Interaction of Duloxetine Hydrochloride with Deoxyribonucleic Acid Measured by Fluorescence Spectroscopy

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ABSTRACT: Interactions with many clinically active therapeutic agents with DNA are well studied and it is necessary to decipher the structure of DNA and to investigate the pathological implications of those molecules in living organism. This study investigated the interaction of antidepressant drug Duloxetine-hydrochloride (DLX) with calf thymus DNA (ct-DNA). The interaction of DLX with ct-DNA was studied employing fluorescence spectroscopy. Hypochromic effect was found in the absorption spectra of duloxetine, and its wavelength had no shift in the presence of DNA indicating external binding mode of duloxetine to DNA. Fluorescence spectroscopic results showed the quenching of fluorescence intensity of DLX in presence of DNA indicating the interaction between DLX and DNA. Hydrophobic interaction and hydrogen bonding played the dominating role in DLX-DNA binding and binding forces also indicate the binding site of duloxetine to be at the minor groove of DNA.

Key words: Calf thymus DNA, affinity, stern volmer constant, fluorescence quenching, hypochromic effect, minor groove

INTRODUCTION

Learning about design principles for targeting of specific deoxyribonucleic acid (DNA) sequences, its interaction with small molecules have been studied for several decades.[1,2] Interactions of many clinically active therapeutic agents with DNA are well studied albeit the exact mechanism of action is not known for all of them.[3] Interest of researchers in that particular area had reduced, but the use of small molecules as therapeutic agents in the field of biotechnology has accelerated the interest again in the last decade.[2] Most of the studies were performed to investigate the interaction of DNA with other small molecules.[3-6] Regions of DNA involved in vital processes such as origin of replication, promotion of transcription, etc. are the major intracellular target in drug development strategies outlined to produce next generation therapeutics for diseases such as cancer. Molecular basis of drug-DNA interaction has been studied in structural, thermodynamic, and kinetic detail not only to promote the clinical efficacy of the existing drug but also to flourish new ones. Investigations on the interaction of small molecules with DNA are necessary to decipher the structure of DNA and also to observe the pathological implication of those molecules in living system.

Duloxetine hydrochloride {N-methyl-γ-(1-naphthyloxy)-2-thiophenepropylamine hydro-chloride} is a selective serotonin-norepinephrine reuptake inhibitor and mainly used to treat major depressive disorder (MDD) and generalized anxiety disorder (GAD).[7] It is also indicated for the management of painful peripheral neuropathy

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especially diabetic neuropathy and approved for use in osteoarthritis and musculoskeletal pain, chronic lower back pain and to control the symptoms of fibromyalgia.[8] Duloxetine potentiates the serotonergic and noradrenergic activity by inhibiting the reuptake of serotonin and norepinephrine in the CNS.[9] The analgesic effect of duloxetine may be due to sodium channel blockade.[10]

Although various researchers have studied the structure and the properties of DNA and their interactions with small molecules such as dyes, drugs and toxic chemicals using the fluorescence method, but there has been no report on the interaction of duloxetine hydrochloride with DNA. In this regard, our work is a detailed and insightful study on the interaction of duloxetine hydrochloride with calf thymus (ct) DNA and was carried out by fluorescence spectroscopy and UV-visible spectroscopy. Ct-DNA was used as DNA model as it is a polymer of alternate sugar phosphate sequence with low protein and high polymerized skeleton.

Investigations have been performed to identify and characterize binding of duloxetine and DNA determining the Stern-Volmer constant, the binding constant, the binding points at different temperatures (298 K and 308 K). Besides, thermodynamic parameters like the enthalpy change (ΔH), the entropy change (ΔS) and Gibb's free energy (ΔG) were calculated to analyze the nature of the binding forces.

MATERIALS AND METHODS

Reagents and materials. Calf thymus DNA was commercially obtained from Sigma–Aldrich Chemicals Co. (USA) and used without further purification. The stock solution of this DNA was prepared by dissolving an appropriate amount of DNA in nuclease-free water overnight at a final concentration of 3.33×10^{-3} mol/L and stored at 4 ºC. The lyophilized DNA should be reconstituted overnight at 2-8 °C to ensure that all materials go into solution. When the product was fully dissolved, the DNA concentration was determined by measuring its UV absorption at 260 nm using a molar absorption coefficient of 6600 L mol^{-1} cm^{-1}.[11] Purity of DNA was checked by monitoring the ratio of the absorbance at 260 nm to that at 280 nm. The solution gave a ratio of A_{260}/A_{280} in the range of 1.8-1.9, indicating that DNA was sufficiently free from protein.[12] Tris-HCl buffer (pH 7.40) was used in this study. 0.05M buffer was prepared by adding an appropriate amount of tris powder (M.W. 121.14) in nanopure water and pH was adjusted by using concentrated HCl which was used for later experiments. 2×10^{-3}M solution of duloxetine hydrochloride was prepared by dissolving stock solution in nanopure water.

Apparatus. All fluorescence spectra were recorded on F-7000 Spectrophotometer (Hitachi, Japan). The absorbance measurements were performed on a UV-1800 Spectrophotometer (Shimadzu, Japan) equipped with 1.0 cm quartz cells. Measurement of pH values of the solutions was performed by using a pH meter (Hanna, Portugal).

Methods

Selection of appropriate concentration of drug. At first different concentrations of Duloxetine hydrochloride were screened in UV spectrophotometer to determine max wavelength and 235 nm was found to be the maximum absorbing wavelength for the drug. In order to determine the effects of different DLX concentration on the interaction of duloxetine hydrochloride with ctDNA, a constant concentration of DNA was treated with different concentrations of the drug. Then fluorescence intensities were measured at the excitation wavelength 235 nm in the presence of fixed DNA concentration at 298 K.

Measurement of absorbance. During absorbance measurement, a constant concentration of the drug was treated with increasing concentration of the DNA. In order to observe the effect of DNA on absorption spectra of drug, the absorbance of drug was measured in the presence of different DNA concentrations in 1cm path length matched quartz cells with continuous stirring throughout the course of the titration.

Effect of DNA on the fluorescence spectra of duloxetine. Fluorescence spectra of the drug in presence of various concentration of DNA were measured at the excitation wavelength of 235 nm. Aliquots of DNA were added to the solution of Duloxetine–HCl in Tris-HCl buffer of pH 7.4 and mixed by stirring the complex for 2-3 minutes.

Fluorescence quenching measurements. A decrease in intrinsic fluorescence intensity of a fluorophore due to various molecular interactions with other molecules is called fluorescence quenching. Dynamic quenching refers to a process in which the fluorophore and the quencher come into contact during the lifetime of the excited state, static quenching, on the other hand, refers to fluorophore-quencher complex formation. To assess the interaction between drug and DNA, the fluorescence quenching data are analyzed by Stern-Volmer equation[13]:

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

Here F_o and F are the fluorescence intensities of drug in the absence and presence of quencher, respectively. [Q] represents the concentration of quencher and K_{SV} is the Stern-Volmer quenching constant.

Thermodynamic parameters and nature of the binding forces. The interaction forces between quencher and bio- molecules may include hydrophobic forces, electrostatic interactions, Van der Waals interactions, hydrogen bonds etc.[14] The thermodynamic parameters namely the enthalpy change, the entropy change and Gibb's free energy are calculated in order to elucidate the interaction forces between the DNA and Duloxetine. The basic reason for carrying out thermodynamic studies of Duloxetine-DNA interaction is to determine the factors responsible for overall binding affinity and specificity of the drug.[15] Thermodynamic parameters can be determined from the Van't Hoff equation:

$$\ln K_a = -(\Delta H/RT) + (\Delta S/R)$$

Where ΔH and ΔS are the enthalpy change and the entropy change respectively; constant K_a is analogous to the Stern-Volmer quenching constants K_{SV} at the corresponding temperature whereas R is the gas constant.[16] The enthalpy change (ΔH) and the entropy change (ΔS) can be determined from the slope and intercept of the fitted curve of ln K_{SV} against 1/T, respectively.

The Gibb's free energy (ΔG) can be estimated from the following relationship:

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

Binding constant and binding points. 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[17]:

$$\log [(F_o - F)/F] = \log K + n \log [Q]$$

Where K and n represent the binding constant to a site and the number of binding per molecule respectively. The values of K and n are calculated from the values of intercept and slope of the plot of log $[(F_o-F)/F]$ versus log [Q], respectively.

Data analysis. All the data were analyzed by using the software Microsoft Excel (MS Excel, 2013).

RESULTS AND DISCUSSION

Effect of DLX concentration on interaction of duloxetine with DNA. Interaction of DNA with DLX was studied first at a fixed DNA concentration of 1×10^{-6} where the concentration of Duloxetine varied from 6.3×10^{-6} to 6.4×10^{-4} in order to observe the effect of DLX concentration on interaction. The result shows (Figure 1) that when the concentration of Duloxetine reaches to 6.4×10^{-5} mol/L, the change of the fluorescence intensity is most obvious. So, this concentration of Duloxetine was chosen for further experiments.

Effect of DNA on absorption of duloxetine. When a small molecule interacts with DNA and forms a new complex, a shift in the absorbance wavelength and/ or a change in the molar absorptivity may occur. In order to determine the effect of increasing concentration of DNA on the absorption spectra of Duloxetine, the absorption of DLX in the presence of different DNA concentrations were measured at the excitation wavelength of 235 nm.

Here the interaction of the Duloxetine with DNA results in changes with hypochromic effect that essentially indicates strong intermolecular interaction (Table 1). At the same time, no bathochromism (red-shift) effect occurs when DLX bound to DNA. If the binding of small molecules and DNA involves a typical intercalative mode, a hypochromism effect coupled with obvious bathochromism for the characteristic peaks of the small molecules will be found due to strong stacking between the chromophore and the base pairs of DNA.[18] Therefore, the hypochromic effect could be the indication of noncovalent groove binding.

Table 1. UV absorbance of DLX in presence of DNA.

[DLX] mol/l	[DNA] mol/l	Absorbance
6.4×10^{-5}	0	0.3
6.4×10^{-5}	3.5×10^{-7}	0.2
6.4×10^{-5}	7.6×10^{-7}	0.2
6.4×10^{-5}	1.0×10^{-6}	0.2
6.4×10^{-5}	3.0×10^{-6}	0.2
6.4×10^{-5}	7.0×10^{-6}	0.2

Analysis of fluorescence quenching mechanism. Molecular rearrangement, energy transfer, ground state complex formation, collisional quenching etc. are the various molecular interactions that can result in fluorescence quenching of excited state fluorophores. To further assess the interaction between DLX and DNA, the fluorescence quenching data are analyzed by Stern-Volmer equation[13]:

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

Figure 2 shows the fluorescence spectra of DLX in the absence and presence of DNA. The quenching of fluorescence of DLX in the presence of different DNA concentration was observed. The drug has an emission spectrum with maxima centered 334 nm when excited at 235 nm. No shift in emission wavelength was found. Here, DNA works as a quencher. This is an indication of strong interaction and energy transfer between DLX and DNA at 298 K. From the value of Stern-Volmer constant (K_{SV}), the pattern of quenching can be determined. K_{SV} is obtained from the slope of the plot of F_o/F against [Q] based on the fluorescence data at different temperatures (298 K and 308 K). Binding constants and binding points are showed in table 2 and table 3 at two different temperatures. Static and dynamic quenching are the two quenching process. In static quenching, formation of non-fluorescent complex with quencher molecule is responsible for quenching of fluorescence intensity of a fluorophore, whereas dynamic quenching refers to a process in which the fluorophore and the quencher come into contact during the lifetime of the excited state.[19] They can be distinguished by their different temperature dependence. Higher temperatures can lead to faster

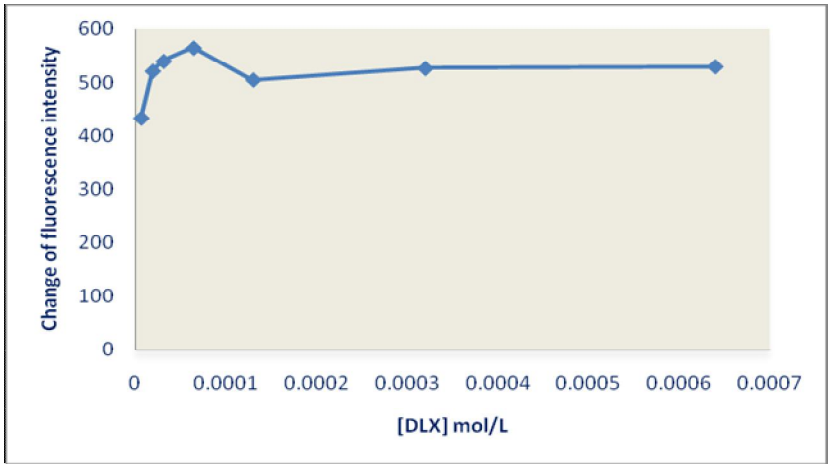

Figure 1. Fluorescence titration curve of effect of duloxetine concentration on the interaction of DLX with ct-DNA.

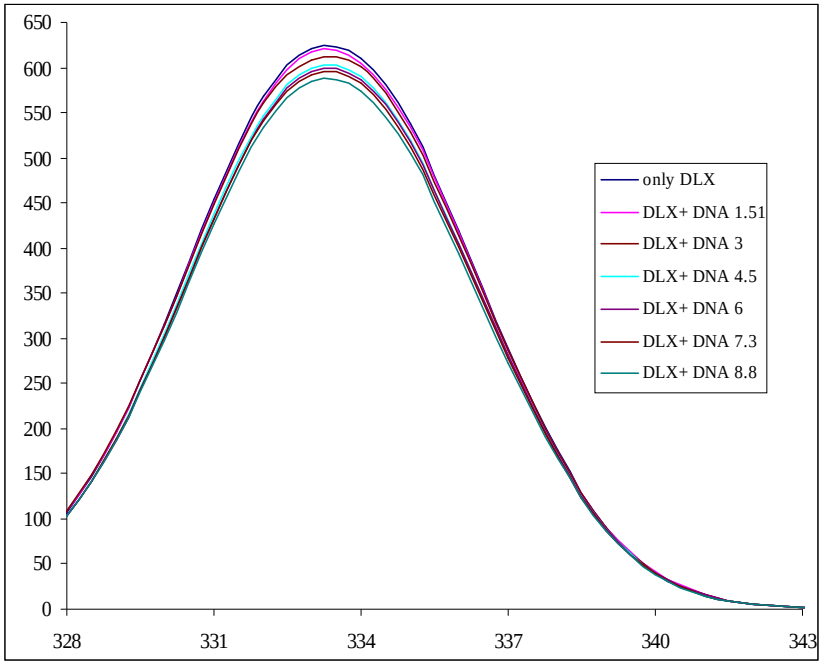

Figure 2. Fluorescence emission spectra of duloxtine (6.4×10^{-5} mol/L) in the presence of different concentration of DNA (λex_{max} = 235 nm, T= 298 K).

Table 2. Data of binding constant and binding points for DLX-DNA system at 298 K.

F_0	F	F_0-F/F	log [(F_0-F)/F]	[DNA] mol/L	log [DNA]
625.1	619.7	0.008714	-2.05978	1.51×10^{-5}	-4.821
625.1	612.0	0.021405	-1.669480	3×10^{-5}	-4.523
625.1	603.9	0.035105	-1.45463	4.5×10^{-5}	-4.347
625.1	599.8	0.042181	-1.37489	6×10^{-5}	-4.222
625.1	595.5	0.049706	-1.3035901	7.3×10^{-5}	-4.137
625.1	588.2	0.062734	-1.202499	8.8×10^{-5}	-4.055

Table 3. Data of binding constant and binding points for DLX-DNA system at 308 K.

F_0	F	F_0-F/F	log [(F_0-F)/F]	[DNA] mol/L	log [DNA]
583.8	575.4	0.0145985	-1.835691	1.51×10^{-5}	-4.821
583.8	571.3	0.0218799	-1.659954	3×10^{-5}	-4.523
583.8	565.2	0.032909	-1.482689	4.5×10^{-5}	-4.347
583.8	561.4	0.039900	-1.399024	6×10^{-5}	-4.222
583.8	556.0	0.05	-1.30103	7.3×10^{-5}	-4.137
583.8	551.0	0.059528	-1.225278	8.8×10^{-5}	-4.055

diffusion and extended collisional quenching, so K_{SV} increases along with increasing temperature. For static quenching, higher temperatures will typically cause the dissociation of weakly bound complexes and K*sv* decreases with increasing solvent temperature.

The fluorescence quenching data at 298 K and 308 K were plotted according to Stern-Volmer equation (Figure 3). The values of Stern-Volmer constant were found to be 710.18 L mol^{-1} and 652.23 L mol^{-1} at 298 K and 308 K, respectively with correlation coefficient of 0.994 and 0.9939. As the K_{SV} decreases with an increase of temperature the

probable quenching mechanism of the DLX-DNA binding reaction is due to static quenching instead of dynamic quenching.

Thermodynamic parameters and nature of the binding forces. In order to elucidate the forces responsible for overall binding affinity and specificity of the drug, thermodynamic parameters are calculated. The value of enthalpy change (ΔH) and the entropy change (ΔS) were determined from the slope and intercept of the fitted curve of ln K_{SV} against 1/T, respectively (Figure 4). The Gibb's free energy (ΔG) can be estimated from the following relationship:

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

The enthalpy change (ΔH), entropy change (ΔS) and Gibb's free energy (ΔG) are the main evidence to characterize the binding mode. The value of ΔH, ΔS and ΔG were -6.43 KJ/mol, 32.97 J/mol/K and -16.26 KJ/mol respectively. Here the free energy change (ΔG) and the enthalpy change (ΔH) are negative and the entropy (ΔS) is positive. The positive ΔS value is considered evidence for hydrophobic interaction from the point of H_2O structure while negative ΔH implies the possibility of hydrogen bonds. The negative sign for ΔG indicates the spontaneity of the binding process.[20] Thus both hydrogen bonding and hydrophobic interactions are present as major forces in the DLX-DNA binding. As hydrophobic interactions and hydrogen bonding are involved in the binding of DLX with DNA, so it can be said probably Duloxetine binds in the minor groove of DNA.

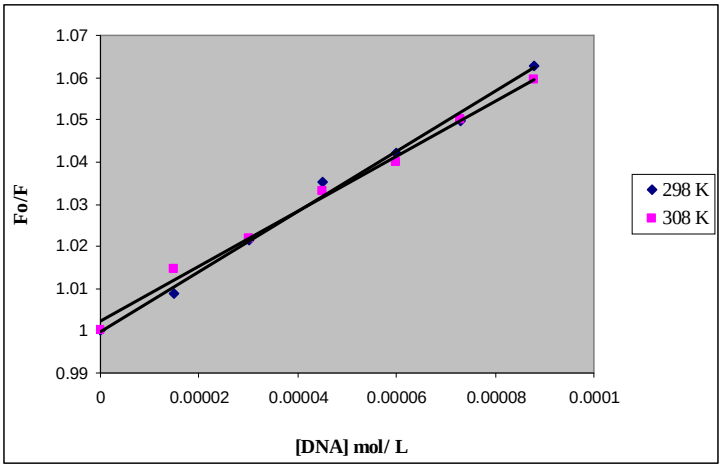

Figure 3. The Stern-Volmer plots for the quenching of DLX by DNA at two different temperatures.

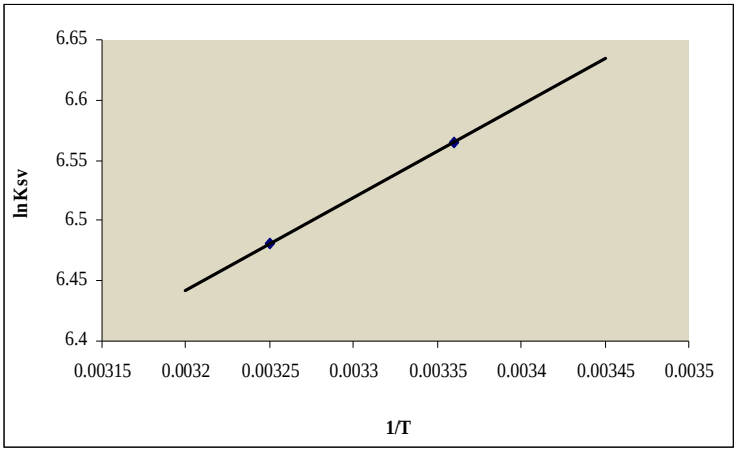

Figure 4. The Van't Hoff Plot for DLX-DNA system.

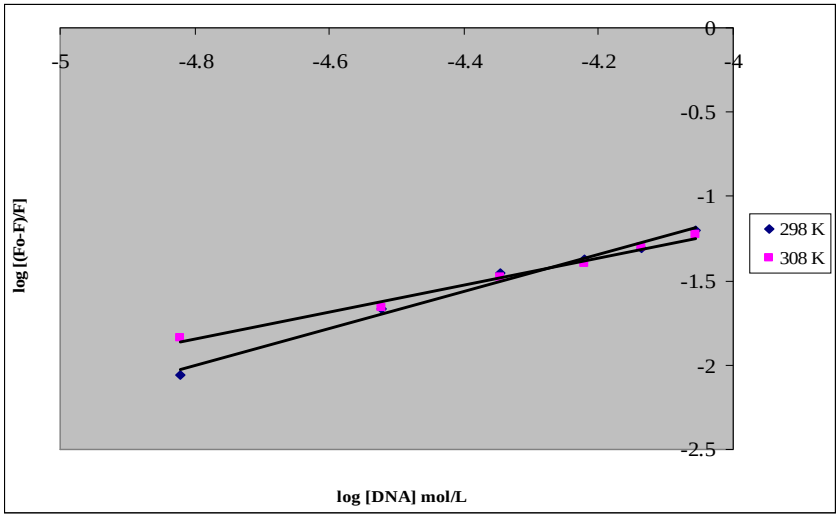

Figure 5. Plot for binding constant and binding points for DLX-DNA system at 298 and 308 K.

Binding constant and binding points. From the plot of log [(F_o- F)/ F] versus log [DNA] (Figure 5) binding constant and binding number for Duloxetine-DNA system were determined. Binding points were found to be 1.095 and 0.8045 at 298 K and 308 K respectively. From corresponding binding constant 1.795×10^3 L/mol and 10.387×10^1 L/mol, it was observed that the binding constant decreases with the increase in temperature of the Duloxetine-DNA complex resulting in the reduction in stability of the DLX-DNA complex, which proved further that the quenching mechanism of DLX-DNA binding reaction is static. The values of *n* remained constant at different temperatures. The Duloxetine-DNA mol ratio of the system is 1:1. That means 1 mol of Duloxetine binds with per mol of DNA.

In this study the nature and magnitude of the interaction of Duloxetine hydrochloride to DNA was investigated in physiological buffer (pH 7.4) by fluorescence and UV absorption spectroscopy. The fluorescence quenching mechanism usually involves static or dynamic quenching. The experimental results demonstrated static quenching involved in the DLX-DNA complex (1:1) formation. The thermodynamic parameters indicated that the interaction between DLX and DNA was spontaneous and hydrogen bonding and hydrophobic interactions were present as major forces in the DLX-DNA binding that dominates the minor groove of binding. The similar observations were found in some other studies.[1,21,22]

CONCLUSION

Fluorescence absorption and quenching of the interaction between Duloxetine and DNA at two different temperatures was observed in this research work. It showed that, duloxetine binds to DNA molecule with high affinity through hydrogen bond and van der Waals force. The quenching pattern was found to be static in this interaction and it was found that duloxetine binds with DNA with a mol ratio of 1:1.

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