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PROFILING OF SOME KNOWN ANTI-MALARIAL DRUGS USING A HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) SEPARATION/EXTRACTION TECHNIQUE

A THESIS DISSERTATION SUBMITTED TO THE

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ABSTRACT

WHO estimates that more than 1.5 to 2.0 million deaths attributed to malaria each year occur in African children (WHO, 1996) The development of synthetic anti-malaria drugs forms one of the most interesting chapters in the history of malaria chemotherapy.

The major obstacle to successful chemotherapy of malaria has been the development of resistant to anti-malarial drugs. Research into drug monitoring has become an increasingly practical preposition (Ayitey-Smith, 1988)

This study has been planned in order to develop a reliable technique of extraction/separation methods for some known antimalarial drugs from blood plasma. For purity sake, only five pure antimalarials have been secured to prepare the serial standards as well as to spike the plasma to be used. These drugs are chloroquine, fansidar, quinine, amodiaquine and pyrimethamine.

Specified amount of each antimalarial drug was used to spike the plasma and aqueous samples. The aqueous was used as control hence both the plasma and the aqueous underwent the same treatment.

Extraction was performed on the spiked samples and the extracts were analyzed by HPLC. Recovery was calculated from the results of all the extracts. Since the recovery only could not give the extent or the degree of the extraction one-way ANOVA with Tukey's multiple comparison test was also performed.

The extractability of chloroquine, amodiaquine and fansidar were effective for both the control and the plasma. The extraction methods for quinine and pyrimethamine were not very effective.

ANOVA and Tukey's multiple comparison test of chloroquine produced $p < 0.0456$ (*) and $p<0.014$ (**) meaning extraction was very effective. However it was clear that at lower concentration, extraction was poor. The method is therefore recommended.

The ANOVA and Tukey's test on the mean recoveries for fansidar gave p values of < 0.0046 (*) and $p < 0.0016$ (**) for peak height and peak area respectively. These values showed that the

extraction was effective and on the other hand fairly effective under the peak height and peak area respectively. In summary, one could say that the extraction of fansidar was effective under peak height; thus this method of extraction is recommended.

For Quinine, Tukey's test and ANOVA of the mean recoveries indicated poor results; $p < 0.0001$ (***). Though the mean recovery (%) for both peak height and peak area looked good, the method of extraction needs to be improved.

ANOVA and Tukey's test performed on mean recoveries for amodiaquine gave $p < 0.046$ (*) and $p < 0.002$ (**) for peak height and peak area respectively. This showed that the extraction was effective under peak height fairly effective under peak area. These results indicated that the extraction was effective. The method is therefore recommended.

When the mean recoveries for pyrimethamine extracts was subjected to ANVOVA and Tukey's multiple comparison test, $p < 0.0001$ was produced for both peak area and peak height. This showed that there was an extremely significant difference hence the extraction was not effective. The method is therefore needs to be improved.

In addition to the above extracts, extractions were performed on double blind test samples, and three (3) samples of volunteers. The blind test was carried out on twenty two (22) pregnant women who were given chloroquine, fansidar and placebo at random. For the volunteers, the first took chloroquine, the second Fansidar and the third took amodiaquine. Blood samples were taken from the women and the volunteers.

Extraction was then performed on the plasma of these samples. HPLC analysis on these extracts was able to identify the double blind test samples. Volunteers' samples were run alongside the spiked control and plasma samples. Using the retention time of the standard antimalarial drugs, six (6) of the blind test samples, proved to be chloroquine and ten (10) as fansidar. Four (4) of the samples results did not show any appreciable peak near the retention time of neither chloroquine nor fansidar. Hence they were classified as placebo and two contaminations were observed.

In conclusion, the extraction methods for Chloroquine, Amodiaquine and Fansidar were effective. But the extraction techniques for Quinine and Pyrimethamine were not effrctive hence, further work needs to be done on them.

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DEDICATION

This work is dedicated to my wife and children for

a shared vision.

DECLARATION

This is to certify that the work herein presented is the product of my own effort, carried out under the able supervision of the Department of Molecular Medicine, SMS, KNUST. I however remain solely responsible for any errors, technical and terminological inaccuracies which are consciously or inadvertently committed. All sited references have been duly acknowledged.

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ABBREVIATIONS

HPLC High performance liquid chromatography

TLC Thin Layer Chromatography

GC Gas chromatography

GLC Gas liquid chromatography

RT Retention time

MP Mobile phase

RPM Revolution per minute

PH Peak height

PA Peak area

CI Confidence interval

CV Coefficient of variation

ANOVA Analysis of variance

RBC Red Blood Cells

GIT Gastrointestinal tract

CSF Cerebrospinalfluid

CHAPTER ONE

GENERAL INTRODUCTION

OVERVIEW

Malaria is a prevalent human disease and is considered one of the oldest infections. It has been mentioned in early writings of Egypt, India and China. Its clinical symptoms were fully described by Hippocrates, 400 years before Christian era (Gilles,1991).

The main break through in the history of malaria was attained in the early seventeenth century when the therapeutic advance was made by the discovery of "Peruvian bark" which was later given the name as *cinchona.* This bark contains the active component Quinine which has schizontocidal effects hence its effectiveness on malaria (Bruce–Chwatt, 1993).

The most important events in the history of malaria took place towards the end of nineteenth century when bacteriologists and pathologists discovered some of the effects of malaria infection; i.e. morbid changes in organs and tissues. In 1880, Alphonse Laveran, a French Army Surgeon in Algeria first saw and described malaria parasite in the red blood cells (RBC) of human beings. This achievement gave Laveran the Noble prize (Gilles, 1991).

World Health Organization (WHO), Pan-American Health Organization (PAHO), United Nations International Children's Emergency Fund (UNICEF) and many other International health departments started the campaign for malaria eradication in 1955 (Bruce-Chwatt 1993). This campaign yielded excellent dividend in most parts of Europe, Middle East, parts

of Asia, Caribbean, the former USSR, Australia, North and South of America but less or poor results in tropical countries and parts of Asia.

The fact still remains that some 1.6 million people throughout the world are still exposed to the risk of malaria infection. In malaria endemic areas this disease causes high mortality of infants and children. This brings to the fore, adverse factors of social and economic advancement in the Third World (Foster & Philips, 1998).

WHO estimates that more than 1.5 to 2.0 million deaths attributed to malaria each year occur in African children (WHO, 1996). Other estimates based on vigorous attempts to calculate the burden of disease in Africa support this level of mortality (Snow *et al.*, 1999). In addition to its burden in terms of morbidity and mortality, the economic effects of malaria infection can be tremendous. These include direct costs for treatment and prevention as well as indirect costs such as lost productivity from morbidity and mortality; time spent seeking treatment and diversion of household resources. The annual economic burden of malarial infection in 1995 was estimated at US$0.8 billion for Africa alone (Foster and Phillips, 1998). This heavy toll can hinder economic and community activities throughout the continent.

Malaria is a widespread disease especially within the tropics and was associated with marshland, thus the former name marsh fever. The causative agent is the malarial parasite *Plasmodium* species. The transmission vector is the female mosquito, *Anopheles*. The

parasite when injected into man enters the red blood cells (RBC) where it destroys the RBC by the release of pyrogenic substances.

There are four main known *Plasmodium* species, which affect man. The most deadly of these is *P. falciparum*. This species is ubiquitous and predominates especially in black Africa (Gilles, 1991).

Chemotherapy has been of great importance, since the controls of malaria were introduced. However, the development of residual insecticides somewhat overshadowed the role of antimalaria drugs around 1960, but with the dramatic resurgence of malaria in many countries, ant malarial drugs regained their importance despite the resistance of some strains (WHO, 1984).

The bark of the "fever trees" known as the "Peruvian bark" had been used to cure malaria in the 1600s in many parts of the world. The spectacular cure in 1682 of the French heir to the throne by an English pyretologist, Robert Tabor enhanced the popularity of the powdered "Peruvian bark". The missionaries also played very important role in the spread of the bark. Thus around 1977, the therapeutic value of the "Peruvian bark" was recognized and was included in the London Pharmacopoeia as *Cortex peruanus* (Bruce-Chwatt, 1993).

The development of synthetic antimalaria drugs forms one of the most interesting chapters in the history of malaria chemotherapy. During the First World War the Germans were cut off from many parts of the world by the supply of quinine, the active component from the "fever trees." Various attempts of synthesising quinine proved futile. From this point on,

using quinoline nucleus of the cinchona alkaloids, led to the first synthetic antimalaria drug, named Pamaquine (Bruce-Chwatt., 1993).

In the course of collective and coordinated study between the armed services scientific institutions, university laboratories and pharmaceutical firms several derivatives of 4-aminoquinoline were found to be superior to all other drugs (Bruce-Chwatt, 1993). Two of these, which underwent further extensive clinical studies, were chloroquine and amodiaquine. Further chemotherapeutic studies have revealed induced drug resistance in some strains of *Plasmodia*, hence the search for better ant malarial continues.

Chromatography is an analytical technique in which two or more compounds can be separated from one another by utilizing differences in their interactions with the surface of a stationary phase such as paper, wax, or silica. To achieve separation, a dynamic equilibrium distribution between the compounds in the mobile phase such as a flowing gas or liquid and the stationary phase is established (Pascal, et al, 2000). The discovery of chromatography is generally credited to Tswett, who in 1903 described his work on using a column of chalk to separate the pigments in green leaves. The term "chromatography" was coined by Tswett to describe the coloured zones that moved down the column (Pascal, et al, 2000).

The dramatic development of chromatographic techniques, especially High Performance Liquid Chromatography (HPLC) has made possible the easy analysis of organic compounds, including drug components for the last two decades. The increased improvement of

analytical methodology with HPLC has enabled researchers and scientists to cope with other scientific and instrumental developments in their fields of work (Pascal, et al, 2000).

PROBLEM STATEMENT

1. Global use of various and combined forms of anti-malarial drugs have historically encountered various degrees of resistance by the plasmodium parasite, the causative agent of Malaria

2. The use of fake anti-malarial drugs calls for a bold-standard technique for validating authentic anti-malarial drugs (WHO, 2006). A high prevalence of sub-standard antimalarials in the African retail sector is of great importance in view of the frequency of their use for fever/malaria treatment (Bate Roger, et al 2008)

HYPOTHESIS:

The development of efficacious and reliable techniques for the separation and quantisation of anti-malarial drugs will be a useful adjunct in the determination of the plasma threshold concentrations of these drugs used in malaria treatment.

JUSTIFICATION

Malaria is an ancient tropical malady and is still African's leading health hazard, accounting for over a million deaths per year and greatly affects the productivity of Africans (Echidna, 1998).

The major obstacle to successful chemotherapy of malaria has been the development of resistance to antimalarial drugs. Research into drug monitoring has become an increasingly practical preposition (Ayitey-Smith, 1988). This will help to establish the most appropriate therapeutic levels of ant malarial drugs and thus serves as a guide in the treatment of malaria.

The efficacy of a drug may be determined by effective plasma separation and quantification taking into consideration such factors as sensitivity, the presence of partial immunity in the human host; the risks of toxic effects, non-compliance of the drug etc (Bate, el al, 2008).

The prevalence of fake anti-malarial drugs calls for a bold-standard technique for validating authentic anti-malarial drugs (Dondorp, *et al*, 2004; WHO, 2006). There is therefore the need to elucidate the aims and objectives of this project. The outcome of such a study will greatly help health scientists to understand therapeutic anti-malarial drug monitoring and thus influence health policies geared towards the control and eradication of malaria in Ghana and for that matter, in the Sub-Saharan Africa.

OBJECTIVE

1. To develop an innovative HPLC technique for the separation of commonly used antimalarial drugs in Ghana.
2. To determine and standardize conditions for the separation of antimalarial drugs by HPLC.
3. To compare the separation profiles of standard antimalarial by HPLC.

4. To recommend standardized data-based threshold producers for separation of antimalarial by HPLC.

CHAPTER TWO

LITERATURE REVIEW

2.1 MALARIA

2.1.1 DEFINITION

The term 'malaria' is common in several languages and is generally used in scientific texts originating from Italy. It refers to the connection between the disease and 'spoiled air' *(mal - aria)* that was believed to be the cause. In French the disease is known as '*paludisme'*, a term indicating the relationship of the disease with marshy areas (*palms – in Latin*) (White, 1982).

2.1.2 ETIOLOGY

Malaria is a mosquito-borne disease of man which is characterized by chills, fever, anaemia, and splenomegaly. It can also cause damage to organs, such as the liver, kidney and the brain. It is the most important parasitic disease in Tropical Medicine and is the first of the six major causes of deaths in tropical disease (Hay et al, 2000).

Malaria is a protozoa disease caused by the genus *Plasmodium* (P). It is transmitted to man through the bite of infected female mosquitoes of the genus, *Anopheles*. Infection can also occur by transfusion of infected donor blood, by injection through the use of contaminated needle and

syringes. Occasionally it occurs congenitally via the placenta of an infected mother (Abel *et al*., 1992).

The microorganisms causing malaria are commonly referred to as malaria parasites. The malaria parasites undergo two types of multiplication. These are asexual divisions (schizogony) in the vertebrate host and single sexual multiplication (sporogony) in the mosquito host. The asexual division occurs in the parenchymal cells of the liver of the vertebrate host which is termed as exo-erythrocytic schizogony (Abel *et al*., 1992). The other multiplication takes place in the mosquito host, the *Anopheles*.

The chief species of human malaria parasites are as follows:

a) *P. falciparum,* which has an erythrocytic cycle of 48 hours in humans and produces *malignant tertian malaria*-'tertian' because the fever was believed to recur every third day, 'malignant' because it is the most severe form of malaria and can be fatal. The parasitized red cells stick to uninfected red cells forming clusters (rosettes). They also adhere to the vessels of the microcirculation, interfering with encephalopathy (cerebral malaria). *P. falciparum* does not have an exoerythrocytic stage, so if the erythrocytic stage is eradicated, relapses do not occur (Cox-Singh et al, 2008).

b) *P. vivax* produces benign tertian malaria-'benign' because it is less severe than falciparum malaria and rarely fatal. Exoerythrocytic forms may persist for years and cause relapses (Cox-Singh, et al, 2008).

c) *P. ovale*, which has a 48-hour cycle and an exoerythrocytic stage, is the cause of a rare form of malaria (Cox-Singh, et al, 2008).

d) *P. malariae* has a 72-hour cycle, causes *quartan malaria* and has no exoerythrocytic cycle (Cox-Singh, et al, 2008).

2.1.3 LIFE CYCLE OF HUMAN MALARIA PARASITE

While it was recognized that the *Anopheles* mosquito played a key role in the transmission of the disease it was not until 1948 that all the stages in its life cycle were identified (Bray and Garnhm, 1992). The malaria parasite undergoes a development stage in the mosquito.The female *Anophelus* species requires a blood meal to mature her eggs. She bites a human and injects material from her salivary glands, which contains primitive malarial parasites called sporozoites, before feeding (Bray and Garnhm, 1992). These sporozoites circulate in the blood for a short time and then settle in the liver where they enter the parenchymal cells and multiply; this stage is known as pre-erythrocytic schizogony. After about 12 days there may be many thousands of young parasites known as merozoites in one liver cell. The cell ruptures and the free merozoites enter red blood cells.

In the red blood cells the parasites develop into two forms, a sexual and an asexual cycle. The sexual cycle produces male and female gametocytes, which circulate in the blood and are taken up by a female mosquito when taking a blood meal. The male and female gametocytes fuse in the mosquito's stomach and form oocysts in the wall of the stomach.

These oocysts develop over a period of 12 days and contain large numbers of sporozoites, which move to the salivary glands and are ready to be injected into man when the mosquito next takes a meal.

In the asexual cycle the developing parasites form schizonts in the red blood cells. The infected red cells rupture and release a batch of young parasites, merozoites, which invade new red cells. The developing forms appear to stick in the blood vessels of the large organs such as the brain and restrict the blood flow with serious consequences (Hay et al, 2000).

All the four species have a haemolytic component. The *P. falciparum* parasites multiply very rapidly and may occupy about 30% or more of the red blood cells causing a very significant level of haemolysis. One reason for this is that *P. falciparum* invades red cells of all ages whereas *P. vivax* and *P. ovale* prefer younger red cells. *P. malariae* seeks mature red cells (Hay et al, 2000).

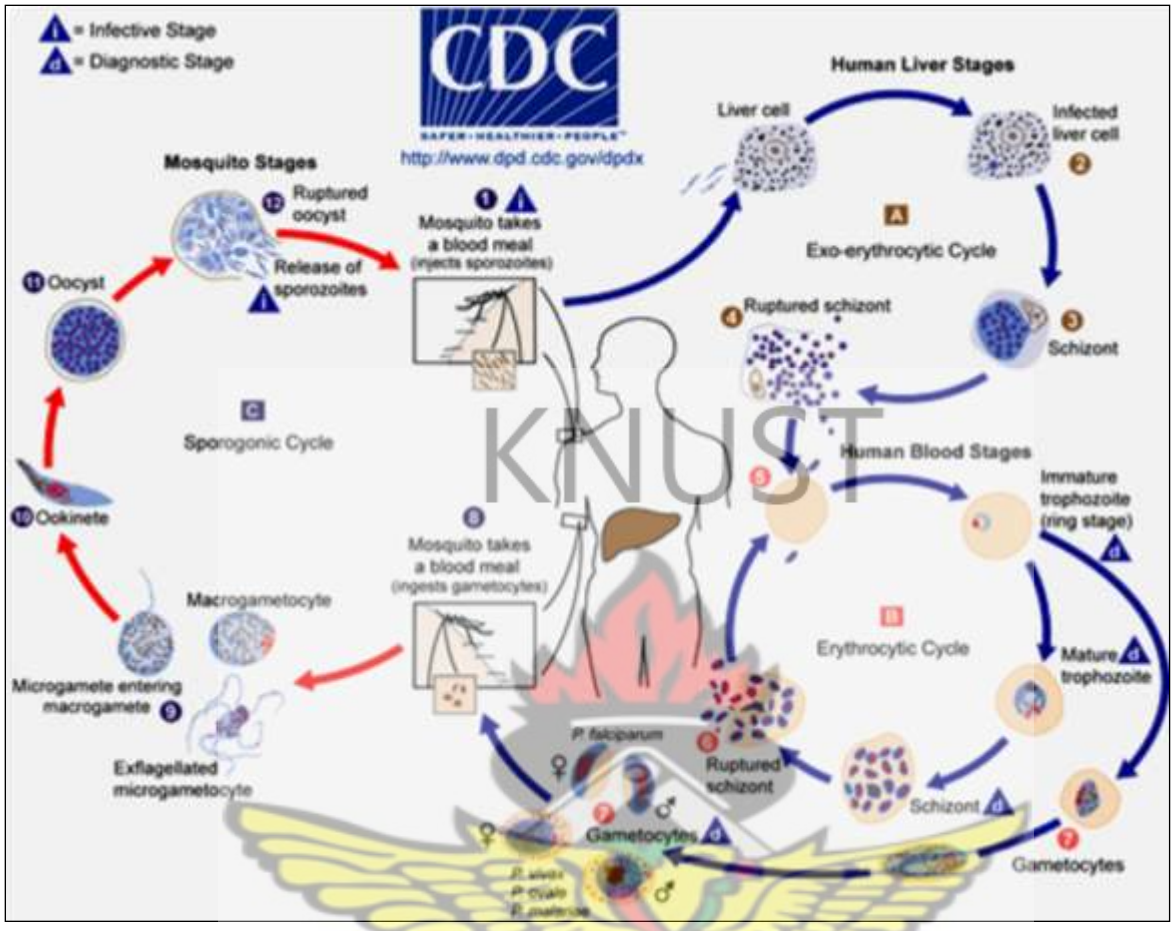

Fig 2.1 Life Cycle of Human Malaria Parasite

2.1.4 CLINICAL FEATURES AND DIAGNOSIS OF MALARIA

Traditionally, complications of severe malaria in Africa children have been considered to fall into two categories. These are anaemia and cerebral malaria. Recent evidence indicates that metabolic acidosis, presenting as clinical syndrome of respiratory distress (particularly deep breathing) is also an important feature of severe malaria (Marsh *et al*., 1998).

Current clinical diagnosis is supposed to offer the advantages of ease, speed, and low cost. However, in many areas where malaria is prevalent, clinical diagnosis usually results in all patients with fever and no apparent other cause being treated for malaria. This approach can identify most patients who truly need antimalarial treatment (Olivar et al, 1991). Over diagnosis considerable contributes to misuse of antimalarial drugs. At times, overlap exists between the signs and symptoms of malaria and other frequent diseases especially acute lower respiratory tract infection (Redd *et al.*, 1992).

Attempts to improve the specificity of clinical diagnosis for malaria by including signs and symptoms other than fever or history of fever have met with only minimal success (Smith *et al*., 1994). By the WHO criteria, every febrile child living in a "high-risk" area of malaria should be considered to have and be treated for malaria. "High Risk" has been defined in Integrated Management of Childhood Illness Adaptation Guild as being any situation where as little as 5% of febrile children between the ages of 2 and 59 months are parasitaemic (WHO, 1996).

2.1.5 SEVERE MALARIA

In a variety of severe disease states, including severe malaria, the presence of acidaemia is associated with a high mortality rate. Acidaemia is a manifestation of severe malaria whether there is an altered consciousness or not (Marsh *et al*., 1998). In a selected group of Malawian children admitted to hospital with primary diagnosis of malaria, 66, (45%) of 145 were found to be either acidaemia with pH less than 7.3 or having compensated metabolic acidosis (Kublin *et al*., 2003); 72% of the children who subsequently died were acidotic on admission. In another

study by Krishna et al 1994, 425 children with cerebral malaria were acidaemic and mortality in these children was found to be 28% compared to 3% in the non-acidaemic group.

Acute renal failure is a common complication of severe *falciparum* malaria and often lethal, and it occurs almost exclusively in adults and older children (Habte, 1990).

Blood glucose less than 2.2 mmol/L (indicating hypoglycaemia) commonly complicates severe malaria in children, and it is associated with an increased risk of dying or suffering sequel from the illness (Koram et al, 2000). Hypoglycaemia is particularly common in young children, less than three years old, in those with convulsions or hyperparasitaemia, and in patients with profound coma (Marsh *et al*., 1998).

2.1.6 CEREBRAL MALARIA

In Nigeria, out of 147 children with fatal *P. falciparum* infections about 69 (46.6%) had Cerebral malaria (Elesha *et al*., 1993). In the Gambia, 43.0% of children admitted in hospital over a period of several years and diagnosed as having severe malaria, were in coma (Elesha *et al*., 1993).

Hypoglycaemia is frequently found in children with cerebral malaria. It is normally associated with low plasma insulin concentrations and is commonly accompanied by hyperlactataemia. Children are more likely prone to hypoglycaemia than adults. Hypoglycaemia was found in 43, (7%) of 603 children admitted to hospital in Mozambique (White *et al*, 1993).

2.1.7 ANAEMIC MALARIA

Anaemia is an important and life-threatening complication of *P. falciparum* malaria in children. Out of 2433 children admitted to a Kenyan hospital 684 (28%) had severe anaemia (haemoglobin < 50.0 g/l), this being strongly associated with *P. falciparum* parasitaemia; 18% of the severely anaemic patients died, compared to 8% of all admissions (van Eijk *et al*., 2002).

The degree of anaemia following the plasmodial infection is by simple destruction of erythrocytes by parasites. The possible mechanisms for these effects are:

a) Production of a toxic factor or auto-antibodies to the erythrocytes.

b) Adherence of circulating antigen-antibody complexes to uninfected erythrocytes and haemolysis through the effect of the complement. (Calis *et al*., 2008).

The mortality among anaemic children in Kenya was greatly increased when associated with respiratory distress (Calis,*et al*., 2008). Among 252 Malawian infants followed-up for the first year of life, 25% had haematocrit below 25% by 2 months of age; anaemia at this age was statistically associated with placental parasitaemia at birth (Redd *et al*., 1994).

Anaemia may develop rapidly during the course of malaria illness, especially if there is an initial hyperparasitaemia (Calis,*et al*., 2008). Anaemia may be present or may develop in a child with cerebral malaria or any other complication of *P. falciparum* infection.

2.1.8 HYPERPYREXIA

Hyperpyrexia is another common feature of falciparum in children. This is normally associated with high fever. Convulsions in children with malaria may occur at any level of body temperature above 38.5°C (Charoenpan, et al, 1990).

2.1.9 JAUNDICE AND RENAL FAILURE MALARIA

Jaundice is common in adult patients with malaria (Warrell et al, 1990). In a study of 330 patients with acute falciparum malaria in Thailand, 124 were clinically jaundiced (total serum

bilirubin ≥ 30 mg/L. Among the jaundiced patients, hyperbilirubinaemia was predominantly unconjugated. Jaundice was associated with cerebral malaria, acute renal failure, pulmonary oedema, shock and other severe complications (Abro et al, 2009).

In Vietnamese adults, 63% of those with acute renal failure were jaundiced compared to 20% of those without renal failure (Warrell et *al*, 1990).

Mild jaundice may result from haemolysis, but very high bilirubin concentrations indicate hepatocyte dysfunction (Warrell et *al*, 1990). In these cases concentrations of unconjugated and conjugated bilirubin are increased.

Malaria induced acute renal failure may be defined as a serum creatinine concentration > 265 μmol/L with 24 hour urine output < 400 ml in spite of dehydration in patients with complication of *P. falciparum*. In 30% of adult patients with cerebral malaria in Thailand serum creatinine rose above 2 mg% and blood urea nitrogen rose above 40 mg % (Warrell et *al*, 1990). These patients had a higher incidence of hypoglycaemia and jaundice, and coma was significantly prolonged.

2.1.10 PATHOPHYSIOLOGY OF MALARIA

The invasion of red-blood cells (RBC) which follows the pre-erythrocytic phase of life cycle is the basic pathological process in malaria infection. The degree of parasitaemia produced by the different species of *Plasmodia* varies considerably. Recent studies in physiopathology of cerebral

malaria indicate that obstruction of the circulation in the capillaries of the brain occurs before the 'plugging' by the infected RBC takes place (Mackintosh, *et al.,* 2004*).*

The spleen is always affected in malaria; the main change being congestion of the spleen which later becomes dark through the accumulation of pigment in parasitized cells (Bonnard et al, 2005). Other organs, which suffer in malaria infections especially *P. falciparum*, are the liver, the kidney and the bone marrow.

2.1.11 IMMUNE RESPONSES IN MALARIA (IRM)

Malaria immunity may be defined as a state of resistance to the infection brought about by all the processes, which are involved in destroying the *plasmodia* or limiting their multiplication. It also includes factors, which modify the effects of invasion of the organism by malaria parasites and aid in the repair of damaged tissues (Perrin *et al*., 1982).

2.1.11.1 NATURAL IMMUNITY (NI)

This is a refractory state or an immediate inhibitory response to the introduction of the malaria parasites. Natural Immunity does not depend on any previous infection of malaria. Observation has revealed that natural immunity relates to race. It has been postulated that the relatives of West African and Black Americans were insusceptible to *P. vivax* infection while other racial groups were susceptible (Doolan et al, 2009).

2.1.11.2 ACQUIRED IMMUNITY (A1)

This immunity could be passive or active. Active acquired immunity is the result of previous encounter of the pathogen with the host. Passive immunity is conferred by the pre-natal or postnatal transfer of protective substances from mother to child or by the injection of such substances contained in the serum of immune persons (Doolan et al, 2009). The immunity acquired following any of the four species of malaria parasites is specie–specific. This means that the protection acquired against one species may not be effective against parasites of another species (Doolan et al, 2009). The physical basis of malaria immunity mainly depends on the activity of both humoral and cellular factors. Antibodies that appear in the blood after infection are carried on the gamma globulins fraction (IgG, IgM, IgA, IgD and IgE) of the blood. The cellular factors are macrophages and other cells produced by the reticulo-endothelial system of the spleen, liver and bone-marrow which undergoes intense proliferation following malaria infection (Cohen *et al*, 1979).

Synthesis of the various immunoglobulins' (Ig) in the sera of malaria infected person's proceeds at different rates. The IgM response occurs early and tends to fall off rapidly, while IgG and IgA antibodies build up over a longer period of time. (Cohen *et al*, 1979).

The four species of human *plasmodia* have a number of common antigens. Within each species the antigens related to the developmental phases are also similar, but not identical. Precise information on the qualitative and quantitative antigenic differences that exist between species and between the phases of life-cycle in one species is not well understood. The most complete

information concerning antigenicity of a parasite is available for *P. falciparum* in which over 30 distinct antigens have been found (Perrin *et al*, 1982). A number of soluble antigen fractions in *P. falciparum* infections have been identified and classified into L (Labile), R (resistant) and S (stable) on the basis of susceptibility to heat (Perrin *et al*, 1982).

Antibodies are found in high prevalence and titre in sera from newborn infants. In the early months of life antibodies decay and the titre becomes low. Around the third year of life, in response to infection, the prevalence and titre of antibodies rise slowly to a peak and maintains a high level in older age groups. Such age related pattern of antibody profiles in various populations forms a valuable method of assessment of malaria endemicity and for evaluation of the efficacy of malaria control activities (Perrin *et al*, 1982).

2.1.12 IMMUNOPATHOLOGY

A number of pathological conditions related to malaria infection are recognized as being the consequences of immune response to the infection. Immunity to malaria occurs and can protect many individuals living in malarious areas. It involves mostly cell-mediated reactions. The immunity is lost if the individual is absent from the area for more than six months (Perrin et al,1982).

2.1.13 NEPHROPATHIES

Immune complexes play an important part in the pathogenesis of adverse effects of malaria infection on the kidneys. Two main lesions have been observed in respect to nephropathy.

These are:

a) Acute and reversible lesions which is of *P. falciparum* infections. At times this leads to severe renal failure with oliguria and retention of urea.

b) Chronic and progressive lesions characteristic of *P. malariae* infections. This is accompanied by persistent proteinuria and hypertension. Immune complexes associated with these lesions are IgG, IgM, and complement (Perrin et al, 1982).

2.1.14 TROPICAL SPLENOMEGALY SYNDROME (T.S.S.)

This syndrome is most often seen in young adults. In addition to an enlarged spleen, there is an enlargement of the liver with a degree of portal hypertension. Normally such patients are anaemic, leucopenia and thrombocytopenia. The immunoglobulin observed in these lesions is IgM (Cohen, 1979).

2.2.1 DIAGNOSTIC METHODS IN MALARIA

A definitive diagnosis of malaria infection is established by the presence of malarial parasites in the blood. In all cases of fever, malaria must be suspected firs,t because several studies in partially immune African children indicate that almost half of clinically diagnosed malaria patients do not have microscopically detectable parasitaemia (Gilles, 1991). On the other hand an examination of the cerebrospinal fluid permits a definite diagnosis.

For simplicity and convenience in routine clinical and epidemiological studies, thin and thick blood films are much preferred in examination for malaria parasite (Gilles, 1991).

2.2.2 BLOOD EXAMINATION FOR MALARIA PARASITES

The only confirmatory test for diagnosing malaria infection is the detection of the *Plasmodia* in the blood by microscopical examination. This examination is the main routine procedure in medical practice.

Advantages of this method include differentiation between species, quantification of parasite density and ability to distinguish between clinically important asexual parasite stages, from gametocytes, which may persist without causing symptoms. The disadvantages of these methods are, slide collection, staining and reading which can be time consuming. The results are often subjective, demanding well-trained personnel.

A modified form of the Giemsa-stained blood films called the quantitative buffy coat method has been established. Quantitative buffy coat has advantage over Giemsa-stained blood film in that the former test is quicker to perform and less training is required. In addition, it has been shown that Quantitative buffy coat is marginally sensitive under ideal condition (Levine et al, 1989; Barat et al, 1999). The high cost of equipment and electricity requirement are the disadvantages of the quantitative buffy coat.

2.2.3 PRESUMPTIVE DIAGNOSIS

Although reliable diagnosis cannot be made on the basis of signs and symptoms because of their non-specific nature, clinical diagnosis of malaria is common in endemic areas. In many of the malaria-endemic world, resources and trained personnel are so scarce that presumptive clinical diagnosis remains the only realistic option. The approach of presumptive clinical diagnosis lead to identifying patients who truly need antimalarial treatment, but it is likely to classify many who do not (Olivar *et al*., 1991).

2.2.4 ANTIGEN DETECTION TEST (ADT)

Antigen detection test is also known as rapid or 'dipstick' test. It involves rapid detection of parasite antigens using rapid immuno-chromatographic techniques. Multiple experimental tests have been developed, targeting parasite antigen (WHO, 1996). Compared with Giemsa-stained blood film and quantitative buffy coat, antigen detection test yielded rapid and highly sensitive diagnosis of *P. falciparum* infection (WHO, 1996). The advantages of this techniques are that, no special equipment is required, minimal training is needed and the reagents are also stable at ambient temperatures. The principal disadvantages are high cost of the reagents and inability to quantify the density of infection (Craig and Sharp, 1997)

2.2.5 MOLECULAR TEST

This technology is known as polymerase-chain reaction (PCR). It detects parasite genetic material and is becoming a more frequently used tool in the diagnosis of malaria, as well as the

diagnosis and surveillance of drug resistance in malaria. One important advantage of PCR over others is that it detects mixed infections (Oliveira et al, 1995).

2.2.6 SEROLOGY

This technology is used for detecting antimalaria antibodies. A positive test indicates a past infection; hence it is not useful for diagnosing acute infections. The reason is that, detectable levels of antimalaria antibodies do not appear until weeks into infection and persist long after parasitaemia has been resolved (Draper and Sirr, 1980).

2.3.1 CHEMOTHERAPY AND CHEMOPROPHYLAXIS OF MALARIA

Anti-malaria drugs are the agents used to inhibit the development of malaria parasites. They are administered with the aim of totally eradicating the parasites. However, other therapeutic measures may be necessary during severe malaria attack (WHO, 1984).

Antimalarial drugs have selective action on the different phases of the parasite life cycle as follows:

a) Drugs used to treat the acute attack of malaria (i.e. for suppressive or clinical cure) act on the parasites in the blood; they can cure infections with parasites (e.g. *P. falciparum)* that have no exoerythrocytic stage.
b) Drugs used for chemoprophylaxis causally called prophylactics act on merozoites emerging from liver cells.
c) Drugs used for radical cure are active against parasites in the liver.

d) Some drugs act on gametocytes and prevent transmission by the mosquito (Bruce-Chwatt et al, 1993).

2.3.2 USES OF ANTIMALARIA DRUGS:

A drug may be put to several uses. The efficacy of a drug may be determined by several factors. The factors may be: the species of malaria parasite concerned; sensitivity of the drug, the presence of partial immunity in the human host; the risks of toxic effects, as well as availability, preference acceptability to the patient and cost (WHO, 1984). The main uses are:

a) Protection (prophylaxis)

b) Cure (therapy)

c) Prevention of transmission.

2.3.2.1 ANTIMALARIAL DRUG RESISTANCE

Antimalarial drug resistance has been defined as the "ability of the parasite strain to survive and multiply despite the administration and absorption of a given drug in doses equal to or higher than those usually recommended but within tolerance of the subject". This definition was later modified to specify that the drug in question must gain access to the parasite or the infected RBC for the duration of the time necessary for its normal action" (WHO, 1996).

In general, resistance appears to occur through spontaneous mutations that confer reduced sensitivity to a given drug or class of drugs. For some drugs only a single point mutation is required to confer resistance, while for other drugs multiple mutations are required. Provided the mutations are not deleterious to the survival or reproduction of the parasite, drug pressure will remove susceptible parasites while resistant parasites survive (White,

1997).

While drug resistance can cause treatment failure, not all treatment failure is due to drug resistance. Many factors can contribute to treatment failure including dosing, non-compliance with duration of dosing regimen, poor drug quality, drug interaction, poor or erratic absorption and misdiagnosis (Foley and Tilley, 1997). Probably all of these factors, while causing treatment failure in the individual, may also contribute to the development and intensification of true drug resistance (Wernsdorfer, 1994). *P. falciparum* has developed resistance to nearly all antimalarials in current use, although the geographical distribution of resistance to any single antimalarial drug varies greatly. *P. vivax* infection acquired in some areas has been shown to be resistant to chloroquine and/or primaquine (Murphy *et al*., 1993).

Chloroquine-resistant *P. falciparum* malaria has been described everywhere including some areas of Central America (north-west of the Panama Canal), the Island of Hispaniola, Middle East and Central Asia (Looareesuwan *et al*, 1996). Sulfadoxine-pyrimethamine (SP) resistance occurs frequently in South East Asia and South America. Sulfadoxine-pyrimethamine resistance is becoming more prevalent in Africa as the drug being relied upon as a replacement for chloroquine. Mefloquine resistance is frequent in some areas of South-East Asia and has been

reported in the Amazon region of South America and sporadically in Africa (Mockenhaupt, 1995). Cross-resistance between halofantrine and mefloquine is suggested by reduced response to halofantrine when used to treat mefloquine failures (Brasseu et al,1992).

2.4 ANTIMALARIAL DRUGS

The major antimalarial drugs are

(1) Quinine

(2) Chloroquine

(3) Mefloquine

(4) Pyrimethamine

(5) Chloroguide

(6) Amodiaquine

(7) Artemisinin

(8) Combination of sulphadoxine-pyrimethamine with trade name as fansidar.

2.4.1. QUININE (Qn)

Quinine was extracted from the bark of the South American cinchona tree, isolated and named in 1820 by French researchers Pierre Joseph Pelletier and Joseph Caventou. The name was derived from the original Quechua (Native American) word for the cinchona tree bark, *"Quina*" or

"*Quina-Quina*", which roughly means "bark of bark" or "holly bark". Prior to 1820, the bark was first dried, ground to a fine powder, and then mixed into a liquid (commonly wine) before being drunk (Barennes *et al*., 1996a).

For more than three centuries, quinine was the only effective drug towards relief of malaria. However, in recent times some new drugs have been developed which are less toxic, hence quinine is not frequently prescribed (Barennes *et al*., 1996a).

Cinchona trees remain the only practical source of quinine. However, under wartime pressure, research towards its artificial production was undertaken. R.B. Woodward and W.E. Doering, American chemists accomplished a formal chemical synthesis in 1944. Since then, several more efficient total syntheses have been achieved (Pukrittayakamee, et al,2003).

2.4.1.1 THE CHEMISTRY OF QUININE (Qn)

Cinchona contains a mixture of more than twenty alkaloids. Out of these, there are four important ones, which are quinine, quinidine, cinchonidine and cinchonine. Quinine is natural white crystalline alkaloid having antipyretic, anti-malarial with analgesic and anti-inflammatory and a bitter taste. It is a stereoisomer of quinidine. Quinine is also used to treat nocturnal leg cramps and arthritis and has also been used to treat people who had been infected by prions (Pukrittayakamee, et al,2003).

The structural formula of Qn is given as below:

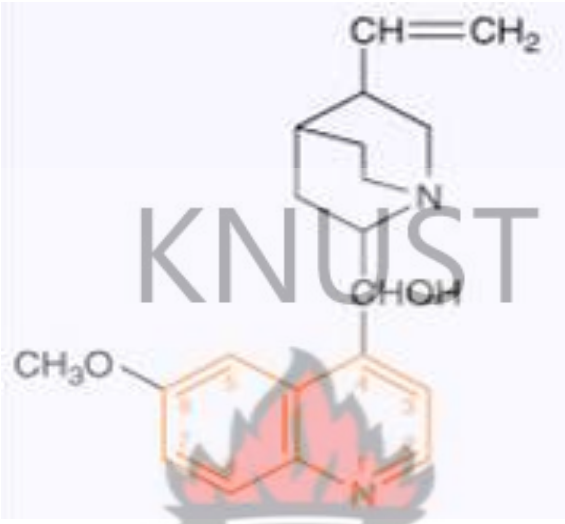

Fig 2.2 Chemical structure of quinine

The systematic (IUPAC) name of quinine is (2-ethyl-4-azabicyclo [2.2.2] oct-5-yl) - (6-methoxyquinolin-4-yl)-methanol. Quinine has empirical formula as $C_{20}H_{24}N_2O_2$ with formula weight of 324.42 g/mol.

Pharmacological action of quinine indicates that the methoxy- and the vinyl- group have no antimalarial activity, rather its potency depends on secondary alcohol – group. Reduction of this group increases the toxicity and reduces the antiplasmodial potency.

2.4.1.2 PHARMACOKINETICS OF QUININE

Oral systemic availability of quinine exceeds 80% of what has been taken by both healthy subjects and uncomplicated malaria patients. The total apparent volume of distribution in healthy subjects is approximately 2L/Kg and its systemic clearance. The predominant urinary metabolites of quinine are 2- and 3- hydroxylated alkaloids (White et al,1982).

Plasma quinine concentrations usually reach a peak on the third day of treatment and fall as the patient recovers. Plasma protein binding on quinine increases from about 80% in healthy subjects to over 90% in severe malaria (White et al,1982). This large increase is the result of increased plasma concentration of $\propto_1$-acid glycoproteins.

Plasma quinine concentrations between 8 and 15 mg/L are clinically effective in severe *P. falciparum* and unlikely to reach toxic levels. Quinine is not concentrated within the erythrocytes unlike the synthetic quinoline antimalarials (White *et al*, 1982).

Mechanism of quinine action is not clear but it is believed that it reduces the ability of malaria parasites feeding within the RBC. Quinine is effective in both suppressing and treating malaria attacks. In addition, it is very useful for *P. falciparum*, ,(e.g. where chloroquine resistance exists). In severe cerebral malaria, parenteral quinine dihydrochloride is the drug of choice and in the case of acute renal failure (ARF) the dosage should be reduced to avoid toxic plasma concentration (White et al,1982).

Quinine is rapidly and completely absorbed from gastrointestinal tract (GIT) with plasma protein binding about 80% in healthy subjects. Quinine is extensively metabolised in the liver and excreted rapidly in urine.

2.4.1.3 ADVERSE EFFECT OF QUININE

Quinine produces a characteristic symptoms complex when plasma concentrations exceed 15 mg/ (Islahudin et l, 2012). The symptoms are called "cinchonism". These include nausea, vomiting, diarrhoea, blurred vision, headache and impaired hearing. Hypoglycaemia is the most important adverse effect of quinine in therapeutic doses. Studiies have indicated that quinine-induced hyperinsulineaemia is one of several factors contributing to the pathogenesis of hypoglycaemia in severe *P. falciparum* (White et al,1982).

2.4.2 CHLOROQUINE (Cq)

Chloroquine belongs to 4-aminoquinoline class of antimalarial drugs and it is still the most important antimalarial drugs in the world and it has marked and rapid schizonticidal activity against all infections of *P. malariae* and *P. ovale* and against chloroquine-sensitive infections of *P. falciparum and P. vivax* (Bloland et al, 1993).

The use of chloroquine as a single first-line drug treatment is now increasingly limited following the evolution of chloroquine-resistant *P. falciparum,* but chloroquine remains the first-line drug of choice in most African countries south of the Sahara where acceptable clinical cure rates can

be obtained (Bloland *et al*, 1993). In areas where it is still used as a first-line drug, persistent parasitemia and lack of haematological recovery in children may be one of the early signs of chloroquine resistance. Even if the frequency of clinical failures is acceptable in the general population, a more effective first-line treatment may be required for vulnerable groups such as young children and pregnant women (Bloland *et al*, 1993).

2.4.2.1 CHEMISTRY OF CHLOROQUININE

Chloroquine has the chemical structure as:

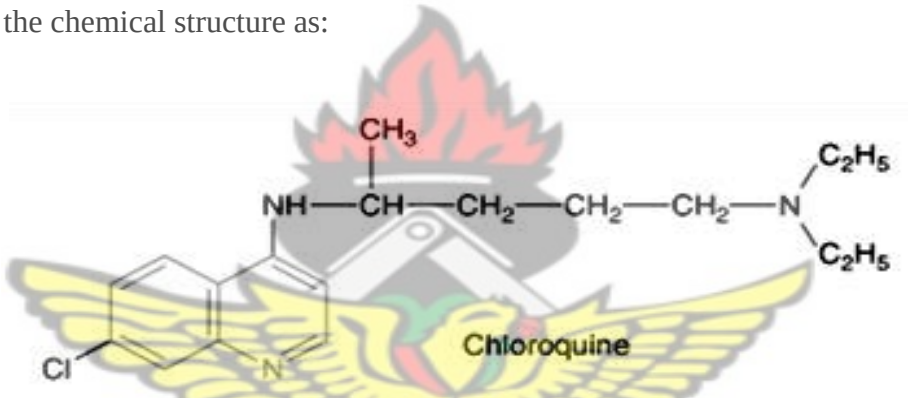

Fig 2.3 Chemical structure of chloroquine

The IUPAC name of Chloroquine is N-(7-chloroquinolin-4-yl)–N, N-diethyl- pentane –1, 4-diamine. Its empirical formula is given as: $C_{18}H_{26}ClN_3$ with molecular mass of 319.9 g/mol. Chloroquine bears close resemblance to pamaquine and pentaquine, which belong to 8-aminoquinoline antimalarials. The antimalarial activity of chloroquine depends on the chlorine-atom in position 7 of the quinoline nucleus. The D- and L- forms of chloroquine are indistinguishable in potency tests but the D- isomer is less toxic than the L-isomer.

2.4.2.2 PHARMACOKINETICS OF CHLOROQUINE

Chloroquine is widely available, cheap, well tolerated, well absorbed by G.I.T and effective with malaria treatment except completed ones (Bergqvist et al, 1983).

Whole blood concentrations are 2-3 times higher than plasma concentrations because chloroquine is concentrated in erythrocytes, platelets and granulocytes. Thus, serum concentration of chloroquine is higher and less reliable than plasma concentrations. Between 50% - 65% chloroquine is bound to plasma protein (Bergqvist *et al*, 1983).

Due to extensive tissue binding and terminal elimination half-life is estimated to be 1-2 months The renal clearance of chloroquine is approximately 5 ml/kg/min which is about half of the total systemic clearance (Frisk-Hokmberg *et al*, 1984).

The pharmacokinetic properties of chloroquine are similar in children and adults unlike quinine. Chloroquine has also been used in the treatment of rheumatoid arthritis but has been discontinued because of its irreversible retinopathy ((Frisk-Hokmberg *et al*, 1984).

2.4.2.3 ADVERSE EFFECTS OF CHLOROQUINE

Generally, chloroquine is well tolerated except few mild symptoms like nausea, dysphoria skin rashes and diarrhoea could occur. With long-term use, either as an antimalarial prophylatic or in

the treatment of rheumatoid arthritis characteristic retinopathy may develop (Bloland e*t al*, 1993).

Chloroquine given rapidly by intravenous route can cause an abrupt fall in blood pressure. Hence intravenous administration of chloroquine should be done slowly in saline drip. Pruritus, blurring of vision and headache are some other toxic effects of chloroquine, but these symptoms disappear soon after the administration is stopped (Verdrager, 1995).

2.4.3 AMODIAQUINE

Chloroquine and amodiaquine are both 4-aminoquinolines and the cross-resistance between these two drugs is well known (Basco, 1991). Structurally, amodiaquine is related to chloroquine and appears to retain some activities against chloroquine -resistant strains of *P. falciparum*. However, this advantage is short lived as one of its metabolite, quinonlimine produces toxic hepatitis and a risk of severe neutropenia of 1 in 2500 (Hatton *et al*, 1986).

2.4.3.1 CHEMISTRY OF AMODIAQUINE

Amodiaquine differ from chloroquine structurally in side chain. While chloroquine has akyl group as the side chain, amodiaquine has aryl group.

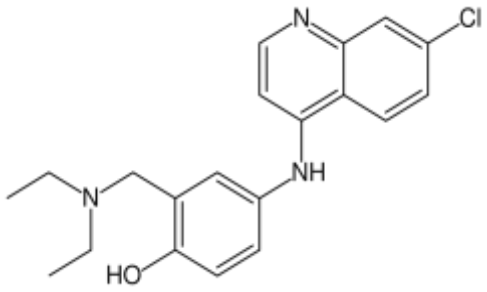

Fig 2.4. Chemical structure of amodiaquine

The chemical name of amodiaquine is 7–chloro-4-(3/-diethylaminomethyl-4/ hydroxyamilino) quinoline. It has empirical formula $C_{20}H_{22}ClN_3$ with molecular mass of 339.5 g/mol

2.4.3.2 PHARMACOKINETICS OF AMODIAQUINE

Amodiaquine is well absorbed by GIT, but can be considered as pro-drug for its active metabolite, monodesethylamodiaquine (Churchill *et al,* 1985). Amodiaquine is used for overt and suppressive malaria.

Amodiaquine has elimination half-life period of about ten hours It has slower terminal elimination period. Both amodiaquine and its metabolite have been detected in urine for months after a single dose(Orell *et al,* 2008).

2.4.3.3 ADVERSE EFFECTS OF AMODIAQUINE

Symptoms most commonly encounter with amodiaquine are diarrhoea, vomiting and vertigo. Long-term use of amodiaquine carries the risk of retinal toxicity (Hatton *et al*, 1986). In severe cases, it is associated with hepatitis (Hatton *et al*, 1986).

2.4.4 CHLOROGUANIDE (Cg)

Another name for chloroguanide is proguanil. It gained a reputation as safe and well tolerated antimalaria drug. The active metabolite, cycloguanil acts on both pre-erythrocytic and erythrocytic stages of the malaria parasite (White, 1997).

2.4.4.1 CHEMISTRY OF PROGUANIL/CHLOROGUANIDE

More popularly known as proguanil, chloroguanide is a white powder with a bitter taste. Its empirical formula is $C_{11}H_{16}ClN_5$ and a molar mass of 253.5g/mol. The IUPAC name of chloroguanide is 1-(p-chloropheny)-5- isopropylbiguanide.

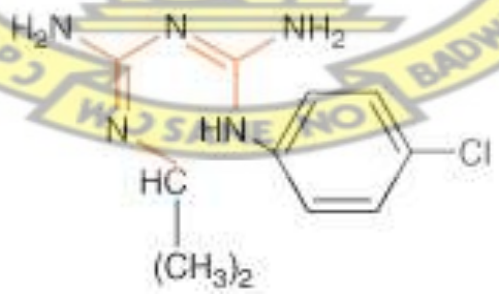

Fig 2.5 The chemical structure of chloroguanide

2.4.4.2 PHARMACOKINETICS OF CHLOROGUANIDE

Chloroguanide is well absorbed in man and its elimination is fairly slow and mainly in the urine. Peak plasma concentration is attained about 5 hours of oral administration (Helsby et al, 1990) It is widely prescribed in combination with chloroquine in the ratio of 200mg : 300mg per day per week as causal prophylactic (Miller et al, 2009).

2.4.4.3 ADVERSE EFFECTS OF CHLOROGUANIDE

At prophylactic dosage, the toxicity of chloroguanide is very low. Some of these are abdominal discomfort, loss of appetite, vomiting and diarrhoea (Miller et al, 2009).

2.4.5 PYRIMETHAMINE (PM)

Pyrimethamine is similar to chloroguanide in structure. Both has chlorophenyl ring. It has pyrimidine ring in addition to the chlorophenyl ring while chloroguanide has biguanide. Both Chloroguanide and pyrimethamine have the same action on malaria parasite as reductase inhibitors.

2.4.5.1 CHEMISTRY OF PYRIMETHAMINE

The chemical name of Pyrimethamine is 2,4-diamino-5-p-chlorophenyl-6-ethylpyrimidine. Pyrimethamine has synergetic ability with sulphones. Pyrimethamine has empirical formula as $C_{12}H_{13}ClN_4$ and molar mass of 248.5g/mol.

The structure of Pyrimethamine is given as:

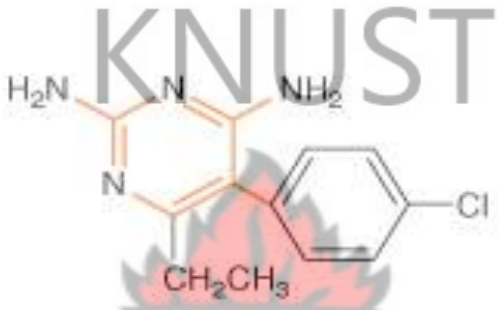

Fig 2.6 Structure of Pyrimethamine

2.4.5.2 PHARMACOKINETIC OF PYRIMETHAMINE

Pyrimethamine is well absorbed with plasma peak concentration reaching 12 hours after administration. This then fall slowly with elimination half-life of 80-95 hours (Dunyo et al, 2006).

In the treatment of malaria, the serum concentration after 50mg oral dose ranged between 300 and 600ng/l. Recent bioassay studies indicate that the plasma level required to inhibit blood schizogony of drug-sensitive *P. falciparum* is between 10µg /L and 100µg/L. A level of 1000µg/L is required for drug-resistant *P. falciparum* (Dunyo et al, 2006).

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2.4.5.3 ADVERSE EFFECTS OF PYRIMETHAMINE

At the recommended dosage of 25 mg/day of pyrimethamine, the toxicity is very low, but for long-term administration, megaloblastic type of anaemia may result. (Phillips et al, 1995). However, remission is rapid when the drug is discontinued.

2.4.6 MEFLOQUINE (MFQ)

Mefloquine is highly active against multidrug resistant strains of *P. falciparum* (Schlagenhauf *et al*, 1996). In order to protect this drug from the development of resistant, mefloquine has been combined with both sulphadoxine and pyrimethamine into a single dose formula.

Mefloquine is a quinoline antimalarial drug and it is structurally similar to the antiarrlythmic agent quinidine. Mefloquine is widely used in both the treatment and prophylaxis of *P. falciparum.* It can also prolong cardiac repolarisation especially when co-administered with halofantrine (Schlagenhauf *et al*, 1996).

2.4.6.1 CHEMISTRY OF MEFLOQUINE

Mefloquine belongs to the group of quinolinemethanols. It is similar to quinine and shows potent activity to schizontocides and effective against all malaria parasites including *P. falciparum* chloroquinine-resistant (Croft and Garner, 2001).

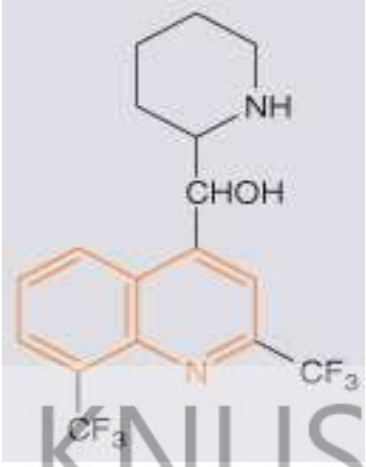

Fig 2.7 Structure of Mefloquine

The chemical name of mefloquine is α-(2-piperidyl)-2, 8-bis (triflomethyl-4-quinolinemethanol).

2.4.6.2 PHARMACOKINETIC OF MEFLOQUINE

Mefloquine is well absorbed. Peak plasma concentrations are achieved between 11-48 hours while its elimination half-life takes about 3 weeks (Karbwang *et al*, 1987a). Mefloquine has proved very effective as a first line treatment of *P. falciparum*. One advantage of mefloquine is its single dosage of 15-25mg/kg body weight in adults and children. It is also effective in single dose of 1.5g against multidrug-resistant strains and as prophylaxis when taken at 250mg/week (Nosten et al, 2000).

2.4.6.3 ADVERSE EFFECTS OF MEFLOQUINE

Mefloquine when given orally in single doses up to 1.5g or in 500mg doses each week is generally tolerated. Side effects such as nausea, vomiting abdominal pains and dizziness are dose

related. Mefloquine is relatively new antimalarial drug which has been associated with a wide variety of adverse effects, including skin reactions (Price *et al*, 2004).

2.4.7 ARTEMISININ

Artemisinin and its derivatives were first discovered, tested and marketed in China. It produces more and rapid resolutions of fever and parasitaemia than all known antimalarial agents (Olliaro *et al*, 1995). Due to these remarkable properties, there are concerns that their uncontrolled and widespread use, particularly as oral formulations, will result in the rapid development of acquired resistance. These concerns were reflected in the recommendation made by WHO Informal Consultations in 1993 and 1995 (WHO, 2001).

Artemisinin, artemether and artesunate, have been used in China for the treatment of uncomplicated malaria for over 20 years and increasingly in the rest of South-East Asia for the last decade. Oral dihydroartemisinin has been introduced recently but there is considerable less data on this compound (Price et al, 1996).

In general, oral formulations of these drugs are rapidly but incompletely absorbed, and their bioavailability is low. There is good evidence that they undergo extensive first-pass metabolism in the liver. Both artesunate and artemether are rapidly transformed into dihydroartemisinin so that the metabolite is generally present at higher levels than the parent compound (Batty et *al*, 1998). Although dihydroartemisinin is the most active derivative on a molar basis, each of the

parent drugs is active in low concentrations (nM) range and is known to achieve levels much higher than the minimum inhibitory concentrations in the plasma. In spite of the rapid clearance and extent of transformation, the parent drugs may contribute a significant proportion of the antimalarial effect in the blood (Batty et al, 1998).

There is some evidence that severe malaria may alter the kinetics of artesunate but further studies are required to confirm this observation and determine whether it has clinically relevant therapeutic consequences (German et al, 2008).

2.4.7.1 CHEMISTRY OF ARTEMISININ

The chemical formula for artesunate is $C_{19}H_{28}O_8$ with a molar mass of 384.421 g/mol.

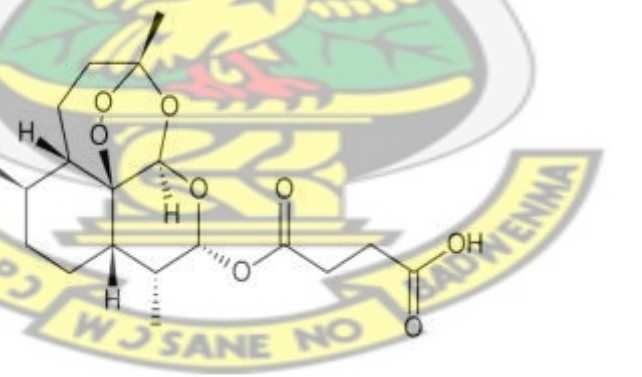

Fig. 2.8 Structure of Artesunate

The chemical formula for artemisinin is $C_{15}H_{22}O_5$ with a molar mass of 282.332g/mol. The systematic name is yl-3,12-epoxy-12*H*-pyrano[4,3-*j*]-1,2-benzodioxepin-10(3*H*)-one

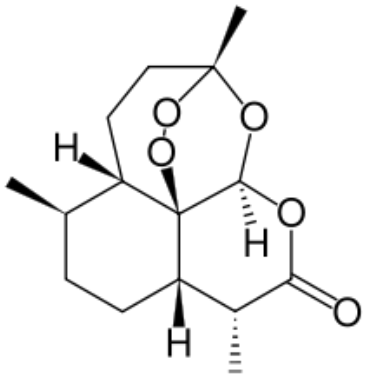

Fig. 2.9 Structure of Artemisinin

2.4.7.2 PHARMACOKINETICS OF ARTEMISININ

No pharmacological interactions with other drugs have been identified although there is a theoretical risk that a pharmacodynamic interaction with desferoxamine might attenuate antimalarial activity. All artemisinin drugs prevent the development of ring stage parasites to the more mature pathogenic stages that rosette and cytoadhere in the capillaries (Nealon *et al*, 2002).

Despite the limitations of many of the reported clinical studies, consistent observation have shown that artemisinin derivatives produce faster relief of clinical symptoms and clearance of parasites from the blood than other antimalarial drugs (Li et al, 2009). In around 90% of the patients given these drugs, the fever resolved and the parasitaemia cleared within 48 hours of treatment. It has been estimated that they reduce the parasite biomass by a factor of approximately 104 for each 36–48 hour asexual cycle of the parasite and by a factor of 106-108 over a 3 day course of treatment (Li et al, 2009).

2.4.7.3 ADVERSE EFFECT OF ARTEMISININ DRUGS

The most common adverse effects reported following the use of artemisinin drugs are headache, nausea, abdominal pain, vomiting and occasional diarrhoea, symptoms that are associated with malaria and which resolve with appropriate treatment (Price et al, 1996).

2.4.7.4 RESISTANCE TO ARTEMISININ DRUGS

There has been no report of clinical resistance to the artemisinin drugs so far although artemisinin-resistant strains of *P. falciparum* have been developed in the laboratory. Clinical isolates and laboratory strains have been shown to vary in their sensitivities to the drug but there is no evidence that this is related to clinical failure (van Vugt *et al*, 1999).

The development of resistance depends in part on the pharmacokinetics and pharmacodynamic characteristics of drugs. Antimalarial drugs with long terminal half-lives are more particularly vulnerable to the development of resistance. There is good evidence that short half-life antimalarial drugs are less vulnerable to the development of resistance (Brockman et al, 2000).

Artemisinin and its derivatives have short half-lives and is the most potent and rapidly acting antimalarial drug known. They reduce the parasite biomass by around 10,000 fold for each asexual cycle and at present resistance to them have not been reported.

Combinations of artesunate and mefloquine and of artemether with benflumetol have both been shown to be highly active against multidrug resistance falciparum infections. There is evidence that the combination of artesunate and mefloquine may have played a role in both slowing down the development of resistance to mefloquine as well as reducing malaria transmission in an area of high mefloquine-resistance (Price *et al*, 2004).

2.4.8 ANTIFOLATE COMBINATION DRUGS

Proguanil, chlorprogual, pyrimethamine and trimethoprim are known as dihydrofolate-reduetase inhibitors while dapsone sulphalene, sulphametho-xazole, sulphadoxine etc. are all referred to as sulphur drugs. Although these drugs have antimalaria activity when used alone, parasitological resistance can develop rapidly (Bloland et al, 2000). When used in combination, they produce a synergistic effect on the parasite even in the presence of resistance to the individual components.

2.4.9 COMBINATION THERAPY WITH ANTIMALARIAL DRUGS

A strategy that has received much attention recently is the combination of antimalarial drugs. An example of this is mefloquine, fansidar or amodiaquine combinations with artemisinin derivatives (WHO, 2001).

The use of combination therapy has been linked to slowing of the development of mefloquine resistance and reduction in overall malaria transmission rates in some part of Thailand and has been recommended for widespread use in sub-Saharan Africa (WHO, 2001).

Generally, resistance appear to occur through spontaneous mutations that confer reduced sensitivity to a given drug or class of drugs. Provided the mutations are not deleterious to the survival or reproduction of the parasite, drug pressure will remove susceptible parasites while resistant parasite survives (Hastings and Donnelly, 2005).

2.4.10 MALARIA TREATMENT FAILURE

A distinction needs to be made between a failure to clear malaria parasitaemia or resolve clinical disease following a treatment with an antimalaria drug and a true antimalarial drug resistance. While drug resistance can cause treatment failure, not all treatment failure is due to drug resistance. Many factors can contribute to treatment failure including incorrect dosing, non-compliance with duration of dosing regimen, poor drug quality, drug interactions, poor erratic absorption, and misdiagnosis (Snow et al, 2003).

2.4.11 FACTORS CONTRIBUTING TO THE SPREAD OF DRUG RESISTANCE

Factors that have been associated with antimalarial drug resistance include human behaviour, vector and parasite biology, pharmacokinetics and economics. Conditions leading to malaria treatment failure may also contribute to the development of resistance (Wernsdorter, 1991).

Factors that decrease the effectiveness of the immune system in clearing parasite residuum after treatment also appear to increase survival of parasites, facilitate development and intensification

of resistance. This mechanism has been suggested as a significant contributor to resistance in South-East Asia, where parasites are repeatedly cycled through the populations on non-immune individuals The non-specific response of non-immune individuals is less effective at clearing parasite residuum than individual (Verdrager, 1995).

Some drug-resistant infections appear to provide a survival advantage or to facilitate the spread of resistance conferring genes in a population. In one study patients experiencing chloroquine treatment failure had recrudescent infections that tended to be less severe or even asymptomatic (Tulu et al, 1996).

2.5 CHROMATOGRAPHY

The most widely used means of performing analytical separations is chromatography, a method that finds application to all branches of science. Separation, identification and determination of a chemical composition are complex. Chromatography has progressed considerably to include a number of variations on the basic separation process. No other separation method is as powerful and as generally applicable as is chromatography, (IUPAC, Nomenclature for Chromatography 1993).

The term chromatography comes from the Greek words *chroma*, meaning "colour" and *graphein*, meaning "to write". Chromatography is generally applied to a wide variety of separation techniques based upon sample portioning between a moving or mobile phase which

can be a gas or liquid and a stationary phase which may be a liquid or solid. By classical definition, chromatography is a separation process that is achieved by distributing the substances to be separated between a mobile phase and a stationary phase. As a consequence the substances are eluted from the column in inverse order of their distribution coefficients with respect to the stationary phase (Laurence et al, 13 June 1989). The discovery of chromatography is generally credited to Russian botanist, Tswett who described his work in the separation of pigment in green leaves (Still et al, 1978).

For maximum or better separation in chromatography, i.e. dynamic equilibrium distribution of compounds in mobile phase and the stationary phase need to be established. Those compounds preferentially distributed in mobile phase would pass quickly through the system while those preferentially distributed in stationary phase would elute later.

Chromatography languished for many years until late 1938 before thin-layer chromatography (TLC) was discovered by Izmailor and Schraiber and was later refined by Martin and Sybger who won the Nobel Prize (Still et al, 1978).

The TLC gave birth to Gas Chromatography (GC) in 1952 and also evolved into more sophisticated analytical tool which later developed into High Performance Liquid Chromatography (HPLC) which can be used to separate non-volatile and polymeric materials including biological substances. Chromatographic processes can be classified according to the type of equilibration process involved. Various bases of equilibration are (1) adsorption, (2) partition, (3) ion exchange, and (4) affinity chromatography (Still et al, 1978).

2.5.1 SOME EXAMPLES OF CHROMATOGRAPHY

2.5.1.1 LIQUID – SOLID CHROMATOGRAPHY (LSC)

LSC is also called adsorption chromatography. This technique depends upon adsorption of a solute on a polar absorbent such as silica gel or alumina. TLC is a form of LSC, which is generally applicable to solutes that are soluble in organic solvents and do not ionize. It is particularly powerful for the separation of isomers (Laurence et al, 1989). In TLC, the stationary phase is a solid on which the sample components are adsorbed. The mobile phase may be a liquid (liquid-solid chromatography). The components distribute between the two phases through a combination of adsorption and desorption processes. TLC is a special example of adsorption chromatography in which the stationary phase is in a form of a solid supported on an inert plate.

2.5.1.2 ION-EXCHANGE CHROMATOGRAPHY

This technique was first used by Taylor and Urey in 1938 to separate lithium (Li) and potassium (K) isotopes using zeolite resins (Laurence et al, 1989). Ion-exchange chromatography is a method where an electrolyte is brought into contact with an ion-exchange resin and the active ions on the resins are replaced by ions of similar charges from the analyte solution.

Ion exchange chromatography uses an ion exchange resin as the stationary phase. The mechanism of separation is based on ion-exchange equilibrium. Ion-exchange chromatography is the method of choice for the analysis of inorganic ions and it is often preferable to reversed-phase methods for the analysis of small organic ions.

2.5.1.3 GAS CHROMATOGRAPHY (GC)

This newest and most selective kind of chromatography utilizes highly specific interactions between one kind of solute molecule and a second molecule, covalently attached (immobilized) to the stationary phase. For example, the immobilized molecule might be an antibody to a particular protein. When a crude mixture containing a thousand proteins is passed through the column, only the protein that reacts with the antibody is bound to the column. After washing all the other solutes off the column, the desired protein is dislodged from the antibody by changing the pH or ionic strength. This is the principle upon which gas chromatography works (IUPAC, Nomenclature for Chromatography, 1993).

It was estimated that 10-20% of the known compounds can be analyzed by GC, and the analyte should have sufficient volatility and thermal stability. The suggestion to use a gas as the mobile phase was made by Martin and Synge in but it was not implemented until the work of James and Martin on gas-liquid chromatography and the work of Cremer, Pilor, Cremer and Muller on gas-solid chromatography (IUPAC, Nomenclature for Chromatography, 1993). Since then, gas chromatography has developed rapidly, particularly during the 1960s and the use of the technique has been applied in almost every area of analytical and biochemical research.

The introduction of chemically bonded fused silica capillary columns was a very recent innovation in gas chromatography, more commonly referred to as high-resolution gas chromatography (HRGC). The carrier gas serves as the mobile phase that elutes the components

of a mixture from a column containing an immobilized stationary phase. In contrast to most other types of chromatography, the mobile phase does not interact with the analyte molecules.

The mobile phase of GC include carrier gases viz; helium, argon and nitrogen which are chemically inert. The stationary phase in gas-solid chromatography is a solid that has a large surface area at which adsorption of the analyte species (solutes) take place. In gas-liquid chromatography, a stationary phase is liquid that is immobilized on the surface of a solid support by adsorption or by chemical bonding. In GC the distribution ratio is dependent on the component vapour pressure, the thermodynamic properties of the bulk component band and affinity for the stationary phase. The equilibrium is temperature dependent hence, the importance of selecting the stationary phase of the column and column temperature programming in optimizing separation.

2.5.1.4 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

For many years, column chromatography with liquid mobile phase, which moves under gravity or by capillary action, has been known. The relative high viscosity of liquid spread by diffusion which leads to poor resolution and low sensitivity often results in slow flow rates and long elution time.

It has been recognized that resolution and sensitivity could be improved if the eluent is pumped through the column at high pressure. With the use of specially designed pump(s), suitable

column(s) and fittings much success has been achieved and this analytical technique is termed as HPLC (Pascal et al, 2000).

The goal of a successful chromatographic analysis is to separate a sample (blood, urine, solutions etc) into its individual components in order to evaluate each component free from interference of the other components. The separation involves making a compromise between resolution, column capacity and analysis time or speed. HPLC is used to analyze liquid samples or the liquid extract of a sample.

The HPLC consists of analyte mixture in a high-pressure solvent (called the mobile phase) through a steel tube, the column packed with sorbents called the stationary phase. As the analytes pass through the column they interact between the two phases mobile and stationary at different rates. The difference in rates is primarily due to different polarities for the analytes. The analytes that have the least amount of interaction with the stationary phase or the most amount of interaction with the mobile phase will exit the column faster. Repeated interactions along the length of the column effect the separation of the analytes. Various mixtures of analytes can be analyzed by changing the polarities of the stationary phase and the mobile phase, (Pascal et al, 2000).

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There are many types of columns on the market today that can help refine your HPLC method. Choosing the right column is essential in obtaining good HPLC results. Obviously, the polarity of the stationary phase can be altered significantly. The stationary phase is typically bonded to a support phase, usually consisting of porous beads. The pore sizes can be varied to allow certain

sized analytes to pass through at different rates. Furthermore, the dimensions of the column can be varied to allow different sample sizes to be analyzed.

A change in the polarity of the mobile phase is another variable that can affect the efficiency of HPLC separation. The mobile phase polarity is generally the opposite of stationary phase. A multi-solvent delivery system allows the polarity of the mobile phase to be changed during the course of the HPLC run. The rate at which the polarity is changed defines the "gradient". This gradient technique helps to further separate mixtures of various polar analytes (Pascal et al, 2000).

As the analytes exit the column, they can be detected by various means. Some of these are:

refractive index, electrochemical, or ultraviolet-absorbance, changes. The amount of analyte leaving the column will determine the intensity of the signal produced in the detector. The detector measures a signal peak as each analyte leaves the column. By comparing the time, called the retention time, for a peak to show up with the retention time of known analyte, the components of unknown sample mixtures can be identified. In addition, by measuring the signal intensity (response) and comparing it to the response of a known amount of that particular analyte, the amount of the unknown analyte in the mixture can also be estimated.

The most popular detector is the photodiode array detector. The photodiode array can continuously scan at various wavelengths of the UV spectrum. As an analyte peak is detected,

the UV spectrum is recorded. This third dimension is useful in identifying compounds and determining the peak consisting of an individual analyte or a mixture of analytes that has not effectively separated.

A computer with Windows-based software can control all aspects of an HPLC system. This software controls an auto sampler, which injects samples at proper intervals. It controls the mobile phase gradient, the solvent flow rate, mobile phase pressure, and it measures the signals produced by the detector. The results of these can then be interpreted and printed in a variety of report formats.

HPLC is usually suited to monitoring drug preparations for quality and purity particularly compounds that are difficult to analyse by GLC due to their thermal instability or poor volatility. As a result of these, HPLC applications are used in most quality control sections of pharmaceutical firms (Pascal et al, 2000).

2.5.2.1 OPTIMUM PERFORMANCE OF HPLC

Peaks emerging from a chromatography column should have a Gaussian shape. Deviation from this suggests that the chromatography conditions are not optimum (Staiger et al, 1981). Usually, the primary cause of tailing is adsorption of analyte to a few highly active binding sites. This effect is greatest for molecules containing highly polar or ionizable groups. It can be reduced or eliminated in HPLC by inclusion of small amount of polar or ionic compounds in the eluent. A

second cause could be the partial ionization of molecules. This can be suppressed by the addition of a trace of acid or base to the mobile phase.

2.5.2.2 RESOLUTION

It is a term used to describe the degree of separation of successive solute bands or peaks. For Gaussian peaks, the separation is given by the ratio of the separation of the peaks maximum to the broadness of the peaks. Resolution depends on two factors, these are the narrowness of the peak and the distance between the peak and maximum (Staiger et al, 1981).

2.5.2.3 COLUMN SELECTIVITY

Column selectivity is a function of the thermodynamics of the exchange process. Another method of improving the resolution in an HPLC is to change the selectivity of the column. Changing the stationary phase could do this and or mobile phase. The efficiency, of course, is a function of the kinetics of the exchange. Column selectivity could be improved by changing the distribution coefficient. Increasing polarity pH and/or ionic strength of mobile phase can also alter the distribution coefficient (Staiger et al, 1981).

In practice, it is seldom necessary to calculate the value of the resolution. From inspection of the peak shapes, one can generally estimate the resolution to a sufficient degree to quickly select the length of a column required for the separation. Generally, the resolution value of 1.0 or greater is for good quantitative or qualitative work (Staiger et al, 1981).

It is possible to improve the resolution by changing the column selectivity, but, if the efficiency is constant, increasing the resolution usually involves longer elution times for a particular system. For example, column selectivity is governed by the thermodynamics of the interaction of the solute with the solvent and the column packing. Increasing the relative amounts of stationary phase will increase resolution at the expense of increased elution time.

2.5.2.4 REVERSE PHASE

This term tends to confuse some people especially novice chromatographers. In reverse phase chromatography, the stationary phase is non-polar while the mobile phase is polar. This means that in this technique, the mobile phase is more polar than the stationary phase hence the term reverse phase (Gitau et al, 2004).

In this method the selectivity is often significantly different from that achieved in normal-phase separations. The elution order is generally related to the increasing hydrophobic nature of the solute. This means the more soluble a solute is in water, the faster it will elute (Snyder et al, 1997).

2.5.2.5 RETENTION CHARACTERISTICS

The overall partition and retention may be related to thermodynamic and kinetics of the separation processes. The volume of the mobile phase required to carry the component solute molecules through the system is termed as retention volume (VR) maximum. This retention volume is frequently recorded as retention time (RT). Partition rate is important quantity in

column chromatography. It relates the equilibrium distribution of the sample in the column to the thermodynamic properties of the column.

2.5.2.6 USE OF PEAK HEIGHTS OR PEAK AREAS

Many schools of thought wonder on which of these techniques is more useful. Both have their uses and selection depends on the type of analysis being performed. In general, peak height is less dependent on flow rate than peak area. Peak broadening can be depended upon neighbouring peaks and thus the use of peak heights for quantification is recommended. Peak areas are less dependent upon the operator and instrument variation (Gitau et al, 2004; Snyder et al, 1997).

2.5.2.7 DETECTORS

The past 30 years has seen massive improvements in the evolution of detection principles in liquid chromatography and today most HPLC analyses are monitored by one of four detection principles: UV absorption, fluorescence, electrical conductivity and refractive index (Jinno, 2002). Typically, a detector consist of two parts: the sensor determines the overall performance of the detector and associated electronics which usually serves as a signal modifier. They are often modified spectrophotometers equipped with a small flow cell that monitors analyte concentration. Detectors are usually classified under one of two broad categories. Bulk property detectors usually measure some bulk property of eluents exiting the column like refractive index and conductivity include the refractive index and electrical conductivity detectors respectively. One the other hand, the solute property detector measures some physical

or chemical property of the solute itself which is expected to be independent of the MP (Kar, 2005). Examples under the solute property detectors category include the UV and the fluorescence detectors.

An ideal detector must exhibit among others, certain desirable characteristics as listed below (McPolin, 2009):

- Analyte detection in the mobile phase either by bulk property detection mechanisms or by solute property mechanisms (specificity and selectivity).
- A good linearity where analyte concentration is proportional to output signal.
- Detection of analyte even in trace amounts.
- Output signal unaffected by factors like temperature and composition of MP.
- Low noise levels

CHAPTER THREE

METHODOLOGY

The main aim of this study was to develop an innovative method of extraction and separation of antimalarial drugs from plasma. This requires the development of an efficient and reproducible method. The method of extractability should be in the neighborhood of 65% (Wu, 2009). Based on this, the following hypotheses were put forward:

1) There will be no difference between the recovery values of the aqueous (control) and the plasma extracts.
2) The recovery means of aqueous and the expected values should be the same.
3) There will be no difference between the recovery values of the plasma and the expected extraction.

3.1.1 METHOD VALIDATION

Method validation provides assurance of reliability during normal use and is sometimes described as the process of providing documented evidence that the method does and what it is intended to do. The specifications under method validation are referred to as "analytical performance parameters" or "analytical figures of merit". Most of these terms are familiar and are used daily; however, some may mean different things to different people hence their elucidation below (Himanshu et al, 2004).

3.1.1.1 ACCURACY

It is the measure of exactness of an analytical method, or the closeness of agreement between the measured value and the value that is accepted either as a conventional, true value or an accepted reference value. Accuracy is measured as the percentage of analyte recovered by assay, by spiking samples in a blind study (Souppart et al, (2002).

3.1.1.2 PRECISION

Precision is the measure of the degree of repeatability of an analytical method under normal operation and is normally expressed as the percent relative standard deviation for a statistically significant number samples (Souppart et al, 2002).

According to International Conference on Harmonization (ICH) or United States Pharmacopeia (USP), precision should be determined from a minimum of nine determinations covering the specified range of the procedure or from a minimum of six determinations at 100% of the test or target concentration.

3.1.1.3 SPECIFICITY

This is the ability to measure accurately and specifically the analyte of interest in the presence of other components that may be expected to be present in the sample matrix.

It is a measure of the degree of interference from such things as other active ingredients, excipients, impurities, and degradation products, ensuring that a peak response is due to only to a single component (Souppart et al, 2002).

3.1.1.4 LIMIT OF DETECTION (LOD)

Limit of detection is defined as the lowest concentration of an analyte in a sample that can be detected, though not necessarily quantitated. It is expressed as a concentration at a specified signal-to-noise ratio. Limit of detection may be calculated based on the standard deviation, (SD) of the response and the slope (S) of the calibration curve. Hence,

LOD=3.3(SD/S) (US Department of Health Services, 2001).

3.1.2.1 THE ZERO BLIND METHOD

This involves a single analyst using samples of known levels of analyte and at the end of the test recovery; accuracy and precision can be demonstrated. This method is general, fast, simple and useful but looks bias to unbiased reviewer of the end result user (Daston, 2005).

3.1.2.2 THE SINGLE BLIND METHOD

The Single blind method involves one analyst preparing samples at varying levels, which are unknown to a second analyst who analyses the samples. This approach looks unbiased at the start but invites biasness when the first analyst compares the two sets of the data into better agreement (Daston, 2005).

3.1.2.3 DOUBLE BLIND METHOD

Double-blind describes an especially stringent way of conducting an experiment, usually on human subjects, in an attempt to eliminate subjective bias on the part of both experimental subjects and the experimenters. In most cases, double-blind experiments are held to achieve a higher standard of scientific rigor (Ranjita et al, 2010). This method involves three analysts and is the most objective approach, an assuming and no bias on the part of the third analyst.

3.2.1 METHOD DEVELOPMENT

Analysis is usually based on prior art or existing literature, using the same or quite similar instrumentation. Method development for an HPLC requires especially the instrumentation, which involves the column, mobile phase, detector and the method of quantification (Himanshu et al, 2004).

There are several valid reasons for developing new methods of analysis. Few of these are:

1) There may not be a suitable method for a particular analyte in the specific sample matrix.
2) Existing methods may be prone to error-, artefact-, and or contamination and may be unreliable.
3) The methods existing may be too expensive, time consuming or energy intensive.
4) Existing methods may not provide adequate sensitivity or analyte selectivity in the samples of interest.

In method development, it is necessary to consider the properties of the analytes that may be used to an advantage and to establish optimal ranges of the analyte parameter values.

Some of these are chromatographic capacity factors, wavelength of detection, flow rate etc. In addition to the above, it is essential that method development be performed using only analytical standards that have been well identified and characterized and whose purity is already known (Himanshu et al, 2004).

3.2.2 METHOD OPTIMIZATION

Optimization of method can be classified under two general headings; Manual and Computer driven.The manual approach involves varying one experimental variable at a time while holding all others constant and recording changes in response. The variable includes flow rate, mobile phase composition, and pH and detection wavelength. This univariate approach to system optimization is slow, time consuming and potentially expensive. However, it may provide a much better understanding of the principles and interaction of the variables (Himanshu et al, 2004). Hence, this approach is applied in this project.

3.3.1 MOBILE PHASE, (MP) PREPARATION

Mobile phase preparation is one of the most important aspects of HPLC. If prepared improperly, it results in poor separation and may even damage the instrument. The following parameters are to be addressed in mobile phase preparation.

a) The pH of mobile phase is one of the parameters, which affect, resolution, flow rate, column selectivity and specificity. It is not possible to measure the pH of mobile phase of

non-polar alone. Hence if the mobile phase is a mixture of non-polar and polar then it is advisable to adjust the pH of the polar solvent before it is added to the non-polar.

b) Degassing. Dissolved oxygen may oxidize components of the eluent or interfere with the operation of the detector (Souppart et al, 2002). It is therefore a standard practice to degas the mobile phase before it is used or stored. This may be achieved by refluxing or vacuum degassing. Vacuum filtration has the advantage of degassing. These techniques may change the composition of eluents containing organic solvents. An alternative is to purge the eluent with helium or nitrogen.

c) Filtration: Particulate matter in the eluent must be avoided at all cost as it can clog the valves in the pump, the Fritz and the column (Souppart et al, 2002). It may therefore cause severe and expensive damage to the equipment. Eluent may be conveniently filtered under vacuum. This has the advantage of achieving a partial degassing and may be required for many applications.

Most biochemical quantitative investigations are carried out on blood. Less often on urine and other materials such as cerebrospinal fluid (CSF), intestinal secretions, faeces, calculi, sweat, aminotic fluid and fluids obtained by paracentesis and occasionally on saliva (Souppart et al, 2002). Carelessness in the collection of blood samples may adversely affect the interpretation or impair the validity of the tests carried out on the specimens. There is therefore the need to consider the following factors in the collection of specimens.

a) Dietary constituents may alter the concentrations of analytes in blood significantly. For example plasma glucose and triglyceride concentrations are affected by carbohydrate and fat-containing meals respectively.
b) Many drugs influence the chemical composition of blood. Such effects of drug treatments have to be taken into account when interpreting test results.
c) The concentrations of many substances in blood vary considerably at different times of the day. Specimens for these analytes must be collected at the times specified by the laboratory (Souppart et al, 2002). When collecting blood specimens caution must be taken. Venous blood specimens should be obtained with minimal stasis. Prolonged stasis can markedly raise the concentration of plasma.

3.3.2 SAMPLE PREPARATION

The greatest single problem a chromatographer encounters in clinical application of HPLC is that of sample preparation. This is the process of partial purification required before a biological sample can be injected onto an HPLC. The samples most commonly encountered by clinical chemists are blood urine, faeces and tissues. Of these, the one most easily handled is urine because of the usual relative absence of protein which presents many problems to the chromatographer (Gitau et al, 2004).

Plasma or serum is much preferred than whole blood by clinical chemists. The major problem here is removing the large amount of proteins.. The simplest sample preparation technique is deproteinisation by denaturation and precipitation with acids such as trichloroacetic acid (TCA).

Protein removal could also be done by addition of some organic solvensts such as methanol or acetonitrile; however, precipitation may be incomplete. In general, large ratios of organic solvent to sample are required to complete the precipitation. Where protein precipitation is incomplete, injection of samples will lead to deterioration of the column.

Ultra filtration is an alternative and does not dilute the sample but not all ultra filters retain albumin effectively and the analyte may bind to the ultra filter (Gitau et al, 2004). In some cases immiscible solvents such as chloroform can be used to extract the analytes. By this technique only centrifugation is done and injection of the organic phase may be all that is required. Two problems that arise are: incomplete recovery of the analyte due to denatured protein and lack of sensitivity due to dilution. The latter could be overcome by evaporation to dryness and reconstitution in a small volume of the mobile phase.

3.4.1 SAMPLE INJECTION

Directly related to chromatography is sample introduction into the pressure system. Errors can arise from leaks, specific gravity differences, sample-solvent incompatibility, precipitation and voids in the column. Sample injection is often a problem since most of the systems are under relatively high pressures. Care must be taken that the column packing is not disturbed during injection since this can lead to a deterioration of column performance. A large dead volume in the injector may lead to tailing and overlapping peaks. Injection directly on to the top of the column is the preferred approach.

3.4.2 QUANTITATION

A chromatogram is the visual output of the chromatography. When a detector that responds to the presence of analytes is placed at the end of the column a series of peaks are obtained called chromatogram. Generally, a chromatogram is a plot of a function of solute concentration versus elution time or elution volume (volume of added mobile phase). Quantitation relies upon relating either the height or area of sample peak to that of a known standard peak. Simple, clean, isocratic separations can be easily and accurately quantitated by peak height measurement alone. Peak height measurement is also much preferred in trace analysis when it is less affected by noise and over lapping peaks than other measurements (Navaratnam et al, 1997).

3.5 MATERIALS AND EQUIPMENT

3.5.1 REAGENTS:

All the reagents below (both solid and solvents) were obtained from BDH Laboratory Supplies Poole, Dorset, England.

(i) 0.1 M sodium dihydrogen phosphate, monobasic, NaH_2PO_4 (assay = 99%, FW = 119.98 g/mol).

(ii) 5% Hydrochloric acid, HCl, (Assay = 37.0% v/v, SG = 1.2 g//ml, FW = 36.46 g/mol)

(iii) 5% sodium hydroxide, NaOH, Analar –pure (Assay = 99.99%, FW = 40.00 g/mol)

(iv) Ethanol, CH_3CH_2OH, Analar –pure (Assay = 99.5% v/v, SG = 0.7897g/ml.

FW = 46.07 g/mol).

(v) Methanol, CH_3OH, Analar – pure (Assay = 99.8% v/v, FW = 32.04 g/mol.

SG = 0.791g/ml.)

(vi) Acetonitrile, CH_3CN, - HPLC grade (Assay = 99.9% v/v, FW = 41.05 g/mol;

SG = 0.786 g/ml)

(vii 1, 2 – Dichloroethane, $ClCH_2CH_2Cl$, - HPLC grade, (Assay = 99.8% v/v,

FW = 98.96 g/mol, S.G. = 1.256 g/ml.)

(viii) Chloroform, $CHCl_3$ - HPLC grade, (Assay = 99.9%; FW = 119.38 g/mol,

S.G. = 1.492 g/ml.)

3.5.2 ANTIMALARIALS AND SAMPLES USED IN THE STUDY

(i) Quinine (Qn)

(ii) Chloroquine (Cq)

(iii) Amodiaquine (Adq)

(iv) Pyrimethamine (Pma)

(v) Sulphadoxine/pyrimethamine, called Fansidar (Fn)

All the above antimalarials were obtained from London School of Hygyiene and Tropical Medicine (UK).

3.5.3 EQUIPMENT

HPLC – Kontron Instrument:

a. Photo Array Detector 440
b. Column, (ODS; C18 reversed phase HPLC column).
c. Pump 442 (Hichroma)

Computer & Accessories

3.5.4 OTHER LABORATORY INSTRUMENTS/EQUIPMENT

3.5.4.1 ITEMS

(i) Analytical balance (Toledo B303)
(ii) pH Meter (Mettler Toledo 320)
(iii) Mixing Roller
(iv) Centrifuge
(v) Oven (0 – 360°)
(vi) Vortex (Graffin George Ltd. U.K.)
(vii) Variable pipettes (10 – 100 µL); (100 - 1000µL); 0.5 – 5 ml)
(viii) Pipette Tips
(ix) Graduated pipettes (0 – 10 ml)
(x) Filter paper (std qualitative diam. size 90 mm)
(xi) Buchner funnel

3.5.4.2 Glassware

(i) Volumetric flasks
(ii) Measuring cylinder
(iii) Beakers
(iv) Glass Test Tubes
(v) Reagent bottles

(vi) Plastic Vials (crimp top)

3.6 REAGENTS PREPARATION

(i) 0.10 M solution of NaH_2PO_4 (buffer):

The buffer was prepared from the Analar compound. The formula weight (FW) of NaH_2PO_4 was 119.98 g/mol.

About 30 g of the pure compound of NaH_2PO_4 was dried at 100°C in an oven to constant weight. It was then cooled in a desiccator. 23.996 g of the salt was accurately weighed on analytical balance. The salt was dissolved in a minimum amount of distilled water. It was then emptied into 2.0 L of volumetric flask. The beaker was rinsed twice and the content added to the flask. The flask was then made up to the mark with distilled water.

Note: 119.98 g of NaH_2PO_4 salt in 1.0 L of solution gives a concentration of 1.0 molar. It implies that 0.1 M concentration requires 11.998 g of the salt in 1.0 L of solution. Therefore 2.0 L of the same concentration needs 23.996 g of the salt.

(ii) 1.00 M HCl

The purity (assay) of the concentrated HCl was 37%. The specific gravity (S.G.) of the acid was 1.2 g/ml and a formula weight (FW) of 36.46 g/mol. Calculation from the above parameters gave 20.5 ml of the concentrated HCl acid to prepare 250 ml of 1.00 M solution. With measuring cylinder 20.5 ml of the acid was measured and poured into about 100 ml of distilled water in a beaker. The solution was stirred and emptied into 250 ml volumetric flask. The beaker was then

rinsed twice and the content added to the flask. The flask was then made up to the mark with the distilled water. The solution was labelled as approximately (≃) 1.000 M HCl.

(iii) 0.100 M HCl.

The 0.100 molar solution was prepared from the 1.000 molar solution by diluting it ten times.

(iv) 0.20 M NaOH

The formula weight of the Analar NaOH salt is 40.0 g/mol.

In a small beaker (50 ml), 8.000 g of NaOH was accurately weighed on analytical balance. The salt was dissolved in a minimum amount of distilled water and emptied into 1.0 L volumetric flask. The beaker was then rinsed twice and the content added to the flask. Using distilled water, the flask was made up to the mark. The solution was then labelled as

≃ 0.20 M NaOH.

(vi) 0.10 M NaOH

The 0.10 M of NaOH was prepared from the 0.20 M NaOH. To prepare 100.0 ml of 0.10 M

of NaOH, 50.0 ml of the 0.20 M was measured with measuring cylinder and made up to 100.0 ml of solution.

3.7 COMPOSITION OF MOBILE PHASE

3.7.1 MOBILE PHASE FOR Cq, Qn AND Adq

From the optimization, (refer 3.3.1) it has been established that a good resolution could be obtained with acetonitrile, ethanol and the buffer (acidic) in the ratio 2:5:4 (v/v) respectively. The mixture was stirred and the pH adjusted with either 0.10 M HCl or 0.20 M NaOH to approximately (≃) 3.50 (ie if above pH 3.5, acid was used and if below pH 3.5, the base was used). After this, the solution was passed through vacuum filtration using standard qualitative filter paper grade No. 3 with buchner funnel and a pump. The solution was then labelled as MP_1.

3.7.2 MOBILE PHASE FOR PYRIMETHAMINE AND FANSIDAR

The established constituents of the mobile phase of the above antimalarial drugs are acetonitrile, methanol and the buffer. They were in the ratio of 3: 5: 4 (v/v) respectively. The mixture was well mixed and its pH adjusted to 3.00 with 0.10 M HCl and 0.20 M NaOH (ie if above pH 3 acid was used and if below pH 3 the base was used). The solution was filtered and then labelled as MP_2.

3.8 PREPARATION OF SERIAL STANDARDS OF ANTIMALARIAL S

3.8.1 FANSIDAR (Fn)

In a small beaker (50 ml) 0.120 g of Fn was accurately weighed on the analytical balance and dissolved in 50% methanol because it was much more soluble in this solvent than water. The

solution was then emptied in 100 ml-volumetric flask. The beaker was rinsed twice and the content added to the flask. With the solvent, the flask was then made up to the mark and labelled as Fn stock solution having a concentration of 1.200 mg/ml.

Below is the dilution of the stock Fn and their corresponding concentrations:

(i) Diluted 120 times = 10.000 ug/ml.

(ii) Diluted 60 times = 20.000 ug/ml.

(iii) Diluted 40 times = 30.000 ug/ml.

(iv) Diluted 24 times = 50.000 ug/ml.

(v) Diluted 16times = 75.000 ug/ml.

(vi) Diluted 12 times = 100.000 ug/ml

3.8.2 CHLOROQUINE (CQ)

In a small beaker 0.150 g of the pure Cq was accurately weighed on analytical balance. It was dissolved in 50% ethanol. The beaker was emptied into 200 ml volumetric flask. The beaker was then rinsed twice and the content added to the flask. The 50% ethanol was used to fill the flask to the mark. The solution was then labelled as stock of Cq with a concentration of 750 ug/ml.

The serial standards were then prepared from the Cq stock as follows:

(i) Diluted 150 times = 5.000 ug/ml.

(ii) Diluted 75 times = 10.000 ug/ml.

(iii) Diluted 30 times = 25.000 ug/ml.

(iv) Diluted 15 times = 50.000 ug/ml.

(v) Diluted 10 times = 75.000 ug/ml.

(vi) Diluted 7.5 times = 100.000 ug/ml..

3.8.3 PYRIMETHAMINE (Pm)

0.150 g of pure PMA was accurately weighed in a small beaker on an analytical balance. It was dissolved in 50% methanol as solvent. The solution was then poured in 250ml-volumetric flask. The beaker was rinsed twice and the content added to the flask. The solvent was used to make up to the mark. The concentration of this solution is 600 ug/ml. It was then labelled as pyrimethamine stock solution.

Diluting the stock Pma at varying ratios, the serial standards were prepared as follows:

(i) 120 times dilution = 5.000 µg/ml.

(ii) 60 times dilution = 10.000 µg/ml.

(iii) 30 times dilution = 20.000 µg/ml.

(iv) 20 times dilution = 30.000 µg/ml.

(v) 12 times dilution = 50.000 µg/ml.

(vi) 8 times dilution = 75.000 ug/ml.

3.8.4 QUININE (Qn)

0.030 g of pure Qn was accurately weighed in a clean small beaker on an analytical balance. 50% ethanol was used as solvent to dissolve the Qn. The dissolved solution was emptied into a 250ml-volumetric flask. The beaker was well rinsed and the content added to the flask. Using the solvent, the flask was made up to the mark and labelled as 120 ug/ml stock solution of Qn.

Dilution of the stock, the following serial standards were prepared:

(i) 120 times dilution = 1.000 µg/ml.

(ii) 80 times dilution = 1.500 µg/ml.

(iii) 60 times dilution = 2.000 µg/ml.

(iv) 48 times dilution = 2.500 µg/ml.

(v) 40 times dilution = 3.000 µg/ml.

(vi) 24 times dilution = 5.000 ug/ml.

3.8.5 AMODIAQUINE (Adq)

In a small beaker 0.150 g of the pure Adq was accurately weighed on analytical balance. It was dissolved in 50% ethanol. The beaker was emptied into 200 ml volumetric flask. The beaker was then rinsed twice and the content added to the flask. The 50% ethanol was used to make up to the mark. The solution was then labelled as Adq stock solution of 750 µg/ml.

The serial standards were then prepared from the stock Adq as follows:

(i) Diluted 150 times = 5.000 ug/ml.

(ii) Diluted 75 times = 10.000 ug/ml.

(iii) Diluted 30 times = 25.000 ug/ml.

(iv) Diluted 15 times = 50.000 ug/ml.

(v) Diluted 10 times = 75.000 ug/ml.

(vi) Diluted 7.5 times = 100.000 ug/ml.

3.9 AVAILABLE SAMPLES

The collected samples were in three categories. These were:

a) Plasma without antimalarial drug.

The blood was collected from the Blood Bank at Komfo Anokye Teaching Hospital (KATH), Kumasi. This sample was screened to be sure that it was free of any antimalaria drugs. This was

done by running the plasma sample collected on the HPLC against all the standards of the antimlaria drugs being used in the study.

b) Volunteers' Samples (VS)

Volunteers who were malaria free were put on antimalarial drugs (e.g. Cq) course. Blood samples were taken on the first day and twenty one (21) days after the completion of the course. These samples were labelled as VS with respect to the type of the antimalarial drug used. For chloroquine (Cq), it was labelled as $CqVS_1$ and $CqVS_{21}$ respectively. Only three volunteers were used, one for chloroquine, another for amodiaquine and the third one for fansidar.

c) Blind Test samples (BTS)

During the execution of this study, there was an antimalaria project, SMAT II (Supervised Monthly Antimalaria Treatment), being carried out at the Department of Community Health, School of Medical Sciences, KNUST. It was a Blind test involving pregnant women. The antimalarial drugs used were chloroquine, fansidar and placebo given to the women at random. Extraction of some antimalarial drugs from blood plasma, an in vivo experiment was the target of this study. SMAT II project, an in vitro test involved similar antimalarials, hence permission was sought to be enrolled in SMAT II for blood samples. As a third party working on these samples, the test was termed as double blind test, DBT (Daston and Lorraine, 2005)

d) Control Samples (Aqueous Solutions).

In addition to the above mentioned samples, aqueous solution (distilled water) was used as control samples. Into these, the serial standards of the various antimalarial drugs were introduced.

3.10 SPIKING PLASMA WITHOUT ANTIMALARIAL DRUG AND THE CONTROL SAMPLES WITH ANTIMALARIAL DRUGS

As the name implies, the plasma without antimalarial drug has no antimalaria compound, hence an antimalarial drug had to be introduced into it before the drug was then extracted. The same procedure was applied to the control samples, the aqueous solutions.

From the method of optimization it was established that the following volumes of reagent and plasma were convenient.

Into labelled glass test tubes (tt) with covers, 2.0 ml each of the plasma without antimalarial drug and control samples was measured. To each tt, 1.0 ml of the prepared serial standards for each antimalarial drug eg. Cq was added. All the test tubes were put on a roller (mixer) for one hour.

3.11.1 INNOVATIVE EXTRACTION PROTOCOL FOR CHLOROQUINE, QUININE AND AMODIAQUINE EXTRACTION

. The first step was to exract the drug from the plasma / aqueous into an organic layer. The

second step was to extract the drug from the organic layer into another aqueous phase.

(a) Organic Phase (Layer) Extraction

Into labelled test tubes, 1.0 ml each of the spiked plasma, control, blind test and volunteers' samples were pipetted. This was followed by the addition of 1.0 ml of 0.20 M NaOH to all the tubes. The mixture was mixed gently by rotating the test tubes. 4.0 ml of 1, 2-Dichloroethane was then added to each tube. The content of each tube was mixed gently to avoid frothing. All the test tubes were put on the roller for one hour.

The samples were removed from the roller and spun for 3 minutes at a speed of 4,000 revolutions per minute (RPM). With 5ml variable pipette, the organic layer was removed from each test tube into new labelled test tubes. For maximum extraction, another 4.0ml of 1, 2-Dichloroethane was added to all the test tubes and the extraction repeated. The second extract was then added to first one for onward second stage of the extraction.

(b) Aqueous Phase (Layer) Extraction

To each test tube of the organic phase, 1.0ml of 0.10M HCl was added. The test tubes were put on the roller for one hour. The samples were spanned for 3 minutes at a speed of 4,000 RPM and the supernatant (aqueous) pipetted into labelled tubes. The procedure was repeated to achieve maximum extraction. The second extract was added to the first one. The extract was well mixed and its pH adjusted to approximately (≃) 3.5 with 0.100 M NaOH. The extracts which looked cloudy were filtered using standard qualitative filter paper grade No. 3.

3.11.2 INNOVATIVE EXTRACTION PROTOCOL FOR PYRIMETHAMINE AND FANSIDAR

(a) Organic Phase (Layer) Extraction

Into labelled test tubes, 1.0 ml each of the spiked plasma, control, blind test and volunteers' samples was pipetted. To each tube 4.0 ml of chloroform ($CHCl_3$) was added. The test tubes were then put on the roller for one hour. The samples were spun for 3 minutes at a speed of 4,000 RPM. The organic layer of each tube was removed into another labelled test tubes using 5.0ml variable pipette. The extraction was repeated using another 4.0 ml of $CHCl_3$. Using the same procedure the second extract was added to the first one.

(b) Aqueous Phase (Layer) Extraction

In the second stage of the extraction, 1.0 ml of 0.20 M NaOH was added to each test tube of the organic layer. All the tubes were again put on the roller for one hour. With suitable pipette the supernatant of each tube was collected into other labelled test tubes. 1.0 ml of 0.20 M NaOH was again added to the organic layer and the extraction repeated. The second extract was added to the first extracts. The pH of the extracts was then adjusted to 4.0 by using 1.0M HCl. The extracts which looked cloudy were filtered using standard qualitative filter paper grade No. 3.

3.12 ANALYSIS OF THE EXTRACTS ON THE HPLC

HPLC analysis of Extracts was carried out after the optimization of all the antimalarial drugs.The HPLC setting and materials which were common for all the antimalarial drugs analysis were:

(i) Reverse phase column

(ii) 20 uL sample loop

(iii) Diode array detector

(iv) HPLC Pump

The following samples were uniquely analysed after setting the required parameters for each antimalarial drug on the HPLC

A) Chloroquine

B) Quinine

C) Amodiaquine

D) Fansidar

E) Pyrimethamine

A) The Parameters for the Analysis of chloroquine were:

(i) Six serial standards, (prepared above)

(ii) Wavelength of 340nm

(iii) Flow rate of 0.8ml/minute

(iv) Mobile phase, MP1 (prepared above)

(v) These parameters produced a retentions time (R.T) of 2.18 minutes.

The column was equilibrated by running the MP_1 for 15 minutes. With the HPLC syringe and needle, each serial standard of chloroquine was injected onto the column through the 20uL loop. The running of the standards normally started from the least concentration. For each standard, the running was repeated for five times. After elution of the six serial standards the column was washed by running the MP_1 for 10 minutes. Under the same conditions and procedure for the standards, the plasma chloroquine extracts, aqueous chloroquine extracts, volunteers' chloroquine extract and blind test extracts were also eluted. Each extract elution was repeated five times.

B) The Parameters for the Analysis of Quinine were as follows:

(i) Six serial standards (prepared above)

(ii) Wavelength 340nm

(iii) Mobile phase, MP_1 (prepared above)

(iv) Flow rate of 1.2 ml / minute

(v) The above parameters produced a retention time (RT) of 3.42 minutes.

Procedure for Quinine Analysis:

The same procedure used for the extraction of chloroquine was used for the extraction and analysis of quinine. Again the values of the chromatograms were recorded for further processing.

The blind test samples were not ran under the above parameters because the test did not involve quinine.

C) For the analysis of Amodiaquine (Adq) the following were the parameters established.

(i) Flow rate of 1.0 ml / minute

(ii) Mobile phase, MP_1

(iii) Serial standards (6)

(iv) Wavelength of 340nm

(v) These parameters produced retention time (RT) of 4.13min

Procedure for Amodiaquine Analysis:

The above parameters were set during the equilibration time of 15 minutes. Following the same procedure for chloroquine analysis as indicated above, amodiaquine extracts were also assayed. The values of the chromatograms produced were retained for onward processing.

D) The following were the established parameters for the analysis of Fansidar (Fn)

(i) Six serial standards (prepared above)

(ii) Flow rate 1.2 ml / minute

(iii) Mobile phase, MP_2 (prepared above)

(iv) Wavelength of 280nm

(v) The above parameters produced retention time of 2.79 min

Procedure for Fansidar Analysis:

The instrument was conditioned for 15 minutes before the ejection of the extracts started. Using the same protocol for chloroquine extracts, each of the fansidar extract was injected onto the column one after the other. The HPLC was then washed using the mobile phase MP_2 for 10 minutes. The blind test sample extracts were then run, one sample after the other. One of the time consuming aspect of the running of these blind test sample extracts was the need for the column to be freed from the previous samples to avoid contamination, hence, thorough washing of the column was carried out after each sample had been ran.

E) The established parameters for Pyrimethamine (Pma) Analysis are as follows;

(i) Flow rate 1.0ml/minutes

(ii) Serial standards (6)

(iii) Wavelength of 220nm.

(iv) Mobile phase, MP_2

(v) The mean retention time (RT) produced by the above parameters was 3.72 mins.

Procedure for Pyrithamine Analysis.

The above parameters were set during the equilibration time of 15 minutes. The six pyrimethamine serial standards were eluted. This was followed by running all the Pyrimethamine extracts. As usual each of the test elution was repeated five times.

3.13.1 CALIBRATION CURVE

A calibration curve is a general method for determining the concentration of a substance in an unknown sample by comparing the unknown to a set of standard samples of known concentrations. A calibration curve is one approach to the problem of instrument calibration; other approaches involve mixing the standard with the unknown which is termed as internal standard addition calibration. (Crouch et al, 2007)

The calibration curve is a plot of how the instrumental response, the so-called analytical signal, changes with the concentration of the analyte (the substance to be measured). Serial standards across a range of concentrations near the expected concentration of analyte in the unknown were prepared. Analyzing each of these standards using the chosen technique will produce a series of measurements. For most analyses a plot of instrument response versus analyte concentrations will show a linear relationship. Measurement of the responses of the unknowns was made and using the calibration curve their concentrations determined.

3.13.2 CALCULATION

Linear regression analysis yields a model described by the equation, $y = mx + C$, where y is the instrument response, m represents the sensitivity, and C is a constant that describes the background. The analyte concentration (x) of unknown samples may be calculated from this equation. Most analytical techniques use a calibration curve. There are a number of advantages to this approach. Calibration curve provides a reliable way to calculate the uncertainty of the

concentration calculated from the calibration curve (using the statistics of the least squares line fit to the data (Crouch et al, 2007).

3.13.3 RECOVERY

The recovery of an analyte in an assay is the detector response obtained from an amount of the analyte added to, and extracted from, the biological matrix, compared to the detector response obtained for the true concentration of the pure authentic standard. Recovery pertains to the extraction efficiency of an analytical method within limits of variability (Wu, 2009).

Recovery of the analyte need not be 100%, but the extent of recovery of an analyte and of the internal standard should be consistent, precise, and reproducible (Wu, 2009). Recovery experiments should be performed by comparing the analytical results for extracted samples at least at three concentrations (low, medium, and high) with unrestricted standards that represent 100% (the concentration of pure authentic standard) (Wu, 2009).

Recovery = (Result found) / (Result expected) × 100%.

High recovery value is not absolutely required, as long as sensitivity, consistency, precision, and reproducibility requirements are met (Wu, 2009). A method with higher recovery is presumably more rugged.

3.14.1 ANALYSIS OF VARIANCE (ANOVA)

In statistics, analysis of variance (ANOVA) is a collection of statistical models and their associated procedures, in which the observed variance is partitioned into components (of difference within a group or between a group – of means). ANOVA is a technique to compare means in three or more groups. It is a common device for partitioning the sum of squares in regression analysis, and hence in providing significance tests for various hypothesis of interest (Lowry, 2008), ANOVA partitions the variability among all the values into one component that is due to variability among group means (due to the treatment) and another component that is due to variability within the groups (also called residual variation). Variability within groups (within the columns) is quantified as the sum of squares of the differences between each value and its group mean. This is the residual sum of squares. Variation among groups (due to treatment) is quantified as sum of squares of the differences between the group means and the grand mean (the mean of all values in all groups). Adjusted for the size of each group, this becomes the treatment sum of squares. Each sum of squares is associated with certain number of degree of freedom (df) and the mean square (MS) which is computed by dividing the sum of squares by the appropriate number of degree of freedom (Lowry, 2008).

3.14.2 TUKEY'S TEST

After performing ANOVA, a follow up test is required to indicate a statistically significant effect. The follow-up test chosen for this study was Tukey's test. Tukey's test, also known as the Tukey range test, Tukey method, Tukey's honest significance test, Tukey's HSD (Honestly

Significant Difference) test (Lowry, 2008) is a single-step multiple comparison procedure and statistical test generally used in conjunction with an ANOVA to find which means are significantly different from one another. This test most commonly compares every group mean treatment with every other group mean treatment. It is based on a studentized range distribution *q* (this distribution is similar to the distribution of *t* from the t-test) (Linton et al, 2007)

3.14.3 THE p VALUE

A p-value in statistics is a probability with a value ranging from zero to one. A p-value is a measure of how much evidence one has against the null hypothesis, (*HO*). It measures consistency by calculating the probability of observing the results from sample data with results more extreme, assuming the null hypothesis is true. The smaller the p-value, the greater the inconsistency while a large p-value means little or no evidence against the null hypothesis (Sellke et al, 2001).

Critics of p-values point out that the criterion used to decide "statistical significance" is based on the somewhat arbitrary choice of confidence interval level which is often set at 0.05 (Sellke et al, 2001). For this study, at confidence level of 95 % and the critical value of p chosen is $p \leq 0.05$.

CHAPTER FOUR

RESULTS AND DISCUSSION

This study is based on extraction/separation of some anti malarial drugs from plasma. After the extraction, an HPLC was used to determine the amount of the drug extracted. Aqueous samples were used as control; hence both the plasma and the aqueous contained the same concentration of the antimalarial drugs.

p Value (SYMBOL)	DEFINITION OF SYMBOL/ EFFICIENCY OF EXTRACTION
< 0.05 (ns)	Not Significant / Very Effective
< 0.046 (*)	Significant / Effective
< 0.002 (**)	Very significant / Fairly Effective
< 0.0001 (***)	Extremely significant / Poorly Effective (In-effective)

Table 4.0 Using p values and symbols to classify the level of extraction /significant diferrence.

Table 4.0 is the summary of ANOVA and Tukey's Multiple Comparison Test performed on data generated for peak height and peak area (e.g for chloroquine refer Appendix Tables 1 – 3).

The following are the explanations of the summary:

1) If there is no significant difference (ns) between two groups (e.g. expected and aqueous) it means that the extraction was very good, and is classified as very effective.
2) If the difference is significant (*), it is classified as effective

3) A very significant difference between two groups (**) means the extraction is fairly effective.
4) An extremely significant difference (***) between two groups is termed poorly effective or the extraction is ineffective.

4.1.1 RESULTS OF CHLOROQUINE (Cq)

Detection parameters for chloroquine extracts on the HPLC

a) The mean and standard deviation (SD) for retention time for:
   (i) Serial standards was 2.815 ± 0.034 minutes
   (ii) Aqueous extract samples was 2.81 ± 0.035 minutes.
   (iii) Plasma extract samples was 2.82 ±0.033 minutes.
b) Wavelength was 340 nm
c) The Flow rate was 0.80 ml/minute
d) The prepared mobile phase consisted of acetonitrile, ethanol, and 0.10 M buffer (NaH_2PO4), MP_1 in the ratio of 2: 5: 4 (v/v) respectively and the pH was adjusted to 3.5

Standard concentration (µg/ml) against Peak Height & Peak Area for calibration curve for chloroquine		
Std Conc. (µg/ml)	Response of Chloroquine (mili-Absorbance)	
	Mean Peak Height (mAb)	Mean Peak Area (mAb)
5.00	3.29	1.98

10.00	10.15	5.26
25.00	35.75	13.81
50.00	67.96	25.00
75.00	94.75	35.02
100.00	133.16	50.07

Table 4.1 Table for plot of standard concentration (µg/ml) against peak height and peak area for calibration curve for chloroquine

Table 4.1 consists of a data of standard concentrations (µg/ml) and their corresponding mean responses of peak height and peak area. From this data, plots of standard curves were drawn. These plots are shown in figure 4.1. For each plot, an equation of the line was derived.

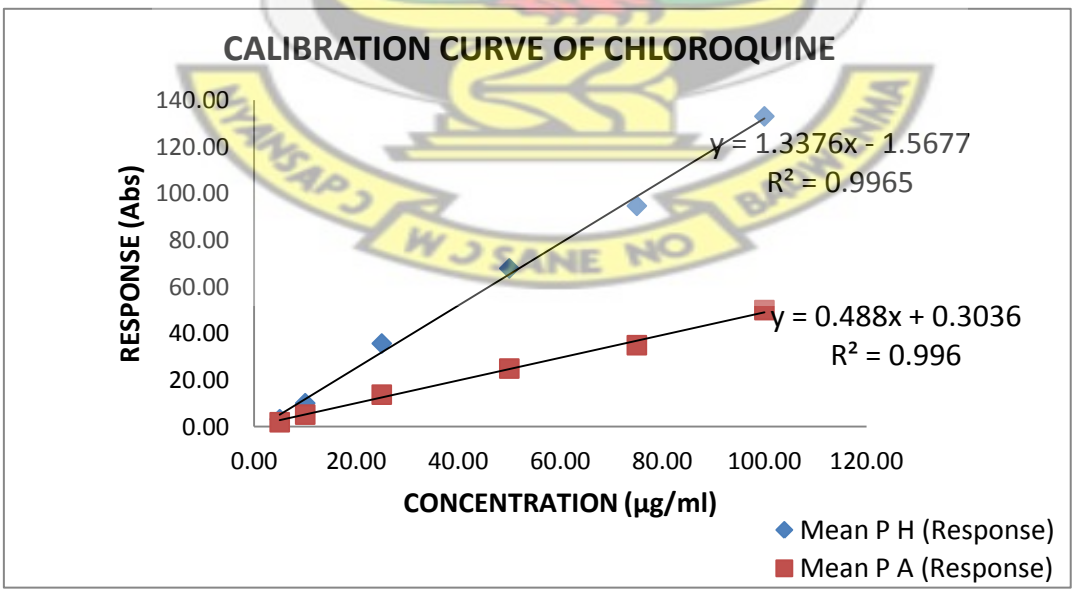

Fig 4.1 Graph of Standard concentration (µg/ml) against PH & PA for plotting

calibration curve for Cq

The equation of each plot was used to calculate the concentrations of the repeated measures of the extract samples of chloroquine. From these calculated concentrations, the Means (m) and their Standard Deviations (SD) for each level of concentrations were derived. These were done for peak height and peak area for aqueous (control) extraction separately and that for plasma also separately. To give more meaning to the results, the mean and standard deviation of recovery were also calculated. The results are illustrated in Tables 1 and 2 of the Appendix.

	Peak height (CV %)	Peak area (CV %)
Aqueous(control)	87.524 ± 13.133 (15.005)	69.214 ± 14.497 (20.945)
Plasma	82.194 ± 14.695 (17.878)	69.622 ± 17.978 (25.822)

Table 4.2 Mean and standard deviation of recovery (%) with their coefficient of variation (C V %) for the six different concentrations of chloroquine extracts

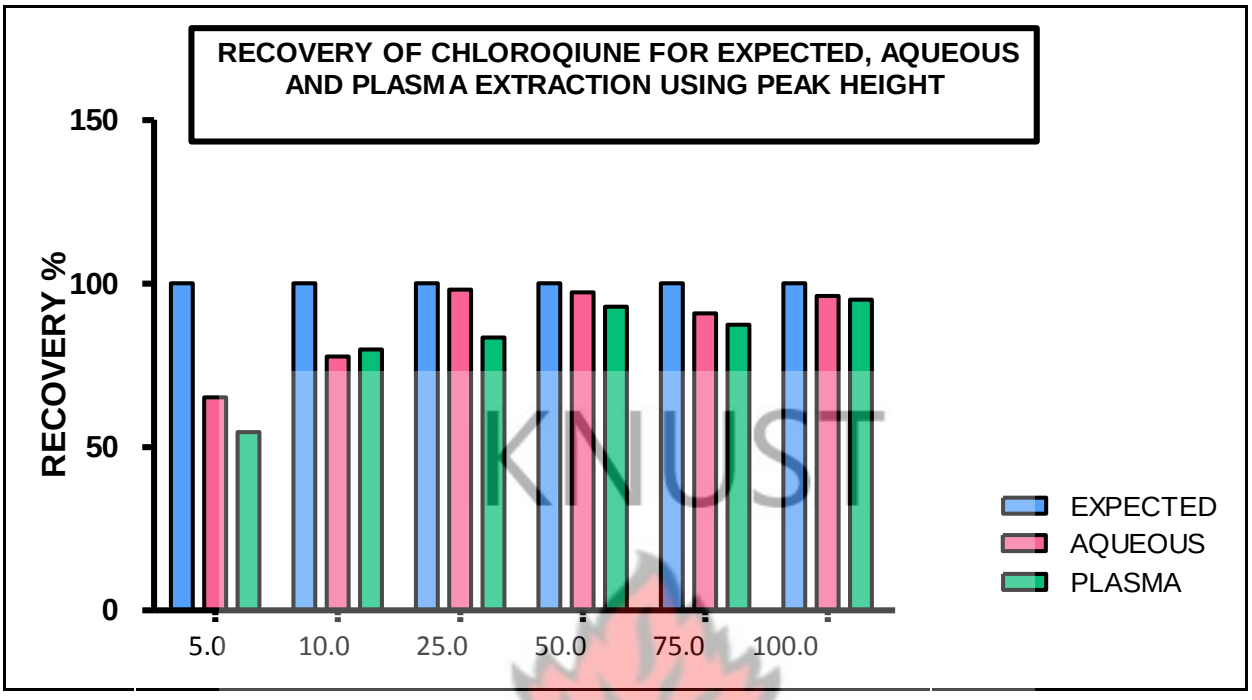

Figure 4.2 Plot of recovery (%) of chloroquine for expected, aqueous, and plasma extraction using PH

Comparison of Recovery (%): Aqueous (control) extracts and Plasma extracts.

Peak Height: The recovery of chloroquine for aqueous extracts ranged from 65.2 % to 96.1% while that of the plasma was from 54.5 % to 95.1%. This was deduced from Tables 1 & 2 of Appendix.

Peak Area: The recovery of chloroquine from plasma varied between 35.6 % and 77.3 %. That of the aqueous extended from 40.0 % to 77.1 %. These results were summarized in

Tables 1 & 2 in the Appendix.

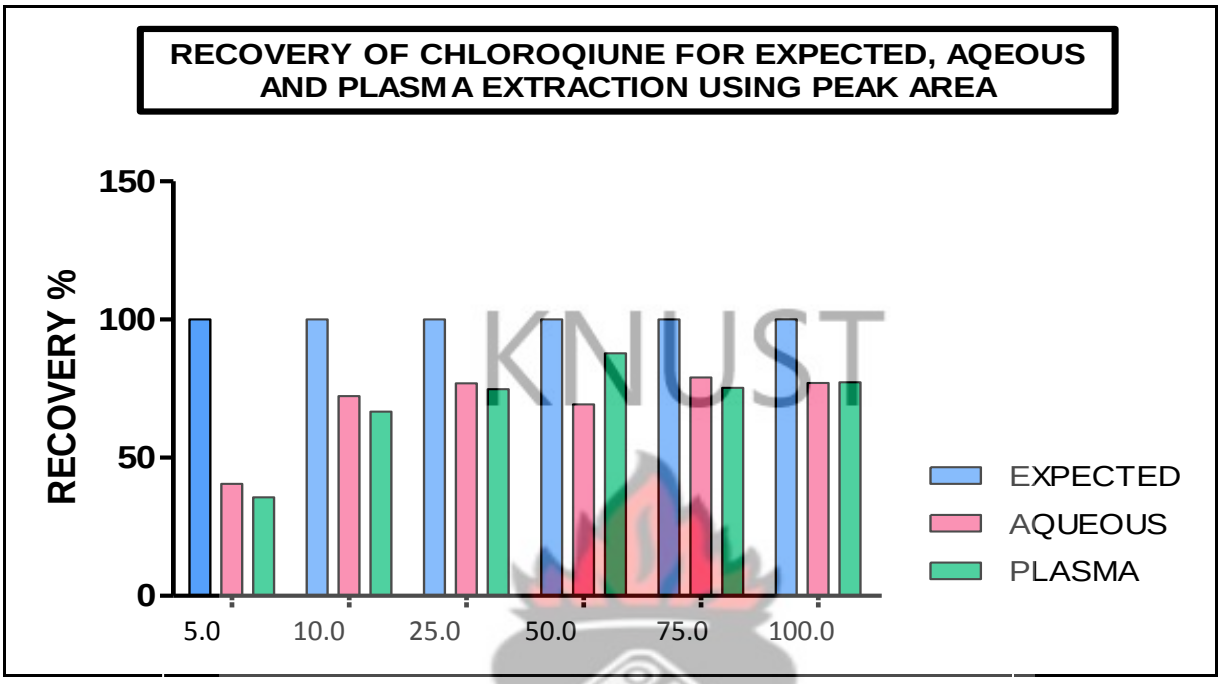

Figure 4.3 Plot of recovery (%) of chloroquine for expected, aqueous, and plasma extraction using PA

Table 4.2 above shows that peak height (PH) and peak area (PA) recoveries were effective since the extractability of both the control and the plasma were almost the same. The main difference was that the extractability of peak height was better than that of peak area (Gitau et al, 2004).

One-way ANOVA and Tukey's test were applied to the recovery results. These were done to see the effectiveness or degree of extraction/separation of the various extraction methods applied to the various antimalarial drugs (All the Tables displayed in Appendix).

Data for chloroquine used for ANOVA for peak height and peak area are respectively shown in Tables 3 and 5 in the Appendix. The standard concentration (Expected) used was taken as

100%, hence the aqueous or control and plasma extracts recoveries were calculated based on the expected extraction of 100%.

ANOVA was carried out on the three groups i.e. the Expected, Aqueous and Plasma under PH and PA separately. These results of the ANOVA and Tukey's test for PH and PA are shown in Tables 4 & 6 respectively in the Appendix and their summary in Table 4.3. The results of Tukey's test clearly showed the significant difference among the groups.Poor recoveries were observed at low concentrations as indicated in figures 4.2 & 4.3.

	Peak height (Efficiency of Extraction)	Peak area (Efficiency of Extraction)
Expected versus Aqueous	ns (Very Effective)	** (Fairly effective)
Expected versus Plasma	* (Effective)	** (Fairly Effective)
Aqueous versus Plasma	ns (Very Effective)	ns (Very Effective)

Table 4.3 Summary of ANOVA and Tukey's test of Chloroquine

The summary of Tukey's test in Table 4.3 showed that the Chloroquine (Cq) extraction was very effective under peak height while fairly effective under peak area. The p values from ANOVA and Tukey's test for Cq were $p < 0.0456$ (*) and $p < 0.0016$ (**) for PH and PA respectively.

Shah et al, (2000) reported a linear calibration curve in the range of 100–1500 ng/ml and coefficient of correlation, r2 > 0.97 for extraction of chloroquine from whole blood. The mobile phase used consisted of water, methanol and triethylamine (83:16:1% v/v), adjusted to pH 2.8. Using UV detector at 340 nm and a flow rate of 3.0 ml/min, retention time of 3.0 minutes was attained. With these parameters, mean recovery of 97 % and CV of less than 15 % were achieved.

For this study, mean and standard deviation of recovery (%) with their coefficient of variations (C V %) for the six different concentrations of chloroquine extracts in plasma and aqueous are displaced in Table 4.2. The mobile phase used consisted of acetonitrile, ethanol, and 0.10 M buffer (NaH_2PO4), in the ratio of 2: 5: 4 (v/v) respectively and the pH was adjusted to 3.5. The detection wavelength was the same as Shah, et al, (2000). Retention time (RT) of 2.8 minutes and flow rate of 0.8 ml/min were obtained. RT which is one of the most important parameters in chromatography depends on flow rate, mobile phase & its pH and the detection wavelength, hence the low value of RT for this study. The calibration curve was in the range 5.0 -100.0 µg/ml with $r^2 = 0.996$.

4.1.2 RESULTS OF FANSIDAR (Fn)

Detection parameters for fansidar extracts on the HPLC

a) The mean and standard deviation for retention time for:
    i. Serial standards was 2.78 ± 0.06 minutes
    ii. Aqueous samples was 2.78 ± 0.06 minutes
    iii. Plasma samples was 2.77 ± 0.07 minutes

b) Wavelength was 280 nm

c) The Flow rate was 1.20 ml/minute

d) The prepared mobile phase consisted of acetonitrile, methanol, and 0.10 M buffer (NaH_2PO_4) in the ratio of 3: 5: 4 respectively and the pH adjusted to approximately 3.0.

Standard concentration (µg/ml) against Peak Height & Peak Area for calibration curve for Fansidar		
Std Conc (ug/ml)	Response of Fansidar (mili-Absorbance)	
	Mean Peak Height (mAb)	Mean Peak Area (mAb)
10.00	9.10	1.29
20.00	17.42	5.09
30.00	29.44	9.38
50.00	52.77	14.60
75.00	72.37	23.22
100.00	98.50	33.20

Table 4.4 Table for plot of standard concentration (µg/ml) against PH and PA for calibration curve

for Fansidar

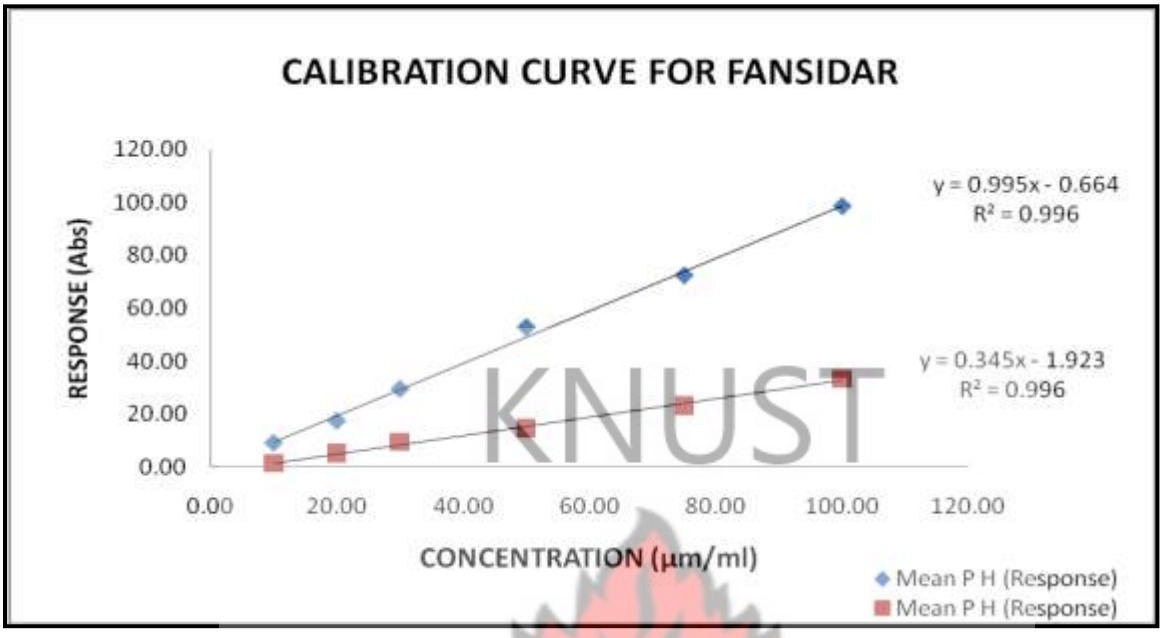

Figure 4.4 Graph of Standard concentrations (µg/ml) against PH & PA for plotting calibration curve

The data of standard concentrations (µg/ml) and their corresponding mean responses of peak height and peak area are displayed in Table 4.4. From this data, plots of standard curves were drawn. These plots are shown in figure 4.4. For each plot, an equation of the line was derived.

Using the equation of each plot, the concentrations of the repeated measures of the extract samples of fansidar were calculated. From these calculated concentrations the means and their standard deviations for each level of concentration were calculated. These were done for peak height and peak area for aqueous extraction (control) separately and that for plasma also separately. The mean and standard deviations of recoveries were also calculated and displayed in Tables 7 and 8 of the appendix.

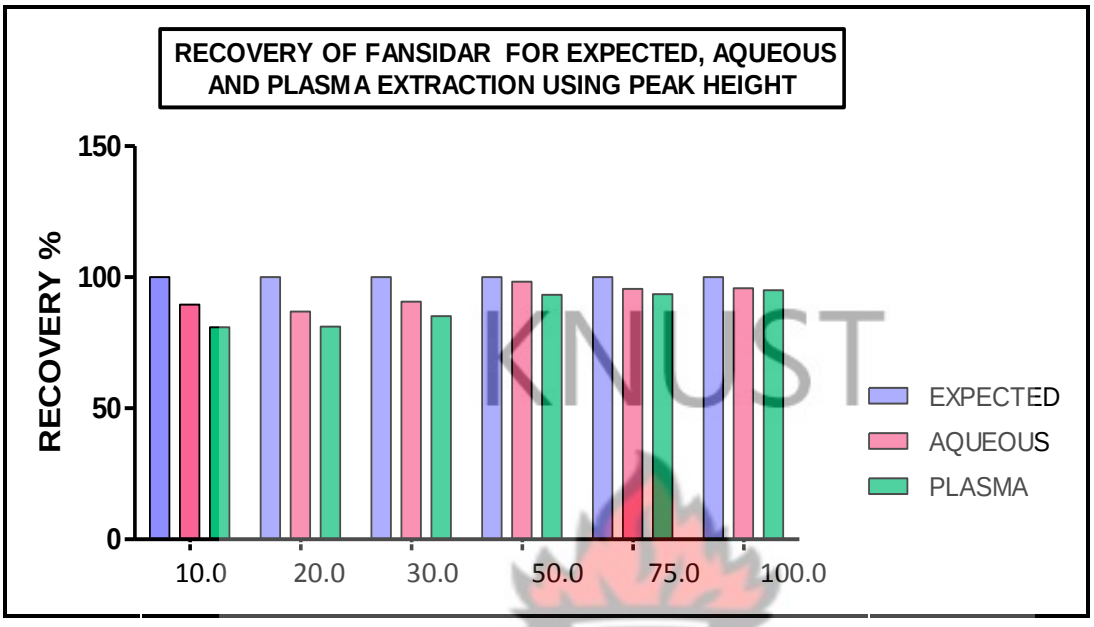

Figure 4.5 Graph of recovery (%) of fansidar for expected, aqueous, and plasma extraction using PH

Comparison of Recovery (%): Aqueous (Control) extracts and Plasma extracts.

Peak Height: Tables 7 and 8 in the Appendix reveal that recovery of fansidar for aqueous extracts ranged from 88.3 % to 96.3 % and the plasma extracts gave values from 88.8 % to 95.3 %.

Peak Area: Aqueous extracts of fansidar produced recovery values from 77.2 % to 82.2 %. while plasma extracts ranged from 74.3 % to 77.5 %.

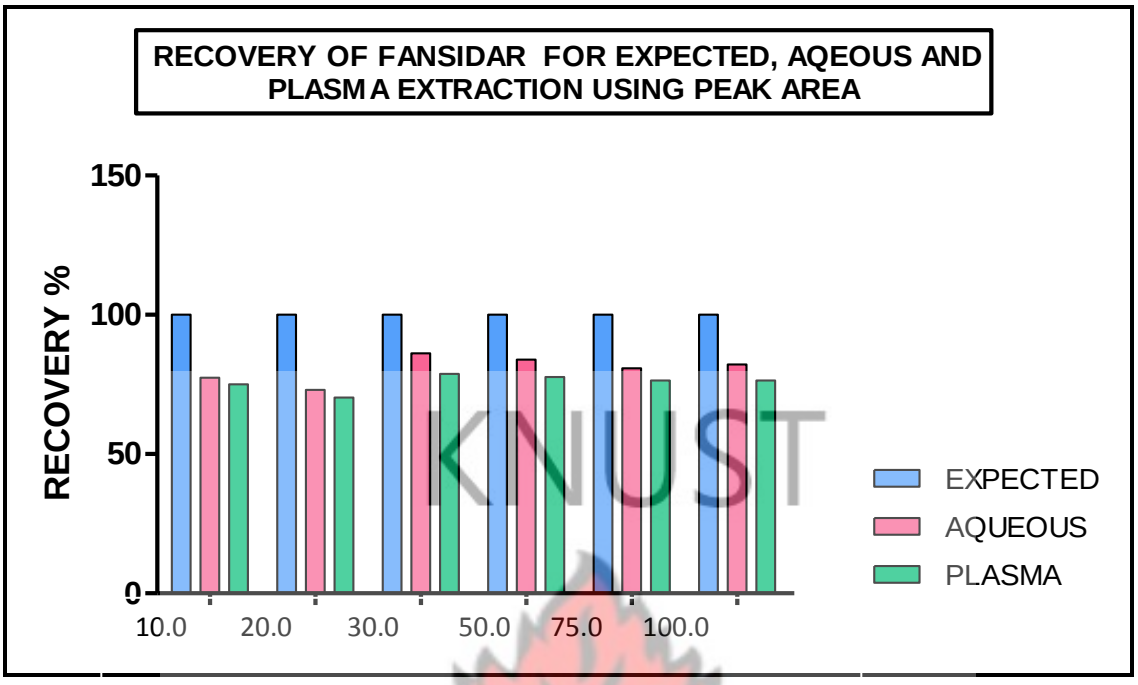

Figure 4.6 Graph of recovery (%) of fansidar for expected,aqueous, and plasma extraction using P A

	Peak height (CV %)	Peak area (CV %)
Aqueous(control)	92.224 ± 4.393 (4.763)	80.590 ± 4.712 (5.847)
Plasma	88.201 ± 6.536 (7.410)	75.804 ± 2.988 (3.942)

Table 4.5 Summary of mean and standard deviation for recovery (%)with their C V % for the six different concentrations of fansidar extracts

	Peak height. (Efficiency of Extraction)	Peak area (Efficiency of Extraction)
Expected versus Aqueous	* (Effective)	** (fairly effective)
Expected versus Plasma	** (Fairly Effective)	** (fairly effective)
Aqueous versus Plasma	ns (Very Effective)	ns (Very Effective)

Table 4.6 Summary of ANOVA and Tukey's test of Fansidar

Recovery values for both the control and the plasma were almost the same (Table 4.5). In addition their coefficient of variation values looked very good. Hence one can say that the extractability method of Fansidar was effective.

Tables 9 and 11 in the Appendix contain data for the ANOVA and Tukey' test for peak height and peak area for fansidar respectively. Figures 4.5 and 4.6 revealed a moderate

variation of extraction of Fn which confirmed the results of ANOVA and Tukey's test.

ANOVA and Tukey's test for Fansidar produced $p < 0.046$ (*) and $p < 0.002$ (**) for peak height and peak area respectively. These were classified as effective and fairly effective extractions under PH and PA respectively. Looking at peak height, expected versus aqueous gave significant difference between the two groups while expected versus plasma gave very significant difference. The aqueous versus plasma produced very effective extraction. Considering peak area, one can conclude that the extractability was fair. This derivation is shown in the summary of Tukey's test in Table 4.6 below.

Dua et al, 1994 reported extraction recoveries averaged 90.6% and coefficients of correlation (r2) > 0.999 for Fansidar. Considering peak height only, this study recorded mean recovery of 90.2% and r^2 = 0.996 with mean coefficient of variation (CV) 6.1%. Statistically, as shown in Table 4.6 the method of extraction of Fansidar was effective.

As first line treatment of falciparum malaria in several resource poor Nations, chloroquine and Fansidar are used but no pharmacokinetic interactions have been detected (Maitland et al, 2004; WHO, 2005) A significant pharmacokinetic interaction between the compounds would have led to suppression or elevation of the bioavailability of any of the components, which was not detected in the case study of (Blessborn, 2009).

Solid Phase Extraction (SPE) technique was used in purification and enrichment of a drug (Blessborn, 2009). This could also help to monitor changes in treatment policy in the Healthcare delivery. For this study, ordinary separation / extraction technique was applied and it also produced good recovery results.

Fansidar is generally well tolerated when used at the recommended doses for malaria therapy. The more severe side effects are related to sulfadoxine and as a sulphur component it can cause hypersensitivity reactions like skin rash or more serious reactions affecting different organ

systems (WHO, 2006). Combination drug like Fansidar is considered safe to use in pregnancy in the first trimester (WHO, 2006), and unfortunately its action is on the same route as chloroquine and its efficacy has become increasingly compromised in several countries (Solomon et al, 2009; Grandesso et al, 2006).

4.1.3 RESULTS OF AMODIAQUINE

Detection parameters for amodiaquine extracts on the HPLC

a) The mean and standard deviation (SD) for retention time for:
    i. Serial standards was 4.23 ± 0.12 minutes
    ii. Aqueous samples was 4.25 ± 0.12 minutes
    iii. Plasma samples was 4.33 ± 0.10
b) Wavelength was 340 nm
c) The Flow rate was 1.0 ml/minute
d) The prepared mobile phase consisted of acetonitrile, ethanol, and 0.10 M buffer (NaH_2PO4) in the ratio of 2: 5: 4 respectively and the pH was adjusted to approximately 3.5

Data of Standard concentration (ug/ml) against Peak Height, & PeakArea, of Amodiaquine		
Standard Conc. (ug/ml)	Response of Amodiaquine (mili-Absorbance)	
	Mean Peak Height (mAb)	Mean Peak Area (mAb)
5.00	6.27	3.81
10.00	11.75	7.20
25.00	33.06	23.00
50.00	64.32	39.89
75.00	92.63	64.97
100.00	129.04	92.86

Table 4.7 Table for plot of standard concentration (µg/ml) against PH and PA for calibration curve for Cq

Table 4.7 consists of a data of standard concentrations (µg/ml) and their corresponding mean responses of peak height and peak area. From this data, plots of standard curves were drawn. These plots are shown in figure 4.7. Using each plot, an equation of the line was derived.

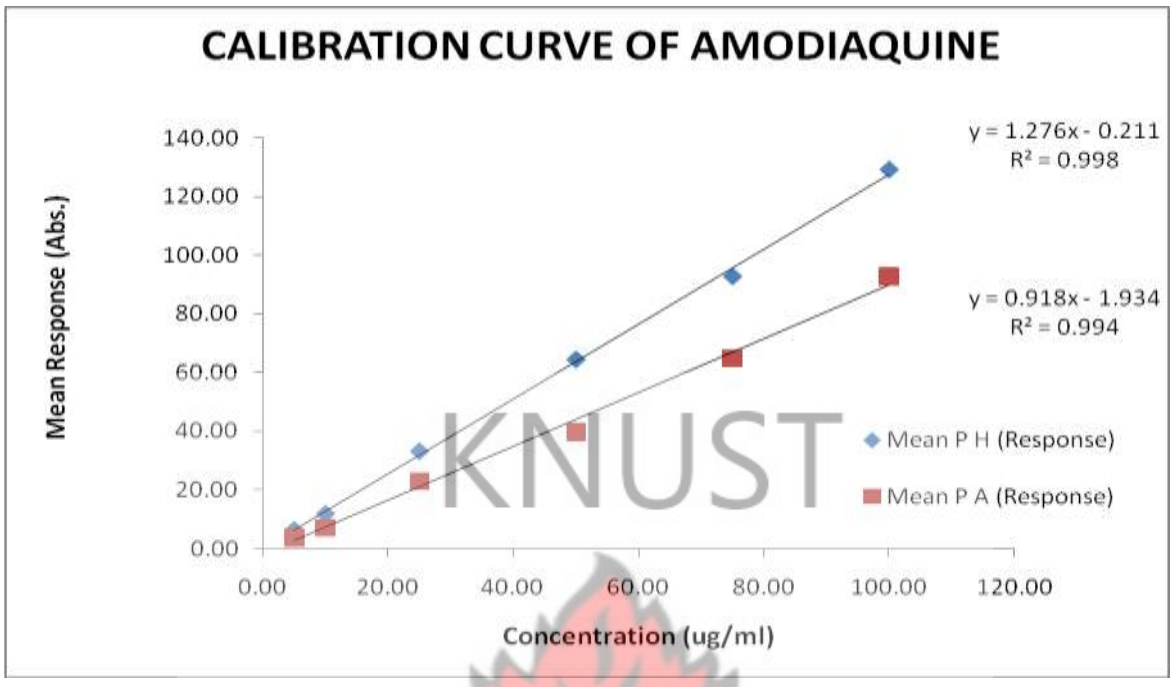

Fig 4.7 Graph of Standard concentration (µg/ml) against PH & PA for plotting calibration curve of amodiaquine

The equation of each plot was used to calculate the concentrations of the repeated measures of the extract samples of amodiaquine. From these calculated concentrations the means and their standard deviations for each level of concentrations were calculated. In addition to these, the mean and standard deviations of each recovery were also calculated. This was done for peak height and peak area for aqueous extraction (control) separately and that for plasma extraction also separately. These results are illustrated in Tables 13 and 14 of the Appendix. Though, there are variations in the recoveries one cannot say whether the extractability was statistically significant, hence, the application of ANOVA and Tukey's test to the results. The data used to perform ANOVA for peak height and peak area respectively are shown in Table 15 & 17 of the Appendix. As usual, the extraction was carried out on six different levels of concentrations with

each level of five replicates. ANOVA was carried out on the expected, aqueous and plasma extractions under peak height and peak area separately. Peak area results of ANOVA and Tukey's test are shown in figure 4.9 and Table 18 of the Appendix while that of peak height shown in figure 4.8 and Table 17 of the Appendix.

Comparison of Recovery %: Aqueous (Control) extracts and Plasma extracts.

Peak Height: The recovery of amodiaquine for aqueous extracts ranged from 79.9% to 97.9% while that of the plasma was from 79.5 % to 92.8 %. (Tables 13 and 14).

Peak Area: The recovery of aqueous extracts of amodiaquine obtained ranged from 87.2 % to 95.1 %, the plasma extracts gave a recovery of 79.2 % to 91.4 % (see Appendix, Tables 13 & 14). The data in Table 4.8 indicates that the extraction method of amodiaquine was effective since the recovery values of both the control and plasma were almost the same. The only difference was that the peak height values were better than peak area.

	Peak height (CV %)	Peak area (CV %)
Aqueous (control)	91.868 ± 6.764 (7.363)	79.375 ± 9.271 (11.680)
Plasma	87.739 ± 4.941 (5.631)	73.138 ± 8.555 (11.697)

Table 4.8 Mean and Standard deviation of recovery (%)with their coefficient of variation (C V %) for the six different concentrations of amodiaquine Extracts

ANOVA with Tukey's test for peak height and peak area are shown in Tables 16 and 18 respectively in Appendix.

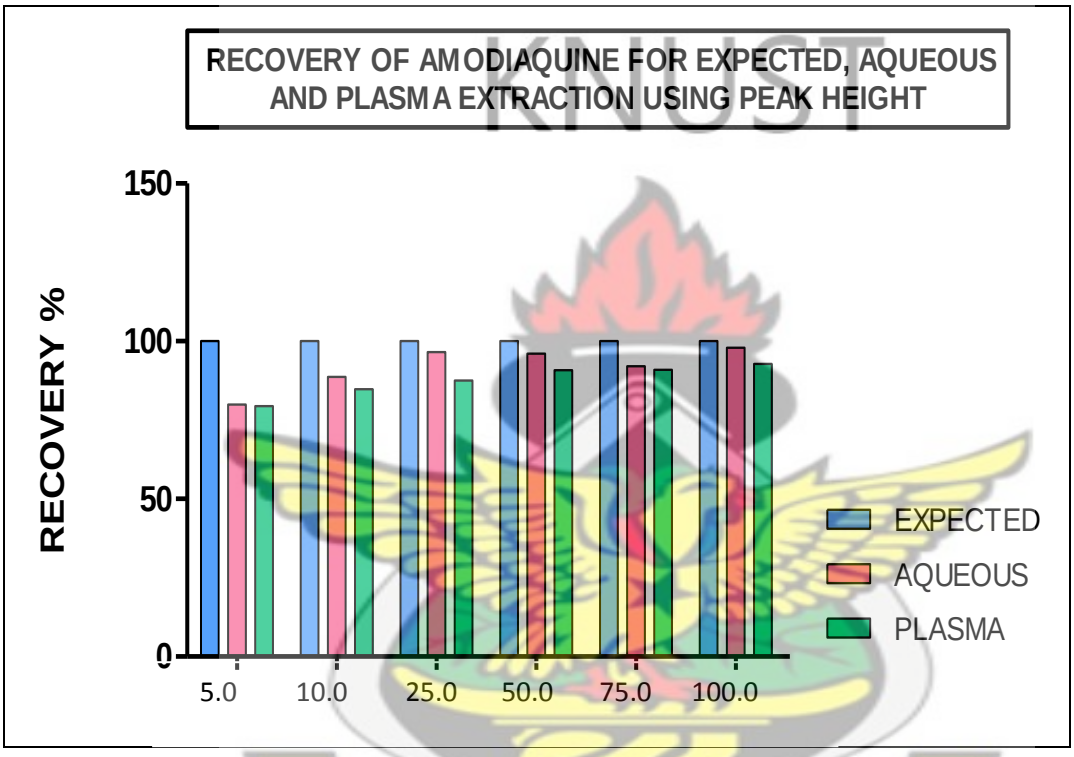

Figure 4.8 Graph of recovery (%) of amodiaquine for expected aqueous, and plasma extraction using PH

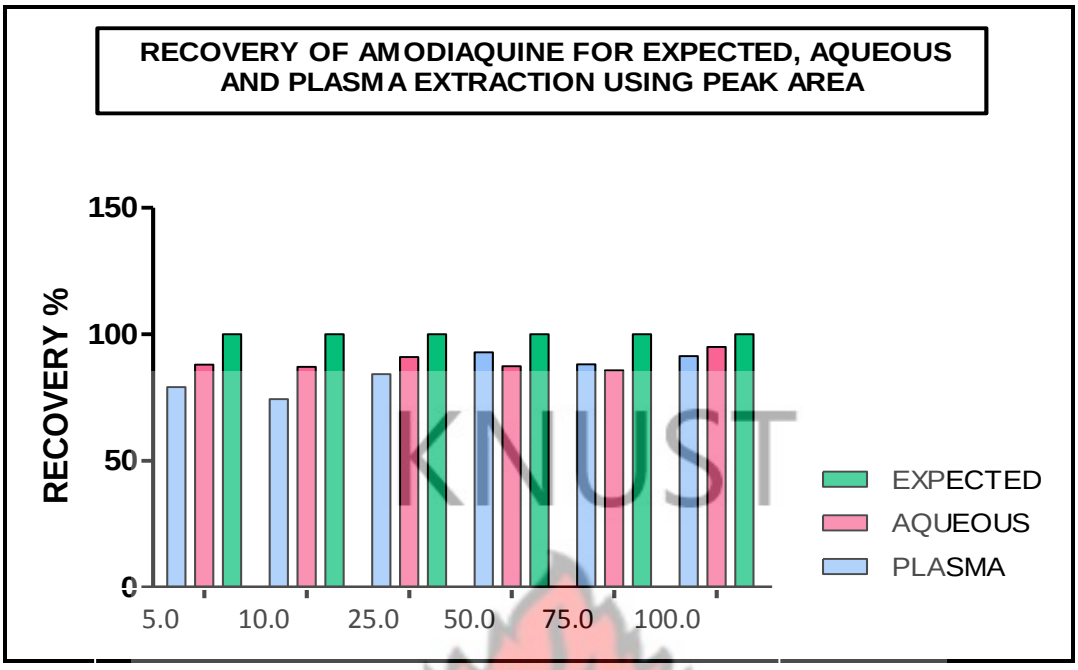

Figure 4.9 Graph of recovery (%) of amodiaquine for expected aqueous, and plasma extraction using PA

Figures 4.8 and 4.9 demonstrate the variation of the recoveries of extraction of amodiaquine, but the degree or extent of the extraction was not clear. Hence, Tukey's test is considered.

	Peak height. (Efficiency of Extraction)	Peak area (Efficiency of Extraction)
Expected versus Aqueous	* (Effective)	** (Fairly Effective)
Expected versus Plasma	** (Fairly Effective)	*** (In-effective)
Aqueous versus Plasma	ns (Very Effective)	ns (Very Effective)

Table 4.9 Summary of ANOV and Tukey's test of Amodiaquine

Under peak Height of Table 4.9, expected versus aqueous gave significant difference between the two groups while expected versus plasma gave very significant difference and the last produced extremely effective extraction. Considering peak area generally, one could say that the extraction method was not very effective.

The established detection wavelenth for amodiaquine in this study was 340nm. Earlier, (Pussard et al, 1986 and Mihaly, et al 1985) also used 340nm in their study because endogenous components peaks of plasma extract produced chromatographic interference at 254 nm. which was more sensitive than the 340nm.

During the study of (Pussard et al 1986 and Mihaly, et al 1985), calibration curves obtained for the serial standard concentrations for amodiaquine, showed linear relationship over the concentration used with the correlation coefficients, $r^2 > 0.99$ while that of this project produced $r^2 = 0.998$.

(Gitau, et al, 2004), have reported 82.48% as mean extraction recoveries for amodiaquine in plasma while mean coefficient of variation for amodiaquine was 4.10 %, (Laurent et al. 1993). The mean recoveries for both aqueous (control) and plasma and their mean coefficient of variation are shown in Table 4.8. The reliability or efficiency of the amodiaquine extraction, an outcome of ANOVA and Tukey's test is displaced in Table 4.9. This showed p values of < 0.05 (ns), < 0.046 (*) and < 0.002 (**) under the peak height but the significant differencies between

the groups under the peak area were too high. Generally, it could be said that the extraction was good uder the peak height.

4.1.4 RESULTS OF QUININE

Detection parameters for quinine extracts on the HPLC

a) The mean and standard deviation for retention time for:
    i. Serial standards was 3.42 ± 0.09 minutes
    ii. Aqueous samples was 3.39 ± 0.11 minutes
    iii. Plasma samples was 3.43 ± 0.14
b) Wavelength was 340 nm
c) The flow rate was 1.2 ml/minute
d) The prepared mobile phase consisted of acetonitrile, ethanol, and 0.10 M buffer (NaH_2PO4) in the ratio of 2: 5: 4 respectively and the pH was adjusted to approximately 3.5

Graph of Standard concentration (µg/ml) against Peak Height & Peak Area		
Std Conc (µg/ml)	Response of Quinine (mili-Absorbance)	
	Mean Peak height (mAb)	Mean Peak area (mAb)
1.00	18.38	8.60
1.50	59.43	34.15
2.00	86.19	50.50
2.50	127.36	69.07
3.00	166.39	96.17
5.00	274.46	142.36

Table 4.10. Table of standard concentration (µg/ml) against Peak Height & Peak Area for plotting

calibration curve of quinine

The data of standard concentrations (µg/ml) and their corresponding mean responses of peak height and peak area of quinine are demonstrated in Table 4.10. From this data, plots of standard curves were drawn. These plots are shown in figure 4.10. For each plot, an equation of the line was derived.

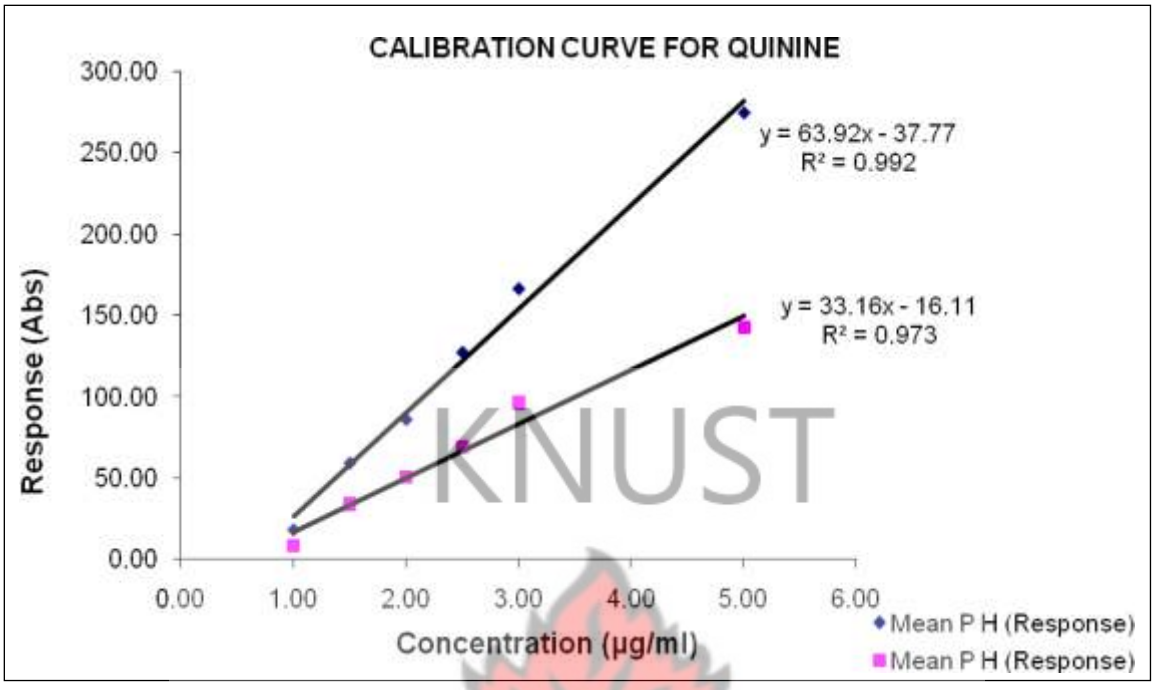

Figure 4.10 Graph of Standard concentrations (µg/ml) against PH and PA for plotting calibration curve of quinine

Using the equation of each plot, the concentrations of the repeated measures of the extract samples of quinine were calculated. From these concentrations the means and their standard deviations for each level of concentration were calculated. These were done for peak height and peak areas for aqueous extraction (control) and that for plasma extracts. The mean and standard deviation for the recoveries were also calculated and shown in Tables 19 and 20 of the Appendix.

Comparison of Recovery (%): Aqueous (Control) and Plasma extracts.

Peak height: The recovery of aqueous extracts of quinine obtained ranged from 85.6% to 96.0%. The plasma extracts gave a recovery of 84.5% to 90.4% (Appendix – Tables 19 & 20).

Peak area: The peak area gave a recovery of 65.8% to 78.9% and 68.0% to 89.4% for aqueous extracts and plasma extracts respectively (Tables 19 and 20 in Appendix).

	Peak height (CV %)	Peak area (CV %)
Aqueous (control)	93.944 ± 4.951 (5.270)	84.163 ± 8.599 (10.217)
Plasma	88.640 ± 2.686 (3.030)	75.562 ± 5.251 (6.949)

Table 4.11 Mean and standard deviations for recovery (%) with their coefficient of variation (C V %) for the six different concentrations of quinine extracts

Comparing the mean control and plasma recoveries for both peak height and peak area of the (Table 4.11), one could say that the extraction method of quinine was good. This not with standing, ANOVA and Tukey's test need to be applied to the results of the recovery.

Peak height ANOVA with Tukey's test for quinine extraction is demonstrated in Table 21 (Appendix) while that for peak area appears in Table 22 (Appendix).

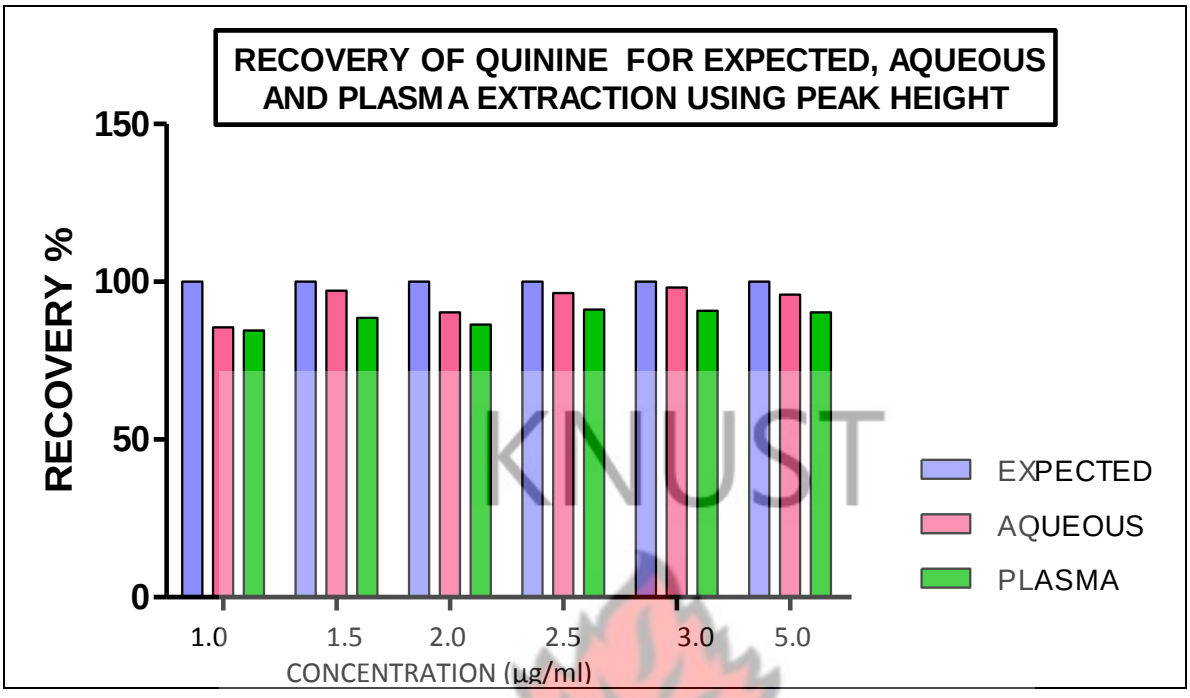

Figure 4.11 Graph of recovery (%) of quinine for expected aqueous, and plasma extraction using PH

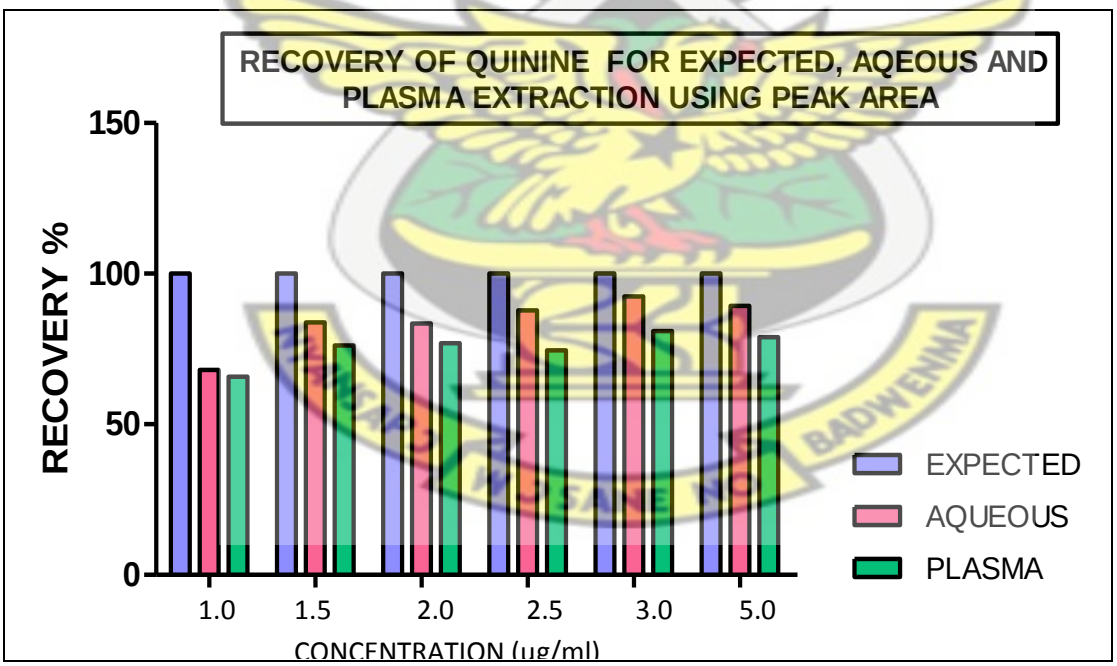

Figure 4.12 Graph of recovery (%) of quinine for expected aqueous, and plasma extraction using PA

The bar charts of figures 4.11 and 4.12 showed the variation of extraction of quinine. These figures produced only general trends of recoveries but not sufficient information on the degree of the extractability of the drug. Hence, ANOVA and Tukey's test were employed.

	Peak height. (Efficiency of Extraction)	Peak area (Efficiency of Extraction)
Expected versus Aqueous	* (Effective)	*** (In-effective)
Expected versus Plasma	*** (In-effective)	*** (In-effective)
Aqueous versus Plasma	* (Effective)	ns (Very Effective)

Table 4.12 Summary of ANOVA and Tukey's test of Quinine

There was a significant difference between the expected and the aqueous under the peak height (Table 4.12). The same significant difference was observed between aqueous and the plasma. The difference between the expected and the plasma extractions were extremely significant. Under the peak area, in exception of plasma and aqueous the rest showed extremely significant. Considering Table 4.12 generally, one could say that the extraction method of quinine was fairly effective.

An earlier work done by Papadoyannis et al, (2005) used a mobile phase consisted of methanol / acetonitrile / 0.1 mol/L ammonium acetate (45:15:40 v/v) for extractiuon of quinine. This was delivered at a flow rate of 1.0 mL/minute at a detection wavelength of 325 nm. A retention time of 5.1 minutes was obtained for quinine. The method of regression equation revealed correlation coefficient of 0.9926. Mean recovery of 92.1% and coefficient of variation values < 10 % were recorded. The statistical evaluation of the method was examined by performing intra-day (n = 8) and inter-day calibration (n = 8) and was found to be satisfactory, with high accuracy and precision results.

For this study, a mobile phase consisted of acetonitrile, ethanol, and 0.10 M buffer (NaH_2PO4) in the ratio of 2: 5: 4 (v/v) respectively and the pH adjusted to approximately 3.5 was used. The assay on the HPLC was performed at detection wavelength of 340 nm. The flow rate was adjusted to 1.2 ml/minute. These parameters produced mean recovery of about 91 % and coefficient of variation of about 9 % at mean retention time of 3.4 minutes (plasma/aqueous) extraction of quinine. Correlation coefficients of 0.992 and 0.973 were obtained for peak height and peak area respectively. The statistical package performed on the results is ANOVA and Tukey's test which produced a mean p value of 0.0001. This showed that the difference between groups were extremely significant meaning the method of extraction was not efficient.

4.1.5 RESULTS OF PYRIMETHAMINE

Detection parameters for pyrimethamine extracts on the HPLC

a) The mean and standard deviation (SD) for retention time for:
    i. Serial standards was 3.75 ± 0.10 minutes

ii. Aqueous samples was 3.70 ± 0.05 minutes

iii. Plasma samples was 3.72 ± 0.08 minutes

b) Wavelength was 220 nm

c) The Flow rate was 1.0 ml/minute

d) The prepared mobile phase consisted of acetonitrile, methanol, and 0.10 M buffer (NaH_2PO_4) in the ratio of 3: 5: 4 respectively and the pH adjusted to approximately 3.0

Standard concentration (µg/ml) versus Peak Height & Peak Area, (Response) of Pyrimethamine		
Standard Conc (ug/ml)	Response of Pyrimethamine (mili-Absorbance)	
	Mean Peak Height (mAb)	Mean Peak Area (mAb)
5	11.083	4.653
10	21.767	9.62
20	41.403	21.55
30	64.177	36.417
50	104.487	62.16
75	154.08	85.59

Table 4.13. Table of standard concentration (µg/ml) against Peak Height & Peak Area for plotting

calibration curve of pyrimethamine

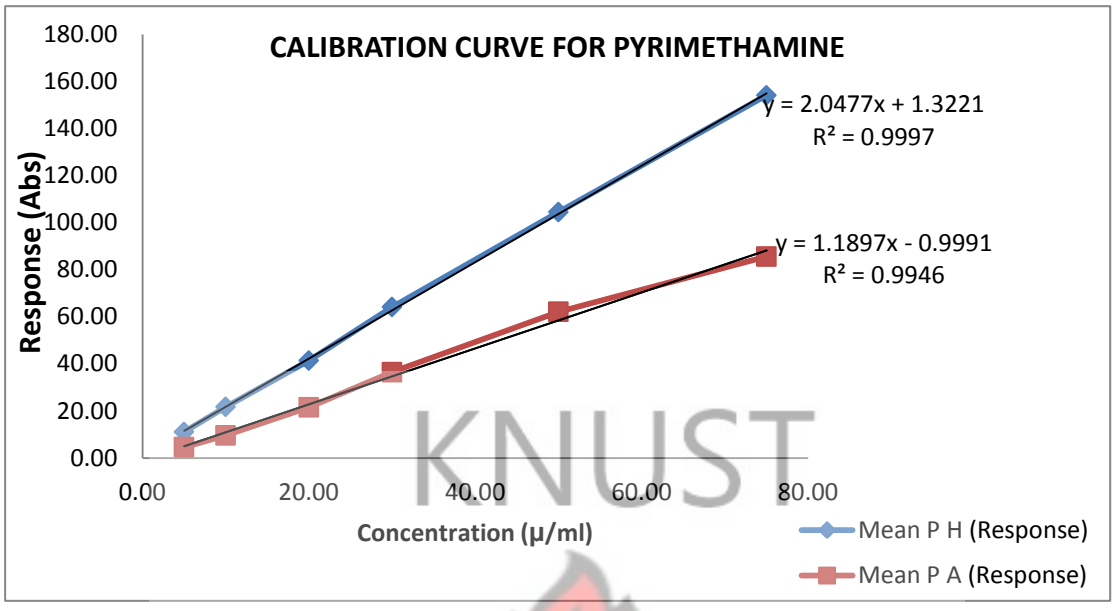

Figure 4.13 Graph of Standard concentrations (µg/ml) against PH and PA for plotting calibration curve of pyrimethamine

The data of standard concentrations (µg/ml) and their corresponding mean responses of peak height and peak area were displayed in Table 4.13. From this data, plots of standard curves were drawn. These plots are shown in figure 4.13. For each plot, an equation of the line was derived.

Using the equation of each plot, the concentrations of the repeated measures of the extract samples of pyrimethamine were calculated. From these calculated concentrations the means and their standard deviations for each level of concentrations were calculated. These were done for peak height and peak area for aqueous (control) separately and that for plasma also separately. The mean and standard deviation recoveries were also calculated (Tables 25 and 26 of the Appendix).

It is well known that drugs in tablets forms with more rapid dissolution in the gastro-intestinal tract usually have more rapid systemic absorption, as measured by the absorption rate constant or time to maximal concentrations (Rowland and Tozer, 1995) Therefore, a rapid absorption for an anti malarial formulations such as Chloroquine, Fansidar and Pyrimethamine could gear towards rapid parasite clearance, while slow absorption may lead to delayed parasite clearance and slower clinical response (Stockley, 2002). On the other hand, impaired absorption may occur when different formulations are given simultaneously (Bergqvist et al, 1985).

Among the various determinants of treatment response, the achievement of sufficient anti-malarial drugs levels in the blood is essential for curing malaria (Tarning et al, 2006). Essential data on the disposition of the anti-malarial drug products in use are lacking especially in children with malaria (Barnes et al, 2007). In addition, the majority of these drugs have not been developed with respect to the selection of doses and dosing regimens based on stringent pharmacokinetics–pharmacodynamics relationships. As a consequence, some patients or patient categories may be under dosed resulting in treatment failures and promoting parasite resistance or over dosed, a cause of toxicity (Barnes et al, 2007).

To improve the current understanding of anti-malarial drugs pharmacokinetics, efficacy and toxicity, a Liquid Chromatography–tandem Mass Spectrometry method was developed (Hodel et al, 2009) This requires only 200ul of plasma for the simultaneous determination of 14 anti-malarial drugs (including Pyrimethamine) and their metabolites which are the components of the

current first-line combination treatments for malaria. This study for Pyrimethamine used High-Performance Liquid Chromatography method for the assay.

Comparison of Recovery (%): Aqueous (Control) extracts and Plasma extracts.

Peak Height: Tables 25 and 26 in the Appendix show that recovery of pyrimethamine aqueous extracts ranged from 81.2 % to 91.8 %. The plasma extracts of pyrimethamine gave values from 81.1 % to 90.2 %.

Peak Area: The recovery for plasma varied between 61.9 % and 83.8 %. That of the aqueous extended from 64.8 % to 83.8 %.These results are summarized in Tables 4.14. and 4.15 and Table 28 in the Appendix. Without statistical analysis, one can say that the extraction method for pyrimethamine was efficient.

.	Peak height (CV %)	Peak area (CV %)
Aqueous (control)	90.523 ± 5.072 (5.603)	79.375 ± 9.271 (11.680)
Plasma	87.297 ± 3.317 (3.800)	73.138 ± 8.555 (11.697)

Table 4.14 Mean and standard recovery (%)with their coefficient of variation (C V %) for the six different concentrations of Pyrimethamine extracts

The results of the ANOVA and Tukey's test for peak height and peak area for pyrimethamine are shown in Table 4.15 as well as Tables 28 & 30 in the Appendix respectively.

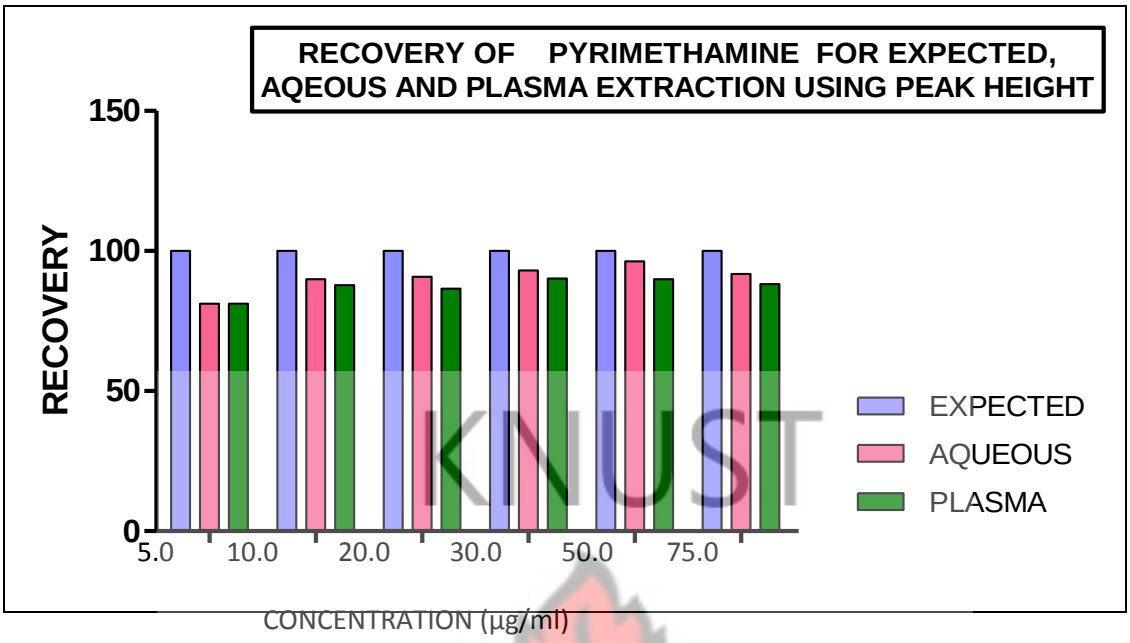

Figure 4.14 Graph of recovery (%) of Pyrimethamine for expected aqueous, and plasma extraction using PH

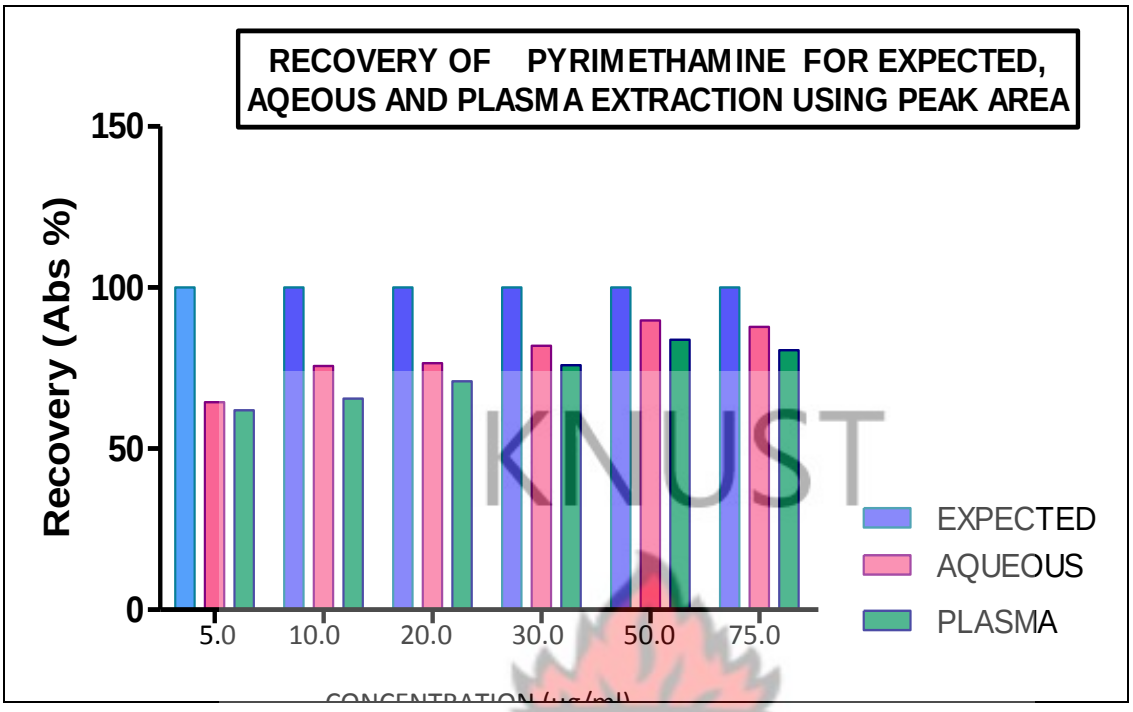

Figure 4.15 Graph of recovery (%) of Pyrimethamine for expected aqueous, and plasma extraction using PA

Figures 4.14 and 4.15 show the variation of extraction of pyrimethamine. These figures produced only general trends of recoveries but not sufficient information on the degree of the extractability of the drug. Hence, ANOVA with Tukey's test was employed.

	Peak height. (Efficiency of Extraction)	Peak area (Efficiency of Extraction)
Expected versus Aqueous	*** (In-effective)	*** (In-effective)
Expected versus Plasma	*** (In-effective)	*** (In-effective)
Aqueous versus Plasma	ns (Very Effective)	ns (Very Effective)

Table 4.15 Summary of ANOVA and Tukey's test of Pyrimethamine

From Table 4.15, it can be deduced that the extraction method for pyrimethamine was not effective since the differences between the Aqueous and the Expected, as well as the Expected and Plasma were both extremely significant. However, there was no significant difference between the Aqueous and the Plasma indicating an efficient extraction.

The recovery values displayed in Table 4.14 with their coefficient of variation have shown that the extraction of pyrimethamine was good but the summary of Tukey's test in Table 4.15 has shown that the extractability was poor. The p value of < 0.0001 (***) means that there was extremely significant diferrence between the groups.

Green et al, (2002) used a method of extraction of pyrimethamine from whole blood dried onto filter paper. The extracts were then assayed on HPLC. The mobile phase consisted of 5.0 mM of 1-octane sulphonic acid (adjusted to pH 2.5), methanol and acetonitrile (30%) in the ratio of 60:10:30 (v/v). The flow rate was adjusted to 3 ml/minute and a detection wavelength of 254 nm was used. The calibration curve was linear in the range 50–1000 ng/ml ($r^2 > 0.95$). The inter-assay and intra-assay CV value < 15 % and the mean recovery was 73.8 %. A retention time of 3.4 minutes was obtained.

This study produced a mean recovery of 93.8 % and 76.2 % for peak height (PH) and peak area (PA) respectively, for pyrimethamine extracts analysis on HPLC. These gave mean coefficient of variations of 4 % for peak height and 9 % for peak area. The prepared mobile phase consisted of acetonitrile, methanol, and 0.10 M buffer (NaH_2PO_4) in the ratio of 3: 5: 4 (v/v) respectively and the pH adjusted to approximately 3.0. With a flow rate of 1.0 ml/minute and a detection wavelength of 220 nm, a mean retention time of 3.7 minutes was achieved. The correlation coefficient, r^2 = 0.999 and 0.994 were obtained for PH and PA respectively. This is displayed in Figure 4.13. The result of p value, < 0.0001 (***) has shown that the extraction method was inefficient.

The aim of this study was to find the possibility/probability of extraction some known antimalarial drugs from blood plasma. For effective work and fulfillment of the aim, it means that there should not be any difference between the means of the comparable groups, hence the null hypothesis.

The confidence interval chosen was 95% level ($P < 0.05$). Thus, at this level there should not be any significant difference between the groups. The p–value which gives more meaning to significant difference does not indicate the size or importance of the observed effect. The two vary together; however, the larger the effect of the significance, the smaller the sample size (Churchill et al, 2000).

The statistical package, ANOVA and Tukey's test performed showed that there were various levels of significant differences. Since the null hypothesis is true, it means that an error has been made. This type 1 error (α error) result is obtained if traditionally p–value is less than 0.05 (5%). Type 1 error can be reduced if sample size is increased, (Churchill et al, 2000).

Type 1 error or α error was pronounced (extremely significant) in the methods of extraction of quinine and pyrimethamine. It was partially observed in the results of the other antimalarials, chloroquine, amodiaquine and fansidar.

The Extraction was carried out on plasma/aqueous samples of six (6) different levels of concentrations with replicates of five (5) times for each, hence the sample size for this study was six (6). If the sample size were to be increased to about twenty (20) better results could have been achieved and Type 1 error could have been avoided.

High-performance liquid chromatography (HPLC) is by far the preferred technique for the assay of antimalarials and in most instances is superior to GC, ELISA and other techniques (Basco LK; 2004). Hence the choice of HPLC for this study.

4.2.1 RESULTS OF DOUBLE BLIND TEST

The double blind test (DBT) was performed on twenty two (22) pregnant women. The results of the tests are shown in Tables 4.16 and 4.17 below:

Chloroquine Results of the Double Blind Test	
No. of days after drug has been taken	Concentration (ug/ml)
10	8.98 ± 0.09
14	4.78 ± 0.03
11	7.32 ± 0.13
3	15.96 ± 0.47
11	9.77 ± 0.13
12	7.32 ± 0.14

Table 4.16 Reasults of chroloquine in Double Blind Test

Fansidar Results of the Double Blind Test	
No. of days after drug has been taken	Concentration (ug/ml)
5	18.35 ± 0.26
7	13.33 ± 0.07
6	16.13 ± 0.34

3	20.93 ± 0.08
4	17.99 ± 0.53
5	16.74 ± 0.37
9	12.36 ± 0.42
10	10.17 ± 0.45
2	17.48 ± 0.41
8	14.37 ± 0.18

Table 4.17 Reasults of Fansidar in Double Blind Test

The DBT samples were chloroquine, fansidar and placebo. They were given to the pregnant women at random. Blood samples of the women concerned were taken after some days of taking the antimalarials. Plasma of these samples were extracted along side the spiked aqueous and plasma samples and their analysis carried out on the HPLC.

Out of the twenty two (22) samples, ten (10) proved to be fansidar and six (6) were chloroquine. Four (4) were classified as placebo with two (2) contaminations. The results were later confirmed at Supervised monthly antimalarial treatment (SMAT II), A project executed at Ejisu Juaben, (Ashanti Region).

4.2.2 RESULTS OF VOLUNTEERS' SAMPLES

Three volunteers were employed. One for Chloroquine, another for Fansidar and the third took Amodiaquine. The results are shown in Tables 4.18, 4.19 and 4.20 below.

No. of days after drug has been taken	Volunteer's Chloroquine Result (Concentration ug/ml)
1	261.36 ± 0.08
21	15.34 ± 0.06

Table 4.18 Results of volunteer' for Chlorioquine

No. of days after drug has been taken	Volunteer's Fansidar Result (Concentration ug/ml)
1	179.94 ± 0.95
21	8.26 ± 0.83

Table 4.19 Results of volunteers' for Fansidar

No. of days after drug has been taken	Volunteer's Amodiaquine Result (Concentration ug/ml)
1	197.94 ± 0.95
21	10.26 ± 0.83

Table 4.20 Results of volunteers' Amodiaquine

Blood samples of the volunteers' were taken on the first day (VS_1) and twenty one days (VS_{21}) of taking the antimalaria drugs concerned. Extraction of these plasma samples were carried out along side the spiked plasma and aqueous samples.The results of $CqVS_1$ & $CqVS_{21;}$ $FnVS_1$ & $FnVS_{21;}$ and $AdqVS_1$;& $AdqVS_{21}$ are shown in Tables 4.18, 4.19 and 4.20 respectively.

Analysis of the volunteers' extracts on the HPLC produced the following retention time of the various antimalarials: 2.87 ± 0.15 minutes for chloroquine, 4.36 ± 0.32 minutes for amodiaquine and 2.83 ± 0.38 minutes for Fansidar.

Recovery was not performed on the DBT and volunteers' sample results because pure samples of various antimalaria drugs were used for the standards as well as the spiked samples. On the other hand, what each participant had taken was purchased from the local market hence, not the same as the pure ones.

CHAPTER FIVE

5.1 CONCLUSION

Analysis on the HPLC normally gives responses (milliabsorbance) in two forms, peak height and peak area. Theoretically, peak height is easier to calculate than the peak area (Gitau et al, 2004). Practically the choice depends on the mobile phase being used and the type of extract being analyzed. Truly there are some other minor considerations for one to adopt a particular response. This study compared both peak area and height to determine the best way of measuring the response.

Recovery (%) of an extract is an essential parameter required in an extraction method for a drug. But to a large extent, it has been observed that one cannot rely on the recovery alone for effective work in analysis. Scientifically, recovery does not clearly indicate the degree of extraction hence the need to compare the group means of the recoveries using ANOVA with Tukey's multiple comparison test. This calls for the introduction of a standard in the form of the pure compound which was used to prepare the serial standards.

ANOVA for chloroquine produced $p < 0.0456$ (*) and $p < 0.016$ (**) meaning extraction was effective. Tukey's multiple comparison tests also gave results that showed that the method of extraction was generally good. However it was clear that at lower concentration, extraction was poor. The method is therefore recommended.

The ANOVA on the mean recoveries for fansidar gave values of $p < 0.046$ (*) and $p < 0.002$ (**) for peak height and peak area respectively. Generally, these values showed that the extraction was effective. Tukey's test classified the extraction as very effective. In summary, one could say that the extraction of fansidar was effective, thus the method of extraction is recommended.

For Quinine, ANOVA with Turkeys multiple comparison test for the mean recoveries indicated poor results; $p < 0.0001$, especially under peak area. Under peak height the extraction was only fairly effective. Though the mean recovery (%) for both peak heights and peak area looked good. The method of extraction therefore needs to be improved.

ANOVA performed on mean recoveries for amodiaquine gave $p<0.0018$ (**) and $p < 0.0001$ (**) for peak height and peak area respectively. This showed that the extraction was only effective under peak height. Tukey's test indicated that the extraction was effective. The method is therefore recommended.

When the mean recoveries for pyrimethamine extracts was subjected to ANOVA and Tukey' test, $p < 0.0001$ was produced for both peak area and peak height. This showed that there was an extremely significant difference hence the extraction was not effective. The method therefore needs to be improved.

Some observations have been made on the entire extractions of the specified antimalarial drugs. These are as follows:

1) The recovery values for the control samples were higher than the blood plasma values. These could be the result of the plasma having some drug binding agents. These agents may tend to adhere to the drug concerned hence, it was not free for thorough extraction. On the other hand, the aqueous had no binding agent. Thus, extraction was much better and the values closer to the standard.

2) The peak height values were higher than the peak area values. This, for some school of thought on chromatography makes the use of peak height much preferred (Gitau et al, 2004).

5.2 RECOMMENDATIONS

1 This study recommends the method employed for the extraction of Chloroquine, Fansidar and Amodiaquine from plasma be adopted, but for Quinine and Pyrimethamine, the extraction methods need further studies.

2 A further study should be carried out to develop the cut-off plasma threshold for anti-malarias employed in this study.

3 The method developed in this study should be extended to cover combined anti-malarials currently in used.

4 Recovery (%) of an extract is an essential parameter required in an extraction method for a drug. But to a large extent, it has been observed that one cannot rely on the recovery alone for effective work in analysis. Scientifically, recovery does not clearly indicate the degree or the effectiveness of the extraction. Hence, there is need to compare the group means of the recoveries using ANOVA with Tukey's multiple comparison test or any statistical package that can give the effectiveness of the extraction.

APPENDIX

CHLOROQUINE

RESULT OF EXTRACTED CHLOROQUINE FROM SPIKED AQUEOUS SAMPLES				
	FROM PEAK HEIGHT		FROM PEAK AREA	
EXPECTED VALUE (ug/ml)	EXTRACTED MEAN (ug/ml)	RECOVERY MEAN (%)	EXTRACTED MEAN (ug/ml)	RECOVERY MEAN (%)
5.00	3.258 ± 0.037	65.157 ± 0.743	2.028 ± 0.102	40.016 ± 2.045
10.00	7.768 ± 0.020	77.684 ± 0.196	7.226 ± 0.044	72.262 ± 0.442
25.00	24.507 ± 0.284	98.097 ± 1.177	19.241 ± 0.508	76.965 ± 2.031
50.00	48.633 ± 0.290	97.265 ± 0.578	34.631 ± 0.327	69.261 ± 0.652
75.00	68.118 ± 0.337	90.824 ± 0.449	59.337 ± 0.521	79.116 ± 0.693
100.00	96.118 ± 0.088	96.118 ± 0.088	77.117 ± 0.397	77.117 ± 0.397

Table 1 Results of extracted chloroquine from spiked aqueous samples

RESULT OF EXTRACTED CHLOROQUINE FROM SPIKED PLASMA SAMPLES				
	FROM PEAK HEIGHT		FROM PEAK AREA	
EXPECTED VALUE(ug/ml)	EXTRACTED MEAN (ug/ml)	RECOVERY MEAN (%)	EXTRACTED MEAN(ug/ml)	RECOVERY MEAN(%)
5.00	2.727 ± 0.037	54.541 ± 0.743	1.782 ± 0.118	35.645 ± 2.351
10.00	7.876 ± 0.152	79.756 ± 1.525	6.666 ± 0.232	66.661 ± 2.320

25.00	20.881 ± 0.111	83.526 ± 0.445	18.712 0.252	74.861 ± 1.408
50.00	46.791 ± 0.130	92.914 ± 0.999	43.934 ± 0.155	87.868 0.311
75.00	65.516 ± 0.237	87.355 ± 0.316	56.513 ± 0.206	75.351 ± 0.273
100.00	95.072 ± 0.281	95.071 ± 0.281	77.342 0.118	77.343 0.118

Table 2 Results of extracted chloroquine from spiked aqueous samples

CHLOROQUINE PEAK HEIGHT (PH) DATA FOR ANOVA ANALYSIS.								
EXPECTED			AQUEOUS EXTRACT			PLASMA EXTRACT		
MEAN (%)	SD	N	MEAN (%)	SD	N	MEAN (%)	SD	N
100	0	5	65.157	0.743	5	54.541	0.743	5
100	0	5	77.684	0.196	5	79.756	1.525	5
100	0	5	98.097	1.177	5	83.526	0.445	5
100	0	5	97.265	0.578	5	92.914	0.999	5
100	0	5	90.824	0.449	5	87.355	0.316	5
100	0	5	96.118	0.088	5	95.071	0.281	5

Table 3 Chloroquine Peak Height (PH) Data for ANOVA

P value	0.0456
P value summary	*
Are means signif. different? (P < 0.05)	Yes
Number of groups	3
F	3.819
R squared	0.3374

ANOVA Table	SS	df	MS
Treatment (between columns)	1002	2	501.1
Residual (within columns)	1968	15	131.2
Total	2971	17	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Expected vs aqueous	12.48	2.668	No	ns	-4.706 to 29.66
Expected vs plasma	17.81	3.808	Yes	*	0.6245 to 34.99
Aqueous vs plasma	5.33	1.14	No	ns	-11.85 to 22.51

Table 4 One-way ANOVA for chloroquinine using peak height

CHLOROQUINE PEAK AREA (PA) DATA FOR ANOVA								
EXPECTED			AQUEOUS EXTRACT			PLASMA EXTRACT		
MEAN	SD	N	MEAN	SD	N	MEAN	SD	N
100	0	5	40.563	2.045	5	35.645	2.351	5
100	0	5	72.262	0.442	5	66.661	2.320	5
100	0	5	76.965	2.031	5	74.861	1.408	5
100	0	5	69.261	0.652	5	87.868	0.311	5
100	0	5	79.116	0.693	5	75.351	0.273	5
100	0	5	77.118	0.397	5	77.343	0.118	5

Table 5 Chloroquine Peak Area (PA) Data for ANOVA

P value	0.0014
P value summary	**
Are means signif. different? (P < 0.05)	Yes
Number of groups	3
F	10.52
R squared	0.5839

ANOVA Table	SS	df	MS
Treatment (between columns)	3742	2	1871
Residual (within columns)	2667	15	177.8
Total	6408	17	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
EXPECTED vs. AQUEOUS	30.79	5.656	Yes	**	10.79 to 50.78
EXPECTED vs PLASMA	30.38	5.581	Yes	**	10.38 to 50.38
AQUEOUS vs PLASMA	-0.4073	0.07483	No	ns	-20.41 to 19.59

Table 6 One-way ANOVA for chloroquinine using Peak Area

FANSIDAR

RESULT OF EXTRACTED FANSIDAR FROM SPIKED AQUEOUS SAMPLES				
	FROM PEAK HEIGHT		FROM PEAK AREA	
EXPECTED VALUE (ug/ml)	EXTRACTED MEAN (ug/ml)	RECOVERY MEAN (%)	EXTRACTED MEAN (ug/ml)	RECOVERY MEAN (%)
10.00	8.962 ± 0.131	89.621 ± 1.309	7.748 ± 0.024	77.481 ± 0.237
20.00	17.377 ± 0.168	86.888 ± 0.838	14.608 ± 0.083	73.040 ± 415
30.00	27.220 ± 0.550	90.734 1.833	25.845 ± 0.336	86.149 ± 1.119
50.00	48.982 ± 0.437	98.0.300 ± 0.403	41.0.951 ± 0.722	83.902 ± 1.444
75.00	71.682 ± 0.258	95.577 ± 0.344	60.618 ± 1.108	80.824 ± 1.477
100.00	95.837 ± 0.586	95.837 ± 0.586	82.144 ± 1.260	82.144 ± 1.260

Table7 Results of extracted Fansidar from spiked aqueous samples

RESULT OF EXTRACTED FANSIDAR FROM SPIKED PLASMA SAMPLES					
FROM PEAK HEIGHT			FROM PEAK AREA		
EXPECTED VALUE (ug/ml)	EXTRACTED MEAN (ug/ml)	RECOVERY MEAN (%)	EXPECTED VALUE (ug/ml)	EXTRACTED MEAN (ug/ml)	RECOVERY MEAN (%)
10.00	7.991 ± 0.267	80.911 ± 2.142	10.00	7.508 ± 0.075	75.085 ± 0.749
20.00	16.282 ± 0.168	81.140 ± 0.814	20.00	14.057 ± 0.166	70.286 ± 0.831
30.00	25.572 ± 0.108	85.240 ± 0.359	30.00	23.661 ± 0.264	78.870 ± 0.879
50.00	46.664 ± 0.192	93.328 ± 0.383	50.00	38.821 ± 0.556	77.641 ± 1.113
75.00	70.450 ±0.529	93.500 ±0.705	75.00	57.352 ± 521	76.469 ± 0.691
100.00	95.089 ±0.377	95.089 ± 0.377	100.00	76.473 ± 1.054	76.473 ± 1.054

Table 8 Results of extracted Fansidar from spiked plasma samples

FANSIDAR PEAK HEIGHT (PH) DATA FOR ANOVA ANALYSIS.								
EXPECTED			AQUEOUS EXTRACT			PLASMA EXTRACT		
MEAN	SD	N	MEAN	SD	N	MEAN	SD	N
100	0	5	89.621	1.309	5	80.911	2.142	5
100	0	5	86.888	0.838	5	81.140	0.841	5
100	0	5	90.734	1.833	5	85.240	0.359	5
100	0	5	98.300	0.403	5	93.328	0.383	5
100	0	5	95.577	0.344	5	93.500	0.705	5
100	0	5	95.837	0.586	5	95.089	0.377	5

Table 9 Fansidar Peak Height (PH) Data for ANOVA

P value	0.0016
P value summary	**
Are means signif. different?(P < 0.05)	Yes
Number of groups	3
F	10.26
R squared	0.5777

ANOVA Table	SS	df	MS
Treatment (between columns)	424.1	2	212.1
Residual (within columns)	310.1	15	20.67
Total	734.2	17	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Expected vs Aqueous	7.174	3.865	Yes	*	0.3542 to 13.99
Expected vs Plasma	11.8	6.356	Yes	**	4.979 to 18.62
Aqueous vs Plasma	4.625	2.492	No	ns	-2.195 to 11.44

Table 10 One-way ANOVA for Fansidar using peak height

FANSIDAR PEAK AREA (PA) DATA FOR ANOVA ANALYSIS.								
EXPECTED			AQUEOUS EXTRACT			PLASMA EXTRACT		
MEAN (%)	SD	N	MEAN (%)	SD	N	MEAN (%)	SD	N
100	0	5	77.481	0.234	5	75.085	0.749	5
100	0	5	73.040	0.415	5	70.286	0.831	5
100	0	5	86.149	1.119	5	78.870	0.879	5
100	0	5	83.902	1.444	5	77.641	1.113	5
100	0	5	80.824	1.477	5	76.469	0.691	5
100	0	5	82.144	1.260	5	76.473	1.054	5

Table 11 Fansidar Peak Area (PA) Data for Anova Analysis

P value	P<0.0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
Number of groups	3
F	94.94
R squared	0.9268

ANOVA Table	SS	df	MS
Treatment (between columns)	1970	2	985.1
Residual (within columns)	155.6	15	10.38
Total	2126	17	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Expected vs Aqueous	19.41	14.76	Yes	***	14.58 to 24.24
Expected vs Plasma	24.2	18.4	Yes	***	19.36 to 29.03
Aqueous vs Plasma	4.786	3.639	No	ns	-0.04546 to 9.617

Table 12 One-way ANOVA for Fansidar using Peak Area

AMODIAQUINE

RESULT OF EXTRACTED AMODIAQUINE FROM SPIKED AQUOEUS SAMPLES					
FROM PEAK HEIGHT			FROM PEAK AREA		
EXPECTED VALUE (ug/ml)	EXTRACTED MEAN (ug/ml)	RECOVERY MEAN (%)	EXPECTED VALUE (ug/ml)	EXTRACTED MEAN (ug/ml)	RECOVERY MEAN (%)
5.00	3.995 ± 0.039	79.903 ± 0.781	5.00	4.405 ± 0.067	88.096 ± 1.342
10.00	8.873 ± 0.096	88.723 ± 0.957	10.00	8.720 ± 0.225	87.198 ± 2.251
25.00	24.129 ± 0.311	96.515 ± 1.242	25.00	22.772 ± 0.137	91.087 ± 0.548
50.00	48.005 ± 0.123	96.010 ± 0.245	50.00	43.744 ± 0.818	87. 489 ± 1.636
75.00	69.085 ± 0.916	92.114 ±1.221	75.00	64.394 ± 0.833	85.859 ± 1.111
100.00	97.941 ± 0.662	97.941 ± 0.662	100.00	95.071 ± 0.314	95.071 ± 0.314

Table 13 Result of Extracted Amodiaquine from Spiked Aqueous Samples

RESULT OF EXTRACTED AMODIAQUINE FROM SPIKED PLASMA SAMPLES					
FROM PEAK HEIGHT			FROM PEAK AREA		
EXPECTED VALUE (ug/ml)	EXTRACTED MEAN (ug/ml)	RECOVERY MEAN (%)	EXPECTED VALUE (ug/ml)	EXTRACTED MEAN (ug/ml)	RECOVERY MEAN (%)
5.00	3.974 ± 0.033	79.486 ± 0.656	5.00	3.958 ± 0.031	79.154 ± 0.624
10.00	8.501 ± 0.103	84.838 ± 0.804	10.00	7.449 ± 0.094	74.489 ± 0.934
25.00	21.884 ± 0.188	87.538 ± 0.751	25.00	21.073 ± 0.430	84.290 ± 1.788
50.00	45.290 ± 0.243	90.779 ± 0.683	50.00	46.448 ± 0.253	92.896 ± 0.506
75.00	68.228 ± 0.351	90.969 ± 0.466	75.00	66.103 ± 0.429	88.138 ± 0.573
100.00	92.823 ± 0.487	92.823 ± 0.487	100.00	91.434 ± 0.354	91.434 ± 0.354

Table 14 Result Of Extracted Amodiaquine From Spiked Plasma Samples

AMODIAQUINE PEAK HEIGHT (PH) DATA FOR ANOVA ANALYSIS.								
EXPECTED			AQUEOUS EXTRACT			PLASMA EXTRACT		
MEAN	SD	N	MEAN	SD	N	MEAN	SD	N
100	0	5	79.903	0.781	5	79.486	0.656	5
100	0	5	88.723	0.957	5	84.838	0.804	5
100	0	5	96.515	1.242	5	87.538	0.751	5
100	0	5	96.010	0.245	5	90.779	0.683	5
100	0	5	92.114	1.221	5	90.969	0.466	5
100	0	5	97.941	0.662	5	92.823	0.487	5

Table 15 Amodiaquine Peak Height (PH) Data for Anova Analysis

P value	0.002
P value summary	**
Are means signif. different? (P < 0.05)	Yes
Number of groups	3
F	9.984
R squared	0.571

ANOVA Table	SS	df	MS
Treatment (between columns)	467	2	233.5
Residual (within columns)	350.8	15	23.39
Total	817.9	17	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Expected vs Aqueous	8.132	4.119	Yes	*	0.8785 to 15.39
Expected vs Plasma	12.26	6.21	Yes	**	5.007 to 19.52
Aqueous vs Plasma	4.129	2.091	No	ns	-3.125 to 11.38

Table 16 One-way ANOVA for Amodiaquine using peak height

AMODIAQUINE PEAK AREA (PA) DATA FOR ANOVA ANALYSIS.								
EXPECTED			AQUEOUS EXTRACT			PLASMA EXTRACT		
MEAN	SD	N	MEAN	SD	N	MEAN	SD	N
100	0	5	64.457	0.420	5	61.879	2.741	5
100	0	5	75.709	0.996	5	65.596	0.378	5
100	0	5	76.601	0.447	5	70.969	0.648	5
100	0	5	81.875	1.086	5	75.992	0.984	5
100	0	5	89.815	0.907	5	83.831	0.732	5
100	0	5	87.792	0.642	5	80.560	0.174	5

Table 17 One-way ANOVA for Amodiaquine using peak Area

P value	1E-04				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	3				
F	16.97				
R squared	0.694				
ANOVA Table	SS	df	MS		
Treatment (between columns)	715.2	2	357.6		
Residual (within columns)	316	15	21.07		
Total	1031	17			
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Expected vs Aqueous	10.87	5.799	Yes	**	3.982 to 17.75
Expected vs Plasma	14.93	7.969	Yes	***	8.048 to 21.82
Aqueous vs Plasma	4.066	2.17	No	ns	-2.818 to 10.95

Table 18 One-way ANOVA for Amodiaquine using peak Area

QUININE

RESULT OF EXTRACTED QUININE FROM SPIKED AQUEOUS SAMPLES					
FROM PEAK HEIGHT			FROM PEAK AREA		
EXPECTED VALUE (ug/ml)	EXTRACTED MEAN (ug/ml)	RECOVERY MEAN (%)	EXPECTED VALUE (ug/ml)	EXTRACTED MEAN (ug/ml)	RECOVERY MEAN (%)
1.00	0.856 ± 0.004	85.55. 0.383	1.00	0.680 ± 0.011	68.041 ± 1.049
1.50	1.458 ± 0.016	97.173 ± 1.047	1.50	1.259 ± 0.008	83.871 ± 0.576
2.00	1.806 ± 0.015	90.307 ± 0.737	2.00	1.668 ± 0.022	83.424 ± 1.105
2.50	2.412 ± 013	96.467 ± 0.534	2.50	2.196 ± 0.003	87.836 ± 0.133
3.00	2.945 ± 0.014	98.179 ± 0.468	3.00	2.774 ± 0.024	92.458 ± 0.805
5.00	4.799 ± 0.003	95.988 ± 0.058	5.00	4.468 ± 0.021	89.350 ± 0.417

Table 19 Result of Extracted Quinine from Spiked Aqueous Samples

RESULT OF EXTRACTED QUININE FROM SPIKED PLASMA SAMPLES					
FROM PEAK HEIGHT			FROM PEAK AREA		
EXPECTED VALUE(ug/ml)	EXTRACTED MEAN (ug/ml)	RECOVERY MEAN (%)	EXPECTED VALUE (ug/ml)	EXTRACTED MEAN (ug/ml)	RECOVERY MEAN (%)
1.00	0.845 ± 0.005	84.505 ± 0.532	1.00	0.658 ± 0.005	65.840 ± 0.553
1.50	1.327 ± 0.004	88.504 ± 0.294	1.50	1.133 ± 0.014	76.220 ± 0.462
2.00	1.729 ± 0.009	86.464 ± 0.422	2.00	1.539 ± 0.003	76.937 ± 0.156
2.50	2.281 ± 0.014	91.218 ± 0.560	2.50	1.863 ± 0.019	74.538 ± 0.765
3.00	2.724 ± 0.008	90.788 ± 0.276	3.00	2.428 ± 0.023	80.928 ± 0.776
5.00	4.451 ± 0.056	90.359 ± 0.205	5.00	3.946 ± 0.024	78.908 ±0.472

Table 20 Result of Extracted Quinine from Spiked Plasma Samples

QUININE PEAK HEIGHT (PH) DATA FOR ANOVA ANALYSIS.								
EXPECTED (STANDARD)			AQUEOUS EXTRACT			PLASMA EXTRACT		
MEAN	SD	N	MEAN RECOVERY (%)	SD	N	MEAN RECOVERY (%)	SD	N
100	0	5	85.552	0.383	5	84.504	0.532	5
100	0	5	97.173	1.047	5	88.504	0.294	5
100	0	5	90.307	0.737	5	86.464	0.422	5
100	0	5	96.467	0.534	5	91.218	0.560	5
100	0	5	98.179	0.468	5	90.788	0.276	5
100	0	5	95.988	0.058	5	90.359	0.205	5

Table 21 Quinine Peak Height (PH) Data for Anova Analysis

P value	P<0.0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
Number of groups	3
F	18.33
R squared	0.7097

ANOVA Table	SS	df	MS
Treatment (between columns)	387.7	2	193.9
Residual (within columns)	158.6	15	10.58
Total	546.4	17	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Expected vs Aqueous	6.056	4.561	Yes	*	1.178 to 10.93
Expected vs Plasma	11.36	8.557	Yes	***	6.483 to 16.24
Aqueous vs Plasma	5.305	3.996	Yes	*	0.4271 to 10.18

Table 22 Quinine Peak Height (PH) ANOVA

QUININE PEAK AREA (PA) DATA FOR ANOVA ANALYSIS.								
Standard			Aqueous Extract			Plasma Extract		
MEAN Expected	SD	N	MEAN Recovery %	SD	N	MEAN Recovery %	SD	N
100	0	5	68.041	1.049	5	65.840	0.553	5
100	0	5	83.871	0.576	5	76.220	0.462	5
100	0	5	83.424	1.105	5	76.937	0.156	5
100	0	5	87.836	0.133	5	74.538	0.765	5
100	0	5	92.458	0.805	5	80.928	0.776	5
100	0	5	89.350	0.417	5	78.908	0.472	5

Table 23 Quinine Peak Area (PA) Data for Anova Analysis

P value	P<0.0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
Number of groups	3
F	27.24
R squared	0.7841

ANOVA Table	SS	df	MS
Treatment (between columns)	1844	2	922
Residual (within columns)	507.6	15	33.84
Total	2352	17	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Expected vs Aqueous	15.84	6.668	Yes	***	7.111 to 24.56
Expected vs Plasma	24.44	10.29	Yes	***	15.71 to 33.16
Aqueous vs Plasma	8.602	3.622	No	ns	-0.1240 to 17.33

Table 24 Quinine Peak Area Data For ANOVA

PYRIMETHAMINE

RESULT OF EXTRACTED PYRIMETHAMINE FROM SPIKED AQUOEUS SAMPLES					
	FROM PEAK HEIGHT			FROM PEAK AREA	
EXPECTED VALUE (ug/ml)	EXTRACTED MEAN (ug/ml)	RECOVERY MEAN (%)	EXPECTED VALUE (ug/ml)	EXTRACTED MEAN (ug/ml)	RECOVERY MEAN (%)
5.00	4.060 ± 0.034	81.209 ± 0.678	5.00	3.223 ± 0.021	64.457 ± 0.420
10.00	8.995 ± 0.144	89.944 ± 1.436	10.00	7.571 ± 0.099	75.709 ± 0.996
20.00	18.172 ± 0.168	90.861 0.840	20.00	15.320 ± 0.089	76.601 ± 0.447
30.00	27.905 ± 0.179	93.017 ± 0.598	30.00	24.562 ± 0.326	81.875 1.086
50.00	48.161 ± 0.594	96.321 ± 1.187	50.00	44.908 ± 0.454	89.815 ± 0.907
75.00	68.837 ± 0.347	91.783 ± 0.463	75.00	65.844 ± 0.481	87.792 ± 0.642

Table 25 Result of Extracted Pyrimethamine from Spiked Aqueous Samples

RESULT OF EXTRACTED PYRIMETHAMINE FROMTHE SPIKED PLASMA SAMPLES				
	FROM PEAK HEIGHT		FROM PEAK AREA	
EXPECTED VALUE (ug/ml)	EXTRACTED MEAN (ug/ml)	RECOVERY MEAN (%)	EXTRACTED MEAN (ug/ml)	RECOVERY MEAN (%)
5.00	4.057 ± 0.084	81.144 ± 1.671	3.094 ± 0.137	61.879 ± 2.741
10.00	8.753 ± 0.091	87.754 ± 0.918	6.596 ± 0.038	65.596 ± 0.378
20.00	17.311 0.119	86.555 ± 0.595	14.194 ± 0.130	70.969 ± 0.648
30.00	27.062 ± 0.112	90.206 ± 0.372	22.797 ±0.295	75.992 ± .984
50.00	44.992 ± 0.160	89.985 ± 0.312	414.916 ± 0.366	83.831 0.732
75.00	66.104 ±0.276	88.139 ± 0.368	60.420 ± 0.130	80.560 ± 0.174

Table 26 Result of Extracted Pyrimethamine from Spiked Plasma Samples

PYRIMETHAMINE PEAK HEIGHT (PH) DATA FOR ANOVA ANALYSIS.								
EXPECTED (STANDARD)			AQUEOUS EXTRACT			PLASMA EXTRACT		
MEAN	SD	N	MEAN Recovery (%)	SD	N	MEAN Recovery (%)	SD	N
100	0	5	81.209	0.678	5	81.144	1.671	5
100	0	5	89.944	1.436	5	87.754	0.918	5
100	0	5	90.861	0.840	5	86.555	0.595	5
100	0	5	93.017	0.598	5	90.206	0.112	5
100	0	5	96.321	1.187	5	89.985	0.312	5
100	0	5	91.783	0.463	5	88.139	0.368	5

Table 27 Pyrimethamine Peak Height (PH) Data for Anova Analysis

P value	P<0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	3				
F	21.37				
R squared	0.7402				
ANOVA Table	SS	df	MS		
Treatment (between columns)	523.2	2	261.6		
Residual (within columns)	183.6	15	12.24		
Total	706.8	17			
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Expected vs Aqueous	9.478	6.635	Yes	***	4.229 to 14.73
Expected vs Plasma	12.7	8.893	Yes	***	7.455 to 17.95
Aqueous vs Plasma	3.225	2.258	No	ns	-2.023 to 8.473

Table 28 One-way ANOVA for Pyrimethamine Peak Height

PYRIMETHAMINE PEAK AREA (PA) DATA FOR ANOVA ANALYSIS.								
Expected			Aqueous Extract			Plasma Extract		
MEAN	SD	N	MEAN	SD	N	MEAN	SD	N
100	0	5	64.457	0.420	5	61.879	2.741	5
100	0	5	75.709	0.996	5	65.596	0.378	5
100	0	5	76.601	0.447	5	70.969	0.648	5
100	0	5	81.875	1.086	5	75.992	0.984	5
100	0	5	89.815	0.907	5	83.831	0.732	5
100	0	5	87.792	0.642	5	80.560	0.174	5

Table 29 Pyrimethamine Peak Height (PA) Data for Anova Analysis

P value	P<0.0001
P value summary	***
Are means signif. Different? (P < 0.05)	Yes
Number of groups	3
F	22.36
R squared	0.7488

ANOVA Table	SS	df	MS
Treatment (between columns)	2372	2	1186
Residual (within columns)	795.7	15	53.05
Total	3167	17	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Expected vs Aqueous	20.63	6.937	Yes	***	9.701 to 31.55
Expected vs Plasma	26.86	9.034	Yes	***	15.94 to 37.79
Aqueous vs Plasma	6.237	2.098	No	ns	-4.687 to 17.16

Table 30 One-way ANOVA for Pyrimethamine Peak area

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