

Investigating the Effects of Formetanate Hydrochloride on Human Hemoglobin by Spectroscopic Analysis and Molecular Docking Modeling

Ali Khatibi Sharifiyeh (PhD)

Department of Biotechnology, Faculty of Biological Sciences, Alzahra University, Sheikh Bahai Square, Tehran, Iran

Elmira Rezaei Pajouhesh (MSc Student)

Department of Biotechnology, Faculty of Biological Sciences, Alzahra University, Tehran, Iran

Zahra Moosavi-Nejad (PhD)

Department of Biotechnology, Faculty of Biological Sciences, Alzahra University, Tehran, Iran

Corresponding author: Ali Khatibi Sharifiyeh

Tel: +98-9124970501

Email: khatibi@alzahra.ac.ir

Address: Department of biotechnology, Faculty of biological sciences, Alzahra University, Sheikh Bahai Square, Tehran, Iran

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ABSTRACT

Background and Objectives: Formetanate hydrochloride is an effective insecticide and acaricide, which is frequently used in citrus gardens. Given its high toxicity, it is necessary to evaluate effects of this insecticide on living factors. In this study, we investigate effects of formetanate hydrochloride on human hemoglobin using spectroscopic analysis and molecular docking modeling.

Methods: Effects of formetanate hydrochloride on human hemoglobin were investigated using molecular docking technique and thermodynamic and spectrophotometric methods such as normal and synchronous fluorescence spectroscopy, UV-Vis absorption spectroscopy and circular dichroism.

Results: Decrement of the soret band without any significant shift proved that the insecticide could penetrate into the heme pocket and directly interact with the porphyrin ring. Results obtained from the intrinsic fluorescence spectroscopy and heme degradation study at 321 and 460 nm indicated that formetanate hydrochloride could potentially destroy the heme prosthetic group and eliminate its filtering effect, which results in fluorescence enhancement. The fluorescence study demonstrated a competitive behavior between hydrophobic probe ANS and formetanate hydrochloride for binding to the hemoglobin hydrophobic patches. Results of circular dichroism and synchronous fluorescence spectroscopy confirmed the structural change and polarity alteration around aromatic fluorophores.

Conclusion: Our findings elucidate that the binding of formetanate hydrochloride to hemoglobin through the heme pocket is energetically much more favorable compared to binding to the hydrophobic central cavity. In addition, formetanate hydrochloride can cause conformational changes in the human hemoglobin and the competitive behavior of this insecticide for the oxygen site can subsequently reduce the oxy form of hemoglobin.

Keywords: Hemoglobin, Insecticides, Protein Degradation, Fluorescence.

INTRODUCTION

Hemoglobin (Hb) plays a crucial role in the vascular system of human beings as a carrier of oxygen. According to its atomic structure, hemoglobin is a tetrameric protein composed of two identical α and two identical β chains each consisting of 141 and 146 amino acid residues, respectively that are held together by non-covalent bonds. They share a common tertiary structure that makes the globin part of each subunit to form a hydrophobic pocket that envelopes the heme prosthetic group. Approximately 75% of the globin chains are found in α -helical structure (1).

The 89th residue in the α chains and the 92nd residue in the β chains are called proximal histidine (his F8), which is attached to a ferrous iron atom through its imidazole nitrogen and links the globin part to prosthetic group (2). In general, the prosthetic heme group plays a key role in cooperative interaction between hemoglobin and its natural ligands (3). Hence, structural properties of Hb makes it a vital factor for electron and proton transport to different parts of body, regulation of blood pH, oxygen and carbon dioxide transport (4). Formetanate hydrochloride [m-(((dimethylamino)methylene)-amino)phenylmethylcarbamate hydrochloride] is an effective insecticide and acaricide which is frequently used in citrus gardens. This compound consists of two functional groups: carbamate moiety that acts as an acetylcholinesterase inhibitor, which primarily affects the peripheral somatic and autonomic nervous systems; and formamidine moiety that inhibits the neurotransmitter octopamine during a competitive process that stimulates insects' nerves, leading to convulsion (5, 6). Formetanate hydrochloride is considered an environmental pollutant with adverse effects on target and non-target creatures. Due to its ionic structure, high water solubility (>50 g 100 mL⁻¹) and poor soil absorption, this insecticide is able to flow on the agricultural fields and contaminate groundwater and surface water. Furthermore, it is resistance against hydrolysis in natural water, which increases its persistence in the environment. Exposure to insecticides, either directly (oral, inhalation or contact with eyes and skin) or indirectly (from agricultural products to body), subsequently threaten the life of creatures, especially human beings. Similar to other carbamate insecticides, formetanate

hydrochloride is widely used around the globe. Considering its high toxicity (LG50 (rat) = 14.8 mg.kg⁻¹), it is essential to investigate effects of this compound on living factors (7). In this study, we investigate effects of this insecticide on human Hb.

MATERIALS AND METHODS

Human Hb and formetanate hydrochloride were purchased from Sigma Aldrich Company. Tris base was obtained from S.R.L (India) and NaCl and HCl were from Merck (Germany). All buffers (Tris [0.05 M] and TBS [0.05 M tris + 0.15 M NaCl]) were prepared using double distilled water in pH=7.4. Stock solution of human Hb (100 μ M) was diluted to 3 μ M for spectroscopic studies. A concentration range of 7.5 to 255 μ M was prepared from the stock solution of formetanate (1.5 mM). After adding the prepared concentrations of formetanate hydrochloride to Hb samples, UV-Vis spectrum was recorded at 200 to 500 nm and 25 °C using a Uv-Vis spectrophotometer (Cary 100, Varian Co., Australia) equipped with 1 cm quartz cells. Uv-Vis absorption measurement is an applicable technique for investigating conformational changes and complex formation (8).

Protein thermal denaturation curvature gives information about structural stability of proteins against increasing course of temperature that can be described by the T_m value. In fact, T_m is a temperature where the protein is semi-folded; therefore, more stable proteins have higher T_m values (9, 10). By studying protein thermal stability at 280 nm in an incremental range of temperature from 25°C to 75 °C at a rate of 2°C min⁻¹, we determined the protein conformational change and subsequently its T_m value using the Cary 100 bio spectrophotometer (Varian Co., Australia).

Protein aggregation potential upon addition of formetanate hydrochloride was assessed by recording the increasing absorption at 360 nm for 1000 seconds at a temperature of 68 °C. Moreover, intrinsic fluorescence spectra were recorded after adding different concentrations of formetanate hydrochloride to Hb samples using a spectrofluorometer (Cary eclipse, Varian Co., Australia) at 25 °C, 33 °C and 37°C from 290 to 450 nm while excitation wavelength was fixed at 280 nm and excitation

and emission slits were 5 and 10 nm, respectively. To detect the insecticide-induced heme degradation products, all samples were excited at 321 and 460 nm and their emission spectra were collected from 345 to 600 nm and 470 to 700 nm, respectively. To identify the ANS competitive binding to Hb hydrophobic patches in the presence of formetanate hydrochloride, 250 μM ANS was added to the samples containing 3 μM Hb and different concentrations of the insecticide. Then, emission spectra were recorded at 25 $^{\circ}\text{C}$ from 430 to 580 nm at the excitation wavelength of 380 nm.

Far UV-circular dichroism (CD) spectra of Hb in the absence and presence of formetanate hydrochloride (5-40 μM) were recorded from 195 to 200 nm using a CD spectrofluorimeter (215, Aviv Co., USA) at 25 $^{\circ}\text{C}$ and pH=7.4.

Generally, like quenching phenomenon, fluorescence enhancement may result from ground-state complex formation, excited state reaction, energy transfer and collisional processes. The Stern-Volmer equation can describe how a ligand interacts with a macromolecule and cause fluorescence quenching or enhancement. In this regard, fluorescence enhancement data were investigated using the following equation: $F_0/F = 1 - K_D[E] = 1 - k_B\tau_0[E]$ (1). According to the Stern-Volmer equation, F_0 and F are fluorescence intensities in the absence and presence of enhancer ligand (formetanate hydrochloride), K_D is the dynamic constant of fluorescence enhancement, k_B is the rate constant of biomolecular fluorescence enhancement, τ_0 is the average lifespan of macromolecule in the absence of enhancer and $[E]$ is the molar concentration of the enhancer molecule. In case of fluorescence enhancement following ligand-macromolecule interaction through equivalent binding sites, the binding parameters can be calculated using the following equation: $\log(F - F_0)/F = \log K_b + n \log [E]$ (2). In this equation, F and F_0 are the maximum fluorescence intensities in the

absence and presence of enhancer ligand $[E]$, K_b is the binding constant and n is the number of binding sites. By plotting $\log(F - F_0)/F$ versus $\log [E]$, n and K_b values can be obtained from the slope and intercept, respectively.

Interaction between small molecules and biological macromolecules can occur through four classical non-covalent forces: hydrogen, electrostatic, hydrophobic and van der Waals bonds (11). The type of binding forces between formetanate hydrochloride and Hb can be determined by calculating the temperature-dependent thermodynamic parameters: enthalpy change, entropy change and Gibbs free energy change. If the ΔH° (enthalpy change) gradually changes over the temperature range studied, both ΔH° and ΔS° (entropy change) values can be obtained from the van't Hoff equation. By graphing $\ln k$ versus $1/T$ as reciprocal absolute temperature, based on the van't Hoff equation, thermodynamic parameters, such as enthalpy and entropy can be calculated: $\ln K_a = -(\Delta H^{\circ}/RT) + (\Delta S^{\circ}/R)$, where K_a and R are the association constant and gas constant, respectively (3). To elucidate the Gibbs free energy change of reaction, ΔH° and ΔS° values were placed in the following equation: $\Delta G^{\circ} = \Delta H^{\circ} - (T\Delta S^{\circ})$ (4).

Molecular docking is an effective computational method used to forecast the probable binding site of drugs or ligands onto a protein. It can help validate the experimental results. To identify the most favorable ligand binding sites on Hb, we carried out molecular docking using the Molegro Virtual Docker software (MVD 2008 version 3.2.1). The crystal structure of human Hb was downloaded from the Protein Data Bank (PDB code: 1GZX) and the atomic structure of formetanate hydrochloride was designed using PRODRG server. After minimizing the energy of the system (Hb and formetanate hydrochloride), docking process was performed by the software.

Figure 1- Absorption spectra of Hb and formetanate-Hb complex. Concentration of Hb was 3 μM . Formetanate concentrations in the formetanate-Hb complex were 30, 90, 120, 150 and 255 μM .

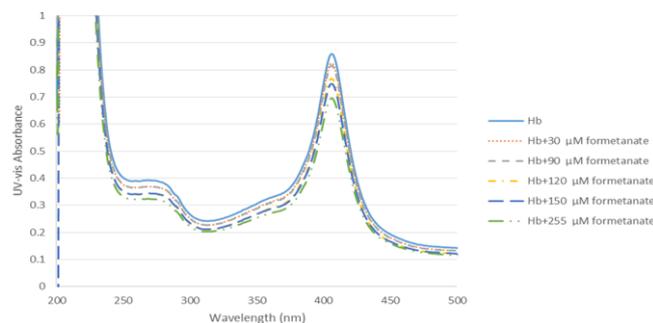


Figure 2- Absorption spectra of Hb at 500-600 nm related to oxy-Hb. Concentration of Hb was 3 μ M. Formetate concentrations in the formetate-Hb complex were 30, 90, 120, 150 and 255

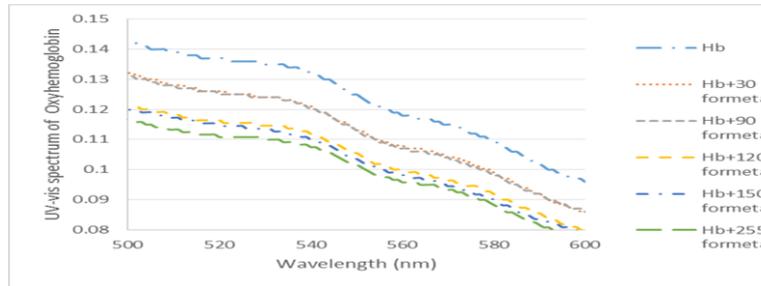


Figure 3- Thermal denaturation profile of Hb with increasing concentrations of formetate at pH 7.4 and 280 nm. Formetate concentrations in the formetate-Hb complex were 45, 52.5 and 60 μ M.

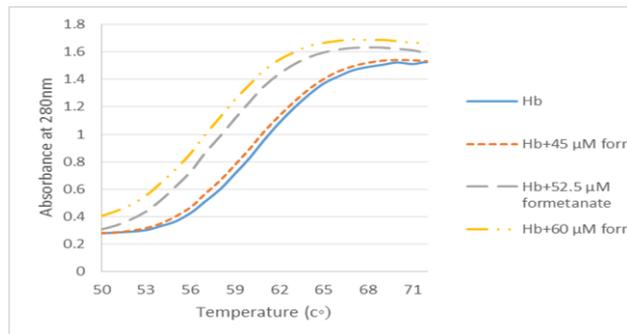
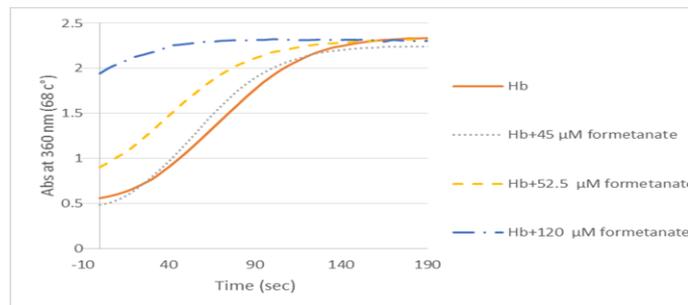


Figure 4- Aggregation potential of Hb in the absence and presence of formetate at concentrations of 45, 52.5 and 120 μ M and pH 7.4.



RESULTS

As shown in figure 1, increasing the concentration of formetate hydrochloride caused hypochromicity in all three main parts of the Hb absorption spectra without any significant shift. The first region (at 285 nm) was related to globin absorption. The second region (Soret band at 406 nm) was related to heme iron porphyrin complex absorption, and the third region (Q band at 550-600 nm) was related to the oxy- and deoxy-forms of heme. However, no shift was observed towards lower or higher wavelengths. Furthermore, diminishing the characteristic peak at 406 nm without any displacement indicated that formetate hydrochlorid penetrated into the heme pocket and directly interacted with the heme group without exposing it to the solvent

, T_m (midpoint transition temperature) was obtained from thermal denaturation curves at a fixed wavelength of 280 nm. As shown in figure 3, with gradual addition of formetate hydrochloride, T_m values decreased (by 4 °C in the sample with higher concentrations of insecticide). Increase in concentration of formetate hydrochloride reduced the lag phase and increased the slope of the logarithmic phase (Figure 4).

Fluorescence intensity measurement gives useful information about protein conformational changes and complex formation. We observed that increasing the concentration of formetate hydrochloride to

the Hb solution introduced a significant red shift in intrinsic fluorescence intensity of free protein. Two different heme degradation products exhibit fluorescence emission at the wavelengths of 465 and 550 nm after excitation at 321 and 460 nm, respectively. As

shown in figures 5 and 6, binding of formetanate hydrochloride to Hb induced heme degradation.

This is also evident from the increase in fluorescence intensity at 465 nm and 550 nm.

Figure 5- Effect of formetanate on fluorescence spectrum of Hb (T = 295 K, pH 7.40, $\lambda_{ex} = 280$ nm). Concentration of Hb was 3 μ M. Formetanate concentrations in the formetanate-Hb complex were 7.5, 22.5, 37, 52.5, 120, 180 and 255 μ M.

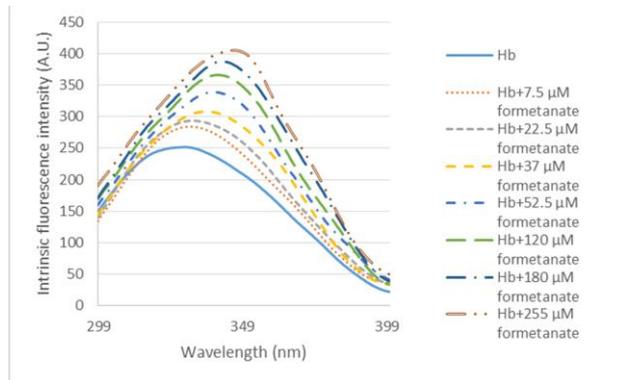


Table 1- Content of Hb secondary structures before and after the addition of formetanate

Secondary structures	Hb	Hb+5.7 μ M Formetanate	Hb+5/22 μ M Formetanate	Hb+45 μ M Formetanate	Hb+52.5 μ M Formetanate	Hb +60 μ M Formetanate
Alpha helix	44.37%	36.28%	35.31%	30.81%	26.86%	26.55%
Antiparallel	3.94%	5.14%	5.19%	7.25%	8.55%	8.39%
Parallel	6.9%	8.28%	8.49%	9.23%	10.02%	10.12%
Beta turns	14.29%	14.76%	14.63%	15.55%	15.73%	15.6%
Random coils	30.47%	35.52%	36.44%	37.13%	38.82%	39.32%

Table 2- Dynamic and bimolecular enhancement constants for the Hb- formetanate interaction at different temperatures

T (K)	K_D (M^{-1})	k_B ($M^{-1} s^{-1}$)	R^2
295	969	969×10^{-8}	0.9
303	664	664×10^{-8}	0.9
310	565	565×10^{-8}	0.9

Table 3- Association constants and the number of binding sites for the Hb- formetanate interaction

T/K	K_a (M^{-1})	n	R^2
295	8.6	0.37	0.96
303	6.1	0.4	0.97
310	6.7	0.5	0.94

Table 4- Thermodynamic parameters for the Hb-formetanate interaction at different temperatures

T/K	ΔH° ($kJ.mol^{-1}$)	ΔS° ($J.mol^{-1}.K^{-1}$)	ΔG° ($kJ.mol^{-1}$)	R^2
295	-45/6	-133/97	-6.36	0.99
303			-5.3	
310			-4.37	

$$F_0/F = 1 - K_D[E] = 1 - k_B \tau_0 [E] \quad (\text{eq.1})$$

$$\text{Log } (F - F_0)/F = \text{log } K_D + n \text{ log } [E] \quad (\text{eq.2})$$

$$\text{Ln } K_a = -(\Delta H^\circ/RT) + (\Delta S^\circ/R) \quad (\text{eq.3})$$

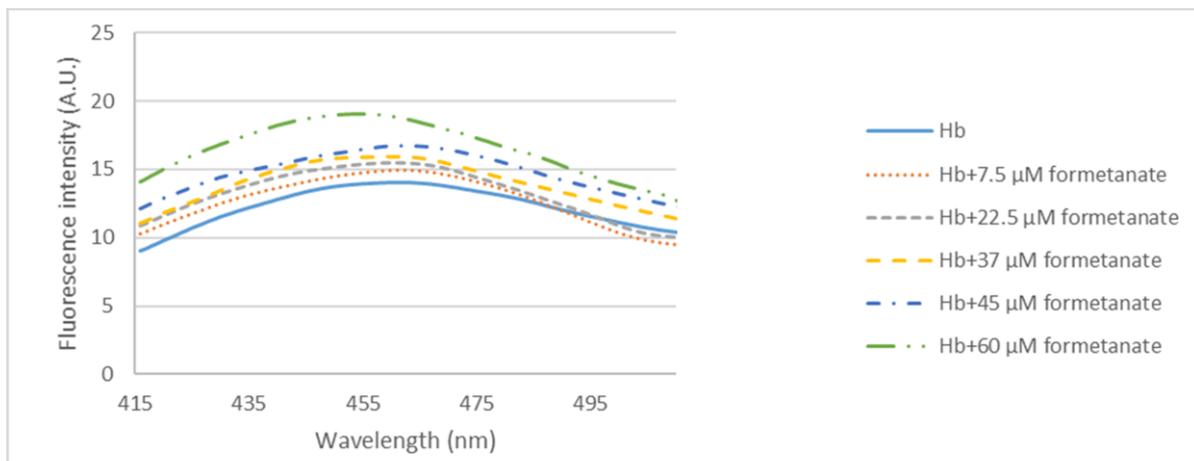
$$\Delta G^\circ = \Delta H^\circ - (T\Delta S^\circ) \quad (\text{eq.4})$$

Figure 7 shows the CD spectra of Hb and Hb-formetate hydrochloride complex. In the far UV-CD spectrum, we observed two negative peaks at 208 and 222 nm that related to $\pi \rightarrow \pi^*$ transition in α -helix and $n \rightarrow \pi^*$ transition in both α -helix and random coil, respectively. Loss of helicity was evident when the concentration of insecticide was raised. The association constant and the number of binding sites were obtained at three different temperatures (295, 303 and 310 K) from the slopes of the $\log(F-F_0)/F$ versus $\log[Q]$ plots (eq.2). As temperature increased, the binding constants reduced due to destabilization of the Hb-formetate hydrochloride complex. It should be noted that unlike K_D , high temperature has a negative effect on K_a and decreases stability of complexes. The number of binding sites was found to be 0.5, indicating a 1:2 ratio between hemoglobin and formetate hydrochloride during the binding process.

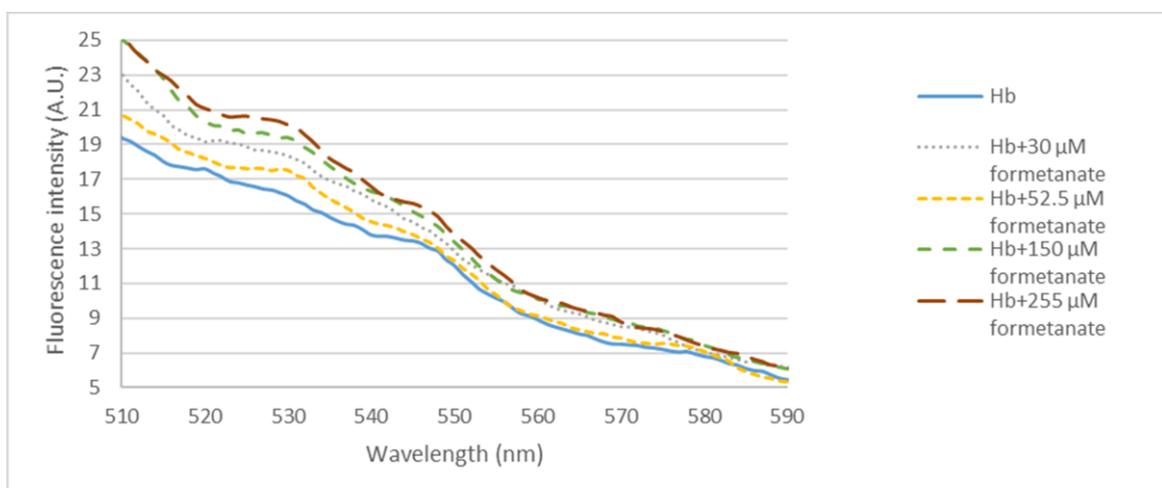
Table 4 represents the thermodynamic parameters for the interaction of formetate hydrochloride with Hb at different temperatures.

According to molecular docking results, although formetate hydrochloride could attach to both Hb central cavity and the hydrophobic pocket, the docking energy for binding of formetate hydrochloride to the hydrophobic pocket (-100/518) is slightly greater than that for binding to the central cavity (-83/875). Figure 8 displays the most favorable binding sites for formetate hydrochloride in the hydrophobic pocket of Hb. In this region, formetate hydrochloride is located near the hydrophobic residues (such as Ala, Trp and Pro) and can easily interact with the prosthetic heme group. This observation is in agreement with the results obtained from UV-Vis and fluorescence spectroscopy and heme degradation study

Figure 6- Fluorescence spectra of heme degradation products at $\lambda_{ex}=321$ nm (A) and $\lambda_{ex}=460$ nm (B), with increasing formetate concentration (pH=7.4 and 295 K).

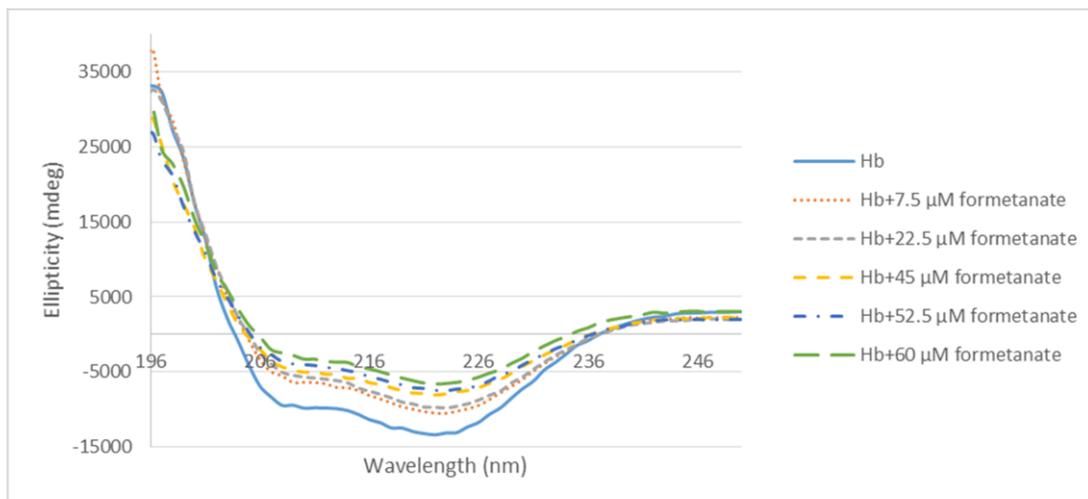


A



B

Figure 7- The far UV-CD spectra of Hb before and after the addition of formetanate (at 25 °C and pH 7.4)



DISCUSSION

The absorbance decrement at 275 nm that arises from aromatic amino acids such as Trp, Thy and Phe demonstrated the occurrence of an interaction between Hb and formetanate hydrochloride (9). Decrement of the oxyHb related peaks at 541 and 577 nm stipulates the degradation of the heme prosthetic group, which impairs functionality in the active sites and reduces Hb oxygen affinity (11, 12). The results of protein aggregation study elucidated that formetanate hydrochloride could aggregate Hb. A thermal aggregation curve comprises of three parts: a lag phase (representing duration of resistance against thermal aggregation), a logarithmic phase (where the protein starts unfolding) and a plateau phase (in which the protein is fully destroyed) (13). A more stable protein has a larger lag phase and its logarithmic phase has a slow slope (12-14). As mentioned previously, hydrophobic interactions have a main role in the formetanate hydrochloride-Hb interaction and protein denaturation. Therefore, the possibility of aggregation was enhanced by the increased hydrophobic forces on the Hb surface resulting from increasing concentrations of formetanate hydrochloride. The heme prosthetic group is able to interfere in the emission spectrum of aromatic residues at high Hb concentrations. In other words, heme-filtering phenomenon is responsible for 99% of non-radiative quenching of aromatic fluorophores emission (15-17). Incremental course of emission intensity could

be explained by the heme degradation process and termination of its filtering effect (18, 19).

Alteration in the polarity around aromatic residues was related to displacement of the maximum peaks towards the longer wavelengths. As explained previously, the interaction between formetanate hydrochloride and Hb exposes hydrophobic patches on protein surfaces to the solvent (20).

Reduction of the CD intensity in the presence of formetanate hydrochloride elucidated that the insecticide may be able to induce conformational changes in the secondary structure of Hb (21, 22)

Based on the results of thermodynamic parameters and the negative values of both ΔH° and ΔS° , it can be concluded that the binding process is enthalpy driven and the hydrogen bonds and van der Waals forces are involved in the binding reaction and complex stability. In addition, the negative amount of ΔG° confirms that the binding process is spontaneous (23).

CONCLUSION

Our findings demonstrate that formetanate hydrochloride can cause conformational changes in the human Hb. Higher affinity of formetanate hydrochloride to penetrate the hydrophobic pocket results in heme destruction and elimination of heme filtering group.

Furthermore, the competitive behavior of this insecticide for the oxygen site can subsequently reduce the oxy form of Hb.

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CONFLICTS OF INTEREST

There is no conflict of interest regarding publication of this article.

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