PROTEIN BINDING INTERACTION STUDY OF OLMESARTAN MEDOXOMIL AND ITS METABOLITE OLMESARTAN BY FLUORESCENCE SPECTROSCOPY

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ABSTRACT

Objective: Determination of the number of binding sites and their properties such as affinity and specificity towards particular ligands is accomplished by obtaining the equilibrium constants between ligands and protein. In this concern fluorescence spectroscopy was employed to investigate the binding of an important AT-II receptor antagonist olmesartan medoxomil and its metabolite to bovine serum albumin (BSA) under the physiological conditions.

Method: The fluorescence quenching of BSA by olmesartan medoxomil and its metabolite olmesartan is a result of the formation of OLM-BSA and OL-BSA complexes, respectively. Fluorescence quenching constants were determined using the modified Stern–Volmer equation at different temperatures to provide a measure of the binding affinity between olmesartan medoxomil and its metabolite and BSA.

Results: The results showed that BSA fluorescence quenched by olmesartan medoxomil and its metabolite through static quenching mechanism with binding constant of 0.1094 and 0.4814M respectively, at 300K temperature for this system.

Conclusion: The fluorescence spectroscopy was found suitable to study the interaction of olmesartan medoxomil and olmesartan with bovine serum albumin and static quenching mechanism was observed.

Keywords: Protein binding, olmesartan medoxomil, olmesartan, fluorescence, bovine serum albumin.

INTRODUCTION

Olmesartan medoxomil (OLM) ([5-methyl-2-oxo-1,3 –dioxolen-4-yl)methoxy –4- (1-hydroxy-1-methyl ethyl)-2-propyl-1-(4-[2-[(tetraol-5-yl)-phenyl] phenyl methyl imidazo-5-carboxylate) is a prodrug and rapidly hydrolyzes in plasma during absorption to form its active metabolite olmesartan (OL) (Fig. 1) [1, 2]. It is a selective AT1 subtype angiotensin II receptor blocker [3, 4] that was recently approved by the US-FDA[5] to treat patients with hypertension.

Serum albumin, in humans, accounts for about 60% of the total serum content. It is the most abundant of the plasma proteins and serves as a transport carrier for numerous endogenous and exogenous compounds [6, 7]. The overall distribution, metabolism and efficacy of many drugs in the body are correlated with their affinities towards serum albumin.[8,9] Bovine serum albumin (BSA) has been one of the most extensively studied proteins, principally because of its structural homology with human serum albumin (HSA). BSA is a small protein with a single polypeptide chain containing 581 amino acids, which is cross-linked by 17 disulfide bonds [7]. Human and Bovine Serum Albumins (HSA and BSA, respectively) are approximately 76% homologous, the main difference between the two proteins is that in HSA there is only one tryptophan amino acid (Trp-214), whereas in BSA there are two tryptophan units (Trp-134 and Trp-212)[10, 11]. Thus, BSA, instead of HSA, was used in this study also because of its low cost and easy availability.

The drug-protein interaction may result in the formation of a stable protein-drug complex, which has important effect on the distribution, free concentration and the metabolism of drugs in the blood stream. Thus, the drug–albumin complex may be considered as model for gaining fundamental insights into drug–protein interactions.

Literature reveals that only study showing the esterase-like activity of human serum albumin (HSA) and the mechanism to hydrolyzes, and activates olmesartan medoxomil has been reported [12]. As per our knowledge there is no any study performing the binding interaction of OLM and OL with aid of fluorescence spectroscopy has been reported. In our previous work we had reported the forced degradation study of olmesartan medoxomil by using chromatographic method [13]. In the sequence of further research on OLM and its metabolite OL, we aimed to investigate the interaction of OLM and its metabolite OL with BSA by using spectrophotometer and their effect on protein binding constant by varying temperatures.

Fig. 1: Chemical structure of olmesartan medoxomil and its metabolite (free carboxylic acid form) olmesartan.

MATERIALS AND METHODS

Materials

Bovine serum albumin (BSA) of purity 99% and Tris Buffer were purchased from HiMidea laboratories and SD fine Chemicals, respectively. All reagents were used without further purification. All solutions were made up with purified water from a Milli-Q Millipore system.

Preparation of standard solutions

Olmesartan medoxomil and olmesartan were accurately weighed and transferred to a 100 ml volumetric flask, dissolved in 10 ml methanol and diluted up to mark with purified water to get 1mM stock solution of both drug. This 1mM stock prepared was transferred and again diluted with water to give working solutions 25-350 µM for OLM and OL.
Preparation of OLM and OL samples to analyze

Working solution (200 µl) of OLM and OL was transferred to micro tubes and volume was made up to 2 ml with BSA (10 µM) to give final concentration of 2.5-35 µM (51-59) respectively. The solutions were vortexed for 2 min to mix it properly. This final mixture of BSA-OLM and BSA-OL was stored in dark at 2-8 °C and was allowed to interact for 12 hr before analyzing them.

Fluorescence Measurement

Fluorescence measurements were performed at three different temperatures on a Shimadzu RF 1501 Spectrofluorophotometer with 1 cm quartz cells equipped with a 150W Xenon lamp. The fluorescence emission spectra were recorded in the wavelength of 300-500 nm, upon excitation at 295 nm, and both excitation and emission bandwidth was 10 nm. The excitation wavelength of 295 nm was chosen since it provides no excitation of tyrosine residues [14]. The concentration of BSA was kept fixed at 10×10⁻⁶ ML⁻¹ and the drug and metabolite concentration was varied from 0 to 35×10⁻⁶ ML⁻¹. Appropriate blank corresponding to the buffer and methanol was subtracted to correct the fluorescence. The results obtained were analyzed by using Stern-Volmer equation or modified Stern-Volmer equation to calculate the binding constant.

RESULTS AND DISCUSSION

This drug-protein interaction could be undertaken with the help of different and independent techniques such as spectroscopy, calorimetry, and potentiometry [15-17]. But, the fluorometric titration approach is the most widely used because of its intrinsic sensitivity and simplicity. Investigations of the interaction of BSA with OLM and OL have been conducted in this study at different temperatures (291, 300 and 309 K) with help of fluorescence spectroscopy.

Fluorescence Spectra

The fluorescence emission spectra of 10×10⁻⁶ ML⁻¹ BSA quenched by OLM and OL at different concentrations in pH 7.4 phosphate buffer solution. Upon addition of OLM and OL (35×10⁻⁶ ML⁻¹), the fluorescence intensity of BSA decreased nearly by 31.9 and 51.0 %, respectively at 300K. Metabolite olmesartan (OL) quench the serum albumin fluorescence stronger than olmesartan medoxomil (OLM) in the same condition. These data indicate that OLM and OL could interact significantly with BSA and quench its intrinsic fluorescence. The interactions between the olmesartan and metabolite studied and serum albumin might be one of reasons leading to their poor bioavailability.

Fluorescence quenching

Fluorescence quenching refers to any process which decreases the fluorescence intensity of a sample. The fluorescence quenching data are usually analyzed by the equation no. (1) Stern-Volmer[18]

\[
\frac{F_0}{F} = 1 + K_{SV} [Q] = 1 + K_a \tau_0 [Q] \quad (1)
\]

\(F\) and \(F_0\) -steady-state fluorescence intensities in the absence and the presence of quencher, \(\Delta F\) - The difference in fluorescence in the absence and presence of the quencher at concentration-[Q], \(K_a\) -Stern-Volmer quenching constant. The \(\tau_0\) value for BSA was found to be 10’s [19].

The different mechanisms of quenching are usually classified as either dynamic quenching or static quenching. Dynamic and static quenching can be distinguished by their differing dependence on temperature and viscosity. Dynamic quenching depends upon diffusion. Since higher temperatures result in larger diffusion coefficients, the bimolecular quenching constants are expected to increase with increasing temperature. In contrast, increased temperature is likely to result in decreased stability of complexes, and thus lower values of the static quenching constants.

In order to confirm the quenching mechanism, the procedure of the fluorescence quenching was first assumed to be a dynamic quenching process. Figure 3 displays the Stern-Volmer plots of the quenching of BSA fluorescence by OLM and OL at three different temperatures. The results in Table 1 and 2 show that the Stern-Volmer constant \(K_a\) is inversely correlated with temperature, which indicates that the probable quenching mechanism of OLM, OL-BSA binding reaction is initiated by compound formation rather than by dynamic collision. According to the literature [20], for dynamic quenching, the maximum scatter collision quenching constant of various quenchers with biopolymers is 2.0×10^9 Lmol⁻¹s⁻¹. Obviously, the rate constant of protein quenching procedure initiated by OLM and OL is noticed to be greater than the \(K_q\) of the scatter procedure. So the quenching process between these drugs and serum albumin was not dynamic quenching but static quenching. Therefore, the quenching data were analyzed according to the modified Stern-Volmer equation: [18]

\[
\frac{F_0 - F}{F} = \frac{1}{f_a K_a} [Q] + \frac{1}{f_a} \quad (2)
\]

In the present condition, \(\Delta F\) is the difference in fluorescence in the absence and presence of the quencher at concentration-[Q], \(f_a\) is the fraction of accessible fluorescence, and \(K_a\) is the effective quenching constant for the accessible fluorophores, which are analogous to associative binding constants for the quencher-acceptor system. The dependence of \(F_0/F\) on the reciprocal value of the quencher concentration \([Q]\) is linear with the slope equaling to the value of \((f_a K_a)^{-1}\). The value \(f_a\) is fixed on the ordinate. The constant \(K_a\) is a quotient of the ordinate \(f_a^{-1}\) and the slope \((f_a K_a)^{-1}\). The corresponding results at different temperatures are shown in Table 1 and 2. The decreasing trend of \(K_a\) with increasing temperature was in accordance with \(K_a\)'s dependence on temperature as mentioned above. It shows that the binding constant between OLM, OL and BSA is considerable and the effect of temperature is small.

![Fig. 2: 1) Stern-Volmer plot and 2) Modified Stern-Volmer plot for the quenching of BSA fluorescence by Olmesartan Medoxomil.](image-url)
Binding constants and number of binding site

For static quenching, the relationship between fluorescence quenching intensity and the concentration of quenchers can be described by Equation No. 3 [21]: where $K_a$ is the binding constant, and $n$ is the number of binding sites. $\log \left( \frac{(F_0 - F)}{F} \right)$ was plotted against $\log [Q]$ and the values of $K_a$ and $n$ were obtained from intercept and slope, respectively, as shown in Table 3. It is evident from results that the values of $K_a$ decrease with increasing temperature to reveal the drug-BSA complex losing its stability with increasing temperature and it is also in line with the results of $K_{SV}$ and $K_a$. The values of $n$ also change with the temperature change, and this may be related to the instability of OLM and OL. The $K_a$ values obtained here indicate the good interaction between OLM, OL and BSA. The values of $n$, approximately equal to 2 and 1 indicate the existence of just two and one high affinity binding site in BSA for OLM and OL, respectively.

$$\log \left( \frac{(F_0 - F)}{F} \right) = \log K_a + n \log [Q]$$  \hspace{1cm} (3)

Table 3: Binding constants ($K_a$) and binding sites ($n$) at various temperatures

| Temperature (K) | $K_a \times 10^4$ (LM$^{-1}$) | $K_a \times 10^13$ (LM$^{-1}$) | $K_a \times 10^6$ (LM$^{-1}$) | $\Delta H$ (kJ M$^{-1}$) | $\Delta S$ (J M$^{-1}$K$^{-1}$) | $\Delta G$ (kJ M$^{-1}$)
<table>
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<tbody>
<tr>
<td>291</td>
<td>2.817</td>
<td>1.747</td>
<td>0.986</td>
<td>1.562</td>
<td>1.059</td>
<td>0.961</td>
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<tr>
<td>300</td>
<td>2.755</td>
<td>1.563</td>
<td>0.990</td>
<td>1.486</td>
<td>0.928</td>
<td>0.990</td>
</tr>
<tr>
<td>309</td>
<td>2.133</td>
<td>1.094</td>
<td>0.982</td>
<td>1.391</td>
<td>0.787</td>
<td>0.941</td>
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The determination of the force acting between OLM, OL and BSA

Analysis of Stern–Volmer plots in this system yields equilibrium expressions for static quenching, $K_a$, which are analogous to associative binding constants for the quencher acceptor system [22]. The interaction forces between drugs and biomolecules may include electrostatic interactions, multiple hydrogen bonds, van der Waals interactions, hydrophobic and steric contacts within the antibody binding site, etc [23].

In order to elucidate the interaction between OLM, OL and BSA, the thermodynamic parameters were calculated from the van’t Hoff plots. If the enthalpy change ($\Delta H$) does not vary significantly in the temperature range studied, both the enthalpy change ($\Delta H$) and entropy change ($\Delta S$) can be evaluated from the van’t Hoff equation [24]

$$\ln K_a = -\frac{\Delta H}{RT} + \frac{\Delta S}{R}$$  \hspace{1cm} (4)

Where $K$ is analogous to the effective quenching constants $K_a$ at the corresponding temperature and $R$ is the gas constant. The temperatures used were 291, 300 and 309 K. The enthalpy change ($\Delta H$) is calculated from the slope of the van’t Hoff relationship. The free energy change ($\Delta G$) is then estimated from the following relationship [25]

$$\Delta G = \Delta H - T \Delta S$$  \hspace{1cm} (5)

The negative enthalpy ($\Delta H$) and positive entropy ($\Delta S$) values of the interaction of OLM, OL and BSA indicate that the electrostatic interactions played a major role in the binding reaction [25].
According to the fluorescence quenching phenomena; we concluded that the thermodynamic parameters calculated from Stern-Volmer quenching constants were reasonable for our studied system.

\[ y = 2260.5x + 8.4713 \quad R = 0.9938 \]
\[ y = 2260.5x + 8.4713 \quad R = 0.9938 \]
\[ y = 3734.5x + 1.9684 \quad R = 0.9489 \]

**Fig. 4: Van’t Hoff plot of OLM and OL- BSA system, pH 7.40**

**CONCLUSIONS**

This study gives information regarding the interaction of BSA with olmesartan medoxomil and its metabolite, using fluorescence spectroscopy. The protein binding constants were determined for olmesartan medoxomil and its metabolite and it evidenced the binding of the drug to BSA. The results showed that BSA fluorescence quenched by olmesartan medoxomil and its metabolite through static quenching mechanism.

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**REFERENCES**