Probing midazolam interaction with human serum albumin and its effect on structural state of protein

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Abstract
The features of midazolam (MDZ) induced structural perturbation of human serum albumin (HSA) has been investigated in detail by Fourier transformation infrared spectra (FT-IR) and circular dichroism (CD). The CD spectra was deconvoluted to quantify the changes in α-helix, β-sheets, β-turns and random coils of the protein. UV-Vis absorption studies suggested the static type quenching procedure for this binding interaction. The mechanism of MDZ-HSA binding was elucidated by steady state and synchronous fluorescence spectroscopy. The binding parameters for the reaction have been calculated according to Stern-Volmer equation at different temperatures. The plots indicated that the binding of HSA to MDZ is characterized by one high affinity binding site with the association constants of the order of 10^4. Employing protein unfolding pathway and site specific marker, site II in domain III of HSA has been assigned as the primary binding site for MDZ. Binding mode was expounded by thermodynamic parameters: enthalpy change (ΔH) and entropy change (ΔS), which imply that hydrogen bonding and hydrophobic interaction play a major role in stabilizing the complex. The molecular distance between donor (HSA) and acceptor (MDZ) was estimated according to Fluorescence resonance energy transfer (FRET).

Keywords: Midazolam, Fluorescence resonance energy transfer, Thermodynamics, FT-IR, Circular dichroism and Human serum albumin

INTRODUCTION
Benzodiazepines are psychoactive agents, much in vogue recently due to their less side effects. Among which midazolam is a powerful anxiolytic, amnestic, hypnotic, anticonvulsant, skeletal muscle relaxant and anesthetic agent alone as well as in combination (Short et al., 1991). Unlike barbiturates, benzodiazepines are almost never fatal and less habit forming; with no side effects at low drug concentrations (Chin et al., 2004). These compounds have short elimination half-life and rapid onset of action, induce greatest amnesia effects (Blin et al., 1999). Midazolam is mostly preferred for patients in an intensive care unit (ICU) for sedation and impairment of psychomotor performance (Newman, 1995). It is also used as a rehabilitating agent in alcoholic withdrawal (Newman, 1995). It does not affect the formation of new associations in shortterm memory, despite the evidence that the drug blocks long-term memory (LTM) retention of associations (Reder et al., 2006). Midazolam has garnered a lot of attention in the recent years as a good pediatric sedative agent (Shashikiran et al., 2006) and preferred tranquilizer used in various regimes of therapy. Human serum albumin is the principal extracellular protein as it is responsible for transporting many exogenous and endogenous substances, including many drugs with a relative constant level of 3.5-4.5% (w/v) (Quevedo et al., 2001). Crystallographic analyses of HSA revealed that the protein, a 585 amino acid residue monomer, contains three homologous α-helix domains (I–III) (He & Carter, 1994; Carter & Ho, 1994). Each domain contain two subdomains (A and B) stabilized by 17 disulphide bridges (Curry et al., 1999). Serum albumin has two well-known ligand binding sites,
Probing midazolam interaction with human serum albumin site I and site II. Site I is located within subdomain II A and is known as azapropazone warfarin site (Fehske et al., 1981). On the other hand, site II, also known as the indole-benzodiazepine site, is located within subdomain III A (Rahim & Aubry, 1995). However, an expanded number of binding studies have shown that not all benzodiazepine (BDZ) derivatives interact with HSA in a similar manner, especially when the molecule contains an asymmetric center in which the inversion of the 1,4-diazepine ring produces a particular, preferential conformer (Noctor et al., 1992; Lucek & Coutinho, 1979). In addition, the moiety at position 7 has been shown in a few studies to influence binding affinity as well as the percentage of BDZ which binds to HSA. This differential binding of benzodiazepines and varied applications of midazolam necessitates the study of this interaction. The binding of a drug to HSA influences its metabolism, distribution, and elimination from the circulation. Co-binding of two drugs or displacement of one drug by another may alter the therapeutic drug level and can lead to serious health conditions. Thus, detailed knowledge of the binding interaction of a drug on albumin and of their relative strengths is important especially for the drugs given in combination regime like midazolam. In the present study, midazolam (MDZ) binding to HSA was studied to explore the drug-induced protein structural alterations at physiological conditions. Hence, this is the first report in regards of investigating midazolam induced protein alteration, first to report binding mode and mechanism of this drug.

MATERIALS AND METHODS

Materials

Human serum albumin (HSA) fraction V was purchased from Sigma Chemical Company, St. Louis, USA and was used as supplied. Midazolam (MDZ) and Diazepam (DZP) were purchased from Ranbaxy, India. The solutions of MDZ and HSA were prepared in 0.1M phosphate buffer of pH 7.4. HSA solutions were prepared based on their molecular weights of 66,500. The protein concentration was determined spectrophotometrically using the extinction coefficient of 36,500 M⁻¹ cm⁻¹ at 280 nm (Painter et al., 1998). All other materials were of analytical reagent grade and double distilled water was used throughout.

FTIR spectroscopic measurements

Infrared spectra of protein solution were recorded on a Nicolet Magna 750FT-IR spectrophotometer (DTGS detector, Ni-chrome source and KBr beam splitter) via the attenuated total reflection (ATR) method with resolution of 4 cm⁻¹ and 60 scans. Spectra processing procedures: spectra of sample solution and buffer solution were collected at the same condition. Then, subtract the absorbance of buffer solution from the spectra of sample solution to get the FT-IR spectra of proteins. The subtraction criterion was that the original spectrum of protein solution between 2200 and 1800 cm⁻¹ was not depicting any significant signal in this region (Dong et al., 1990).

Circular dichroism (CD)

CD measurements of HSA in presence and absence of MDZ was made in the far-UV (200–250 nm) region on a Jasco-J820 spectropolarimeter coupled to a microcomputer using a quartz cell of 0.1 cm. All the spectra were recorded at 298 K and the temperature was maintained constant by a thermostatically controlled Neslab RTE-110 circulating water bath. A stock solution of each of 150 µM HSA was prepared in 0.1M phosphate buffer. The molar ratio of HSA to drug concentration was 1:2, 1:4, 1:8 and 1:10 CD spectra.

Absorption Spectroscopy

The UV measurements of HSA were recorded on a Shimadzu double beam spectrophotometer model-UV 1700 using a cuvette of 1 cm path length. Absorbance value of protein in the presence and absence of MDZ were made in the range of 250–300 nm and HSA concentrations was fixed at 12 µM while the drug concentration was varied from 12 to 48 µM.
Fluorescence spectroscopy

Fluorescence measurements were made on a Shimadzu spectrofluorometer, model RF-5301PC (Shimadzu, Japan) equipped with a 150W Xenon lamp. The fluorescence quenching of HSA at increasing molar ratios of MDZ to HSA was recorded in the wavelength range 300–450 nm after exciting the protein solution at 280 nm, using 3nm/3nm as slit widths. The HSA concentration was fixed at 3 µM unless mentioned and the drug concentration was varied from 3 to 30 µM. To evaluate the effect of temperature at MDZ-HSA interaction, fluorescence was recorded at three different temperatures i.e. 298, 306 and 310 K. The instrument was thermostatically controlled by a Neslab RTE-110 circulating water bath.

Stoichiometric analysis

The stoichiometry of interaction of MDZ with HSA was determined by the method of continuous variations (Rahman et al., 1993). The fluorescence change ($\Delta F = F_{\text{protein}} - F_{\text{protein}+\text{drug}}$) of a series of protein drug mixture was done, keeping the molarity of the mixture constant while varying the mole fraction of each.

Synchronous Fluorescence Spectroscopy

Synchronous Fluorescence spectra of HSA in the absence and presence of increasing amount of MDZ (0–27 x 10⁻⁶ mol/L) were recorded λex: 290–390 nm and a constant difference of $\Delta \lambda = 60$ nm was maintained.

Energy transfer between MDZ and HSA

The absorption spectrum of MDZ (3 µM) was recorded in the range of 300–400 nm. The emission spectrum of HSA (3µM) on excitation at 295nm was also recorded in the range of 300–400 nm. The overlap of the UV absorption spectrum of MDZ with the fluorescence emission spectrum of protein was used to calculate the energy transfer as per the Forster’s theory (Forster & Sinanoglu, 1996 ).

RESULTS

FTIR measurements

To understand the structural alterations in HSA and gain a view of physicochemical properties of HSA after addition of MDZ in the surroundings, FT-IR spectroscopic measurements were performed on HSA and HSA-MDZ. Since infrared spectra of proteins exhibit a number of the so-called amide bands, which represent different vibrations of the peptide moiety. Of all the amide modes of the peptide group, the single most widely used one in studies of protein secondary structure is amide I. This vibration mode originates from the C=O stretching vibration of the amide group (coupled to the in-phase bending of the N–H bond and the stretching of the C–N bond) and gives rise to infrared bands in the region between approximately 1600 and 1700 cm⁻¹(Dong et al., 1990). The protein amide bands have a relationship with the secondary structure of protein, and amide I band is most sensitive to the change of protein secondary structure than other
amide bonds (Witold et al., 1993). Figure 2 showed the FT-IR spectra of the MDZ free and MDZ-bound form of HSA with its difference absorption spectrum. The spectrum in Figure 2a was obtained by subtracting the absorption of phosphate buffer from the spectrum of protein solution. Difference spectrum in present paper (Fig. 2b) was obtained by subtracting the spectrum of the MDZ + buffer from that of the MDZ-bound form. The evident peak shift of amide I band from 1642.60 to 1648.01 cm⁻¹. These results indicate that MDZ interacts with the C=O and C–N groups in the protein structural subunits. This interaction results in the rearrangement of the polypeptide carbonyl hydrogen bonding pattern and reflects reliable alteration in antiparallel β-sheets of the protein secondary structure (He et al., 2005).

Table 1: Alterations in protein secondary structures induced by Midazolam binding with HSA

<table>
<thead>
<tr>
<th>Midazolam: HSA (molar ratio)</th>
<th>% Structure change</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>54.8 23.1 17.1 5.0</td>
</tr>
<tr>
<td>1</td>
<td>48.3 26.2 17.9 7.5</td>
</tr>
<tr>
<td>4</td>
<td>36.3 30.1 24.3 9.2</td>
</tr>
<tr>
<td>8</td>
<td>28.2 31.4 30.2 10.1</td>
</tr>
<tr>
<td>10</td>
<td>27.0 31.7 31.8 10.5</td>
</tr>
</tbody>
</table>

Circular Dichroism (CD) analysis

For more adequate prediction in protein secondary structure changes CD spectroscopy was employed due to its sensitive prediction. In this work, the molar ratios of 1:2, 1:4, 1:6 and 1:8 for HSA: MDZ were used for the CD measurements. The CD spectra of HSA in the absence (line a) and presence (lines b and e) of MDZ are shown in Figure 3. The CD results were expressed in terms of mean residue ellipticity (MRE) in deg cm² dmol⁻¹ according to the following equation:

\[
MRE = \frac{\text{observed CD (mdeg)}}{Cpnl \times 10}
\]

Where \(Cp\) is the molar concentration of the protein, \(n\) the number of amino acid residues and \(l\) is the path length. The instrument was installed with software based on Yang equation (Chen & Yang, 1971) to calculate the four components in the secondary structure of the protein. The CD spectra of HSA exhibited two negative bands in the UV region at 208 and 220 nm (Fig.3), characteristic of an α-helical structure of protein (Shobini et al., 2001). First titration of MDZ resulted in diminution of the peak intensity (curve b), indicating slight deduction of the helical structure at this concentration. On further addition of the MDZ from (c, d & e), resulted in the drastic variation in the protein helical content inferred by the significant change in the spectral pattern. The alteration in ellipticity is accompanied by the transformation of secondary structures (Table.1), illustrating the destabilization of the protein secondary structure on MDZ-HSA interaction. It is important to quote that saturable binding of some ligands to HSA induce alterations in the structure and function of this protein. However, competitive binding displayed by different ligands may result from allosteric effects, whereby binding of ligand A at a certain site causes a conformational change in the protein so that binding of ligand B at a different site is altered (Miles et al., 1962).
Absorption Spectroscopy

UV-Vis absorption studies were performed to ascertain whether the changes in the secondary structure of HSA was either simply due to the collision of this drug to protein along with its strong electronegative atoms or due to some complex formation between them. The UV absorbance showed an increase with the increase in drug in concentration (Fig.4). Also, a distinct blue-shift in peak could be observed on interaction of HSA with MDZ. These two changes are indicative of a complex formation between the protein and drug (Hu et al., 2004; Cui et al., 2004).

Steady state Fluorescence spectroscopy

The fluorescence spectroscopy was used to measure the change in the tertiary structure of protein induced by different concentrations of MDZ. At an excitation wavelength of 280 nm intrinsic fluorescence spectra of HSA in the absence and presence of increasing concentration of MDZ were recorded in the range of 290-600 nm. A significant blue shift in protein fluorescence spectra was observed upon the interaction of HSA with MDZ, especially at higher drug concentration (fig.5). The shift of the emission maximum wavelength from 345.61 to 331.23 nm is observed. This implies that the changes in the environment of aromatic fluorophores of the protein resulted into a more folded structure on interaction with MDZ. To further elaborate the fluorescence quenching mechanism the Stern-Volmer equation was utilized for data analysis:

\[ \frac{F_0}{F} = 1 + K_{SV} [Q] \]  

where \( F_0 \) and \( F \) are the steady-state fluorescence intensities in the absence and presence of quencher, respectively. \( K_{SV} \) the Stern-Volmer quenching constant and \([Q]\) is the concentration of quencher (MDZ). However, the Stern–Volmer curve showed upward curvature toward y axis at higher drug concentrations (Fig.6). The procedure of quenching was further confirmed from the values of bimolecular quenching rate constants, Kq, which are evaluated using the equation:

\[ K_q = \frac{K_{SV}}{\tau_0} \]

where \( \tau_0 \) is the lifetime of protein without the quencher. Various values of fluorescence lifetime for HSA were reported but average fluorescence lifetime used was about 5 ns. (Gelamo et al., 2002). The bimolecular quenching rate constant was calculated to be \( 6.28 \times 10^{12} \text{L mol}^{-1} \text{s}^{-1} \). Which is largely greater then the maximum limiting diffusion constant \( K_{diff} \) of the biomolecule \( (K_{diff}=2.0 \times 10^{10} \text{Lmol}^{-1} \text{s}^{-1}) \) (Maurice & Camillo, 1981). Which suggested the high affinity and specificity of this interaction and that the quenching was mainly arisen by the formation of non-fluorescent complex i.e. static quenching. The procedure of quenching was further confirmed by temperature dependence of quenching. Studying the dependence pattern of quenching parameters can differentiate between the dynamic and static quenching. The \( K_{SV} \) values decrease with an increase in temperature for static quenching and the reverse will be observed for dynamic quenching. The trend in the present study (Table-2) indicates that the probable quenching...
mechanism of HSA fluorescence by MDZ is a static type.

**Table 2: Temperature effect on quenching constants of HSA–Midazolam system**

<table>
<thead>
<tr>
<th>T(K)</th>
<th>$K_{sv} \times 10^4$ (Lmol$^{-1}$)</th>
<th>$K_q \times 10^{15}$ (Lmol$^{-1}$s$^{-1}$)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>298</td>
<td>3.14 ± 0.04</td>
<td>3.14 ± 0.04</td>
<td>0.984</td>
</tr>
<tr>
<td>306</td>
<td>2.13 ± 0.05</td>
<td>2.13 ± 0.05</td>
<td>0.998</td>
</tr>
<tr>
<td>310</td>
<td>1.97 ± 0.02</td>
<td>1.97 ± 0.02</td>
<td>0.992</td>
</tr>
</tbody>
</table>

**Analysis of binding**

When ligand molecules bind independently to a set of equivalent sites on a macromolecule, the equilibrium between free and bound molecules is given by the equation (Gao et al., 2004):

\[
\log \left( \frac{[F_0 - F]}{F} \right) = \log K + n \log [Q] \quad (4)
\]

where $K$ and $n$ are the binding constant and the number of binding sites, respectively (Table 3). Thus, a plot of $\log \left( \frac{[F_0 - F]}{F} \right)$ versus $\log [Q]$ can be used to determine $K$ as well as $n$. The values of $K$ were found to be $(0.39 \pm 0.03) \times 10^4$, $(0.32 \pm 0.07) \times 10^4$ and $(0.34 \pm 1.56) \times 10^4$ M$^{-1}$ for HSA at 298, 306 and 310 K, respectively. The values of $n$ were noticed to be $(0.96 \pm 0.05)$, $(0.86 \pm 0.01)$ and $(0.82 \pm 0.04)$ respectively, at 298, 306 and 310 K. It was found that the binding constant increased with an increase in temperature, resulting in the stabilization of the MDZ–HSA complex. Meanwhile, from the data of $n$ it may be inferred that there is single class of binding sites for MDZ.

**Table 3: Comparative assessment of quenching constants and Midazolam binding Parameters to HSA conformers.**

<table>
<thead>
<tr>
<th>HSA Isomers</th>
<th>$K$ ($\times 10^4$ Lmol$^{-1}$)</th>
<th>$n$</th>
<th>$\Delta G^\circ_{binding}$ (KJmol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>native (N)</td>
<td>0.39 ± 0.26</td>
<td>0.96</td>
<td>$-20.44 \pm 0.037$</td>
</tr>
<tr>
<td>fast moving (F)</td>
<td>0.13 ± 0.03</td>
<td>0.49</td>
<td>$-17.76 \pm 0.013$</td>
</tr>
<tr>
<td>urea induced (I)</td>
<td>0.09 ± 0.04</td>
<td>0.45</td>
<td>$-16.85 \pm 0.006$</td>
</tr>
</tbody>
</table>

**Stoichiometric analysis**

Binding of drugs to HSA is especially significant as it affects the distribution and elimination of the drug as well as duration and intensity of its physiological action. Hence it is important to know the concentration of bound midazolam at plasma concentrations. This was calculated using following methodology. If $P_t$ is the total protein concentration and $n$ is the number of binding sites, the total number of sites on protein is given by $nPt$ and the concentration of bound sites on protein is given by $n\theta Pt$ ($\theta = \Delta F/\Delta F_{max}$) (Ward, 1985), which is also equal to the concentration of the bound drug ($Db$). $Df$, the number of moles of free drug, was obtained from the difference, $Dt - Db$, where $Dt$ is the total drug added. The amount bound was expressed as moles of drug bound per mole protein, $r$ ($= Db/Pt$). The percentage of drug bound ($\beta = Db/Dt \times 100$) was calculated from the association constants using the relationship (Martin, 1965):

\[
\beta = \frac{1}{\left(\frac{1}{K_a} + [Df] / ([Pt] + (1/K_a) + [Df])\right)} \times 100 \quad (5)
\]

The strongly bound drug at low concentration is concentrated primarily in the blood plasma compartment. There is dose range within which small increase in dose result in relatively large increase in the amount of unbound drug. When $[Df] \rightarrow 0$, $\beta$ becomes proportional to $1/K_a$

\[
\beta = \frac{1}{([Pt] + (1/K_a))} \quad (6)
\]

At a plasma concentration of HSA $(6.7 \times 10^{-4}$M), the fraction of bound MDZ was calculated according to eq.6 and was found to be $~27\%$. The stoichiometry of interaction was calculated by the method of continuous variation. The analysis and interpretation are simpler if, as in the present case, the drug molecule has no fluorophore in the range of wavelength scanned here. Hence the relative fluorescence observed is directly proportional to the protein concentration as present in the complex. As is evident from the figure 7 the Job’s plot (Job, 1928), implies that the stoichiometric ratio of MDZ: HSA at 298 K and pH 7.0 is 1:1.

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**Figure 7: Job's plot based on the method of continuous variation. Depicting stoichiometry of the HSA-MDZ interaction.**

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Probing midazolam interaction with human serum albumin

Figure 8. The plot of log ([F₀-F]/F) versus log [Q] for N (○), F (●) and I (▼) conformation of HSA for binding constant and binding sites.

Allocation of Binding site

Figure 9. Quenching of intrinsic fluorescence of HSA by DZP (▼) and MDZ (○) and the fluorescence of HSA-MDZ complex DZP (●) under identical conditions.

HSA unfolding pathways

The acid and urea induced unfolding pathway was employed to locate the binding site for MDZ on HSA. As HSA undergoes N-F and N-I transitions induced by the acidic pH between 7.0-3.5 and the urea concentration in the range of 4.8-5.2 M respectively (Khan, 1986; Chmelik & Kalous, 1982). The F isomer which predominates at pH 3.5, is characterized by unfolding and separation of domain III, and The I isomer is characterized by unfolding of domain III and partial but significant loss of native conformation of domain I. Domain II is known persist in both N-F or N-I transitions. Figure 8 shows conformation dependent binding of MDZ; most distinguish in N-conformer than in F- or I-conformation. The reduction in the number of binding sites from N-F and N-I transitions is suggestive of the presence of binding site for MDZ on domain III. Since the loss in binding site from N-F transition due to unfolding of domain III was almost equivalent to the loss binding site in I conformer, which was characterized with the partial loss of domain I in addition to domain III. This reflects negligible role of domain I in MDZ binding. The reduction in the binding sites inspite of the presence of domain II in all conformers, rule out the possibility for allocation of binding sites on it. Small increase in the (n) and K values (Table 3) in F and I forms are expected due to the loss of inter domain interactions.

Site specific probe

Displacement experiment was carried out using site specific probe to further augment the allocation of binding site of MDZ. The MDZ to HSA ratio was kept 1:1 in order to keep the non-specific binding. The quenching of the complex fluorescence on titration of the specific binder of site II i.e. DZP was measured at 560 nm (Khan et al., 2002). The diminution in the fluorescence of the complex on addition of the DZP indicates the displacement of MDZ by a more tight binder of that site, which suggest the location of binding sites of MDZ on site II. Relative fluorescence intensity (F/F₀, where F₀ and F are the fluorescence intensity of the HSA-MDZ system, in the absence and presence of the probe i.e. DZP) has been plotted against the concentration of DZP in Figure 9. In another set of experiment, the quenching trend of HSA intrinsic fluorescence by DZP and MDZ were compared and the similarity in trend further advocate the same binding pocket i.e. site II for both the ligands. Crystallographic analyses have assigned site II to subdomain IIIA, which corroborates with the assigning of primary binding sites of midazolam to domain III applying the protein unfolding models. Hence, recommending the binding of MDZ primarily to site II located in subdomain IIIA. More specifically, among the individual amino acid residues in this subdomain, 410Arg and 411Tyr are usually assumed to be important (Sugio et al., 1999). 410Arg usually helps in binding of the acidic drugs as the –COOH group electrostatically interacts with the guanidino group of 410Arg (Wanwimolruk et al., 1983). Since MDZ does not possess any –COOH group so it appears that the phenolic group of 411Tyr helps in binding of drug by hydrogen bonding. This can be explained as strong electronegative groups are present in the drug.
molecule, which is in accordance with our thermodynamic data.

![Figure 10: Synchronous fluorescence spectrum of HSA (T = 298 K, pH 7.40), [HSA] = 6.0 x 10^-6 M [MDZ] = 0.0, 3.0, 6.0, 9.0, 12.0, 15.0, 18.0, 21.0, 24.0 and 27.0 x 10^-6 M, respectively. λ ex: 290–390; Δλ = 60 nm.](image)

**Structure ascribed synchronous fluorescence**

Intrinsic fluorescence of HSA was studied to evaluate the change in the microenvironment of protein induced as a result of the interaction between MDZ and HSA. Synchronous mode of fluorescence spectroscopy introduced by Llody (Lloyd, 1971) was applied to infer the conformational changes of the protein due to this binding reaction. Simultaneous scanning of excitation and the emission monochromators with fixed wavelength difference between them was set. It provides several advantages like spectral simplification, reduction in the spectral noise and spectral area, over other modes of fluorescence study. According to Miller (Miller, 1979) the characteristic information of tryptophan residue is obtained when Δλ difference is maintained at 60 nm. Figure 10 shows the effect of addition of MDZ on the synchronous fluorescence spectrum of HSA when Δλ = 60 nm. The addition of the drug results in the strong fluorescence quenching of tryptophan with the maximum emission wavelength at 340 nm. It is reported that the maximum emission wavelength (Δλ) at 330–332 indicated that tryptophan residues are located in the nonpolar region, that is, they are buried in a hydrophobic cavity in HSA; Δλ at 350–352 nm shows that tryptophan residues are exposed to water, that is, the hydrophobic cavity in HSA is disagglomerated and the loosening of the HSA structure (Miller, 1979). Figure 10 with its λ max shift suggests that MDZ mainly bound to the hydrophobic cavity of HSA. This is in accordance with the result from binding mode, suggesting the presence of hydrophobic forces in this interaction.

**Binding mode**

Considering the dependence of binding constant on temperature, a thermodynamic process was considered to be responsible for this interaction. Therefore, the thermodynamic parameters dependent on temperatures were analyzed in order to further characterize the acting forces between MDZ and HSA. The acting forces between a small molecule and macromolecule mainly include hydrogen bonds, van der Waals forces, electrostatic forces and hydrophobic interaction forces. The thermodynamic parameters, enthalpy change (ΔH°), entropy change (ΔS°) and free energy change (ΔG°) are the main evidences to determine the binding mode. The thermodynamic parameters were evaluated using the following equations:

\[
\log K = -\frac{\Delta H^o}{2.303R} + \frac{\Delta S^o}{2.303R} \quad (7)
\]

\[
\Delta G^o = \Delta H^o - T\Delta S^o \quad (8)
\]

where \(K\) and \(R\) are the binding constant and gas constant, respectively. The results obtained are shown in Table 4 suggest that the process is entropically driven. The positive entropy change occurs because the water molecules that are arranged in an orderly fashion around the ligand and protein acquire a more random configuration as a result of hydrophobic interactions. Negative \(\Delta H^o\) value cannot be attributed to electrostatic interactions because for electrostatic interactions, \(\Delta H^o\) is almost zero (Ross & Subramanian, 1981; Aki & Yamamoto, 1989). Negative \(\Delta H^o\) value is observed whenever there is hydrogen bonding in the binding (Seedher et al., 1999). The negative \(\Delta H^o\) and positive \(\Delta S^o\) values in case of MDZ, therefore, showed that both hydrogen bonds and hydrophobic interactions play a role in the binding of MDZ to HSA (Seedher et al., 1999; Epps et al., 1999).

**Table 4**: Thermodynamic parameters for HSA–Midazolam system.

<table>
<thead>
<tr>
<th>T(K)</th>
<th>ΔG° (kJmol⁻¹)</th>
<th>ΔH° (kJmol⁻¹)</th>
<th>ΔS° (Jmol⁻¹K⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>298</td>
<td>-20.44±0.037</td>
<td>-8.34±0.04</td>
<td>40.58±0.031</td>
</tr>
<tr>
<td>306</td>
<td>-20.76±0.022</td>
<td>-8.34±0.04</td>
<td>40.58±0.031</td>
</tr>
<tr>
<td>310</td>
<td>-20.92±0.046</td>
<td>-8.34±0.04</td>
<td>40.58±0.031</td>
</tr>
</tbody>
</table>
Fluorescence resonance energy transfer

Figure 11. The overlap of the fluorescence spectrum of HSA λex = 295nm (a); and the absorbance spectrum of MDZ (b); ([HSA]/[MDZ] = 1:1).

The spectral studies suggested that HSA form complex with MDZ. The distance r between the protein residue (donor) and the bound MDZ (acceptor) could be determined using fluorescence resonance energy transfer (FRET). The distance between the donor and acceptor and extent of spectral overlaps determines the extent of energy transfer. Generally, FRET occurs whenever the emission spectrum of a fluorophore (donor) overlaps with the absorption spectrum of another molecule (acceptor). The overlap of the UV absorption spectrum of MDZ with the fluorescence emission spectra of HSA are shown in Figure 11. The distance between the donor and acceptor can be calculated according to Föster’s theory of dipole-dipole energy transfer (Forster & Sinanoglu, 1996). The efficiency of energy transfer, E, is related to R, the distance between the donor and the acceptor by

$$E = 1 - \frac{F}{F_0} = \frac{R_0^6}{R_0^6 + r^6}$$  (9)

where F and F0 are the fluorescence intensities of HSA in the presence and absence of MDZ, r the distance between acceptor and donor and R0 is the critical distance when the transfer efficiency is 50%.

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

where $k^2$ is the spatial orientation factor of the dipole, $\eta$ the refractive index of the medium, $\Phi$ the fluorescence quantum yield of the donor and $J$ is the overlap integral of the fluorescence emission spectrum of the donor and the absorption spectrum of the acceptor. $J$ is approximated by the given equation:

$$J = \sum \frac{F(\lambda) \varepsilon(\lambda) \lambda^4 \Delta \lambda}{\sum F(\lambda) \Delta \lambda}$$  (11)

where $F(\lambda)$ is the fluorescence intensity of the fluorescent donor of wavelength, $\lambda$ and $\varepsilon(\lambda)$ is the molar absorption coefficient of the acceptor at wavelength, $\lambda$. In the present case, $k^2 = 2/3$, $\eta = 1.336$ and $\Phi = 0.118$ for HSA (Hu et al., 2004). From Eqs. (9) to (11), we were able to calculate that $J = 1.09 \times 10^{-15}$ cm$^3$ L mol$^{-1}$, $R_0 = 1.69$ nm, $E = 0.13$ and $r = 2.31$ nm for HSA. The donor-to-acceptor distance, $r < 8$nm (Valeur & Brochon, 1999; Hu et al., 2005) is in accord with the Forster’s non-radiative energy transfer and reveals the presence of static type quenching mechanism (Gao et al., 2004).

Table 5: The binding constants $K$ (L mol$^{-1}$) between midazolam and HSA at 25°C in the presence of [Na$^+$] ions

<table>
<thead>
<tr>
<th>NaCl (mM)</th>
<th>$K$ (x 10$^4$)</th>
<th>R</th>
<th>$K'/K$</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>1.68</td>
<td>0.997</td>
<td>0.43</td>
</tr>
<tr>
<td>100</td>
<td>4.03</td>
<td>0.999</td>
<td>10.33</td>
</tr>
<tr>
<td>150</td>
<td>4.54</td>
<td>0.999</td>
<td>11.64</td>
</tr>
</tbody>
</table>

Effect of presence of salt

The binding of MDZ to HSA was also analyzed in the presence of increasing salt concentrations. The binding parameters are given in Table 5. The presence of salt causes a strong increase in binding constant (50-100mM). As the increase in ionic environment screened electrostatic interaction, resulting in lower binding constant. Its vice versa suggest the presence of other interactive forces rather than the electrostatic interaction. This also suggested that people with low ionic concentration have low binding affinity than those having high ionic plasma concentration, which implies that more pharmacologically active (unbound) drug is available with them. This augments a need of optimizing doses in case of diseases inducing salt or electrolytic imbalances. The increase in binding constant with the increased ionic strength excluded the possibility of electrostatic interactions, which is in accord with our thermodynamic data.
CONCLUSION

The interaction of MDZ with HSA has been investigated in vitro under simulated physiological conditions (pH 7.4, ionic strength 0.1M) using different optical techniques. Experimental results showed that the binding of MDZ to HSA induced a conformational change of HSA, which was further proved by the quantitative analysis data of CD spectrum. The data of fluorescence and synchronous fluorescence spectra indicated the changes in microenvironment of HSA induced by the binding of MDZ. The thermodynamic analysis also suggested that MDZ could bind HSA through the hydrophobic force and hydrogen bond between MDZ and HSA residue. According to competitive binding experiments and protein unfolding model, the binding site is located in the hydrophobic pocket of subdomain IIIA. The distance $r = 2.31$ nm between HSA and MDZ was obtained according to fluorescence resonance energy transfer. The binding study of drugs with HSA is of great importance in understanding chemico-biological interactions for drug design, pharmacy, pharmacology and biochemistry. This study is expected to provide important insight into the interactions of the physiologically important protein HSA with an important drug used in various therapeutic regimes. Information is also obtained about pharmacologically active free drug and the effect of environment on HSA structure, which may be correlated to its physiological activity.

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References


