Experimental, computational and chemometrics studies of BSA-vitamin B6 interaction by UV–Vis, FT-IR, fluorescence spectroscopy, molecular dynamics simulation and hard-soft modeling methods

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Abstract

The interaction of pyridoxine (Vitamin B6) with bovine serum albumin (BSA) is investigated under pseudo-physiological conditions by UV–Vis, fluorescence and FTIR spectroscopy. The intrinsic fluorescence of BSA was quenched by VB6, which was rationalized in terms of the static quenching mechanism. According to fluorescence quenching calculations, the bimolecular quenching constant (kq), dynamic quenching (kSV) and static quenching (kSd) at 310 K were obtained. The efficiency of energy transfer and the distance between the donor (BSA) and the acceptor (VB6) were calculated by Foster’s non-radiative energy transfer theory and were equal to 41.1% and 2.11 nm.

The collected UV–Vis and fluorescence spectra were combined into a row-and-column-wise augmented matrix and resolved by multivariate curve resolution-alternating least squares (MCR-ALS). MCR-ALS helped to estimate the stoichiometry of interactions, concentration profiles and pure spectra for three species (BSA, VB6 and VB6-BSA complex) existed in the interaction procedure. Based on the MCR-ALS results, using mass balance equations, a model was developed and binding constant of complex was calculated using non-linear least squares curve fitting. FT-IR spectra showed that the conformation of proteins was altered in presence of VB6. Finally, the combined docking and molecular dynamics (MD) simulations were used to estimate the binding affinity of VB6 to BSA. Five-nanosecond MD simulations were performed on bovine serum albumin (BSA) to study the conformational features of its ligand binding site. From MD results, eleven BSA snapshots were extracted, at every 0.5 ns, to explore the binding affinity (GOLD score) of VB6 using a docking procedure. MD simulations indicated that there is a considerable flexibility in the structure of protein that affected ligand recognition. Structural analyses and docking simulations indicated that VB6 binds to site I and GOLD score values depend on the conformations of both BSA and ligand. Molecular modeling results showed that VB6–BSA complex formed not only on the basis of electrostatic forces, but also on the basis of π–π stacking and hydrogen bond. There was an excellent agreement between the experimental and computational results. The results presented in this paper, will offer a reference for detailed and systematic studies on the biological effects and action mechanism of small molecules with proteins.

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1. Introduction

Nowadays, drug–serum albumin (SA) interactions play an important role in pharmacokinetics and pharmacodynamics. Solubility, distribution, half-life and metabolism of many biologically active compounds in the body have been correlated with their affinities toward SA [1–3]. Because of clinical and pharmaceutical importance [4], the investigations of interaction of SA with small molecules such as vitamins, hormones, natural or synthetic drugs have attracted the attention of the biologists, chemists, pharmacists and therapists [5,6]. The most important property of SA is that they serve as transporters for a variety of compounds such as fatty acids, hormones and drugs. Bovine serum albumin (BSA) has been one of the most extensively studied of this group of proteins, particularly because of its structural homology with human serum albumin (HSA). Like other serum albumins, BSA possesses a wide range of physiological functions associated with the binding, transport and distribution of biologically active compounds [7–9].
Vitamin B6 is an essential co-factor in numerous enzymatic reactions involved primarily in amino acid metabolism. All living organisms require vitamin B6 and they must either synthesize it or, like humans, derive it from nutrients [10]. It helps body to convert food into fuel, metabolize fats and proteins, maintain proper functioning of nerves, and produce red blood cells. It is a medicine for treating acne, sunburn, roughness and itch of the skin [11–14]. 2-Methyl-3-hydroxy-4,5-bis (hydroxymethyl) pyridine hydrochloride (C₈H₁₂O₃NCl) which is known as, Pyridoxine hydrochloride, is the alcohol form of vitamin B6 (Fig. 1). The studies on the interaction of VB6 and BSA in scientific literature are limited to binding constant and thermodynamic parameters [15] and the detailed and systematic investigations are still to be reported.

In recent years, many research have been reported about protein-ligand interactions by spectroscopic [16,17], electrochemistry [18,19], HPLC [20], NMR spectroscopy [21], X-ray diffraction [22], capillary electrophoresis [23], density functional theory (DFT) calculations [24], isothermal titration calorimetric (ITC) [25], scanning electron microscope (SEM) and transmission electron microscopy (TEM) analyses [26]. Among these various methods for studying the interactions of ligands with proteins in solution, fluorescence detection because of its sensitivity, selectivity, convenience and relatively low costs is a powerful method to provide more information [16]. Also, UV–Vis spectrophotometry is a simple method and applicable to exploration of the structural change and the complex formation [27]. Spectroscopic FTIR technique could be useful for evaluation of protein secondary structure [5]. The features of these techniques are crucial from the point of view of quality control in pharmaceutical industry.

A good approach for studying the interactions of ligands with proteins is modeling the multivariate data with chemometric tools [28,29]. Chemometrics modeling methods in this context can be divided to: soft-modeling (model free) and hard-modeling (model-based) methods [30–32]. The goal of model-based analysis methods is to facilitate the ’translation’ from original data to a set of values for the parameters that quantitatively describe the measurement, within the limits of experimental noise. Information provided by soft modeling methods can be very useful in supplying preliminary information about the system under investigation and ultimately could guide the researcher toward selection of the correct model [28]. Soft-modeling multivariate curve resolution (MCR) techniques can be used to detect the number of independent chemical species, their absorption spectra as well as their concentration profiles [33].

Molecular docking is a key tool in structural molecular biology and computer-assisted drug design [34–37]. The goal of ligand-protein docking is to predict the predominant binding mode(s) of a ligand with a protein of known three-dimensional structure [38], but this procedure does not take into consideration the important role that the whole protein movement plays in the ligand recognition process. Molecular dynamics (MD) simulations can treat both ligand and protein in a flexible way, allowing for an induced fit of the receptor-binding site around the newly introduced ligand [39]. In addition, effect of explicit water molecules can be studied directly, and very accurate binding free energies can be obtained [40]. Also, MD can mimic physiological conditions including ion content, temperature, and pH. Therefore, fast and inexpensive docking protocols can be combined with accurate but more costly MD techniques to predict more reliable protein–ligand complexes [41]. The strength of this combination lies in their complementary strengths and weaknesses.

This work reports a detailed study on the interactions between VB6 and BSA for the first time, which provide some useful clues for clinical and pharmaceutical application of these biologically important molecules. The interaction between VB6 and BSA was investigated in vitro using fluorescence, UV–Vis and FTIR techniques under physiological conditions. Binding parameters, such as quenching constant, number of binding sites, and binding distance were obtained from fluorescence data. Effects of VB6 on BSA secondary structure changes were studied using FTIR spectra. MCR-ALS method helped to further understand the formation of complex and extract the equilibrium profiles of reacting species. The non-linear curve fitting was used for fitting the predefined complexation model to spectral mole ratio data in order to calculate the binding constant. Moreover, MD and molecular docking techniques were combined to evaluate the binding of VB6 on 11 BSA snapshots. Combination of these techniques in a protocol facilitated evaluation of contribution of BSA conformational changes under physiological conditions in binding to VB6 and calculation of accurate energies.

2. Experimental

2.1. Chemicals and solutions

Bovine serum albumin fraction V (free fatty acid) and Pyridoxine hydrochloride (VB6) were purchased from Sigma Chemical Co. All other reagents were of analytical grade, and doubly distilled water was used in all experiments. Weekly prepared stock solutions of BSA, and VB6 were prepared by dissolving proper amount of their solid powder in an aqueous solutions of Tris–HCl buffer (0.05 mol L⁻¹, pH = 7.4). The stock solutions of BSA was kept in dark at 0–4°C.

2.2. Instruments and software

FP–6500 Spectrofluorimeter (JASCO, Japan) equipped with a 1.0 cm quartz cell and a thermostat bath was used for recording the fluorescence spectra. Agilent-8453 UV–Vis diode-array spectrophotometer with 1.0 nm spectral band-pass equipped with a thermostat bath was used for recording the absorption spectra. Agilent UV–Vis Chem-Station software for data acquisition was used throughout. Infrared spectra were recorded on a Nicolet FTIR spectrometer (Magna-IR 550) equipped with a liquid-nitrogen-cooled HgCdTe (MCT) detector and a KBr beam splitter, using ZnSe windows. The FTIR spectroscopic measurements analysis was performed using FTIR software (OMNIC). AMTAST pH-meter equipped with combined Ag/AgCl electrode was used. MCR-ALS was implemented using the MCR-ALS 2.0 MATLAB toolbox [42]. MATLAB (Version 7.12.0) was used for pretreatment and analysis of the data. The Stability Quotients from Absorbance Data (SQUAL) program written in FORTRAN by Leggett et al. [31,43,44], was used for fitting the complexation model. The chemical structure of the VB6 was sketched in Gauss View 5 and optimization steps were carried out with b3lyp/6-311g(d,p) basis set in Gaussian 09W [45] molecular modeling package. The crystal structure of BSA (4JK4) [46] was taken from RCSB protein databank [47]. GROMACS 4.5.3 simulation package [48] using the Amber99sb-ildn force field [49] was applied for classical MD simulations. Discovery Studio 2.5 and GOLD program were used to dock V-B6 into the protein [50].

![Fig. 1. Chemical structure of VB6.](image-url)
3. Methods

3.1. Titration procedure

Fluorometric and UV–Vis titrations were carried out in Tris–HCl buffer (0.05 mol L\(^{-1}\)) of pH 7.4 at 310 K. Two separate experiments were performed by the mole-ratio method. In one case the VB6 solution was used as a titrant (titration 1) and in other case, BSA solution was used as titrant (titration 2). Titration 1 was done as follows: A 3.0 mL portion of BSA solution (8.0 × 10\(^{-6}\) mol L\(^{-1}\)) was transferred accurately to the quartz cell and then titrated by successive additions of VB6 solution (1.0 × 10\(^{-3}\) mol L\(^{-1}\)) using a 5.0 μL micro-syringe to attain a series of total final concentrations for VB6 (0.0–4.5 × 10\(^{-3}\) mol L\(^{-1}\)) with an interval of 1.7 × 10\(^{-6}\) mol L\(^{-1}\). Titration 2 was performed as follows: A 3.0 mL portion of VB6 solution (1.6 × 10\(^{-3}\) mol L\(^{-1}\)) was transferred accurately to the quartz cell, and different amounts of BSA (range: 0.0–2.3 × 10\(^{-3}\) mol L\(^{-1}\) with an interval of 8.3 × 10\(^{-7}\) mol L\(^{-1}\)) were added to the solution. The total added volume of BSA or VB6 in titration 1 and 2 were 0.15 mL. In both cases after addition of titrant in each step, the solutions were mixed thoroughly and then absorption spectra were recorded in the 240–360 nm range and fluorescence spectra were recorded at 305–530 nm range by 1 nm intervals. The excitation wavelength was 295 nm and the width of emission slits were adjusted to 5 nm. In total, 2 absorption spectra matrices (D\(_{\text{BA}}\) and D\(_{\text{VB6}}\)) and two fluorescence spectra matrices (D\(_{\text{UV}}\) and D\(_{\text{VB6}}\)) were recorded. D\(_{\text{UV}}\) is the absorption spectra matrix of titration 1 and D\(_{\text{VB6}}\) is its fluorescence spectra matrix. D\(_{\text{VB6}}\) is the absorption spectra matrix of titration 2 and D\(_{\text{VB6}}\) is fluorescence spectra matrix. In the fluorescence experiments, fluorescence intensities were corrected for absorption of the exciting light and reabsorption of the emitted light to decrease the inner filter effect [51,52].

3.2. Fluorescence quenching studies

Fluorescence quenching may result from various processes, such as molecular collision, ground-state complex formation, excited-state reaction, molecular rearrangement, or energy transfer. The mechanisms of these processes are usually classified through both dynamic and static quenching. Dynamic quenching generally depends on molecular collision, but static quenching is usually associated with complex formation between the protein and the quencher [53,54]. Fluorometric measurements from titration 1 were used for quenching studies. The intensities of emission maxima were used for calculation of dynamic and static quenching constant, the fraction of fluorophore accessible to the quencher, binding constant, and occupation of binding sites. The Stern-Volmer and Lineweaver-Burk equations [55] can be used to describe the fluorescence quenching process. These equations are commonly used in describing dynamic quenching and static quenching, respectively.

The Stern-Volmer equation is [55]:

$$\frac{F_0}{F} = 1 + K_{SV}[Q] = 1 + k_{q}t_{0}[Q]$$

(1)

where \(F\) and \(F_0\) are the fluorescence intensity in the presence and absence of quencher, respectively, \(k_{q}\) is the bimolecular quenching rate constant (L mol\(^{-1}\) s\(^{-1}\)), \(K_{SV}\) is the Stern–Volmer constant with the unit of L mol\(^{-1}\), \([Q]\) is the concentration of quencher, and \(t_{0}\) is the fluorescence lifetime of molecule in the absence of any quencher. The fluorescence lifetime of the biopolymer (protein) and the maximum scatter collision quenching constant of various quenchers with the biopolymers are 10\(^{-8}\) s and 2 × 10\(^{16}\) L mol\(^{-1}\) s\(^{-1}\), respectively [56]. \(K_{SV}\) can be obtained by plotting \(F_0/F\) versus \([Q]\).

The static quenching constant and fraction of fluorophore accessible to the quencher can be determined by using the Lineweaver-Burk equation (Eq. (2)) [55]:

$$\frac{F_0 - F}{F} = \frac{1}{F_0} + \frac{1}{F_{0}K_{SB}[Q]}$$

(2)

where \(K_{SB}\) is the static quenching constant (L mol\(^{-1}\)), which describes the binding efficiency of micromolecules to biological macromolecules at ground state and \(f_{a}\) is the fraction of fluorophore accessible to the quencher. The plot of \(\frac{F_0}{F_0 - F}\) versus \(1/[Q]\) is linear and \(K_{SB}\) and \(f_{a}\) can be estimated from the site binding model which assumes the existence of \(n\) independent binding sites for a quencher (Q) in a biomolecule and is expressed using Hill equation [57]:

$$\log \left( \frac{F_0 - F}{F} \right) = \log K_0 + n \log [Q]$$

(3)

The plot of \(\log \left( \frac{F_0 - F}{F} \right)\) versus \(\log [Q]\) is linear and yields \(K_0\) as the intercept, and \(n\) as the slope.

3.2.1. Fluorescence resonance energy transfer

Fluorescence resonance energy transfer (FRET) is a distance-dependent interaction between the different electronic excited states of molecules. In this phenomenon, excitation energy is transferred from one donor (BSA) to acceptor (VB6) through direct electro-dynamic interaction, without emission of a photon from the former molecular system [58]. Energy transfer may occur under the following conditions: when (a) the donor can produce fluorescence; (b) the absorption spectrum of the acceptor overlaps enough with the donor’s fluorescence emission spectrum and (c) the distance between donor and acceptor is less than 8 nm [59]. Non-radiative energy transfer can be explained and determined by Foster’s energy transfer theory and this theory is often used to calculate the distance between amino acid residues on proteins and drugs in binding sites [60]. According to Foster’s non-radiative energy transfer theory, the energy transfer efficiency (E) can be defined by the following equations:

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

(4)

where \(F_0\) and \(F\) are the fluorescence intensities of the BSA in the absence and presence of VB6, \(r\) is the distance between acceptor (VB6) and donor (BSA), and \(R_0\) is the critical distance when the transfer efficiency is 50%. The value of \(R_0\) is calculated using the following equation:

$$R_0^6 = 8.79 \times 10^{-25} \text{ K}^2 \text{ n}^{-4} \phi J$$

(5)

where \(K^2\) is the orientation factor related to the geometry of the donor–acceptor dipole and \(K^2 = 2/3\) for random orientation as in fluid solution; \(n\) is the average refractive index of medium in the wavelength range where spectral overlap is significant; \(\phi\) is the fluorescence quantum yield of the donor; and \(J\) is the spectral overlap between the emission spectrum of donor and the absorption spectrum of acceptor, given by:

$$J = \int_\lambda \frac{F(\lambda)\varepsilon(\lambda)\lambda^4d(\lambda)}{\int_\lambda F(\lambda)d\lambda}$$

(6)

where \(F(\lambda)\) is the fluorescence intensity of the donor at wavelength \(\lambda\), and \(\varepsilon(\lambda)\) is the molar absorption coefficient of the acceptor at wavelength \(\lambda\).
3.3. FTIR spectra experiments

FTIR spectroscopic measurements were carried out in the Tris–HCl buffer (0.05 mol L\(^{-1}\)) of pH 7.4. The solutions of VB6 with different concentrations (0.03, 0.50 and 1.00 mM) were added dropwise to the BSA solution (0.50 mM) with constant stirring to ensure the formation of homogeneous solution. The final concentrations of VB6 in the complex mixtures were 0.015, 0.250, and 0.500 mM with a final protein concentration of 0.25 mM. Spectra were recorded after 30 min of incubation of BSA with drug solution at 310 K, using hydrated films in the range 4000–600 cm\(^{-1}\) with a nominal resolution of 2 cm\(^{-1}\) and 100 scans. The difference spectra [(protein solution + ligand solutions) – (protein solution)] were generated using the water combination mode around 2300 cm\(^{-1}\), as the standard [61]. When producing difference spectra, this band was adjusted to the baseline level, in order to normalize difference spectra.

Analysis of the secondary structure of BSA and complexes was carried out according to the previously reported method [62]. The protein secondary structure is determined from the shape of the amide I band, located around 1660–1650 cm\(^{-1}\). The FTIR spectra were smoothed, and their baselines were corrected automatically using routine FTIR software on the equipment. Thus, the root-mean square (rms) noise of every spectrum was calculated.

The major peaks for BSA and the complexes were resolved by means of the second derivative in the spectral region 1700–1600 cm\(^{-1}\). The above spectral region was deconvoluted by the curve-fitting method with the Levenberg–Marquadt algorithm and peaks corresponding to \(\alpha\)-helix (1658–1651 cm\(^{-1}\)), \(\beta\)-sheet (1640–1610 cm\(^{-1}\)), random coil (1648–1641 cm\(^{-1}\)), and \(\beta\)-antiparallel (1692–1680 cm\(^{-1}\)) were adjusted and the area was measured with the Gaussian function. The area of all component bands assigned to a given conformation were then summed up and divided by the total area [53,63].

3.4. Soft-hard modeling

MCR-ALS is a popular soft-modeling chemometric method which is applicable for resolution of multiple component responses in unknown unresolved mixtures [64]. MCR methods are focused on extracting relevant responses through a bilinear model decomposition of experimental data matrix \(D\) into product of matrices \(C\) and \(S\) that contain pure components profiles in a mixture system. This bilinear model can be written as:

\[
D = CS^T + E
\]  

(7)

where \(D\) \(\times r \times c\) is the matrix of experimental data with \(r\) titration steps and \(c\) detection channels (wavelengths that are used for monitoring the titration). The matrix, \(C\) \(\times r \times n\), describes the concentration profiles of \(n\) species involved in the given titration. The matrix, \(S\) \(\times n \times c\), is the pure signal profiles of involved species in titration. \(E\) \(\times r \times c\) is the matrix of residuals not explained by bilinear modeling. Simultaneously analysis of data matrices obtained from UV–Vis and fluorescence from titration 1 and titration 2 were done by column and row wise augmentation (data fusion) according to Eq. (8) [65–67].

\[
\begin{bmatrix}
D_{BSA}^{UV} & D_{F}^{BSA} \\
D_{VB6}^{UV} & D_{F}^{VB6}
\end{bmatrix}
= 
\begin{bmatrix}
C_{BSA} & S_{BSA}^T \\
E_{BSA}^{UV} & E_{F}^{BSA} \\
C_{VB6} & S_{VB6}^T \\
E_{VB6}^{UV} & E_{F}^{VB6}
\end{bmatrix}
\]

(8)

According to Eq. (8), fluorescence spectra of two titration methods were column-wise augmented, i.e., their wavelengths were kept constant. The same procedure was followed for UV–Vis spectra. Then, augmented fluorescence and UV–Vis matrices were row-wise augmented with each other [68].

3.5. Molecular dynamic simulations

MD simulations were performed with GROMACS 4.5.3 simulation package [48] using Amber99sb-ildn force field [49]. Starting BSA coordinates were taken from 2.65 Å resolution crystal structure (PDB ID: 4JK4) [46]. The B chain, 3,5-diodosalicylic acid (ligand), and water molecules of the crystal structure were removed. There was one missing residue in the crystal structure of BSA, and hence, its pdb file was corrected by MOE 2009.10 (Molecular Operating Environment (MOE) from chemical computing group Inc., Montreal, QC, Canada, 2013). The protein structure was solvated in a cubic box of size 1309.02 nm\(^{-3}\) with 40,577 simple point charge (SPC) water molecules [74]. The minimum distance between the macromolecule and edge of the box was set to 1 nm. In order to neutralize the charge of the system, 17 Na\(^+\) ions were added. An energy minimization step was performed for full system without constraints using steepest descent integrator for 50,000 steps, until a tolerance of 1.0 kJ mol\(^{-1}\). This was followed by a short (100 ps) position restrained equilibration simulation at...
310 K. Finally, MD simulations were performed for a period of 5 ns with a time step of 2 fs. The LINCS algorithm (Linear Constraint Solver), which is three to four times faster than SHAKE algorithm, was used to constrain the length of covalent bonds [75]. The particle-mesh Ewald (PME) summation technique was used to compute long-ranged electrostatic interactions [76]. The Coulomb and van der Waal’s cut-offs were set to 1.0 and 1.4 nm, respectively. The constant temperature and pressure (NPT) ensemble at 310 K with periodic boundary conditions was used to perform simulation. Initial velocities were assigned from Maxwell distribution at 310 K. Berendsen thermostat was applied to keep the temperature constant. The MD simulation output over 5 ns provided several BSA structures that were sampled every 0.5 ns to study the ligand recognition energetic and binding modes of VB6.

### 3.6. Molecular docking

Molecular docking simulations were carried out with Discovery Studio 2.5. The GOLD program was used to dock VB6 into the protein [50]. GOLD is a genetic algorithm for docking of flexible ligands into protein binding sites that accounts for the side chain flexibility of the target protein. From the MD simulation output over 5 ns, 11 BSA snapshots were extracted every 0.5 ns, and applied to explore the binding affinity of VB6 using GOLD program. For the preparation step of the conformational features of BSA, the CHARMM force field was applied, and the pH of protein was adjusted to almost neutral, 7.4, using the protein preparation protocol. The chemical structure of the VB6 was constructed by Gauss View 5 and optimization steps were carried out in Gaussian 09W (www.gaussian.com) molecular modeling package. The resulting structure was imported into Discovery Studio, and typed with CHARMM force field and partial charges were calculated using the Momany-Rone option [77]. Charges were then minimized with Smart Minimizer, which performs 1000 steps of steepest descent with a RMS gradient tolerance of 3, followed by conjugate gradient minimization. Other parameters were set as the default protocol settings. The protein active site was defined as a sphere with a radius of 35 Å around the bounded ligand to allow free motions to ligand atoms and side-chains of residues of receptor within 35 Å radius from center of the binding site. Then bounded ligand was removed from binding site. To validate docking reliability, root-mean-square distance (RMSD) value was calculated between bounded ligand and redocked ligand. The RMSD was 1.725 Å, which confirmed that GOLD method results were reliable in this study. The Gold score was used to evaluate the affinity of VB6 ligand toward the candidate pocket of BSA. The VB6 with the largest GOLD score value was selected.
4. Results and discussion

4.1. Interpretation of UV–Vis and fluorescence spectra

Fig. 2 shows the fluorescence and spectrophotometric spectra obtained from titration 1 and 2 (Section 3.1). Fig. 2A and C displays the UV–Vis spectra obtained from titrations 1 and 2, respectively. Fig. 2B and D are fluorescence emission spectra of these titrations. In Fig. 2A, with addition of VB6 into BSA solution, the hypochromism is observed and BSA absorbance at 278 nm increases gradually, and two new intensive absorption peaks at 259 and 324 nm appear. In Fig. 2C, VB6 shows two characteristic absorption bands at 252 and 324 nm that with addition of BSA into VB6 solution, the absorption at 252 nm is increased and shifts toward 259 nm, while absorbance peak at 324 shows no major spectral shifting. These alterations in UV–Vis spectra are probably related to binding of VB6 to BSA. Fluorescence of BSA mainly originates from tryptophan residue, while tyrosine and phenylalanine residues also take part [78]. Tryptophan fluorescence may change when other molecules interact with BSA depending on impact of such interaction on the fluorophore-quencher complex [79]. In Fig. 2B, BSA exhibits a strong emission at 345 nm and its fluorescence intensity is specifically quenched with successive addition of VB6 to BSA solution. Also, fluorescence maxima have shifted from 345 nm to 356 nm, because after addition of VB6, the FRET mechanism, so dynamic collision effects, if any, should have been negligible. Fig. 2A in supplementary data, indicating that BSA has only one site for binding of VB6 to BSA molecule.

4.2. Fluorescence quenching study

Table 1 shows the results of fluorescence quenching study. The values in the parentheses show the standard deviation of measurements. Based on Stern-Volmer equation (Eq. (1)), KSV value is obtained from slope of the plot of the F0/F versus [VB6] (Fig. S1 in supplementary data). The calculated KSV value is larger than the limiting diffusion rate constant of the biopolymers, (i.e. $2.0 \times 10^{10}$ L mol$^{-1}$ s$^{-1}$) in aqueous medium. It suggests that fluorescence quenching is caused by an interaction between BSA and VB6, rather than dynamic collision. In other words, the BSA fluorescence quenching that was caused by VB6 resulted from specific complex formation, so dynamic collision effects, if any, should have been negligible. Fig. 2B in the supplementary data, shows that plot of KSV versus 1/(ligand concentration) is linear. The KSV and fQ can be estimated from the intercept and slope of this plot, respectively. The KSV value is far exceeding the Stern–Volmer constant (Table 1). Also, value of fQ at 310 K was 0.82 ± 0.07 indicating that VB6 could easily bind to the hydrophobic pocket of site I of subdomain IIA and that ~80% of the total fluorescence of Trp 213 is accessible to the quencher. As mentioned, it has been proven that quenching is not initiated by dynamic collision but originates from formation of a complex [81]. For static quenching interaction, the value of $K_0$ is essential to our understanding of drug distribution in plasma, since weak binding can lead to a short lifetime or poor distribution, while strong binding can decrease concentrations of free drug in plasma. As shown in Fig. S3 of supplementary data, experimental values of log($F_0/F$) plotted against log($Q$) based on Eq. (3) gives a straight line. The quantity of $K_0$ value indicates moderate binding between VB6 and BSA, compared to strong ligand–protein complexes, with binding constants ranging from $10^6$ to $10^8$ L mol$^{-1}$ [82,83]. The binding site number n is about 1 (Fig. S3 in the supplementary data), indicating that BSA has only one site for binding of VB6 to BSA molecule.

4.3. Fluorescence resonance energy transfer

Overlap of the absorption spectrum of VB6 with the fluorescence emission spectrum of BSA is shown in Fig. 3A. The value of J could be evaluated by integrating the overlap spectra at $\lambda = 305–455$ nm. According to Eqs. (467) and using $n = 1.36, \varphi = 0.15$ for BSA [84], the following parameters were obtained: $E = 0.411, J = 2.41 \times 10^{-15}$ cm$^{-1}$ L mol$^{-1}$, $R_0 = 1.989$ nm and $r = 2.112$ nm for the VB6-BSA system. Fig. 3B shows that energy transfer efficiency is dependent on the distance between amino acid residues on proteins and VB6 in the binding sites and energy transfer efficiency is 50% when $R_0 = 1.989$ nm. Since the average distance was $r < 8$ nm and 0.5 $R_0 < r < 1.5R_0$ [85], the energytrans-
fer from BSA to VB6 occurred with high probability. Furthermore, larger value of \( r \) in comparison with \( R_0 \) supported the idea of static quenching mechanism for the interaction of VB6 with BSA as it has been proven in the literature for other protein–ligand interactions [54,86].

4.4. Soft-hard modeling

Further investigations of the interaction between VB6 and BSA on the basis of direct observations of measured spectra was difficult, therefore, soft-hard modeling chemometric approach was used to extract more useful information. The individual data matrices obtained from UV–Vis and fluorescence measurements (Fig. 2) were combined into an augmented data matrix (56 objects \( \times \) 220 variables, first term of Eq. (8)) and in order to extract the concentration and pure spectral profiles of the various reactant and product species and determining the stoichiometry of complexation, the augmented matrix was analyzed by MCR-ALS (Eq. (8)). Initial estimates of the concentration and spectral profiles were obtained by SIMPLISMA method [87]. The applied constraint were, non-negativity in the concentration and spectral profiles and unimodality of concentration profiles. Singular value decomposition (SVD) analysis of the augmented data matrix revealed that 3 singular values explain more than 99% of variance in the data. The same results were obtained from SVD analysis of individual data matrices. It is reasonable to say that two singular values account for free protein and free drug and third one account for complex species. Also, from fluorescence quenching studies, it was concluded that the formed complex has 1:1 stoichiometry, because as it is proved above, the BSA has one binding site.

Fig. 4A and B shows the concentration profiles of free BSA, free VB6 and BSA-VB6 complex in the binding procedure that were obtained by MCR-ALS method, which otherwise, were difficult to obtain by conventional methods. The x-axis in Fig. 4A represents the \( C_0/C_p \) and in Fig. 4B it represents the \( C_0/C_p \), where \( C_p \) is the total concentration of protein and \( C_b \) is the total concentration of VB6 in each step of titration. In titration 1, total concentration of added titrant (VB6) was 5 times the total concentration of titrand, and in titration 2, total concentration of the titrant (BSA) was 1.5 times the total concentration of the titrand (VB6). The legends show the species that are present in the equilibrated under study BSA-VB6 system. Fig. 4A shows that in titration 1, concentration of BSA is decreasing upon addition of VB6 and the complex specie concentration is increasing. Concentration of the free VB6 increases slowly, because some portion of the added VB6 gets consumed due to complex formation. Fig. 4B shows the concentration profiles of species in titration 2. In titration 2, the BSA is added to the VB6 and the 1:1 complex specie appears from beginning of the titration and increases steadily. Upon step-by-step addition of BSA, concentration of free VB6 decreases and concentration of free BSA increases.

Fig. 4C and D shows the pure UV–Vis and fluorescence spectra simulated for different species, which correspond to \( S^T_{UV} \) and \( S^T_{F} \), respectively. These pure spectral profiles provided qualitative information about the nature of the complex species. According to Fig. 4C, when complex is formed, intensity at 278 nm (\( \lambda_{max} \)) decreases and two new additional bands appear at 259 and 324 nm. These results suggested that conformation and/or microenvironment of BSA are altered in the presence of VB6. In Fig. 4D, decrease in the intensity of fluorescence of BSA is due to formation of VB6–BSA complex and red shift in their maximum emission wavelength indicates that the microenvironment of tryptophan residues on BSA has altered. Red shift is because of the enhancement of polarity around the tryptophan residues and decreasing of hydrophobicity [88]. Also, the widening of

Fig. 4. The concentration and spectral profiles of equilibrium species obtained by MCR-ALS analysis of augmented data matrix: (A) and (B) concentration profiles of species in titration 1 and 2, respectively. (C) and (D) Spectral profiles of species involved in UV–Vis and fluorescence, respectively. The legends show the involved equilibrium species.
fluorescence peak indicated that the stretch of BSA molecule after binding \(^{[89]}\). Change in the microenvironment of tryptophan residues can be due to an unfolded rather than the originally folded structure of the BSA protein \(^{[29]}\). Therefore, the formation of VB6-BSA complexes causes unfolding of the BSA structure.

The presence of rotation ambiguity related uncertainty in the profiles of species was evaluated by MCR-BANDS method. The differences between the maximum and minimum of the objective function optimized by MCR-BANDS (relative contribution) for every source indicated that 3.5% of rotation ambiguity (uncertainty) was present in the VB6 profile, while the rotation in the profiles of the BSA and BSA:VB6 complex were negligible. In this study, the MCR results were used for determination of the stoichiometry of VB6 binding to BSA. The stoichiometry number was further used to define the chemical model of interaction between VB6 and BSA based on the law of mass action. Then the binding constant was calculated by the model-based non-linear least squares, which for sure there is no rotation ambiguity related uncertainty in results of model based method. Therefore, existence of rotation ambiguities in the MCR-ALS derived concentration and spectral profiles of VB6 doesn’t affect the calculated binding constants. But, it is always important to be aware of the rotation ambiguity related uncertainties in the MCR-ALS results, anyway.

As previously mentioned, the quality and reliability of the MCR-ALS solution may be assessed using the lack of data fit (LOF, Eq. (9)) and explained data variance (\(R^2\), Eq. (10)) parameters that allow assessing the dissimilarity among the experimental data matrix (\(D\)) and the data modeled by MCR-ALS. In this work, the lof and \(R^2\) value were obtained to be 3.22% and 99.98% which implied that almost all of the variability in the experimental data are modeled. The lof value of less than 5% is favorable, since assumption of the 5% of error in experimental procedure and spectra recording is almost all of the variability in the experimental data are modeled. At the 0.015–0.250 mM range of VB6, the intensity of these bands decreased very sharply, but, when the concentration of VB6 increased to 0.500 mM, the decrease in the intensities was mild. Negative features in the difference spectra for amide I and II bands at different concentrations were located at 1655 cm\(^{-1}\) and 1543 cm\(^{-1}\) (0.015 mM, Fig. S5A in supplementary data), 1654 cm\(^{-1}\) and 1540 cm\(^{-1}\) (0.250 mM, Fig. 5), and 1654 cm\(^{-1}\) and 1538 cm\(^{-1}\) (0.500 mM, Fig. S5B in supplementary data). These negative features are due to VB6 binding to C=O, C=N, and N–H

### Table 2

<table>
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<th>Analyzed matrix</th>
<th>Stoichiometry</th>
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</tr>
</thead>
<tbody>
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<tr>
<td>(D_{V6}^{C_2})</td>
<td>1:1</td>
<td>1.09 (±0.06) (\times 10^4)</td>
</tr>
<tr>
<td>(D_{BSA}^{C_2})</td>
<td>1:1</td>
<td>1.59 (±0.01) (\times 10^4)</td>
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<tr>
<td>Titration 2</td>
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<td></td>
</tr>
<tr>
<td>(D_{W}^{V6})</td>
<td>1:1</td>
<td>2.44 (±2.31) (\times 10^4)</td>
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<tr>
<td>(D_{W}^{BSA})</td>
<td>1:1</td>
<td>3.63 (±1.44) (\times 10^4)</td>
</tr>
</tbody>
</table>

Fig. 5 shows the FTIR spectra of free BSA, free VB6 and its complex. Since there was no major spectral shifting for the protein amide I band at 1654 cm\(^{-1}\) (mainly C=O stretch) and amide II band at 1540 cm\(^{-1}\) (C–N stretching coupled with N–H bending modes) \(^{[62,90]}\) upon VB6 interaction, in order to monitor the intensity variations, difference spectra [(protein solution + VB6 solutions) – (protein solution)] were obtained. At different concentrations of VB6 (Section 3.3), the intensities of amide I (1654 cm\(^{-1}\)) and amide II bands (1540 cm\(^{-1}\)) of BSA were decreased. At the

**4.5. FTIR study of the secondary structures**

The nonlinear least squares curve fitting (SQUAD computer program) was used for fitting the spectral data to the previously defined complexation model to obtain the binding constants. The chemical model of 1:1 stoichiometry ratio was used for fitting to the spectral data (Section 3.5). Stoichiometric ratio was inferred from MCR-ALS results. The calculated binding constants are shown in Table 2. As can be seen, there is a small differences in the binding constant obtained from fluorescence and spectrophotometry. This is probably because of the fact that fluorescence and absorption spectroscopy techniques reveal the relatively different interactions between the BSA and VB6. And also because the UV–Vis and fluorescence spectra of each titration are recorded by doing each titration 2 times, one for recording the UV spectra and other for recording the fluorescence spectra. The differences are somehow related to the experimental error in the results. There is a small and almost negligible discrepancy between binding constants obtained by the multivariate hard modeling and Hill equation. It is reasonable to say that hard modeling results are more accurate, since hard modeling methods use the whole domain of spectra for calculation of binding constants.
groups and changing in the secondary structures of the protein. Therefore, this observation suggested a major reduction of protein \( \alpha \)-helical structure at different VB6 concentrations in complex. Similar infrared spectral changes were observed for protein amide I and amide II bands in several ligand/BSA complexes, where major protein conformational changes occurred [5,91].

A quantitative analysis of the protein secondary structure for free BSA and their VB6 complexes in hydrated films has been carried out and results are shown in Fig. 6 and Table 3. Amide I is the most sensitive probe for detecting changes in the protein secondary structures and its absorption band is located at 1700–1600 cm\(^{-1}\). Information about the different types of secondary structures such as \( \alpha \)-helix, \( \beta \)-sheets, turns and random coil can be obtained by comparing the spectra of BSA and ligand-BSA complexes in this region [92]. The curve-fitting results show that free BSA (Fig. 6A) contains \( \alpha \)-helix 65\% (1654 cm\(^{-1}\)), \( \beta \)-sheet 11\% (1616 and 1625 cm\(^{-1}\)), turn 15\% (1671 cm\(^{-1}\)), \( \beta \)-antiparallel 4.0\% (1683 cm\(^{-1}\)) and random coil 5.0\% (1636 cm\(^{-1}\)), consistent with the conformation of BSA [6]. Upon VB6 interaction with BSA, a major decrease of \( \alpha \)-helix from 65\% (free BSA) to 46\% (VB6-BSA, 0.25 mM), turn from 15\% to 14\% with an increase in the \( \beta \)-sheet from 11\% to 21\% (complex), random from 5.0\% to 7.0\% (complex) and \( \beta \)-antiparallel from 4.0\% to 12\% were observed (Fig. 6B and Table 3). These results are consistent with the decrease in intensity of the protein amide I band discussed above. The conformational changes observed for BSA suggest a partial protein unfolding in the presence of VB6. Similar results were observed for the protein in several ligand–protein complexes [5,6].

Fig. 6. Second derivative resolution enhancement and curve-fitted amide I region (1700–1600 cm\(^{-1}\)) of IR spectra for (A) free BSA, and (B) VB6-BSA complex (0.25 mM). The legends show the percent of each structure present in the system.

**Table 3**
Percent contributions of different secondary structures (IR spectra) of free BSA and VB6-BSA complexes in hydrated film at pH = 7.4.

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<thead>
<tr>
<th>Amide I (cm(^{-1}))</th>
<th>Free BSA (%)</th>
<th>VB6-BSA (%)</th>
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</thead>
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<td>( \beta )-sheet 1614–1625</td>
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</tr>
<tr>
<td>( \beta )-antiparallel 1680–1691</td>
<td>4.0</td>
<td>10</td>
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</table>

Fig. 7. The changes of RMSD of BSA versus MD simulation time (picoseconds).

Fig. 8. (A) View of the superimposed structures of BSA at 1 and 5 ns of MD simulation. (B) BSA binding site I at 1 and 5 ns. The greenish-blue coloring shows binding site I at 1 ns and purple coloring shows binding site I at 5 ns.
4.6. Molecular dynamics studies

The combined docking and molecular dynamics simulations were used to estimate the binding affinity of VB6 toward BSA. Before investigation of protein-ligand interaction using docking, a 5-ns molecular dynamics simulation was performed for the protein system using GROMACS 4.5.3 simulation package [48] to relax the structure and dynamics of protein. The procedure was carried out while accounting for certain physiological and environmental factors that occur on proteins, which are not considered in simpler docking protocols [93]. Relaxation of the 3D structure is required when the solid-state X-ray structure is used in order to obtain conformations adequate for performing the required docking simulations. This can be achieved by MD simulation of protein taking into account its flexibility and conformational changes in the binding site region, due to the mobility of its neighboring protein regions and intrinsic backbone flexibility of some amino acid residues [41,94]. It is important to consider this fact for ligand recognition processes, to improve theoretical binding energies by computational docking methods. However, theoretical methods do not consider many factors involved in ligand–target recognition, which are certainly beyond the chemical composition of ligand [95]. The drift of each simulated structure from its initial crystallographic conformation provides information on the quality of the simulations. The drift was measured in terms of the root mean square deviation (RMSD) of the backbone (Cα atoms) from the initial structures as a function of time. The RMSD of trajectories from initial structure of BSA was used to examine whether the structure of protein remained stable under the simulation conditions or not. Fig. 7 shows the plot of the RMSD of backbone α-carbon (a structural transition) during the 5 ns of MD simulation for the whole protein. The RMSD values of the protein backbone ranged from 0.15 to 0.25 nm, reaching to a stable value of ~0.3 at the end of the simulation, as shown in Fig. 7. This is indicative of a stable simulation.

The overall goal of MD simulation was to provide a more refined and flexible BSA structure model to use for docking procedure, as shown by Alonso et al. [40]. It is important to consider the conformations of protein receptor to optimize the structures of the final complexes, and calculate accurate energies. Fig. 8 shows the

<table>
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<th>t (ns)</th>
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<th>H bond</th>
<th>pi-pi</th>
<th>pi-sigma</th>
<th>pi-cation</th>
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superimposition of snapshots taken at 1 and 5 ns as a whole (Fig. 8A) and in the binding site I (Fig. 8B) of BSA that illustrate the mobility of protein and intrinsic backbone flexibility of some amino acid residues. MD simulations indicated that there is significant movement for BSA (Fig. 8). These BSA movements are very valuable for understanding the dynamic behavior of proteins at different timescales, from fast internal motions to slow conformational changes or even protein folding processes [96] and modified binding site behavior.

The results of this work suggested that BSA flexibility underlies the preferential affinity for several binding modes by this protein. Therefore, MD simulation's results (snapshots) were used for further investigation of BSA-VB6 interaction for subsequent docking simulations.

4.7. Molecular docking

The GOLD program was used to realize the binding mode of VB6 in the active site of BSA. 11 BSA structures, sampled every 0.5 ns during 5 ns of MD simulation, were used in docking studies with VB6. Table 4 shows GOLD score values of VB6 docked in BSA, type of interactions, H bond distance, and amino acid residues involved in binding of VB6 to BSA. The GOLD score values were used to evaluate the affinity of VB6 toward BSA. Parameters in Table 4 were unequal at different simulation times, which indicated that BSA binding affinity toward VB6 depend on both protein and ligand movements. The relative docking orientations of VB6 with respect to five MD snapshots of BSA binding site that sampled at 1, 2, 3, 4 and 5 ns are shown in Fig. 9. Also, Fig. 9 shows the backbone and side chain movements of residues involved in the ligand recognition at different times (ns), with respect to the native BSA structure (pdb core, 4JK4) [46]. It is evident that the binding site shows considerable flexibility and there is a mutual dependence between BSA binding site conformation and ligand orientation. These BSA domain and binding site movements enabled VB6 to adopt to different structural binding modes and, consequently, different GOLD score values were obtained (Table 4). This process is facilitated by changes in the relative orientations of amino acids at binding sites. Subsequently, binding site movements induced changes in local physicochemical properties, such as hydrophobicity, steric hindrance effects, and electronic distributions [95,97]. Therefore, it is apparent that net effect is modification of the ligand affinities toward protein. The docking results shown in Table 4 indicated that VB6 binds to BSA binding site I through several interactions including: π-cation interactions between pyridine moiety of VB6 and the basic side chain residue of Arg198, at snapshots taken at 2, 2.5, 3, and 4.5 ns, Arg217 and Arg194, at snapshots taken at 0, 1.5, 3.5, 4 ns, and Lys221, at snapshots taken at 3.5 ns; π-π interactions with the aromatic system of Trp213, at snapshots taken at 2, 2.5, 3 and 4.5 ns; and hydrogen bonding interactions between some of residues (Arg194, Arg198, Arg217, Arg256, Ala290, Glu291, Ser286, Ser191, Pro 440, Asp 450) and N, H, O atoms of VB6, during snapshots taken in the range of 0–5 ns. It was concluded that snapshot taken at 3 ns have favorable interactions with site I through π-cation interactions with Arg198, π-π interactions with Trp213, and hydrogen bonding interactions with Arg194 and Arg198. Also, as shown in Table 4, the best GOLD score values were obtained when VB6 interacted with BSA through tryptophan residue, along with other amino acids. This observation suggested that principal regions of ligand binding to BSA are located in hydrophobic cavities of subdomain IIA. This finding provided a good structural basis to explain the efficient fluorescence quenching of BSA in the presence of the VB6. Furthermore several ionic and polar residues in the proximity of the ligand (VB6) play an important role in stabilizing the molecule via H–bonds and electrostatic interactions.
5. Conclusions

In this paper, the interactions of VB6 with bovine serum albumin (BSA) in aqueous solution were investigated by fluorescence, UV–Vis, FTIR and molecular modeling methods under physiological conditions. Study on stability of BSA-VB6 complex in a quantitative way was done by calculating binding constants. The stability is studied in molecular level by molecular modeling methods, and the mechanism of interaction is studied by spectroscopic methods. The information reported in this paper paves the way for further pharmacokinetics studies of this system.

The fluorescence spectra showed that quenching of BSA by VB6 is a result of the formation of BSA-VB6 complex. Fluorescence quenching study indicated the quenching mechanism mainly arises from the static quenching rather than dynamic quenching. Moderately strong binding exist between the BSA and VB6 and the efficiency of energy transfer was equal to 41.1%. FTIR spectra revealed that VB6 interaction alters protein secondary structure for BSA causing a partial protein unfolding.

The MCR-ALS analysis of the augmented data matrix of UV–Vis and fluorescence measurements produced the estimates of the pure signals of BSA and VB6 which were in good agreement with the measured responses. The stoichiometry of interaction was obtained from MCR-ALS analysis. The extension of the rotation ambiguity in MCR-ALS results was studied by MCR-BANDS method. From MCR-ALS derived stoichiometry numbers, the model of the interaction between BSA and VB6 was developed using mass balance equations and then the experimental fluorescence and UV–Vis data were fitted based on this models by nonlinear least squares curve fitting to obtain the binding constants. Besides of the negligible discrepancies, the binding constants obtained by band modeling and curve fitting were close to the values obtained by the Hill equation.

Also, this work has demonstrated that a combination of docking and MD simulations in a protocol is a powerful approach for further study of the protein–ligand binding studies. Molecular modeling results showed that VB6–BSA complex formed on the basis of electrostatic forces, π–π stacking and hydrogen bond. The results indicated that the whole protein movement and the BSA conformational changes influence the ligand recognition in different binding geometries which leads to different GOLD score value of binding due to the changes in overall chemical environment. Minimum protein movement caused different binding site properties, which allowed several ligand affinities with the same ligand at different snapshots. This study showed that these computational techniques are powerful approaches to provide an accurate and useful picture of ligand–protein interactions at the molecular level.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biore.2016.07.014.

References