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Alprazolam induced conformational change in hemoglobin

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Abstract

Alprazolam (ALP) is a widely prescribed sedative and antidepressant benzodiazepine group of drugs. The wide uses of this drug lead us to investigate its possible interaction with hemoglobin (Hb). Spectrophotometric and spectofluorimetric studies showed strong binding of ALP with Hb. Circular dichroic spectra showed that α-helical structure of Hb-subunits has been largely changed. On ALP treatment partial pressure of O_2 is increased in the blood indicating release of O_2 from erythrocytes. Further, the binding of ALP-induced conformational changes in Hb resulting in larger Hb particle size was demonstrated by dynamic light scattering experiment. Thus, the present study unambiguously raises question of danger of random usage of ALP, which binds with and changes the function of Hb.

Keywords: Alprazolam; Hemoglobin; Dynamic light scattering; Spectrophotometry; Spectrofluorimetry; Circular dichroic spectra

1. Introduction

The most frequently prescribed benzodiazepine (BDZ) drug is alprazolam {8-chloro-1-methyl-6-phenyl-4H-s-triazolo(4,3-a)(1,4)benzodiazepine} and it belongs to the class of anxiolytic, sedative and hypnotic anticonvulsant [1–3]. Alprazolam (ALP) is the most commonly used anti-anxiety drug because they are believed to be fairly safe and it rapidly reduces the symptoms of anxiety.

BDZs with their proven efficacy in panic disorder exerted through control of the central nervous system (CNS) excitability by a selective and potent enhancement of inhibitory gamma-amino butyric acid (GABA) mediated neurotransmission [4]. Also it has been reported that apart from $GABA_A$ receptors in the CNS where BDZs mainly act, there are other peripheral receptors. These are called as peripheral benzodiazepine receptors (PBRs) [5]. PBR is both structurally and pharmacologically distinct from the $GABA_A$ receptors. PBR is highly expressed in steroid producing tissues, e.g. ovary, testis, adrenal and placenta [6]. They are also located in heart, liver, lymphocytes, mast cells, etc. [2]. Skeletal muscle, gastrointestinal (GI) tract contain relatively lower number of PBRs [5]. Within the cell PBR is mainly located in mitochondria, plasma membrane and also in nuclear membrane [5,7,8].

BDZs are administered orally and are rapidly absorbed in lower intestine. Changes in absorption, distribution, metabolism and excretion (ADME) of BDZs have been reported in a variety of disease states including alcoholism, impaired hepatic and renal function [9]. In a report blood samples from 13 volunteers ($n = 13$) were collected for the determination of plasma level of BDZs after their administration and ALP was found to sustain for a longer period of time than other BDZs [10]. A mean half-life of ALP was found to be 11.0 h (range: 6.3–15.8 h, $n = 16$) in healthy adult people compared to 16.3 h (range: 9.0–26.9 h, $n = 16$) for healthy elderly people. Thus, the removal rate of ALP in elderly people decrease, particularly after long-term uses [11]. In patients with alcoholic liver the half-life of ALP increases up to 19.7 h (range: 5.8–65.3 h, $n = 17$) whereas in an obese group of people the average half-life of ALP is 21.8 h (range: 9.9–40.4 h, $n = 12$) [9]. Thus, depending on age and health conditions ALP sustains inside the body and may get more chance to interfere with different physiological processes. Consistent with that the manifestations of ALP overdose or withdrawal includes somnolence, confusion, impaired coordination, diminished reflexes

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and coma [12]. Death has been reported in association with overdoses of ALP by itself, as it has with other benzodiazepines. The acute oral dose (LD_{50}) of ALP in rats was reported to be 331–2171 mg/kg and massive intravenous doses of ALP (over 195 mg/kg, 975 times the maximum recommended daily human dose of 10 mg/day) in laboratory animals was found to induce cardiopulmonary collapse [13].

The overuse and abuse of BDZs was shown to have adverse effect on human [14–16] and ALP has been reported to be more toxic than other BDZs [17]. ALP absorbs quickly in the human body and found to be deposited in blood, urine and even hair samples [18,19]. Thus, ALP after absorption though intestinal mucosa may interact with several biomolecules mainly in the blood and influence their effects. The interaction of peripheral blood lymphocytes with ALP was documented as it enhances the antibacterial activity of the lymphocytes [20] and also enhances the immune response in mice [21]. While most of the work on the adverse effect of ALP has been done on either laboratory animals or human samples, little or no efforts have been made to study the effect of this drug on biomolecules particularly on hemoglobin.

This made us promptly to explore the interaction of ALP with hemoglobin, the major constituents of RBC. We have seen that ALP binds with Hb and induces its de-oxygenated state. The binding was evaluated by spectrofluorimetric observation followed by Stern–Volmer plots. The conformational change of Hb in presence of ALP was observed by CD spectroscopy. Further, dynamic light scattering experiment showed aggregation of Hb induced by ALP. Thus, our in vitro data suggest the strong interaction between Hb and ALP, which may have significant implications in vivo.

2. Methods

2.1. Hemolysate preparation

Human erythrocyte suspension was prepared from fresh blood collected into heparinized tubes and was centrifuged for 10 min at $1500 \times g$. The plasma and buffy coat were then removed. The red cell preparations were washed twice in Buffer G (50 mM Tris, 50 mM HEPES, 10 mM $MgCl_2$, 2 mM EDTA, 10 mM D-glucose, 10 mM $CaCl_2$, 50 mM NaCl, 5 mM KCl and 0.1% bovine serum, pH 7.4) and then resuspended in the same buffer maintaining initial hematocrit conditions. No appreciable cell lysis was observed [22]. Erythrocyte suspension in Buffer G was centrifuged at $1500 \times g$ for 10 min and the supernatant was discarded. Hemolysis was done by adding hypotonic water followed by heavy centrifugation at $10000 \times g$ for 30 min. Hemoglobin concentration in the hemolysate was determined by taking optical density at 415 nm.

2.2. Hemoglobin solution preparation

Some experiments were done with hemoglobin powder (Himedia), which was dissolved in 100 mM Tris–Cl buffer (pH 9.0) kept overnight followed by filtration.

2.3. Alprazolam stock solution preparation

Alprazolam (Torrent Pharmaceuticals Ltd., India) was dissolved at a concentration 10 mM in 0.24N HCl and stored at 4 °C in dark. The stock was diluted freshly before each experiment.

2.4. pO_2 measurement

Fresh venous blood from non-smoker human volunteers were taken in a heparinized disposable syringe in vacuum vials and treated with different concentrations of ALP and incubated for 30 min. The pH of the samples was 7.0. The partial pressure of free oxygen in the treated and mock treated whole blood was analyzed by a blood gas analyzer (SP3 NOVA).

2.5. Fluorescence spectroscopic study of hemoglobin after alprazolam treatment

A 3 μM of hemoglobin solution was incubated with different concentrations of ALP for 30 min. The solutions were excited separately at 270 nm, 285 nm and 295 nm, respectively and the emission spectra were taken using a spectrofluorimeter (Hitachi 3010, excitation and emission band pass 5 nm each, scan speed 60 nm/min and spectral response 2 s).

2.6. CD spectroscopic study

CD spectra (Jasco-J-600) of hemoglobin and hemoglobin treated with different concentrations of ALP were taken after incubation for half an hour at room temperature. Scan speed 50 nm/min; bandwidth 1 nm; spectral response 0.2 nm; data points-211 (taken on an average of three).

2.7. Dynamic light scattering

Hemolysate of 10 μM was treated with different concentrations of drug and hydrodynamic radius of the particles was measured using a PCS (photon correlation spectrophotometer, Protein Solutions/DynaPro).

3. Results

ALP first encounters with blood cells after absorption in intestinal mucosa. The earlier reports showed that ALP functionally interacts with blood PBMC [20], and so, attempts were made to study the interaction between Hb, the major constituent of RBC and ALP. We first use the absorption spectra of hemoglobin in the presence of ALP. Hemoglobin has three characteristic absorption maximas at 415 nm, 540 nm and 580 nm, respectively. The first peak, i.e., soret peak is obtained due to the heme-group whereas the later two peaks are due to vinyl group and porphyrin ring system, respectively. All these three peaks are due to π–π^* transition. Oxyhemoglobin shows the presence of the later two peaks (at 540 nm and 580 nm) distinctly. But in deoxyhemoglobin both the peaks flatten out to give only a broad peak [17]. Our spectrophotometric studies with increasing ALP concentration showed in Fig. 1a and

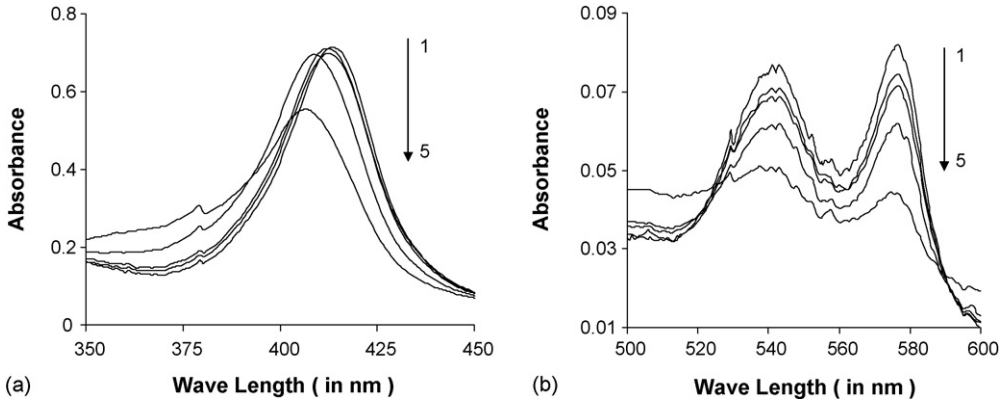

Fig. 1. Absorption spectra of hemoglobin in the presence of different concentrations of ALP. Hemoglobin solution (3 μM) was incubated with ALP (1 → 5 in the figure, 0 μM, 10 μM, 20 μM, 30 μM, 70 μM, respectively) for 30 min and absorption spectra was scanned from 350 nm to 450 nm (a), and 500 nm to 600 nm (b). Three absorption maxima were observed at 413 nm, 541 nm and 576.5 nm. Three isosbestic points were also observed at 462 nm, 523 nm and 587.5 nm.

b, the absorbance of all the three absorption maximas are decreased after ALP treatment. Three isosbestic points were also observed at 462 nm, 523 nm and 587.5 nm, which suggest that Hb has some common mode of complexation with ALP. At soret region we further analyzed the ground state complex formation between Hb and ALP. In the absorption spectra, second and third peak at 541 nm and 576.5 nm were gradually flattened out which strongly indicates the conversion of oxy-state to deoxy-state of hemoglobin. The binding affinity constant "*K*" (on the heme basis) was determined using the relation [23]:

$$\frac{A_0}{\Delta A} = \frac{A_0}{\Delta A_{\max}} + \left(\frac{A_0}{\Delta A_{\max}}\right) \times \frac{1}{K} \times \frac{1}{L_t}$$

where $\Delta A = A_0 - A$, $\Delta A_{\max}$ = maximum reduced absorbance, A_0 = maximum absorbance of Hb (without any ligand), A = reduced absorbances of Hb (in presence of ligand), and L_t = ligand (ALP) concentration.

The linear plot of $A_0/\Delta A$ versus $1/L_t$ (Fig. 2a) shows that $K = 1.823 \times 10^3\ M^{-1}$ (on heme basis). This means that the binding affinity of ALP with Hb is more stronger than the binding affinity of O_2 with Hb, $1.3 \times 10^3\ M^{-1}$ [24]. This suggests that ALP can induce oxygen release from Hb. The oxygen release was further determined by observing the change in partial pressure of whole blood sample treated with different concentrations of ALP. As shown in Table 1, the partial pressure of oxygen in the blood samples (collected and treated with ALP in vacuum) enhanced with increasing concentrations of ALP indicating O_2 release from RBC. The pH of the solutions (~7.0) did not change much. As calculated from Table 1, the partial pressure of free oxygen in the blood sample was increased upto 69.88% in the presence of 70 μM ALP. We had also calculated the possible number of binding sites "b" on the heme basis from the plot of $1/(1-\theta)$ versus L_t/θ (Fig. 2b) for Hb–ALP interaction following

Table 1
Change in partial pressure of oxygen in blood in presence of ALP

Alprazolam concentration (μM)	pO_2 (mm Hg)
0	100.267 ± 1.100
10	107.767 ± 1.600
30	118.567 ± 2.200
40	151.400 ± 1.200
50	169.300 ± 0.800
60	169.667 ± 0.800
70	170.333 ± 0.500

Oxygen release by ALP treated human red blood cells. Blood was collected in vacuum vials, treated with different concentrations of ALP and incubated for 30 min. After incubation partial pressure of oxygen was measured by a blood gas analyzer.

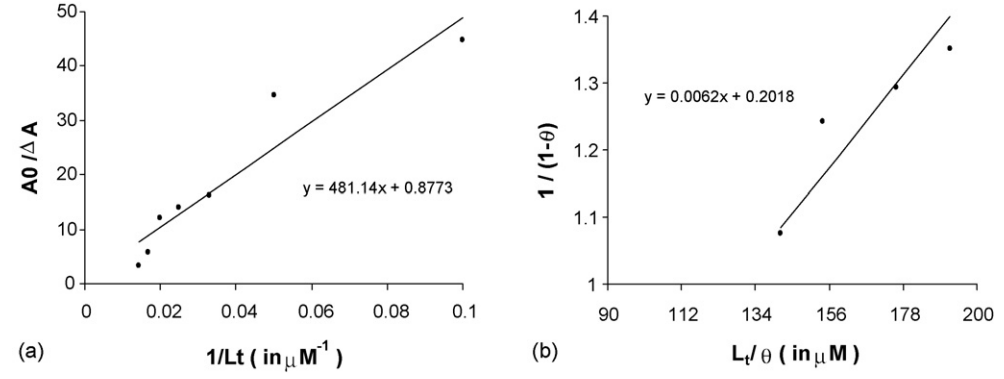

Fig. 2. Determination of ground state binding constant between hemoglobin (3 μM) with different concentrations of ALP (see text for detail). The calculated value of binding affinity constant (on heme basis) was $1.823 \times 10^3\ M^{-1}$.

the relation [23]:

$$\frac{1}{1-\theta} = K\frac{L_t}{\theta} - Kbp$$

where $\theta = \Delta A/\Delta A_{max}$ and p = protein (Hb) concentration = 3 μM.

The calculated number of binding sites per Hb molecule was found to be 11 (on heme basis).

Thus, absorption spectroscopic studies of hemoglobin confirmed the interaction between ALP and hemoglobin. In order to explore the interaction one step further, detailed spectrofluorimetric experiments with ALP and Hb was carried out. Hemoglobin contains three intrinsic fluorophores—tryptophan, tyrosine and phenylalanine (weak fluorophore). In our study, 3 μM hemoglobin solution was excited in the presence of increasing concentrations of ALP at excitation wavelengths 270 nm (for Trp), 285 nm (for Tyr) and 295 nm (for both Trp and Tyr) and emission spectra were taken. The fluorescence emission spectrum (Fig. 3a–c) showed considerable quenching in all these three excitation wavelengths. It was found that the fluorescence intensity at saturation level quenched up to 45.6%, 33.1% and 32.1% at 270 nm, 285 nm and 295 nm, respectively. All three experiments were carried out in same day under identical experimental conditions. The top most spectrums in all these figures corresponds to untreated control hemoglobin and the spectra following it corresponds to hemoglobin samples treated with 10 μM, 20 μM, 30 μM, 40 μM, 50 μM, 60 μM, 70 μM, 80 μM, 90 μM and 100 μM ALP concentrations, respectively. It is evident from all the spectra that a saturation of binding occurs at an ALP concentration of 70 μM. Furthermore, the association constants between ALP and hemoglobin were determined from Stern–Volmer plots (Fig. 3d–f). The values of the affinity constants agreed well with each other for all the three excitation wavelengths. The values are $1.67 \times 10^4\ M^{-1}$ (at 270 nm), $1.02 \times 10^4\ M^{-1}$ (at 285 nm) and $1.09 \times 10^4\ M^{-1}$ (at 295 nm), respectively. We have also seen that after the saturation binding of ALP (70 μM), further addition of acrylamide (a neutral quencher) reduces the fluorescence intensity upto 64% at 270 nm (data not shown). This indicates strongly that after saturation binding of ALP other sites of Hb are still available for quenching. However, the possible number of binding sites "b" was also determined by using the Scatchard plot (Fig. 4),

$$\frac{1}{1-\theta} = \frac{K[Q]}{\theta} - Kbp$$

where $\theta = \Delta F/\Delta F_{max}$, $\Delta F = (F_0 - F)$, $\Delta F_{max} = (F_0 - F_{saturation})$, $[Q]$ = quencher (ALP) concentration, K = affinity constant, p = protein (Hb) concentration, and b = number of binding sites on each protein (Hb) molecule.

The number of binding sites of ALP per Hb molecule was calculated to be 17 (on tryptophan basis).

Thus, the binding of ALP induces a certain conformational change of Hb. This was further confirmed by circular dichroic (CD) spectra of hemoglobin treated with different concentra-

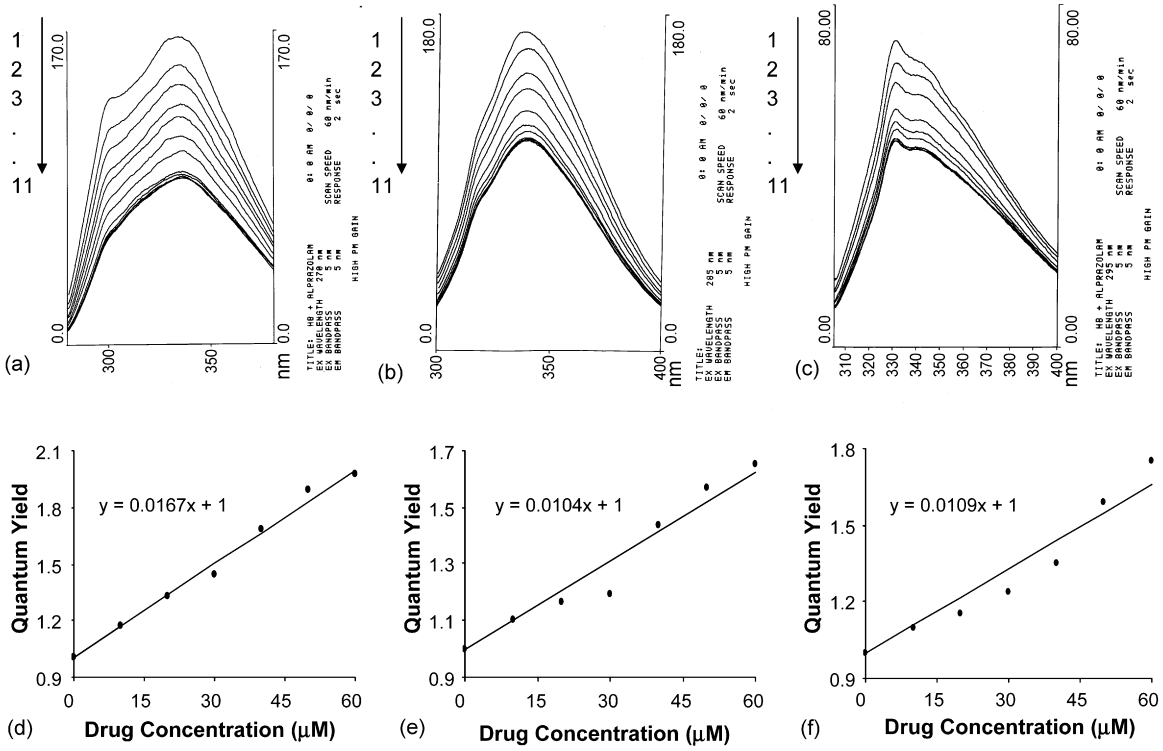

Fig. 3. Fluorescence spectra of hemoglobin at different excitation wavelengths (a) 270 nm, (b) 285 nm (c) 295 nm. The top most spectrums correspond to mock treated hemoglobin (3 μM) and the spectra following it corresponds to hemoglobin (3 μM) treated with ALP concentrations (2 → 11 in the figure) 10 μM, 20 μM, 30 μM, 40 μM, 50 μM, 60 μM, 70 μM, 80 μM, 90 μM and 100 μM, respectively. The saturation of quenching of hemoglobin was obtained at 70 μM of ALP concentration for all the three wavelengths. Stern–Volmer plots obtained from the above set of experiments at 270 nm (d), 285 nm (e) and 295 nm (f), respectively. The affinity constants estimated from the graphs were $1.67 \times 10^4\ M^{-1}$ (at 270 nm), $1.02 \times 10^4\ M^{-1}$ (at 285 nm) and $1.09 \times 10^4\ M^{-1}$ (295 nm). See text for detail.

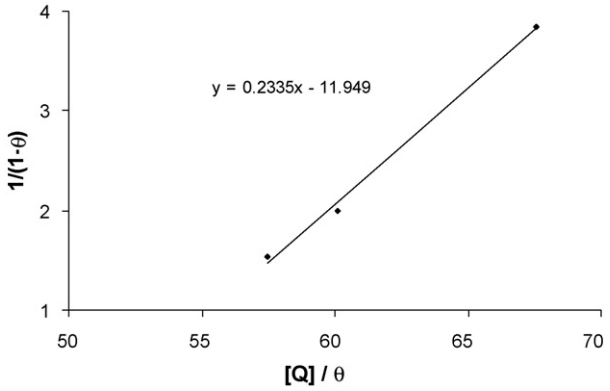

Fig. 4. Scatchard plot for determination of number of binding sites: plot of $1/(1-\theta)$ vs. $[Q]/\theta$ (see text for detail).

Table 2
Percentage decrease in α-helicity of hemoglobin in presence of ALP

Alprazolam concentration (μM)	Angular rotation (m degree)	% decrease in α helicity
0	−11.29	–
30	−8.947	20.753
70	−6.625	41.319
90	−5.477	51.488
110	−3.626	67.883
140	−3.497	69.026
200	−2.294	79.681

A 3 μM of hemoglobin was incubated with different concentrations of ALP for 30 min. Then the samples were analyzed for circular dichroism in Jasco-J-600 instrument. Percentage decrease in α-helicity was calculated using the formula described elsewhere [24].

tions of ALP (Fig. 5). It shows that both the negative peaks at 222.2 nm (due to $n \rightarrow \pi^*$), 210 nm (due to $\pi \rightarrow \pi^*$) and the positive peak at 195 nm (due to $\pi \rightarrow \pi^*$) gradually decreases with increasing concentrations of ALP, which indicates unambiguously that the α-helicity of hemoglobin subunits [24] are gradually lost and was calculated to be approximately 79.681% when treated with maximum 200 μM of ALP concentration (shown in Table 2). CD spectroscopic studies with powder hemoglobin (data not shown) and hemoglobin isolated from pricked blood samples showed identical results.

As oxygenation and deoxygenation of Hb may be related with Hb aggregation [25] we also performed scattering experiment at 660 nm (where Hb has no considerable absorption) to determine the possible aggregation of Hb. Different concentrations of hemoglobin were treated with 50 μM ALP. We have selected 50 μM, as beyond this concentration of ALP no considerable increase in oxygen release from Hb was observed (Table 1). We carried out a graphical extrapolation in Fig. 6a to determine the optimum concentration of Hb required to be aggregated when ALP concentration is 50 μM. The threshold concentration of hemoglobin to achieve the aggregation was found to be 169 μM. Conversely, a reverse titration method was done in order to find out the threshold concentration of ALP, if any. Thus, scattering study was done by adding increasing concentration of the ALP to the threshold hemoglobin concentration (169 μM). Using the same methodology the threshold ALP concentration was found to be 2.33 μM (Fig. 6b).

This conformational change in Hb influences its hydrodynamic volume [26] as we have observed by dynamic light scattering experiment (DLS). The hydrodynamic radius depends on both mass and shape of the molecule. In this study, 5 μM Hb solution was treated with different concentrations of ALP and particles size of the Hb molecule and mass percentage abundance was measured by DLS study. The bar diagram (Fig. 7) shows increase in the hydrodynamic radius of the scattering particles with increasing concentrations of ALP. Further the result also showed that these larger particles contribute more than 85% of the entire mass of the solution.

4. Discussion

The increasing uses of ALP either in medication or abuses lead us to investigate the possible effects of ALP on Hb. Due to the presence of three aromatic moieties in ALP, it is perhaps susceptible to stack with the aromatic amino acids present

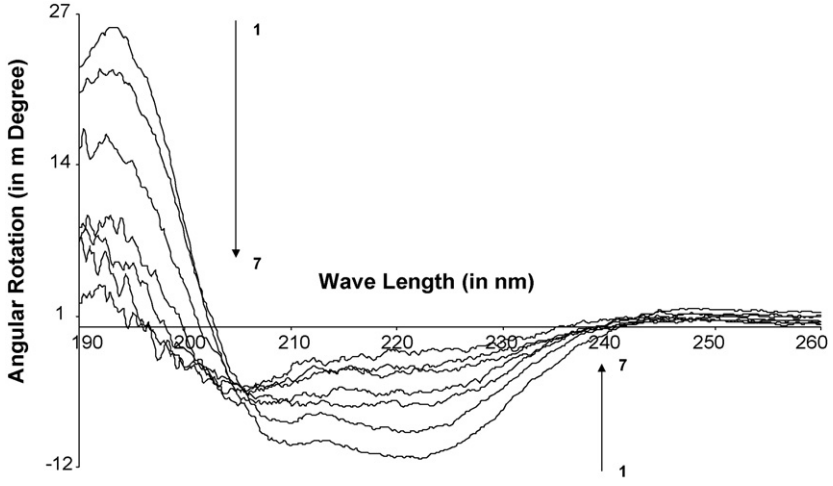

Fig. 5. Representative circular dichroic spectra of prick hemoglobin (3 μM) in absence and presence of different concentrations of ALP (1 → 7 in the figure) 0 μM, 30 μM, 70 μM, 110 μM, 140 μM, 170 μM and 200 μM.

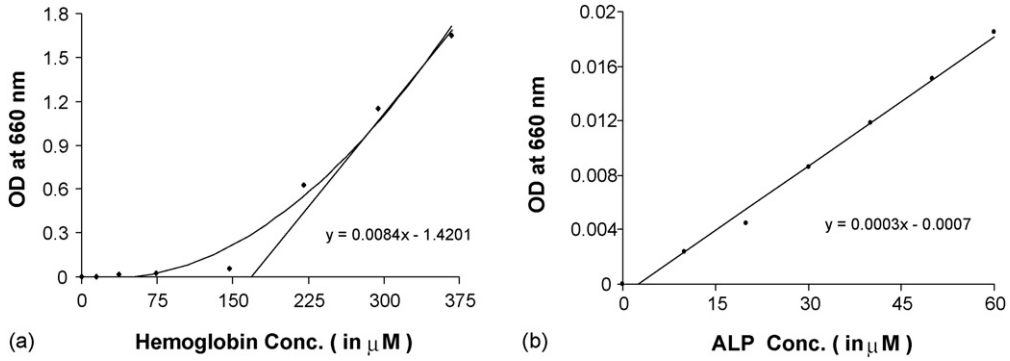

Fig. 6. Light scattering experiment of hemoglobin at 660 nm. (a) 50 μM of ALP were treated with different concentrations of hemoglobin, the critical concentration of hemoglobin was estimated from the graph to be aggregated was 169 μM; (b) 169 μM of hemoglobin was treated with different concentrations of ALP, the threshold ALP concentration estimated from the graph was 2.33 μM to aggregate 169 μM of hemoglobin.

in Hb. Though there are several reports about the mode of action of BDZ group of drugs particularly on the PBR receptors [5], there are no reports so far about their action on the Hb molecule. Bearing in mind that after absorption through the intestinal mucosa ALP may encounter with the peripheral cells like RBC, we have investigated the biophysical and functional changes associated with Hb after the treatment of ALP. We have seen that in the presence of the ALP the oxygen carrying capacity of Hb is reduced significantly. We have performed different experiments using different techniques to demonstrate the interaction between ALP and Hb. Our results on spectrophotometric and spectrofluorimetric observations indicate a strong interaction between ALP with Hb. In the absorption spectra, second and third peak at 541 nm and 576.5 nm (Fig. 1b) flattened out gradually indicating that ALP induces deoxygenated state of Hb. Also, the binding constants for all the three fluorophores of Hb are strong enough which may be responsible for the enhancement in oxygen release from Hb by ALP. The nature of binding between Hb and ALP is static as they produce ground state complex (Fig. 1a). Further, we have also seen that the slope of the Stern–Volmer plot decreases with increasing temperature [27] (data not shown), which strongly support our spectrophotometric observation of producing ground state complex. At the excitation wavelength of 270 nm, the fluorescence spectra of Hb in the presence of ALP indicates energy transfer from tyrosine to tryptophan as the hump at 307 nm (Fig. 3a) due to tyrosine is gradually reduced. Thus, in the altered conformation of Hb tyrosine and tryptophan residues may gradually comes within close proximity. Similarly at 295 nm, the energy is being transferred from tryptophan to phenylalanine (Fig. 3c). So, ALP-induced conformational change in Hb may bring the different moieties closer to each other so that the energy is readily transferred. Alternatively, the ALP might itself mediate the energy transfer between different fluorophores. Whatever the mechanism be, this conformational change may influence the functional activity of Hb, i.e., oxygen carrying capacity as we have observed. Our studies on CD spectra showed that the α-helical conformation of Hb is distorted significantly. Either this conformational change induced by ALP or the release of oxygen from Hb due to interaction with ALP may cause the formation of larger particle size of Hb, as determined by the dynamic light scattering experiments.

Taken together it seems likely that ALP being a sedative drug may interfere with the normal Hb function particularly in long-term use and may alter different physiological response related with Hb mediated catabolism. Hence, our present study unambiguously raises the question of the danger of using, overusing and abusing of ALP.

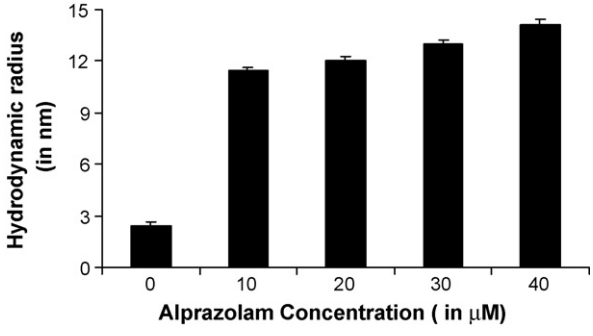

Fig. 7. Photon correlation spectroscopy (dynamic light scattering experiment) with hemoglobin (5 μM) treated with different concentrations of ALP. Average hydrodynamic radius of hemoglobin is plotted against ALP concentrations (see text for detail).

Acknowledgments

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