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Binding of Drug Pamabrom to Human Hemoglobin: Structural and Thermodynamic Characterization Studies

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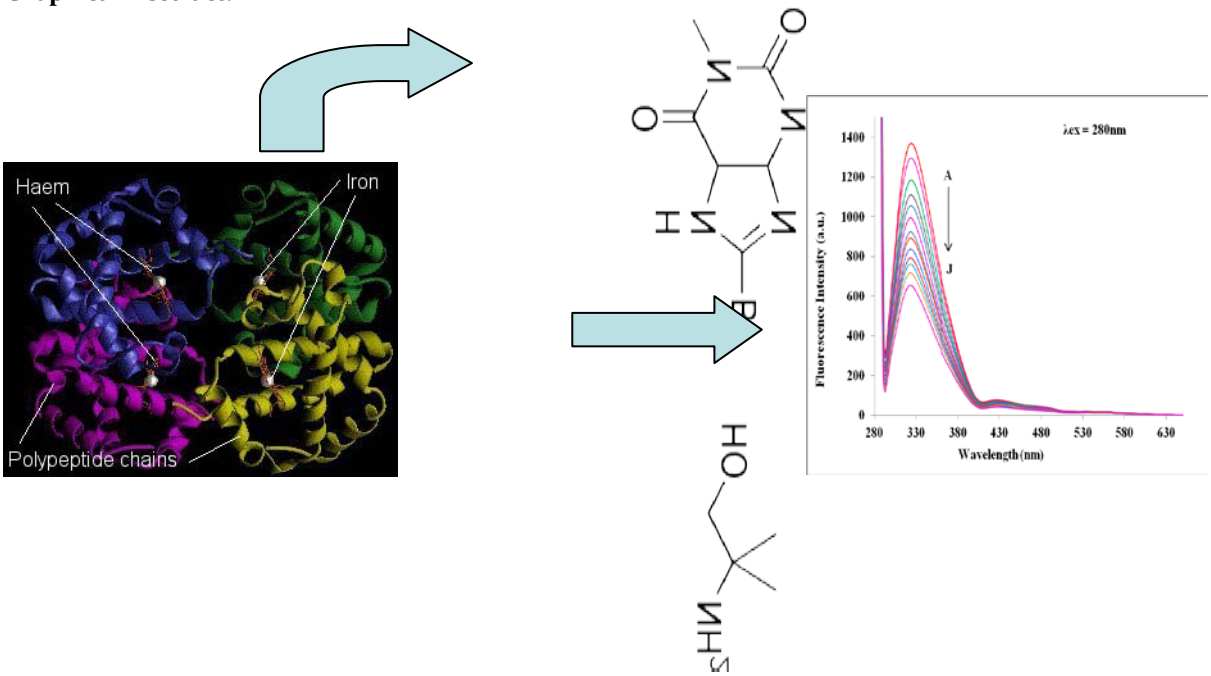
Abstract: ***Binding of drug with human hemoglobin (HHb) and Pamabrom (PABr) have been systematically investigated by fluorescence spectroscopy, UV-visible absorption spectroscopy and synchronous spectroscopy under similar to human physiological conditions. The quenching mechanism was investigated with the quenching type, the association constants, the number of binding sites and basic thermodynamic parameters. The present paper review the drug–HHb interactions, their types and applications of experimental techniques used to study interactions between HHb and small ligand molecules that are potentially of pharmaceutical interest.***

Keywords: Human hemoglobin, pamabrom, static quenching, Synchronous spectroscopy, Fluorescence spectroscopy

Graphical Abstract:



Highlights

a) The thermodynamic parameters are indicated by this process is spontaneous, entropy driven.
b) The values of n indicate the existence of a single binding site in HHb for PABr.

1. Introduction

Human hemoglobin (HHb) is rich of α- helix in resident state and widely known to act as a hydrogen peroxide (H_2O_2) biosensor [1]. HHb reversibly binds many endogenous and exogenous molecules including various drugs [2]. Thus the knowledge of the binding features of therapeutically important drug with HHb and other applicable functionally important cellular proteins is crucial for understanding the mechanism of their drugs actions. However, to date very little is known about the precise interactions of drug with HHb. The adult HHb molecule is a tetramer of two α and two β chains arranged in two identical $\alpha_1\beta_1$ and $\alpha_2\beta_2$ subunits in each α β subunit, there are three tryptophan residues i.e. α 14 Trp. The structure of HHb as shown in fig.1.

Pamabrom (PABr) is a diuretic (water pill). It works by rising urination. PABr is used to treat bloating, puffiness, and other signs of water weight gain related to menstrual symptoms. PABr may also be used for other purposes not listed in this medication guide. Do not use this medication if you are allergic to PABr or if you are unable to urinate. Before using PABr, tell your doctor if you take any other diuretic (water pill), or if you have high blood pressure, heart disease, or kidney disease. If you have any of these conditions, you may need a dose adjustment or special tests to safely take PABr. Use this medication as directed on the

label, or as your doctor has prescribed. Do not use the medication in larger amounts or for longer than recommended. Do not take more than 4 tablets in one day (24 hours).

PABr (trade name Diurex) is a diuretic product included in retail drugs available in over-the-counter medications. The active diuretic ingredient in PABr is 8-bromotheophylline. PABr is available in combination with acetaminophen (paracetamol) for various conditions such as back pain and menstrual relief. The acetaminophen helps reduce menstrual pains and the PABr reduces associated bloating. The combination is available in a number of products from various brands under different names. The dosages are essentially the same for each brand, including generic drug store varieties. Fig.(2) indicates structure of PABr. Bio macromolecules, including nucleic acids, proteins, and carbohydrates, arein dispensable to life, and mediate nearly all biological processes. The surface properties of proteins, and especially their surface electrical properties, have an important influence on their ionic interactions with other biomacromolecules. The surface charge of proteins arises primarily from ionization of surface groups [***]

Nucleic acids, proteins, and carbohydrates are also deeply involved in the pathology of many diseases. The development of innovative chemical methods for selectively 20 controlling specific biomacromolecule function is thus of considerable importance in the fields of chemistry, biology, and medicine. In this context, much attention has been given to development of organic photoactivatable agents capable of target selective degradation of bio macromolecules.

2. Experimental Procedures

2.1. Reagents

HHb was purchased from Sigma-Aldrich Chemical Company and used as it is without further purification. PABr was obtained from. Tris Buffer Tris(hydroxymethyl) aminomethane puris AR Spectrochem Pvt Ltd Mumbai (India).

2.2. Procedures

A 1.0 mL solution containing appropriate concentration of HHb (3×10^{-5}M) was titrated by addition of (0-5.0 mL) 1×10^{-4} M stock solution of PABr. The double distilled water used throughout the experiment.

2.3. Methods

2.3.1. Fluorescence spectra
All fluorescence spectra were recorded on spectrofluorometer (Jasco, Japan FP-8300) equipped with 1.0 cm quartz cell. The width of the excitation and the emission slits were set to 5-5 nm and thermostatically controlled cell holder for HHb-PABr system respectively.

2.3.2 .UV – vis spectrum
The UV-vis spectrum was recorded at room temperature on a UV-vis-NIR spectrophotometer [Shimadzu UV-3600]

2.3.3. Synchronous fluorescence spectra
The synchronous fluorescence were recorded by scanning simultaneously the excitation and emission wavelength at $\Delta\lambda$= 15nm and $\Delta\lambda$= 60nm [7].

2.3.4. Three dimensional spectra
The three dimensional spectra were recorded on spectrofluorometer (Jasco, Japan FP-8300) equipped with 1.0 cm quartz cell..

3. Result and discussion

3.1. Effect of drug on the steady state quenching mechanism

The biomedical application of fluorescence often utilizes intrinsic protein fluorescence [8]. Fluorescence studies have been widely used to understand the interaction of small molecules to protein [9-10]. Changes in the intrinsic fluorescence of HHb may provide information about the nature and mode of interaction [11]. HHb contains three intrinsic fluorophores as tryptophan, tyrosine and phenylalanine. Among the six tryptophan residues, two α β dimers have three Trp residues each as α-Trp-14, β-Trp-15,β-37Trp [12].Moreover, there are also five tyrosine residues in two α β dimer as α-Tyr- 24, α- Tyr-42, α-Tyr-140, β-Tyr-34 and β-Tyr-144 [13]. A valuable feature of intrinsic fluorescence of protein is the high sensitivity of tryptophan to its local environment.

HHb solution excited at 280 nm emit fluorescence attributable mainly to β-37Trp. Fluorescence measurements of proteins can provide considerable information about the accessibility of ligands to the fluorophores [14].

Fluorescence quenching is usually classified into dynamic and static quenching by different mechanisms, which are dependent on temperature [15]. To further elucidate the fluorescence quenching mechanism, the fluorescence data have been analysed by stern – Volmer equation [16]

$$F_0/F = 1 + k_q \tau_0 [Q] = 1 + K_{sv} [Q] \quad (1)$$

where F_0 and F are the fluorescence intensities before and after the addition of the quencher respectively. Kq, Ksv, τ_0 and [Q] are the quenching rate constant of the biomolecules, the Stern-Volmer dynamic quenching constant, the average life time of the biomolecules without quencher [$\tau_0=10^{-8}$ s], the fraction of accessible fluorophores, binding constant and the concentration of quencher respectively [17]. Ksv is acquired from a slope of F_0/F versus [Q]. Fig. shows that the fluorescence quenching curve of HHb by PABr follow the stern –Volmer plots [18] and has a good linearity. The quenching constant Ksv and Kq at different temperatures are collected in table 1.It indicates that the value of K_{SV} decreases as temperature increases.

For static quenching a complex is formed between ground state of fluorescence substance and quencher, therefore the absorption spectra of fluorescence substance would be influenced [19]. Hence the result shows that type of fluorescence quenching is static.

Fig.3 indicates that in fluorescence spectra intensity decreases with concentration gives blue shift elevate that interaction between HHb and PABr.

$$F_0/F = F_0/F_0 - F = 1/f_a + 1/f_a K_a[Q] \quad (2)$$

According to modified stern – Volmer equation as shown in figure (4,5) linearity is present from which concluded that static fluorescence quenching which obey the equation . Where F_0 and F are the fluorescence intensities before and after the addition of the quencher respectively. f_a, Ka and [Q] are the quenching rate constant of the biomolecule, the average life time of the biomolecules without quencher [$\tau_0=10^{-8}$ s], the fraction of accessible fluorophores, binding constant and the concentration of quencher respectively.

In order to confirm the quenching mechanism, the fluorescence quenching was [20] analysed according to the Scatchered's equation

$$\frac{r}{D_f} = nK - rK \quad (3)$$

Where r is the moles of drugs bound per moles of protein , D_f is the molar concentration of free drug, n is binding site multiplicity per class of binding site and K is the association binding constant. A possible quenching mechanism was evident from the Fig.6 that fluorescence quenching type is static.

3.2. Binding model and number of binding sites

Noncovalent bio molecular interactions numerously exists in nature and their binding parameters are extremely important in all binding studies of protein drug nature [21].Understanding the binding process by estimating the binding parameters such as binding constant, binding domain and structural changes of protein is essential for realizing complexation in the biological system[22].

When ligand molecule bind independently to a set of equivalent sites on a macromolecules, the equilibrium between free and bound ligand molecules is given by the following relation

$$\log(F_0 - F/F) = logK + nlog[Q] \quad (3)$$

where K is the binding constant, n is the number of PABr bound to a macromolecule HHb. The plot of log (F_0-F/F) versus log [Q] slope is equal to n and intercept on Y-axis is equal to log K (Fig. 7). In Table 2, the binding constants K and number of binding sites n are listed for HHb associated with PABr. The interaction between drug and HHb are intrinsically complex process involving different type of intermolecular forces. The results showed that the binding constant increases with the rising temperature which may indicate that forming a stable complex between HHb and PABr. The stable complex could not be partly decomposed with the increasing temperature. The values of n indicate the existence of a single binding site in HHb for PABr.

3.3. Thermodynamic analysis and the nature of binding force

The thermodynamic parameters enthalpy (ΔH) and entropy (ΔS) of reaction are important for conforming the acting force [23]. For this reason, the temperature dependence of the binding constant was studied. The temperature chosen were 301, 311 and 321K so that HHb does not undergo any structural degradation from the van't Hoff equation

$$\ln K = -\frac{\Delta H}{RT} = \frac{\Delta S}{R} \quad (4)$$

The value of ΔH and ΔS were obtained from linear van't Hoff plot [Fig.9] and are presented in table [3]. The value of ΔG calculated from the relation [5].

$$\Delta G = \Delta H - T\Delta S \quad (5)$$

The value of ΔG is negative indicate that spontaneous reaction. ΔH and ΔS values are positive in which value of ΔH is small indicates hydrophobic interaction.

As a rule negative value of ΔG indicates spontaneous reaction. The value of ΔH and ΔS is positive and ΔH value is smaller gives hydrophobic interactions positive value of entropy obeys entropy becomes driven in this reaction. The bar diagram as shown in Fig. (10) indicates the values of ΔG , ΔH and ΔS at temperature 301K . Table (4) indicates the interaction between HHb and PABr is spontaneous.

3.4. Energy Transfer Study

The energy transfer is important in biochemistry, because the efficiency of transfer can be used to evaluate the distance between the ligand and the tryptophan residues. So it has been used as a ''spectroscopic ruler'' for distance measurement over several nanometres (Lakowicz, 2006; de Silva et al., 1997). According to Förster's non-radiative energy transfer theory (FRET), if the emitted fluorescence from a donor can be absorbed by an acceptor, energy may transfer from the donor to the acceptor. FRET is an interaction that depends strongly on the distance between the donor and an acceptor. But it is not the only factor influencing the efficiency of the process.

According to Förster's non-radiative energy transfer theory (FRET) (Förster, 1996), the energy transfer will take place under the following conditions: (i) the donor can produce fluorescent light that has sufficiently long lifetime; (ii) the fluorescence emission spectrum of the donor and the UV–vis absorbance spectrum of the acceptor have sufficient overlap; (iii) the distance between the donor and the acceptor is less than 8 nm. The efficiency of energy transfer between the donor and the acceptor, E, could be calculated by the following equation

The donor and acceptor can be calculated according to the Förster'stheory of energy transfer [24]. The efficiency, E was calculated using the equation given below:

$$E = 1 - \frac{F}{F_0} = \frac{R_0^6}{R_{0+r}^6} \quad (6)$$

where R_0 is the critical distance when the transfer efficiency is 50% and it is calculated using the equation shown below:

$$R_0 = 8.79 \times 10^{-25} K^2 n^{-4} \emptyset J \quad (7)$$

Where R_0 is a factor describing the relative orientation in space of the emission and absorption transition dipoles of the donor and acceptor, respectively, n is the average refractive index of the medium in the wavelength range where spectral overlap is significant, Φ is the fluorescence quantum yield of the donor, J is the overlap integral, which expresses the degree of spectral overlap between the donor emission and the acceptor absorption, is calculated using the equation indicated below:

$$J = \frac{\int_0^\infty F(\tau)\epsilon(\tau)\tau^4 d\tau}{\int_0^\infty F(\tau) d\tau} \quad (8)$$

In the present case, K^2 = 2/3, n = 1.36 and Φ = 0.062 for HHb. From Eqs. we were able to calculate that J = 86800.192 cm3 L mol−1, and r = 4.2 nm for HHb. The calculated binding distance 'r' was found to be much lower than 8 nm indicating that energy transfer from HHb to PABr has occurred with high probability.

Fluorescence resonance energy transfers (FRETs) is a nonradiative process whereby an excited state donor (D) transfers energy to a ground state acceptor (A). FRET mainly occurs over distances comparable to most biological macromolecules i.e. about 10–100A ˚ . The rate of energy transfer is highly dependent on the extent of spectral overlap between the relative orientation of the transition dipoles and the distance between the donor and acceptor molecules (Fig.11).

3. 5. Conformation Investigations

Conformational investigation of hemeprotein in which structural changes takes place includes UV-vis spectroscopy, synchronous spectroscopy, three-dimensional spectroscopy and circular dichroism.

3.5 .1.UV-visible spectroscopic studies

Absorption spectrum of HHb exhibited two peak at 275 and 410 nm .The band at 275 nm appears due to phenyl group of tryptophan and tyrosine residues while the sharp peak at 410 nm corresponding to the characteristics absorption of the porphyrin soret band (Fig.12) . This band originates from the heme groups, embedded in a hydrophobic pocket formed by the proteins back-bone through appropriate folding [25].

HHb has four heme group located in the crevices near the surface of the molecule. Fig. shows that two peaks at 275 and 410nm in its absorption spectrum. The sharp peak was just the characteristic absorption of the porphyrin soret band [26].The rich information on the change of HHb conformation from soret band of HHb. However the absorption spectrum of soret band in presence of PABr did not show any significant spectral change, which indicated that all four heme groups bound to HHb. The absorption peak at 270 nm of HHb-PABr system showed a slight shift towards lower wavelength.

The UV absorption spectra of fluorescence substance in the presence of quencher as shown in Fig. 13. For dynamic quenching, the absorption spectra of fluorescence substance was not changed only excite-state fluorescence molecule was influenced by quenchers; but for static quenching, a compound is formed between ground state of fluorescence substance and quencher, therefore the absorption spectra of fluorescence substance would be influenced [27] PABr The UV absorption spectrum of HHb was changed due to form a ground state complex. From this it could be deduced that the fluorescence quenching type of HHb initiated by PABr was static quenching.

It is a powerful technique for studying conformational distribution and dynamics of biological molecules such as DNA, protein etc., which play a key role in maintaining human health. Donor–acceptor combination has tremendous impact in hemeprotein analysis [28].

3.5.2. Synchronous fluorescence spectroscopy

Generally there is only one emission band in the normal fluorescence spectrum of a protein.However synchronous fluorescence spectra can supply characteristic information about the tyrosine and tryptophan residues in the vicinity of the chromophores. Synchronous fluorescence spectroscopy has been applied to a variety of multicomponent system. The main advantages of synchronous fluorescence spectra are simplified spectra narrowed bandwidth, high sensitivity and selectivity. The excitation and emission monochromators are synchronously scanned separated by a constant wavelength interval (Δλ) .

As it is well known synchronous fluorescence spectra can provide information on the molecular microenvironment, particularly in the vicinity of the fluorophore's functional groups [29]

Fluorescence of HHb may be due to the presence of tyrosine, tryptophan and phenylalanine residues. Hence spectroscopic methods are usually applied to the study of conformation of hemeprotein. In synchronous fluorescence spectroscopy, according to Miller, the difference between excitation wavelength and emission wavelength reflects the spectra of a different nature of chromophores, with large Δλ values such as 60 nm, the synchronous fluorescence of HHb is characteristic of tryptophan residue and with small Δλ values such as 15nm is characteristic of tyrosine . The synchronous fluorescence spectra of HHb with various amounts of PABr were recorded at Δλ =15nm (Fig. 13) and Δλ = 60 nm (Fig.14), respectively.

3.5.3. Three-dimension spectroscopy

Three-dimension fluorescence contour maps are a rising fluorescence analysis technique in recent years. The excitation wavelength, the emission wavelength and the fluorescence intensity can be used as the axes in order to investigate the synthetically information of the samples and the contour map spectra can also provide a lot of important information [30].

Fig.(16 a) represent the three dimensional spectra of HHb-PABr system. Contour spectra employ information about binding of HHb and PABr. The spectra becomes as bird's eye view (Fig.16 b) which indicates interaction between HHb and PABr .

4. Conclusions

In summary the experimental observations indicates that interaction between HHb and PABr from complex with one binding site. In binding process hydrophobic interaction provides major role. The binding study of drug receive information about study of drug binding to physiologically important hemeprotein HH b is greatly important for regarding in pharmacy, biochemistry which provides some references for the rational use of drugs in the clinic and helpful for the clarifying the function of HHb as drug storage. Thermodynamic parameters indicate that interaction reaction is spontaneous and entropy is utilised. The study of interaction between PABr and HHb is of significant and scientific and technological important. Drugs interactions at protein binding level would significantly affect the apparent distribution volume of the drugs and also affect the elimination rate of drugs. Therefore, investigating the interaction of drugs and hemeprotein was significant for knowing the transport and distribution of drugs in body, and for clarifying the action mechanism and pharmaceutical dynamics. The biological significance of this work was evident since HHB serves as a carrier molecule for multiple drugs and the interaction of HHb and drug. Hence, the report had a great significance in pharmacology and clinical medicine as well as methodology. Several instrumental techniques are used to study such interactions. In the present review, we will discuss UV–Visible spectroscopy, fluorescence spectroscopy.

The applications of spectroscopic techniques are reviewed and we have discussed the type of information (qualitative or quantitative) that can be obtained from the use of each technique. Not only have novel techniques been applied to study drug–HHb interactions but such interactions may also be the basis for the development of new assays. The interaction between HHb and drugs can cause chemical and conformational modifications and thus variation of the electrochemical properties of nucleobases .

5. Acknowledgments

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Figure Captions:

Figure 1: Molecular structure of HHb.

Figure 2: Molecular structure of PABr.

Figure 3: The fluorescence quenching effect of PABr on the spectra of HHb in the presence of, $C_{HHb} = 3.0x10^{-5}$ mol L^{-1}; C_{PABr} / (10^{-5} mol L^{-1}) a–j: 0,1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, 12.0 (T = 303 K, pH= 7.40, (λex= 280 nm).

Figure 4: Stern-Volmer and modified Stern-Volmer plot describing HHb quenching caused by PABr at three different temperatures, $C_{HHb} = 3.0x10^{-5}$ mol L^{-1}; C_{PABr} / (10^{-5} mol L^{-1}) a–j: 0,1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, 12.0 (T = 303 K, pH= 7.40, (λex= 280 nm).

Figure 5: The plots of log $(F_0 – F)/F$ versus log [Q] at three different temperatures $C_{HHb} = 3.0x10^{-5}$ mol L^{-1}; C_{PABr} / (10^{-5} mol L^{-1}) a–j: 0,1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, 12.0 (T = 303 K, pH= 7.40, (λex= 280 nm).

Figure 6: The Scat chard, plots of r/D_r versus r at three different temperatures $C_{HHb} = 3.0x10^{-5}$ mol L^{-1}; C_{PABr} / (10^{-5} mol L^{-1}) a–j: 0,1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, 12.0 (T = 303 K, pH= 7.40, (λex= 280).

Figure 7: Van't Hoff plot for the binding of HHb to PABr .

Figure 8: Thermodynamic parameters of the interaction of HHb with PABr at 303K.

Figure 9: Overlay spectra for the binding of HHb to PABr.

Figure 10: UV-vis absorption spectra of HHb in the absence and presence of PABr .

Figure 11: Effect of PABr on the UV-vis absorption of HHb.

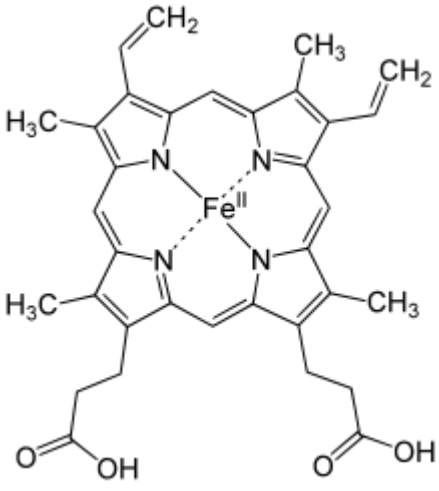
Figure 12: Synchronous fluorescence spectrum of HHb (T = 303 K pH = 7.40),$C_{HHb} = 3 x10^{-5}$ mol L^{-1}; C_{PABr} / (10–5 mol L–1): 0, 1.0, 2.0, 3.0,4.0, 5.0, 6.0, 7.0, 8.0, 9.0 at Δλ = 15 nm.

Figure 13: Synchronous fluorescence spectrum of HHb (T=303 K, pH = 7.40),$C_{HHb} = 3 x10^{-5}$ mol L^{-1}; C_{PABr} / (10–5 mol L–1): 0, 1.0, 2.0, 3.0,4.0, 5.0, 6.0, 7.0, 8.0, 9.0 at Δλ = 60 nm.

Figure 14: The slope diagram of Δλ = 15 nm and Δλ = 60 nm.

Figure 15: The three-dimensional projections and the corresponding excitation-emission matrix fluorescence diagrams of HHb before and after PABr addition.

Figure 1:



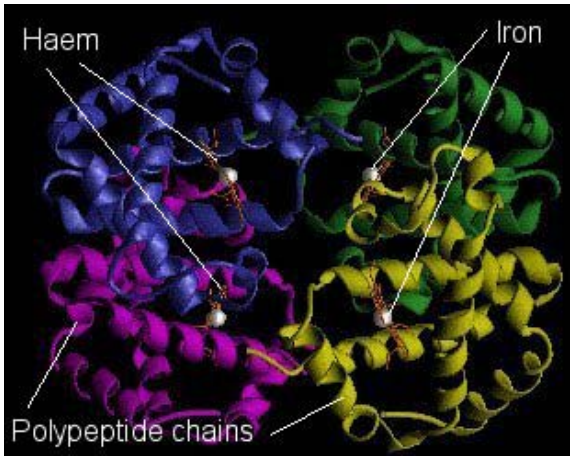
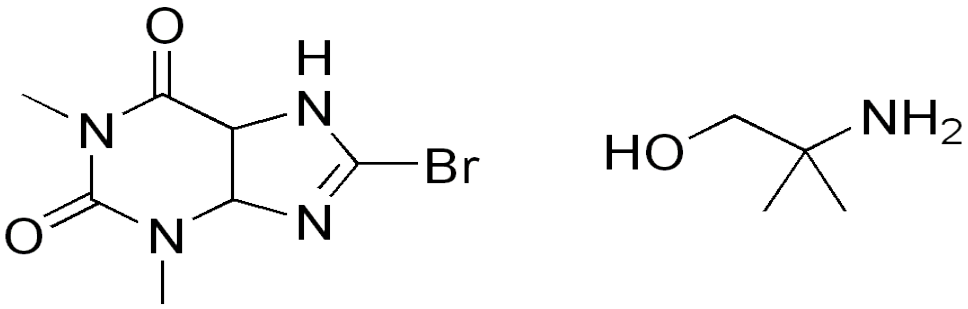


Fig. 2:



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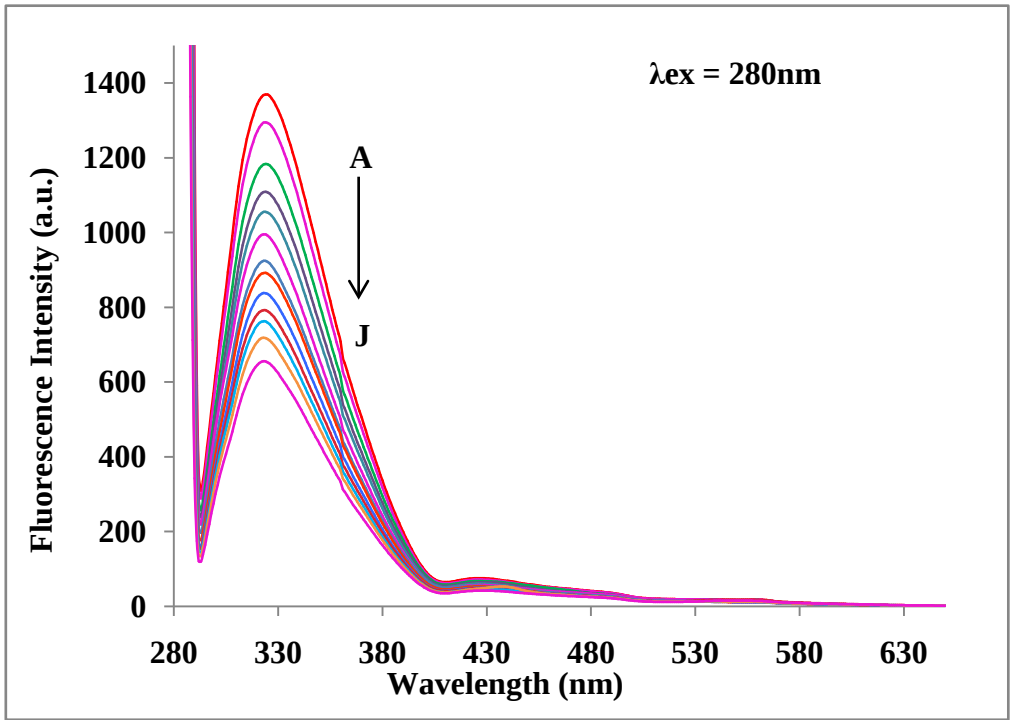

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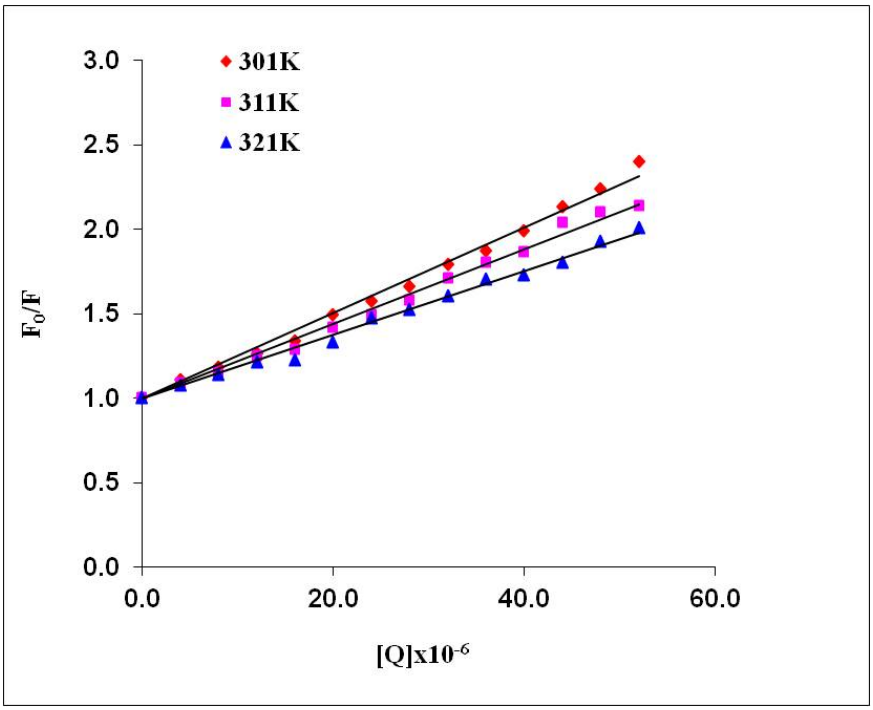


Fig. 5:

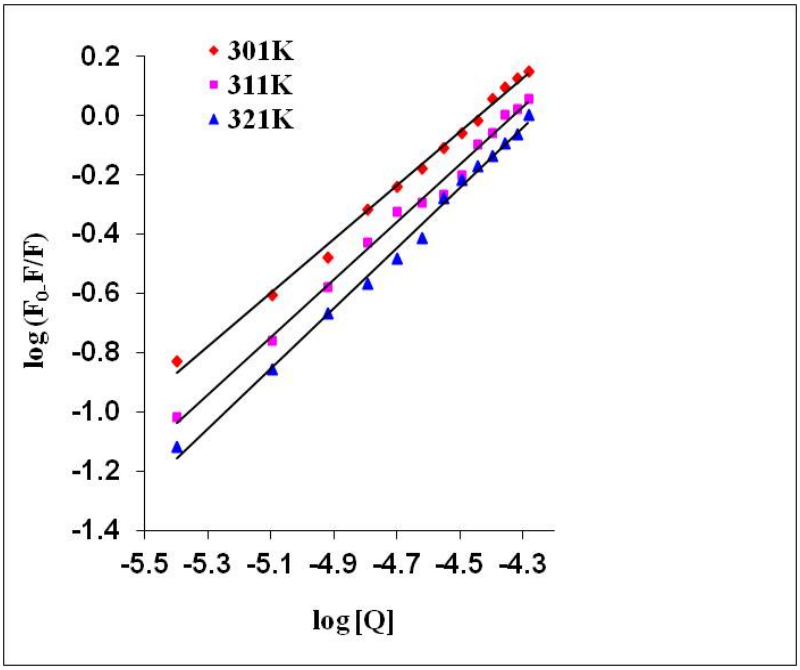
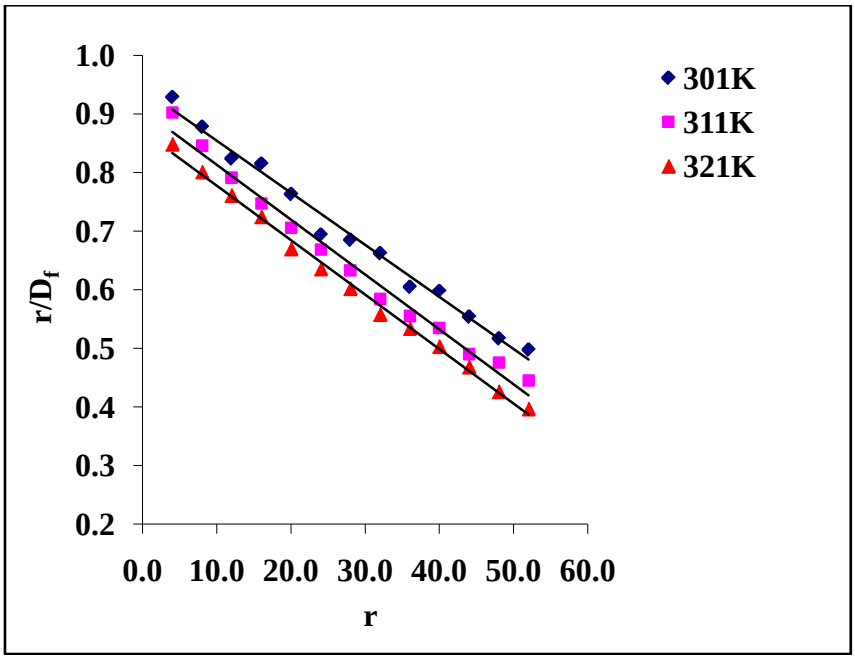


Fig. 6:



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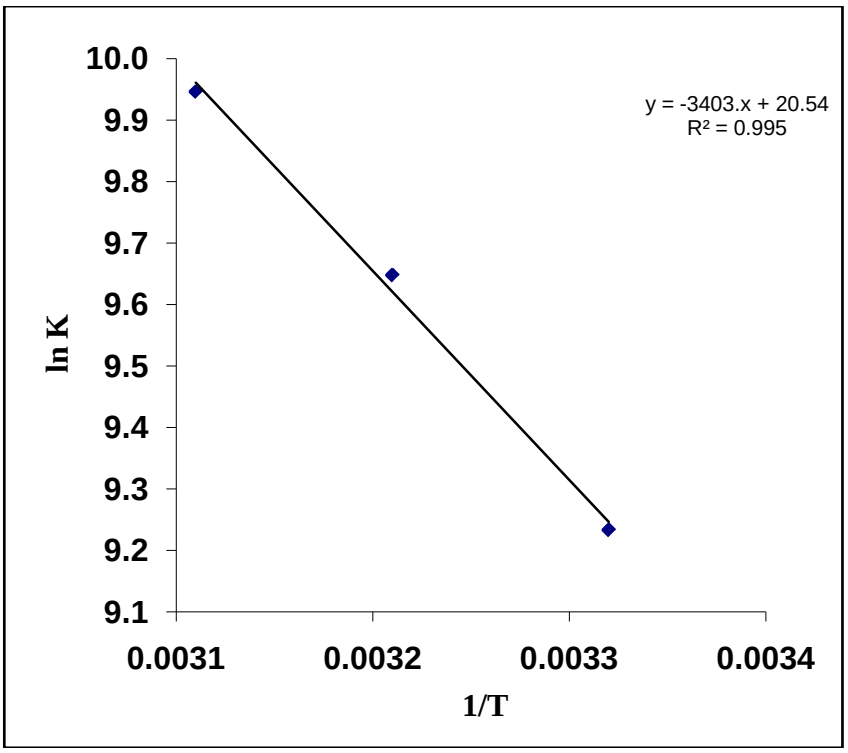

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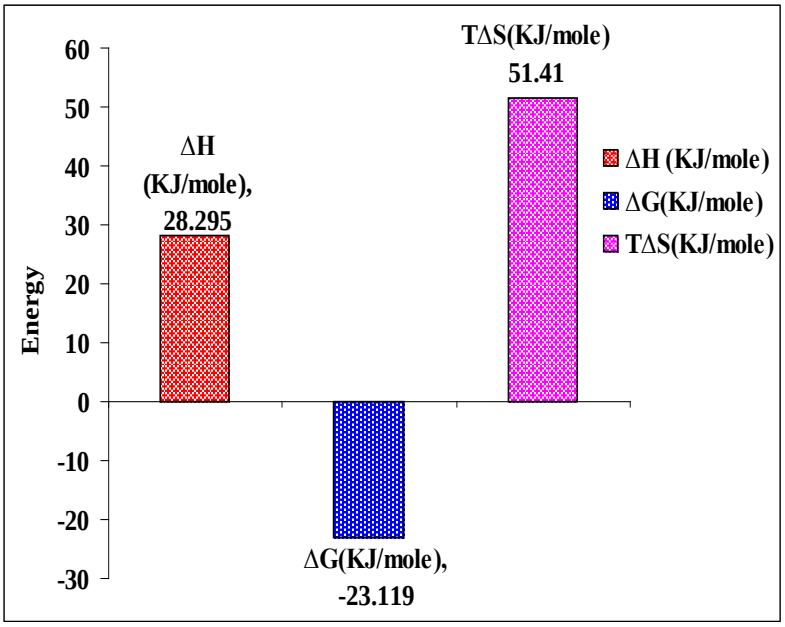

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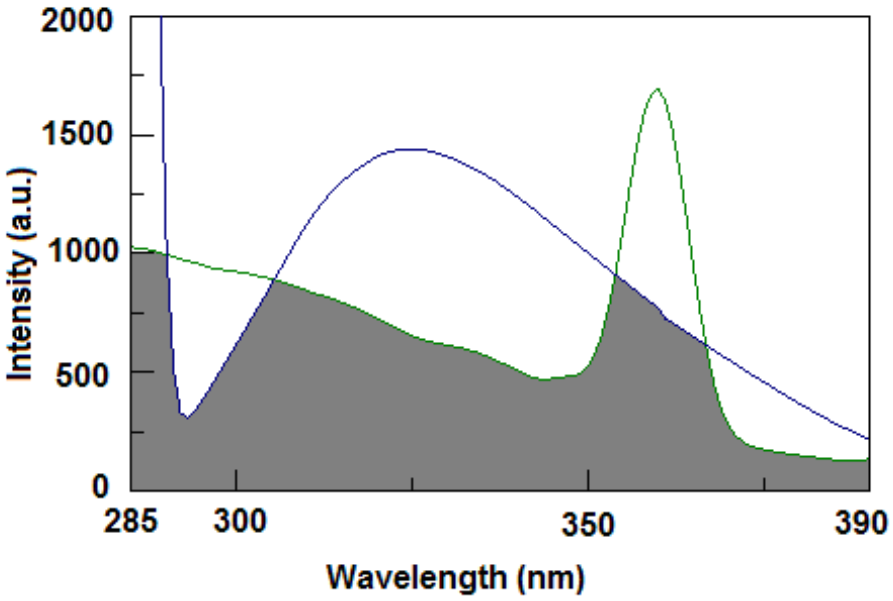

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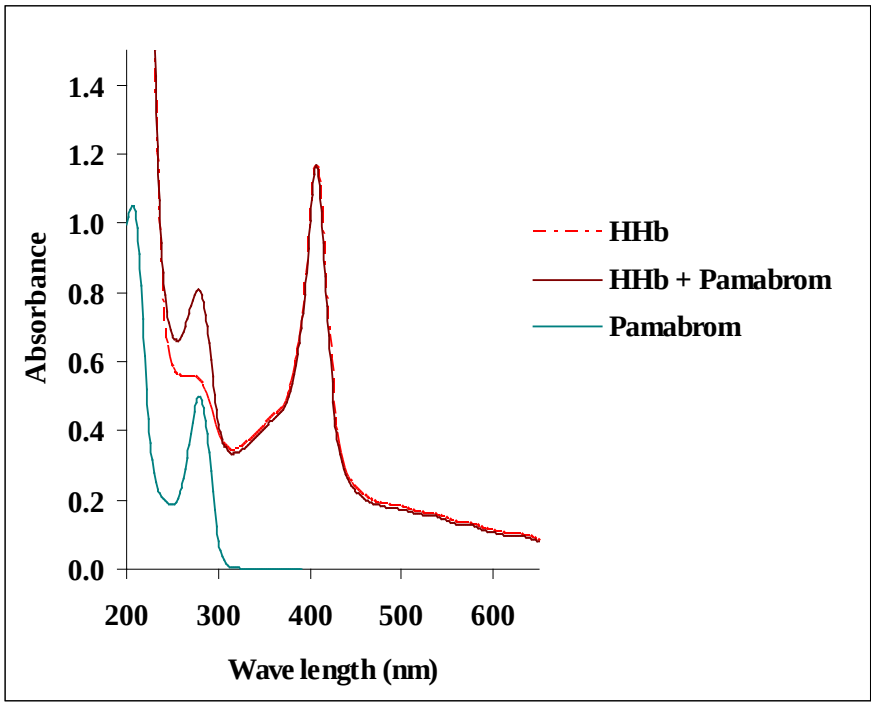

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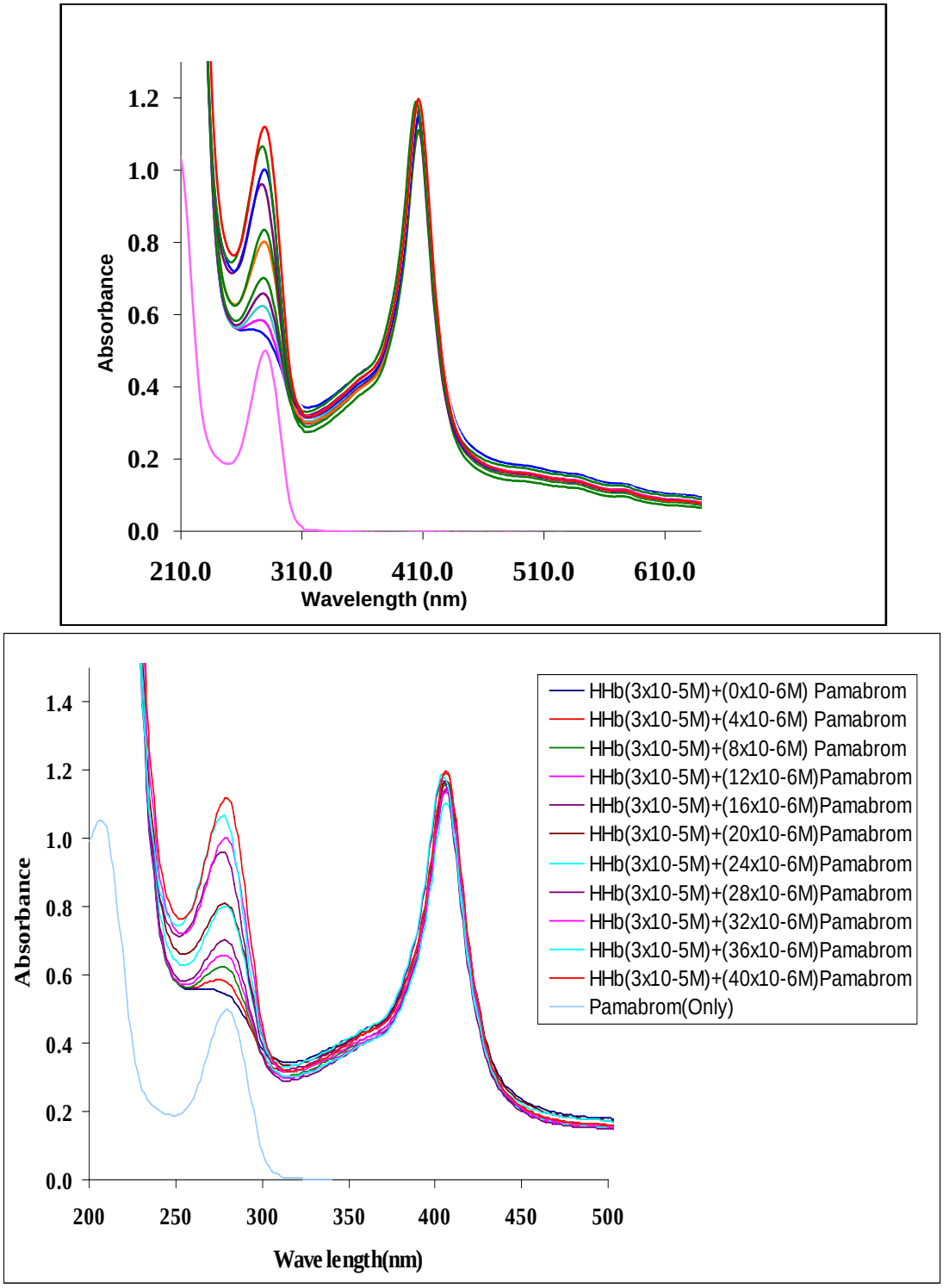

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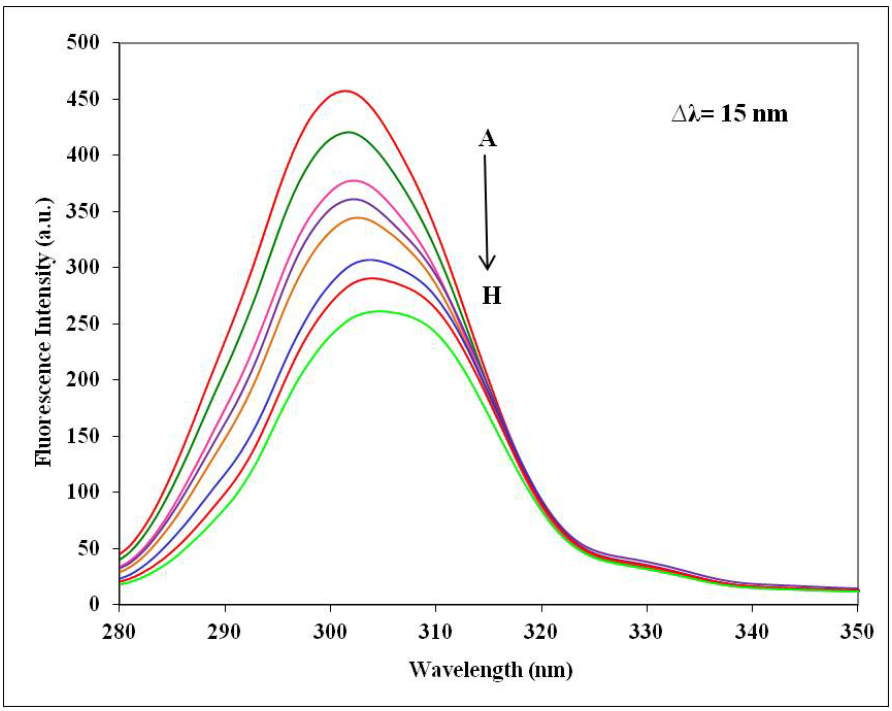

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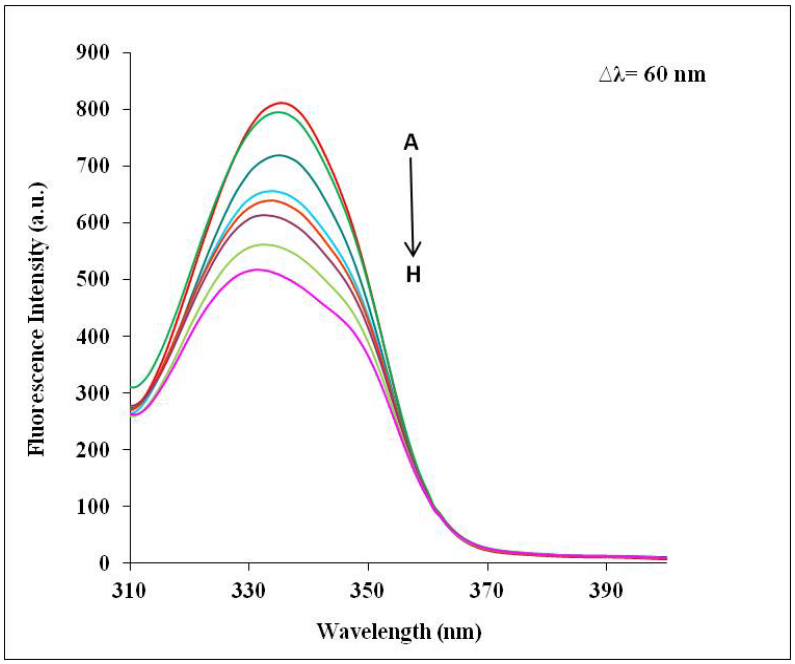

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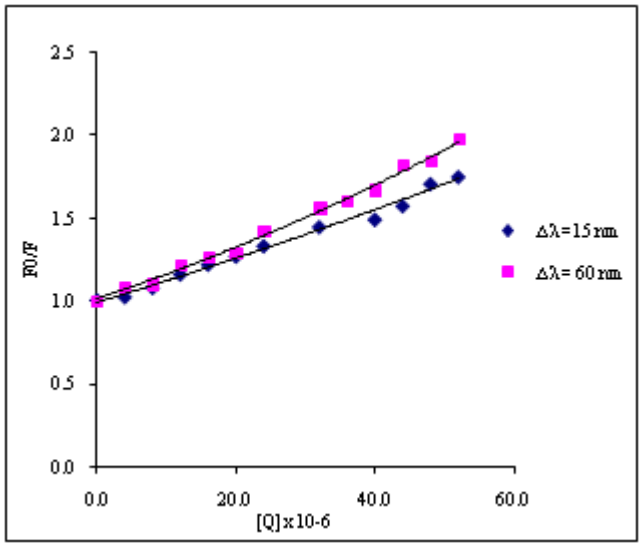

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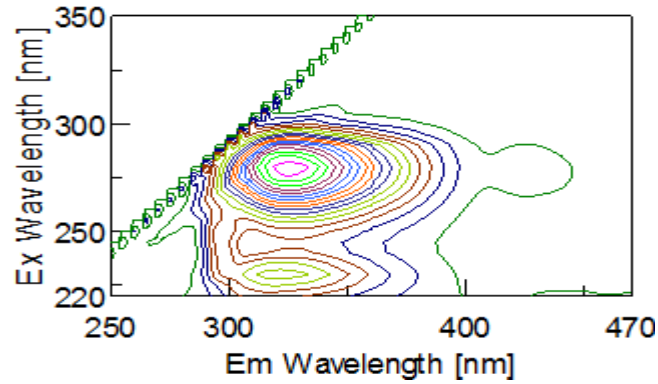
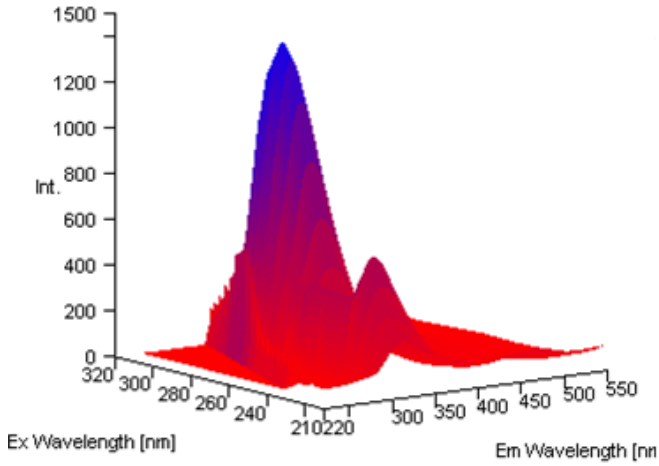
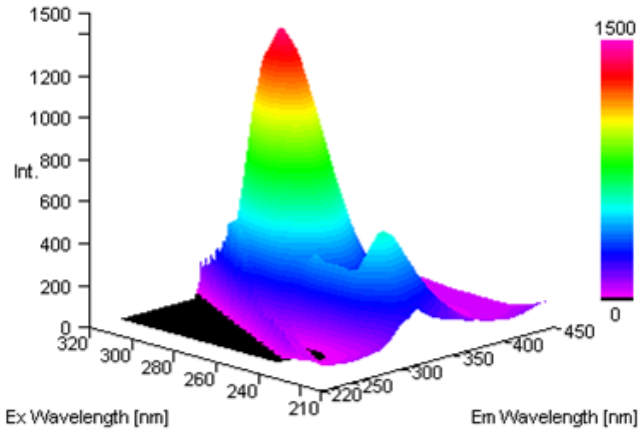
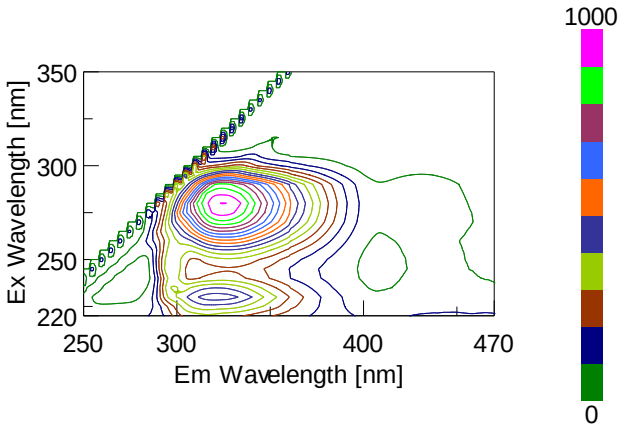


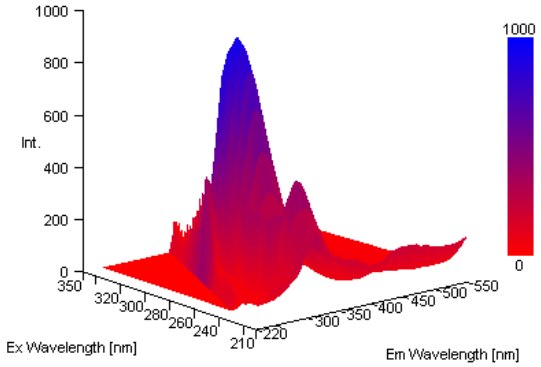


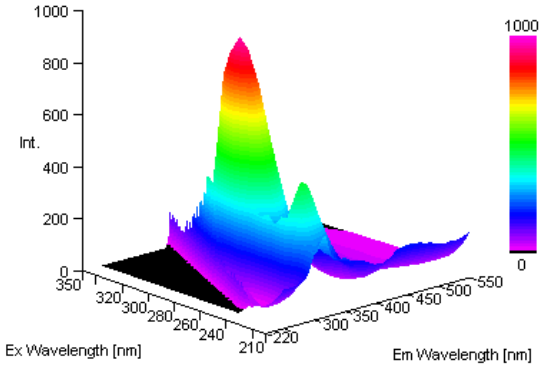


HHb + PABr

Figure 16:







Tables

Table 1: Stern-Volmer quenching constants and bio-molecular quenching rate constants for HHb-PABr at various temperatures

pH	T (K)	10^{-4} K sv /(L mol^{-1})	10^{-12}Kq /(Lmol-1 s^{-1})	R
7.4	301	2.53	2.53	0.9958
	311	2.21	2.21	0.9961
	321	1.88	1.88	0.9955

R = correlation constant

Table 2: Binding constants (K) and number of binding sites (n) of competitive experiment of HHb- PABr system.

T(K)	10^{-4} K/(Lmol^{-1})	n	R
301	1.0452	0.9056	0.9977
311	1.5743	0.9694	0.9967
321	2.0883	1.0143	0.9971

R = correlation constant

Table 3: Thermodynamic parameters of HHb- PABr interaction at pH = 7.4.

T (K)	*ΔH (KJ mol^{-1})*	*ΔG (KJ mol^{-1})*	*ΔS (J$mol^{-1}K^{-1}$)*	*R*
301	28.295	-23.119	170.81	0.9978
311		-24.8271		
321		-26.5353		

R = correlation constant

Table 4: Bar diagram showing thermodynamic parameters of the HHb with PABr at 303 K.

ΔH(KJ/mole)	**Δ G (KJ/mole)**	**TΔ S (KJ/mole)**
28.295	- 23.119	51.41

R = correlation constant