TITLE

INTERACTION OF ANTIMALARIAL DRUGS (PYRIMETHAMINE AND SULPHADOXINE) WITH NORMAL AND SICKLE HAEMOGLOBINS: A UV-VISIBLE STUDY

A DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE (M.Sc) IN BIOCHEMISTRY (INDUSTRIAL BIOCHEMISTRY AND BIOTECHNOLOGY) OF THE UNIVERSITY OF NIGERIA, NSUKKA

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CERTIFICATION

Nnolim, Nonso E, a postgraduate student of the Department of Biochemistry with the registration number of PG/M.Sc/12/64408 has satisfactorily completed the research work requirement for the degree of Master of Science (M.Sc) in Biochemistry (Industrial Biochemistry and Biotechnology). The work embodied in this project (dissertation) is original and has not been submitted in part or full for any other diploma or degree of this or any other university.

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EXTERNAL EXAMINER

DEDICATION

This work is dedicated to the God Almighty for abundant grace and inspiration.

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ABSTRACT

Crude haemoglobins were extracted from blood samples of identified individuals of normal (AA), sickle trait carrier (AS), and sickle (SS) by employing centrifugation techniques. The crude haemoglobins were dialysed at $4^{o}C$ for 12hr against 50mM Tris-HCl buffer of pH 7.2. The effects of pyrimethamine and sulphadoxine on the haemoglobins in the presence and absence of sodium dodecyl sulphate (SDS) were studied at pH 5.0 and 7.2 with uv-visible titration spectrophotometry. The study showed that sodium dodecyl sulphate at pH 5.0 unfolded the studied proteins. These can be related to destabilization of haemoglobin structure by proteases such as plasmepsins and falcipains in the acidic environment of malaria parasite food vacuole due to malaria parasite infection. Pyrimethamine and sulphadoxine at pH 5.0 and 7.2 decreased the concentration of oxyhaemoglobin and increased the concentrations of methaemoglobin and deoxyhaemoglobin of the studied proteins. The results also show how haemoglobins are deoxygenated due to interaction with sodium dodecyl sulphate. Deoxygenation of haemoglobin as a result of their interaction with SDS can be likened to pathological condition whereby malaria parasites infection reduced the oxygen tension of erythrocytes of their host. HbS had the highest interaction with sulphadoxine and pyrimethamine followed by HbAS while HbA had the least interaction. Formation of methaemoglobin is associated with lipid oxidation. Increase in absorbance at 275 nm observed in this study refers to dynamic motion of the studied proteins and their deviation from normal structure and function. The interaction of haemoglobins with sulphadoxine-pyrimethamine combination at pH 5.0 caused a large perturbation of the protein conformation that was reflected in modest spectral shift of the soret band.

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CHAPTER ONE

INTRODUCTION

One of the main causes of death today is malaria, especially in numerous parts of Asia, Sub-Saharan Africa and the America (Esparza, 2005). Of the four *Plasmodia* that cause malaria, *Plasmodium falciparum* is responsible for the majority of illness and death in mankind (Duraisingh and Refour, 2005; Idro *et al*., 2005; Okie, 2005; Worrall *et al.,* 2005). In Sub-Saharan Africa, this disease has a profound impact on children and infants, whilst millions have already died from AIDS (Acquired Immunodeficiency Syndrome) (Esparza, 2005; Harms and Feldmeier, 2005). In addition to this, malaria adds in mortality while the spread of chloroquine resistant strains of the *plasmodium* parasites across Africa increases (Farooq and Mahajan, 2004; Mahajan *et al.,* 2005). Approximately, three million people, of whom more than half are children, die of malaria caused by *P. falciparum* annually (Duraisingh and Refour, 2005). Mortality and morbidity increase every year with over 500 million people infected with *P. falciparum,* presenting clinical symptoms of mild to severe malaria.

There exist several reasons for the increase in the occurrence of malaria including:

i An increase of the protozoan parasite's resistance to anti-malarial drugs,

ii The development of the anopheles mosquito vectors' resistance to numerous insecticides

iii The growth and the widespread migration of vulnerable populations to vastly endemic areas (Abdel-Hameed, 2003; Gregson and Plowe, 2005).

Malaria can be treated with various anti-malarial drugs. Sulphadoxine and pyrimethamine combination therapy was introduced into clinical practice for the prevention and treatment of malaria in the late 1960s as a follow-up on the drug chloroquine (Schultz *et al*., 1994). The first product by the name of Fansidar® containing sulphadoxine and pyrimethamine in combination was produced by Roche in 1971. Since the expiration of the Fansidar® patent, numerous generic products have been produced worldwide, contributing towards cheaper anti-malaria therapy.

1.1 Malaria

1.1.1 A Health Problem

Of all the communicable illnesses, Malaria, known as the globe's greatest tropical parasitic infection, claims the most lives aside from tuberculosis (TB). Being a health problem in over 90 countries and inhabited by approximately 2400 million people, an expected 300 - 500 million clinical cases occur per annum and more than one million lives are claimed yearly (Greenwood *et al*., 2005). In the year 2001, the three illnesses TB, HIV and malaria contributed to approximately 5.7 million deaths, of which the majority was young children together with men and women of their productive years, and in developing countries, deemed liable for 23% of fatalities (Theobald *et al.,* 2006).

The great impact of these diseases and the globally inadequate responses has counteracted health gains produced over the last ten years and contributes towards poverty considerably in numerous low- and middle-income countries (Theobald *et al.,* 2006). The World Health Organization (WHO) states that 90% of fatalities worldwide are in Africa, mainly children under the age of 5 years.

1.1.2 History of Malaria

Scientific studies on this parasitic infection made their first major advance in 1880. Charles Louis Alphonse Laveran, a doctor in the French armed forces working in Algeria, observed the parasites in red blood cells obtained from malaria patients, claiming that this protozoan causes malaria, making it the first time for protozoa to be identified as the cause of disease. The two Italian scientists Angelo Celli and Ettore Marchiafava named the protozoan *Plasmodium.* A year later, the Cuban doctor Carlos Finlay who was treating patients in Havana for yellow fever, was the first to suggest that the disease was transmitted to humans by mosquitoes. But it was Sir Ronald Ross from Britain, working in India during that time, who in 1898, showed that mosquitoes transmitted malaria. He showed that malaria was transmitted to birds via certain species of mosquitoes and the malaria parasites were isolated from mosquitoes' salivary glands that fed on birds infected with the parasites (Wikipedia, 2013).

1.1.3The Life Cycle of the Malaria Parasite

Protozoa of the genus *Plasmodium* cause malaria. Four species of *Plasmodium* are responsible for the disease in humans: *P. falciparum*, *P. malariae*, *P. ovale* and *P. vivax*. Of these, *P. falciparum* may cause the condition known as cerebral malaria, which is responsible for the majority of fatal outcomes. The pathogenesis and life cycle of the malaria parasite are complex (Miller *et al*., 2002), consisting of two stages: a sexual stage (sporogony), which occurs within the mosquito, and an asexual stage (schizogony), which occurs in the host (Frederich *et al.,* 2002). The illness results when an infected female anopheles mosquito feeds on the blood of an uninfected vertebrate host. Mosquitoes inject parasites (sporozoites) into the subcutaneous tissue, less frequently directly into the bloodstream. In less than one hour, the sporozoites travel to the liver, invade the hepatocytes and undergo exoerythrocytic schizogony. After some time, depending on the plasmodium species, the schizonts are transformed into merozoites, which after release into the blood stream invade erythrocytes. After significant reorganization of the membrane proteins of an occupied erythrocyte (Parker *et al.,* 2004), the merozoites undergo erythrocytic schizogony, which comprises young rings (12 hr after erythrocyte infection), mature rings (18 hr), early trophozoites (24 hr), mature trophozoites (30 hr), early schizonts (36 hr) and mature schizonts (42 hr). At the end of erythrocytic schizogony, the parasites return into the merozoite form but enormously multiplied causing splattering of the erythrocyte. The released merozoites invade new red blood cells and start a new erythrocytic schizogony cycle. Erythrocytic schizogony occurs every 2ï 3 days, depending on the plasmodium species. Each cycle is accompanied by typical malaria symptoms, such as fever, chills, headache and exhaustation. After several cycles, some of the merozoites undergo sexual development and are transformed into gametocytes. The gametocytes remain in the erythrocytes and are consumed by an anopheles mosquito. In the mosquito, female and male gametocytes join and form zygote. Within 18 to 24 hr, the zygote transforms into a slowly motile ookinete. Between 7 and 15 days, depending on the plasmodium species and the ambient temperature, a single oocyst forms more than 10,000 sporozoites. The motile sporozoites migrate into the salivary glands and accumulate in the acinar cells. When infected, the mosquito bites a susceptible vertebrate host; a new parasite cycle commences (Opsenica and Solaja, 2009).

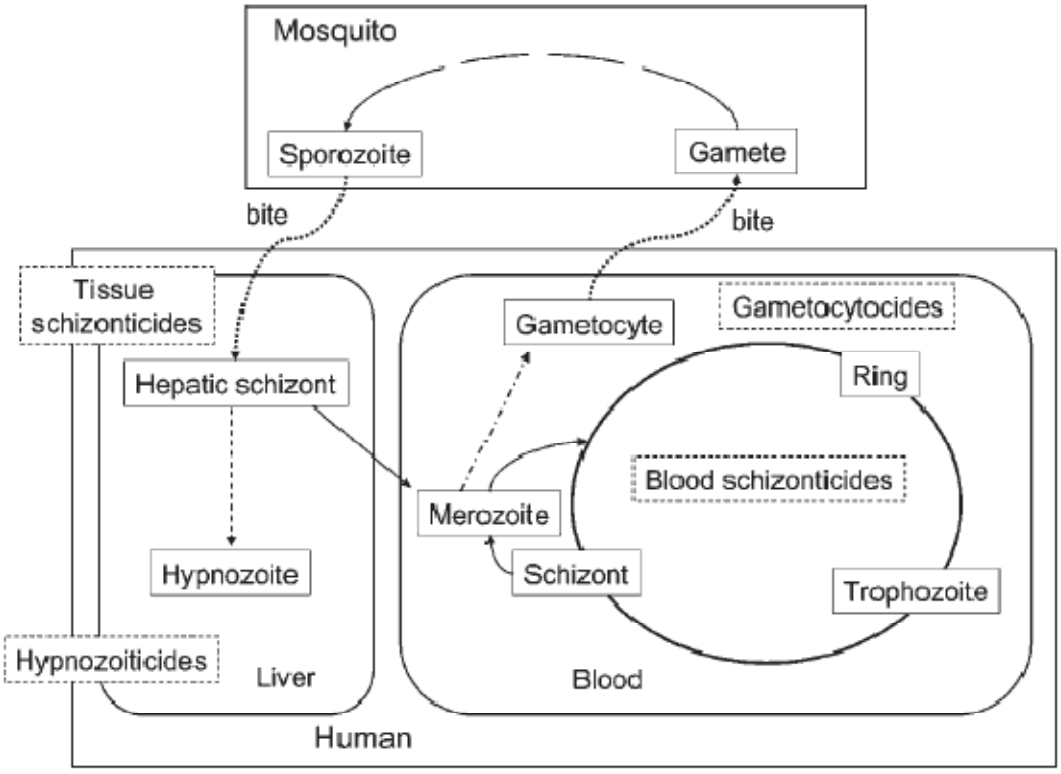


Figure 1.1: The life cycle of malaria parasite (Schlitzer, 2007).

1.1.4 Signs and Symptoms of Malaria

The signs and symptoms of malaria typically begin 8ï 25 days following infection (Fairhurst and Wellems, 2010). However, symptoms may occur later in those who have taken antimalarial medications as prevention (Nadjm and Behrens, 2012). Initial manifestations of the disease common to all malaria species are similar to flu like symptoms (Bartoloni and Zammarchi, 2012), and can resemble other conditions such as septicemia, gastroenteritis, and viral diseases.The presentation may include headache, fever, shivering, arthralgia (joint pain), vomiting, hemolytic anemia, jaundice, hemoglobinuria, retinal damage (Beare *et al.,* 2006), and convulsions. Approximately 30% of people however will no longer have a fever upon presenting to a health care facility. Owing to the non-specific nature of disease presentation, diagnosis of malaria in non-endemic countries requires a high degree of suspicion, which might be elicited by any of the following: recent travel history, splenomegaly (enlarged spleen), fever without localizing signs, thrombocytopenia, and hyperbilirubinemia combined with a normal peripheral blood leukocyte count (Beare *et al.,* 2006).

The classic symptom of malaria is paroxysm; a cyclical occurrence of sudden coldness followed by rigor and then fever and sweating, occurring every two days (tertian fever) in *P. vivax* and *P. ovale* infections, and every three days (quartan fever) for *P. malariae*. *P. falciparum* infection can cause recurrent fever every 36ï 48 hours or a less pronounced and almost continuous fever (Ferri, 2009). Severe malaria is usually caused by *P. falciparum* (often referred to as *falciparum* malaria). Symptoms of *falciparum* malaria arise 9ï 30 days after infection. Individuals with cerebral malaria frequently exhibit neurological symptoms, including abnormal posturing, nystagmus, conjugate gaze palsy (failure of the eyes to turn together in the same direction), opisthotonus, seizures, or coma (Bartoloni and Zammarchi, 2012).

1.1.5 Diagnosis and Treatment of Malaria

Malaria is usually diagnosed by the microscopic examination of blood films or by antigen-based rapid diagnostic tests (RDT) (Abba *et al.,* 2011; Kattenberg *et al.,* 2011). Microscopy is the most commonly used method to detect the malaria parasite, about 165 million blood films were examined for malaria in 2010 (Wilson, 2012). Despite its widespread usage, diagnosis by microscopy suffers from two main drawbacks: many settings (especially rural) are not equipped to perform the test, and the accuracy of the results depends on both the skill of the person examining the blood film and the levels of the parasite in the blood. The sensitivity of blood films ranges from 75ï 90% in optimum conditions, to as low as 50%. Commercially available RDTs are often more accurate than blood films at predicting the presence of malaria parasites, but they are widely variable in diagnostic sensitivity and specificity depending on manufacturer, and are unable to tell how many parasites are present (Wilson, 2012).

Numerous classes of antimalarials are available and these drugs are categorized according to the selective actions they have on different stages of *plasmodium's* life cycle. The tissue schizonticides are drugs with the action of eliminating dormant or developing liver forms; while blood schizonticides are those acting on the erythrocytic parasites; and the last group, called the gametocides are responsible for preventing transmission of the parasite to mosquitoes as well as exterminating the sexual stages (Rosenthal and Goldsmith, 2001).

The foundation in the prophylaxis of malaria is preventing mosquito bites. Even during the use of chemoprophylactic agents, non-medication measures must be strictly applied. These measures include the following:

ÅEndemic areas should be visited in the dry season.

Å Wear ankle protectors, long trousers, long sleeves and light-coloured clothing when outdoors between dusk and dawn.

ÅInsect repellants that contain diethyltoluamide should be applied to clothing and skin that is exposed.

ÅCoils, screens and mosquito nets can also be used.

ÅImpregnate clothing and nets with the insecticide known as Peripel® (contains pyrethroid) (Gibbon, 1997).

Any person who is at risk of having a severe malaria attack should not enter a malaria area and must be discouraged from doing so. High risk groups include: Children under the age of five years, pregnant women, elderly and immunity impaired patients, AIDS patients and immune-compromised patients on chemotherapy or long-term steroid therapy (Gibbon, 1997).

1.2 Antifolates

In most species, tetrahydrofolic acid plays a key role in the biosynthesis of thymine, purine nucleotides, and several amino acids (Methionine, Glycine, Serine, Glutamate, and Histidine). Whereas humans depend on dietary intake of pre-formed dihydrofolic acid as an essential nutrient, which is then reduced to tetrahydrofolic acid, pathogenic microorganisms including Plasmodia can synthesize dihydrofolic acid from simple precursors. Furthermore, *P. falciparum* is able to use exogenous dihydrofolic acid via a salvage pathway (Hyde, 2005). Inhibitors of two key enzymes of the folate biosynthetic pathway, dihydropteroate synthase (DHPS) and dihydrofolate reductase (DHFR), have long been used in the treatment of bacterial and protozoal infections. Whereas dihydropteroate synthase is completely absent in humans, bacterial and protozoal dihydrofolate reductases are sufficiently different from the human enzyme to allow the development of selective inhibitors. In *P. falciparum*, both enzymes are present not as monofunctional proteins, but the DHPS and DHFR activities are present on specific domains of bifunctional proteins. In the case of DHPS, the preceding enzymatic activity of hydroxymethyldihydropterin pyrophosphokinase (Kasekarn *et al.,* 2004), is located on the same polypeptide. DHFR, in turn, is collocated with the subsequent thymidylate synthase activity on a single protein. The use of antifolates against malaria (Anderson, 2005; Gregson and Plowe, 2005; Chan and Anderson, 2006), and the possibility of using other enzymes along the

folate biosynthetic pathway as drug targets has already been reviewed (Nzila *et al.,* 2005). The first antifolate to be used against malaria was the wellknown DHPS inhibitor sulphachrysoidine. It was developed in 1932 by Domack as an antibacterial agent. Later it was found that sulphanilamide, arising from the reductive cleavage of the azo substructure, is the active component.

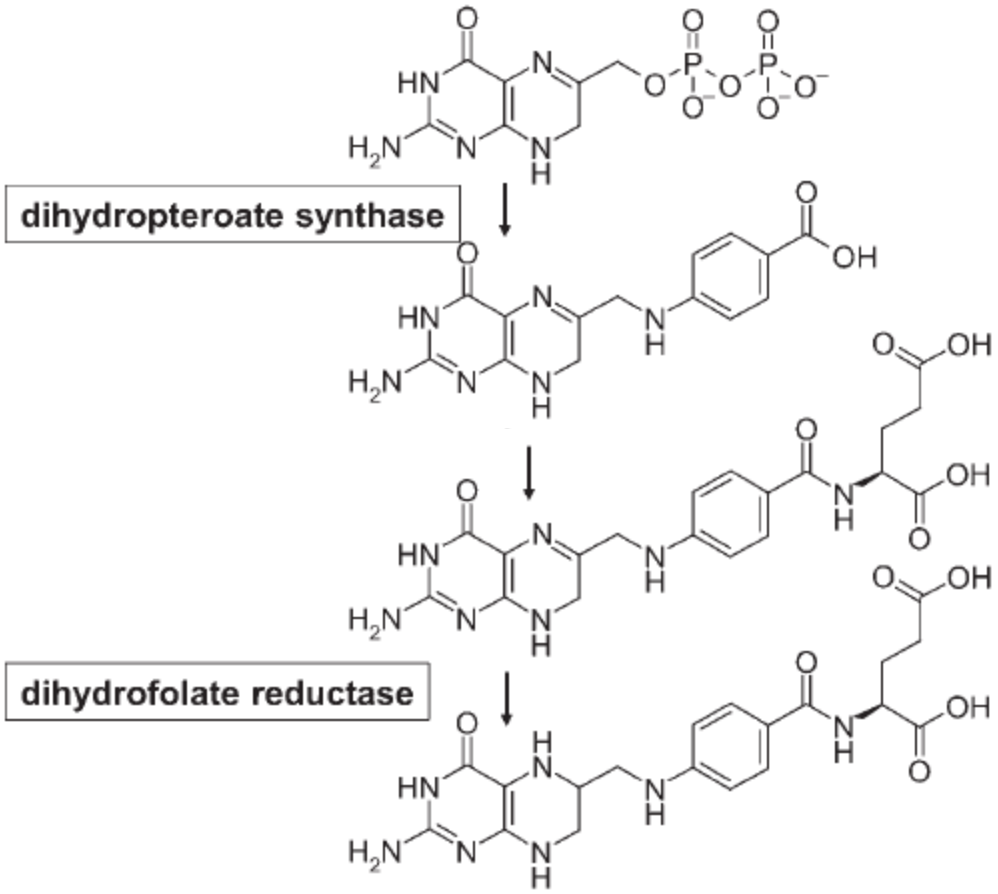


Figure 1.2: The folate pathway (simplified) showing the targets of the antifolates (Schlitzer, 2007).

1.2.1 Combination of DHPS and DHFR Inhibitors

In the late 1950s and early 1960s several studies revealed the synergistic effect of combining sulfonamides that inhibit DHPS activity with a DHFR inhibitor. This synergism strongly depends on functional DHPS; however, the precise mechanism is not completely understood. Sulfonamides act as competitive antagonists of p-aminobenzoic acid (PABA), which is condensed to hydroxymethyldihydropteridine diphosphate to form dihydropteroate. In

addition, sulfonamides react with hydroxymethyldihydropteridine diphosphate as false substrates to form covalent adducts, commonly called sulfa-dihydropteroates. While it has been shown that these adducts inhibit the growth of *P. falciparum* (Nzila, 2006), their intracellular targets are unclear. There is certain evidence that sulfa-dihydropteroates inhibit DHFR. The combination of the sulphadoxine with the DHFR inhibitor pyrimethamine, known under its brand name Fansidar, became the most important antimalarial next to chloroquine. The combination of DHPS and DHFR inhibitors shows little effect during the first 24 h of the parasiteôs life cycle because the combination inhibits parasite DNA synthesis. This event peaks in the late erythrocytic schizont stage, at which antifolates exert their toxic effect. Treatment regimes have generally been regarded as sufficiently safe, but with prolonged prophylactic use, toxicity of the sulphonamide combination partner becomes significant, resulting in an increased risk of agranulocytosis and toxic epidermal necrolysis (a disease condition where the epidermis gets detached from the dermis) - Stevensï Johnson syndrome (Taylor and White, 2004).

1.2.2 Sulphadoxine-Pyrimethamine Combination Therapy

Numerous studies indicate that children in Africa can be protected against malaria by means of chemoprophylaxis, using antimalarials frequently, occasionally in doses lower than that of the therapeutic range (McGregor *et al.,* 1956; Greenwood *et al.,* 1988; Allen *et al.,* 1990; Menendez *et al.,* 1997, Geerligs *et al.,* 2003). Generally, the mortality in Gambian children decreased by approximately 35%, following treatment with pyrimethamine and dapsone taken in combination fortnightly during the transmission period of malaria (Greenwood *et al.,* 1988).

In pregnant women, preventive treatment, as the above mentioned, initially proved to be a successful approach in the management of malaria. Placental malaria was reduced by 72% in Malawi when the sulphadoxine-pyrimethamine combination was administered (Schultz *et al.,* 1994). In some regions of Tanzania, where the transmission of the disease is perennial, a related approach was modified in two studies to prevent malaria in infants (Schellenberg *et al.,* 2001; Massaga *et al.,* 2003). Clinical episodes of anaemia and malaria, and the frequency thereof, were reduced by approximately two thirds (Verhoef *et al.,* 2002; Desai *et al.,* 2003).

1.2.3 Sulphadoxine and Its Pharmacological Classification

Sulphadoxine is a long acting sulphonamide with a half life of 7 - 9 days and acts as an antifolate agent. It is absorbed well after oral intake and the urinary excretion is extremely slow. In serum this results in drug levels that are prolonged. The slow excretion of sulphadoxine is partially due to extensive tubular reabsorption and in part due to a protein binding exceeding 85%. Available as Fansidar®, sulphadoxine is used in combination with pyrimethamine as second-line treatment of malaria (Chambers, 2001).

1.2.4 The Mechanism of Action of Sulphonamides

As competitive antagonists and structural analogs of para-aminobenzoic acid (PABA), sulphonamides act in the synthesis of pteroylglutamic acid (folic acid) by preventing PABA's normal bacterial utilization. More particularly, sulphonamides act as competitive inhibitors of the bacterial enzyme dihydropteroate synthase, liable for PABA's incorporation into dihydropteroic acid (folic acid's immediate precursor). Sensitive microorganisms have to produce their own folic acid, while unaffected bacteria are those that use preformed folate. Sulphonamide induced bacteriostasis is competitively counteracted by PABA. Since mammalian cells cannot produce their own folic acid, they are not affected by the mechanism of sulfonamides and can therefore be compared to sulfonamide insensitive bacteria, which make use of preformed folate (Mandell and Petri, 1996).

1.2.5 Physico-Chemical Properties of Sulphadoxine

Sulphadoxine is an odourless, white or creamy-white, crystalline powder. Other names for sulphadoxide include sulphormethoxine, sulphorthodimethoxine, sulphorthomidine and sulphadimoxinum. Sulphadoxine is chemically known as 4-amino-N- (5,6-dimethoxy-4-pyrimidinyl) benzenesulphonamide (Kapoor, 1988).

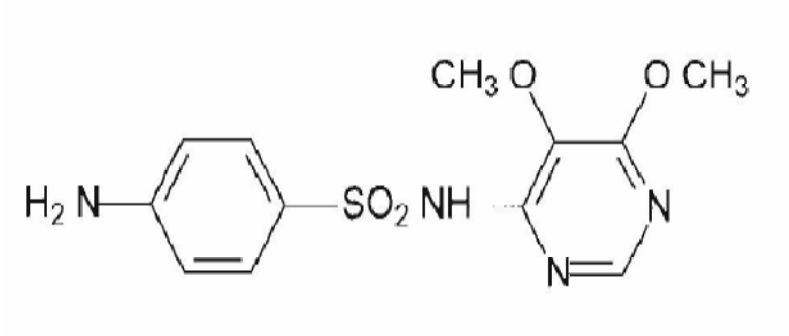


Figure 1.3: The chemical structure of sulphadoxine (Kapoor, 1988)

Sulphadoxine is slightly soluble in methanol and alcohol and its solubility in water is very slight. It is basically insoluble in ether but soluble in alkali solutions, i.e. carbonates and hydroxides, as well as diluted mineral acids. Suggested solvents for sulphonamides include both mono- and di-lower alkyl glycerol ethers. Sulphadoxine has an acidic nature and it melts between 197°C and 200°C (Kapoor, 1988).

1.2.6 Pyrimethamine and its Pharmacological Classification

Categorized as a blood schizontocide the slow acting antimalarial, pyrimethamine, has equivalent *in vivo* effects to that of chloroguanide. Pyrimethamine antimalarial potency is greater though because it targets malarial parasites directly, and apart from that its active metabolite has a much shorter half-life than pyrimethamine. Pyrimethamine is of a pharmacological class called the diaminopyrimidines (Tracy and Webster, 1996).

1.2.7 The Mechanism of Action of Diaminopyrimidines

In a series of investigations, it was shown that the 2,4-diaminopyrimidines inhibit plasmodia's dihydrofolate reductase at much lower concentrations than required for similar inhibition of mammalian enzymes. The difference has been shown between plasmodial dihydrofolate reductase and its mammalian counterparts in that the latter do not possess both thymidylate synthetase and dihydrofolate reductase activities. Two steps are inhibited in a vital metabolic pathway, and this inhibition explains pyrimethamine's synergism with sulphones and sulphonamides. The first step is the utilization of PABA in dihydropteroic acid's synthesis (inhibited by sulphonamides), while the second step consists of dihydrofolate's reduction to tetrahydrofolate (inhibited by pyrimethamine). Antifolates inhibit nuclear division during the

formation of schizonts in the liver and erythrocytes. This occurs late in the malaria parasite's life cycle. Compared to quinoline antimalarials, the antifolates have a slow onset, causing a consistent mechanism (Tracy and Webster, 1996).

1.2.8 Binding Mechanism of DHFR Inhibitors

In contrast to many other antimalarials, the interaction of DHFR inhibitors with their target is known at the molecular Level (Rathod and Philips, 2003; Parenti *et al.,* 2004). The main interactions are illustrated in Figure 1.4 with pyrimethamine as an example. The negatively charged carboxylate group of Aspartate 54 interacts with the positively charged NH moiety of the pyrimidine ring and the NH_2 group at position 2, whereas the NH_2 hydrogen atoms at position 4 form hydrogen bonds with the backbone carbonyl groups of Isoleucine14 and Isoleucine164. Another hydrogen bond is formed between the hydroxyl group of the Serine108 side chain and NADPH. The dihydropyridine ring undergoes a charge transfer interaction with the chlorophenyl residue of pyrimethamine.

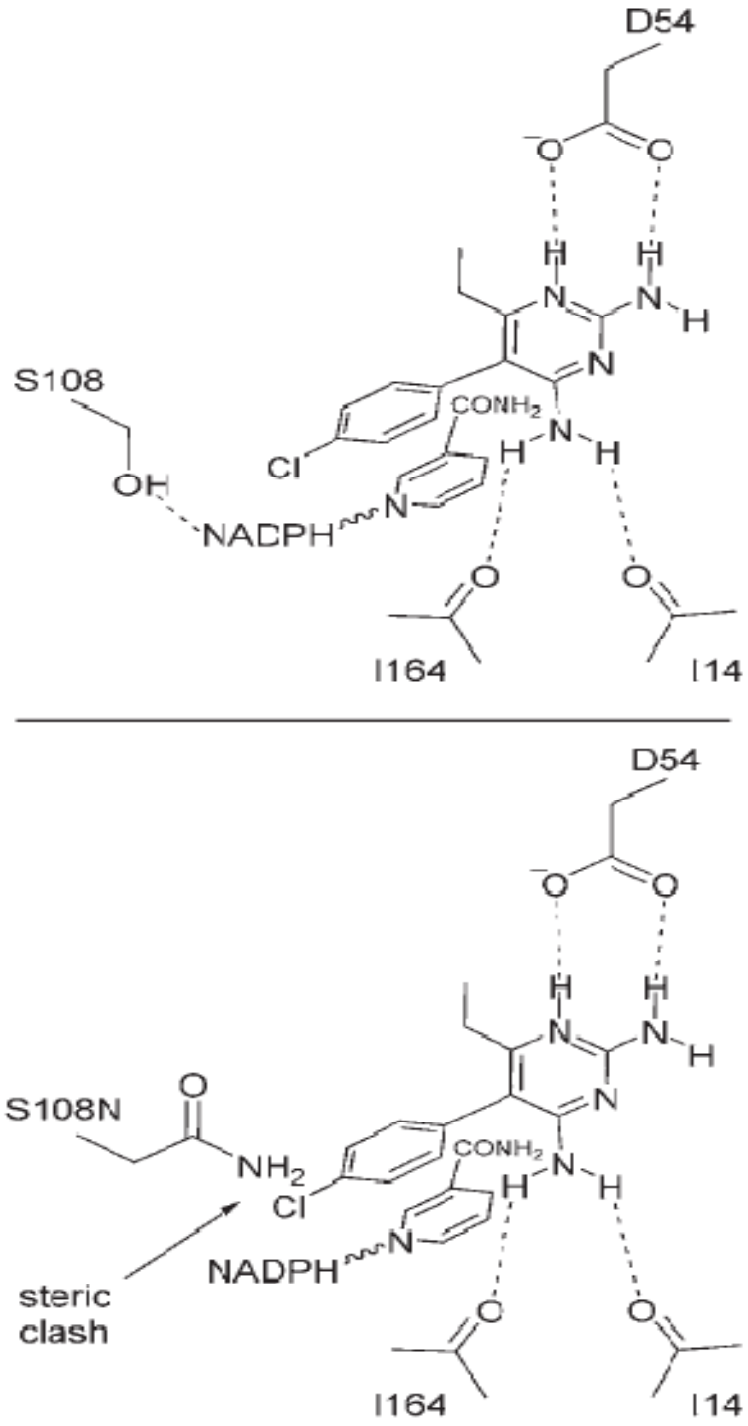


Figure 1.4: Binding of pyrimethamine to *plasmodium falciparum* DHFR (PfDHFR). The S108N mutation (bottom) results in a steric clash between the arginine side chain and the terminal chloro substituent of the DHFR inhibitor (Schlitzer, 2007).

1.2.9 Physico-chemical Properties of Pyrimethamine

Pyrimethamine is a white, tasteless, odourless, crystalline powder. The chemical name for pyrimethamine is 2,4-diamino-5-(p-chlorophenyl)-6-ethylpyrimidine and in combination with sulphadoxine, it is known as Fansidar® (Loutfy and Aboul-Enein, 1983).

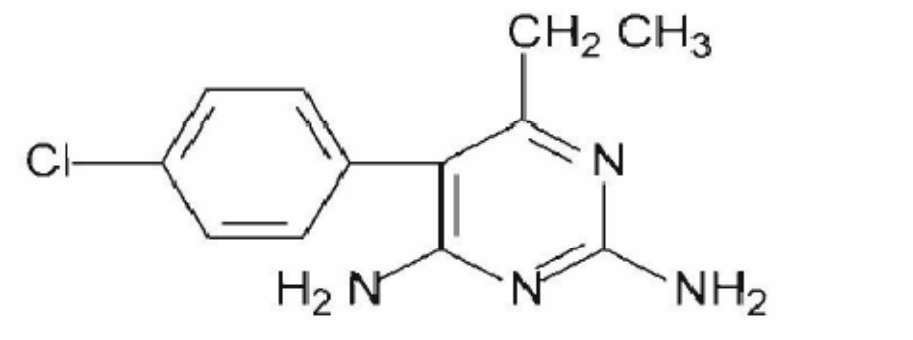


Figure 1.5: The chemical structure of pyrimethamine (Loutfy and Aboul-Enein, 1983).

In water, pyrimethamine is basically insoluble, however in ethanol, chloroform, acetone and dilute hydrochloric acid (HCI) it is slightly soluble (Loutfy and Aboul-Enein, 1983).

1.2.10 Clinical Uses and Adverse Effects of the Combination

The sulphadoxine-pyrimethamine combination is used in the treatment of uncomplicated *P. falciparum* malaria resistant to chloroquine. In adults, a single oral dose of 50/1000 mg to 75/1500 mg pyrimethamine/sulphadoxine should be taken (that is 2 - 3 Fansidar tablets). The paediatric dose for children under the age of four is half a tablet; for 4 - 8 years, a tablet and for children 9 - 14 it is two tablets also as a single oral dose (Gibbon, 1997).

The contraindications for this combination include the following:

ÅSulphonamide hypersensitivity.

ÅFolate deficiency.

ÅMegaloblastic anaemia.

ÅG6PD deficiency.

ÅBlood dyscrasias.

ÅSevere hepatic or renal impairment.

ÅConvulsive disorders (Gibbon, 1997).

Fansidar® is also contraindicated in neonates; pregnant women, as it crosses the placenta; lactating women, because of its excretion in breast milk and in patients with porphyria (Gibbon, 1997).

Drug interactions with Fansidar® include:

ÅIncreased anti-folate effects with folate antagonists.

ÅIncreased phenytoin levels with phenytoin.

ÅEnhanced hypoglycaemic effects with sulphonylureas.

ÅHigh risk of fatal skin reactions, predominantly in HIV patients, with chloroquine.

ÅPotentiated anticoagulant effects with warfarin (Gibbon, 1997).

Frequent adverse effects for the combination sulphadoxine-pyrimethamine are abdominal discomfort, nausea and vomiting, dizziness, headaches, skin reactions and photosensitivity. Leukopenia, megaloblastic anaemia, thrombocytopenia and hepatitis are rare adverse effects while more severe effects include toxic epidermal necrolysis, fatal skin reactions and Stevens-Johnson syndrome (Gibbon, 1997).

1.3 Chloroquine

Chloroquine (CQ) has been the most successful single drug for the treatment and prophylaxis of malaria. Chloroquine is a safe and affordable drug, and it was effective before resistant strains began to emerge in the 1960s. It was the drug of choice in the World Health Organization (WHO) Global Eradication Programme. Landscaping measures, vector control with dichlorodiphenyl trichloroethane (DDT), and the prophylactic use of CQ led to a considerable containment of malaria, which had once been endemic as far north as 648 N (Hay *et al.,* 2004; Guinovart *et al.,* 2006). Chloroquine is a relatively well tolerated drug as long as it is used in therapeutic regimes. The therapeutic index is rather small, with the therapeutic dose being 10mgkg^{-1} b.w.; a dose of 20 mgkg^{-1} causes serious toxic effects and 30 mgkg^{-1} is potentially lethal (Taylor and White, 2004). When CQ is used in long term prophylaxis, serious and irreversible side effects such as neuromyopathy, retinopathy, erythema multiform, and bone marrow toxicity may occur. However, these reactions are rare. Retinopathy has been associated with a cumulative total dose of 50ï 100 g. Despite its overwhelming importance, the mechanism

of action of chloroquine is still a matter of debate (Egan, 2003; OôNeill *et al.,* 2006). There is common consent that CQ interacts with the parasiteôs ability to digest haemoglobin. Chloroquine, a dibasic compound (pKa values: 8.1 and 10.2), is trapped in the acidic digestive vacuole (pH 5.0ï 5.4) as a dication where it accumulates by some orders of magnitude. Similar to the other 4-aminoquinolines, CQ forms a complex with ferriprotoporphyrin IX and thereby prevents its polymerization into haemozoin. This has been recently confirmed by spinning-disc confocal microscopy of live intraerythrocytic malaria parasites (Gligorijevic *et al.,* 2006).

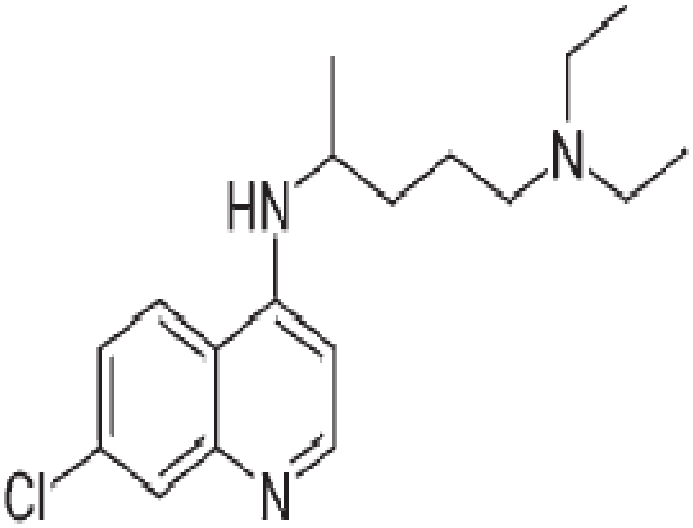


Figure 1.6: The Chemical Structure of Chloroquine (Schlitzer, 2007).

1.4 Lumefantrine

Lumefantrine (also known as benflumetol) is structurally similar to halofantrine. It was developed in the 1970s by the Academy of Military Sciences in Beijing, China. It shows lower antimalarial activity than halofantrine. IC50 values against three different laboratory strains were 8.9ï 9.9 nM for halofantrine and 34ï 44 nM for lumefantrine. In a study with parasites collected from Cameroonian patients, the median IC50 value was 11.9 nM for lumefantrine and 1.6 nM for halofantrine. Chloroquine-resistant parasites are slightly more susceptible than chloroquine-sensitive strains (Basco *et al.,* 1998). Similar to halofantrine, the oral bioavailability of lumefantrine is variable. Oral absorption of this highly lipophilic drug is enhanced 16-fold if taken with a fatty meal. The most significant difference from halofantrine is the absence of the dangerous cardiac side effect (Ezzet *et al.,* 2000). Lumefantrine displays in vitro synergism with

artemether (Alin *et al.,* 1999). This combination is currently used under the brand name Riamet (Omari *et al.,* 2004). Desbutyllumefantrine is one putative metabolite, although it has not been detected in humans. It has about four fold higher antimalarial activity than its parent drug (Noedi *et al.,* 2001).

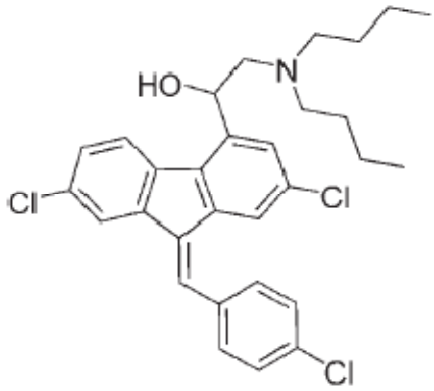


Figure 1.7: The chemical structure of Lumefantrine (Schlitzer, 2007).

1.5 Artemisinins and Synthetic Peroxides

Extracts of the herb known as sweet wormwood have been used in China for the treatment of fever for as long as 2000 years. In 1971 the active ingredient, the sesquiterpene lactone artemisinin was isolated, which has been used in China for the treatment of malaria since 1972 (Haynes and Vonwiller, 1997). Artemisinin is a highly active antimalarial agent. In assays with 40 wild isolates from northwestern Thailand, a mean IC50 value of 12.1 nM (8.2ï 17.9 nM) has been reported (Ramharter *et al.,* 2002). In a different study, a slightly higher value of 21nM (15.5ï 28.3 nM) was observed (Tanariya *et al.,* 2000). A key structural feature of all artemisinins is the 1,2,4-trioxane substructure or, more precisely, the endoperoxide, which is mandatory for antimalarial activity. Despite the growing importance of artemisinins, their exact mechanism of action is still unresolved and remains a matter of intense debate. It has been proposed that iron (II) mediated cleavage of the endoperoxide leads to the formation of different C-centered radicals which may be primary or secondary in nature. For a long time it was thought that the formation of C radicals takes place in the digestive vacuole and that ferrousprotoporphyrin IX is the activating species. The reactive C radicals are thought to subsequently react more or less indiscriminately with different protein targets as well as with ferriprotoporphyrin IX itself, thus preventing haem detoxification and inhibiting a multitude of enzymes (Posner and OôNeill, 2004; Haynes and Krishna, 2004).

1.5.1 Artemether

Because artemisinin is only poorly soluble in water and in oil, semisynthetic derivatives have been developed. The reduction of the lactone substructure of artemisinin leads to the hemiacetal-containing compound dihydroartemisinin. Alkylation of the hemiacetal produces artemether and arteether, both characterized by an acetal moiety (and not by an ether as the names might indicate). Arteether is used in India and the Netherlands (Artemotil) but the more prevalent substance is artemether (Woodrow *et al.,* 2005). Artemether displays high antimalarial activity, with reported mean IC50 values against wild isolates of 1.54, 2.5, 5.3, and 16.2 nM (Pradines *et al.,* 1999; Brockmann *et al.,* 2000). Oxidative demethylation starting with the well-known hydroxylation in the Ŭ-position to the exocyclic acetal oxygen atom rapidly leads to dihydroartemisinin, which contributes approximately 50% of the effect of artemether. Dihydroartemisinin itself undergoes rapid hydroxylation at positions 5, 7, and 14, and glucuronidation at the hemiacetal OH group to yield highly water soluble metabolites, resulting in an elimination half-life of 40ï 60 min (Haynes, 2001). Because of this high clearance rate, artemisinins have to be administered over a period of five to seven days, which leads to poor compliance and ultimately to recrudescence (Ashley *et al.,* 2006). Therefore, artemisinins are combined with antimalarial drugs that have prolonged half-lives (Kremsner and Krishna, 2004; Mutabingwa, 2005). The modification of dihydroartemisinin to artemether led to a more lipophilic molecule, which is better absorbed from the gastrointestinal tract, thus allowing oral administration. Blood levels after application of an oily solution have shown to be unpredictable and sometimes undetectable (Woodrow *et al.,* 2005). Currently, the application of artemether with lumefantrine (Coartem or Riamet) is the only artemisinin based combination therapy available manufactured under Good Manufacturing Practice (GMP) standards.

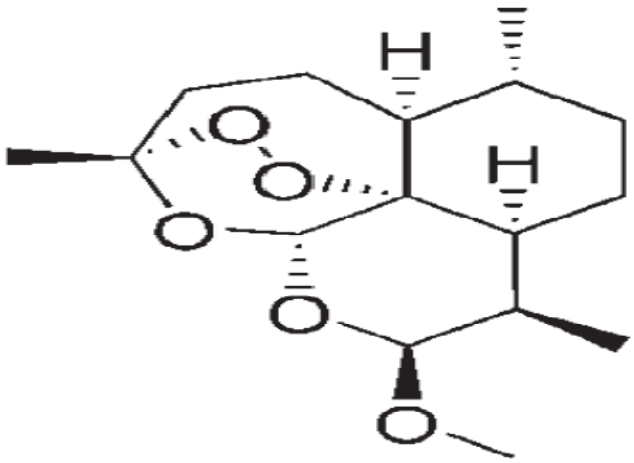


Figure 1.8: The chemical structure of artemether (Schlitzer, 2007).

1.6 Oxygen-Carrying Protein in the Blood: Haemoglobin

Haemoglobins are tetrameric conjugate proteins comprised of pairs of two different polypeptide subunits and prosthetic haem group which contains iron (Nelson and Cox, 2005). Greek letters are used to designate each subunit type. The subunit composition of the principal haemoglobins are $Ǔ_2ɓ_2$ (HbA; normal adult haemoglobin), $Ǔ_2ɔ_2$(HbF; foetal haemoglobin), $Ǔ_2S_2$(HbS; sickle cell haemoglobin), and $Ǔ_2ŭ_2$ (HbA_2; a minor adult haemoglobin). The primary structures of the ɓ, ɔ, and ŭ chains of human haemoglobin are highly conserved. The ferrous iron is coordinated to four pyrrole nitrogen of protoporphyrin IX and to imidazole nitrogen of a histidine residue of porphyrins (Rodwell and Kennelly, 2003). The sixth coordinate position is available for binding to small molecules such as O_2, CO or CO_2 (Scott and Eagleson, 1988). A mutation which results in the replacement of glutamate with valine at position six of the two ɓ-chains of HbA gives rise to mutant form of haemoglobin called sickle haemoglobin (HbS) (Nelson and Cox, 2005). It is well known that the main physiological function of haemoglobin is transport of oxygen. Other functions of haemoglobin are enzymatic such as lipoxygenase, peroxidase, oxygenase as well as catalase- like activities (Paco *et al.,* 2009).

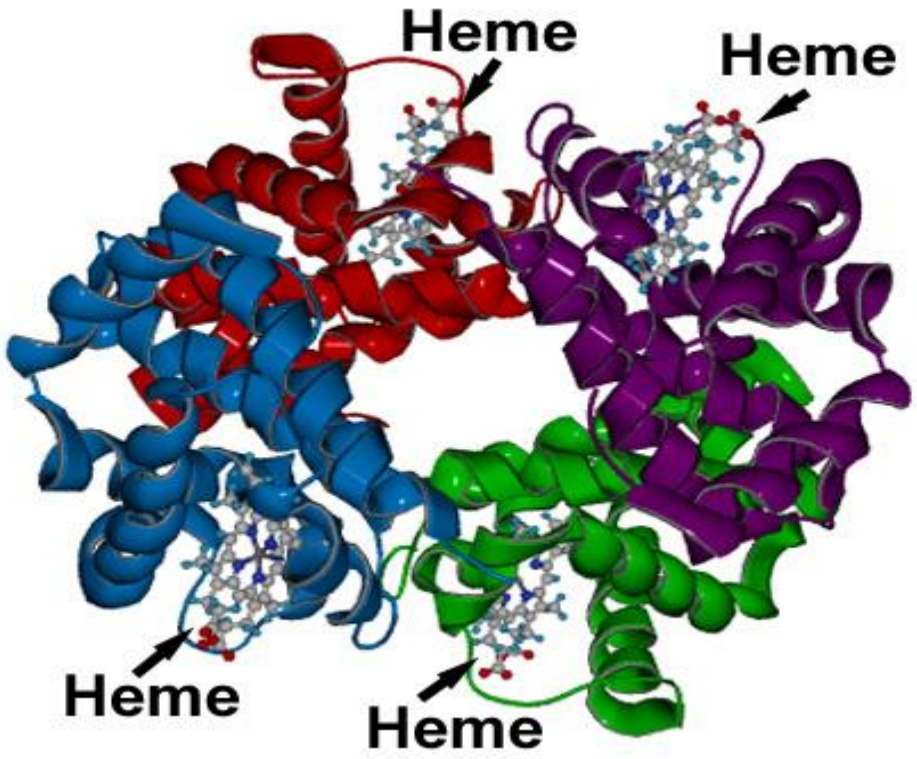


Figure 1.9: Three dimensional structure of haemoglobin (Wikipedia, 2013)

1.6.1 Haemoglobin Variants

Haemoglobin variants are mutant forms of haemoglobin in a population (usually of humans), caused by variations in genetics. Some well-known haemoglobin variants such as sickle cellanemia are responsible for diseases, and are considered haemoglobinopathies. Other variants cause no detectable pathology, and are thus considered non-pathological variants. Some normal haemoglobin types are; Haemoglobin A (HbA), which is 95-98% of haemoglobin found in adults, Haemoglobin A_2 (HbA_2), which is 2-3% of haemoglobin found in adults, and Haemoglobin F (HbF), which is not found in adults and is the primary haemoglobin that is produced by the fetus during pregnancy (Bonavetura and Riggs, 1968).

Haemoglobin variants occur when there are genetic changes in specific genes, or globins that cause changes or alterations in the amino acid. They could affect the structure, behaviour, the production rate, and/or the stability of that specific gene. Usually there are four genes that code for alpha globin and two genes that code for beta globin. If the genes for alpha chains are mutated, the most common condition that occurs is alpha thalassemia, which causes a decrease in production of that gene. The level of severity of alpha thalassemia is determined by the number

of genes that are affected. With lengthy list of common haemoglobin variants, there are some variants that are less common. These variants are considered silent, which means that they have no signs or symptoms. They usually affect the functionality and/or the stability of the hemoglobin molecule. With most of these variants are mutations in the alpha globin gene that results in an abnormally long alpha chain and an unstable haemoglobin molecule (Bonavetura and Riggs, 1968).

1.6.2 Haemoglobin Digestion by Malaria Parasite: Role of Multiple Proteases

The malaria parasite depends on the host to fulfill its requirements of amino acids for synthesis of proteins and other metabolic functions. Haemoglobin is the most abundant protein in the erythrocytes cytoplasm (5 mM) and provides the major source of amino acids (Krugliak *et al.,* 2002). The malaria parasite, during intra-erythrocytic development and proliferation, ingests more than 75% of the hostôs haemoglobin. As an estimate, at 20% parasitemia about 110 g of haemoglobin would be consumed by the parasite during 48 h. The parasite ingests haemoglobin by the process of pinocytosis via the cytostome (Slomianny *et al.,* 1985). Ingested haemoglobin is degraded inside the acidic digestive vacuoles through a specific semiordered sequential process, which involves multiple proteases (Goldberg *et al.,* 1991). Digestive vacuoles in the parasite are acidic organelles with a pH estimated at 5.0-5.4. Different aspartic proteases (plasmepsins), cysteine proteases (falcipains) and a metalloprotease (falsilysin) have been characterized from the digestive vacuoles of *P. falciparum*. Both plasmepsin I and plasmepsin II are able to cleave native non-denatured haemoglobin (Banerjee *et al.,* 2002). The degradation of haemoglobin is initiated by plasmepsin I, which hydrolyses the 33Phenylalanine-34Leucine bond in the hinge region that appears to be vital for integrity of the haemoglobin tetramer. Eight additional plasmepsin genes have been identified in the *P.falciparum* genome. A histo-aspartate protease (HAP) and plasmepsin IV are homologous to plasmepsin I and II. HAP and plasmepsin IV were also found to be active in *P.falciparum* food vacuole and may be the additional aspartate proteases involved in haemoglobin digestion (Banerjee *et al.,* 2002). As soon as the haemoglobin molecule is relaxed the plasmepsins (I, II) and the falcipains can further cleave at other sites of the protein.

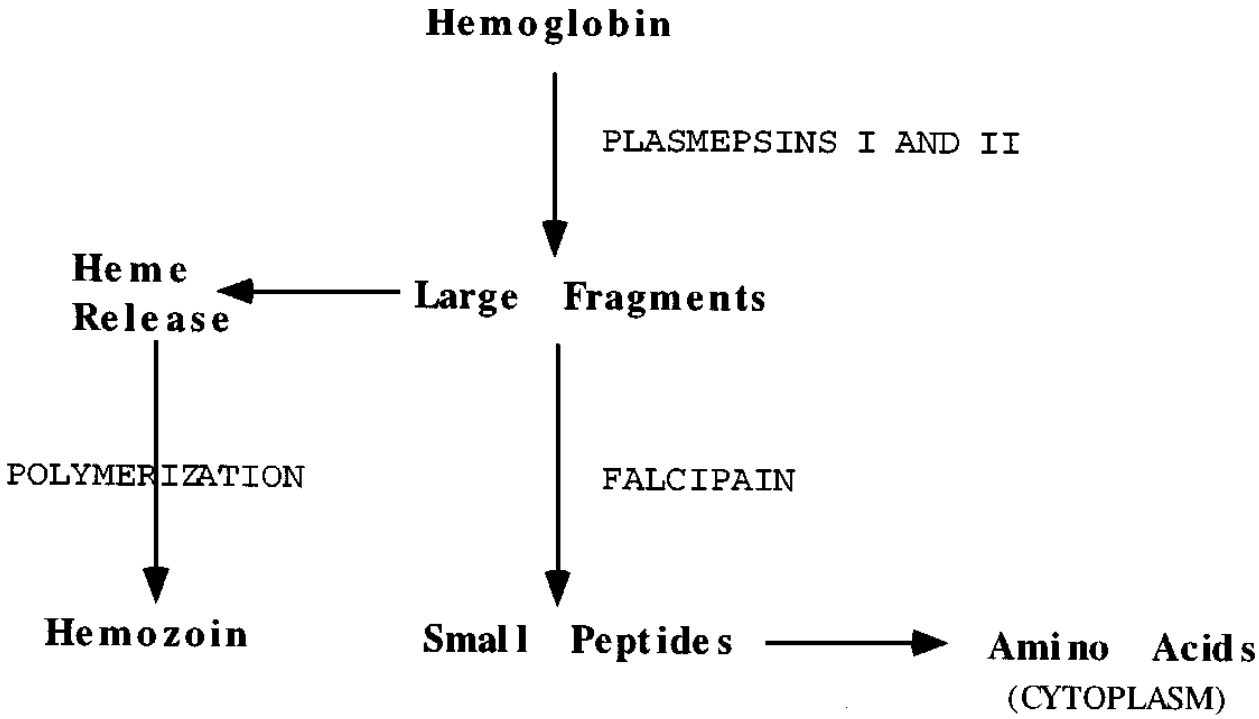


Figure 1.10: Proposed pathway for haemoglobin degradation in *P. falciparum* (Francis *et al.,* 1997).

1.6.3 Oxidative Stress Generated by Haemoglobin Degradation

In the erythrocyte, haem iron is almost entirely in the ferrous state. Upon degradation in the parasite digestive vacuole, the iron is oxidized to the ferric state. Electrons liberated by oxidation combine with molecular oxygen to produce reactive oxygen species: superoxide anions ($O_2^{\bar{\cdot}}$), hydroxyl radicals (OH^-), and hydrogen peroxide (H_2O_2) (Rosenthal, 2002). This appears to be a major source of oxidative stress to the parasite. Oxidative damage to the digestive vacuole is prevented by the action of superoxide dismutase (SOD) which converts superoxide radicals to hydrogen peroxide. Hydrogen peroxide is then cleaved by catalase. Both SOD and catalase are found in the digestive vacuole (Srivastava and Pandey, 1995; Egan *et al.,* 2002), and they are likely obtained from the host when erythrocyte cytoplasm is ingested. In addition, a parasite-derived SOD gene has been described from *P. falciparum* (Krugliak *et al.,* 2002). Its role as a vacuolar antioxidant is uncertain, because the enzyme that is encoded by the cloned gene has not been localized. Reduced glutathione, another erythrocyte antioxidant, is present in red blood cell cytoplasm at 2.2 mM and may also provide some relief from oxidative stress after ingestion. Reactive oxygen species that are generated by the parasite during haemoglobin degradation have

been proposed to be responsible for further damage to the host cell by creating oxidative stress (Tekwani and Walker 2005). Reactive oxygen species that originate in the parasite have been detected in infected erythrocyte cytoplasm (Rosenthal, 2002). Their role in malarial pathogenesis is not yet known. Interestingly, conditions which predispose erythrocytes to oxidative stress (like glucose 6-phosphate dehydrogenase deficiency) partially protect the host against infection by *P. falciparum* (Sulliavan *et al.,* 1996). This is possibly because parasite-derived oxidants overwhelm the hostגs antioxidant system, resulting in damage to the infected cell and in clearance by the reticuloendothelial system (Sulliavan *et al.,* 1996).

1.7 Haem Detoxification Pathways of the Malaria Parasite

The intra-erythrocytic asexual stage of the malaria parasite resides in environment rich in haemoglobin and is exposed to toxic amounts of haem when haemoglobin undergoes degradation, as occurs spontaneously in haemolytic anemia with the production of Heinz bodies (Sills and Zinkham, 1994). It was shown that concentrations of haem as low as 20 mM will lyse malaria parasites within 10 minutes; this amount of haem can be produced by the destruction of only 0.1% of total haemoglobin present in erythrocytes (Fitch, 1983). The trophozoite stage of malaria parasite consumes almost 75% haemoglobin of erythrocyte, with concomitant release of free haem. With haem as a prosthetic group of haemoglobin, the iron is in the ferrous state, but once haem is free from its protein scaffold it tends to lose one electron and assumes the ferric state (Hargrove *et al*., 1997). This ferric haem could promote membrane damage via its peroxidative properties (Stojiljkovic *et al.,* 2001), and may also interfere with the haemoglobin degradation pathway. The cysteine protease, falcipain has been shown to be very sensitive to free haem (Pandey *et al.,* 1999). To avoid the damaging properties of free haem it is necessary for the parasite to convert it to non-toxic metabolites. Several properties of haemozoin, which is structurally similar to ß-haematin, make it an ideal excretory product of haem detoxification. First it is dense and insoluble under physiological conditions and this may be an irreversible link between haemoglobin degradation and release of haem. Due to the coordinated nature of haem in ß- haematin it does not contribute to oxygen radical stress and auto-oxidation. Detoxification of haem occurs through different pathways. The most important and the predominant mechanism is sequestration of haem into ñhaemozoinò, also known as the malarial pigment. Haem is incorporated into a crystalline black brown pigment which is accumulated within the parasite

food vacuole. Haemozoin may be visualized as regular fibrillar crystals by electron microscopy (Saliba *et al*., 1998). Appearance of haemozoin crystals may be used as evidence for the presence of malaria parasite within blood and may have importance in diagnosis of the disease (Hanscheid *et al.,* 2001). After schizont maturation when the erythrocyte ruptures, haemozoin is released into the circulation. It is initially ingested by macrophages, where it appears to diminish phagocytic capacity and also alter cytokine secretion pathways. Haemozoin has been shown as a potent proinflammatory agent *in vivo*, which could contribute to the immunopathology related to malaria. The hemozoin is predominantly accumulated in liver and spleen of the host, where amount of haemozoin increases with the progress of parasitaemia (Pandey *et al*., 1994).

1.8 Haemozoin: The Antimalarial Drug Target

Synthesis of haemozoin by the malaria parasite as a non-toxic metabolite of haem detoxification process is the most distinct characteristic of the parasite. Malaria pigment was discovered even before the discovery of the parasite. Even though several questions, regarding exact mechanism of haemozoin synthesis by the malaria parasite, still remain unanswered, convincing evidences have been produced on inhibition of haem detoxifications functions of the malaria parasite as a target for action of most of the blood schizontocidal antimalarials, which are currently in clinical use (Ziegler *et al.,* 2001; Egan, 2002; Sullivan, 2002). Inhibition of haem detoxification function of the malaria parasite would lead to accumulation of toxic haem which would kill the parasite due to membrane lysis and interference with other vital functions of the parasite (Francis *et al.,* 1997). Quinoline antimalarials have been found to be the most consistent inhibitors of haemozoin synthesis. Several novel antimalarials also have been found to interfere with the process of haemozoin formation (Kurosawa *et al.,* 2000).

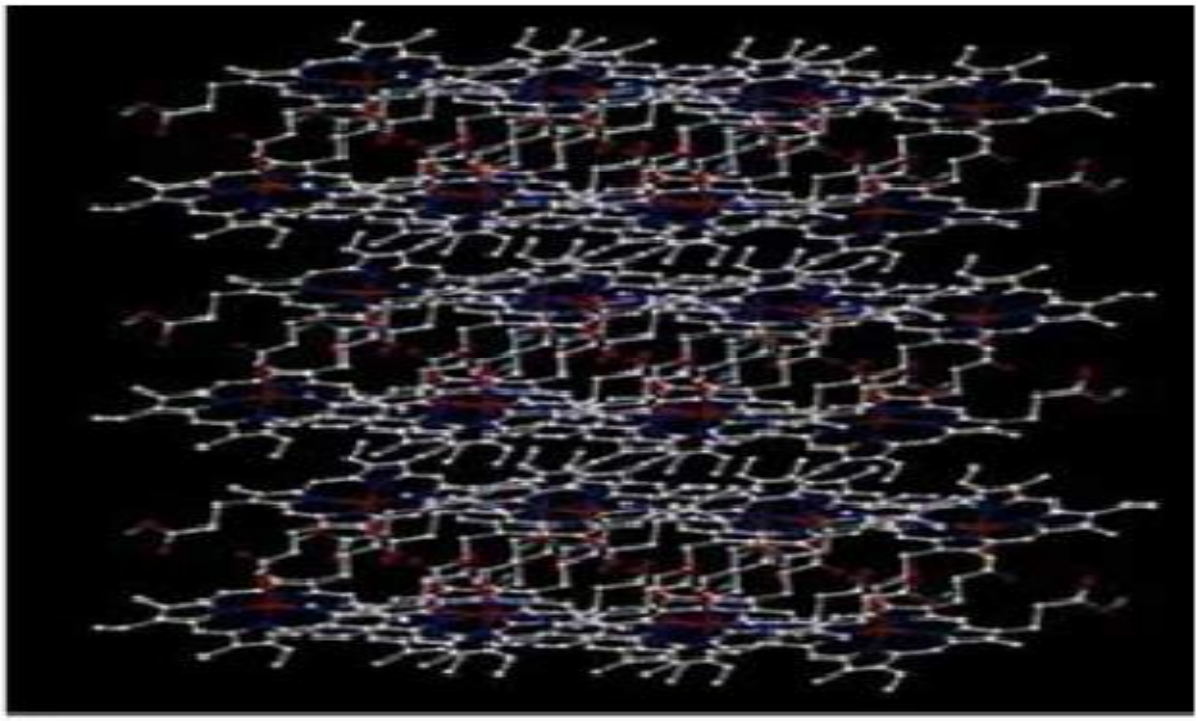


Figure 1.11: Proposed structure of haemozoin (Tekwani and Walker, 2005)

1.8.1 Mechanism of Haemozoin Formation in the Malaria Parasite

This is the most important and intriguing aspect related to haemozoin synthesis pathway, but is still under extensive debate. Several theories have been proposed to explain the mechanism of haemozoin formation by the malaria parasite. The concept of mechanism of haemozoin synthesis has been shifting along with the more accurate understanding of the structure of the haemozoin. The very early idea of formation of the malaria pigment was simply the association and sequestration of free haem, released as a result of haemoglobin degradation, with a protein (either a partially digested peptide derived from haemoglobin or a parasite derived protein) (Yamada and Sherman, 1979). Later, when haemozoin was characterized as a haem polymer, the proposal was made of an enzymatic reaction termed as ðhaem polymerizationô and a parasite derived proteinaceous factor named ñhaem polymeraseò was proposed to catalyze this reaction (Slater and Cerami, 1992). Formation of a product similar to haemozoin could be demonstrated in the presence of various factors related and unrelated to malaria parasites and based on these reports different mechanisms of formation of malaria pigment have been proposed. The reports demonstrating *in vitro* synthesis of ß-haematin may be mainly categorized into four groups

namely chemical synthesis and spontaneous formation of ϐ-haematin; role of different proteins in initiation of ϐ-haematin formation; formation of haemozoin by autocatalysis and finally initiation of haem polymerisation by different lipids.

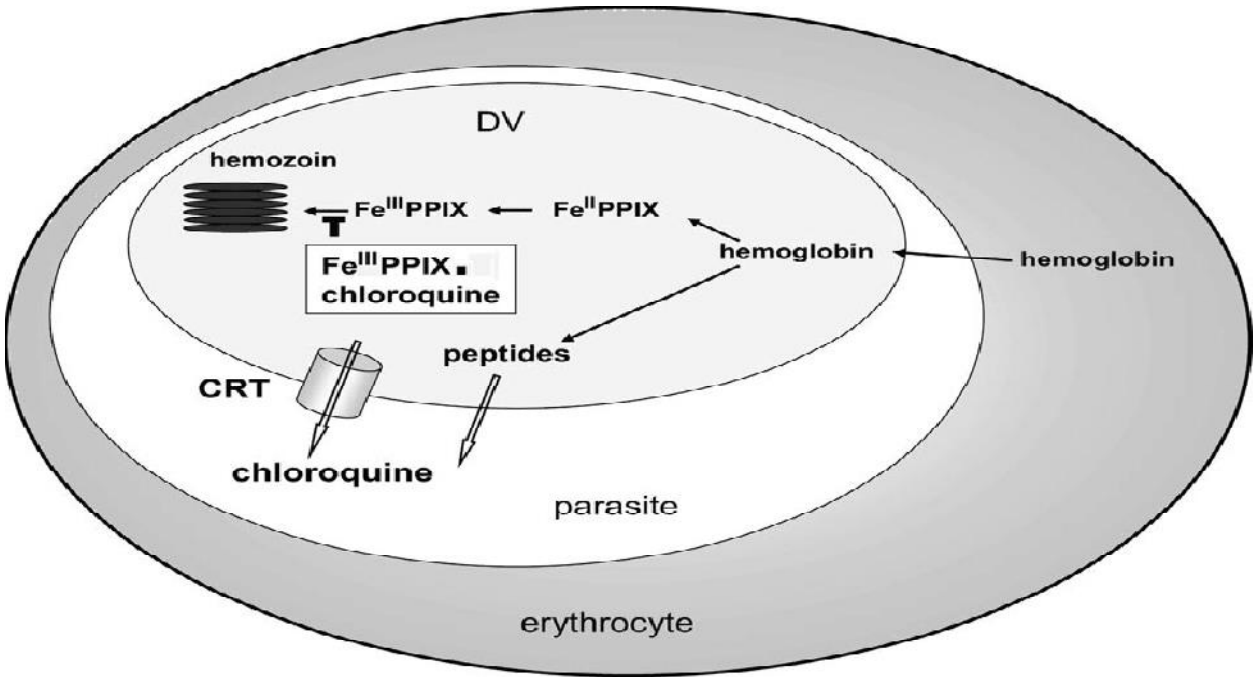


Figure 1.12: The proposed mechanism of haemozoin formation in digestive vacuole of malaria parasite (Francis *et al*., 1997)

1.9 Spectral Properties of Haemoglobin

The haem group consists of a porphyrin ring with a ferrous or ferric iron co-ordinated centrally. The conjugated double bond system of the porphyrin ring causes a strong absorption in haemprotein termed Ŭ, ϐ, ɔ-bands. Typically, Ŭ-bands occur at the longest wavelengths (550-650), ɔ-bands at shortest wavelengths (also called Soret bands, after the Swiss scientist who first examined the near UV region of cytochromes and the ϐ-bands lie between.

Solution of oxyhaemogloin is bright red, and tends to a yellowish colour at high dilutions. A dark red colour indicates the presence of some ferric haemoglobin. Oxyhaemoglobin shows two visible absorption bands with maxima at 577 nm and 542 nm from mammalian haemoglobin; these bands are shifted towards the red (591 and 542 nm) in myoglobin. The Soret band of oxyhaemoglobin lies near 415nm (Antonin and Brunori, 1971). Solutions of

deoxygenated haemoglobin and myoglobin have a typical red violet colour which becomes greenish at high dilutions. The absorption spectrum of deoxygenated haemoglobin is characterized by a single, broad and asymmetrical band in the visible, with a maximum at about 555 nm; the soret band, in the near ultraviolet shows a maximum at 430 nm in deoxygenated haemoglobin and 435 nm in myoglobin. The spectrum of iron (III) haemoglobin or myoglobin is pH sensitive. Acidic iron (III) haemoglobin and myoglobin appear dark brown in concentrated solution and yellow-green in dilute solution. The spectrum shows two bands in the visible region with maxima at about 500 and 625 nm; the soret band lies at about 405 nm. Alkaline iron (III) haemoglobin has two bands in the visible region with maxima at about 540 and 580 nm. The soret band of the alkaline form has a maximum at 412 nm. Iron (III) haemoglobin usually has two forms (states) known as low and high-spin forms (states). There are absorption bands characteristic for absorption spectra of haemoglobin molecule: Ferryl haemoglobin is distinguished from methaemoglobin by its lack of showder at 630 nm; oxyhaemoglobin at 577 and 542 nm bands; haem-haem interaction band (Soret band) at 420 nm; globin-haem interaction band at 340 nm and constant globin at 275 nm which is characteristic of dynamic motion of haemoglobin (Samir, 2006; Ibrahim *et al.,* 2008).

A decrease in the ration (A_{577}/A_{542}) Indicates conversion of oxyhaemoglobin to methaemoglobin and can be observed in anemia. Ibrahim *et al.* (2008) showed that decrease in absorbance ratio (A_{577}/ A_{542}) and absorbance of soret band at 420 nm with a concomitant appearance of a new band at 630nm means conversion of oxyhaemoglobin to methaemoglobin. Nabil, (2008) revealed that decrease in the absorbance ratio (A_{577} / A_{542}) shows an imbalance of the two bands at 542 and 577 nm which indicate a defect in the bonds between haem iron and nitrogen in porphyrin ring and haem-haem interaction bands, respectively. Generally, the degree of conversion of oxyhemoglobin to methaemoglobin depends on the degree of unfolding and leads to the existence of a hybrid of low spin and high spin states as it appears like shift toward shorter wavelength of the soret band. The degree of this conversion depends on haem-haem interaction at 577 nm and the elevation in absorbance at the band detected at 630 nm (Ibrahim *et al*., 2008).

Decrease in absorbance band at 340 nm refers to the stretching or weakness of the non-covalent bond between histidine of globin and haem iron (Ibrahim *et al,* 2008). Absorbance of haemoglobin at 275 nm corresponds to constant globin. Proteins are dynamic systems and their

motions are essential to their functions. Enhancement in the absorbance at 275 nm, as an indication for its abnormal motion, reflects its deviation from a normal structure and function depending on the degree of globin unfolding and random motion of the haemoglobin molecule under different degrees of oxidative conditions (Ibrahim *et al.,* 2008). Globin chain imbalance has been observed to be an important determinant of the clinical severity of anemia, especially thalassemia. It has been reported that the band at 275 nm (Jetsrisuparb *et al.,* 2006) is assigned to **Π – Π*** transitions in the aromatic amino acids. Elevation in the half soret band width and shifting towards shorter wavelength indicate the stretching of iron and nitrogen bond in porphyrin ring and the imbalance between protein and haem in the haemoglobin molecule. These are due to coupling with a new band at 630 nm.

1.10 Aim and Objectives of Study

1.10.1 Aim of the Study

This study is aimed at determining the interaction of antimalarial drugs: pyrimethamine and sulphadoxine with normal and sickle haemoglobins.

1.10.3 Specific Objectives of the study

1. Titration of pyrimethamine and sulphadoxine with haemoglobins
2. Titration of pyrimethamine and sulphadoxine combinations with haemoglobins

CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

All chemicals used in this work were of analytical grade

Sodium dodecyl sulphate (BDH, England)

Tris salt [tris-(hyfroxylmethyl) amino] methane (Mw = 121.114) (Sigma, Germany)

Absolute ethanol

Hydrochloric acid (Sigma, Germany)

Sodium acetate (BDH, England)

Glacial acetic acid (May and Baker)

2.1.2 Equipment

Equipment	**Make**	**Model**
UV-Visible spectrophotometer	Jenway, USA	6405
Magnetic stirrer	USA	AM- 3250B
pH meter	Singapore	ECOSAN 601
Weighing balance	China	Metter H51
Centrifuge	PAC pacific, USA	2030E

2.2 Methods

2.2.1 Collection of Blood Samples

Four millilitres (4 ml) of blood samples were collected from two identified individuals of genotype AA and AS after informed consent. A measured volume, 2 ml of blood sample was collected from an identified individual of genotype SS after informed consent and when the individual was not in crises. In each case, the blood sample was collected with an ethylene diamine tetracetic acid (EDTA) bottle.

2.2.2 Isolation and Purification of Haemoglobin

A known volume, (4 ml) each of the collected blood samples was combined with 6ml of cold normal saline in 50 mM Tris ï HCl pH 7.2 (wash buffer) and kept in the fridge (4 ^{o}C) for 10 min. In the case of the 2ml blood sample, 3ml of cold normal saline in 50mM Tris ï HCl pH 8.5 was added and was also kept in the fridge for 10 min. The resulting solutions were centrifuged for 10 min at 4000 rpm (Denninghoff *et al.,* 2006). Thereafter, the supernatants were removed by aspiration. The same amount of wash buffers were appropriately introduced into the pellet and kept in the fridge for 20 min. The above steps were repeated for 2-4 times until a clear supernatant appeared in each case. The clear supernatants were removed and the resulting pellets in the case of 4 ml blood sample were made up to 5ml while that of 2 ml blood sample was made up to 2.5ml using 50 mM Tris - HCl buffer, pH 7.2. The samples containing 50 mM Tris - HCl were kept in the freezer in order to lyse the red cells. After lysing, four millilitres of 5 % NaCl was added to the resulting volume and centrifuged for 10 min at 4000 rpm to remove inorganic phosphates and other ions from the sample. After the centrifugation the resulting supernatants (crude haemoglobin) were collected into separate vials and labelled appropriately. Each of the crude haemoglobin (i.e HbA, HbAS and HbS) was dialysed at 4 ^{o}C for 12 h against 50 mM Tris ï HCl buffer, pH 7.2. The dialyzed haemoglobin samples were collected and stored at -20 ^{o}C for further experiments.

2.2.3 UV - Visible Titration

A known volume, (100 εl) of 0.01mM of each of the haemoglobin samples calculated on haem basis by using $\varepsilon_{415} = 1.25\times10^{5}\ M^{-1}\ cm^{-1}$ (Gebicka and Banasiak, 2009) was scanned from 250 nm to 700nm using JENWAY 6405 UV ï VIS Spectrophotometer in the absence and presence of SDS and different concentrations of drugs in 50 mM buffers of pH 5.0 and 7.2 after appropriate buffer baseline. The titrations were done by fixing 0.1 ml of the haemoglobins in 1.5 ml cuvette containing a fixed volume of the buffer (0.7 ml) then various volumes (10 to 130 εl) corresponding to different concentrations of the drugs (0 to 0.025 mM for pyrimethamine and 0 to 0.013mM for sulphadoxine) were added in stepwise manner from stock solution of the drug (0.25 mM for pyrimethamine and 0.117 mM for sulphadoxine). This was mixed and scanned from 250 to 700 nm. Spectral readings were recorded at each titration point (after each addition of the drug solutions). The results were analyzed by monitoring absorbance changes at different wavelengths of the haemoglobin spectra (275, 340, 415, 542, 560, 576 and 630 nm) and concentrations of Oxy-, Deoxy- and Methaemoglobin were calculated according to equation 1-3 below as reported by Reza *et al.* (2002). In the equations below, A_{576} means absorbance at 576 nm, A_{630} means absorbance at 630 nm etc.

[Oxy]= $(1.0154A_{576}$ ï $0.2772A_{630}$ ï $0.742A_{560}) \times 10^{-4}$Mol é é é é é é é é .(1)

[Deoxy]= $(1.335A_{560}$ ï $0.7356A_{576}$ ï $0.6254A_{630}) \times 10^{-4}$Mol é é é é é é é .(2)

[Met]= $(2.6828A_{630} + 0{,}174A_{576}$ ï $03414A_{560}) \times 10^{-4}$Mol é é é é é é é ..(3)

2.2.4 Data Analysis

The spectral data were plotted with Microsoft excel and peaks of haemoglobin absorbance at different wavelength corresponding to different chemical species were recorded. Qualitative spectral analysis of haemoglobin ï drug interaction was obtained by visual inspection.

CHAPTER THREE

RESULTS

3.1 Absorption spectra of hemoglobin A

3.1.1 Absorption spectra of haemoglobin A in varying concentrations of pyrimethamine.

Fig. 3.1 shows that pyrimethamine at pH 5.0 and 7.2 increased the absorbance of the aromatic band (275 nm), with a concomitant decrease at the haemï globin band (340 nm), and soret band (415 nm) in a concentration dependent manner. There was an initial slight increase in the absorbance of the oxyhaemoglobin bands (542 and 577 nm) at lower concentration of pyrimethamine, but as the concentration increased, the bands decreased.

3.1.2 Absorption spectra of haemoglobin A in varying concentrations of pyrimethamine, in the presence of SDS.

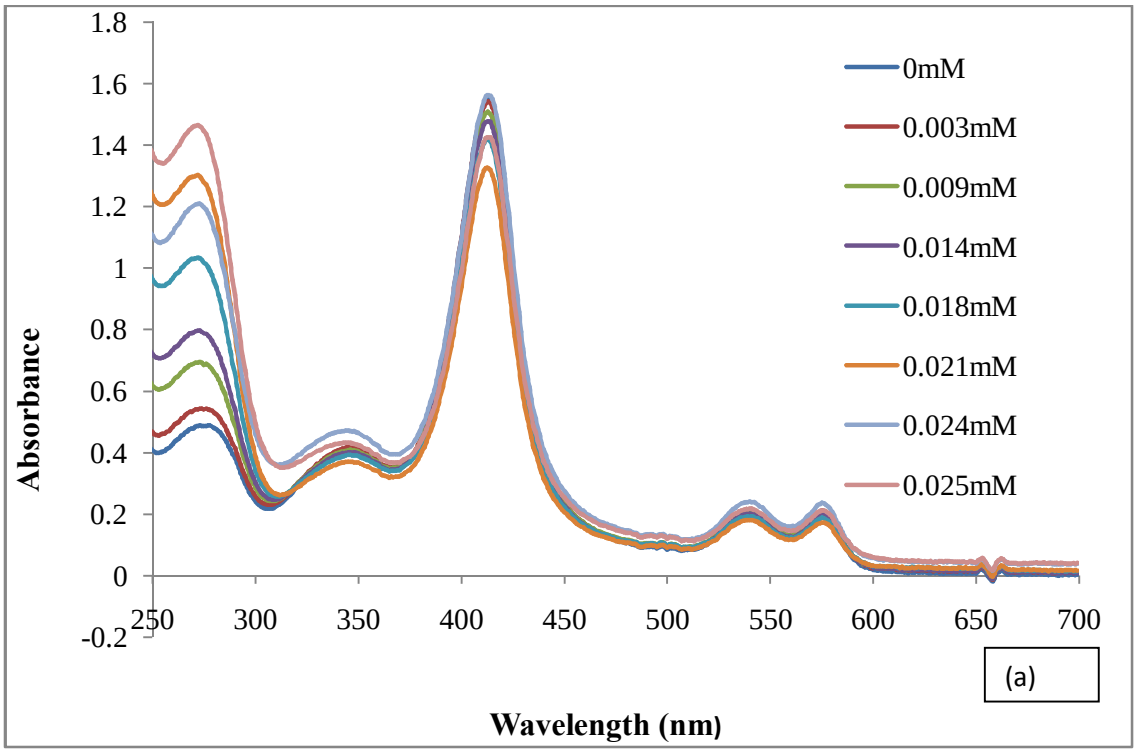
Fig. 3.2 shows the effect of sodium dodecyl sulphate on haemoglobin A both in (a) acidic pH and (b) physiological pH. The SDS caused denaturation of HbA. In Fig. 3.2a, there was a decrease in absorbance of the soret band and disappearance of the oxyhaemoglobin bands. In Fig. 3.2a Pyrimethamine increased the absorbance of the aromatic band and soret band. While in 3.2b, SDS decreased the absorbance of the aromatic band and soret band. Also there was a

stretch of the haemï globin band (340 nm) and disappearance of the oxyhaemoglobin bands (542 and 577 nm). In Fig. 3.2b, pyrimethamine increased the absorbance of aromatic band (275 nm).

3.1.3 Absorption spectra of haemoglobin A in varying concentrations of pyrimethamine and constant concentration of sulphadoxine.

Fig. 3.3 shows that pyrimethamineï sulphadoxine combination increased the absorbance of the aromatic band with a concomitant decrease of the haemï globin band, soret band and oxyhaemoglobin bands of HbA in a concentration dependent manner but the effect was more at pH 5.0 than at pH 7.2. Also, the combination of both drugs caused a hypsochromic (blue) shift of the soret band at pH 5.0 (Fig. 3.3a).

Figs. 3.1 to 3.2 show the absorption spectra of haemoglobin A in varying concentration of pyrimethamine at pH 5.0 and 7.2.



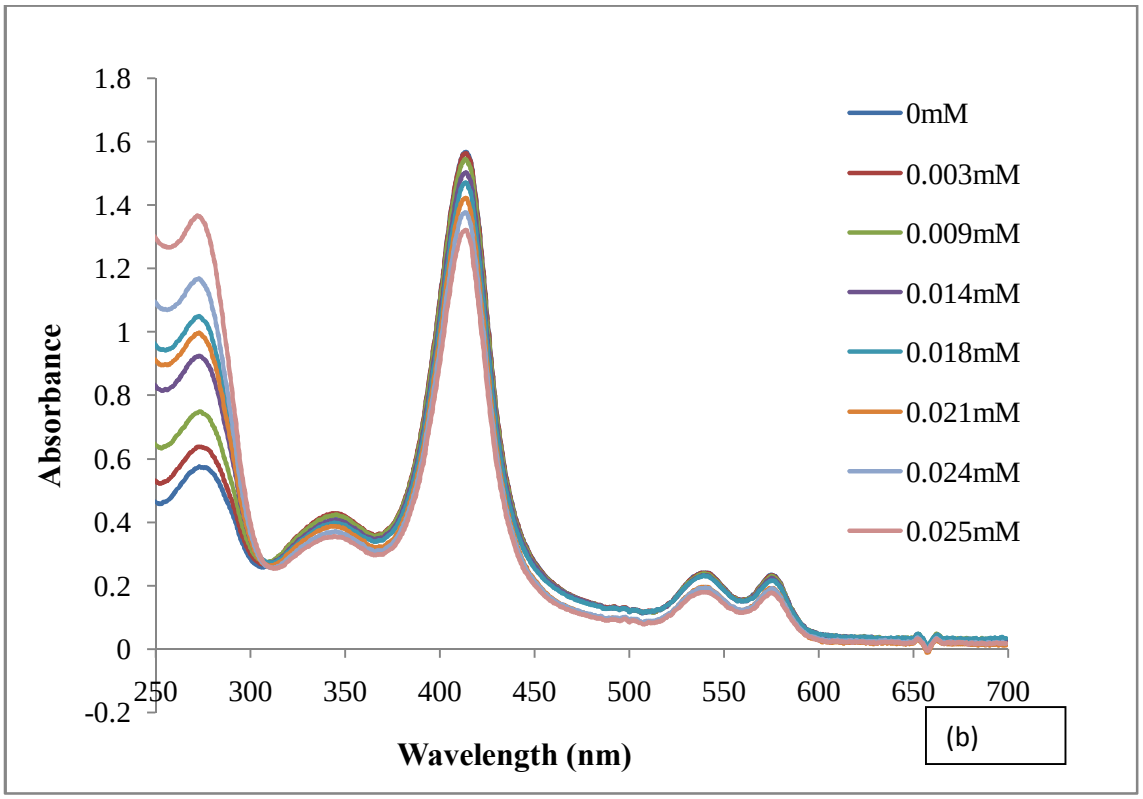


Figure 3.1: Absorption spectra of haemoglobin A in varying concentrations (0-0.025 mM) of pyrimethamine, at (a) pH 5.0 and (b) pH 7.2

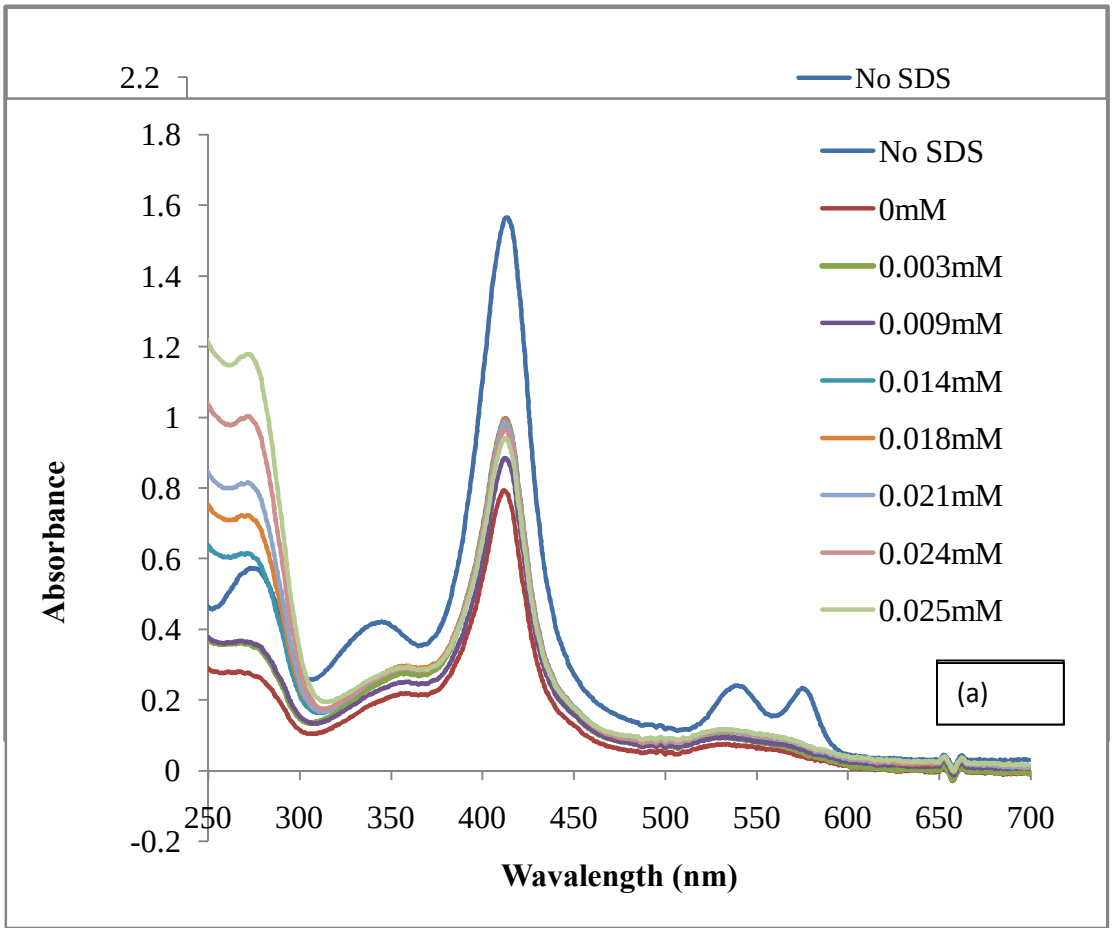
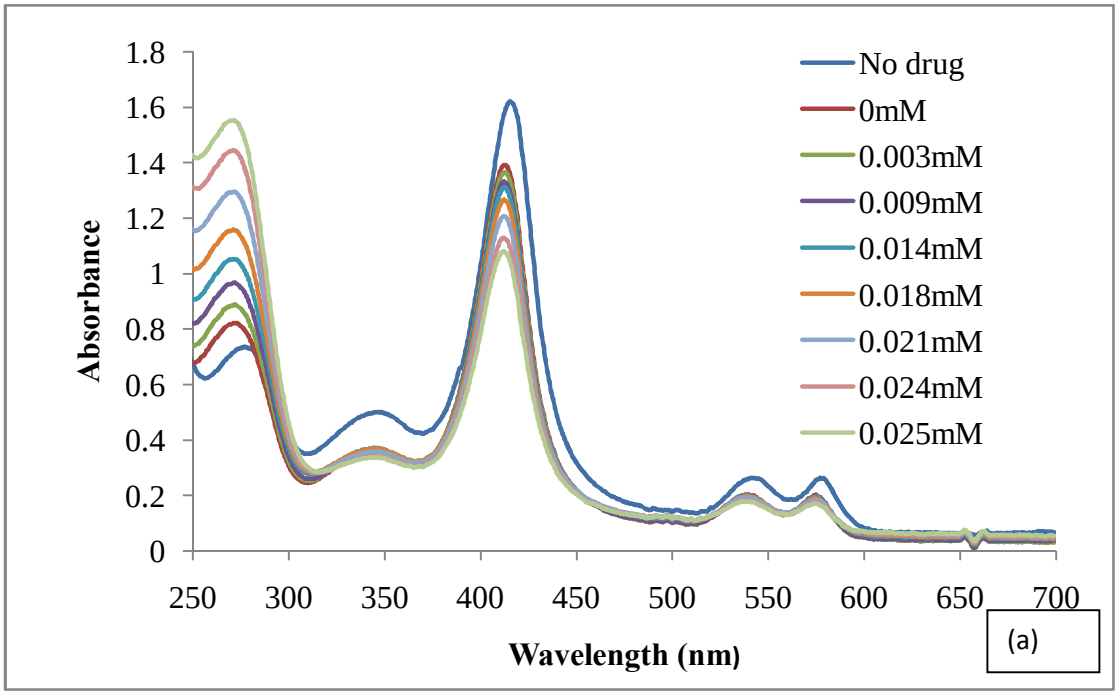


Figure 3.2: Absorption spectra of haemoglobin A in varying concentrations (0-0.025 mM) of pyrimethamine, in the presence of SDS, at (a) pH 5.0 and (b) pH 7.2

Figs. 3.3 to 3.4 show the absorption spectra of haemoglobin A in varying concentrations of pyrimethamine and constant concentration (0.008 mM) of sulphadoxine, at pH 5.0 and 7.2



(b)

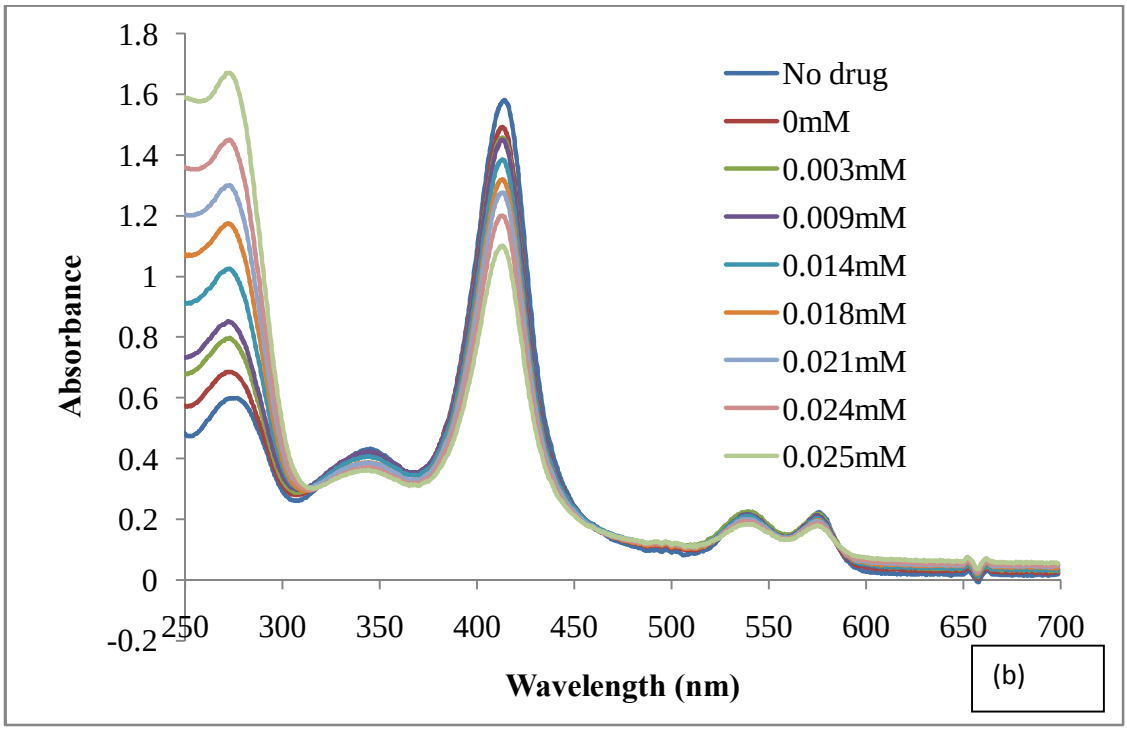


Figure 3.3: Absorption spectra of haemoglobin A in varying concentrations (0-0.025 mM) of pyrimethamine and constant concentration (0.008 mM) of sulphadoxine, at (a) pH 5.0 and (b) pH 7.2.

3.1.4 Absorption spectra of haemoglobin A in varying concentrations of pyrimethamine and constant concentration of sulphadoxine in the presence of SDS.

Fig. 3.4 shows that pyrimethamine-sulphadoxine combination increased the absorbance of the aromatic band and globin-haem band (340 nm) of HbA in a concentration dependent manner (Fig. 3.4a). At pH 7.2 (Fig. 3.4a), there was an increase in the absorbance of the aromatic band (275 nm) with a concomitant decrease at the soret band. Both Figs. 3.4a and 3.4b showed disappearance of the oxyhaemoglobin bands (542 and 577 nm).

3.1.5 Absorption spectra of haemoglobin A in varying concentrations of sulphadoxine.

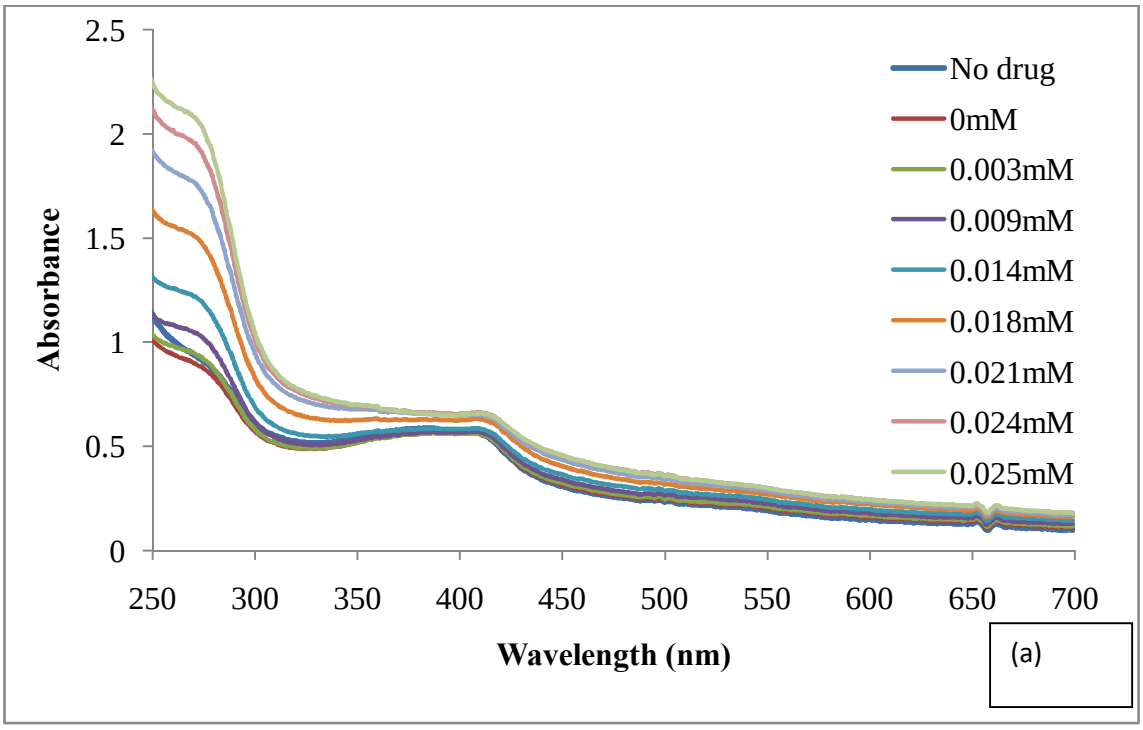
Fig. 3.5a shows that sulphadoxine increased the absorbance of the aromatic band (275 nm) of HbA. While there was a decrease at the globin-haem interaction band (340 nm), soret band (415 nm) and oxyhaemoglobin bands. Fig. 3.5b shows increased absorbance at the aromatic band and a slow decay of the soret band (415 nm).

3.1.6 Absorption spectra of haemoglobin A in varying concentrations of sulphadoxine in the presence of SDS.

Fig. 3.6 shows that sulphadoxine increased absorbance of the aromatic band (275 nm) both at pH 5.0 and 7.2. Also there was disappearance of the oxyhaemoglobin bands (542 and 577 nm) of HbA. At pH 7.2, there was a decrease in absorbance of the soret band (415 nm).

3.1.7 Absorption spectra of haemoglobin A in varying concentrations of sulphadoxine and constant concentration of pyrimethamine.

Fig. 3.7 shows that sulphadoxine-pyrimmethamine combination increased absorbance of the aromatic band (275 nm) and decreased absorbance of the soret band (415 nm) of HbA. In Fig. 3.7a, there was a decrease in absorbance of the globin-haem interaction band (340 nm) and oxyhaemoglobin bands (542 and 577 nm). Also there was a shift in soret band towards shorter wavelength.



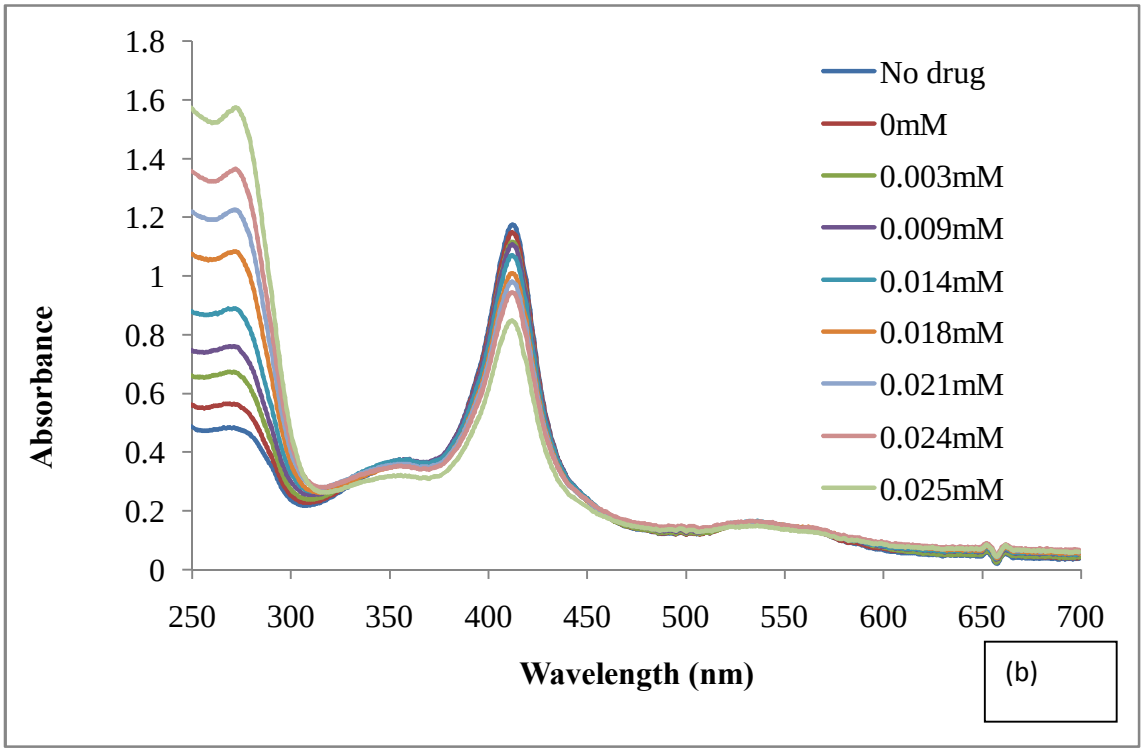
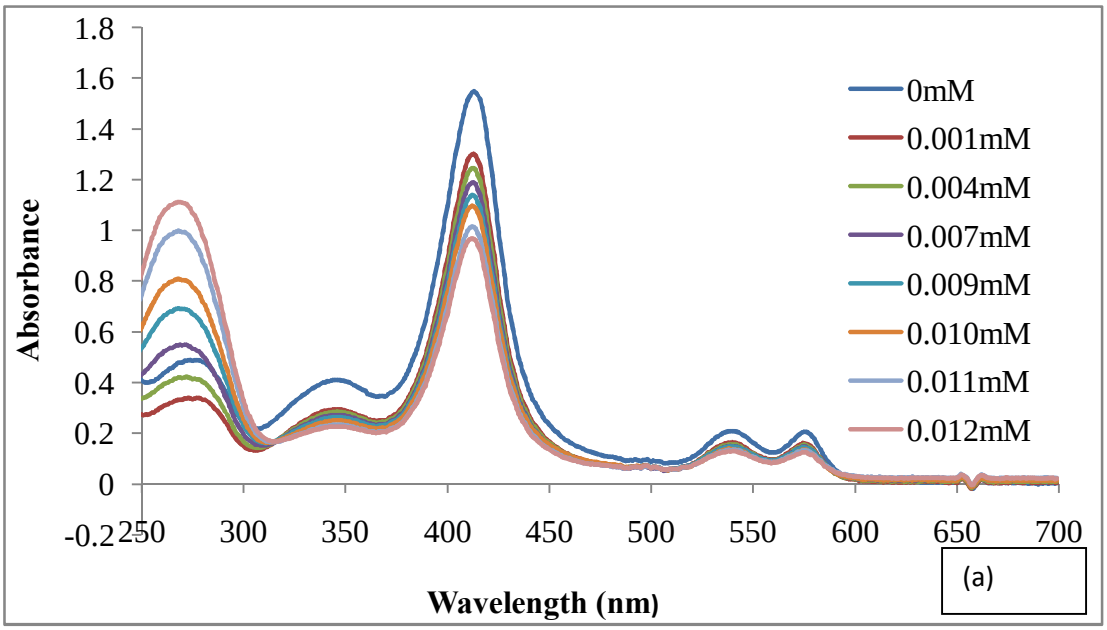


Figure 3.4: Absorption spectra of haemoglobin A in varying concentrations (0-0.025 mM) of pyrimethamine and constant concentration (0.008 mM) of sulphadoxine in the presence of SDS, at (a) pH 5.0 and (b) pH 7.2.

Figs. 3.5 to 3.6 show the absorption spectra of haemoglobin A in varying concentrations of sulphadoxine at pH 5.0 and 7.2



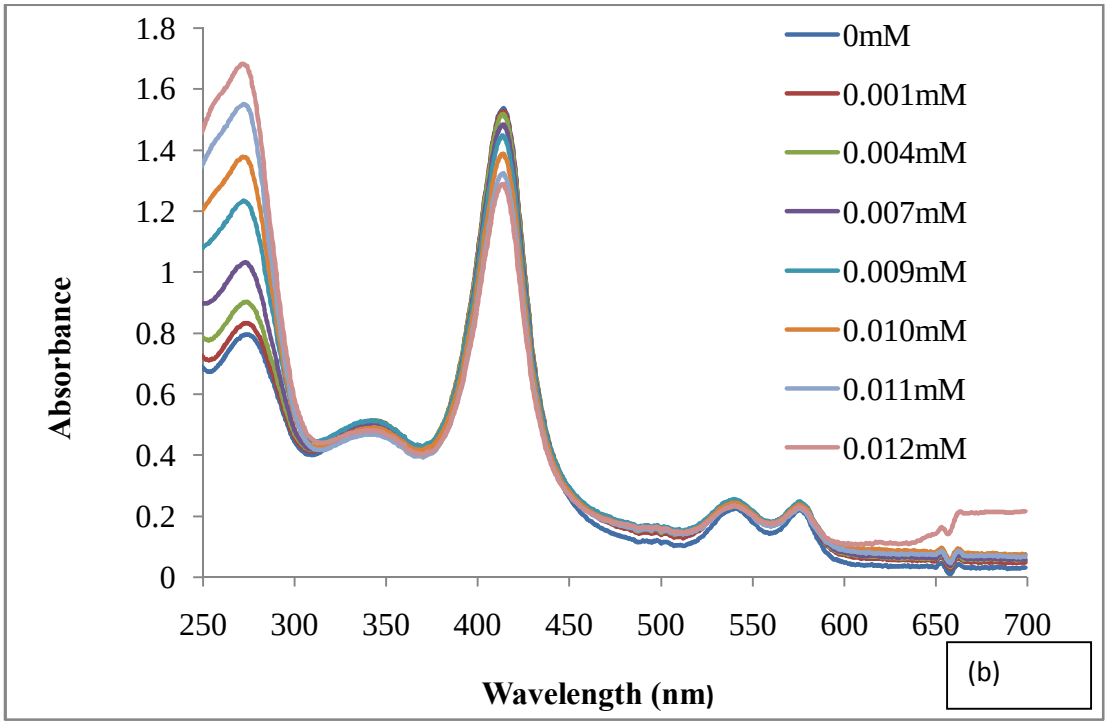
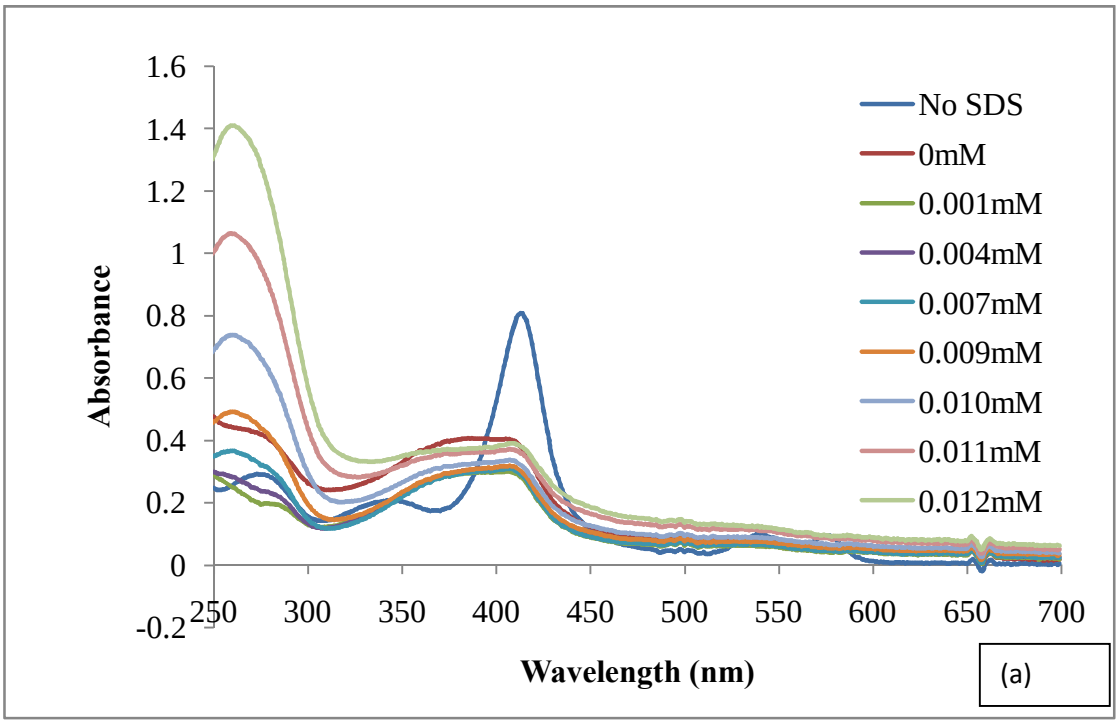


Figure 3.5: Absorption spectra of haemoglobin A in varying concentrations (0-0.012 mM) of sulphadoxine, at (a) pH 5.0 and (b) pH 7.2.



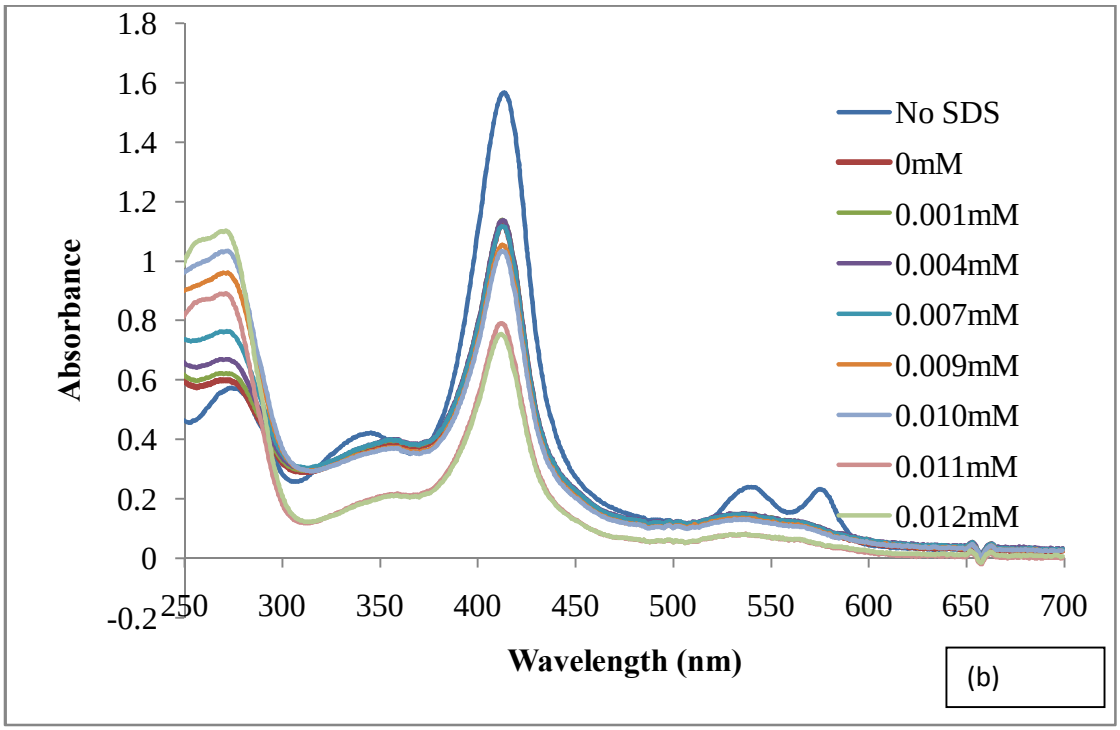


Figure 3.6: Absorption spectra of haemoglobin A in varying concentrations (0-0.012 mM) of sulphadoxine in the presence of SDS, at (a) pH 5.0 and (b) H 7.2.

Figs. 3.7 to 3.8 show the absorption spectra of haemoglobin A in varying concentrations of sulphadoxine and constant concentration (0.017 mM) of pyrimethamine, at pH 5.0 and 7.2

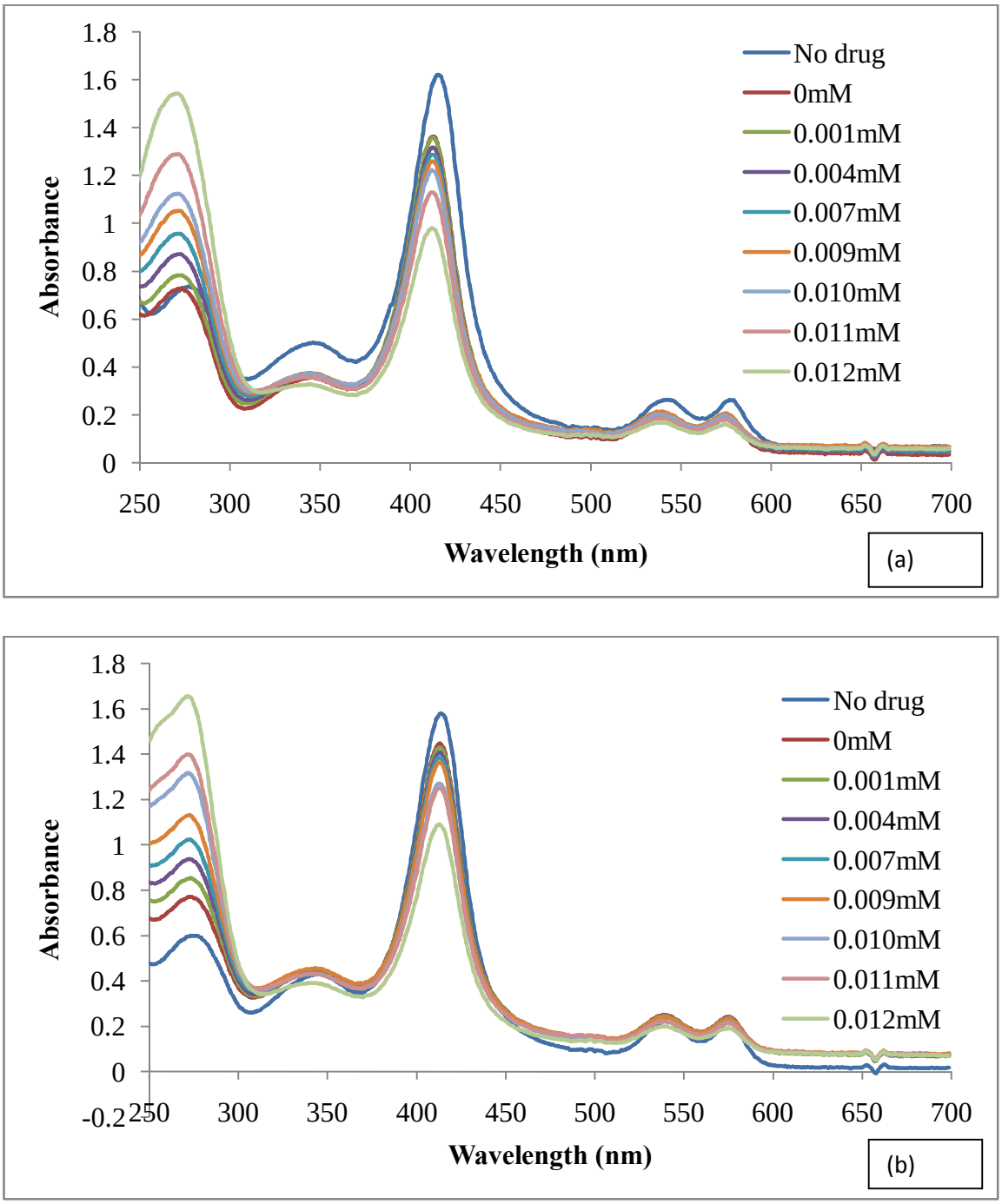
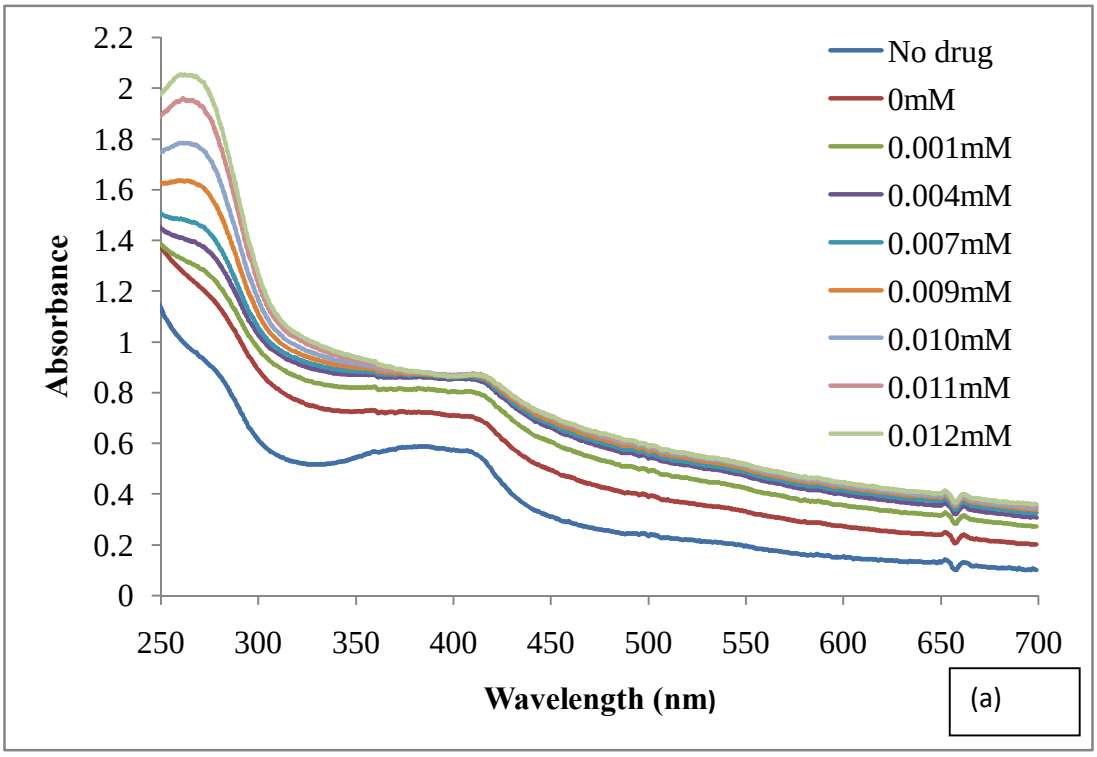


Figure 3.7: Absorption spectra of haemoglobin A in varying concentrations (0-0.012 mM) of sulphadoxine and constant concentration (0.017 mM) of pyrimethamine, at (a) pH 5.0 and (b) pH 7.2

3.1.8 Absorption spectra of haemoglobin A in varying concentrations of sulphadoxine and constant concentration of pyrimethamine.

Fig. 3.8 shows that sulphadoxine-pyrimethamine combination increased absorbance of the aromatic band (275 nm). There was a disappearance of the oxyhaemoglobin bands (542 and 577 nm). In Fig 3.8a, absorbance of the soret band (415 nm) increased at increasing concentration of sulphadoxine to a point where there was no noticeable change in absorbance even when the concentration was increased.


Absorbance
Wavelength (nm)
No drug
0mM
0.001mM
0.004mM
0.007mM
0.009mM
0.010mM
0.011mM
0.012mM
(a)

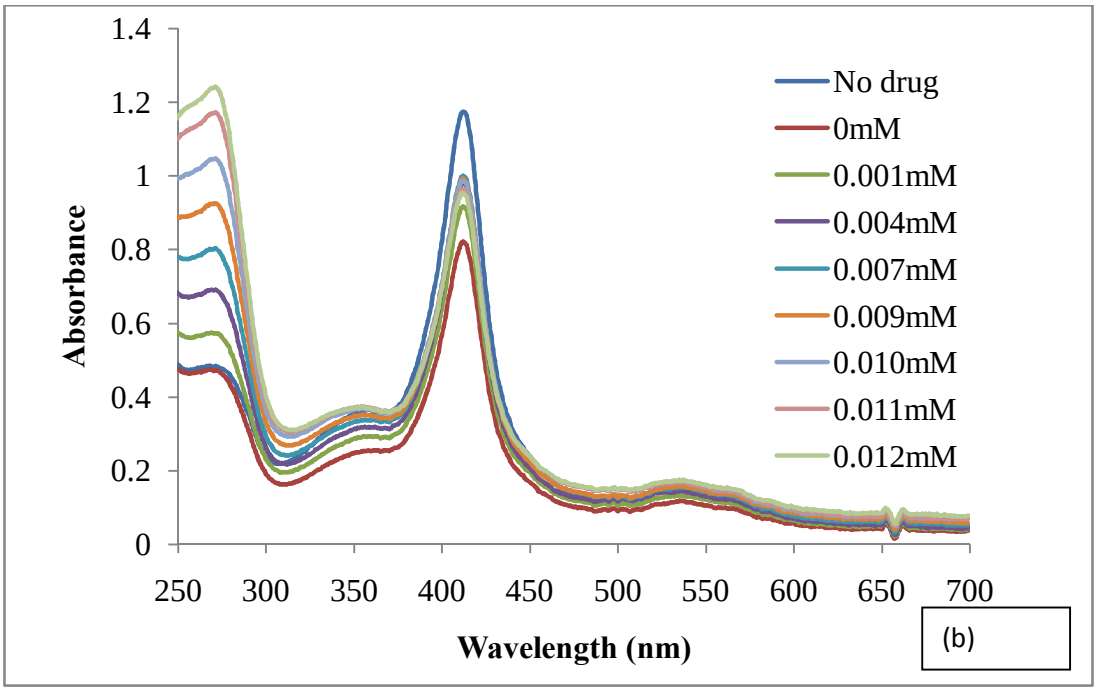

Absorbance
Wavelength (nm)
No drug
0mM
0.001mM
0.004mM
0.007mM
0.009mM
0.010mM
0.011mM
0.012mM
(b)

Figure 3.8: Absorption spectra of haemoglobin A in varying concentrations (0-0.012 mM) of sulphadoxine and constant concentration (0.017 mM) of pyrimethamine in the presence of SDS, at (a) pH 5.0 and (b) pH 7.2.

3.2 Absorption spectra of haemoglobin AS

3.2.1 Absorption Spectra of Haemoglobin AS in Varying Concentrations of Pyrimethamine.

Fig. 3.9 shows that pyrimethamine increased absorbance of the aromatic band (275 nm) and decreased absorbance of the soret band (415 nm) of HbAS both at pH 5.0 and 7.2

3.2.2 Absorption spectra of haemoglobin AS in varying concentrations of pyrimethamine in the presence of SDS.

Fig. 3.10 shows that pyrimethamine increased absorbance of the aromatic band (275 nm) and stretching of globin-haem interaction band (340 nm) of HbAS. The increment at aromatic band was higher at acid pH (Fig. 3.10a). There was an increase in absorbance of the soret band at pH 5.0 and decay in soret band at pH 7.2. Also there was disappearance of the oxyhaemoglobin bands (542 and 577 nm).

3.2.3 Absorption spectra of haemoglobin AS in varying concentrations of pyrimethamine and constant concentration of sulphadoxine.

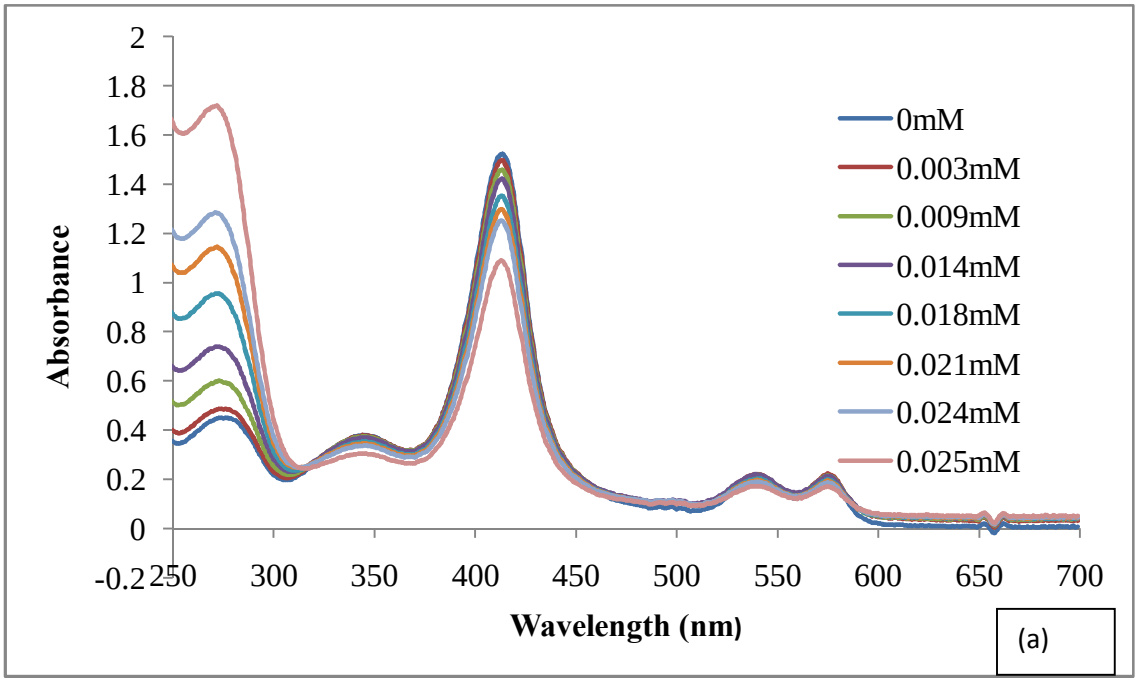
Fig. 3.11 shows that pyrimethamine-sulphadoxine combination increased the absorbance of aromatic band (275 nm) and decreased absorbance of the soret band (415 nm) of HbAS in a concentration dependent manner.

3.2.4 Absorption spectra of haemoglobin AS in varying concentrations of pyrimethamine and constant concentration of sulphadoxine in the presence of SDS.

Fig. 3.12 shows that pyrimethamine-sulphadoxine combination increased absorbance of the aromatic band (275 nm) of HbAS. There was a stretch of the globin-haem interaction band

(340 nm) and disappearance of oxyhaemoglobin bands (542 and 577 nm). Fig. 3.12b shows that the combination decreased absorbance of the soret band (415 nm).

Figs. 3.9 to 3.10 show the absorption spectra of haemoglobin AS in varying concentrations of pyrimethamine at pH 5.0 and 7.2



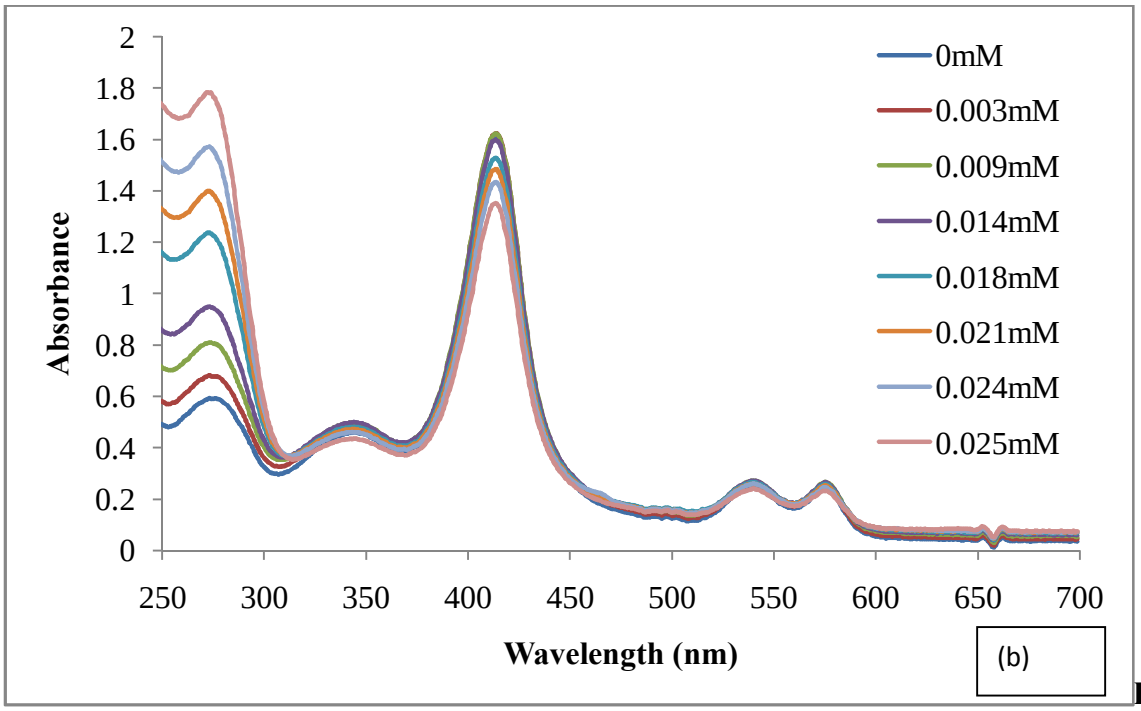


Figure 3.9: Absorption spectra of haemoglobin AS in varying concentrations (0-0.025 mM) of pyrimethamine, at (a) pH 5.0 and (b) pH 7.2.

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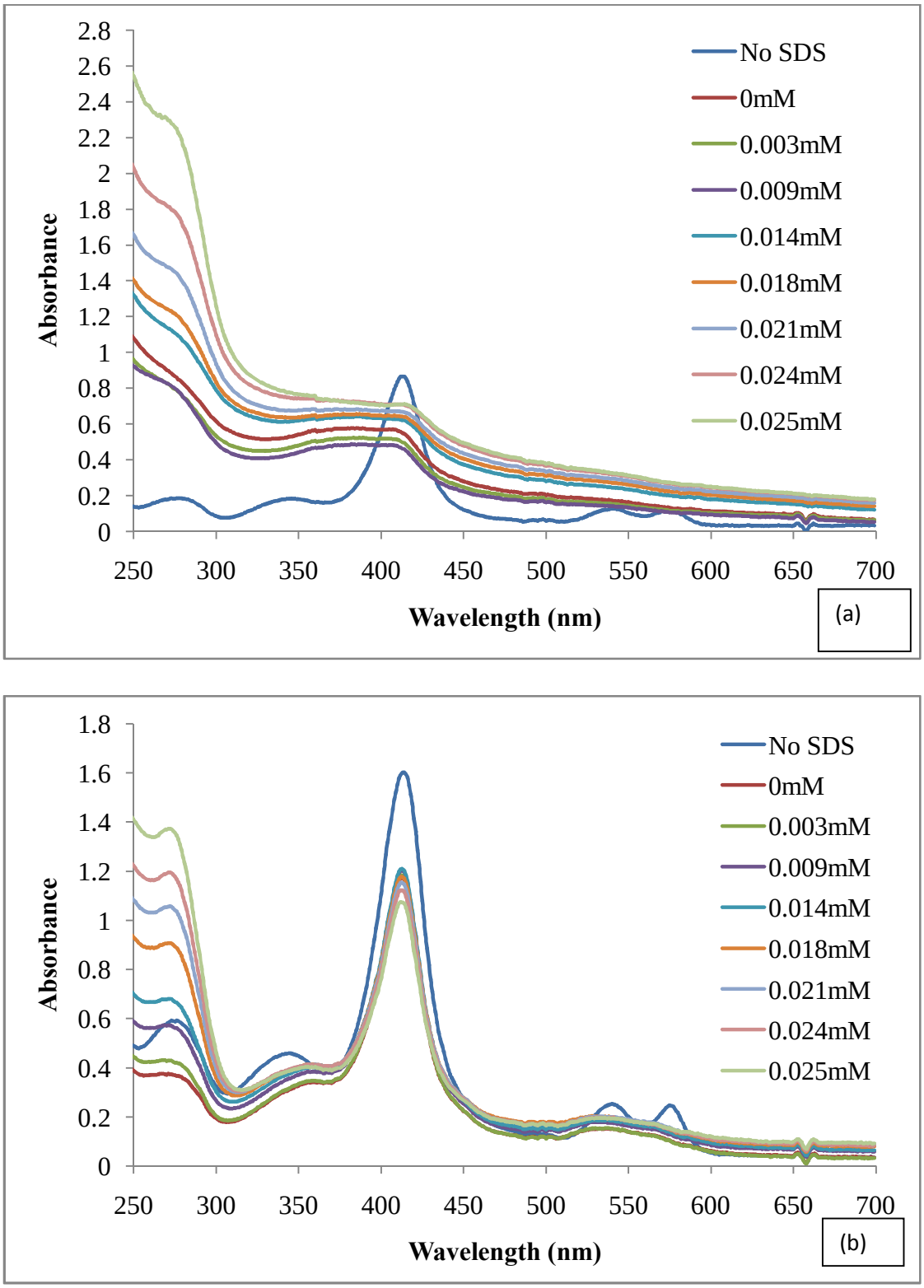
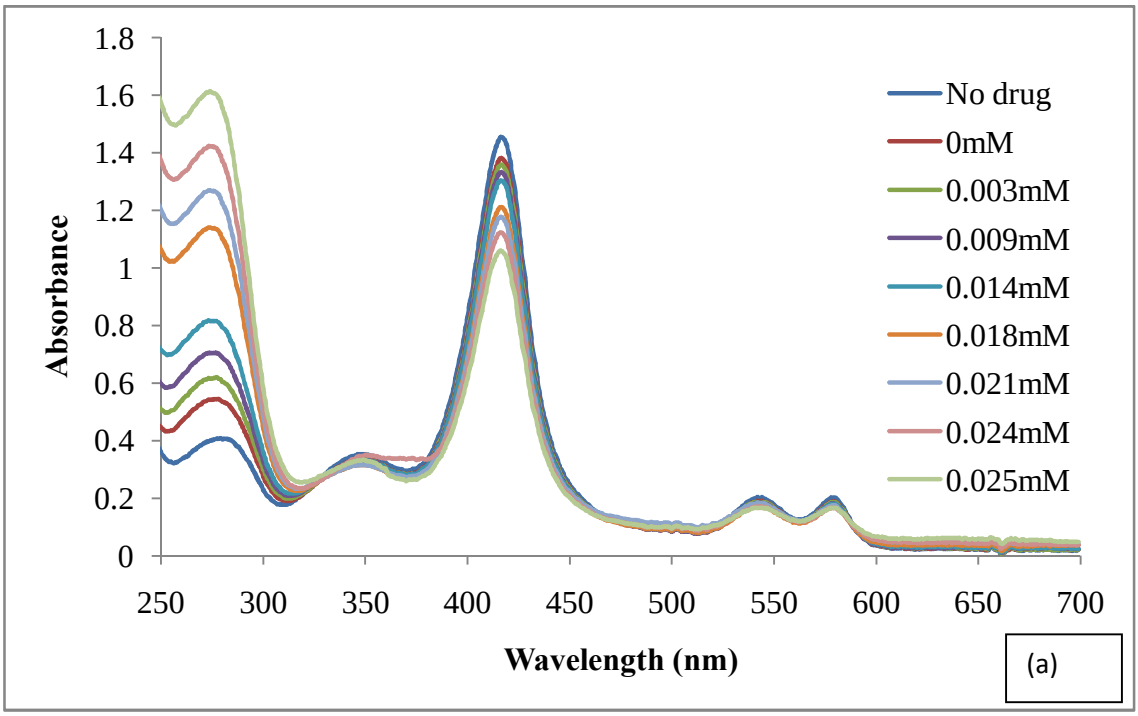


Figure 3.10: Absorption spectra of haemoglobin AS in varying concentrations (0-0.025 mM) of pyrimethamine in the presence of SDS, at (a) pH 5.0 and (b) pH 7.2.

Figs. 3.11 to 3.12 show the absorption spectra of haemoglobin AS in varying concentrations of pyrimethamine and constant concentration (0.008 mM) of sulphadoxine, at pH 5.0 and 7.2



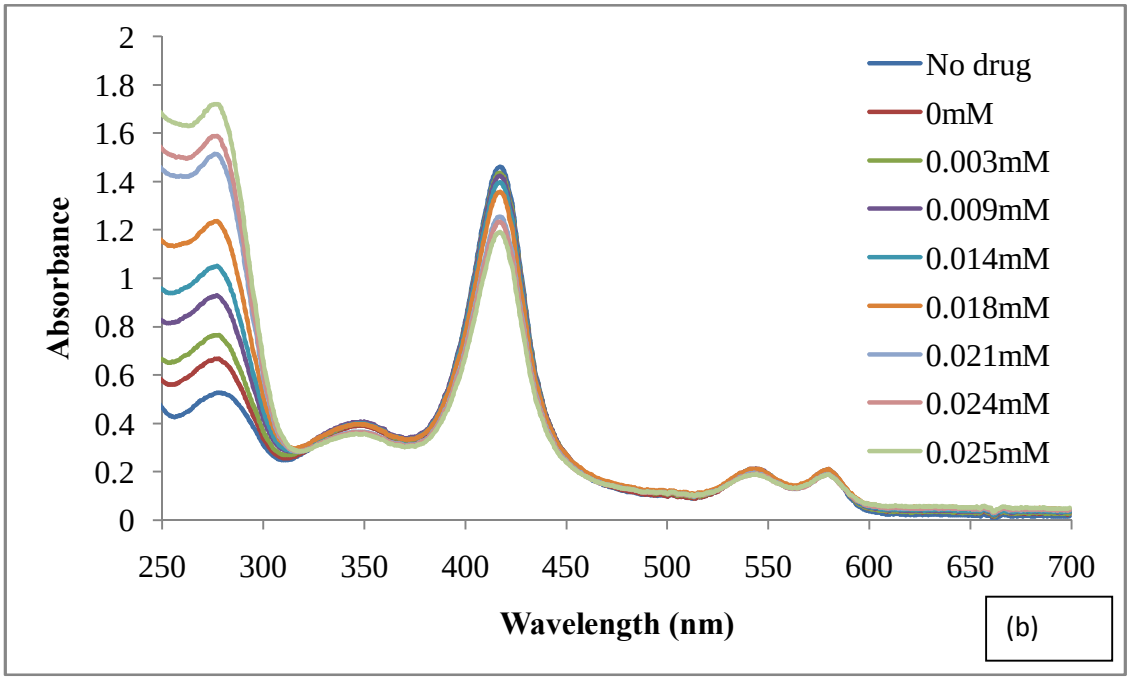
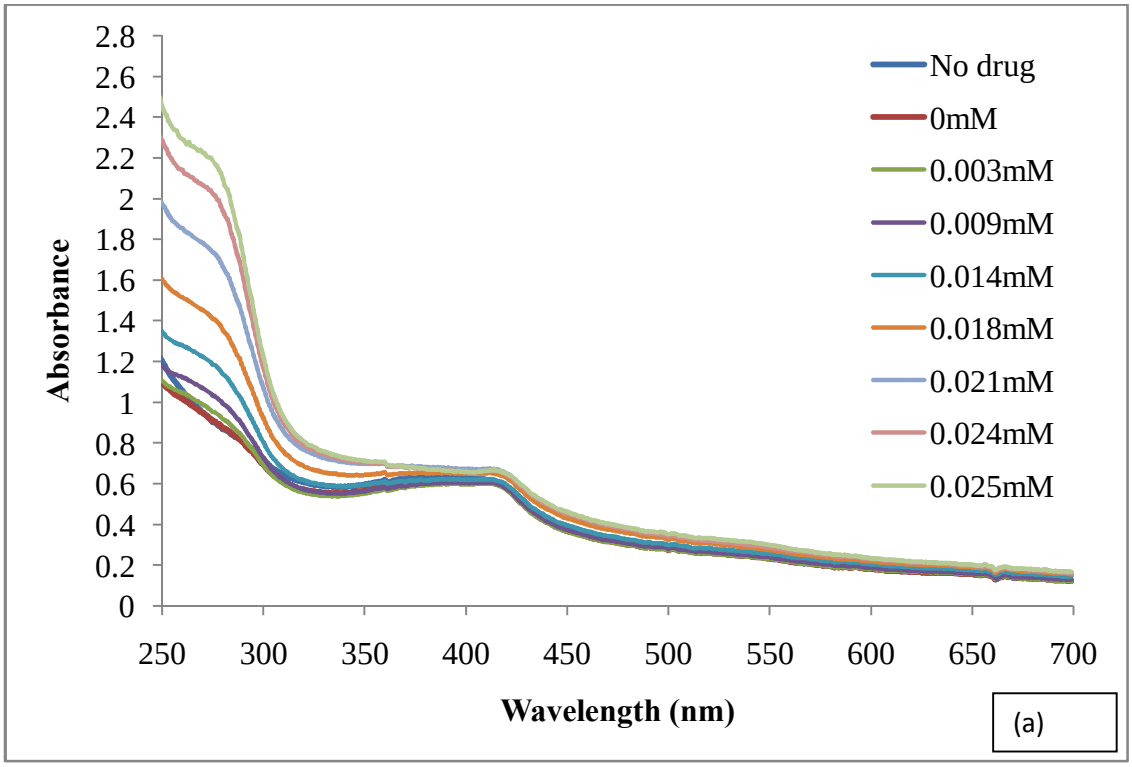


Figure 3.11: Absorption spectra of haemoglobin AS in varying concentrations (0-0.025 mM) of pyrimethamine and constant concentration (0.008 mM) of sulphadoxine, at (a) pH 5.0 and (b) pH 7.2.



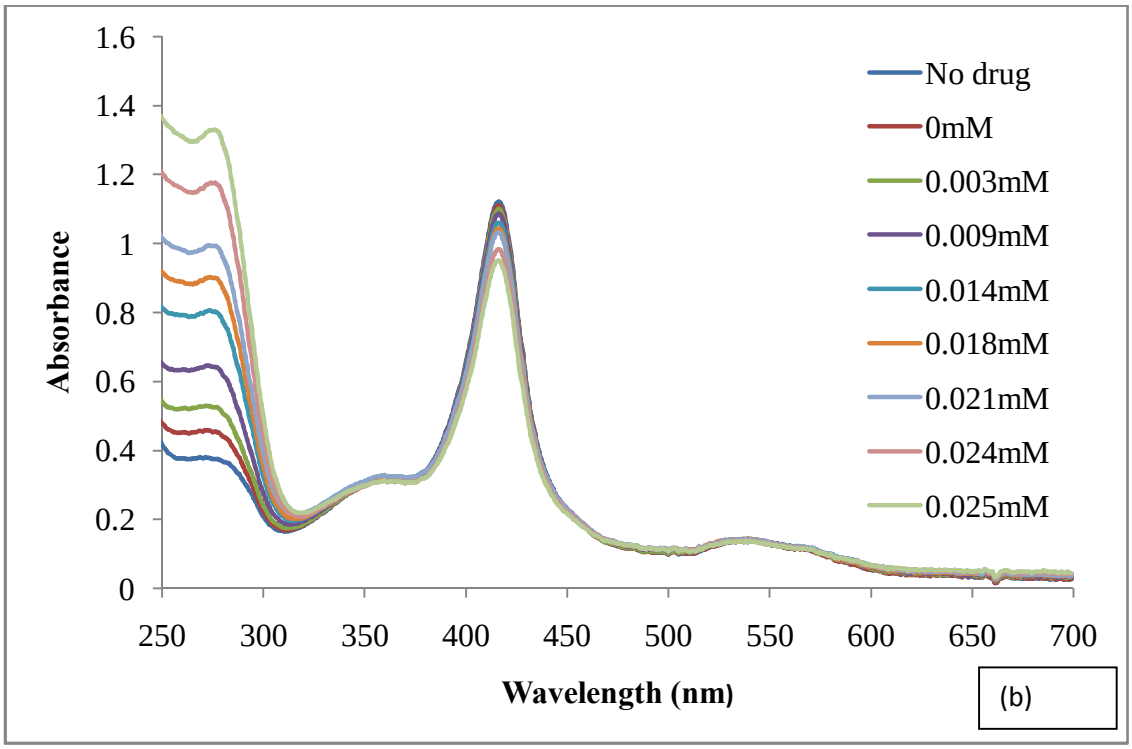


Figure 3.12: Absorption spectra of haemoglobin AS in varying concentrations (0-0.025 mM) of pyrimethamine and constant concentration (0.008 mM) of sulphadoxine in the presence of SDS, at (a) pH 5.0 and (b) pH 7.2.

3.2.5 Absorption spectra of haemoglobin AS in varying concentrations of sulphadoxine.

Fig. 3.13 shows that sulphadoxine increased absorbance of the aromatic band (275 nm) and decreased absorbance of the soret band (415 nm) in a concentration dependent manner.

3.2.6 Absorption spectra of haemoglobin AS in varying concentration of sulphadoxine in the presence of SDS.

Fig. 3.14 shows that sulphadoxine increased absorbance of the aromatic band (275 nm) and disappearance of the oxyhaemoglobin bands (542 and 577 nm). At pH 7.2, there was a decrease in absorbance of the soret band (415 nm).

3.2.7 Absorption spectra of haemoglobin AS in varying concentrations of sulphadoxine and constant concentration of pyrimethamine.

Fig.3.15 shows that sulphadoxine-pyrimethamine combination increased absorbance of the aromatic band (275 nm) and decreased absorbance of the soret band (415 nm), globin-haem interaction band (340 nm) and oxyhaemoglobin bands (542 and 577 nm). There was also a spectra shift in the soret band towards the shorter wavelength (Fig. 3.15a).

3.2.8 Absorption spectra of haemoglobin AS in varying concentrations of sulphadoxine and constant concentration of pyrimethamine in the presence of SDS.

Fig. 3.16 shows that sulphadoxine-pyrimethamine combination increased absorbance of the aromatic band (275 nm). There was disappearance of the oxyhaemoglobin bands (542 and 577 nm), stretching of the globin-haem interaction band (340 nm) of HbAS. Also there was a decrease in absorbance of the soret band (415 nm) (Fig. 3.16b).

Figs. 3.13 to 3.14 show the absorption spectra of haemoglobin AS in varying concentration of sulphadoxine at pH 5.0 and 7.2

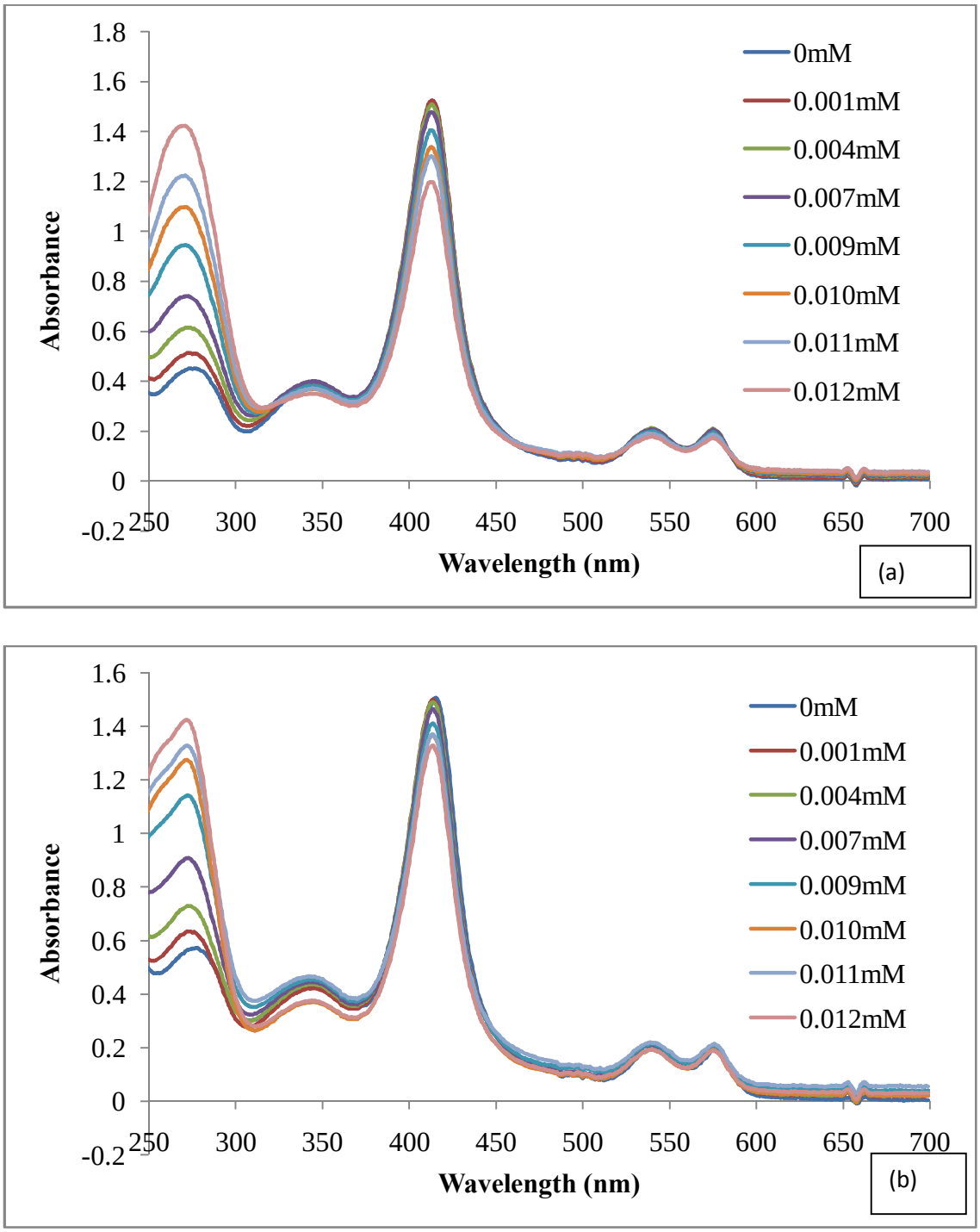
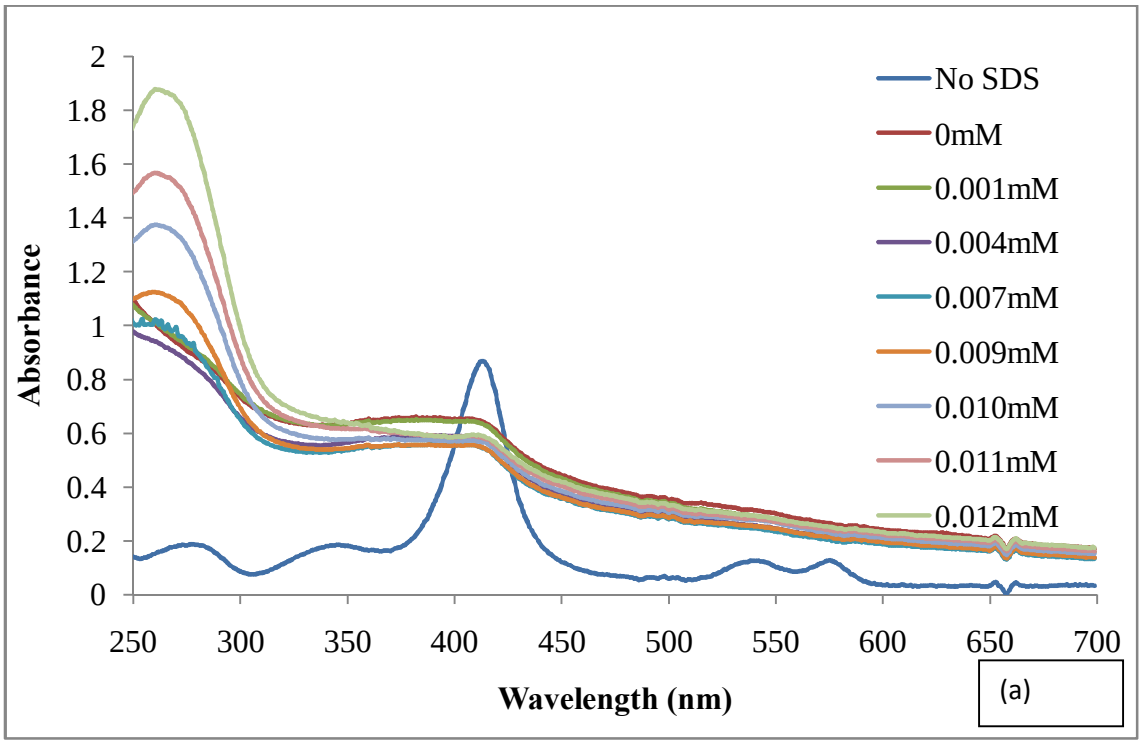


Figure 3.13: Absorption spectra of haemoglobin AS in varying concentrations (0-0.012 mM) of sulphadoxine, at (a) pH 5.0 and (b) pH 7.2.


Absorbance
Wavelength (nm)
No SDS
0mM
0.001mM
0.004mM
0.007mM
0.009mM
0.010mM
0.011mM
0.012mM
(a)

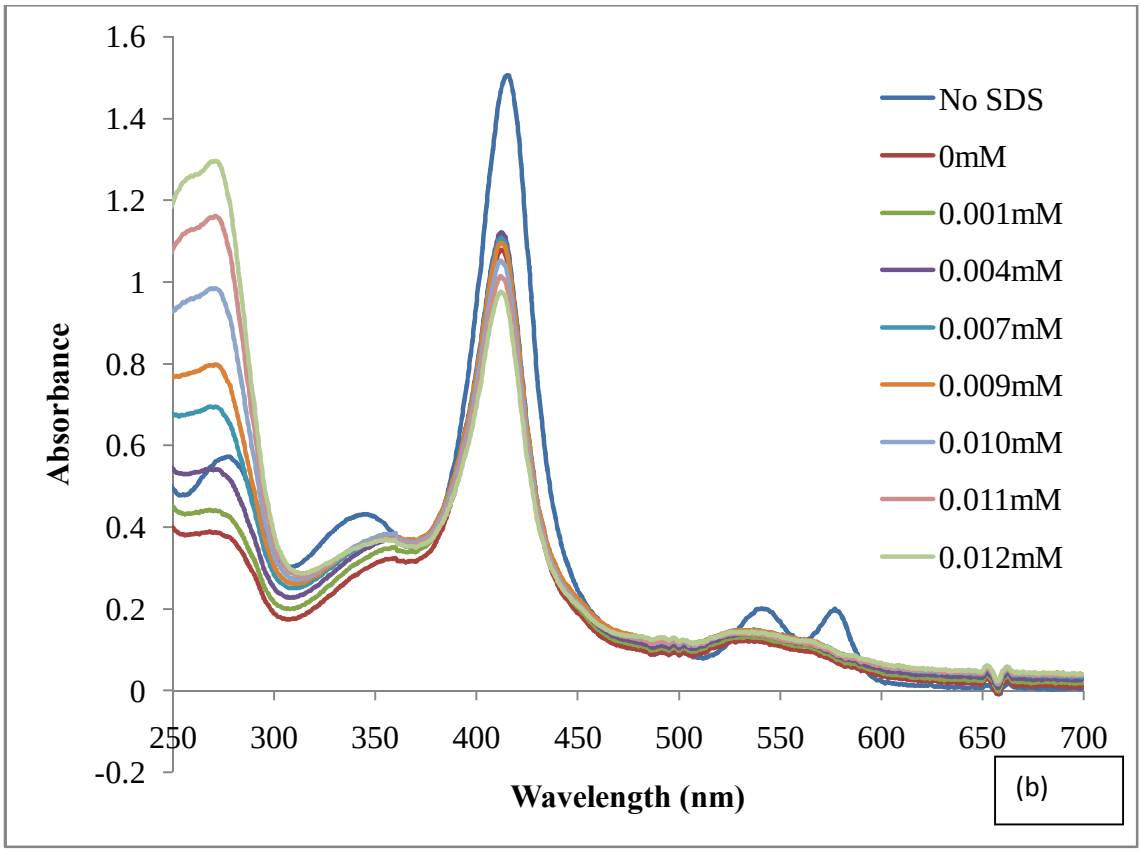
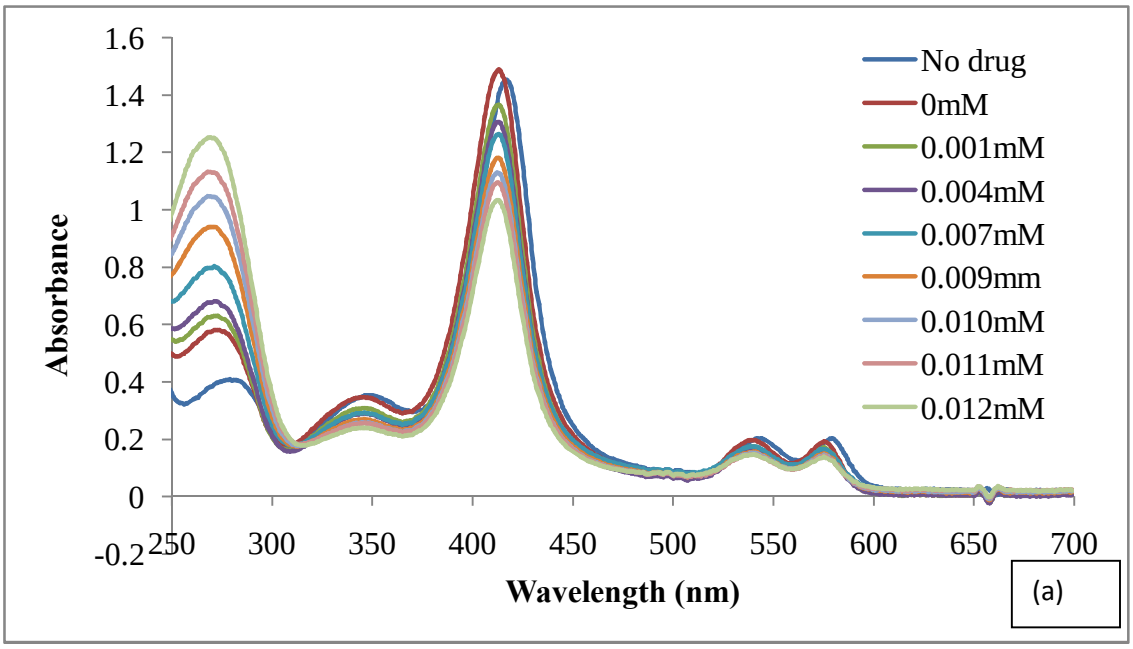

Absorbance
Wavelength (nm)
No SDS
0mM
0.001mM
0.004mM
0.007mM
0.009mM
0.010mM
0.011mM
0.012mM
(b)

Figure 3.14: Absorption spectra of haemoglobin AS in varying concentration (0-0.012 mM) of sulphadoxine in the presence of SDS, at (a) pH 5.0 and (b) pH 7.2.

Figs. 3.15 to 3.16 show the absorption spectra of haemoglobin AS in varying concentrations of sulphadoxine and constant concentration (0.017 mM) of pyrimethamine, at pH 5.0 and 7.2



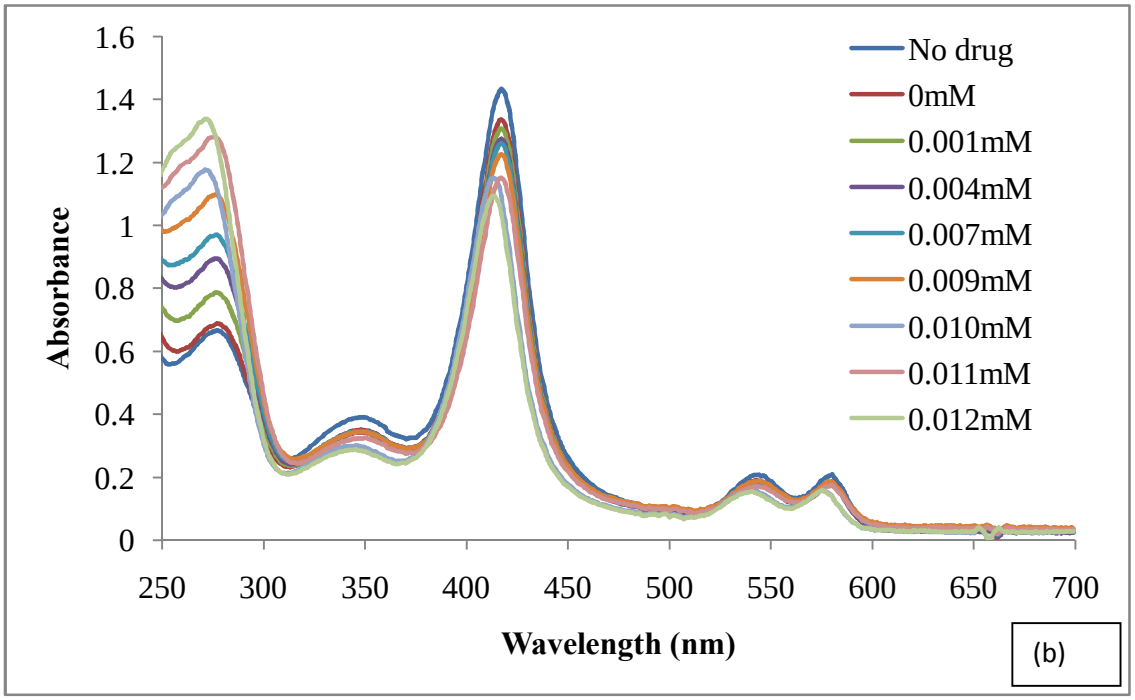
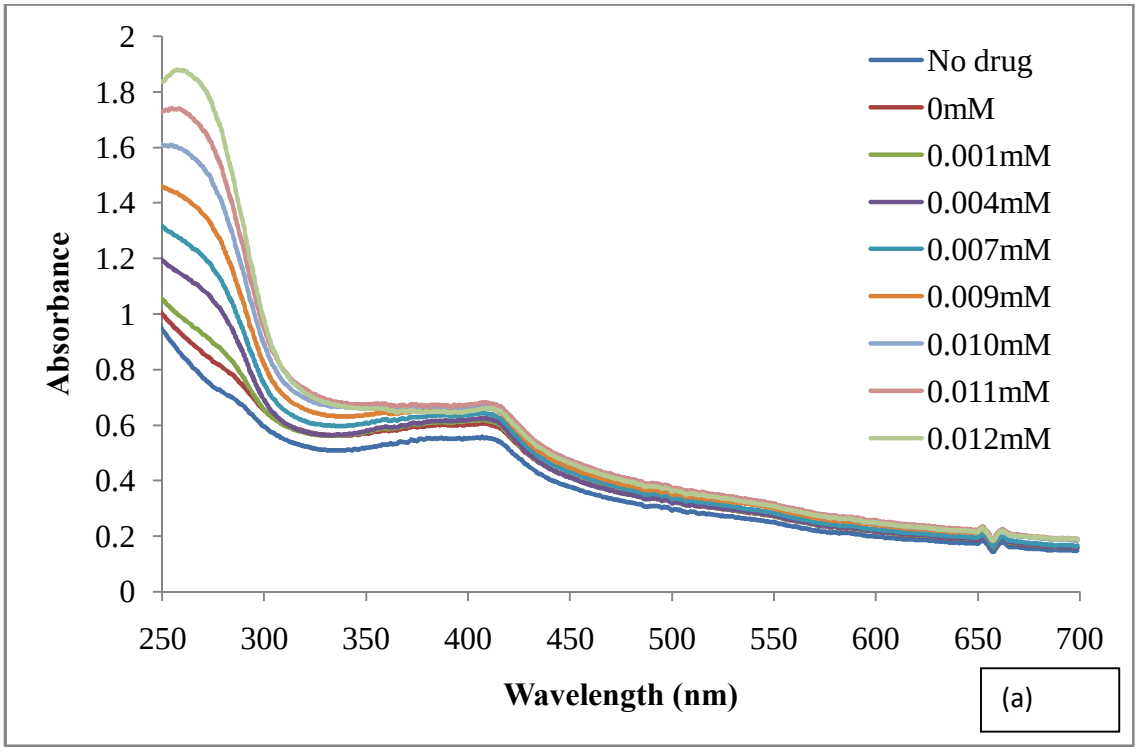


Figure 3.15: Absorption spectra of haemoglobin AS in varying concentrations (0-0.012 mM) of sulphadoxine and constant concentration (0.017 mM) of pyrimethamine, at (a) pH 5.0 and (b) pH 7.2.



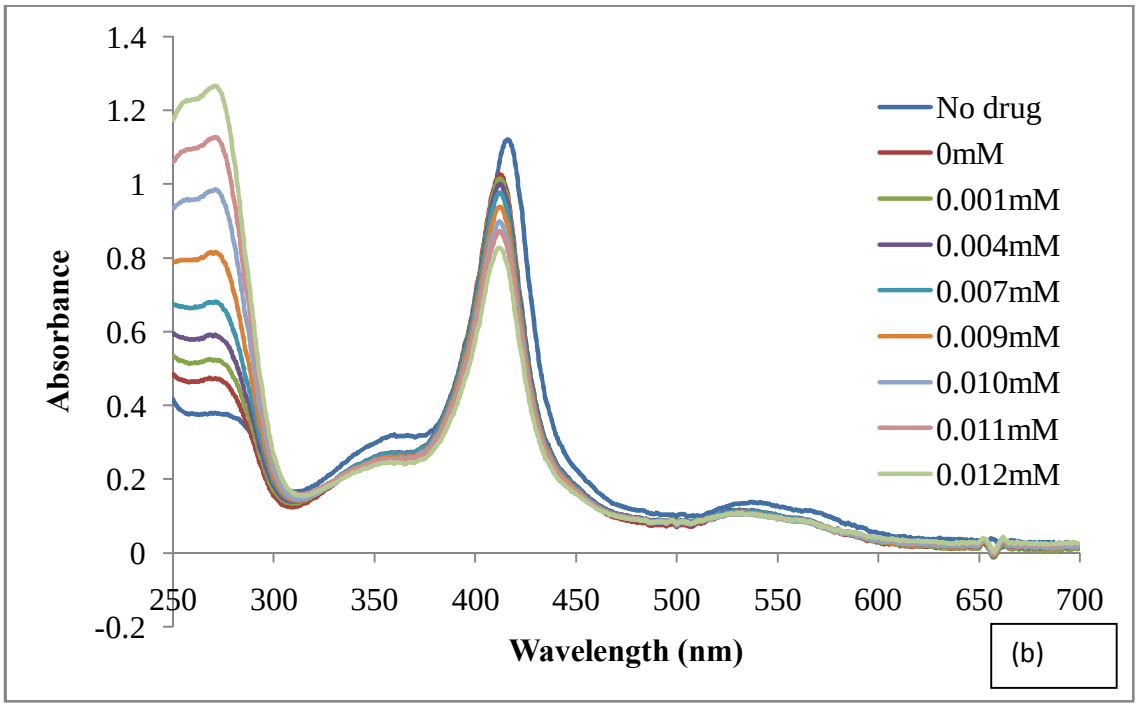


Figure 3.16: Absorption spectra of haemoglobin AS in varying concentrations (0-0.012 mM) of sulphadoxine and constant concentration (0.017 mM) of pyrimethamine in the presence of SDS, at (a) pH 5.0 and (b) pH 7.2.

3.3 Absorption Spectra of Haemoglobin S

3.3.1 Absorption spectra of haemoglobin S in varying concentrations of pyrimethamine.

Fig. 3.17 shows that pyrimethamine increased absorbance of the aromatic band (275 nm) and decreased absorbance of the soret band (415 nm) of HbS.

3.3.2 Absorption spectra of haemoglobin S in varying concentrations of pyrimethamine in the presence of SDS.

Fig. 3.18 shows that pyrimethamine increased absorbance of the aromatic band (275 nm) of HbS. There was stretching of the globin-haem interaction band and disappearance of the

oxyhaemoglobin bands. Also there was an increase in absorbance of the soret band at pH 5.0 (Fig. 3.18a) and a decrease in absorbance of the soret band at pH 7.2 (Fig. 3.18b).

3.3.3 Absorption spectra of haemoglobin S in varying concentrations of pyrimethamine and constant concentration of sulphadoxine.

Fig. 3.19 shows that pyrimethamine-sulphadoxine combination increased absorbance of the aromatic band (275 nm) and decreased the absorbance of the soret band (415 nm) in a concentration dependent manner.

3.3.4 Absorption spectra of haemoglobin S in varying concentrations of pyrimethamine and constant concentration of sulphadoxine in the presence of SDS.

Fig. 3.20 shows that pyrimethamine-sulphadoxine combination increased absorbance of the aromatic band (275 nm) of HbS. There was a stretch of the globin-haem interaction band (340 nm) and disappearance of oxyhaemoglobin bands (542 and 577 nm).

Figs. 3.17 to 3.18 show the absorption spectra of haemoglobin S in varying concentrations of pyrimethamine at pH 5.0 and 7.2

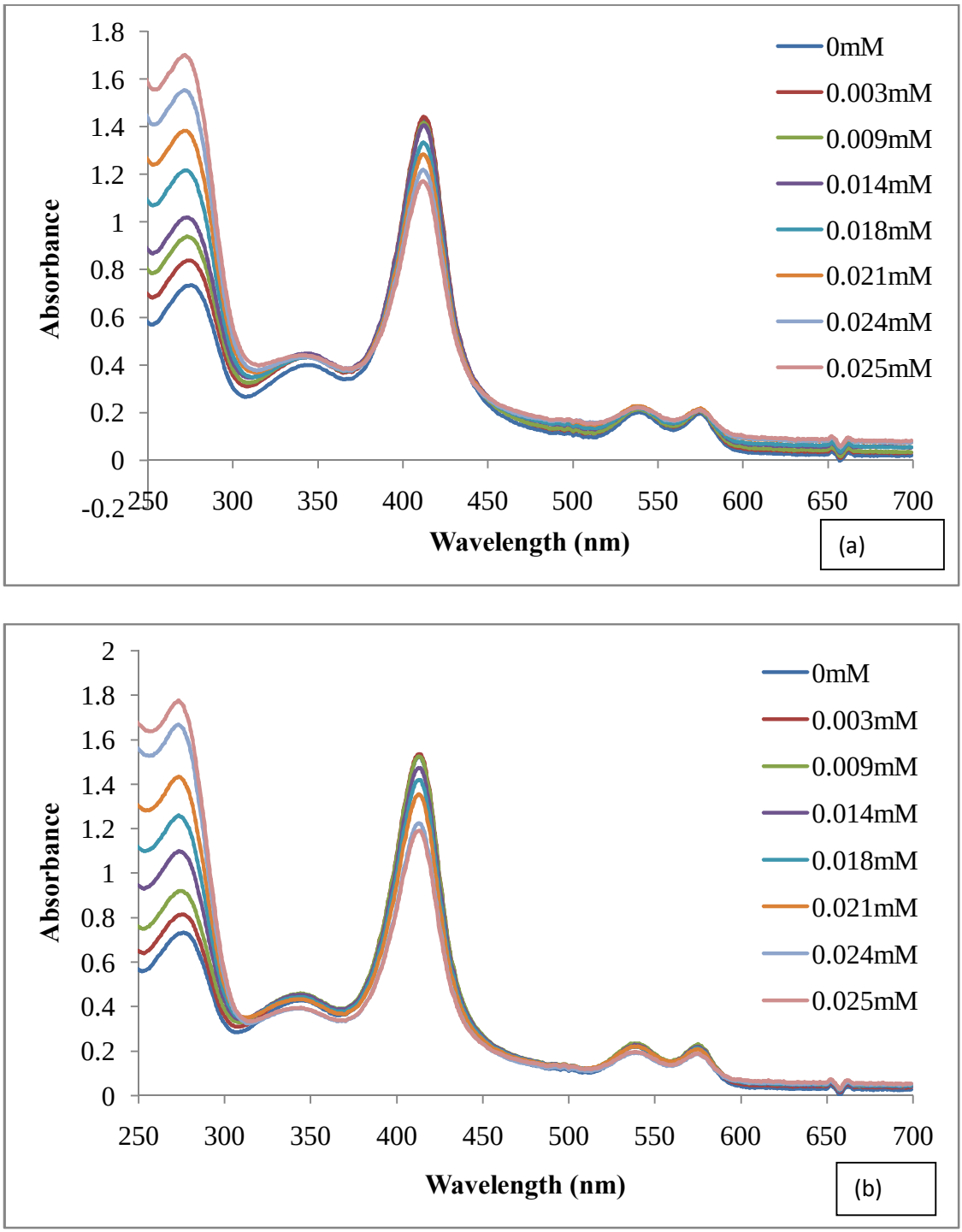
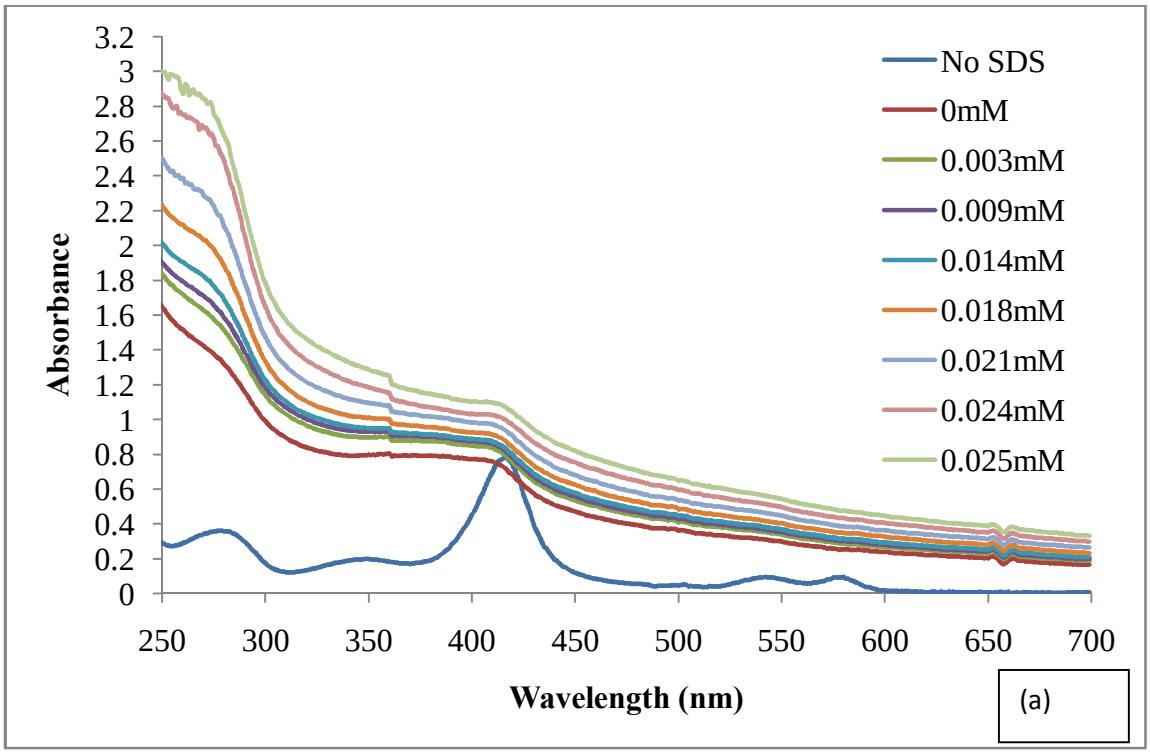


Figure 3.17: Absorption spectra of haemoglobin S in varying concentrations (0-0.025 mM) of pyrimethamine, at (a) pH 5.0 and (b) pH 7.2.



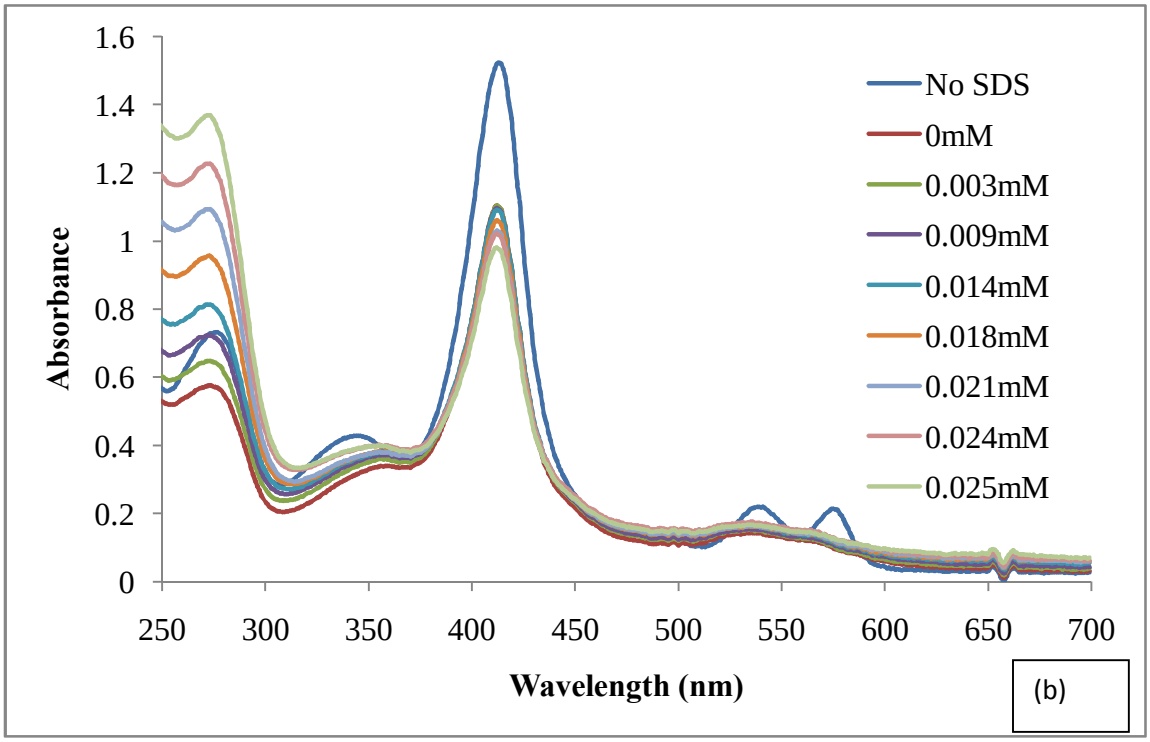
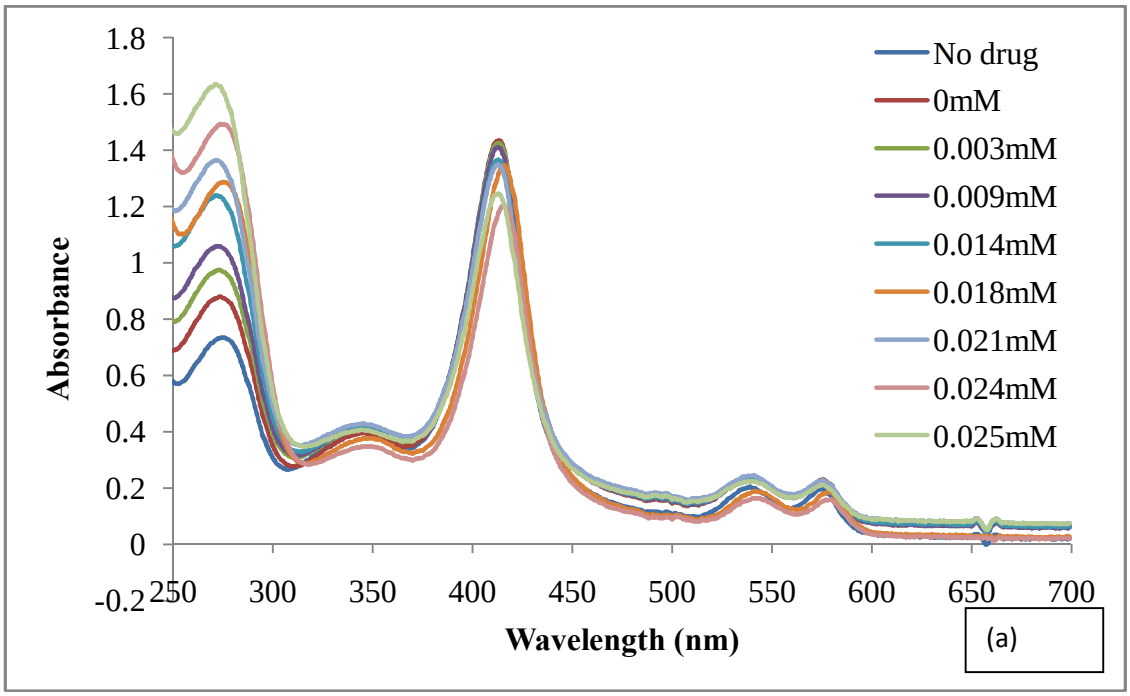


Figure 3.18: Absorption spectra of haemoglobin S in varying concentrations (0-0.025 mM) of pyrimethamine in the presence of SDS, at (a) pH 5.0 and (b) pH 7.2.

Figs. 3.19 to 3.20 show the absorption spectra of haemoglobin S in varying concentrations of pyrimethamine and constant concentration (0.008 mM) of sulphadoxine, at pH 5.0 and 7.2



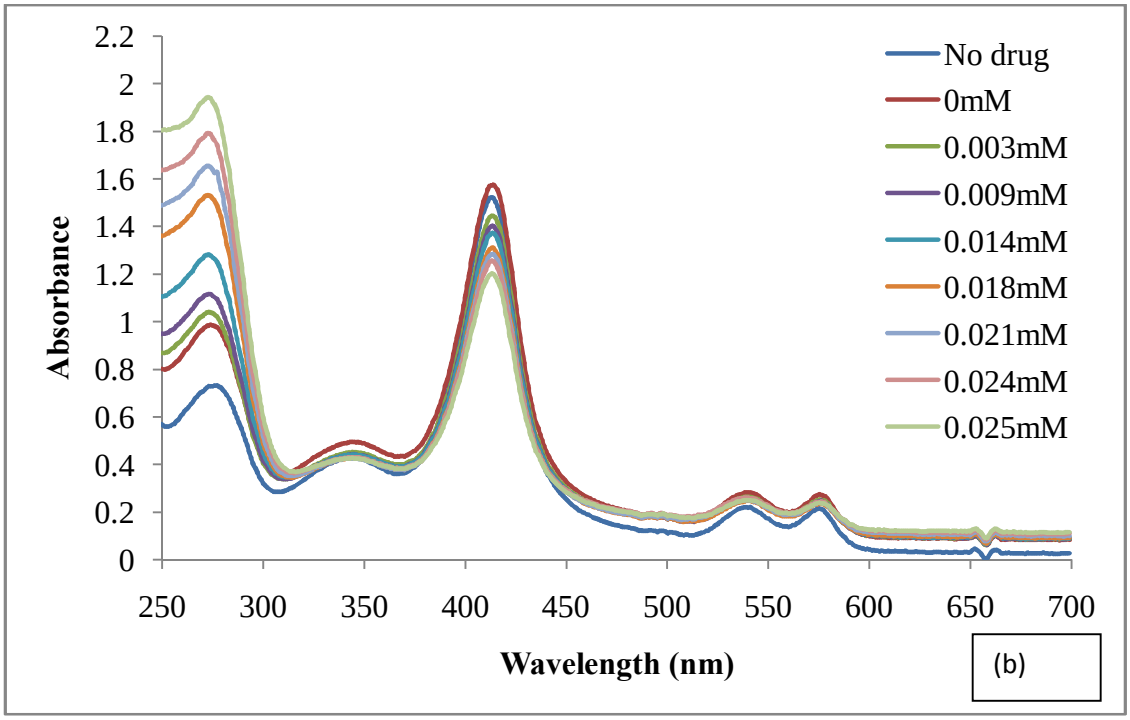
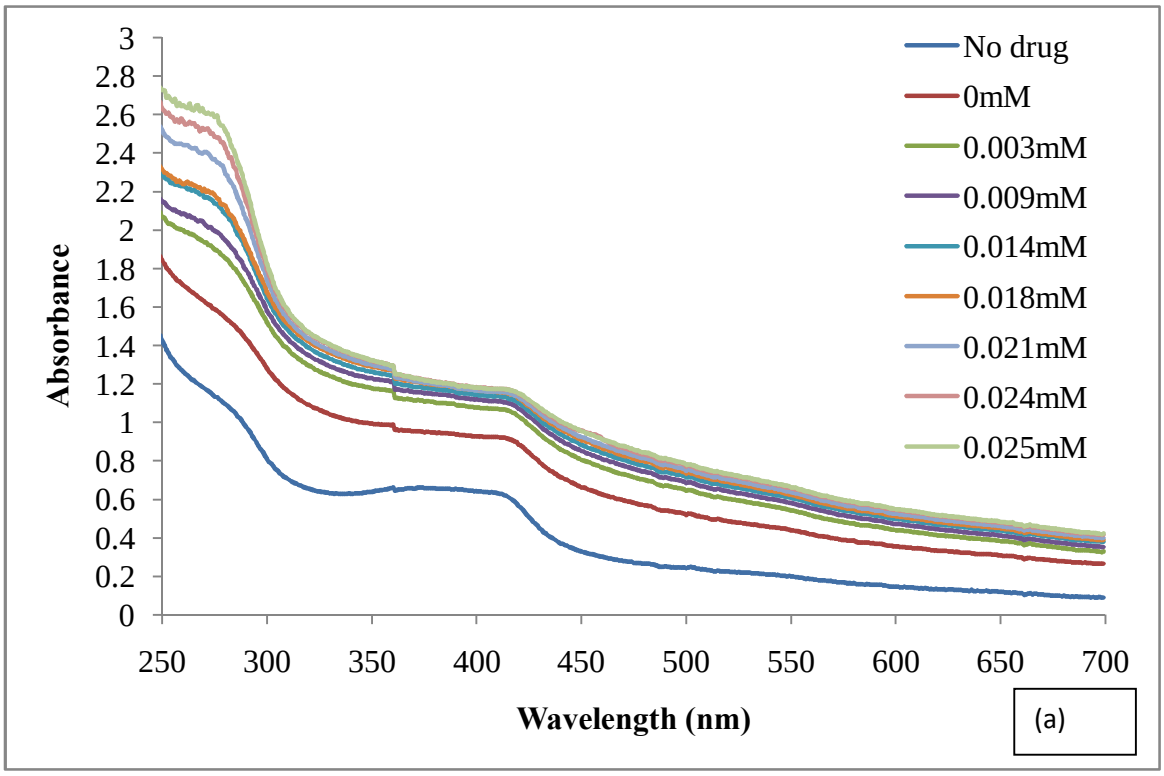


Figure 3.19: Absorption spectra of haemoglobin S in varying concentrations (0-0.025 mM) of pyrimethamine and constant concentration (0.008 mM) of sulphadoxine, at (a) pH 5.0 and (b) pH 7.2.



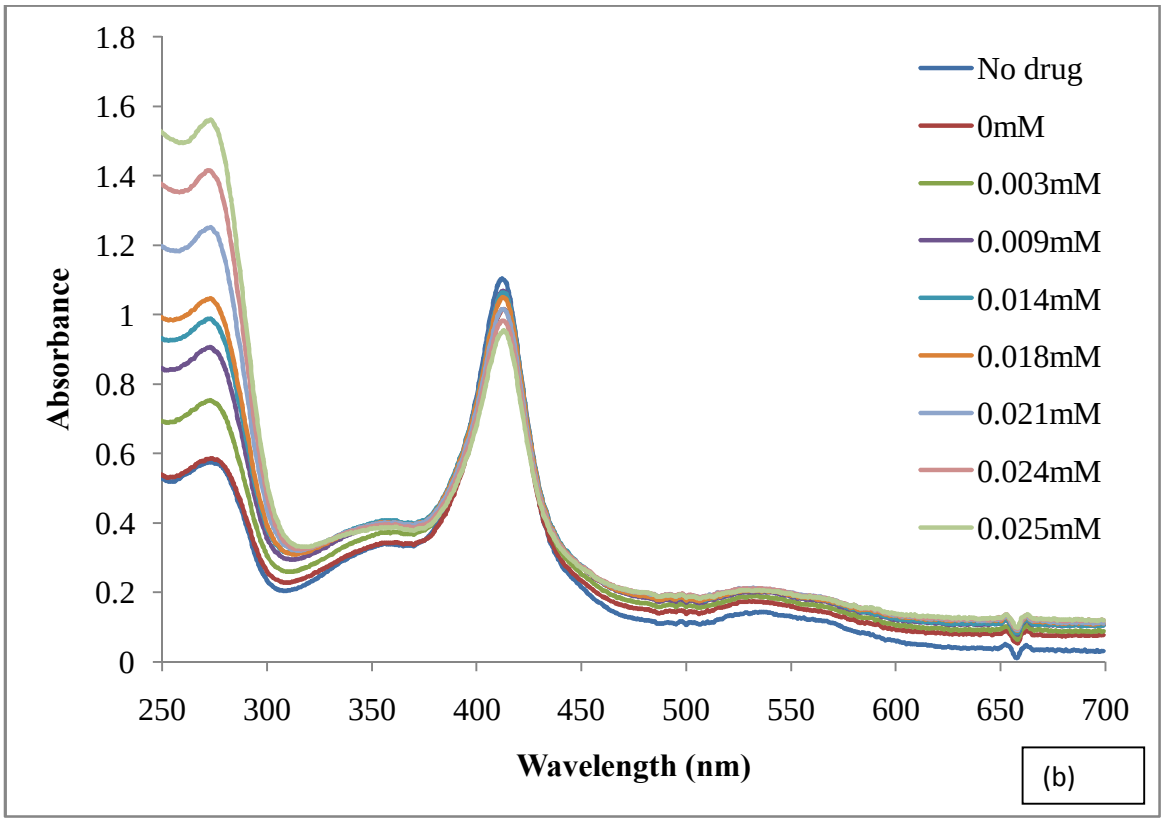


Figure 3.20: Absorption spectra of haemoglobin S in varying concentrations (0-0.025 mM) of pyrimethamine and constant concentration (0.008 mM) of sulphadoxine in the presence of SDS, at (a) pH 5.0 and (b) pH 7.2.

3.3.5 Absorption spectra of haemoglobin S in varying concentration of sulphadoxine

Fig. 3.21 shows that sulphadoxine increased absorbance of the aromatic band (275 nm) and caused decay of the soret band (415 nm) of HbS at both pH 5.0 and 7.2.

3.3.6 Absorption spectra of haemoglobin S in varying concentrations of sulphadoxine in the presence of SDS.

Fig. 3.22 shows that sulphadoxine increased absorbance of aromatic band (275 nm) of HbS. There was a stretch of globin-haem interaction band (340 nm) and also disappearance of oxyhaemoglobin bands.

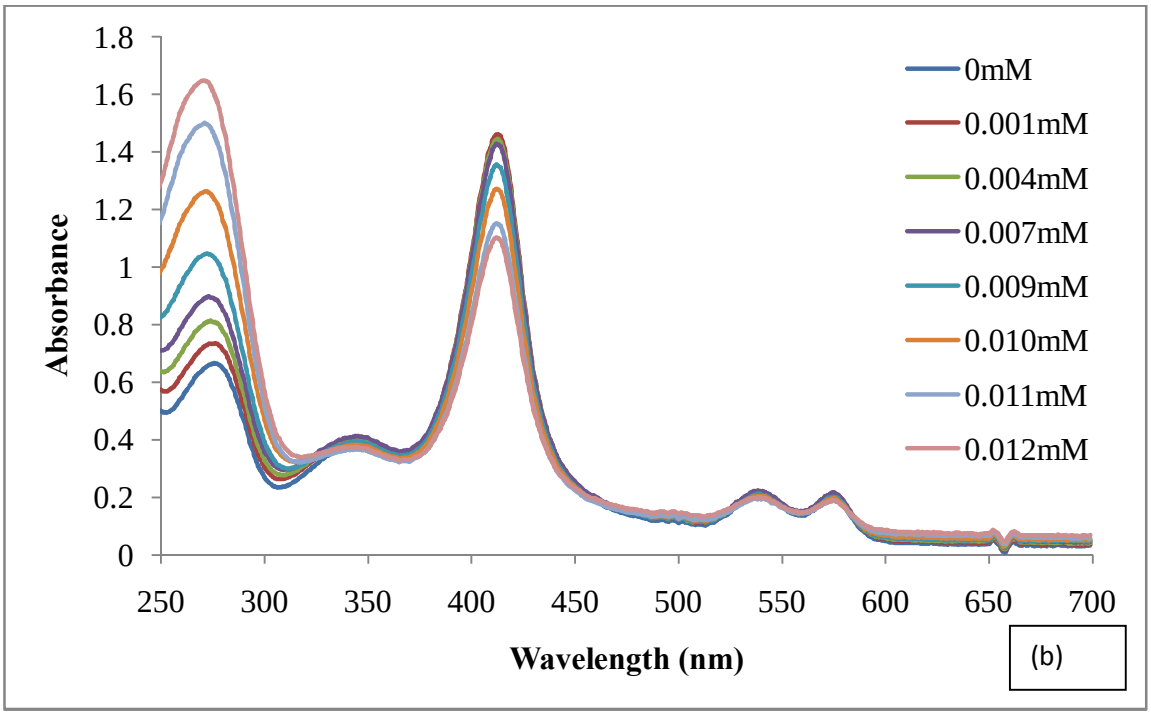
3.3.7 Absorption spectra of haemoglobin S in varying concentration of sulphadoxine and constant concentration of pyrimethamine.

Fig. 3.23 shows that sulphadoxine-pyrimethamine combination increased absorbance of the aromatic band (275 nm) and decreased absorbance of the soret band (415 nm) of HbS.

3.3.8 Absorption spectra of haemoglobin S in varying concentrations of sulphadoxine and constant concentration of pyrimethamine in the presence of SDS.

Fig. 3.24 shows that sulphadoxine-pyrimethamine combination increased absorbance of aromatic band (275 nm) of HbS. There was a stretch of the globin-haem interaction band (340 nm) and disappearance of the oxyhaemoglobin bands (542 and 577 nm). In Fig. 3.24a, the absorbance of soret band increased as the concentration was increasing and became constant at a point even when the concentration was increased.

Figs. 3.21 to 3.22 show the absorption spectra of haemoglobin S in varying concentrations of sulphadoxine, at pH 5.0 and 7.2.



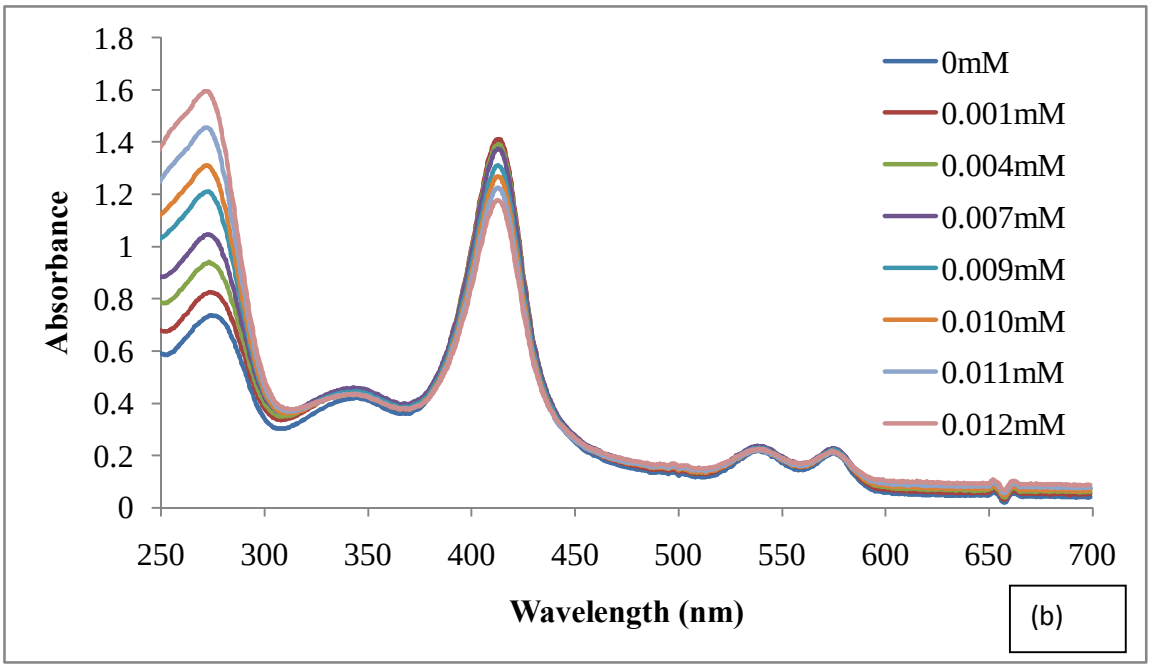
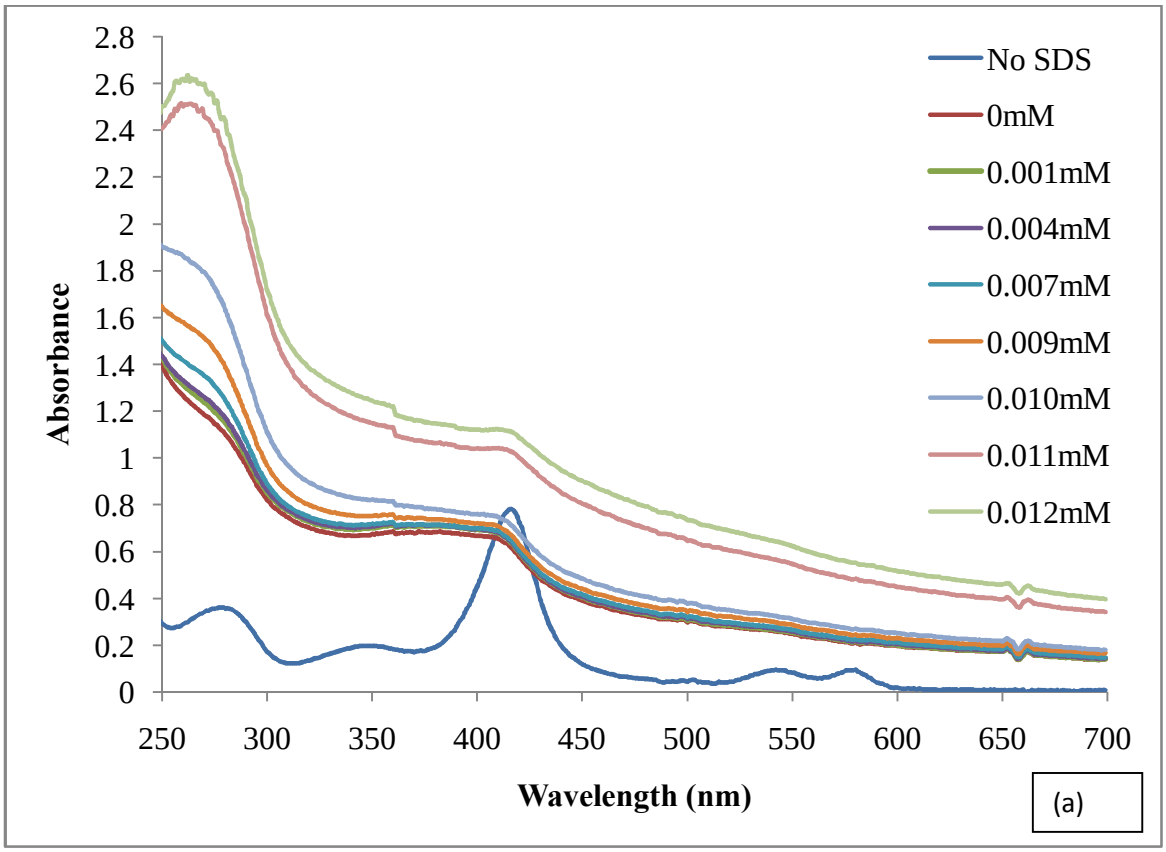


Figure 3.21: Absorption spectra of haemoglobin S in varying concentrations (0-0.012 mM) of sulphadoxine, at (a) pH 5.0 and (b) pH 7.2


Absorbance
2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
250
300
350
400
450
500
550
600
650
700
Wavelength (nm)
No SDS
0mM
0.001mM
0.004mM
0.007mM
0.009mM
0.010mM
0.011mM
0.012mM
(a)

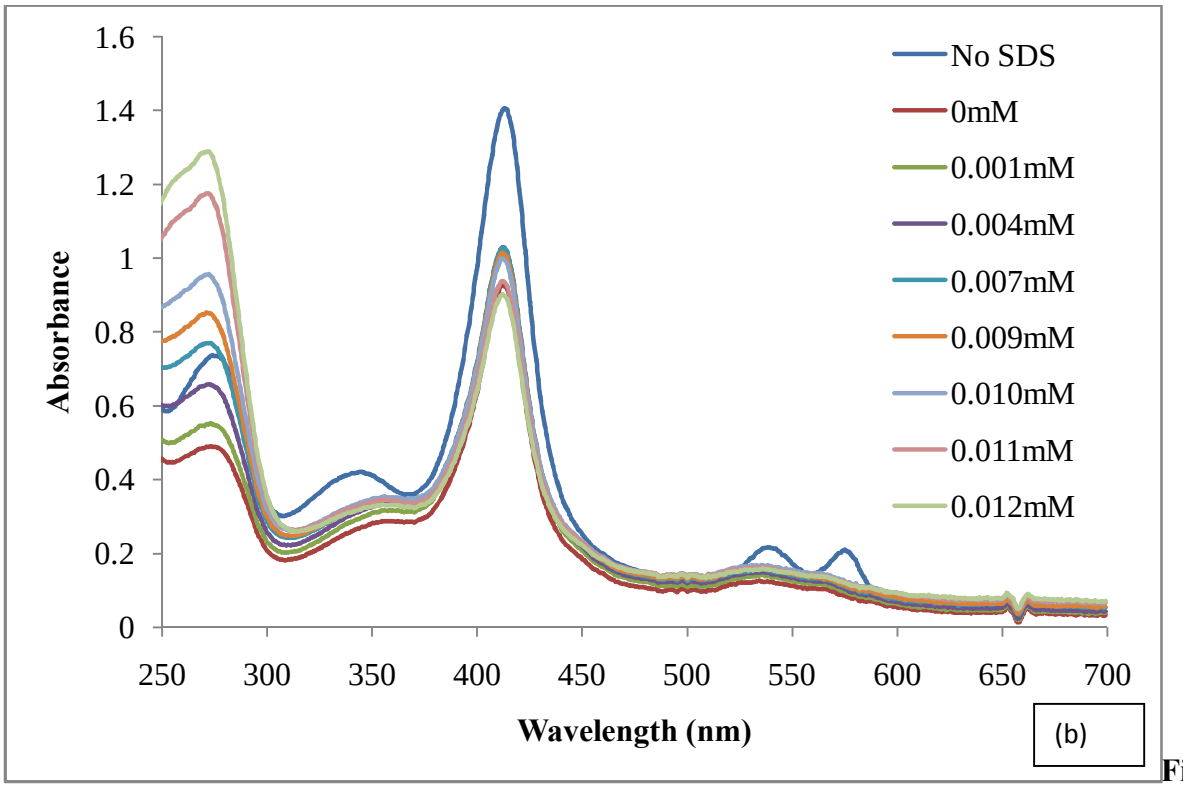
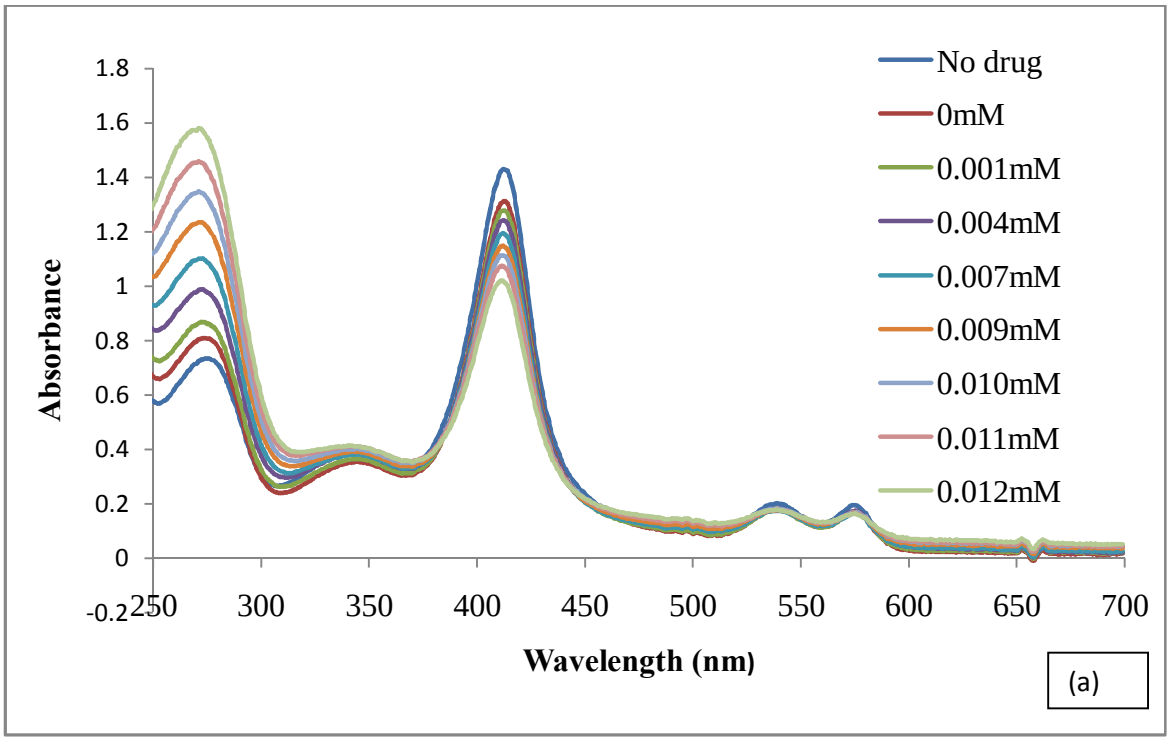


Figure 3.22: Absorption spectra of haemoglobin S in varying concentration (0-0.012 mM) of sulphadoxine in the presence of SDS, at (a) pH 5.0 and (b) pH 7.2.

Figures 3.23 to 3.24 show the absorption spectra of haemoglobin S in varying concentrations of sulphadoxine and constant concentration (0.017 mM) of pyrimethamine at pH 5.0 and 7.2.



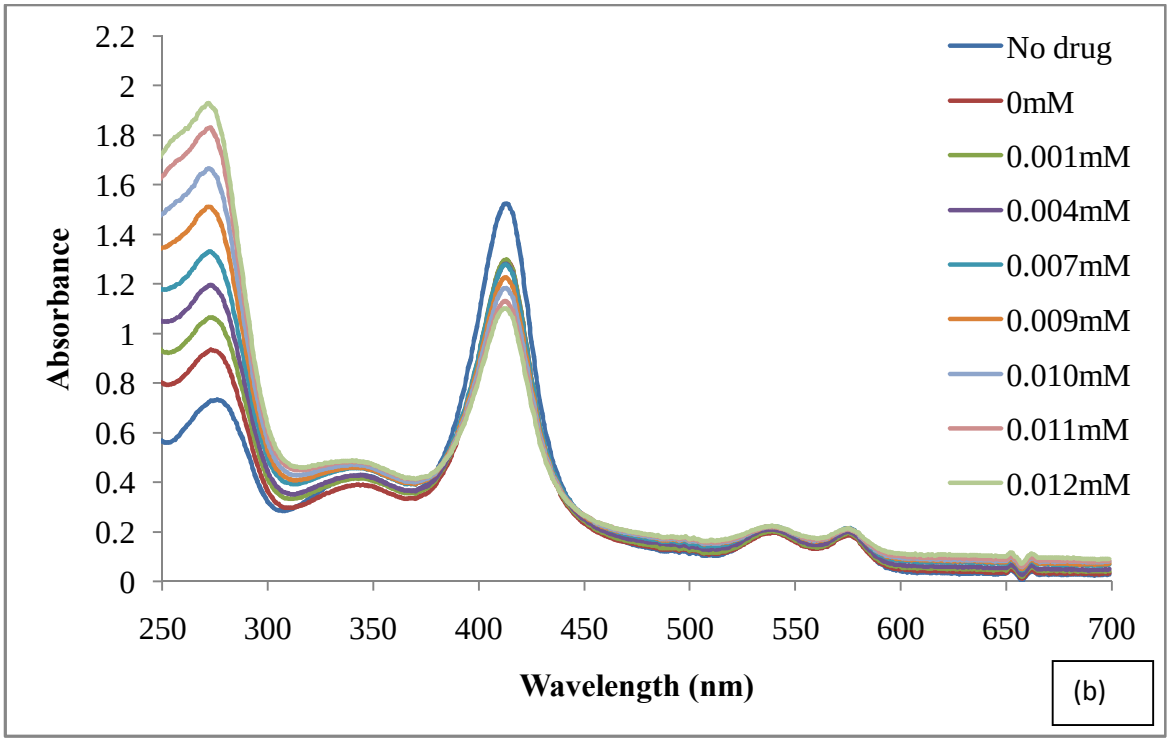
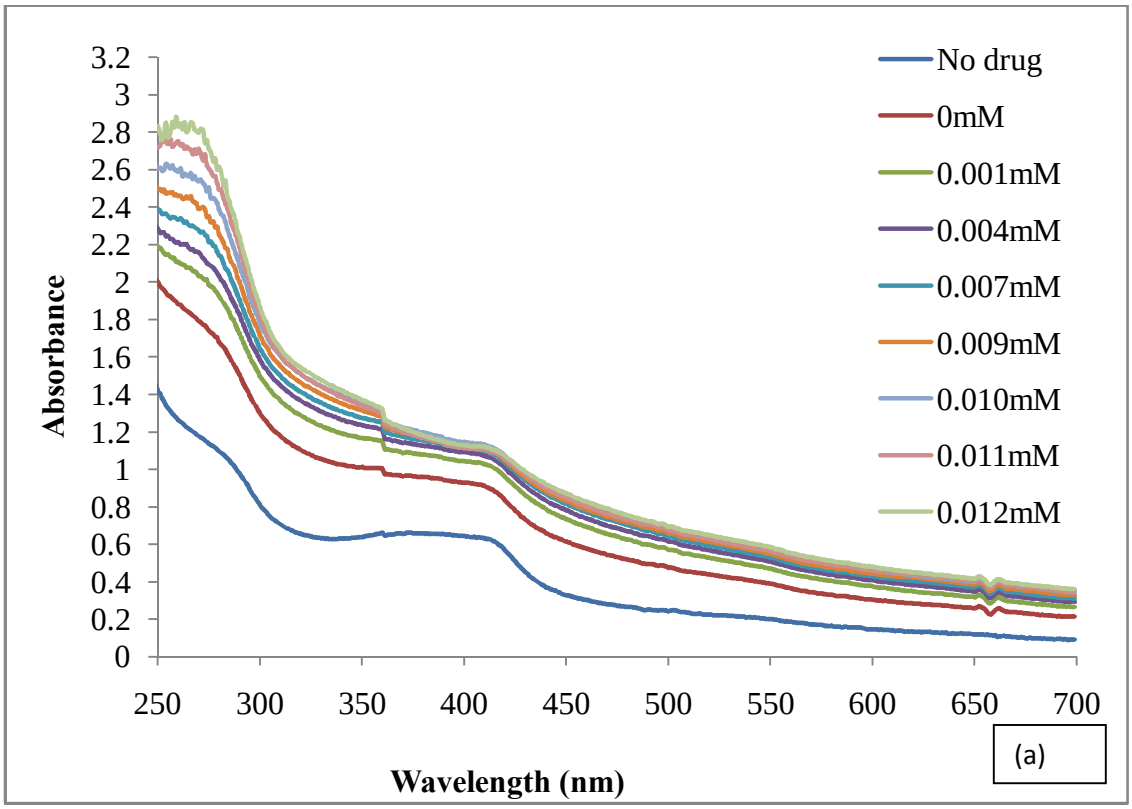


Figure 3.23: Absorption spectra of haemoglobin S in varying concentrations (0-0.012 mM) of sulphadoxine and constant concentration (0.017 mM) of pyrimethamine, at (a) pH 5.0 and (b) pH 7.2



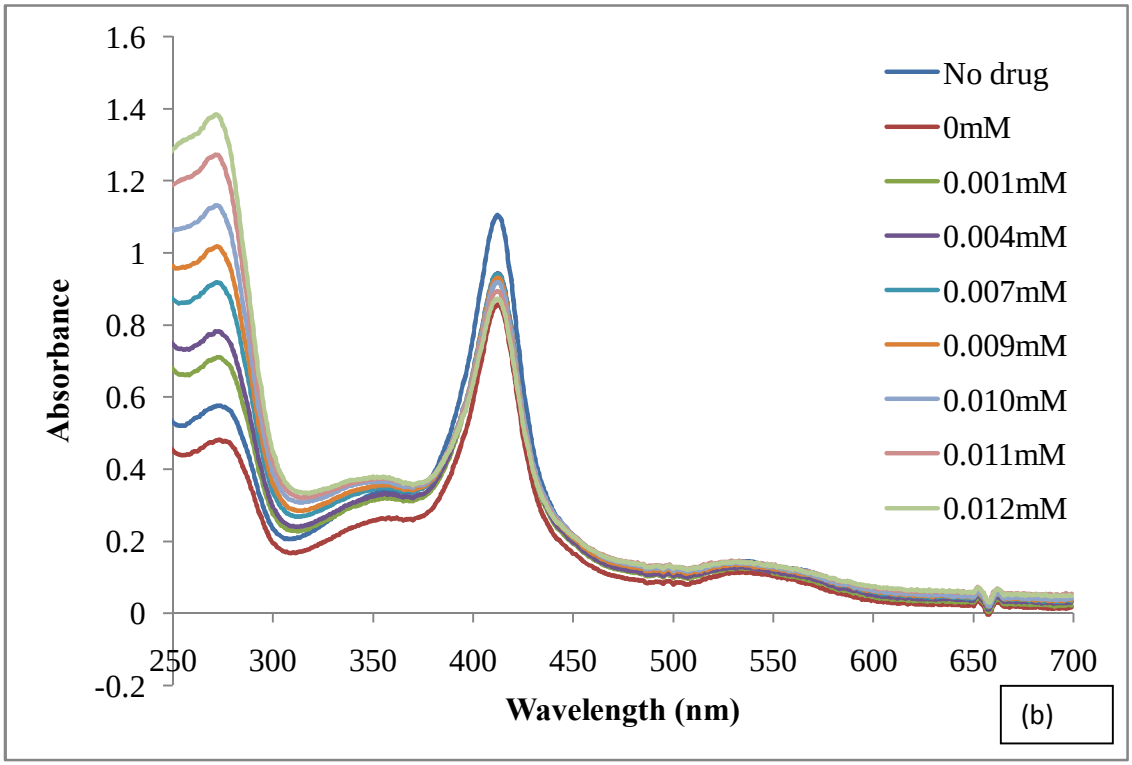


Figure 3.24: Absorption spectra of haemoglobin S in varying concentrations (0-0.012 mM) of

sulphadoxine and constant concentration (0.017 mM) of pyrimethamine in the presence of SDS, at (a) pH 5.0 and (b) pH 7.2.

CHAPTER FOUR

DISCUSSION

Interaction of pyrimethamine or sulphadoxine with haemoglobin caused changes on the spectra of haemoglobin. These changes involved increase or decrease in absorbance at wavelength(s) corresponding to aromatic band (275 nm), soret band (415 nm), globin-haem interaction band (340 nm), oxyhaemoglobin bands (542 and 577 nm), methaemoglobin band (630 nm) and spectral shift.

SDS caused total unfolding and denaturation of the studied haemoproteins. This can be related to destabilization of haemoglobin structure by proteases such as plasmepsins and falcipains in the acidic environment of malaria parasite food vacuole as a result of malaria parasite infection (Tekwani and Walker, 2005). This unfolding exposes the haem moiety and buried aromatic amino acid residues of the proteins which explain the effect of SDS on aromatic and soret bands of the studied proteins. The iron (II) ion (Fe^{2+}) of the haem moiety can oxidize to iron (III) ion (Fe^{3+}) to form methaemoglobin (Voet and Voet, 2004) or a prooxidant that can catalyze the production of reactive oxygen species.

The increase in absorbance of the aromatic band of the haemoproteins when varying concentrationsof pyrimethamine were added at pH 5.0 and 7.2 refers to dynamic motion of the molecule and its deviation from normal structure and function (Ibrahim *et al.,* 2008) or unfolding of the haemoproteins (Matsui *et al*., 2008). There was a slow decay of the soret band which suggests a protein with less haem content. The effect of pyrimethamine on the studied proteins was more pronounced on the aromatic band both at the pH 5.0 and 7.2. This could be possible because the drugôs target in malaria parasite is the globin part of haemoglobin. The effect was not much on the globin-haem interaction band and oxyhaemoglobin bands in the presence of SDS both at pH 5.0 and 7.2. This may be due to the hardness of the non-covalent bond between histidine of globin and haem iron of the studied proteins and disappearance of oxyhaemoglobin bands as a result of interaction of SDS with haemoglobin and its conversion to methaemoglobin or deoxyhaemoglobin.

The effect of sulphadoxine on haemoglobins was assessed on the basis of the increased absorbance of the aromatic band and decreased absorbance of the soret band, a condition that is achieved as a result of haem alkylation and subsequent disruption of the pie electron delocalized system (Messori *et al*., 2003). The slow decay of the soret band as observed in the results shows that all four haem ferrous centres of native haemoglobin are targeted; eventual oxidative degradation of the iron porphyrin chromophores takes place with progressive loss of the soret band. The haemoglobin spectra showed that sulphadoxine greatly perturbed the solution structure of the haemoproteins. In the presence of SDS, the effect of sulphadoxine on the studied proteins was the same with pyrimethamine except that the effect in absorbance of the aromatic band was markedly higher.

The interaction of pyrimethamine-sulphadoxine combination with haemoglobins was investigated spectrophotometrically at pH 5.0 and 7.2 in the presence and absence of SDS. It was observed that at increasing concentration of pyrimethamine at constant concentration (0.008mM) of sulphadoxine or increasing concentration of sulphadoxine at constant concentration (0.017mM) of pyrimethamine (which are the equivalent administerable doses of both drugs), the absorbance of the aromatic band (275 nm) increased in a concentration-dependent manner. At pH 5.0 in the presence of SDS, the addition of increasing concentration of pyrimethamine at constant concentration of sulphadoxine did not cause appreciable increase in absorbance of the soret band (415 nm) of the studied haemoproteins. But when sulphadoxine was increased in concentration at constant concentration of pyrimethamine, the absorption spectra of the haemoglobins increased to a certain point where it got saturated and remained constant in spite of the addition of increasing concentrations of sulphadoxine. This interaction showed positive cooperativity with HbS-sulphadoxine. At pH 7.2 in the presence of SDS, the increase in absorbance of the aromatic band also led to a decrease in absorbance of the soret band and stretching of globin-haem interaction band (340nm).

It is worth remembering that the two drugs, i.e pyrimethamine and sulphadoxine are fairly hydrophobic. Pyrimethamine and sulphadoxine are incapable of docking to protein-bound haem unless the milieu flanking the haem excludes water. It is believed that the haems in haemoglobin are anchored in such hydrophobic pockets that exclude water, facilitating the partitioning of pyrimethamine and sulphadoxine into the haem pockets. Such partitioning may trigger strong reaction between the drugs and haem.

The pyrimethamine-sulphadoxine combination caused a modest blue shift in absorbance of the soret band. Deformations of porphyrin are known to result in extreme changes in the chemical and spectroscopic properties of the porphyrin macrocycle. The chemical properties of porphyrin that are modified by distortion include oxidation potential, basicity of the inner nitrogen atoms, and axial ligand binding affinity (Haddad *et al*., 2003). All of these can influence the biological functions of porphyrin cofactors in proteins.

CONCLUSION

Action of SDS on haemoglobins can be likened to the way proteases secreted by malaria parasites destabilize host haemoglobin for their homeostasis. Haemoglobins are being deoxygenated in the presence of SDS. The Fe^{2+} ion of the deoxygenated haemoglobin can oxidize to Fe^{3+} to form methaemoglobin and formation of methaemoglobin is correlated to lipid oxidation. Deoxygenation of haemoglobin as a result of interaction with SDS can be related to pathological condition where by malaria parasites infection reduces the oxygen tension of erythrocythes of their host which can lead to production of membrane-associated haemin secondary to repeated formation of sickle cell in sickle cell trait individuals but not in individuals of genotype AA. This membrane-associated haemin can oxidize membrane lipids and proteins or may play a role in destroying malaria parasite. The increase in absorbance observed at the aromatic band may suggest that the proteins have deviated from normal structure and function and HbS having the highest absorption maxima could be due to the two fewer negative charges as a result of replacement of glutamic acid with valine at the sixth position of the beta subunit of haemoglobin A. The blue shift when the combination of pyrimethamine-sulphadoxine reacted with haemoglobins could be as a result of formation of new chemical species or that their presence promotes quick oxidation of haemoglobin to methaemoglobin.

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APPENDICES

PREPARATION OF CHEMICAL SOLUTIONS

Preparation of Stock buffer solution

Tris - HCl buffer 0.1 M (pH 7.2): A measured volume, 500 ml each of 0.1 M Tris - Hcl buffer, pH 7.2 was prepared. 6.055 g of tris-[hydroxyl methyl amino] methane was dissolved in

300 ml of distilled water and titrated with a dilute solution of hydrochloric acid to adjust the pH. After which the solution was made up to 500 ml with distilled water.

Sodium Acetate buffer 0.1 M (pH 5.0): A known weight, 4.1 g of sodium acetate was dissolved in about 300 ml of distilled water and titrated with a dilute solution of acetic acid to adjust the pH. After which the solution was made up to 500 ml with distilled water.

Preparation of dilute (Working) buffer solutions

Tris-HCl buffer 0.05 M, pH 7.2 and sodium acetate buffer 0.05 M, pH 5.0: The working buffer solution was prepared from stock buffer solution appropriately in the ratio of 1:1 of the buffer to distilled water.

Wash buffer: The wash buffer was prepared as normal saline (0.9 g NaCl) in 100 ml of 0.05 M Tris- HCl buffer, pH 7.2

Preparation of 5 mM Sodium dodecyl sulphate (SDS) solutions

Exactly 0.0721 g of sodium dodecyl Sulphate was separately dissolved in 30ml of distilled water. The solution was made up to 50 ml to give 50 ml of 5 mM sodium dodecyl sulphate solution.

Preparation of 40 % Ethanol solution

In preparing 100 ml of 40 % ethanol, 40 ml of 99.7 % of ethyl alcohol was measured in a graduated measuring cylinder and then added 60 ml of distilled water to give 40 % ethanol solution.

Preparation of Drug Solutions

Preparation of 0.25 mM Pyrimethamine Solution

A kown weight, 0.0622 g of pyrimethamine was dissolved in 30 ml of 40 % ethanol. The resulting solution was made up to 50 ml with ethanol to give 0.25 mM pyrimethamine solution.

Preparation of 0.117 mM Sulphadoxine Solution

Sulphadoxine (0.0363 g) was dissolved in 30 ml of ethanol (40%). The resulting solution was made up to 50 ml with ethanol to give 0.117 mM sulphadoxine solution.

Table 1: Titration of Haemoglobin with varying concentrations (0-0.025 mM) of Pyrimethamine at pH 5.0 and 7.2

Volume of 50 mM buffer pH 5.0 and 7.2 (mI)	Volume of Haemoglobin (ml)	Volume of 0.25 mM Pyrimethamine stock solution (ml)	Volume of reaction mixture (ml)	Concentration of pyrimethamine in reaction mixture (mM)
0.7	0.1	0.000	0.800	0.000
0.7	0.1	0.010	0.810	0.003
0.7	0.1	0.030	0.840	0.009
0.7	0.1	0.050	0.890	0.014
0.7	0.1	0.070	0.960	0.018
0.7	0.1	0.090	1.050	0.021
0.7	0.1	0.110	1.160	0.024
0.7	0.1	0.130	1.290	0.025

Table 2: Titration of Haemoglobin with varying concentrations (0-0.012 mM) of Sulphadoxine at pH 5.0 and 7.2

Volume of 50 mM buffer pH 5.0 and 7.2 (mI)	Volume of Haemoglobin (ml)	Volume of 0.117 mM Pyrimethamine stock solution (ml)	Volume of reaction mixture (ml)	Concentration of sulphadoxine in reaction mixture (mM)
0.7	0.1	0.000	0.800	0.000
0.7	0.1	0.010	0.810	0.001
0.7	0.1	0.030	0.840	0.004
0.7	0.1	0.050	0.890	0.007
0.7	0.1	0.070	0.960	0.009
0.7	0.1	0.090	1.050	0.010
0.7	0.1	0.110	1.160	0.011
0.7	0.1	0.130	1.290	0.012

Table 3: Changes in haemoglobin absorbance at 275 nm and 415 nm in varying concentrations (0-0.025 mM) of pyrimethamine in the absence of SDS.

[Pyrimethamine] (mM)	275 nm HbA pH 5.0	275 nm HbA pH 7.2	415 nm HbA pH 5.0	415 nm HbA pH 7.2
0.000	0.488	0.574	1.547	1.555
0.003	0.543	0.638	1.541	1.549
0.009	0.695	0.748	1.508	1.530
0.014	0.797	0.924	1.477	1.490
0.018	1.031	1.049	1.418	1.455
0.021	1.298	1.109	1.326	1.405
0.024	1.399	1.168	1.298	1.359
0.025	1.461	1.365	1.196	1.302

Table 4: Changes in haemoglobin absorbance at 275 nm and 415 nm in varying concentrations (0-0.025 mM) of pyrimethamine in the presence of SDS.

[Pyrimethamine] (mM)	275 nm HbA pH 5.0	275 nm HbA pH 7.2	415 nm HbA pH 5.0	415 nm HbA pH 7.2
0.000	0.425	0.275	0.365	0.763
0.003	0.420	0.360	0.364	0.950
0.009	0.471	0.365	0.367	0.966
0.014	0.537	0.614	0.366	0.975
0.018	0.766	0.721	0.381	0.971
0.021	1.076	0.813	0.422	0.963
0.024	1.406	1.001	0.469	0.942
0.025	1.816	1.179	0.507	0.917

Table 5: Changes in haemoglobin absorbance at 275 nm and 415 nm in varying concentrations (0-0.012 mM) of sulphadoxine in the absence of SDS.

[Sulpadoxine] (mM)	275 nm HbA pH 5.0	275 nm HbA pH 7.2	415 nm HbA pH 5.0	415 nm HbA pH 7.2
0.000	0.488	0.795	1.532	1.528
0.001	0.339	0.832	1.275	1.522
0.004	0.421	0.901	1.218	1.508
0.007	0.544	1.032	1.160	1.474
0.009	0.685	1.232	1.110	1.437
0.010	0.797	1.377	1.070	1.377
0.011	0.982	1.548	0.987	1.313
0.012	1.094	1.678	0.942	1.276

Table 6: Changes in haemoglobin absorbance at 275 nm and 415 nm in varying concentrations (0-0.012 mM) of sulphadoxine in the presence of SDS.

[Sulphadoxine] (mM)	275 nm HbA pH 5.0	275 nm HbA pH 7.2	415 nm HbA pH 5.0	415 nm HbA pH 7.2
0.000	0.425	0.599	0.365	1.122
0.001	0.203	0.622	0.271	1.138
0.004	0.245	0.669	0.282	1.137
0.007	0.332	0.763	0.280	1.117
0.009	0.452	0.957	0.290	1.054
0.010	0.683	1.029	0.310	1.033
0.011	0.984	1.052	0.350	0.788
0.012	1.316	1.090	0.372	0.750

Table 7: Changes in haemoglobin absorbance at 275 nm and 415 nm in varying concentrations (0-0.025 mM) of pyrimethamine in the absence of SDS.

[Pyrimethamine] (mM)	275 nm HbAS pH 5.0	275 nm HbAS pH 7.2	415 nm HbAS pH 5.0	415 nm HbAS pH 7.2
0.000	0.450	0.592	1.508	1.595
0.003	0.487	0.681	1.481	1.615
0.009	0.600	0.809	1.441	1.615
0.014	0.740	0.949	1.405	1.590
0.018	0.954	1.236	1.334	1.518
0.021	1.140	1.398	1.280	1.472
0.024	1.279	1.572	1.233	1.421
0.025	1.709	1.782	1.072	1.341

Table 8: Changes in haemoglobin absorbance at 275 nm and 415 nm in varying concentrations (0-0.025 mM) of pyrimethamine in the presence of SDS.

[Pyrimethamine] (mM)	275 nm HbAS pH 5.0	275 nm HbAS pH 7.2	415 nm HbAS pH 5.0	415 nm HbAS pH 7.2
0.000	0.883	0.374	0.539	1.149
0.003	0.810	0.429	0.488	1.158
0.009	0.814	0.571	0.451	1.178
0.014	1.124	0.680	0.617	1.183
0.018	1.230	0.907	0.639	1.153
0.021	1.466	1.056	0.664	1.128
0.024	1.798	1.194	0.706	1.098
0.025	2.279	1.372	0.709	1.052

Table 9: Changes in haemoglobin absorbance at 275 nm and 415 nm in varying concentrations (0-0.012 mM) of sulphadoxine in the absence of SDS.

[Sulphadoxine] (mM)	275 nm HbAS pH 5.0	275 nm HbAS pH 7.2	415 nm HbAS pH 5.0	415 nm HbAS pH 7.2
0.000	0.450	0.567	1.508	1.506
0.001	0.513	0.635	1.511	1.491
0.004	0.615	0.730	1.491	1.485
0.007	0.740	0.908	1.462	1.453
0.009	0.941	1.141	1.384	1.403
0.010	1.091	1.271	1.316	1.359
0.011	1.213	1.327	1.281	1.359
0.012	1.412	1.421	1.1768	1.317

Table 10: Changes in haemoglobin absorbance at 275 nm and 415 nm in varying concentrations (0-0.012 mM) of sulphadoxine in the presence of SDS.

[Sulphadoxine] (mM)	275 nm HbAS pH 5.0	275 nm HbAS pH 7.2	415 nm HbAS pH 5.0	415 nm HbAS pH 7.2
0.000	0.920	0.386	0.635	1.050
0.001	0.938	0.439	0.628	1.079
0.004	0.945	0.540	0.568	1.092
0.007	0.957	0.690	0.538	1.078
0.009	1.074	0.794	0.540	1.067
0.010	1.317	0.978	0.563	1.024
0.011	1.499	1.154	0.577	0.984
0.012	1.809	1.287	0.582	0.947

Table 11: Changes in haemoglobin absorbance at 275 nm and 415 nm in varying concentrations (0-0.025 mM) of pyrimethamine in the absence of SDS.

[Pyrimethamine] (mM)	275 nm HbS pH 5.0	275 nm HbS pH 7.2	415 nm HbS pH 5.0	415 nm HbS pH 7.2
0.000	0.732	0.729	1.403	1.504
0.003	0.838	0.813	1.414	1.516
0.009	0.938	0.920	1.386	1.507
0.014	1.019	1.099	1.375	1.455
0.018	1.217	1.259	1.301	1.398
0.021	1.383	1.433	1.252	1.334
0.024	1.549	1.667	1.186	1.204
0.025	1.695	1.777	1.138	1.170

Table 12: Changes in haemoglobin absorbance at 275 nm and 415 nm in varying concentrations (0-0.025 mM) of pyrimethamine in the presence of SDS.

[Pyrimethamine] (mM)	275 nm HbS pH 5.0	275 nm HbS pH 7.2	415 nm HbS pH 5.0	415 nm HbS pH 7.2
0.000	1.397	0.576	0.729	1.069
0.003	1.599	0.648	0.804	1.071
0.009	1.682	0.723	0.830	1.064
0.014	1.795	0.814	0.845	1.061
0.018	2.022	0.957	0.886	1.028
0.021	2.262	1.093	0.947	0.998
0.024	2.635	1.226	1.007	0.991
0.025	2.825	1.369	1.079	0.949

Table 13: Changes in haemoglobin absorbance at 275 nm and 415 nm in varying concentrations (0-0.012 mM) of sulphadoxine in the absence of SDS.

[Sulphadoxine] (mM)	275 nm HbS pH 5.0	275 nm HbS pH 7.2	415 nm HbS pH 5.0	415 nm HbS pH 7.2
0.000	0.660	0.734	1.420	1.384
0.001	0.734	0.824	1.434	1.390
0.004	0.811	0.940	1.414	1.370
0.007	0.897	1.046	1.397	1.354
0.009	1.045	1.209	1.325	1.290
0.010	1.258	1.308	1.239	1.246
0.011	1.492	1.450	1.121	1.202
0.012	1.640	1.592	1.073	1.157

Table 14: Changes in haemoglobin absorbance at 275 nm and 415 nm in varying concentrations (0-0.012 mM) of sulphadoxine in the presence of SDS.

[Sulphadoxine] (mM)	275 nm HbS pH 5.0	275 nm HbS pH 7.2	415 nm HbS pH 5.0	415 nm HbS pH 7.2
0.000	1.165	0.491	0.626	0.899
0.001	1.218	0.552	0.648	0.972
0.004	1.240	0.657	0.650	0.996
0.007	1.329	0.770	0.655	0.996
0.009	1.486	0.850	0.679	0.982
0.010	1.758	0.952	0.723	0.967
0.011	2.434	1.170	1.033	0.907
0.012	2.560	1.283	1.116	0.871