Original Research Article

INTERACTION OF ANTIMALARIAL DRUG, PYRIMETHAMINE AND SULPHADOXINE WITH SICKLE HAEMOGLOBIN AT pH 5.0 and pH 7.2: A FOURIER TRANSFORM INFRARED (FTIR) STUDY

ABSTRACT

Aggregation of sickle haemoglobin (HbS), upon deoxygenation, is responsible for an inherited genetic disorder in the human red blood cells (RBCs) known as Sickle cell disease (SCD). On the suggestion that this phenomenon could arise from some form of conformational changes in the structure of HbS arising from a pH-induced rearrangement of secondary structural elements of the haemoglobin, Fourier Transform Infrared (FTIR) spectroscopy (in the mid-infrared region) was used to measure the amino acid side chain absorbance and also, the secondary structural transitions in proteins in a comparative study between the interaction of HbS with Pyrimethamine and sulphadoxine at pH 5.0, (the acidic pH of malarial parasites' digestive vacuole during the intra-erythrocytic development and proliferation), and the body's physiological pH of 7.2. The amide I region (1700 – 1600 cm^{-1}) comprising the amino acids side-chains absorbance and the proteins secondary structural components (α-helix, β-sheets, random and turn structures) showed that at pH 5.0 for Pyrimethamine-sulphadoxine combination therapy, there were significant changes in absorbance for HbS while at pH 7.2, little or no significant absorbance changes were observed. The results suggest that HbS is easily destabilized at pH 5.0 than pH 7.2 on interaction with Pyrimethamine and sulphadoxine. These interactions may hinder the development of the Plasmodium falciparum at the intra-erythrocytic stage and may account for the novel strategies of monitoring HbS aggregation during malarial infection as well as improvements in administering effective antimalarial treatment.

***Keywords**: Antimalarial, FTIR spectroscopy, sickle haemoglobin, spectral peak assignments, 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 utilised 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 favoured 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 Shaoning, 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

After informed consent, four millilitres (4 mL) of venous blood were collected from an identified individual of genotype SS at the Haematology Unit, University of Nigeria Medical Centre, Nsukka. The blood samples were 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.

Isolation and Purification of Haemoglobin

Crude haemoglobin was extracted from the blood sample by employing differential centrifugation technique as described by Denninghoff *et al.* (2006). 4 mL of the blood samples - SS 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 red cells were lysed 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. HbS) was dialyzed at 4 ^{o}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 ^{o}C for further experiments.

Solubility of the Drugs

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

0.1 ml of 0.01 mM of haemoglobin S at pH 5.0 and pH 7.2 were scanned within the frequency range of 4000 - 200 cm $^{-1}$ in the absence and presence of the drugs using 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. 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. 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 a few minutes to remove the water bands. To minimize problems from voidable baseline shifts, the spectra were baseline corrected and normalized.

RESULTS

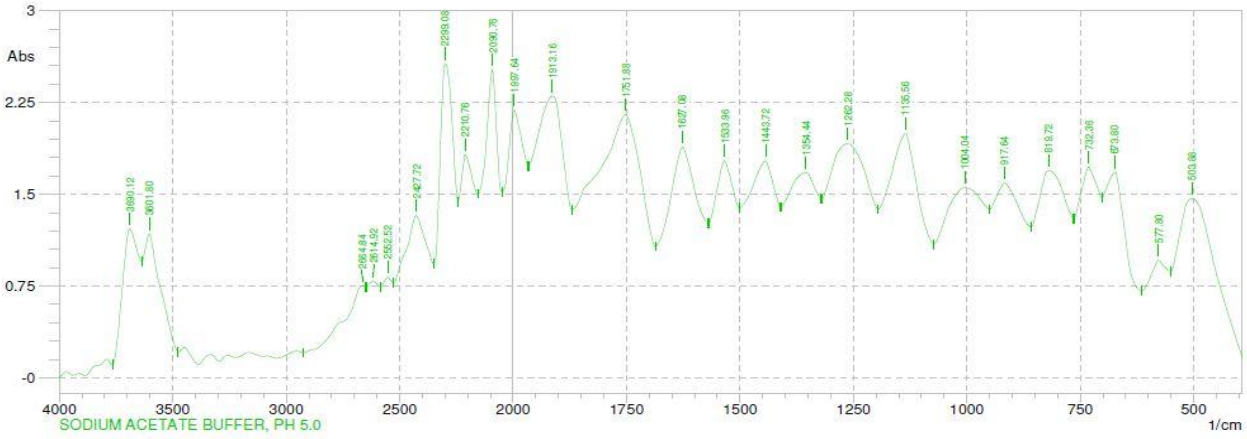

Figure 1*: Absorption Spectra of Sodium Phosphate Buffer, pH 5.0*

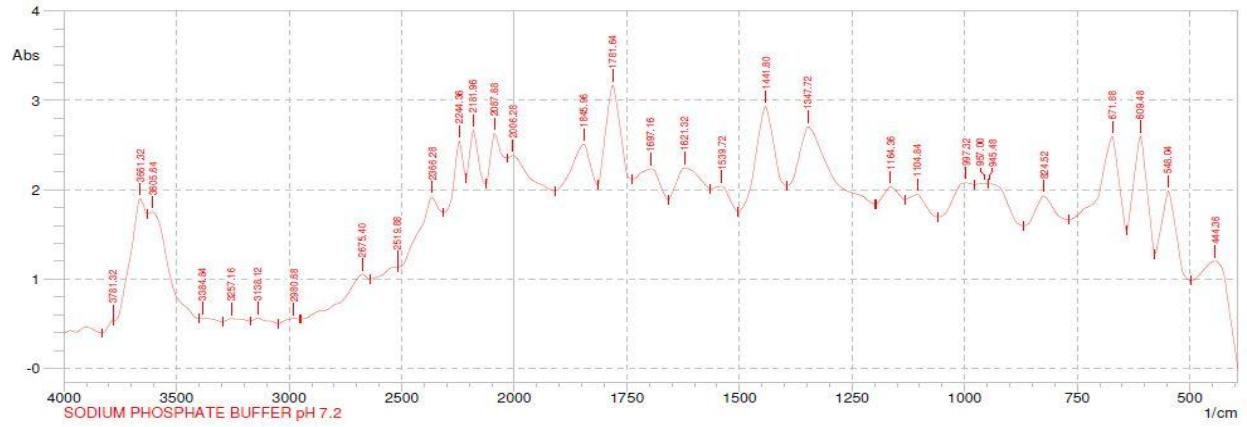

Figure 2*: Absorption Spectra of Sodium Phosphate buffer, pH 7.2*

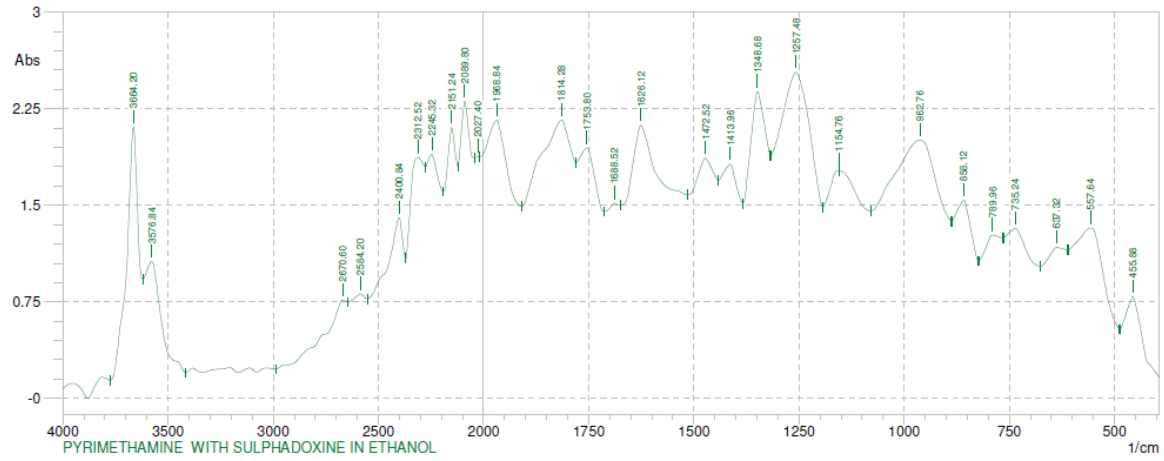

***Figure 3**: Absorption Spectra of Pyrimethamine and Sulphadoxine Combined Therapy*

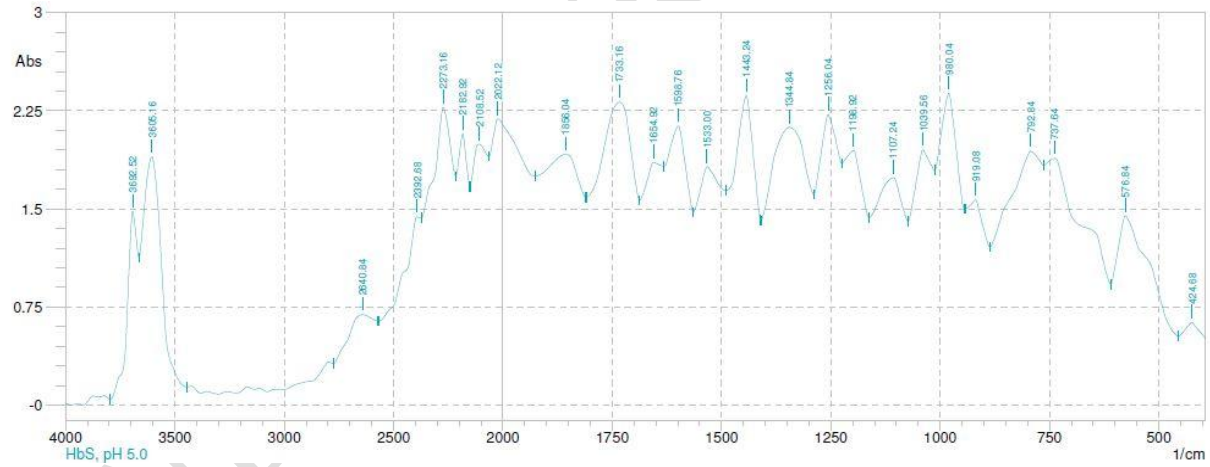

***Figure 4**: Absorption Spectra of HbS at pH 5.0*

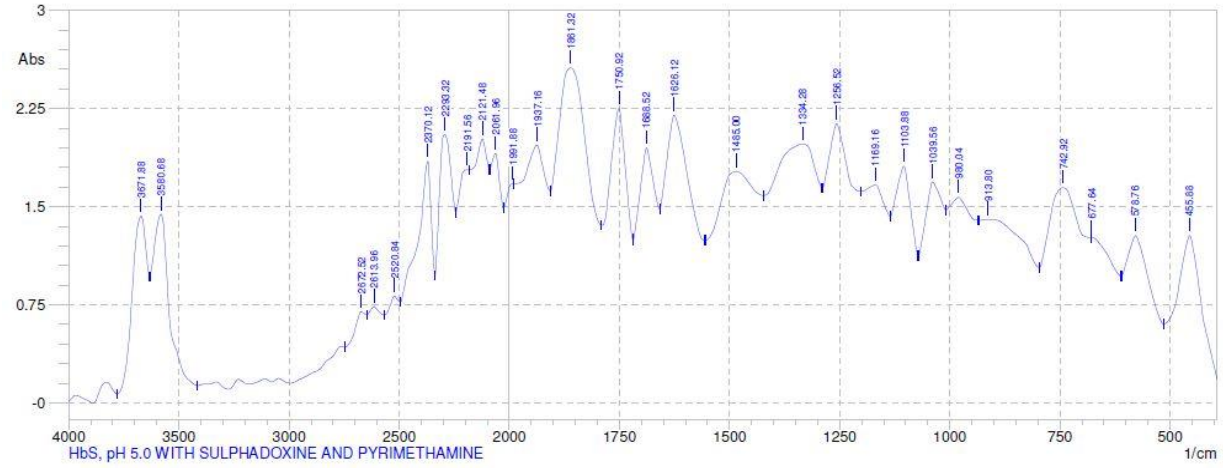

***Figure 5**: Absorption Spectra of HbS with Pyrimethamine and Sulphadoxine, pH 5.0*

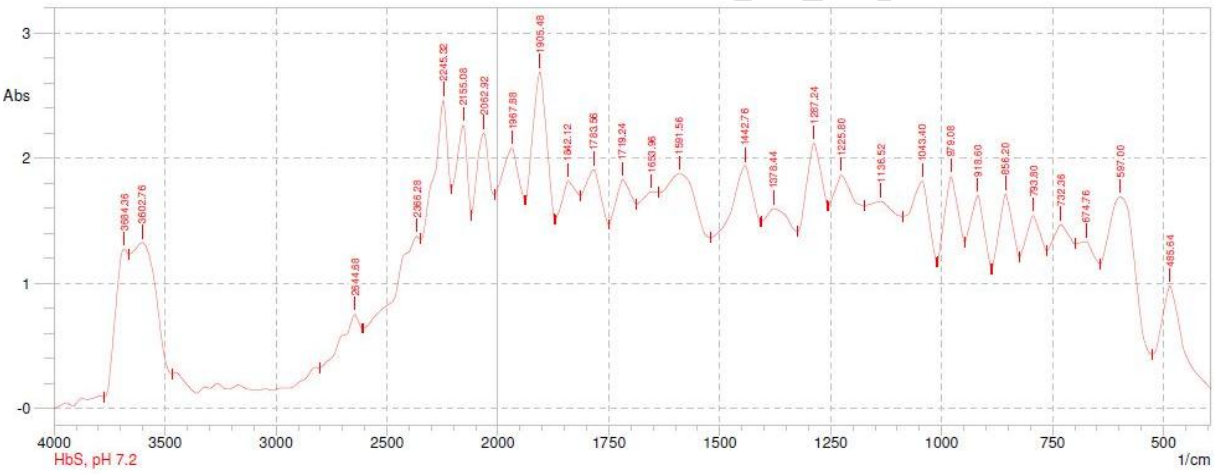

***Figure 6**: Absorption Spectra of HbS at pH 7.2*

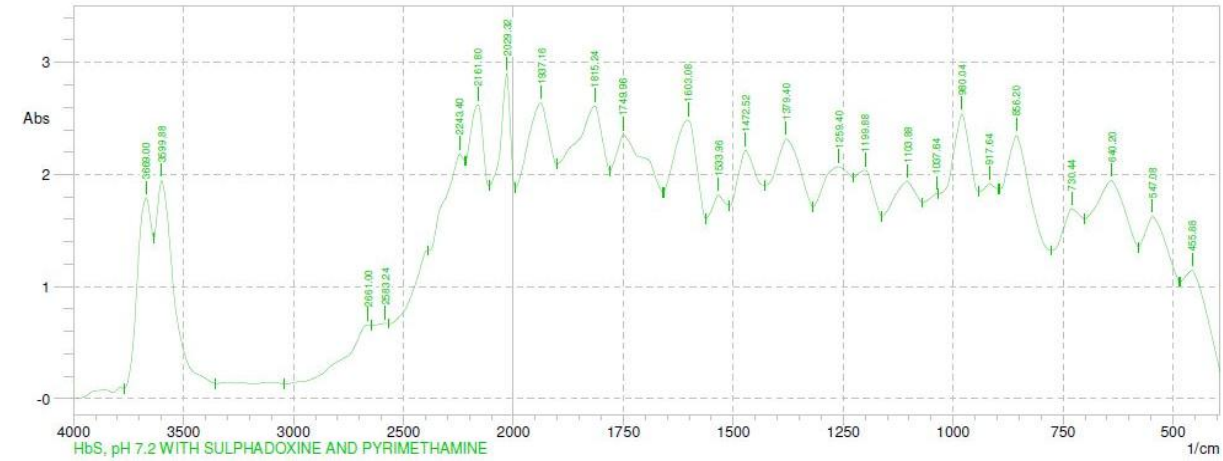

***Figure 7**: Absorption Spectra of HbS with Pyrimethamine and Sulphadoxine, pH 7.2*

DISCUSSION

In the FTIR analysis, the samples were subjected to IR radiation. The primary reason for employing FTIR over other techniques for this comparative study is that the absorption spectra of proteins can be obtained over a wide range of environments, and it can furnish the direct correlations between the IR frequencies and the proteins secondary structural components. In addition, it can be used to study conformational changes – effects of ligand binding, temperature, pH and pressure, structural stability and protein aggregation. A known volume, (0.1 ml) of 0.01 mM of HbS was titrated with Pyrimethamine and sulphadoxine in combined therapy and FTIR spectral analysis 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 analysed 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 Pyrimethamine and sulphadoxine in combined therapy as shown in figure 3 contained the characteristic peaks at 3664 and 3576 cm^{-1} indicative of stretching vibrations of O-H, N-H, and aromatic ring of C-H functional groups, 2400 – 2027 cm^{-1} which depicts the presence of C≡C and C≡N functional groups. The spectrum also displayed absorbance bands in the 1626 and 1472 cm^{-1} which correspond to the stretching vibrations of the C=C, C=O, N=N, and C=N functional groups respectively from the aromatic rings in the drug.

The several absorption bands as observed in Figures 4 and 6 depicted that HbS 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 HbS showed vibrations in the range of 3650 – 3500 cm^{-1} indicating the presence of hydrogen bonds 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 HbS 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 HbS 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 bands at 1485 cm^{-1}, amide III bands at 1256 cm^{-1}, amide V and VI bands at 742 and 578 cm^{-1} respectively.

At pH 5.0, the interaction of the drugs with HbS caused significant perturbation in the amide I region when compared to pH 7.2. This could be because of decreased oxygen affinity and the formation of Methaemoglobin. Thus, at the acidic pH, Pyrimethamine-sulphadoxine interaction increases the tendency for the protein's aggregation in comparison to the physiological pH, 7.2.

CONCLUSION

The amide I region (1700 – 1600 cm^{-1}) comprising the amino acids side-chain absorbance and the proteins secondary structural components (α-helix, β-sheets, random and turn structures) showed that at pH 5.0 for Pyrimethamine-sulphadoxine combination therapy, there were significant changes in absorbance for HbS while at pH 7.2, little or no significant absorbance changes were observed. The results suggest that HbS is easily destabilized at pH 5.0, than at pH 7.2, on interaction with Pyrimethamine and sulphadoxine. 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 HbS aggregation during malarial infection as well as improvements in administering effective antimalarial treatment.

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