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INTERACTION OF FANSIDAR WITH NORMAL HAEMOGLOBIN AT pH 5.0 and pH 7.2: A FOURIER TRANSFORM INFRARED (FTIR) STUDY

*Amalunweze, A.E., Okoye, O.G., and Ezumezu, C.P.

Science Laboratory Technology Department, Federal Polytechnic, Oko, Anambra State

**Author's corresponding email: adaude.amalunweze@gmail.com*

Abstract

On the assumption that resistance to malaria is related to the structure of the haemoglobin molecule, a comparative study was designed to determine the interaction of antimalarial drug, Fansidar with normal haemoglobin (HbA) at pH 5.0 and pH 7.2 using Fourier Transform Infrared (FTIR) Spectrophotometry in the mid-infrared region (4000-200 cm^{-1}). Fansidar, a combination of Pyrimethamine (Dihydrofolate reductase inhibitor) and Sulphadoxine (PABA inhibitor) is active against the asexual erythrocytic stages of *Plasmodium falciparum* and may be effective against strains of *P. falciparum* resistant to chloroquine. Genotype AA blood sample was collected from the University of Nigeria, Medical Centre, Nsukka. The blood was centrifuged and purified to obtain crude haemoglobin A. The crude haemoglobin was dialyzed at 4 ^{o}C for 12 hours against 50mM Tris-HCl buffer of pH 7.2. The FTIR results showed that at pH 5.0, in the amide I region (1700 – 1600 cm^{-1}) comprising the proteins secondary structural components (α-helix, β-sheets, random and turn structures), there were significant changes in absorbance for HbA while at pH 7.2, little or no significant change in absorbance were observed. The results suggest that HbA is easily destabilized at pH 5.0, than at pH 7.2, on interaction with Fansidar. These haemoglobin-drug interactions may hinder the development of the Plasmodium falciparum at the intraerythrocytic stage and may account for the novel strategies of monitoring HbA aggregation during malarial infection as well as improvements in administering effective antimalarial treatment. The interaction of Fansidar with HbA at pH 5.0 and pH 7.2 can be likened to the iron-ligand interactions between α and β subunits of haemoglobin. Understanding the interaction of antimalarial drugs with haemoglobin has allowed the identification of essential processes and metabolic weak points that could be exploited to combat this scourge to humanity.

Keywords: *Malaria, Antimalarial, FTIR, Haemoglobin, ligand, Plasmodium falciparum*

Introduction

Malaria is a mosquito-borne infectious disease caused by the eukaryotic protists of the genus, *Plasmodium* (Sutherland *et al.*, 2010). Of the four species of *Plasmodium* that cause malaria, *Plasmodium falciparum* is responsible for the majority of illness and death in humans by extensive degradation of haemoglobin (Hb) (Duraisingh and Refour, 2005). The main symptoms of severe malaria include low blood sugar, low blood haemoglobin, breathing difficulties and coma (Beare *et al.*, 2006). The clinical manifestations of malaria primarily result from the proliferation of the parasite within the hosts' erythrocytes (Ezebuo *et al.*, 2013). During this process, haemoglobin is utilized as the predominant source of nutrition. Haemoglobins are tetrameric conjugate proteins comprised of pairs of two different polypeptide subunits and a prosthetic haem group, which contains iron (Nelson and Cox, 2008). Individuals heterozygous for the HbS gene are reported to be resistant to malaria (Chotivanich *et al.*, 2002; Williams *et al.*, 2005), thus are favored to survive in a malaria-endemic environment, such as in sub-Saharan Africa. Attempts have been made to correlate possession of sickle haemoglobin (HbS) with resistance to malaria especially the underlying biochemical mechanisms (Chilaka and Moosavi-Movahedi, 2005; Nwamba and Chilaka, 2010; Chilaka *et al.*, 2011; Nwamba *et al.*, 2011; Akwudike *et al.*, 2011; Ezebuo *et al.*, 2012; Nwamba *et al.*, 2013). Fourier transform infrared spectroscopy (FTIR) is a measurement of wavelength and intensity of the absorption of infrared radiation by a sample and in the case of proteins, FTIR can be used to determine the number of different types of secondary structures present.

The IR spectral data of high polymers are usually interpreted in terms of the vibrations of a structural repeat unit (Krimm and Banderkar, 1986). The polypeptide and protein repeat units give rise to nine characteristic IR absorption bands, namely, amide A, B, and I–VII. Of these, the amides I and II bands are the two most prominent vibrational bands of the protein backbone (Krimm and Banderkar, 1986). The most

sensitive spectral region to the protein secondary structural components is the amide I band (1700–1600 cm^{-1}), which is due to the C=O stretch vibrations of the peptide linkages (Kong and Yu, 2007). Chloroquine and its derivatives are antimalarial drugs that have been effective in the treatment of malaria. Recently, many strains of the parasite have been identified, which possess the ability to become resistant to most, if not all, of the antimalarial agents presently available (Banerjee *et al.*, 2002; Hanspal *et al.*, 2002). It becomes imperative, therefore, to seek new modalities in the treatment of malaria. Sulphadoxine and Pyrimethamine, the constituents of Fansidar, are folic acid antagonists that inhibit the activity of dihydropteroate synthase and dihydrofolate reductase respectively. These enzymes are important in the asexual erythrocytic stages of *Plasmodium falciparum*. Fansidar may also be effective against strains of *P. falciparum* resistant to chloroquine. The parasite digests haemoglobin within its digestive vacuole with an estimated acidic pH of 5.0 - 5.5 through a sequential metabolic process involving multiple proteases and Fansidar has been shown to repress malaria symptoms by inhibiting the growth and reproduction of the parasite, it is therefore expected to interact with haemoglobin. This haemoglobin-drug interaction can be studied using FTIR.

Materials and Methods

Materials

Pure samples of the antimalarial drug, Pyrimethamine and Sulphadoxine were obtained from Juhel Nigeria Limited, Enugu State. Other chemicals used in this work obtained from BDH, England and Sigma, Germany are of analytical grade.

Methods

Collection of Blood Samples

After informed consent, four millilitres (4 mL) of venous blood were collected from each of the identified individual of genotype AA at the University of Nigeria Medical Centre, Nsukka. Each of the blood samples was collected and stored in an ethylene-di-amine-tetra acetic acid (EDTA) vial.

Activation of Sephadex G-25

A known quantity, 20g of Sephadex G -25 was weighed and heated to boiling in excess distilled water. The heated gel was soaked in 300 ml of distilled water and allowed to stand for 3 days to swell up. After that, it was packed into a chromatographic column of 2.5 x 70 cm and equilibrated with 50 mM sodium acetate buffer, pH 7.0.

Isolation and Purification of Haemoglobin

Crude haemoglobin was extracted from the blood samples by employing differential centrifugation technique as described by Denninghoff *et al.* (2006). Four mL of the blood samples - AA was combined with 6 mL of normal saline in 50 mM Tris-HCl pH 7.2 (wash buffer) and centrifuged at 4 ºC for 10 minutes at 4000 rpm. Supernatants were removed via aspiration. The centrifugation was repeated 2-4 times until a clear supernatant was obtained. The clear supernatants were removed and the resulting pellets were made up to 5 mL with 50 mM Tris-HCl. The samples containing 50 mM Tris-HCl were kept in the freezer in order to lyse the red cells and 5% NaCl was added to the resulting volume and centrifuged for 10 minutes at 4000 rpm to remove inorganic phosphates and other ions present in the sample. After the differential centrifugation, the resulting supernatants (crude haemoglobin) were collected into separate vials and labelled appropriately. The crude haemoglobin (i.e. HbA) was dialyzed at 4 °C for 12 h against 50 mM Tris–HCl buffer, pH 7.2. The dialyzed haemoglobin samples were purified using the Sephadex G-25 gel and stored at -20 °C for further experiments.

Solubility

The drug samples were solubilized by the method of Kim *et al.* (2012). The drug dissolved in ethanol and low-molecular-weight succinoglycan dimer (D3) dissolved in water were mixed. The mixture was magnetically stirred at 25ºC for 24h, shielded from light to prevent degradation of the molecules. After equilibrium was reached, ethanol was evaporated using N_2 gas, and the mixture was lyophilized. The sample was dissolved in 0.5 mL water filtered and freeze-dried. The FTIR studies were carried out at pH 5.0 and pH 7.2.

FTIR Sample Preparation and Scan

The titration of the drugs with the haemoglobins was carried out by the method of Chilaka *et al.* (2011). Fourier transform infrared spectra of samples were recorded within the frequency range 4000 - 200 cm^{-1} on a Buck Model 500 EZ Scan Software Version 2.10 11212003 at National Centre for Energy Research and Development, University of Nigeria, Nsukka, Enugu State. As the background for each spectrum, infrared transparent potassium bromide material without the protein was scanned. Fourier transform infrared spectra were obtained by spreading a small volume of sample on a potassium bromide plate (IR transparent material) and allowed to dry for few minutes to remove the water bands. To minimize problems from voidable baseline shifts, the spectra were baseline corrected and normalized.

Results and Discussion

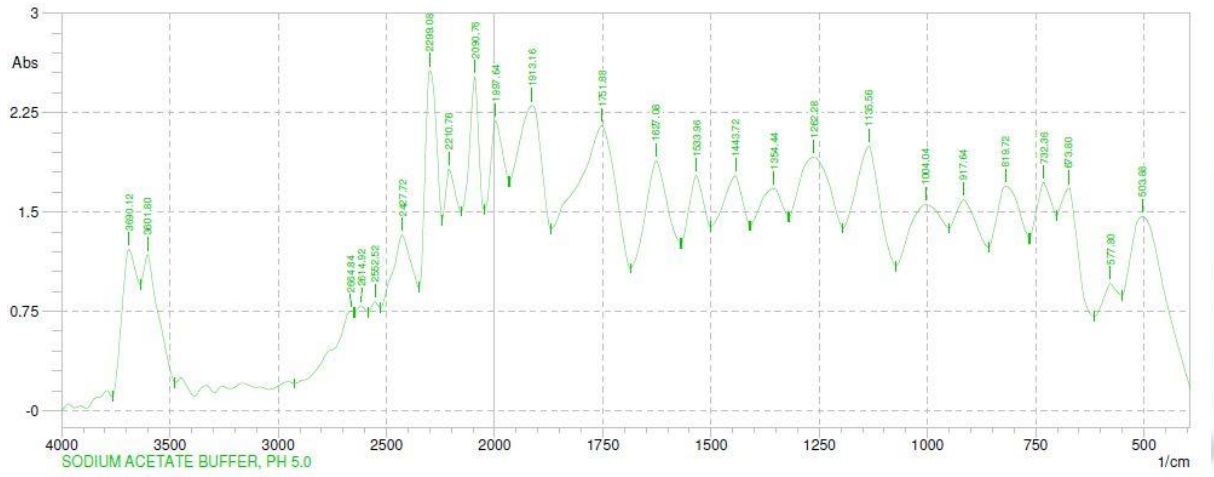

Figure 1: Absorption Spectra of Sodium Phosphate Buffer, pH 5.0

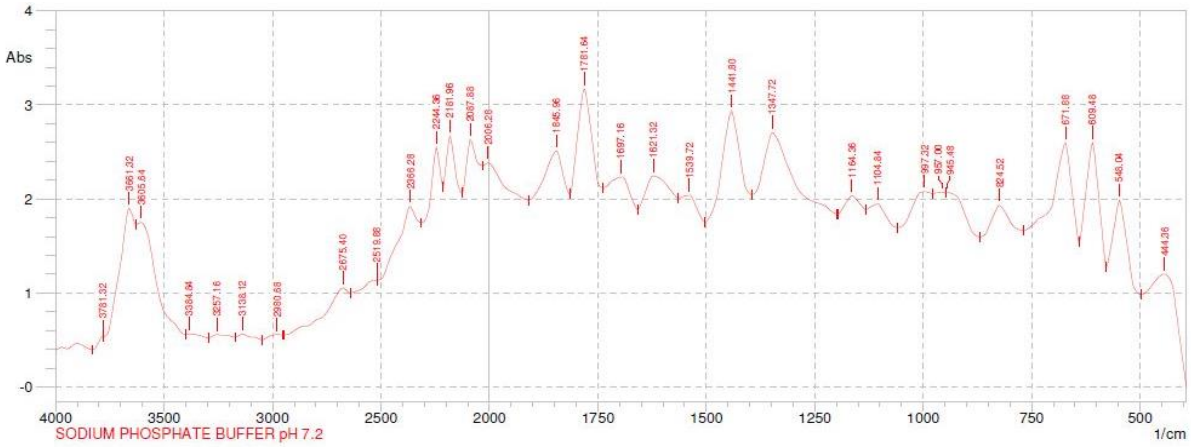

Figure 2: Absorption Spectra of Sodium Phosphate buffer, pH 7.2

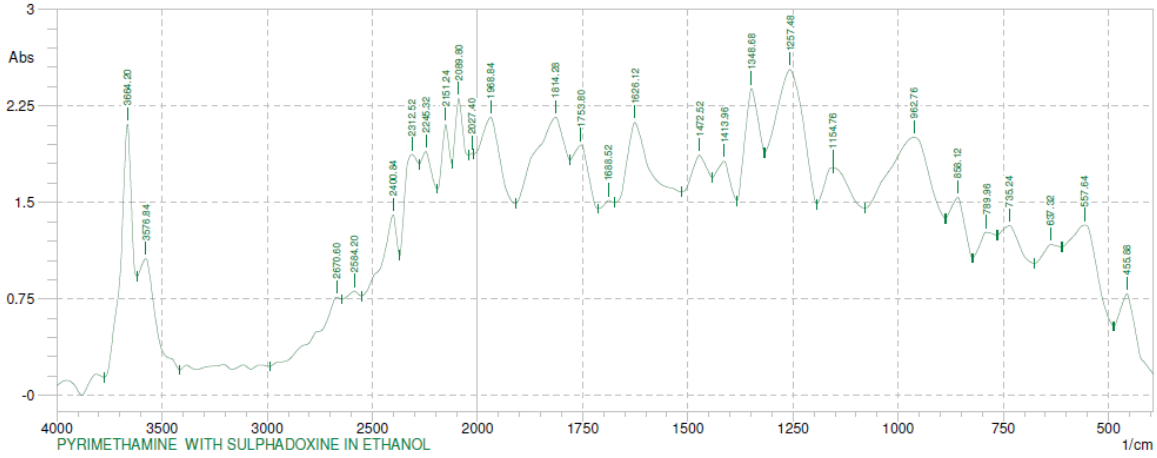

Figure 3: Absorption Spectra of Fansidar (Pyrimethamine and Sulphadoxine)

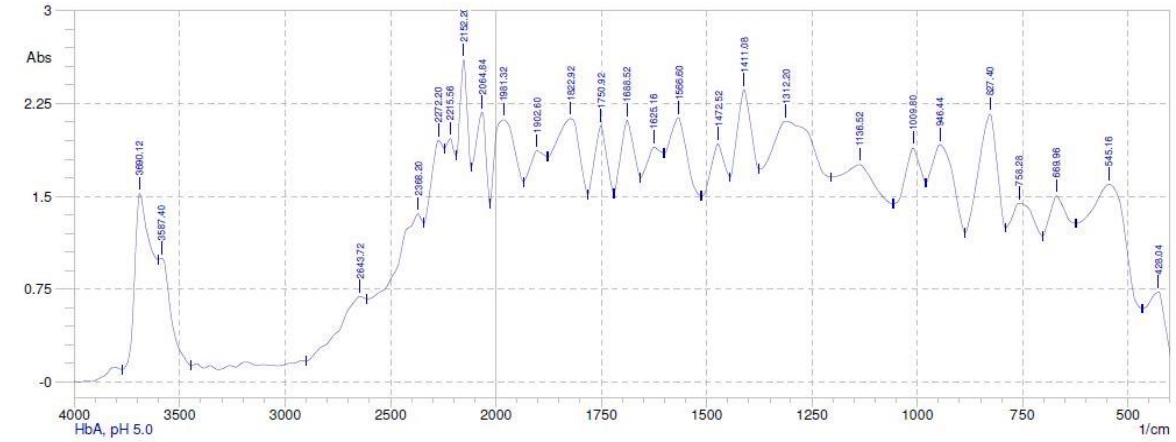

Figure 4: Absorption Spectra of HbA at pH 5.0

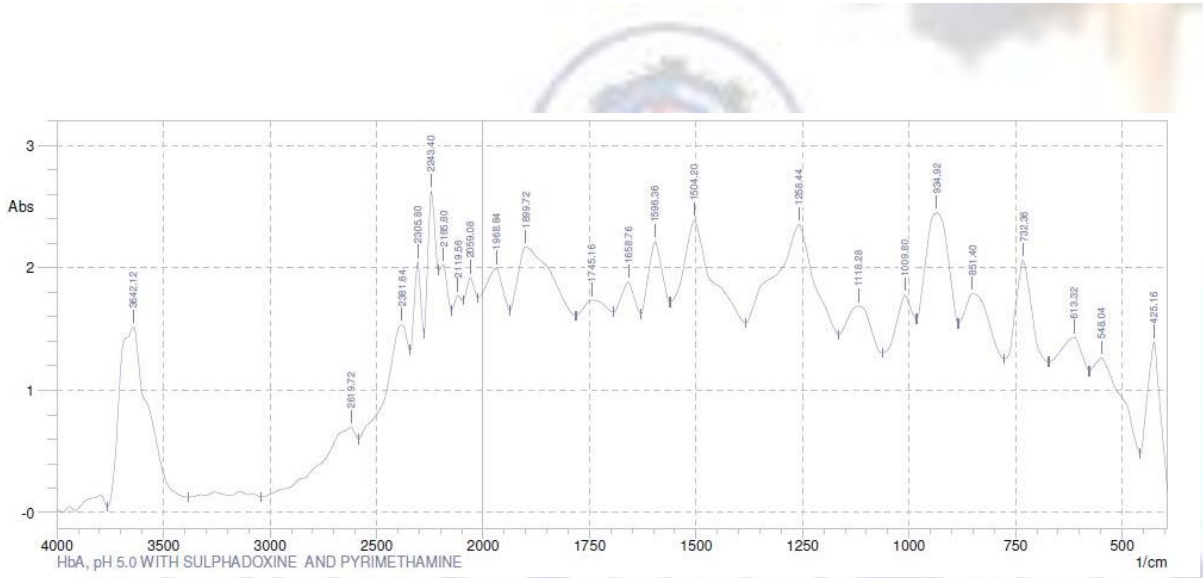

Figure 5: Absorption Spectra of HbA with Fansidar, pH 5.0

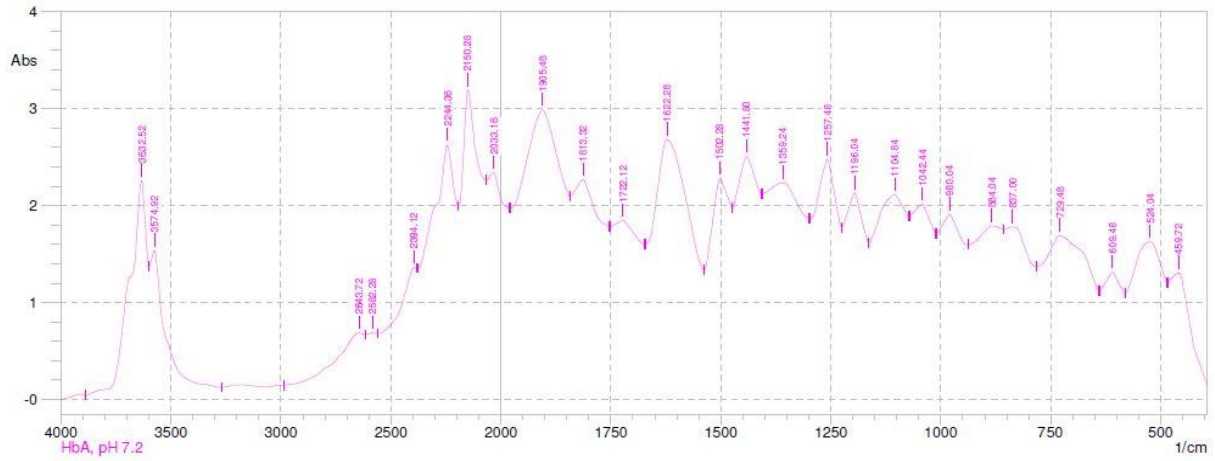

Figure 6: Absorption Spectra of HbA at pH 7.2

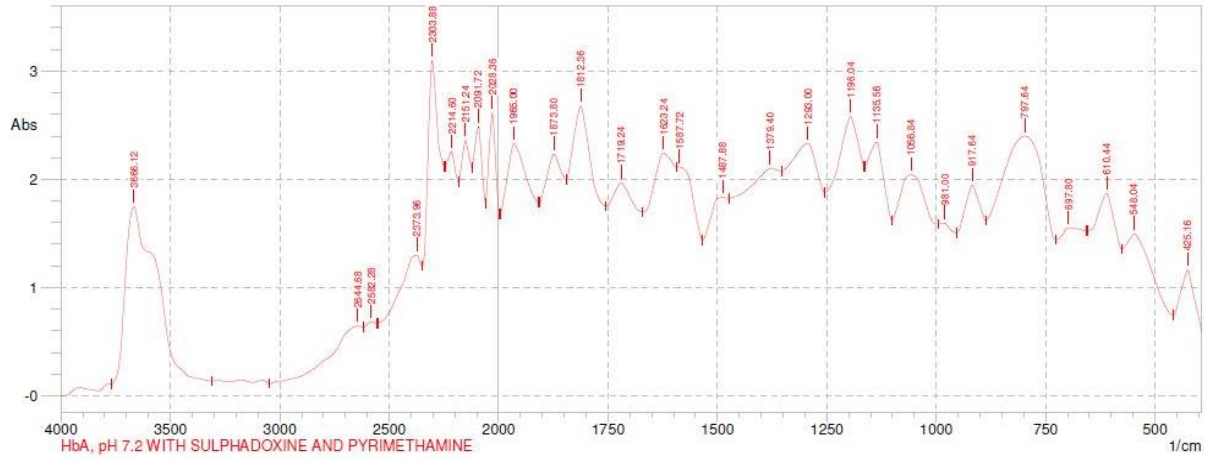

Figure 7: Absorption Spectra of HbA with Fansidar, pH 7.2

In the FTIR analysis, the samples were subjected to IR radiation. Titration of 0.1ml of HbA with Fansidar was performed at pH 5.0 and pH 7.2 respectively. Potassium bromide (KBr) was the window material of choice. It has a transmittance of 100% in the range of 4000 – 400 cm^{-1} region, thus it does not exhibit absorption in this range. The results of the FTIR spectra obtained were represented as Absorbance versus wavenumber. The IR spectrum analyzed under the mid-IR spectrum can be segregated into four regions namely 4000 – 2500 cm^{-1}, 2500 – 2000 cm^{-1}, 2000 -1500 cm^{-1}, and 1500 - 400 cm^{-1} representing the single bond region, the triple bond region, the double bond region and the fingerprint region respectively (Coates, 2000). Absorption spectra of Fansidar (figure 3) showed peaks around 4000 – 3576 cm^{-1} comprising of O-H, N-H, C-H functional groups, 2400 – 2027 cm^{-1} which depicts the presence of C≡C and C≡N functional groups, peak absorption in the 2000 – 1472 cm^{-1} region represented by the C=C, C=O, N=N, and C=N functional groups respectively.

The several absorption bands as observed in Figure 4 and 6 depicted that HbA is a complex macromolecular protein whose polypeptide and protein repeat unit produces nine characteristic IR absorption bands namely amide A, B, and I – VII. At pH 5.0, the absorption spectra of HbA showed vibrations in the range of 3650 – 3500 cm^{-1} indicating the presence of hydrogen bond and amino groups. This is followed by a weak absorption peak at 1654 cm^{-1}, strong absorption peaks at 1443 cm^{-1}, 1344 cm^{-1}, 792 cm^{-1}, 737 cm^{-1} and 576 cm^{-1} which may indicate the amide I (C=O stretching), amide II (CN stretching, NH bending), amide III (CN stretching, NH bending), amide IV (OCN bending), amide V (out-of-plane NH bending) and amide VI (out-of-plane C=O bending) bands respectively. At pH 7.2, the absorption spectra of HbA in a buffer as represented in figure 6 showed weak absorption peaks at 3300 and 3100 cm^{-1} depicting the amide A and amide B bands respectively. A weak absorption peak was observed at 1653, 1443, 1287- 1225, 732, 793, and 597 cm^{-1} which may be assigned to the amide I, amide II, amide III, amide IV, amide V and amide VI bands respectively.

On the interaction of HbA with Pyrimethamine and sulphadoxine combination therapy, sharp absorption peaks were observed at 1603, 640 and 547 cm^{-1} at pH 7.2 while at pH 5.0, amide I band were observed around 1688 and 1626 cm^{-1}, amide II band at 1485 cm^{-1}, amide III band at 1256 cm^{-1}, amide V and VI bands at 742 and 578 cm^{-1} respectively.

The FTIR results showed that at pH 5.0, in the amide I region (1700 – 1600 cm^{-1}) comprising the proteins secondary structural components (α-helix, β-sheets, random and turn structures), there were significant changes in absorbance for HbA while at pH 7.2; little or no significant absorbance changes were observed. The results suggest that HbA is easily destabilized at pH 5.0, than at pH 7.2, on interaction with Fansidar.

Conclusion

The interaction of Fansidar with HbA at pH 5.0 and pH 7.2 can be likened to the iron-ligand interactions between α and β subnits of haemoglobin. These ligands include O_2, CO and NO bound to Fe^{2+} and CN^-, N_3, OCN^-, SCN^-, and NO that can bind to Fe^{3+}. Some of these ligands when combined with HbA may alter the physiological function of the protein. From the results, the increase in absorbance observed at pH 5.0 on interaction of HbA with Fansidar may suggest that the protein has deviated from its oxidation state; Fe^{2+} to Fe^{3+}, also normal structure and function as a result of change in the environment from the physiological pH 7.2 to pH 5.0. These haemoglobin-drug interactions may hinder the development of the *Plasmodium falciparum* at the intraerythrocytic stage and may account for the novel strategies of monitoring HbA aggregation during malarial infection as well as improvements in administering effective antimalarial treatment. Understanding the interaction of antimalarial drugs with haemoglobin has allowed the identification of essential processes and metabolic weak points that could be exploited to combat this scourge to humanity.

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