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Electrochemical and spectroscopic studies on the interaction of Ketoconazole with Herring Sperm DNA

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ABSTRACT

The interaction between ketoconazole (KTZ) and Herring Sperm DNA (HsDNA) have been studied by cyclic voltammetry, differential pulse voltammetry, UV-vis absorption and fluorescence spectral studies and viscometric methods. The measurements were performed in 0.1 mol L^{-1} PBS buffer solution at pH = 7.4. The voltammetric behaviour of KTZ in the presence and absence of HsDNA was investigated at glassy carbon electrode using Cyclic Voltammetry and Differential Pulse Voltammetry. The calculated binding quenching constants (K_{SV}) were 1.057 x 10^4, 5.9268 $\times 10^3$, and 3.039 $\times 10^3$ L mol^{-1} at 298, 303 and 308 K respectively. The Stern-Volmer quenching rate constant (k_q), binding site number (n) and corresponding thermodynamic parameters (ΔH, ΔG and ΔS) were calculated.

Keywords: Ketoconazole (KTZ), Herring Sperm DNA (HsDNA), spectroscopic, voltammetry

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INTRODUCTION

Ketoconazole (**Figure 1**) is one of the most famous antifungal medications and a potent inhibitor against the enzyme cytochrome P450 (CYP3A4); several statins, including simvastatin and lovastatin, interact with this hepatic microsomal enzyme, which is responsible in significant part for statin clearance [1]. It is often used to treat fungal infections that can spread to different parts of the body through the bloodstream such as yeast infections of the mouth, skin, urinary tract, and blood, and certain fungal infections that begin on the skin or in the lungs and can spread through the body. KTZ works by slowing the growth of fungi which may cause infection. It is used to treat a variety of fungal infections such as candida infections of the skin or mouth (thrush), blastomycosis, histoplasmosis, coccidiomycosis, and others. Protein is an important component of cell and the executor of life activities. It is a frontier topic to study the function of protein in life science. Studying the thermodynamics characteristics and mechanism of the interaction of a small organic molecule such as medicament with biological macromolecules is an important component of life sciences.

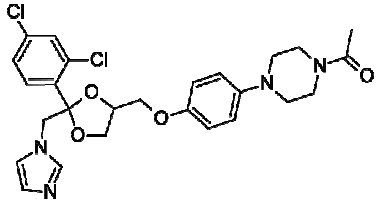


Fig.1 Chemical structure of Ketoconazole

Serum albumins are the most abundant proteins in plasma [2]. As the major soluble protein constituents of the circulatory system, they have many physiological functions. They contribute to colloid osmotic blood pressure and are primarily responsible for the maintenance of blood pH [3, 4]; they can play a dominant role in drug metabolism,

efficacy and disposition [5]. Many drugs and other bioactivity small molecules bind reversibly to albumin [6-8], which implicates a serum albumin role as carriers. Consequently, it is important to study the interactions of drugs with this protein. These studies may provide information about the structural features that determine the therapeutic effectiveness of drugs, and have become an important research field in the life sciences, chemistry and clinical medicine.

As far as we know, the interaction between KTZ and Hs-DNA has not been investigated. In this work, Hs-DNA was selected as our DNA model because of its medical importance, low cost, ready availability, unusual ligand binding properties.

In recent years, there has been a growing interest in the CV investigations of the interactions between drugs and proteins. Observing the pre- and post-electrochemical signals of proteins provides good evidence for determining the interaction mechanism. An electrochemical approach can provide new insight into rational drug design and would lead to increased understanding of the interaction mechanism between drugs and proteins.

Spectroscopy (fluorescence and UV-vis. spectroscopy) is also a powerful tool for the study of the reaction of chemical and biological systems since it allows non-intrusive measurements of substances at a low concentration under physiological conditions [9]. Quenching measurement of albumin fluorescence is an important method to study the interactions of drugs with protein [10, 11]. The effectiveness of drugs depends on their binding ability with albumins, so it is significant to study the interactions of drugs with proteins [12]. These studies can reveal the accessibility of quenchers to DNA, help to understand DNA binding mechanisms to drugs, and provide clues to the nature of the binding phenomenon.

In the present work, we demonstrated the interactions between KTZ and HsDNA by cyclic Voltammetry, differential pulse Voltammetry, spectroscopic and viscometric methods. The aim of our work was to determine the affinity of KTZ for HsDNA, and to investigate the thermodynamics of their interaction.

MATERIALS AND METHODS

1.1 Materials

Herring sperm DNA, approximately 99% pure were obtained from Sigma Aldrich, India. Ketoconazole was obtained as gift sample from Cipla, India. All other materials were of analytical reagent grade. The solutions of drug and Hs-DNA were prepared in 0.1 M PBS buffer of pH 7.4 containing 0.15 M NaCl. Hs-DNA solution was prepared based on its molecular weight. Water used was distilled and deionized.

1.2 Equipments and spectral measurements

Electrochemical experiments were performed in a conventional three-electrode cell powered by an electrochemical system comprising a CH electrochemical analyzer. All fluorescence measurements were carried out on an F-4500 spectrophotometer (Hitachi, Tokyo, Japan) equipped with a 150W Xenon lamp source and 1.0 cm quartz cells. A double beam Ellico UV-Vis spectrophotometer (India) equipped with 1.0 cm quartz cells was used for scanning the UV spectrum. Viscosity measurements were performed using a viscometer, which was immersed in a thermostat water-bath at room temperature.

RESULTS AND DISCUSSION

3.1. Voltammetric behaviour of KTZ at a GCE

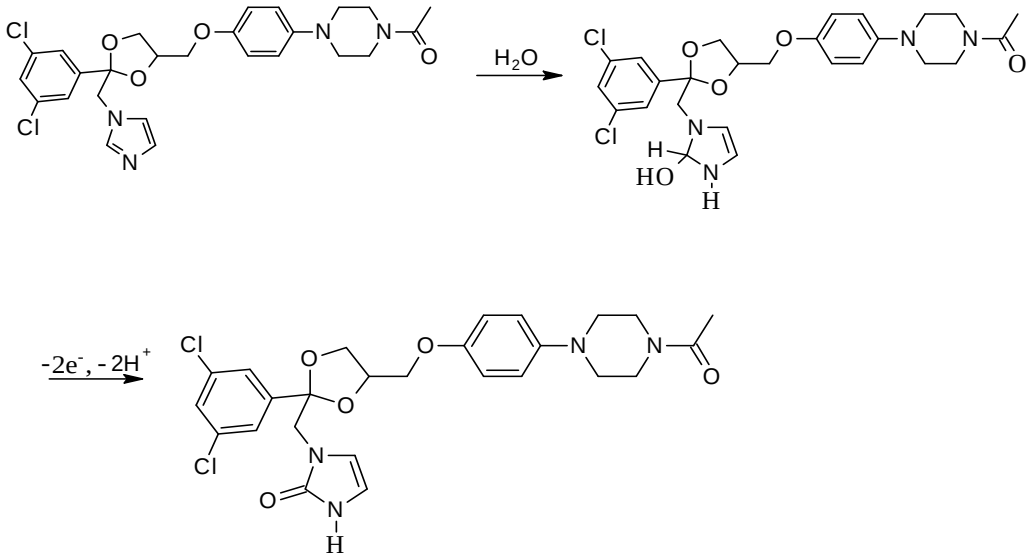
The electrochemical behaviour of KTZ (1.0×10^{-4} M) at a GCE was investigated by CV and DPV. KTZ showed one oxidation peak at 0.667 V with corresponding peak current -2.202 µA in PBS buffer of pH 7.4 with a scan rate of 50 mV s^{-1} (**Figure 2a**). No peak was observed in the reverse scan, suggesting that the oxidation of KTZ on the GCE was irreversible. Multi-sweep cyclic voltammogram of KTZ (data not shown) revealed a significant decrease in peak current, indicating fouling of the electrode surface due to adsorption of KTZ or its oxidation product.

For an irreversible process, the number of electron transfer (n) could be obtained by Eq. (1) [13].

$$\Delta E_{pa} = E_{pa} - E_{pa/2} = (47.7/\alpha n)\ \text{mV (at 298 K)} \qquad (1)$$

Where $E_{pa/2}$ is the half peak potential, α represents the electron transfer coefficient (generally, $0.3 < \alpha < 0.7$), α is assumed to be 0.5 for a totally irreversible process. In this study, a value of 47 mV for $|Epa\text{-}Epa_{/2}|$ is obtained from equation 1 and the value of n is 2.029 (≈ 2) is yielded referring to Eq. (1) [14]. Therefore, the electrochemical oxidation of KTZ undergoes $2e^-$ transfer process.

Based on the above results, it is proposed that during electrooxidation, KTZ involves two protons and two electrons oxidation to yield substituted ketone adduct, due to an oxidation mechanism (**Scheme 1**) similar to caffiene. This is also in agreement with an earlier report [15].

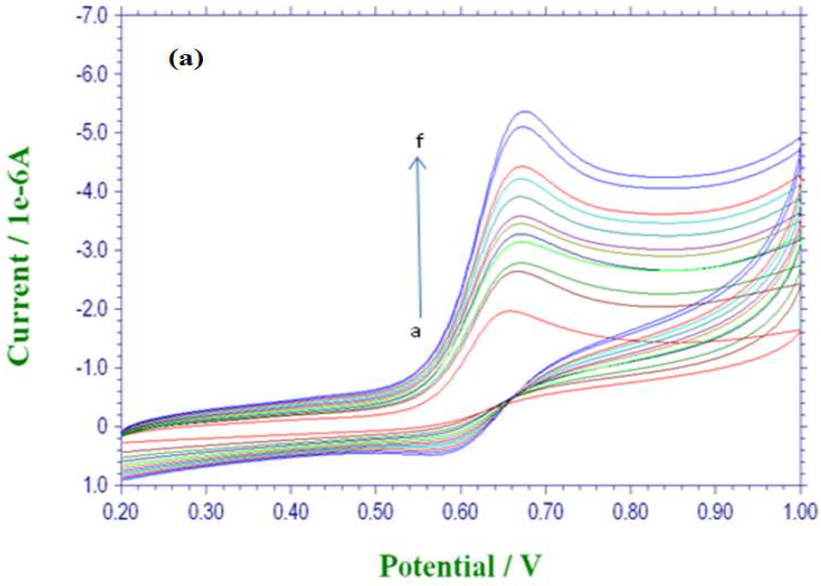


Scheme 1. Probable reaction mechanism for electrooxidation of KTZ

For an irreversible oxidation reaction, the following equation was used to calculate standard rate constant (k_0) [16, 17].

$$E_p = E^0 + (RT/\alpha nF)\,[ln\,(RTk_0/\,\alpha nF)] - lnv \qquad (2)$$

Where E_0 is the formal potential, R is the universal gas constant (8.314 J K^{-1} mol^{-1}), T (K) was the Kelvin temperature, α is the transfer coefficient, k_0 (s^{-1}) is the electrochemical rate constant and F was the Faraday constant (96,487 C mol^{-1}). The value of E^0 was obtained from the intercept of E_p *vs ln v* plot by the extrapolation to the vertical axis at ν = 0. The k_0 value, 1.27 x 10^2 s^{-1} was evaluated from the plot of E_p *vs* ln ν.



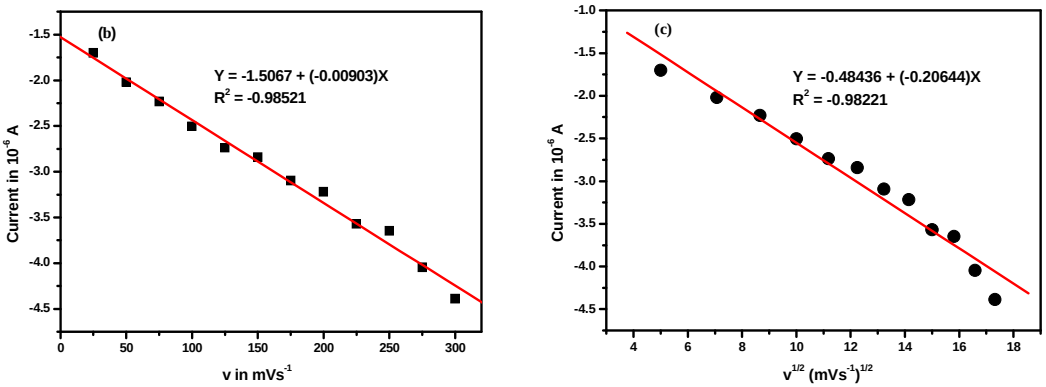


Fig. 2 (a) Cyclic voltammogram for the oxidation of 1.0 x 10^{-4} M of KTZ at different scan rates: 25, 50, 75, 100, 125, 150, 175, 200, 225 250, 275 and 300 mV s^{-1} (a–f). (b) Plot of I_{pa} versus ν (c) Plot of I_{pa} versus $ν^{1/2}$ of KTZ.

3.2. Influence of scan rate on electrooxidation of KTZ

We examined the influence of the scan rate on the electrochemical behaviour of KTZ, to understand the nature of the electrode process. For this, we recorded cyclic voltammogram of 1.0 x 10^{-4} M KTZ at a GCE at different scan rates (**Figure 2a**). The oxidation peak current of KTZ was noted to increase with increasing scan rate, with a positive shift in the peak potential. The plot of values of log I_p versus log ν in the scan rate range of 25 - 300 mV s^{-1} yielded a straight line with a slope of 0.532. This value is close to the theoretical value of 0.5 expected for an ideal reaction condition for a diffusion-controlled electrode process [13]. In addition, the graphs of I_{pa} versus ν and I_{pa} versus $ν^{1/2}$ (**Fig. 2b and 2c**) have good linearity. In the range from 25 to 300 mVs^{-1}, the anodic peak currents were proportional to the scan rate. The correlation coefficient was found to be -0.997981 (n = 12) and -0.98221 (n = 12), which indicates the electrode reaction was diffusion controlled.

3.3. Voltammetric studies of KTZ–HsDNA interaction

CVs and DPVs of 1.0 x 10^{-4} M KTZ in absence and presence of HsDNA on GCE in PBS buffer of pH 7.4 are shown in **Fig 3(a) and 3(b)**. The voltammogram without HsDNA shows stable anodic peak in the potential range of 0.2 - 1.0 V. The anodic peak was appeared at 0.667 V versus SCE corresponding peak current is -2.202 µA. By the addition of 10 - 180 µM of HsDNA the anodic peak potential was shifted to negative direction and also anodic peak current (I_{pa}) was decreased. The significant decrease in peak current is attributed to the formation of slowly diffusing KTZ- HsDNA complex due to which the concentration of the free drug, which is responsible for the transfer of current is lowered. The calculated value of k_0 is found to be 1.27 x 10^2 s^{-1} in the absence of HsDNA and 1.38 x 10^2 s^{-1} in presence of HsDNA. In this way, appreciable difference in the value of k_0 in presence and absence of HsDNA was not observed indicating that HsDNA did not alter the electrochemical kinetics of KTZ oxidation.

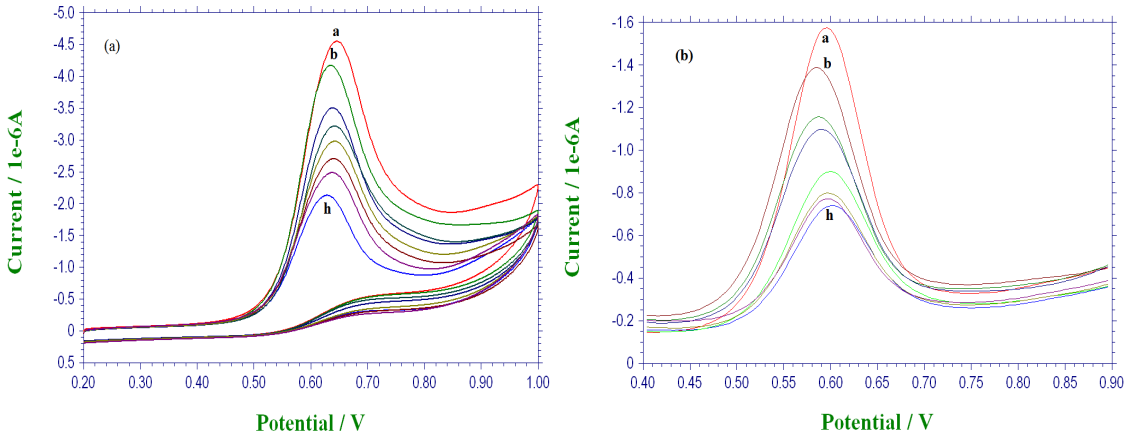


Fig. 3 (a) CV's and (b) DPV's of 1.0 x 10^{-4} M KTZ in absence of HsDNA (a), and in presence of C_{HsDNA} = 10.0, 50.0, 70.0, 100.0, 120.0, 150.0 and 180 µML^{-1} (b to h) in PBS buffer of pH 7.4 at 50 mVs^{-1}

In general the positive shift in potential is caused by the intercalation of the drug into HsDNA [18], while negative shift is observed for the electrostatic interaction of HsDNA [19]. So the obvious negative peak potential shift in the CV and DPV of KTZ by the addition of HsDNA is attributable to the electrostatic interaction of KTZ with HsDNA.

The cathodic peak potential shift indicates the easier oxidation of KTZ in presence of HsDNA because its oxidized form is more strongly bound to HsDNA than its reduced form.

According to the decrease in peak current of KTZ by the addition of different concentration of HsDNA, the binding constant was calculated according to the following equation [20]:

$$\frac{1}{[BSA]} = \frac{K\,(1\text{-}A)}{1 - (I/I_o)} - K \qquad (3)$$

Where, K is the binding constant, I and I_0 are the peak currents with and without HsDNA and A is the proportionality constant.

A plot of 1/ [1- (I_0/I)] versus 1/ [DNA] was constructed (**Fig. 4a**) and from the ratio of the intercept to slope, the value of K is calculated to be 1.24 x 10^4 L mol^{-1} (R^2 = 0.9999).

The binding constant and binding site size was determined using the following equation [21]:

$$\frac{C_b}{C_f} = K \left\{ \frac{[\text{Free base pairs}]}{S} \right\} \qquad (4)$$

Where, S is the binding site size in terms of base pairs. Measuring the concentration of HsDNA in terms of [KTZ], the concentration of the base pairs can be expressed as [DNA]/2. Therefore, Eq. (4) can be written as:

$$\frac{C_b}{C_f} = K \left\{ \frac{[BSA]}{2S} \right\} \qquad (5)$$

Therefore, C_f and C_b represent the concentrations of the free and HsDNA-bound species respectively. The C_b/C_f ratio was determined by the equation given below [22],

$$\frac{C_b}{C_f} = \frac{I_0 - I}{I} \qquad (6)$$

Where, I and I_0 represent the peak currents of the drug in the presence and absence of HsDNA respectively.

Putting the value of 1.24 x 10^4 L mol^{-1} as calculated according to Eq. (3), the binding site size of 0.89 ≈ 1.0 bp was obtained from the plot (**Fig. 4b**) of C_b/C_f versus [HsDNA]. The small value of 'S' indicates electrostatic interaction of KTZ with HsDNA.

3.4. Fluorescence spectral studies

The interaction between KTZ and HsDNA was investigated by evaluating fluorescence intensity of HsDNA before and after addition of KTZ in a physiological condition (pH = 7.4) upon excitation at 260 nm. Here the concentration of HsDNA was stabilized at 1.0 x 10^{-5} M and the concentration of KTZ varied from 10 to 180 $\mu M L^{-1}$. The effect of KTZ on HsDNA fluorescence intensity is shown in **Figure 5a**. The fluorescence intensity of HsDNA decreases progressively but the emission maximum did not move to shorter or longer wavelength, due to the interaction of KTZ with HsDNA and quench its intrinsic fluorescence [23], but there was no alteration in the local dielectric environment of HsDNA.

In order to confirm the quenching mechanism, fluorescence quenching mechanism was analysed according to stern – volmer equation [24]

$$F_0 / F = 1 + k_q \tau_o [Q] = 1 + K_{sv} [Q]$$

In this equation, F_0 and F are the fluorescence intensities of HsDNA in absence and presence of KTZ respectively, [Q] is the KTZ concentration, k_q is the quenching rate constant, τ_o is the average excited-state lifetime and K_{sv} is the Stern-Volmer quenching constant.

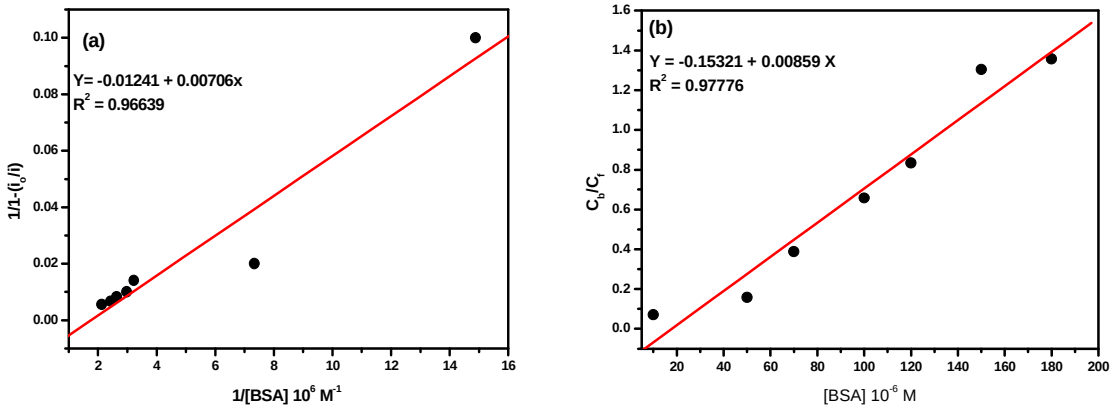
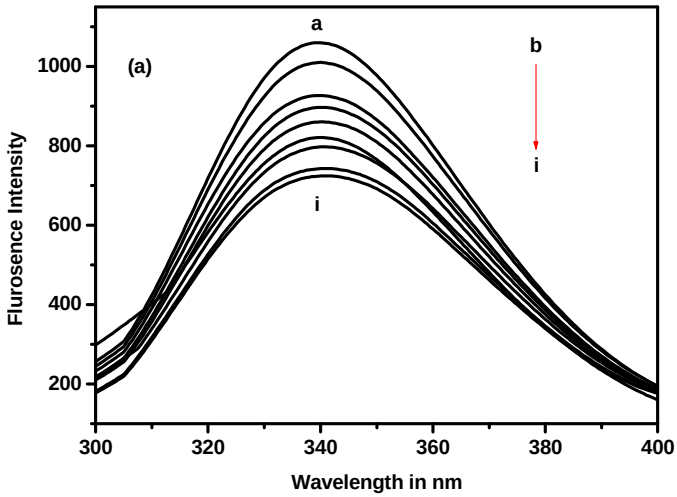


Fig. 4 (a) Plot of 1/ [1- (i_0/i)] versus 1/ [1- HsDNA]; (b) Plot of C_b /C_f versus [HsDNA]



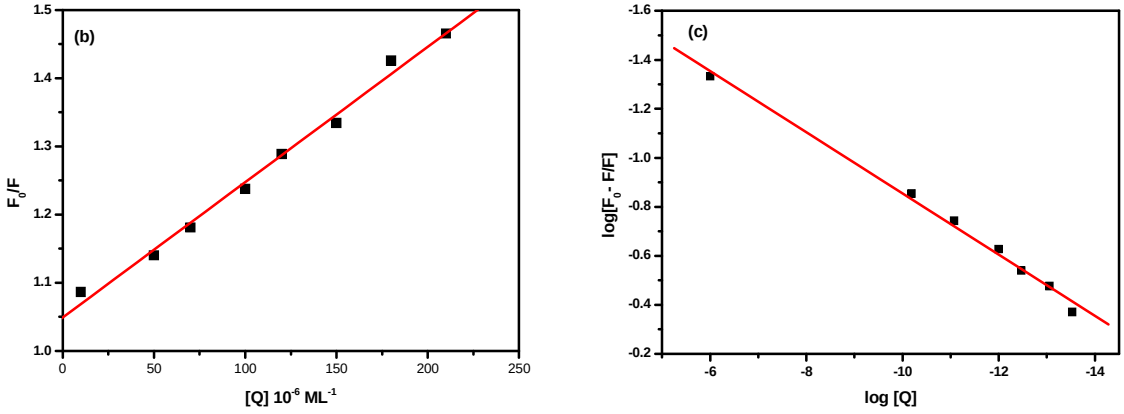


Fig. 5 (a) Florescence spectra of 1x10^{-5} M HsDNA in the absence of HsDNA and in presence of C_{KTZ} = 10, 50, 70, 100, 120, 150, 180 µML^{-1} (b to i) in PBS of pH 7.4, (b) Plot of F_0/F versus [HsDNA] and (c) Plot of log [(F_0-F)/F] versus log [HsDNA]

The quenching mechanism of KTZ with HsDNA was probed using the Stern-Volmer equation [24], which can be applied to determine K_{sv} by linear regression from the Stern-Volmer plot of F_0/F, against [KTZ] (**Fig. 5(b)**). **T**he value of Stern-Volmer quenching constant decreases with increase in temperature. According to the literature [25] for dynamic quenching, the maximum scatter collision quenching constant of various quenchers with the biopolymer is 2.0 x 10^{10} L mol^{-1} s^{-1} and the fluorescence lifetime if the biopolymer is 10^{-8} s. From **Fig. 5(c),** the values of K_{SV}

and k_q (= K_{SV}/τ_0) were calculated. The obtained values of k_q (1.0527 x 10^{12} L mol^{-1} s^{-1}) were larger than the limiting diffusion rate constant of the biomolecule (2.0 x 10^{10} L mol^{-1} s^{-1}), which indicate that the fluorescence quenching is caused by a specific interaction between HsDNA and KTZ. Therefore, the quenching mechanism mainly arises from the formation of HsDNA-KTZ complex rather than dynamic quenching. So it was implied that the static quenching was dominant in the system. From the plot of log [(F_0-F)/F] *vs* log [KTZ] binding constant K and the number of binding sites 'n' were calculated from the intercept and slope.

3.5. Determination of thermodynamic parameters

In order to elucidate the interaction between KTZ and HsDNA, the thermodynamic parameters were calculated from the van't Hoff's plot **Fig. 6(b).** If the enthalpy change (ΔH) does not vary significantly over the temperature range studied then the thermodynamic parameters, ΔH, ΔG and ΔS can be determined from the following equations respectively.

$$\ln K = \Delta H/RT + \Delta S/R \quad (7)$$

$$\Delta G = -RT \ln K \quad (8)$$

$$\Delta S = (\Delta H - \Delta G)/T \quad (9)$$

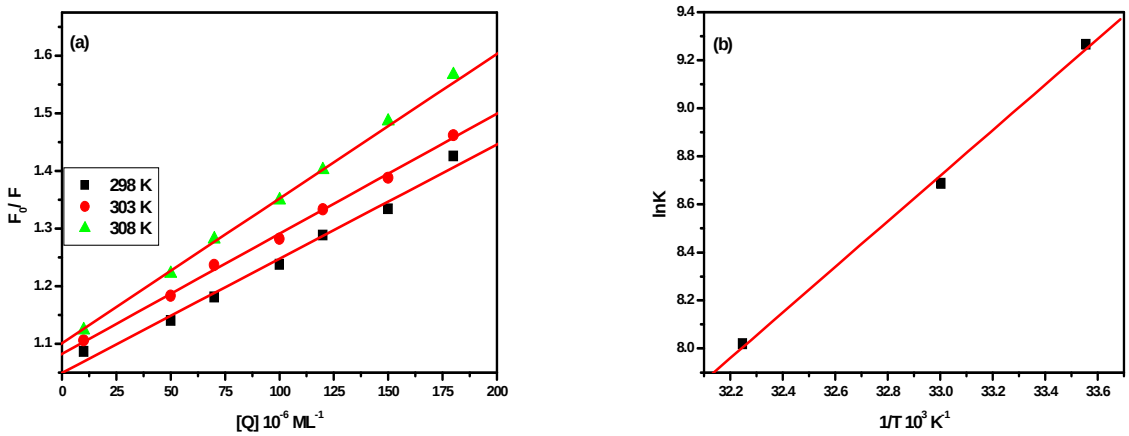


Fig. 6 (a) Stern-Volmer plots for (KTZ- HsDNA) at different temperatures and (b) Van't Hoff plots of *ln*K vs. 1/T

According to the theory of Ross, the positive enthalpy change (ΔH) and entropy change (ΔS) are associated with hydrophobic interaction. The negative values of ΔH and ΔS are associated with hydrogen binding and van der Waals interactions whereas the very low positive or negative ΔH and positive ΔS values are characterized by electrostatic interactions. From the values of ΔH, ΔG and ΔS (-16.87, -22.958 and 20.49 respectively), it was found that there is electrostatic interaction between HsDNA and KTZ [26].

3.6. Binding sites

The binding reaction in HsDNA molecules happened for the static quenching interaction. According to the relationship of

$$\lg\left[\frac{F_0 - F}{F}\right] \textit{ vs. } \lg [Q]$$

the fit to the fluorescence data using equation

$$\lg\left[\frac{F_0 - F}{F}\right] = \lg K + z\lg[Q]$$

for KTZ and HsDNA was found by setting z = 0.848 ± 0.037.

3.7. UV/Vis absorption spectroscopy

To explore the structural change of HsDNA after the addition of KTZ, we have measured the UV-vis absorbance spectra of KTZ with various amounts of HsDNA. **Figure 7a** displays the UV-vis absorbance spectra of KTZ at 248 nm at different concentrations of HsDNA. The intercalative binding mode of molecule to HsDNA has been considered by appreciable shift in the wavelength due to interaction between HsDNA stack with ligand, while

outside binders (groove binder) display a slighter red shift [27,28]. The absorbance of KTZ decreased with increasing concentration of HsDNA and the peak was slightly red shifted. The decrease in absorption spectra is mostly attributed to the interaction between electronic states of the compound and the HsDNA bases. Frome these results non-intercalation or groove binding mode may be ruled out as a major binding mode of KTZ with HsDNA.

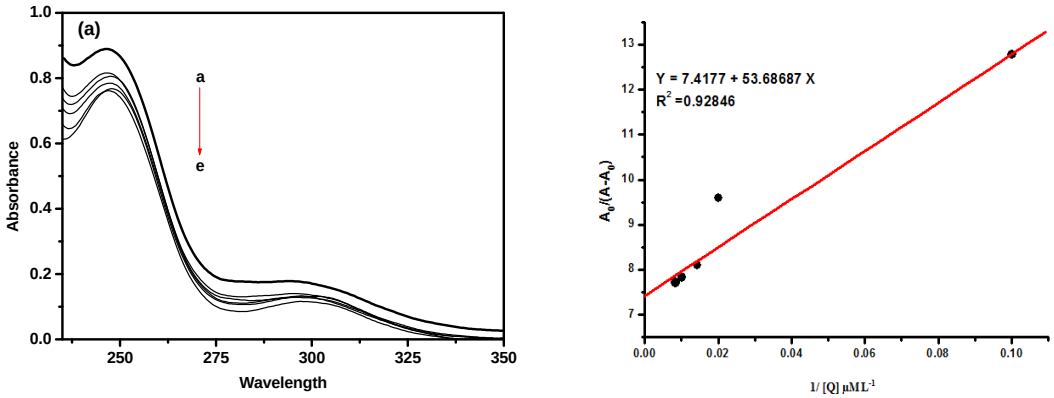


Fig. 7 (a) UV-visible spectra of 1.0 × 10^{-4} M KTZ in the absence and in presence of C_{HSDNA} = 10, 50, 70, 100, 120 µML^{-1} HsDNA in PBS of pH 7.4 and (b) Plot of (A_0/ (A-A_0) versus 1/ [Q]

3.8. Determination of viscosity

One indication of HsDNA binding mode is the change in viscosity when a small molecule associates with HsDNA. Intercalative binding increases the length of HsDNA and the viscosity significantly, whereas groove binding typically has a smaller effect on viscosity [29]. **Fig. 9** reveals that the viscosity of KTZ decreased considerably by the addition of HsDNA at concentrations more than 40.0 mmol L^{-1}. It supports the electrostatic or groove mode via hydrophobic interaction.

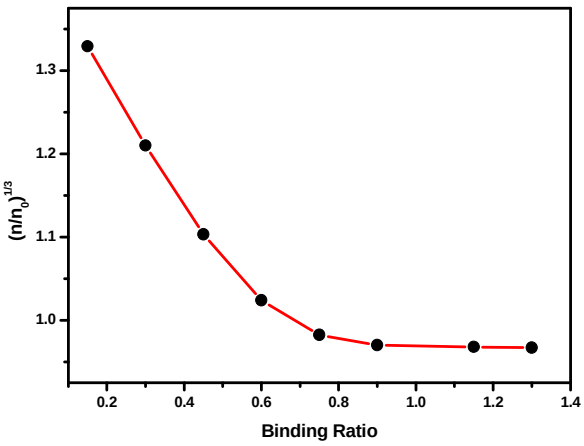


Fig. 9. Effect of HsDNA on the viscosity of KTZ solution at 100 µmol L^{-1}. C_{HSDNA} = 0, 10, 20, 30, 40 and 50 µmol L^{-1} phosphate buffer (0.1 mol L^{-1}, pH 7.4) at 298 K

CONCLUSION

In this paper, the interaction of KTZ with HsDNA was studied by cyclic voltammetry, differential pulse voltammetry spectroscopic (fluorescence spectroscopy and UV-Vis absorption spectroscopy) and viscometric methods. The results show that the modified Stern-Volmer quenching constant K_{sv} is inversely correlated with temperature, which indicates that the probable quenching mechanism of KTZ-HsDNA binding reactions is initiated by complex formation. The results of fluorescence spectroscopy and ultraviolet spectra indicate that the secondary structures of albumin molecules are changed dramatically in the presence of KTZ. The experimental results also indicate that the probable quenching mechanism of fluorescence of HsDNA by KTZ was static quenching procedure. The binding reaction of KTZ with HsDNA is mainly enthalpy driven; the electrostatic interactions play a major role in the reaction, in addition to the hydrophobic association.

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