent acting forces. Ross and Subramanian have summed up the thermodynamic laws to determine the types of binding forces [24]. The enthalpy change (ΔH^0), free-energy change (ΔG^0) and the entropy change (ΔS^0) for the interaction between BSA and DOX were calculated based on the van't Hoff equation (6)

$$\log K = -\frac{\Delta H^0}{2.303\ RT} + \frac{\Delta S^0}{2.303R} \qquad (6)$$

where K is the binding constant at the corresponding temperature , R is the gas constant and T is the temperature. From the slope and intercept of plot of log K vs 1/T (SI Figure 2), the values of ΔH^0 and ΔS^0 for the binding process were obtained. The value of ΔG^0 was calculated using the Gibbs Helmholtz equation (7)

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 \qquad (7)$$

The values of ΔH^0, ΔS^0 and ΔG^0 are listed in Table 1. It can be seen the negative value of ΔG^0 indicates that the binding process was spontaneous and it was exothermic reaction. The positive values of enthalpy (ΔH^0) and entropy (ΔS^0) indicate that the binding is mainly entropy driven and the enthalpy is unfavorable for it, the hydrophobic forces played effective role in the reaction [25].

3.5. UV-Absorption spectroscopic studies

UV–visible absorption spectroscopy allows to structural analysis of substances; it is a simple, useful technique to investigate conformational changes of proteins, even at the low concentrations. This method is applicable to know the change in hydrophobicity [26] and the interaction between drug and protein [27]. The λ_{max} of BSA observed at around 280nm was mainly due to the presence of amino acid residues of tryptophan and tyrosine in BSA. It was evident from the UV-spectrum (Figure 5) that the absorption intensity of BSA increased regularly with increasing concentration of DOX. The maximum peak position of BSA–DOX was shifted slightly towards lower wavelength region (blue shift). The change in λ_{max} indicates the change in polarity around the tryptophan residue and the change in peptide strand of BSA molecules and hence the change in hydrophobicity. These above observations signify that with the addition of DOX, the peptide strands of BSA molecules were extended more and hydrophobicity was decreased.

3.6. Energy transfer between DOX and BSA

Fluorescence energy transfer is an effective tool for the measurement of distance between the drug and protein. The overlap of the UV-absorption spectra of DOX with the fluorescence emission spectra of BSA is

shown in Figure 6. The energy transfer process is very important in biochemistry, therefore energy transfer phenomena have wide applications [28]. According to Forster's non-radiative energy transfer theory [29], the rate of energy transfer depends on (i) the relative orientation of the donor and acceptor dipoles, (ii) the extent of overlap of fluorescence emission spectrum of the donor with the absorption spectrum of the acceptor and (iii) the distance between the donor and the acceptor. The energy transfer effect is related not only to the distance between the acceptor and donor, but also to the critical energy transfer distance R_0 and the efficiency of energy transfer *E*. According to Förster's energy transfer theory, the energy transfer efficiency E can be calculated by using the equation (8)

$$E = \frac{R_0^6}{R_0^6 + r^6} = \frac{F_0 - F}{F_0} \qquad (8)$$

where F and F_0 are the fluorescence intensities of BSA in the presence and absence of DOX, r is the distance between the acceptor and the donor and R_0 is the critical distance when the transfer efficiency is 50%.The value of R_0 was evaluated using the equation (9)

$$R_0^6 = 8.8 \times 10^{-25}\, k^2\, N^{-4}\, \Phi J \qquad (9)$$

where k^2 is the spatial orientation factor of the dipole, N is the refractive index of the medium, Φ 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. J is given by the following equation (10)

$$J = \frac{\Sigma F(\lambda)\varepsilon(\lambda)\lambda^4 \Delta\lambda}{\Sigma F(\lambda)\Delta\lambda} \qquad (10)$$

where F(λ) is the fluorescence intensity of the fluorescent donor of wavelength, λ, ε(λ) is the molar absorption coefficient of the acceptor at wavelength, λ. For ligand–BSA interaction, k^2=2/3, N = 1.336 and Φ = 0.15 [30]. The values of J, R_0, E and r were calculated to be $J = 2.41\times10^{-15}$ cm^3 L mol^{-1}, R_0 = 1.31nm, E = 0.046 and r = 2.17nm respectively at 298 K. The average distance between the donor and acceptor distance, r < 7nm indicated that the energy transfer from BSA to DOX occurred with high probability [31].

3.7. Synchronous fluorescence spectra

Synchronous fluorescence is a common spectral technique. This study is helpful to understand the molecular environment in the vicinity of chromophore molecules, such as tyrosine or tryptophan residues, and has several merits, such as spectral simplification, reduction of the spectral bandwidth and avoidance of different perturbing effects [32].

When the difference in wavelength (Δλ) between excitation and emission wavelength were set at 15 nm or 60nm, ($\Delta\lambda=\lambda_{em}-\lambda_{ex}$) the synchronous fluorescence spectra can provide the characteristic interactions of tyrosine residue or tryptophan residue in BSA respectively. The spectra are shown in Figure 7((A) and (B)).

From the Figure 7 spectral evidence for maximum emission wavelength at Δλ=15nm, a red shift (270 to 286 nm) was observed which indicates the conformational changes in the vicinity of tyrosine residue. Similarly, a red shift (265 to 272 nm) was also observed at Δλ=60nm, which represents conformational changes in the vicinity of tryptophan residue. Therefore, the interaction of BSA with DOX system confirms change in the molecular micro environment for both the tyrosine and tryptophan residues. Similar results have been found in the literature [33].

3.8. Three dimensional fluorescence spectra (3D)

Three dimensional fluorescence spectra (3D) can provide more detailed information about the conformational changes of proteins. The contour ones and three-dimensional fluorescence spectra's of BSA are shown in Figure 8 A and BSA–DOX in Figure 8 (B-D), respectively. The contour map displayed a bird's eye view of the fluorescence spectra. Further, from 3D fluorescence spectra, peak (a) is the Rayleigh scattering peak (λex=λem) [34]. Whereas peak (b) mainly reveals the spectral characteristic of tryptophan and tyrosine residues. The gradual decrease in the BSA fluorescence intensity upon regular addition of DOX suggests the interaction between BSA and DOX and the maximum emission wavelength of the peak (b) shifted towards lower wavelength (blue shift) indicating that the conformations of the tryptophan and tyrosine residues of BSA were altered which is in accordance with UV-visible and synchronous spectral studies . Hence, we conclude that molecular micro-environment and the conformational changes of protein were occurred after interaction with DOX [35].

3.9. Fourier transform infrared spectroscopic measurements

FT-IR spectroscopy has long been used as a powerful tool for investigating the secondary structure of proteins and their dynamics [36,37]. Additional evidence for BSA–DOX interaction was obtained from FT-IR spectra as shown in SI Figure 3((A) and (B)). Infrared spectrum of protein exhibited a number of amide bands due to different vibrations of the peptide moiety. Amide I band is the most intense absorption band in protein. The exact position of this band can be determined by backbone conformation and hydrogen bonding pattern. Since, amide I band is more sensitive to the changes in protein secondary structure compared to amide II, amide I band is more important [38]. The amides I and II peaks occurred in the region of 1600–1700 cm^{-1} and 1500–1600cm^{-1}, respectively. Hence, the amide bands are more useful for studies of secondary structure of protein. The FT-IR spectrum reveals that the peak position of amide I was shifted from 1655 cm^{-1} to 1652 cm^{-1} and the amide II was shifted from 1546 cm^{-1} to 1543 cm^{-1} with remarkable decrease in intensity. This indicated that the DOX interacted with BSA and the secondary structure of BSA was changed.

3.10. Differential pulse voltammetric study

Differential pulse voltammetry (DPV) is very sensitive and sophisticated analytical technique to analyze the various drugs in clinical and pharmaceutical laboratories. So DPV was applied to investigate the interaction between BSA and DOX at glassy carbon electrode. At first, the voltammogramms of blank phosphate buffer solution (PBS) (pH) and 5 μM BSA was taken which showed no electrochemical activity. Upon addition of 5 μM DOX into BSA there obtained two oxidation peaks (peak (a) & (b)) whose peak current increased with increasing concentration of DOX (5μM to 45μM) as shown in Figure.9A. This confirms the binding in BSA-DOX system. The effect of BSA on DOX was also studied by fixing the concentration of drug and varying the concentration of BSA (Fig.9B). In the absence BSA, peak current of drug (5μM) produced a well defined voltammogram with two oxidation peaks. After successive addition BSA, the intensities of both the peaks decreased, however peak (b) slowly diminished. Thus, it confirms the change in the molecular micro environment of BSA. Therefore DPV studies also support fluorescence quenching interactions between BSA and DOX.

3.11. Site probe study

In order to know the binding site in BSA for DOX, competitive displacement experiments were performed using site probes, warfarin, ibuprofen and digitoxin for sites I, II and III respectively as per Sudlow et al [39]. The specific binding site of drug on BSA was found from the fluorescence quenching of BSA after adding a drug into the probe–BSA system [40]. For this, emission spectra of BSA-site probe system at different concentrations of DOX were recorded. The calculated binding constant values are listed in Table 2. The calculated binding constant of BSA–DOX value decreased remarkably in presence of warfarin, which revealed that warfarin displaced DOX from the binding site, but in presence of ibuprofen and digitoxin had a slight effect on the binding of BSA-DOX system. Hence, we conclude that DOX is mainly located in the hydrophobic pocket of sub domain IIA of site I of BSA.

3.12. Effect of some metal ions on the interactions of DOX with BSA

In plasma, there are some metal ions, which can affect the interactions of the drugs and serum albumins. Trace metal ions, especially the bivalent type are essential in the human body and play an important structural role in many proteins. It is reported [41] that Ca^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+} and other metal ions can interact with proteins at 298K. Hence, the effects of some metal salt solutions viz., $CaCl_2$, $CoCl_2$, $CuCl_2$, $NiCl_2$ and $ZnCl_2$ on the binding of BSA with DOX were investigated in the present study. Under the experimental conditions, none of the cations gave the precipitate in phosphate buffer. The binding constants of BSA with DOX in presence of above ions were examined and the results are shown in Table 3. In presence of all the above metal ions the binding constant of DOX−BSA system were increased because of stronger molecular interaction. This was likely to be caused by a conformational change in the vicinity of the binding site. Thus, the storage time of the drug in blood plasma was prolonged and the maximum effectiveness of the drug was enhanced. As a result, DOX could be stored, transferred and slowly released

better by protein in the presence of above ions [12, 33]. This shows the less amount of drug is enough to achieve the desired therapeutic effect in presence of above metal ions.

4. Conclusion

In this present study, interaction of doxycycline with bovine serum albumin has been investigated under the physiological conditions using different spectroscopic techniques. The interaction between BSA and DOX was dynamic quenching and was confirmed by time resolved life time measurements. This work also reports the distance between BSA and bound DOX based on Forster's energy transfer theory and thermodynamic parameters involved in the mechanism. The binding interaction of BSA-DOX is mainly entropy driven and hydrophobic interaction forces are predominant. The results of UV-absorption spectra, FT-IR, synchronous fluorescence spectra, 3D fluorescence spectra and differential pulse voltammetry shows the changes in secondary structure, molecular micro-environment and the conformational changes of protein. The binding of drugs to proteins is an important factor in determining their pharmacokinetics and pharmacological effects, such works are useful for pharmaceutical industries and clinical laboratories.

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Table 1. Stern-volmer quenching constant, binding constant and thermodynamic parameters of DOX−BSA system

Temp (K)	$K_{SV}\times10^{-5}$ (M)	$k_q\times10^{-13}$ ($Lmol^{-1}s^{-1}$)	$K\times10^{-8}$ ($Lmol^{-1}$)	Bind sites (n)	ΔH^0 ($kJmol^{-1}$)	ΔS^0 ($JK^{-1}mol^{-1}$)	ΔG^0 ($kJmol^{-1}$)
288	3.563	3.563	0.413	1.710			-28.93
298	6.300	6.300	3.411	1.858	25.65	189.5	-30.84
308	12.717	12.717	41.88	1.935			-32.72

K_{SV}, K_q and K are Stern Volmer quenching constant, quenching rate constant and binding constant respectively

Table 2. The comparison of binding constants of BSA−DOX system (at pH 7.4, 298K) upon addition of site probes (warfarin, ibuprofen and digitoxin)

System	Binding constant K $x10^{-8}$ ($Lmol^{-1}$)	Binding site (n)	R^2
BSA−DOX	3.411	1.858	0.9992
BSA + warfarin+DOX	0.056	1.851	0.9893
BSA+ ibuprofen+DOX	4.892	1.764	0.9827
BSA + digitoxin+DOX	2.349	1.815	0.9813

Table 3. Effect of some common metal ions on binding constant of BSA−DOX system

System	Binding constant K $x10^{-8}$ ($Lmol^{-1}$)	R^2
BSA−DOX	3.411	0.9992
BSA+ Ca^{2+}+DOX	32.35	0.9778
BSA+ Co^{2+}+DOX	24.04	0.9818
BSA+ Cu^{2+}+DOX	17.82	0.9802
BSA+ Ni^{2+}+DOX	31.14	0.9801
BSA+ Zn^{2+}+DOX	28.18	0.9795