Kinetic and Conformational Studies of Adenosine Deaminase Upon Interaction with Oxazepam and Lorazepam

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Abstract: Oxazepam and lorazepam inhibit the adenosine deaminase (ADA) differently. In the case of lorazepam temperature increment causes an increase in the inhibition potency whereas higher temperature reduces the inhibitory effect of oxazepam; which proposes the overall profounder structural changes in the case of lorazepam relative to those caused by oxazepam.

Keywords: Adenosine deaminase, oxazepam, lorazepam, kinetics, structures.

INTRODUCTION

Adenosine deaminase (ADA), (E.C. 3.5.4.4.), is an important enzyme which is involved in the purine metabolic pathway and in the maintenance of a competent immune system. ADA is a monomeric protein (34.5 kDa), that catalyzes the irreversible hydrolytic deamination of adenosine and 2'-deoxyadenosine nucleosides to their respective inosine derivatives and ammonia with a rate enhancement of 2×10^{12} relative to the nonenzymatic reaction [1]. Catalysis requires a Zn^{+2} cofactor [2]. The enzyme is widely distributed in vertebrates, invertebrates and mammals including humans. The enzyme is present in virtually all human tissues, but the highest levels are found in the lymphoid system such as lymph nodes, spleen, and thymus [3]. Aberrations in the expression and function of ADA have been implicated in several disease states such as severe combined immunodeficiency (SCID), which is characterized by impaired B- and T-cell based immunity resulting from an inherited deficiency in ADA [4, 5 and 6]. Higher levels of ADA in the alimentary tract and decidual cells, developing fetal-maternal interface put ADA among those enzymes performing unique roles related to the growth rate of cells, embryo implantation, and other undetermined functions [7, 8]. ADA is widely distributed in the brain, and one important function of this enzyme is probably associated with regulation of the extracellular level of adenosine and 2'-deoxyadenosine in contact with cerebral blood vessels. The inhibition of adenosine deaminase in brain would allow an accumulation of adenosine, which would produce vasodilation and increase in cerebral blood flow. Therefore, the decrease in enzyme activity would potentiate the sedative actions of adenosine in interneuronal communication of the central nervous system [9].

ADA can hydrolyze the free amino substituent on the 6-position of a variety of substituted purine nucleosides. The enzyme hydrolytic capabilities have been exploited to convert lipophilic 6-substituted purine nucleosides to products, which show anti-HIV (human immunodeficiency virus) activity [10, 11].

ADA is a glycoprotein, sequenced in 1984 [12], that consists of a single polypeptide chain of 311 amino acids. The primary amino acid sequence of ADA is highly conserved across species [13]. ADA has a (α/β) barrel structure motif and the active site of ADA resides at the C-terminal end of the β barrel, in a deep oblong-shaped pocket; a penta-coordinated Zn^{+2} cofactor is embedded in the deepest part of the pocket. The zinc ion is located deep within the substrate binding cleft and coordinated in a tetrahedral geometry to His 15, His 17, His 214, and Asp 295. A water molecule, which shares the ligand coordination site with Asp 295, is polarized by the metal giving rise to a hydroxylate ion that replaces the amine at the C6 position of adenosine through a stereo-specific addition–elimination mechanism [14, 15]. Mutation studies of amino acids in the proposed active site near the zinc-binding site in adenosine deaminase confirmed the essential role of these residues in catalysis [16, 17, and 18].

It is well cited in the literature that some drugs with neuroregulatory effects influence the level of adenosine deaminase [19]. Based on such a hypothesis it was predicted that drugs with anxiolytic, anticonsulvant and sedative char-

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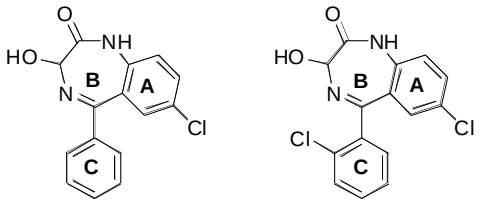
acteristics (such as the members of the benzodiazepine family), might also be capable to regulate the levels of the enzyme adenosine deaminase.

To investigate the structure and activity of the enzyme, we have compared the effect of two widely used drugs, oxazepam and the lorazepam [Scheme **1**], which differ only in a chlorine atom on the C-ring of lorazepam.



Oxazepam *Lorazepam*

Scheme 1. Two members on the benzodiazepine family of drugs; lorazepam and oxazepam; the drugs differ only in the additional O-chlorophenyl moiety in the lorazepam, which is absent in the case of oxazepam (note the ring (c)).

MATERIAL AND METHODS

Materials

Adenosine deaminase (type IV, from calf intestinal mucosa), adenosine, and the drugs oxazepam and lorazepam were obtained from Sigma. The other related chemicals, of the highest grade, were obtained from different industrial sources. The solutions were prepared in doubly distilled water.

Methods

Enzyme Assay

Enzymatic activities were assayed by UV-Vis spectrophotometry with a Shimadzu-3100™ instrument, based on The Kaplan Method to follow the decrease in absorbance at 265 nm resulting from the conversion of adenosine to inosine [20]. This method uses the change in extinction coefficient of adenosine ($8400 M^{-1}cm^{-1}$), on conversion to inosine by the catalytic activity of the enzyme. The concentration of enzyme in the assay mixture, 50 mM sodium phosphate buffer, pH 7.5, was 1.5 nM with a final volume of 1 ml [20, 21]. Activities were measured using at least six different concentrations of lorazepam and five different concentrations of oxazepam. The ligand-enzyme incubation time was three minutes in each case. The assays were at least in triplicate. The adenosine concentration range used is between 0.25–2.5 Km. Care was taken to use experimental conditions where the enzyme reaction was linear during the first minute of the reaction. The experiments in each case were also carried out in the absence of drugs as the control. The rates were measured, individually, at 27 °C and 37 °C.

Fluorescence Spectroscopy

Fluorescence spectroscopy was performed using a Varian fluorescence spectrophotometer, Carey Eclipse model 100 ™ and a 1-cm path length fluorescence cuvette; thermostated to maintain the temperature at 27 and 37 °C. Substrate addition followed after incubation of enzyme with different concentrations of the two drugs. The excitation wavelength was adjusted to 290 nm and emission spectra were recorded for all of the samples in the range of 300 to 500 nm. Samples of 1.2 μM ADA were in 50 mM standard phosphate buffer, pH 7.5. All spectra were normalized for protein concentration. The mixture was incubated for three minutes. The "three minutes", is the time required for the equilibrium between the ligands and the protein. Within three minutes the system was found to be stable, before that i.e. $t < 3$ min; the system is far from equilibrium, and as far as the steady-state assumptions are in effect (in derivation of equations), the results obtained would not be eligible. At longer times, however no significant changes in the results were observed; that confirm the fact that 3 minutes is a reasonable time span for the incubation of the ligands and the enzyme.

Circular Dichroism (CD) Measurements

CD spectra were recorded on a JASCO J-715 spectropolarimeter ™. The solutions for far-UV investigations contained two constant concentrations of ligands as well as protein (0.25 mg/ml), compared to those obtained in the absence of these ligands as control. The ligand-enzyme incubation time was three minutes in each case. "The three minutes" is the time at which we ascertain that the system has reached equilibrium as mentioned earlier. The results were expressed in molar ellipticity [θ] (deg cm^2dmol^{-1}) based on a mean amino acid residue weight of 111 (MRW). The molar ellipticity was determined as $[\theta]_\lambda = (100 \times \theta_{obs}/cl)$; where θ_{obs} is the observed ellipticity in degrees at a given wavelength, c is the protein concentration in mg/ml and l is the length of the light path in cm. The noise in the data was smoothed using the JASCO J-715 software. This software uses the fast Fourier-transform noise reduction routine that allows enhancement of most noisy spectra without distorting their peak shapes. The JASCO J-715 software was used to predict the secondary structure of the protein according to the statistical method [22-25].

Docking Simulations

Docking simulations were performed with AutoDock 3.0.5 using a Lamarckian genetic algorithm (LGA) [26] during standard flexible docking procedure. Three dimensional coordinates of ADA structure (PDB ID: 1krm) was obtained from RCSB. After removing co-crystalled ligand (the transition-state analogue, 6-hydroxy-1,6-dihydropurine riboside (HDPR)) from protein complex in the PDB file using a plain text editor, a short minimization (100 steepest descent steps using AMBER95 force fields with a gradient convergence value of 0.05 kcal/mol Å) was performed using HyperChem 7™ in order to release any internal strain. AutoDockTools package was used to prepare docking files. The grid maps were calculated using AutoGrid (part of AutoDock package; version 3.0.5). A grid map of 40, 40, and 40 points in x, y, and z directions, with grid-point spacing of 0.375 Å was built, centered on the center of the mass of the zinc atom on the catalytic site of the protein (1krm). 100 independent runs with the step sizes of 0.2 Å for translations and 5° for orientations and torsions were performed. The clustering tolerance for the root-mean square positional deviation (RMSD) was 0.5 Å, and the crystallographic coordinates on the co-crystalled ligand were used as the reference structure. De-

fault settings were used for all other parameters. At the end of docking, ligands with the most favorable free energy of binding were selected as the resultant complex structures. All calculations were carried out on PC-based machines running Linux x86 as operating systems. The resultant structure files were analyzed using Rasmol [27] visualization programs.

RESULTS

Kinetic Studies of Oxazepam

Fig. (**1a**) shows the double reciprocal Lineweaver-Burk plot for the ADA adenosine system in which five different concentrations of oxazepam (0.05–0.25 mM) are incubated (three minutes) with the enzyme-substrate complex at 27 °C. These plots show a set of straight lines, which intersect on the left hand side of the vertical axis, and a little further from the horizontal axis, which confirms mixed inhibition. The apparent maximum velocity ($V_{max\ app}$) and apparent Michaelis constant (K'_m) values as well as the slope values of these straight lines (K'_m / $V_{max\ app}$) can be obtained at different fixed concentrations of the inhibitor as shown in Fig. (**1b**). A secondary plot of the slope against the concentration of inhibitor gives a straight line with an abscissa-intercept of –Ki (Fig. (**1b**)) and also another secondary plot of the reciprocal apparent maximum velocity against the concentration of inhibitor gives a straight line with an abscissa-intercept of –αKi (Figs. (**1c**)), where Ki is the inhibition constant and α is the interaction factor between the substrate and inhibitor sites (K_i = 0.317 mM, α = 4.02).

The experiment was further conducted at the same conditions of phosphate buffer 50 mM with pH=7.5 and the same concentrations of oxazepam, but this time at 37°C as the independent variable. The value of Michaelis constant ($K'm$) is unchanged by increasing the oxazepam concentrations, but the V_{max} values are decreased Fig. (**2a**). This confirms the non-competitive inhibition of adenosine deaminase by oxazepam (K_i = 0.476 mM), indicating that the inhibitor does not interfere with the binding of substrate to the active site. The value of the inhibition constant or the Ki is determined via secondary plot by plotting $1/V_{max\ app}$ against the different concentrations of oxazepam at 37 °C and obtaining the corresponding abscissa-intercept Fig. (**2b**).

Kinetic Studies of Lorazepam

Fig. (**3**) shows the double reciprocal Lineweaver-Burk plot for the ADA adenosine system in which seven different concentrations of lorazepam (0.05–0.35 mM) are incubated (three minutes) with the enzyme-substrate complex at 27°C.

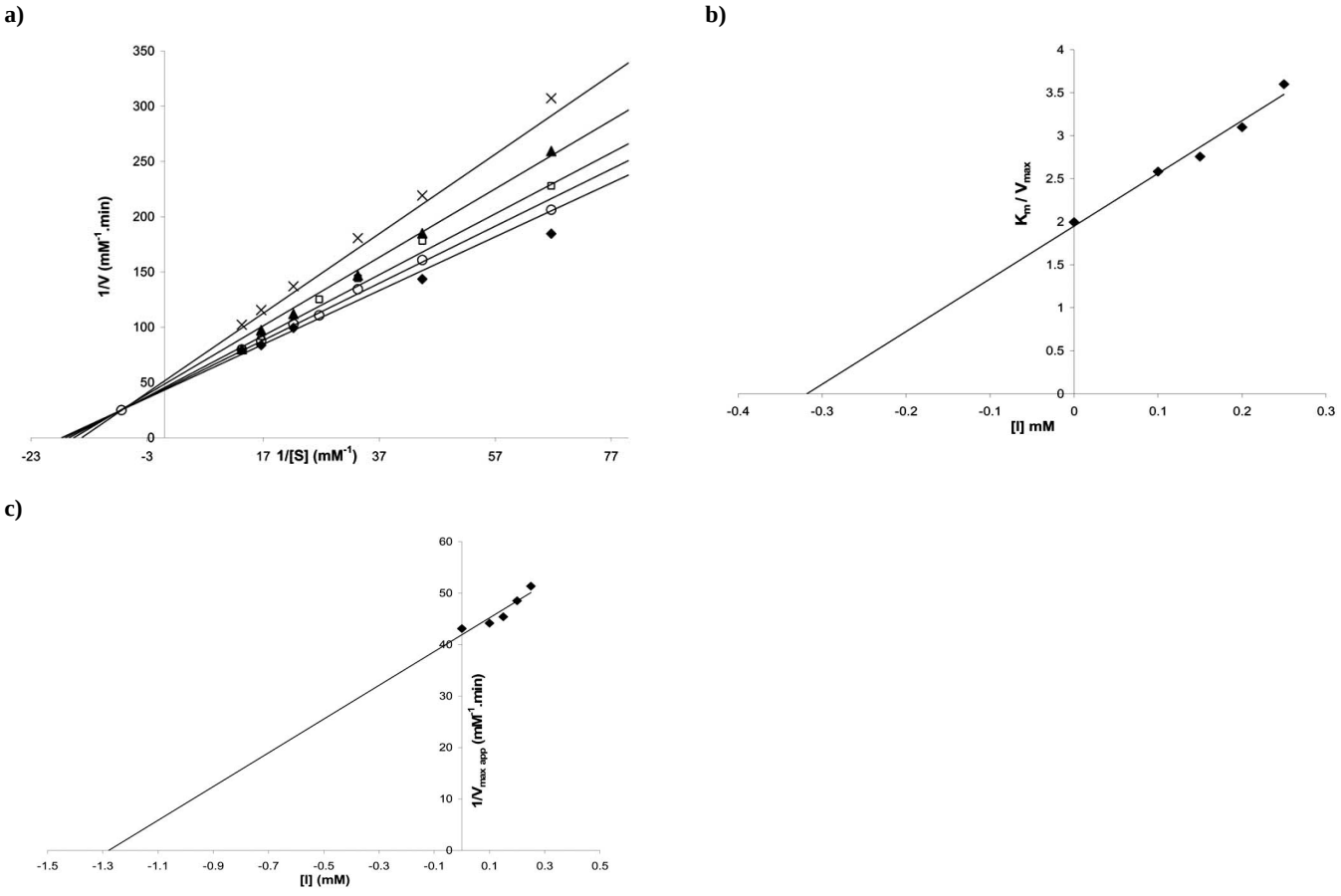


Figure 1. (a) The double reciprocal Lineweaver-Burk plot for the ADA adenosine system in which five different concentrations of oxazepam (0.1–0.25 mM; Absence of oxazepam (♦), 0.1 mM (○), 0.15 mM ( ), 0.20 mM (▲), 0.25 mM (×)) are incubated (three minutes) with the enzyme-substrate complex at 27 °C. **(b)** Secondary plot of the slope ($K'_m/V_{max\ app}$) against the concentration of inhibitor, which gives the -Ki from the abscissa-intercept. **(c)** Secondary plot of $1/V_{max\ app}$ versus concentration of inhibitor, which gives -αKi from the abscissa intercept.

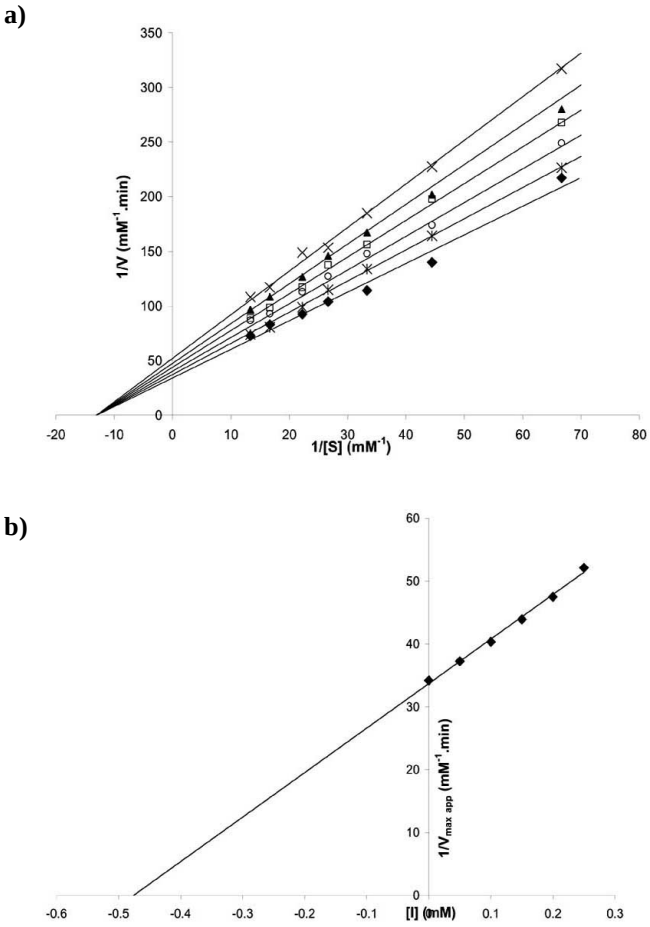


Figure 2. (a) The double reciprocal Lineweaver-Burk plot for the ADA adenosine system in which five different concentrations of oxazepam (0.05–0.25 mM; Absence of oxazepam (⬩), 0.05 mM (∗), 0.1 mM (○), 0.15 mM ( ), 0.20 mM (▲), 0.25 mM (×)) are incubated (three minutes) with the enzyme-substrate complex at 37°C. **(b)** Secondary plot of the slope ($1/V_{max\ app}$) against the concentration of inhibitor, which gives the -Ki from the abscissa-intercept.

The experiment was also performed at 37 °C Fig. (**4**), the techniques used were the same as those applied to the oxazepam. This time at both temperatures non-competitive inhibition were observed (K_i = 0.250 mM at 27˚C and K_i = 0.163 mM at 37˚C). These results are summarized in Table **1**.

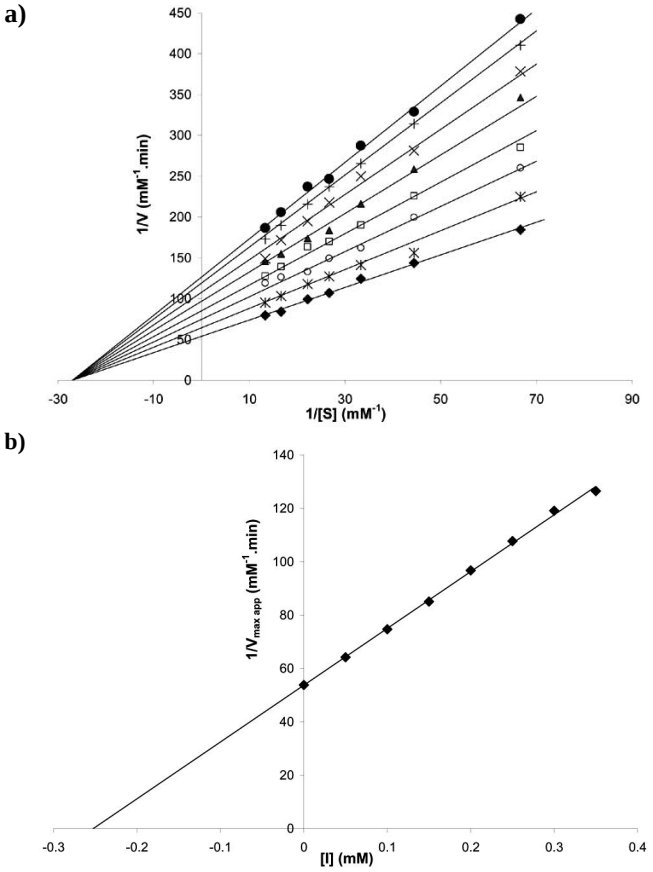


Figure 3. (a) The double reciprocal Lineweaver-Burk plot for the ADA adenosine system in which seven different concentrations of lorazepam (0.05–0.35 mM; Absence of oxazepam (⬩), 0.05 mM (∗), 0.1 mM (○), 0.15 mM ( ), 0.20 mM (▲), 0.25 mM (×), 0.30 mM (+), 0.35 mM (●)) are incubated (three minutes) with the enzyme-substrate complex at 27°C. **(b)** Secondary plot of the slope (1/Vmax app) against the concentration of inhibitor, which gives the -Ki from the abscissa-intercept.

Structural Studies

Fluorescence Technique

Herein, fluorescence technique is used for determination of temperature-induced conformational changes occurring on the ADA structure at the tertiary level. Fig. (**5**) shows the fluorescence spectra of the native as well as those of other states of enzyme structure, formed upon incubation of the protein with three different concentrations of oxazepam at 27 and 37 °C.

Table 1. Kinetic Parameters and Estimated Docking Energy (kcal/mol) of Adenosine Deaminase Upon Interaction with Lorazepam and Oxazepam

Drugs	Estimated Docking energy (kcal/mol)	K_i (mM) at 27°C (calculated)	K_i (mM) at 27°C	K_i (mM) at 37°C	V_{max} (mM.min $^{-1}$) at 27°C	V_{max} (mM.min $^{-1}$) at 37°C
Lorazepam	-7.85	0.148	0.250	0.163	0.018	0.029
Oxazepam	-7.95	0.177	0.317 α = 4.02	0.476	0.023	0.029

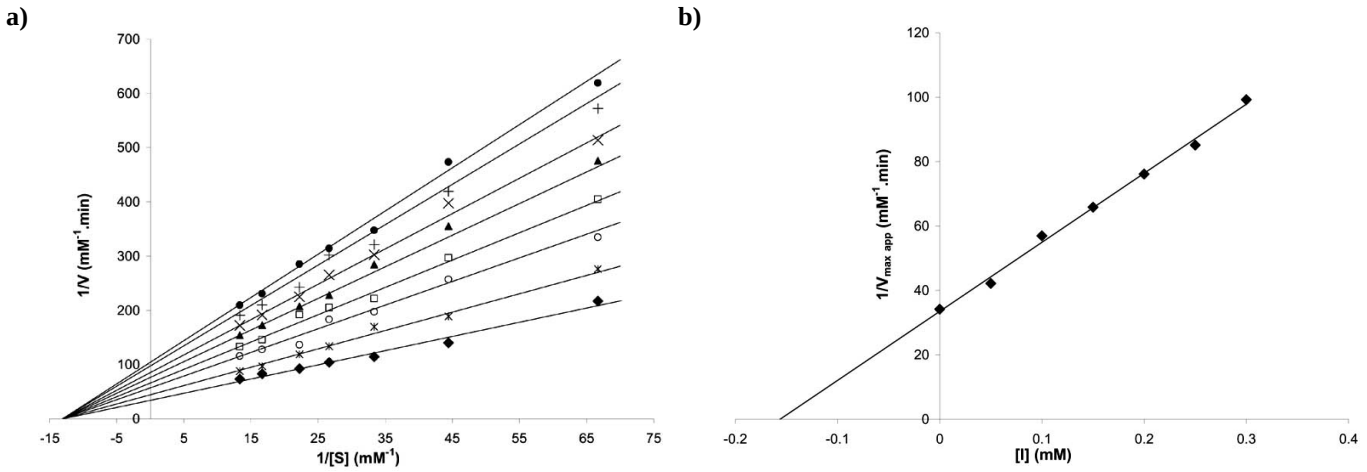


Figure 4. (a) The double reciprocal Lineweaver-Burk plot for the ADA adenosine system in which seven different concentrations of lorazepam (0.05–0.35 mM; Absence of oxazepam (⬥), 0.05 mM (∗), 0.1 mM (○), 0.15 mM ( ), 0.20 mM (▲), 0.25 mM (×), 0.30 mM (+), 0.35 mM (●)) are incubated (three minutes) with the enzyme-substrate complex at 37°C. **(b)** Secondary plot of the slope (1/Vmax app) against the concentration of inhibitor [I], which gives the -Ki from the abscissa-intercept.

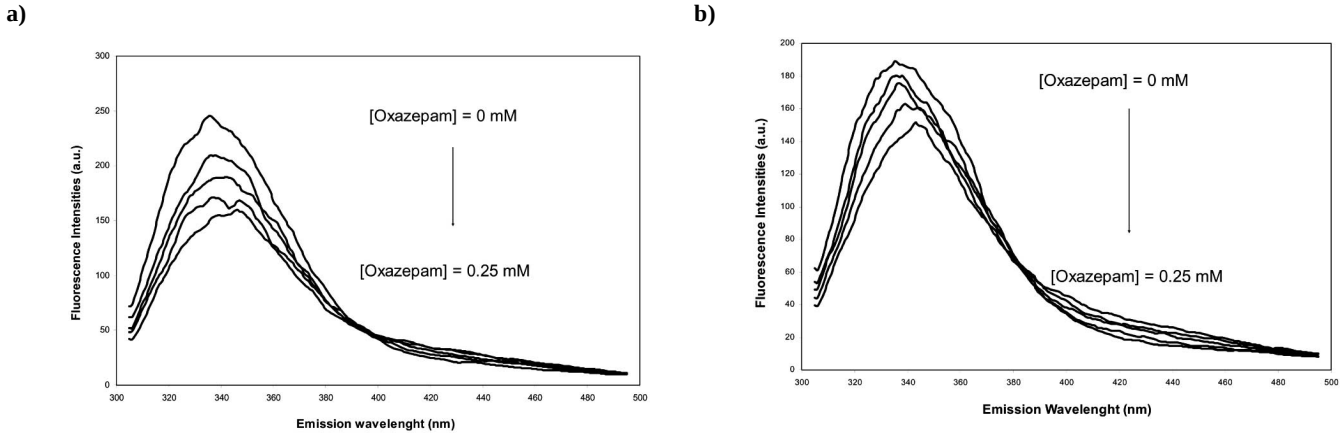


Figure 5. (a) The fluorescence spectra of ADA (1.2 μm) at pH =7.5 and T=27 °C in the presence of different fixed concentrations of oxazepam (from top to bottom: 0.05 mM, 0.10 mM, 0.15 mM, 0.20 mM, 0.25 mM). All spectra are normalized for protein concentration. the mixture was incubated for three minutes. **(b)** The spectra obtained under the identical conditions, but at 37°C.

The fluorescence technique has revealed that the increase in the temperature causes a slight quenching due to the migration of tryptophan residues and their exposure to the aqueous solvent. The increase of temperature show a shift of emission intensity of about 10 nm but the fluorescence quenching seems to be the comparable. The increase in the concentration of the inhibitor causes a more dramatic shift and decrease in emission intensity; which is more pronounced in the case of lorazepam (Fig. (**6**)); in comparison to that of the oxazepam, which is in full consistency with the stronger potency of lorazepam as an inhibitor. Interestingly, similarity can be seen to the findings of the circular dichroism (CD) investigations.

CD Investigations

To find out the nature of the induced structural changes i.e. secondary or tertiary, spectra of the far UV-CD were recorded at 27 °C for both drugs. The results confirmed a more pronounced secondary structure conformational change in the case of lorazepam Fig. (**7b**), Table **2** with respect to that of observed in the case of oxazepam Fig. (**7a**), Table **2**.

DISCUSSION

Due to higher lipophilicity of lorazepam, the hydrophobic interactions with ADA are generally stronger at both temperatures and lorazepam is generally a more potent inhibitor at both temperatures (Scheme (**2**) and (**3**)).

In addition in the case of lorazepam, the increase in temperature abolishes the steric hindrance caused by the large van der Waals radius of the O-chlorophneyl moiety, which leads to the greater inhibitory potency and hence a reduced Ki at 37°C. The mixed system in the case of oxazepam could be attributed to the lower lipophilicity (higher hydrophilicity) that can lead to a better establishment of the electrostatic interactions between the enzyme and the oxazepam. The increment in the temperature from 27°C to 37°C is expected to lead in abolishment of such electrostatic interactions as a result of which the system of inhibition would be noncom-

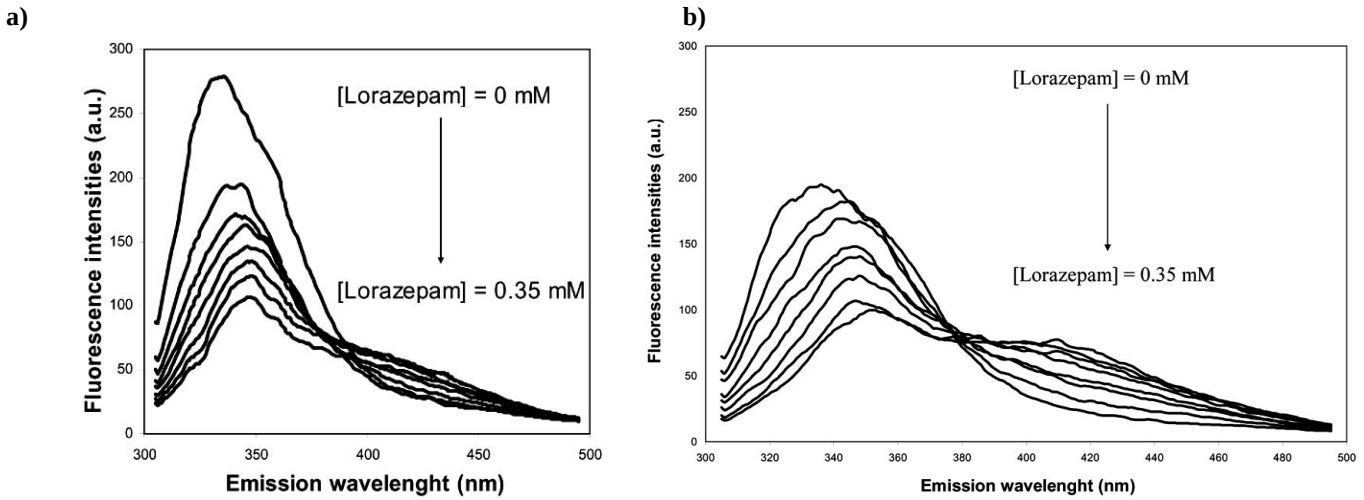


Figure 6. (a) The fluorescence spectra of ADA (1.2 μm) at pH =7.5 and T=27 °C in the presence of different fixed concentrations of lorazepam (from top to bottom: 0.05 mM, 0.10 mM, 0.15 mM, 0.20 mM, 0.25 mM, 0.30 mM, 0.35 mM), the mixture was incubated for three minutes. **(b)** The spectra obtained under the identical conditions, but at 37 °C.

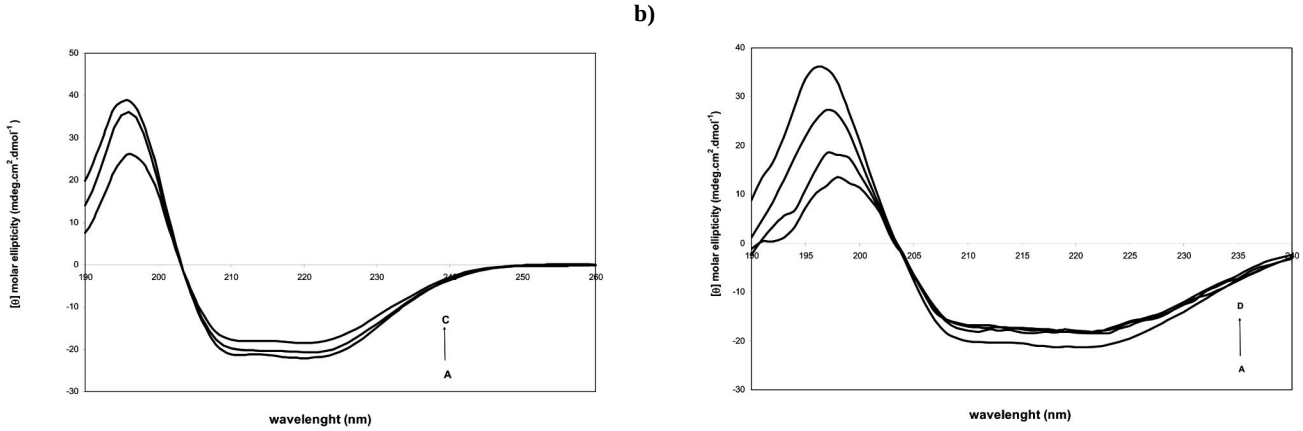


Figure 7. (a) The UV–CD spectra for the native structure of ADA (0.25 mg/ml) at pH =7.5 and 27 °C in the presence of a two different concentrations of oxazepam (from top to the bottom: The arrow shows the order of increase in oxazepam concentration ; In the absence of oxazepam (A), 0.125 mM (B), 0.250 mM (C). **(b)**, The UV–CD spectra for the native structure of ADA (0.25 mg/ml) at pH =7.5 and 27 °C in the presence of a three different concentrations of lorazepam (from top to the bottom: In the absence of lorazepam (A), 0.125 mM (B), 0.250 mM (C), 0.350 mM (D).

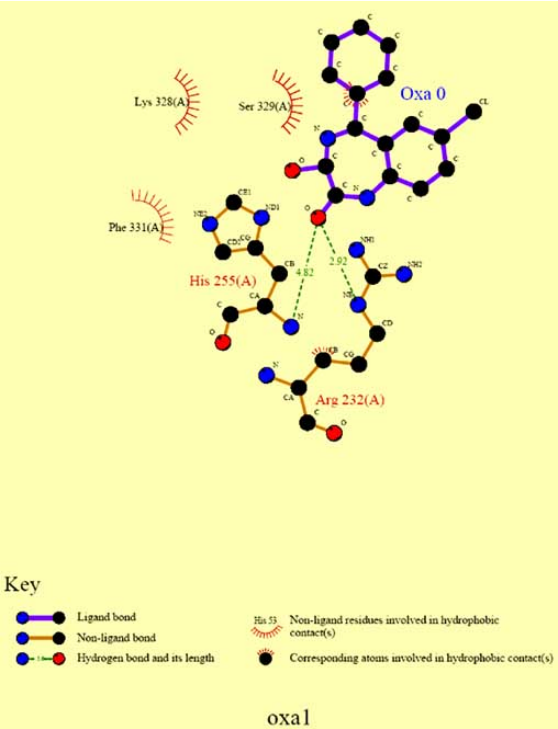
Table 2. Circular Dichroism Informations Regarding the Secondary Structure of ADA upon Incubation (3 Minutes) with Different Concentrations of Oxazepam, and Lorazepam at 27°C

Secondary Structure at 27°C	ADA	ADA+0.125mM [Oxazepam]	ADA+0.250mM [Oxazepam]	ADA+0.125mM [Lorazepam]	ADA+0.250mM [Lorazepam]	ADA+0.350mM [Lorazepam]
α Helix	33.74%	30.87%	24.19%	37.70%	37.70%	23.9%
β Sheet	12.04%	13.51%	18.25%	7.90%	7.90%	0.00%
Turn	16.05%	17.15%	18.25%	33.50%	33.50%	36.90%
Random Coil	38.17%	38.46%	38.27%	20.90%	20.90%	29.90%

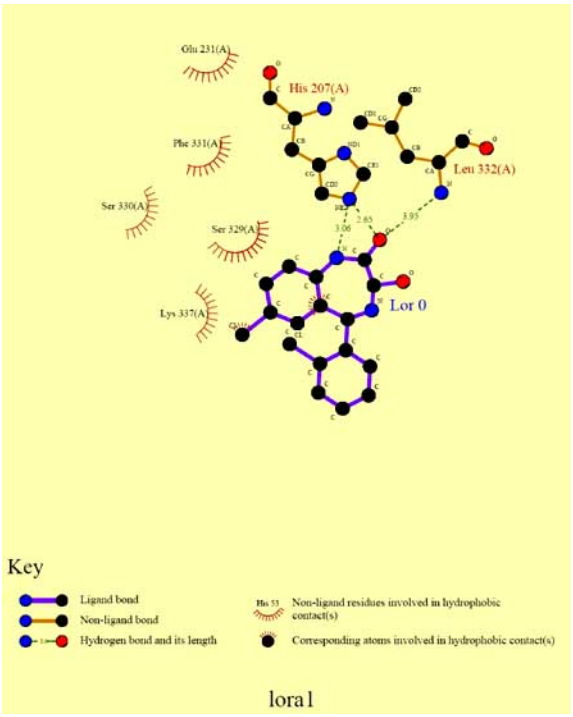
petitive. It is also well cited in the literature that the inhibition potency increases with the hydrophobicity of the ligand, which proposes a profounder involvement of the hydrophobic interactions in the noncompetitive inhibition of a variety of enzymes [28, 29].

The data of macromolecular docking have indicated that both ligands situate themselves at the same site within the enzymatic macromolecular structure (Scheme (**4**)).

The sole difference is the slight localization of the (C) ring at the site of interaction between the enzyme and the

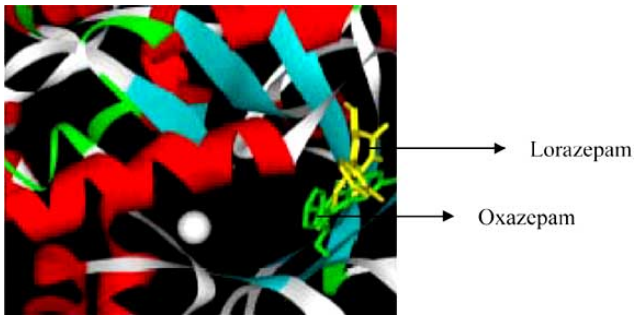


Scheme 2. Interaction of ADA by oxazepam (green) and lorazepam (yellow) as revealed by the data of macromolecular docking.



Scheme 3. LIGPLOT for lorazepam, depicting five hydrophobic contacts and three hydrogen bonds.

ligands. Therefore, it might be plausible to propose that the more hydrophobic ligands would be better candidates for ADA inhibition. From the fluorescence data in the range of 300-500 nm, lorazepam seems to be more able to adapt into the enzyme catalytic pocket. Moreover, the higher stability of lorazepam–ADA complex may depend from larger number of H-bond formed (Scheme **3**).



Scheme 4. Interaction of ADA by oxazepam (green) and lorazepam (yellow) as revealed by the data of macromolecular docking

Taking the fact in to consideration that lorazepam is a far more potent therapeutic than oxazepam, it exerts its intended effect at a far lower concentration (Table **3**) [30].

The potent concentration for the selective action at the benzodiazepine receptor; is ultimately low in the steady-state serum consideration to inhibit the ADA. In other word to inhibit the ADA, copious concentrations of lorazepam should be administrated which is of course toxic. On the other hand oxazepam, in its intended use, is a far less potent drug, allowing a much higher steady state concentration to be achieved, considering the mentioned facts and the Ki of both ligands which is quite similar; it seems reasonable to propose that oxazepam is a better candidate for the therapy in patients with elevated levels of the ADA; whereas lorazepam is advantageous in those who are ADA-deficient.

As confirmed by the partition coefficient data [31], it is evident that both drugs have near similar degrees of hydrophobicity; yet lorazepam is more hydrophobic and can establish a better interaction with the enzyme, which is validated by both the kinetic and structural results. The mixed inhibition system, observed in the case of oxazepam at 27 °C conforms to the fact that oxazepam is more hydrophilic (has a smaller octanol/water partition coefficient) and can establish a minute degree of interaction with the polar residues of the enzyme; the increase in the temperature leads to the increased hydrophobicity; which in turn leads to the observation of noncompetitive inhibition in the case of oxazepam at 37 °C. Lorazepam however due to it's higher hydrophobicity exhibits noncompetitive inhibition at both temperatures. These results were confirmed by the theoretical studies conducted on both drugs and are consistent to the aforementioned structural studies (Schemes **2** and **3**).

Docking investigations have shown that the calculated K_i values and the estimated docking energy are less for lorazepam in comparison with oxazepam (Table **1**); which implies higher inhibitory potency and tighter binding, respectively.

The results are in good agreement with the experimental data mentioned earlier at both temperatures (Table **1**). Furthermore the LIGPLOT schemes of the interaction between ADA and ligands show a higher number of interactions in the case of lorazepam in comparison with that of oxazepam

Table 3. Steady State Therapeutic and Toxic Concentration for Lorazepam and Oxazepam

Drug	[Therapeutic] mgL^{-1}	[Toxic] mgL^{-1}	[Therapeutic] μM	[Toxic] μM
Lorazepam	0.3	1	0.93	3.11
Oxazepam	1	5	3.48	17.4

(5 hydrophobic bonds and 3 hydrogen bonds versus 2 and 3 in the case of oxazepam)(Scheme **2** and **3**). Therefore it might further be suggested that in addition to lipophilicity; the mode of interaction between the ligands and the enzyme is also important.

Oxazepam and lorazepam are effective on their target benzodiazepine receptor at sub-micromolar concentrations, which is caused by their structure activity relationships (SAR); whereas indicated by the experimental as well as theoretical results, these drugs are effective on ADA at micromolar concentrations. Therefore for inhibition of ADA at higher concentrations of these drugs should be administrated to achieve the saturation as implicated by the Michaelis-Menton equation.

The ligands (drugs); oxazepam and lorazepam do not cause any adverse effect; i.e. inhibition of adenosine deaminase (ADA) at clinical dose, however the increase of ligand concentration caused an inhibition. Taking the non-nucleoside structure of the ligands (oxazepam and lorazepam) into consideration; it might be plausible to block or alleviate the effect of ligands on their intended benzodiazepine receptor and there it might provide an insight into the design of a new class of non-nucleoside inhibitors of the ADA with reduced central nervous system (CNS) toxicity and higher ADA inhibitory potency.

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