CHAPTER ONE

1.0. INTRODUCTION

1.1. STATEMENT OF PROBLEM

The occurrence of resistance to chloroquine and sulfadoxine/pyrimethamine by *Plasmodium falciparum* in parts of South-Eastern Asia, South America and Africa stimulated new interest in quinine as an alternative drug for treating multi-resistant *falciparum* infection (White & Warrell 1983, Imbert & Gendrel 2002). Parenteral quinine is the gold treatment in the management of severe and complicated malaria. This is because quinine, a cheap drug with a long history of use is still effective in cerebral and severe malaria. If untreated, cerebral malaria is fatal within 24-72 hours. This therefore suggests the need for early initiation of treatment.

One of the current strategies for the management of malaria is the encouragement of initiation of treatment in the home The effectiveness of home treatment will depend upon early diagnosis, prompt, appropriate treatment and proper health education about malaria. Early commencement of appropriate treatment will ensure better outcome and prevent the progression to severe malaria. An antimalarial drug to be used at home must be safe, effective, affordable, easy to administer and preferably in single dosage packs (Simoes *et al;* 2003, FMOH, 2005). This can be done through the availability of an easy to administer, effective and stable formulation. A rectal formulation will serve this purpose and meet the above conditions. The intra-rectal formulation will also be useful in pre-referral management procedures in primary health facilities, homes and rural areas where health facilities are not located. In order to enhance early treatment in cases of severe malaria, a suppository formulation of quinine is proposed. This suppository formulation will also be life-saving in hospitals in cases of circulatory collapse. The suppository formulation is of particular interest in children since it is non-

invasive, painless and easy to administer and can therefore be administered by untrained caregivers.

Quinine is also used in the management of severe malaria in pregnancy (FMOH 2005). Quinine is safe in pregnancy but the teratogenic effects of the Artemisinin Combination Therapies have not been fully assessed. The suppository formulation will provide an easy to administer, non-invasive alternative for pregnant women who may not be able to take oral formulations.

National and global efforts to treat malaria have focused largely on provision of effective antimalarial treatment, mainly through public health services. The cost of artemisinin-based combination treatments (ACTs) is far beyond the reach of the average family in Africa, let alone the poorer populations (Talisuna *et al;*2009). Suppositories of Artemisinin derivatives in the market cost about $5.00 as at November 2010, while the estimated cost of the quinine suppository is $1.00. Quinine suppository will provide a more affordable alternative. On the basis of cost, a therapeutically effective suppository formulation of quinine would be more cost-effective for the many living in the rural zones of malaria endemic regions.

A study of quinine concentration in the main sections of the mouse brain will confirm availability in the brain and shed more light on the mechanism of action of the drug in cerebral malaria. This work is designed to formulate such suppository that will ensure the availability of quinine in the brain so that it can be used to initiate treatment in the management of severe malaria.

1.2 OBJECTIVES OF THE STUDY:

The objectives of the study are to:

i. Evaluate the suitability of Cocoa butter and FattiBase™ as suppository bases for quinine suppository.

ii. Investigate the effect of varying concentration of surface active agents (polysorbate 80) on the release profile of the suppository formulations

iii. Assess the availability of quinine from the suppository formulation through release profile

iv. Determine the site(s) of, and time-related concentration of quinine in the different sections of the brain using mice as the animal model.

1.3 SIGNIFICANCE OF STUDY

The study will provide a major contribution to the management of severe malaria which is a fatal disease that could kill within 24 hours if left untreated. The success of malaria chemotherapy is dependent on early treatment. A suppository dosage form will provide such a formulation that can be administered even in the absence of trained health workers. The mouse brain section/site studies will provide the needed evidence of availability of quinine from a suppository formulation in the brain and contribute to the knowledge of mechanism of action of quinine in cerebral malaria. It will therefore provide the confirmation that the suppository can be used in the management of cerebral malaria.

1.4 LIMITATIONS OF STUDY

A limitation of this study was the lack of literature reference for the fluorescence of quinine crystals. Quinine is simply reported as having green fluorescence. Non-availability of a fluorescence detector in the high performance liquid chromatography is another limitation.

1.5 RESEARCH QUESTIONS

1. Will the local base, cocoa butter be suitable for the formulation of an available quinine suppository?
2. Will quinine from the synthetic base FattiBase^{TM} suppository be more available through dissolution than from cocoa butter base suppositories?
3. Will quinine uptake be higher in the brain of parasitized mice than in brain of non-parasitized mice?
4. Will the uptake of quinine in the mice brain be affected by time?
5. Will uptake of quinine vary in the four brain sections?

1.6 OPERATIONAL DEFINITION OF TERMS/ABBREVIATIONS

Cerebral malaria (CM) – Complication of *Plasmodium falciparum* malaria affecting the central nervous system

Suppository – Solid dosage forms intended for administration of medicine via the rectum that melts, soften, or dissolve in the body cavity

ECM – Experimental cerebral malaria

PRBC – Parasitized red blood cells

NPRBC – Non-parasitized red blood cells

DV – Displacement value

HPLC – High pressure liquid chromatography

ACT – Artemisinin Combination Therapy

RBM – Roll Back Malaria

2.0. LITERATURE REVIEW

2.1. Malaria

2.1.1. Overview of malaria

Malaria is a life - threatening parasitic disease transmitted by the female *Anopheles* mosquito. The disease is caused by blood parasites of the genus Plasmodium. Of the 156 species of Plasmodium, the four that are identified as human parasites are P. falciparum, P. vivax, P. ovale and P. malariae. The parasites are transmitted to humans through mosquito bites, and malaria is common in tropical and subtropical areas where the Anopheles mosquito is present.

The Life Cycle of Malaria – Malaria goes through two major stages: asexual stage in the human and the sexual stage in the mosquito.

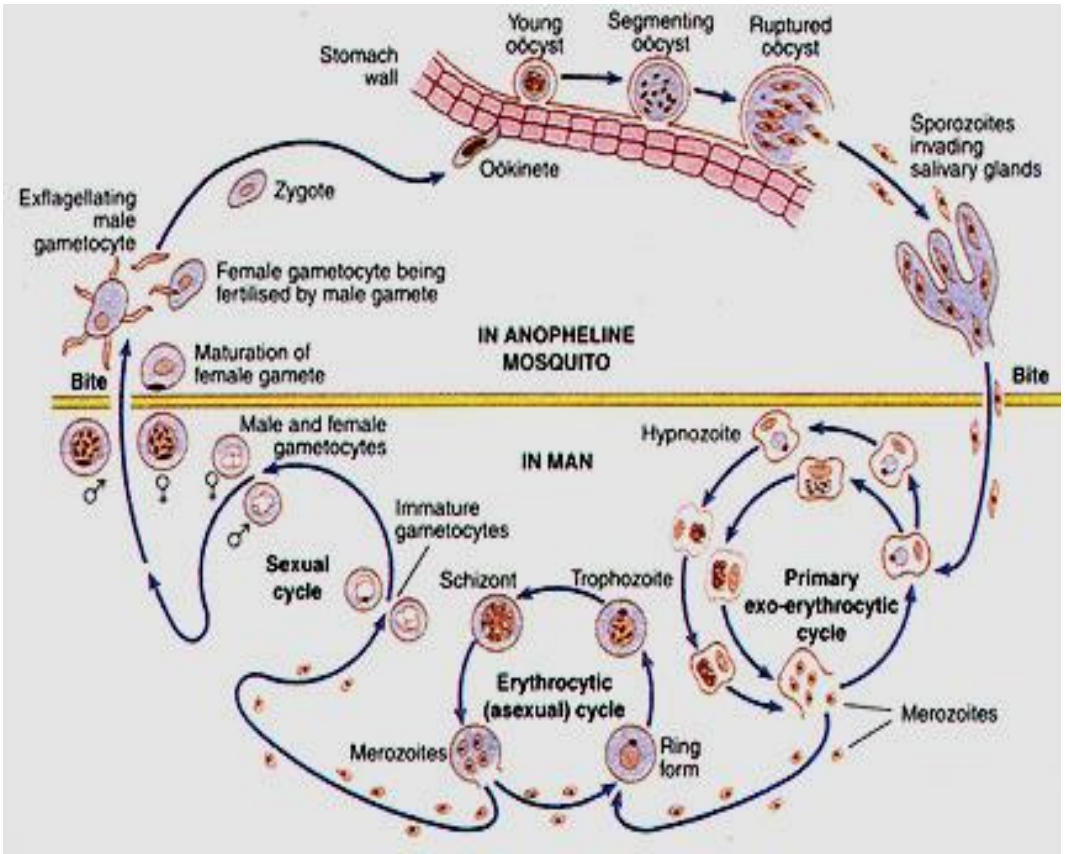

Fig. 1 The life cycle of human malaria
(Asexual phase in human body and sexual phase in the mosquito)
source:www.malariasite.com/malaria/evolution.html

Asexual phase in the human host

Tissue schizogony (Pre- erythrocytic schizogony): This phase starts with the inoculation of the parasite into the human blood by the bite of a female anopheles mosquito. Within half an hour, the sporozoites reach the liver and invade the liver cells. Within the liver cells, the trophozoites start their intracellular asexual division. At the completion of this phase, thousands of extra erythrocytic merozoites are released from each liver cell. The time taken for the completion of the tissue phase is variable, depending on the infecting species; *(8 - 25 days for P. falciparum, 8 - 27 days for P. vivax, 9 - 17 days for P. ovale, 15 - 30 days for P. malariae)* and this interval is called as *pre-patent period.*

In case of *P. vivax* and *P. ovale*, some sporozoites may go into hibernation - the *cryptobiotic phase*- in which they are called *hypnozoites.* They can lie dormant for months or years and on reactivation they cause clinical relapse.

Erythrocytic schizogony: The merozoites released from the liver cells attach onto the red blood cell membrane and by a process of invagination, enter the red cell. Within the red blood cell, the asexual division starts and the parasites develop through the stages of rings, trophozoites, early schizonts and mature schizonts; each mature schizont consisting of thousands of erythrocytic merozoites. These merozoites are released by the lysis of the red blood cell and they immediately invade uninfected red cells. This repetitive cycle of invasion - multiplication - release - invasion continues. The intra erythrocytic cycle takes about 48 hours in *P. vivax*, *P. ovale* and *P. falciparum* infections and 72 hours in case of *P. malariae* infection. It occurs synchronously and the merozoites are released at approximately the same time of the day. The contents of the infected cell that are released with the lysis of the RBC stimulate Tumor Necrosis Factor and other cytokines, which results in the characteristic clinical manifestations of the disease.

A small proportion of the merozoites in the red blood cells undergo transformation into gametocytes - male and female. Mature gametocytes appear in the peripheral blood after a variable period and enter the mosquito when it bites an infected individual. *(Appear on the 5th day of primary attack in P. vivax and P. ovale, and thereafter become more numerous; appear at about 5 - 23 days after primary attack in P. malariae; appear after 8 - 11 days of the primary attack in P. falciparum, rising in number until 3 weeks and falling thereafter, but may circulate for several weeks).*

Sexual phase in the mosquito

Sporogony: The gametocytes continue their development in the mosquito. The male and female gametes fuse and form into a zygote. This transforms into an ookinete which penetrates the gut wall and becomes an oocyst. The oocyst divides asexually into numerous sporozoites which reach the salivary gland of the mosquito. On biting a man, these sporozoites are inoculated into human blood stream. The sporogony in the mosquito takes about 10 - 20 days and thereafter the mosquito remains infective for 1 - 2 months.

Prevalence

The disease is predominant in the tropical regions of the world, affecting more than 240 million people (over 40% of the world's population). The tropics provide ideal breeding and living conditions for the *Anopheles* mosquito in the world. The global burden of malaria remains enormous, though progress in malaria control has accelerated dramatically since 2006. Over three billion people are at risk with an estimated 247 million malaria cases which caused nearly a million deaths. 91% were in Africa while 85% were children under 5 years of age. In addition, half a billion African people became ill and unable to work. Malaria was endemic in 109 countries in 2008 and 45 of them were within the African region. 80% of the cases in Africa were detected in 13 countries and over half were in Nigeria, the Democratic Republic of the Congo, Ethiopia, the United Republic of Tanzania and Kenya. The figures however only cover

malaria transmission in areas and periods of endemicity. In Africa, there are more than 12 million malaria episodes and 155,000–310,000 malaria deaths per year attributable to epidemics. This is equivalent to some 4% of estimated annual malaria cases worldwide and 12-25% of estimated annual worldwide malaria deaths (Worrall *et al;* 2004). There are evidences that prevention and treatment can alleviate the burden imposed by the disease. A major concern is that children remain by far the most vulnerable and likely to die of the disease (World Malaria Report 2009). Use of nets, especially ITNs and access to treatment has been consistently lower in rural than in urban areas and coverage with principal interventions was far below the 2005 target of 60% set for Africa at the outset of the Roll Back Malaria. Fig 2 shows the median for 30 countries for fever, 28 countries for fever treatment, 29 countries for mosquito nets, and 28 for insecticide-treated nets (ITNs) (Monasch *et al;* 2004).

Assuming 80% insecticide-treated net coverage by 2010, the estimate of total malaria incidence would have been reduced to about 25million children by end of 2010 and further down to about 8 million per annum by 2013 (Fig 3).

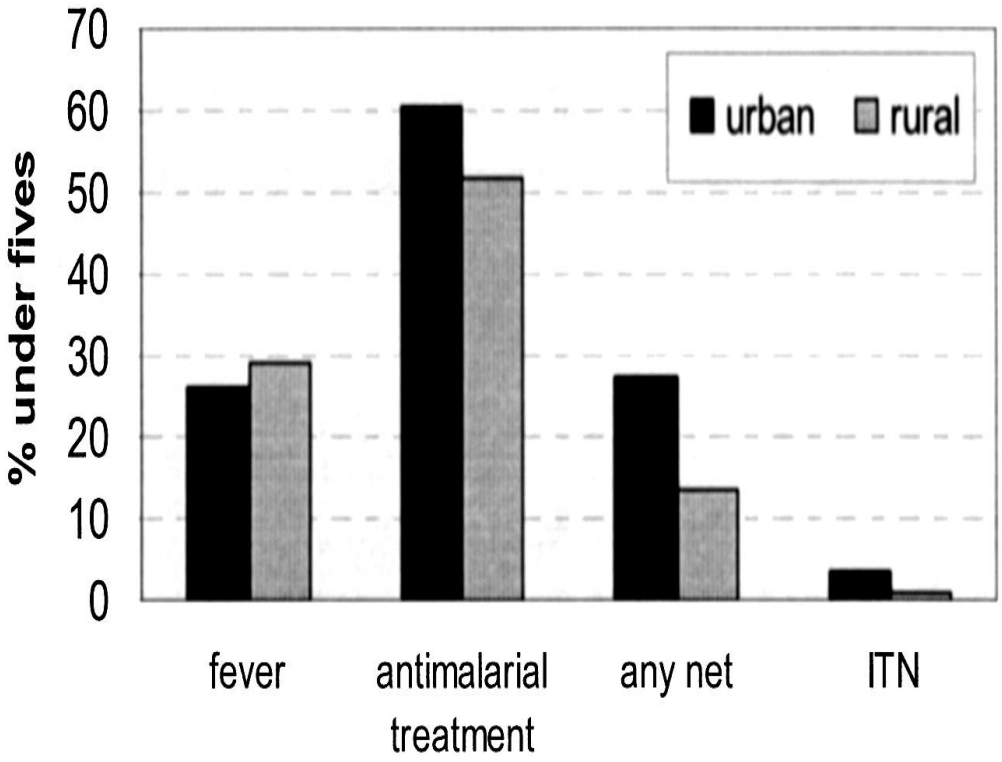

Fig. 2 Urban-rural differentials in < 5s with fever, coverage with mosquito nets and those with antimalarial treatment.

Source: (Monasch *et al;* 2004)

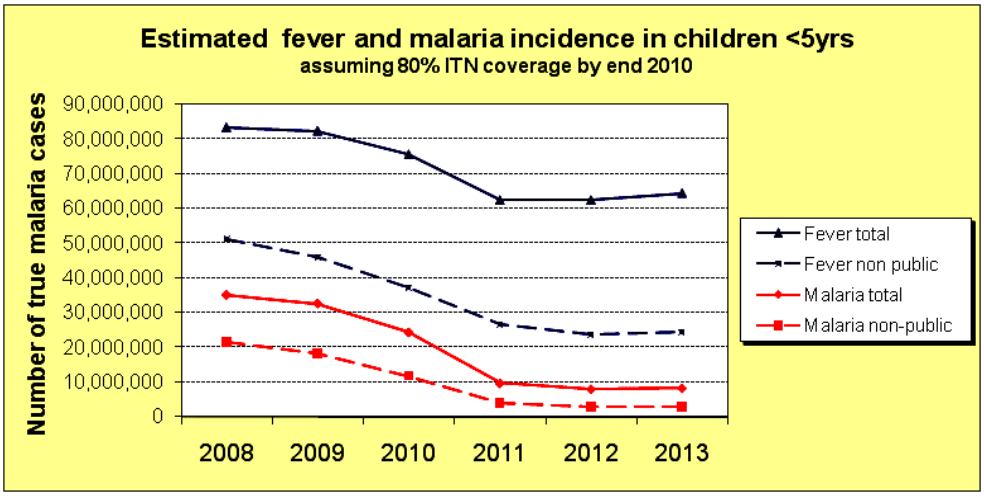

Fig. 3 Estimated fever and malaria incidence in children < 5yrs

Source: Roll Back Malaria, 2008

The estimated annual expenditure on global malaria research, prevention and treatment is $84 million while estimated worldwide expenditure per malaria fatality is put at $65 as compared to $3274 for HIV/AIDS and $789 for Asthma. Currently, the total 'cost' of malaria-health, care, treatment, lost production time etc is estimated to be more than $1,800 million for Tropical Africa (World Malaria Report 2009).

2.1.2. Malaria Situation in Nigeria

Malaria is the commonest cause of hospital attendance in all age groups in all parts of Nigeria and every case of fever is presumptively treated as malaria. It is also one of the four commonest causes of childhood mortality in the country, the other three being acute respiratory infection (pneumonia), diarrhoea and measles (unicef.org 2008). It is estimated that 50% of the population has at least one episode of malaria each year while children under 'five' have on the average of 2 – 4 attacks in a year. However, estimates are usually based on attendance in public and private health facilities and may not have captured cases treated at home and within the community. For every case of febrile illness seen in the health facilities, there may be approximately 4-5 in the community. This has been demonstrated in studies on malaria management carried out in two African countries: Ghana and Kenya (Agyepong & Kangeya-Kayonda 2004). Cibulskis *et al.*, (2004), suggested that sentinel surveillance from public and private health facilities, selected according to risk stratification, combined with occasional household surveys and other population-based methods of surveillance, may provide better assessments of malaria trends.

Malaria has severe negative effects on maternal health and birth outcomes. It causes maternal anaemia, increases miscarriages and low birth weight. *P. falciparum* is the most predominant parasite species accounting for about 98% of malaria cases in the country. *P. malariae* usually

occurs as a mixed infection with *P. falciparum*. *Anopheles gambiae* is the main vector of malaria in Nigeria, but *An. funestus* and *An. arabiensis* are also commonly encountered. *An. melas* is found in the coastal areas. Malaria is characterized by a stable, perennial, transmission in all parts of the country. Transmission is higher in the wet season than in the dry season. This seasonal difference is more striking in the northern part of the country.

Many of the WHO-recommended intervention strategies have been incorporated into the malaria management policy in Nigeria (FMOH 2005). Malaria disproportionately affects poor people with 58% of malaria cases occurring in the poorest 20% of the world's population (Malaria Fact sheet, DFID 2009). However, a growing body of evidence from benefit-incidence analyses has demonstrated that many public health interventions that were designed to aid the poor are not reaching their intended target. If malaria control interventions are to achieve their desired impact, they must reach the poorest segments of the population (Barat *et al*; 2004, Onwujekwe *et al;* 2007).

2.1.3. Economic Burden

Malaria impedes human development and is both a cause and consequence of under development. It is presently one of the most significant public health problems in Nigeria. On an annual basis, it is estimated that 60% of Nigeria's formal and informal medical sector resources is consumed by the disease which results in 300,000 child deaths and 11% of maternal deaths. Every year, the nation loses over N132 billion from cost of treatment and absenteeism from work, schools and farms (www.unicef.org/infobycountry/nigeria_49472)

As far back as 2000, the direct cost that a family had to incur for an episode of severe malaria in Nigeria was estimated at US $ 28.9 (WHO/AFRO Bulletin 2001). The cost estimate covered transport to hospital, drugs, laboratory tests, admission fees, food and accommodation for caretaker's etc. In 2007, a country-wide survey put the average cost per malaria treatment per month at ₦685.00 while cost per month for control within the family was estimated as

₦7,324.00. This translates into ₦7340.00 per head per year. For a country with a population of about 120 million, the expenditure on malaria management within the country translates to about ₦880,801.00 million per annum. This represents about 12.0 per cent of Nigeria's Gross Domestic Product (Jimoh *et al;* 2007).

Cost is a major deterrent in treatment seeking. The poor members of the population have problems paying for their medicines and may resort to purchases and payment through installments or seek help from alternatives in the community (Helmut *et. al;* 1987, Asenso-Okyere *et. al;* 1998 Onwujekwe *et.al;* 2007). Malaria constitutes a heavy burden on the human and financial resources with multiplying negative effects on economic development.

2.1.4. Malaria Control Policy and Strategy

Within the last ten years, advocacy, political awareness and commitment to malaria control has continued to improve. In 1996, Nigeria developed its first National Malaria Control Policy. A yearly Plan of Action was developed for 1997 and 1998 and a three-year Plan of Action was also developed for 1999 – 2001. The highest advocacy between 1996 and 1998 was the celebration of the National Social Mobilization Day when the Malaria Control logo was launched. The National Technical Committee, a body consisting of National, State and some LGAs, Malaria Programme Managers and representatives from the private sector and international agencies was resuscitated in 1998. The committee meets at the end of each year and is responsible for reviewing the activities of the previous year and planning those of the next year. Series of trainings have been carried out nationally on management of severe and uncomplicated malaria.

The strategy for the implementation of the national malarial treatment policy is that of Roll Back Malaria (RBM). This strategy seeks to establish a social movement in which the local communities, public and private sectors, all tiers of government and non-governmental

development agencies etc come together in a partnership and network to implement malaria control interventions. RBM is an initiative to improve malaria control in the context of health sector reform. It was initiated in 1998 through a joint partnership of WHO, UNICEF, UNDP and the World Bank. Nigeria drew attention of the world to problems of malaria control in Africa by hosting and co-financing the African Heads of State Summit on RBM in April 2000. Forty-four of the fifty malaria-affected countries in Africa attended the summit. Nineteen country delegations were led by the Heads of State while the remaining delegations were led by senior government officials. The Summit was also attended by the senior officials from each of the four founding agencies (WHO, UNICEF, World Bank & UNDP) and other development partners. The Summit concluded with the signing of the Abuja Declaration and Plan of Action through which the African leaders re-dedicated themselves to the principles and targets of the Harare Declaration of 1997 and gave commitment to intensify efforts to halve the malaria mortality in Africa by the year 2010 through implementing strategies and actions of Roll Back Malaria. The partnerships are on-going in 15 countries in sub-Saharan Africa, 11 in North Africa/Middle East and another 8 in Asia and the South America (Amazon district). April 25th was declared Africa Malaria Day. Since then, the Africa Malaria Day has been celebrated with public events, education and campaigns demonstrating the commitment of resources to the fight against malaria.

2.1.5 Roll Back Malaria (RBM)

The RBM intervention strategy has four key elements:

i. Patients with malaria should have access to appropriate and adequate treatment within 24 hours of the onset of symptoms

ii. Pregnant women particularly in their 1st and 2nd pregnancies should have access to effective antimalarial prophylaxis and treatment

iii. Insecticide treated nets and other materials should be available and accessible to persons at risk of malaria particularly pregnant women and children under 5 years of age.

iv. Epidemics of malaria should be recognized and steps initiated for their containment within one week of their onset.

The United Nations Special Envoy for Malaria, Ray Chambers, released a report highlighting the advances that have been made in expanding malaria control interventions. The report cites notable progress in achieving Secretary-General Ban Ki-moon's goal of providing universal distribution of proven malarial interventions to all endemic African countries by the end of 2010. He noted the expansion of prevention and treatment options through global commitment to fight malaria, including over $3 billion in pledges for malaria funding and underscores last year's announcement that the world can reach near-zero deaths from malaria by 2015 with increased momentum. According to the report, more than 40 percent coverage of endemic African populations with long-lasting, insecticidal bed nets and more than 60 percent coverage for 18 specific countries have been achieved. In sub-Saharan Africa, where a vast majority of malaria deaths occur, there has been more than 140 million bed nets distributed to those in need in the past three years.

RBM consists of two phases - the inception phase and the implementation phase. After the Consensus Building Meeting for countries in West Africa in March 1999, Nigeria started the RBM inception phase. Sensitization and advocacy on RBM were carried out at the level of Commissioners of Health in the States and Federal Capital Territory, Abuja, press briefing to enlighten the public about the importance of RBM and workshops for executives of media houses.

In line with RBM approach and Abuja Declaration, series of activities have been carried out to complete the inception process and begin the implementation phase in Nigeria. Some of these activities include:

- Consensus building meeting in all the six geopolitical zones and the development of a three year National Plan of Action.
- Development of partnerships with stakeholders (private and public sectors) and NGOs and International developmental agencies (WHO, UNICEF, DFID USAID, etc.)
- Malaria situation survey to assess the actual situation of malaria in the country to fill the gap created from the deskwork
- Development of national strategic plan to guide implementation from 2001 to 2005

It is obvious that achieving the goal of RBM would require the regular availability of appropriate antimalarial drugs through proper financial provisions at costs that the people can afford. The consumers and providers have to be properly educated on malaria and its treatment and an effective monitoring and evaluation system set up to ensure that objectives are being properly pursued. There will also be a need for new understanding of old problems and new problems requiring clarification will also arise. These will require continued strategic and operational research. (RBM document FMOH 2000)

Various intervention programmes that are currently in operation worldwide alongside their principal goals are listed in Table 1

Table 1 Intervention to control malaria and principal goals

Principal Goal	**Interventions**
Treatment	• Outpatient antimalarial treatment for uncomplicated malaria following active or passive case detection. • treatment for severe and complicated malaria • Home treatment
Prevention: Inhibit mosquito breeding	• Source reduction e. g. drainage, filling of ditches • Chemical larviciding • Management of agricultural, industrial and urban development to avoid the creation of breeding sites
Kill adult mosquitoes in order to reduce survival rate (and hence vectorial capacity) of adult mosquito population	• Indoor residual spraying • Repellents and domestic insecticides, e.g. sprays, coils, burning traditional herbs
Isolate humans from biting by vector mosquitoes	• Insecticide treated materials e.g bednets, curtains etc.
Prevent malaria infection in human	• Chemoprophylaxis for non-Immune groups e.g. children, pregnant women, and migrants
Prevent malaria morbidity in humans	• Intermittent treatment of pregnant women

2.2. SEVERE AND COMPLICATED MALARIA AND TREATMENT

2.2.1. Severe and Complicated Malaria

Severe malaria is the commonest cause of death, particularly in rural areas not serviced by the formal health systems. In response to the severity of the problem, the WHO revised the practical handbook on "Management of Severe and Complicated Malaria" published in 1991 with a release of the updated version in 2000.

A patient has severe malaria when there is *P. falciparum* asexual parasitaemia and no other confirmed cause of symptoms, in the presence of the following clinical or laboratory features: Prostration (i.e. generalized weakness or inability to sit, stand or walk without support; Impaired consciousness (confusion or drowsiness or coma; Respiratory distress (difficulty in breathing, fast deep breath, Multiple convulsions (>2 generalized seizures in 24hrs with regaining of consciousness); Severe anaemia (Hb <5 gm/dl); Circulatory collapse (shock), Pulmonary oedema (respiratory distress /radiology, Abnormal bleeding (disseminated intravascular coagulopathy); Jaundice (yellow discoloration of the eyes); Haemoglobinuria (Coca-Cola coloured urine); Hyperparasitaemia (Density of asexual forms of *P.falciparum* in the peripheral smear exceeding 5% of erythrocytes, i.e. more than 250,000 parasites per µl at normal red cell counts); Renal failure (Urine output of less than 400 ml in 24hours or <12ml/kg per 24 hours in children and a serum creatinine of more than 265 µ mol/l [> 3.0mg/dl], failing to improve after rehydration, (WHO, The Liaison Bulletin of the Malaria Programme 2000).

Severe malaria can therefore be described as the acute form of malaria that is accompanied with complications. When complications manifest as neurological, this progression of the infection is categorized as cerebral malaria. Result of a multicenter study on severe malaria in ten selected districts of ten countries in African region (1999-2000) revealed that the direct cost that a family has to incur for an episode of severe malaria ranged between US$ 14.5 to US $ 58.4, median

value US$ 36.4. The cost for Nigeria was estimated at US $ 28.9. The cost estimate covered transport to hospital, drugs, laboratory tests admission fees, food and accommodation for caretaker's etc (WHO/AFRO 2001). The study also revealed that for most children, treatment is started at home, it is imperative that efforts be directed at improving quality of care at home. This can be done through the availability of easy to administer effective formulation such as rectal antimalarials.

2.2.2 Cerebral Malaria

Cerebral malaria is the clinical manifestations of *Plasmodium falciparum* infection that induces changes in mental status and may eventually lead to coma and death. It is an acute widespread disease of the brain which is accompanied by fever and the mortality rate is between 25-50%. If untreated, cerebral malaria is fatal within 24-72 hours

WHO's definition of cerebral malaria is based on the Glasgow coma score. It lists three key elements for the confirmation of cerebral malaria (Newton and Warrell, 1999; Wassmer *et al;* 2003)

1. Unrousable coma: this implies no localizing response to pain, persisting for more than six hours if the patient has experienced a generalized convulsion (scores 2 or less in the Glasgow coma scale) or inability to localize a painful stimulus, with or without convulsion.
2. Asexual form of *Plasmodium falciparum* found in blood.
3. Exclusion of other causes of encephalopathy i.e. viral or bacterial.

Newton *et al; (2000)* suggested a strict definition of cerebral malaria, requiring "a deep level of unconsciousness" or coma. Such restricted criteria might be justified in studies comparing treatment protocols. Neurological manifestations have been found to fall into five broad groupings (Daroff 1999): disturbance of consciousness (ranging from extreme lethargy to

coma); delirium, movement disorders (tremor, myoclonus, chorea), unilateral cerebral hemispheric syndrome, acute personality changes (manifesting as a paranoid psychosis or delusional state).

Lactic acidosis (a metabolite derangement) and hypoglycemia have also been identified as common (Olumese *et al;* 1999). This is caused by both the abnormal metabolism of the red blood cells and the liver. The hypoglycemia is of particular concern with children and pregnant women. It happens as a result of problems with the liver's glucose-producing cycles and from increased use of glucose by both the body and the parasite. Unfortunately, quinine which is commonly used in the treatment of severe malaria stimulates the release of insulin which drives the blood glucose down still further (Sharma *et al;* 1986). In *falciparum* malaria, 10% of all admissions and 80% of deaths are due to the involvement of the central nervous system. CNS manifestations are fairly common in malaria and it could be due to high grade fever and even certain antimalaria drugs. It is therefore important to differentiate between these so as to avoid unnecessary anxiety and improper treatment. Other encephalopathies must also be excluded. In *falciparum* malaria, 10% of all admissions and 80% of deaths are due to the involvement of the central nervous system as manifested in cerebral malaria. However, all patients with *P. falciparum* malaria with neurological manifestations of any degree should be treated as cases of cerebral malaria.

2.2.2.1 Pathophysiology

The pathophysiology of Cerebral Malaria (CM) is not completely understood. The basic defect seems to be the clogging of the cerebral microcirculation by the parasitized red cells. These cells develop increased cytoadherent properties as a result of which they tend to adhere to the endothelium of capillaries and venules. This results in sequestration of the parasites in these

deeper blood vessels. Also, rosetting, a clustering of the parasitized and red cells in the blood vessels and deformation of the red cells further increase the clogging of the microcirculation.

Two major hypotheses have been postulated in explaining this etiology. These are the Mechanical and the Humoral hypotheses (Brown *et al;* 1999). The mechanical, asserts a specific interaction between *Plasmodium falciparum*, an erythrocyte membrane protein (PfEMP-1) and ligands on the cell wall causing sequestration which reduces micro vascular blood flow thereby inducing hypoxia. The tiny knobs that cover the surface of infected erythrocytes leading to sticking were characterized as a unique protein called KAHRP (Kaul *et al;* 1998). Without the protein the *Plasmodium* is powerless to make infected blood cells sticky. Immunohistochemical studies show that a large number of possible sequestration reports are expressed on brain endothelium during cerebral malaria. Cerebrovascular endothelial expression of the putative cytoadherence receptors ICAM-1, VCAM-1, E-selectin, and chondroitin sulfate and also HLA class II is increased in cerebral malaria (Sein *et al;* 1993). However, these receptors are also expressed in other tissues, and not just in severe malaria but also mild malaria and other septic conditions. This implies that other factors may influence the receptor mediated parasite sequestration (Reeder *et al;* 1999).

In cerebral malaria patients, the proportion of parasitized erythrocytes is higher in the brain than in other organs and the cerebral vessels are more tightly packed with red blood cells than in non-cerebral malaria patients (MacPherson *et al;* 1985, Tufail *et al;* 2002). Sequestration of parasitized red blood cells (PRBCs) in cerebral microvessels is significantly higher in the brains of patients with CM compared with those with non-cerebral malaria (NCM) in all parts of the brain (cerebrum, cerebellum, and medulla oblongata). The hierarchy of sequestration is more in the cerebrum and cerebellum than the brain stem with 26.6 times more PRBCs in the brain microvasculature than in the peripheral blood (Emsri *et al;* 2003). Differential sequestration of PRBC occurs in the microvessels of the cerebrum and cerebellum. In the cerebellum, there is a statistically significant higher percentage of microvessels with PRBC sequestration than that in

the cerebrum. There is a higher degree of vascularity in the cerebellum (7vessels/mm^2) than in the cerebrum (5vessels/mm^2). Perivascular hemorrhages also occur more frequently in the cerebellum than in the cerebrum. This may explain the varied neurologic manifestations that result from cerebral and cerebellar dysfunction in human cerebral malaria. Within the same brain, different vessels had discrete but different populations of parasites, indicating that the adhesion characteristics of cerebrovascular endothelium change asynchronously during malaria and also that significant recirculation of parasitized erythrocytes following sequestration is unlikely (Sein *et al,* 1993). All developmental stages of *P. falciparum* are sequestered in the brain in severe malaria and the clinical severity of cerebral malaria depends on the level of PRBC sequestration in the brain and it is quantitatively associated with pre-mortem coma *(Silamut et al, 1999).* Neutrophils play a role in the pathogenesis of ECM via enhancement of the expression of Th1 cytokines in the brain (Chen *et al,* 2000). Early neutrophil depletion also prevents the development of ECM and dramatically decreases the sequestration of monocytes and microhaemorrhage in the brain. CM appears to involve minimal intraparenchymal inflammatory responses compared with other neurologic infections. This focuses attention on local events within and around the cerebral microvasculature in CM, rather than indicating widespread parenchymal disease.

The humoral hypothesis suggests that a malaria toxin may be released that stimulates macrophages to release cytokines. The cytokines may then induce additional and uncontrolled production of nitric oxide. Nitric oxide would then diffuse through the blood brain barrier and impose similar changes on synaptic functions as do general anesthetics and high concentrations of ethanol leading to a state of reduced consciousness (Newton *et al;* 1996). The nature of this interaction would explain the reversibility of coma. Impairment of transport within nerve fibers could induce neurological dysfunction and may have the potential either to resolve or to progress to irreversible damage. Axons are vulnerable to a broad range of cerebral insults that

occur during *P. falciparum* malaria infection. Findings suggest that disruption in axonal transport may represent a final common pathway leading to neurological dysfunction in cerebral malaria (Medana *et al;* 2002). Other issues like disruption of the blood brain barrier, local cytokines release and neurotoxicity contribute to the pathophysiology of CM (Paco *et al;* 2000, Reed & Weina, 2000, Brown *et al;* 2001, Adams *et al;* 2002). The cerebral hemodynamics is also disturbed in children leading to increased cerebral blood flow velocity (usually associated with seizures), hemiparesis and severe intracranial hypertension (Newton *et al;* 1996; Reed and Weina 2000).

There is compelling evidence for major hemodynamics dysfunction in cerebral malaria. Edema further worsens ischemia by compressing cerebral arteries, which subsequently leads to a collapse of the blood flow that ultimately represents the cause of death. The coexistence of inflammatory and ischemic lesions and the preponderant role of edema in the fatal outcome of experimental cerebral malaria have been demonstrated (Wassmer *et al;* 2003, Penet *et al;* 2005).

Platelets can act as bridges between PRBC and endothelial cells (Wassmer *et al;* 2008) allowing the binding of PRBC to endothelium devoid of cytoadherence receptors. Furthermore, platelets also potentiated the cytotoxicity of PRBC for brain endothelial cell by inducing an alteration of the integrity of their monolayer. Researchers have found that a high level of the hormone erythropoietin (EPO) produced in response to anaemia may help protect children from long term brain damage caused by severe malaria (Causals-Pascual *et al;* 2009). The researchers found that children whose blood contain a high level of the hormone recover better than those who have less. EPO is made by the kidney in response to low oxygen levels in the blood. It encourages multiplication and production of new red blood cells. EPO has been shown to protect nerve cells in the brain from long term damage. The results suggest that EPO could be used as an adjuvant in the management of cerebral malaria in children.

A new adjunctive strategy using erythropoietin at high doses combined with quinine was tried in CM patients (Picot *et al;* 2009). None of the expected side effects of erythropoietin were observed during the seven days follow-up providing the first evidence of the short-term safety of EPO-Quinine combination. A multicentre study is needed to assess the potential of EPO as an adjunctive therapy to increase the survival rate during cerebral malaria.

An artificial version of EPO is a banned substance that enhances athletic performance. Administration of the low-molecular-weight thiol pantethine prevented the cerebral syndrome in *Plasmodium berghei* ANKA-infected mice. The protection was associated with a decrease of circulating microparticles and preservation of the blood–brain barrier integrity. Parasite development was unaffected. Pantethine modulated one of the early steps of the inflammation–coagulation cascade, i.e., the trans bi-layer translocation of phosphatidylserine at the cell surface. Pantethine mimicked the inactivation of the ATP-binding-cassette transporter A1 (ABCA1), which also prevents the cerebral syndrome in this malaria model. However, pantethine acts through a different pathway, because ABCA1 activity was unaffected by the treatment. The mechanism of pantethine action is through the disulfide group (oxidized form). Thio-sensitive mechanisms are also involved in the impairment of microparticle release by TNF-activated endothelial cells. Pantethine is well tolerated and it has already been administered in other contexts to man with limited side effects. Therefore, trials of pantethine treatment in adjunctive therapy for severe malaria are recommended (Penet *et al;* 2005).

Changes in the retina, known as malarial retinopathy have been found to accompany cerebral malaria. Because the retina can be considered as an extension of the central nervous system, it has been used previously as a "window into the brain", allowing for swifter diagnosis of cerebral malaria. Beare *et al;* (2003) using a technique known as fluorescein angiography showed impaired blood flow in the eyes of patients with cerebral malaria. Adults and children have whitening to areas of the retina where blood did not appear to reach, implying that the parasites were disrupting the supply of oxygen and nutrients (Maude *et al;* 2009). Malaria

retinopathy can be a useful diagnostic sign in severe malaria. The retinopathy provides an opportunity to visualize an infected microvasculature in-vivo (Beare *et al;* 2006) Drugs that improve circulation e.g. ibuprofen and aspirin and limit the damage caused by the lack of oxygen could help prevent many deaths. The retinal changes however do not affect visual acuity (Beare *et al;* 2004) after recovery from cerebral malaria. Quinine infusion administered in the treatment of severe malaria produced changes in the electro-retinogram of patients (Lochhead *et al;* 2003).

2.2.2.2 Murine Mice Model

Several workers have presented murine mice models of CM (Hearn *et al; 2000*, Adesina *et al;* 2009, Sein *et al;* 2007, Lackner *et al;* 2006, 2008). Genetically susceptible mice are infected with *Plasmodium berghei* ANKA (PbA) and the mice developed a neurologic syndrome 6–14 days after infection with features of CM in humans (parasite sequestration and inflammation in the brain). The mouse model mimics most parts of human CM, with clogging and rupture of vessels clearly visible in both cerebral and cerebellar regions in the brain. This murine model of experimental cerebral malaria is the one most widely used and the same was used in this work.

2.2.3 Chemotherapy of Malaria

Treatment of malaria depends on: type of infection (causative parasite), severity of infection, status of the host and other associated conditions/diseases. Malaria is probably the only disease of its kind that can be easily treated in just 3 days but if diagnosis and proper treatment are delayed, can be fatal very quickly and easily. The fatality of malaria is attributed to the accompanying complications, especially those of the central nervous system (CNS) as observed in cases of severe malaria. All cases of malaria accompanied with CNS complications are presumed to be due to *Plasmodium falciparum* infection and given adequate evaluation and treatment.

The occurrence of resistance to chloroquine and sulfadoxine/pyrimethamine of *Plasmodium falciparum* in parts of South -Eastern Asia and South America stimulated new interest in quinine as an alternative drug for treating multi-resistant falciparum. Chemotherapy for cerebral malaria has primarily involved the use of quinine. A patient with severe cerebral malaria even after initial treatment must be assumed to have chloroquine resistance since chloroquine was commonly the first line drug of choice.

Anti-malaria drugs act preferentially on different stages of parasite development. It is therefore highly conceivable that a drug that caused faster clearance of parasites from the peripheral blood might cause slower clearance of sequestrated parasites. This is of particular importance in cerebral malaria since parasitic sequestration has been responsible for much of the pathology.

The gold standard therapy for cerebral malaria is intravenous administration of quinine. WHO recommends the use of a loading dose in commencement of treatment (Imbert & Gendrel 2002; Lesi and Meremikwu 2004). Parenteral quinine is also the drug of choice in management of malaria in pregnant women. This is because quinine, a cheap drug is still effective in severe malaria. The advent of the artemisinin group of drugs has presented an alternative in the

treatment of severe malaria. Use of single dose artemisinin has been discouraged in order to protect them from resistance. Several countries in the malaria endemic zone including Nigeria have adopted the artemisinin combination therapy (ACT) as first line treatment for uncomplicated malaria (FMOH 2005).

In vitro evidence shows that quinine acts mainly against older parasites which are usually sequestered, whereas artemether has a broad range of action hence the rapidity of parasite clearance observed with this class of drugs. When parasitemia rises after the commencement of treatment it is often assumed that this is because of drug resistance. This may simply indicate that a large portion of the parasites was initially sequestered (White & Warrell 1983). Artemisinin and its derivatives act to suppress parasitemia more rapidly than quinine. This has however been questioned by several studies and no significant reduction in mortality and neurological sequelae was demonstrated when artemether was compared with quinine (Newton and Krishna 1998, Gravenor *et al;* 1998). Lubell *et al;* (2009) however posited that artesunate is superior for treatment of severe malaria from an economic as well as a clinical perspective. This position was based on the incremental cost per death averted with the use of artesunate instead of quinine. However, quinine is currently still the drug of choice in the treatment of severe and complicated malaria. The average African family purchases drugs out-of-pocket and may not be able to afford the artesunate. The benefits of cost-effectiveness will be lost through lack of access to medical facilities where the ACTs could be obtained free or at subsidized rate. Artemisinins and the combinations are still undergoing comparative studies with quinine in African patients. They are also undergoing registration appraisals in the USA.

In order to enhance early treatment in cases of severe malaria in the rural zones of endemic regions, intra-rectal formulations of antimalarials have been proposed. Suppositories of artemisinin derivatives are currently in the market. On the basis of cost, an intra-rectal formulation of quinine would be required for the many living in the rural zones of these regions.

The intra-rectal formulation is of particular interest in children since it is non-invasive, painless and easy to administer and can therefore be administered by untrained caregivers.

This work is therefore designed to formulate such suppository and ensure its availability in the brain so that it can be used for the initiation of treatment in the management of severe malaria.

2.3. QUININE

2.3.1. Monograph of Quinine

Quinine is one of the four main alkaloids obtained from the bark of the cinchona tree. Despite its long history of use, the drug still remains largely effective for treating the disease. Quinine is currently available in oral and parenteral forms. The various salts have different base contents. Most of the quinine salts contain equally active hydroquinone in concentrations of up to 10%, and low concentrations of quinidine, cinchonine and cinchonidine.

Quinine is chemically 6-methoxy-alpha(5-vinyl-2-quiniclidinyl)- 4- quinoline-methanol

Molecular formula – $C_{20}H_{34}N_2O_23H_2O$; Molecular weight = 378; PKa 4.1; 8.5

2.3.2. Quinine bisulfate

Molecular formula. $C_{20}H_{24}N_2O_2,H_2SO_4,7H_2O$

Relative molecular mass. 548.6

Graphic formula.

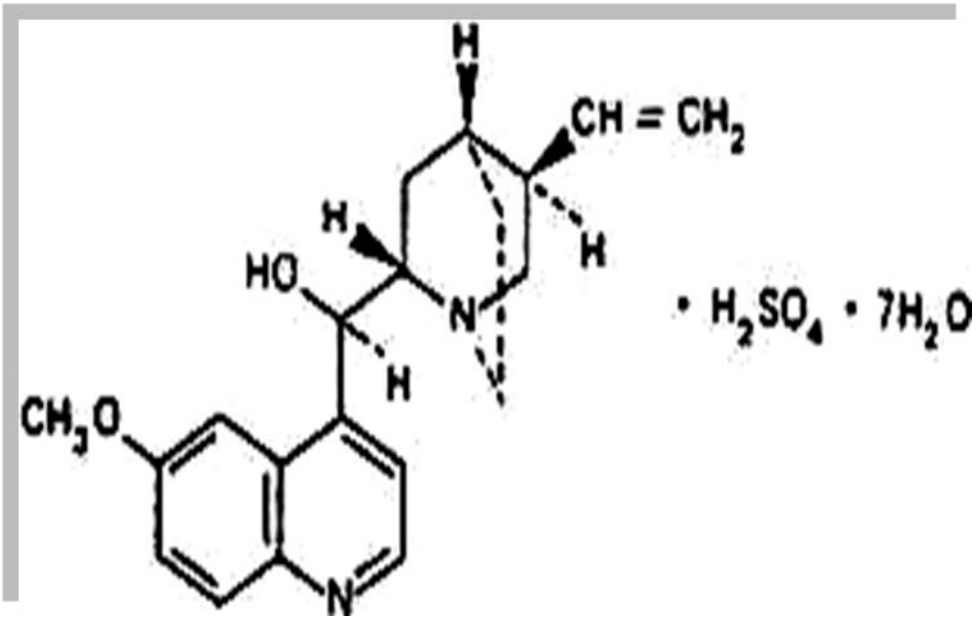

Fig.4 Quinine bisulphate (-9-hydroxy-6'-methoxycinchonan sulfate (1:1) heptahydrate

2.3.3. Definition and Description.

Quinine bisulfate contains not less than 98.5% and not more than 101.5% of total alkaloids, calculated as $C_{20}H_{24}N_2O_2$, H_2SO_4 and with reference to the dried substance. The pH of a 10 mg/ml solution is 2.8-3.4. (International Pharmacopoea 2009) Quinine bisulphate comes in the form of colourless crystals or a white, crystalline powder; odourless and freely soluble in water; but sparingly soluble in ethanol (~750 g/l). Quinine bisulfate effloresces in dry air. Even in the absence of light, it is gradually degraded on exposure to a humid atmosphere, the decomposition being faster at higher temperatures.

2.3.4. Pharmacology of Quinine

Quinine has a negative inotropic action, it slows the rate of depolarization and conduction, and increases the action potential duration and the effective refractory period in the myocardium (Bateman & Dyson, 1986). Prolongation of the PR, QT, and QRS intervals, ST and T waves changes may be observed. The drug also has an alpha-adrenergic blocking effect and hypotension may occur as a result of vasodilation, myocardial depression, or dysrrhythmias (Bateman & Dyson, 1986). Bradycardia rarely occurs due to vagolytic activity. Quinine overdose may result in the delayed onset of cardiac ventricular conduction defects, ectopy, and torsade de pointes and subsequent cardiac arrest 25 hours after ingestion. Quinine administered intravenously may produce venous thrombosis.

Skeletal and smooth muscle – Quinine increases the tension response to a single maximal stimulus delivered to the muscle directly or through the nerve, but it also increases the refractory period of the muscle so that the response to tetanic stimulation is diminished. The excitability of the motor end-plate region decreases so that responses to repetitive nerve stimulation and to acetylcholine are reduced. Thus, quinine can antagonize the actions of

physostigmine on skeletal muscle as does curare. The same mechanism, however, may provoke alarming respiratory distress in patients with myasthenia gravis (Webster, 1990). On uterine muscle it may be oxytocic to the pregnant uterus (Rollo, 1975).

Quinine acts at extrasynaptic autoreceptor sites and presynaptic junction (Clement *et al;* 1998). Quinine blocks uptake and release of dopamine, 5-HT and nor-adrenaline in the brain of mice through the blocking of K^+ channel. Quinine has also been reported to block gap junctions, specifically the neuronal connexin 36 (Margineanu and Klitgaard 2006). This is the major receptor for parasite sequestration in the microvasculature of infected subjects. The disruption of sequestration of parasites by quinine may be mediated by this mechanism. A patent has provided a method for treatment of Parkinson's disease or controlling movement of a Parkinsonian patient by administering an ATP-sensitive potassium channel blocker, such as a sulfonyl urea, or quinine (US Patent 5236932). In Parkinson's disease, a portion of the neurons in the brain which is important in the regulation of movement has been found to degenerate. This portion of the neurons contains a pore or channel in the membrane that lets potassium out of the cell, under certain conditions relating to the metabolism in the neuron ("K ATP channel"). In accordance with the present invention, by administering to the brain of a Parkinsonian patient, especially the substantia nigra portion, thereof, a substance which blocks the K-ATP channel, otherwise uncontrollable movements of the patients may be controlled.

Quinine and quinidine also potentiate the behavioral effects of serotonergic agents and monoamine uptake inhibitors. An investigation into the presynaptic action of quinine and its stereoisomer quinidine on monoamine uptake and release in rat brain tissue *in vitro* showed that both quinine and quinidine evoked the release of 5-HT, noradrenaline and dopamine from pre-loaded rat brain slices in a concentration dependent manner. Both quinine and quinidine also inhibited the active uptake of 5HT, noradrenaline and dopamine into the rat brain (Takahito *et al;* 2008). The potency of inhibition was significantly higher for 5HT than the other two

monoamines. There was no correlation between the potencies to either induce release of monoamines or to inhibit their uptake. This suggests that these effects are mediated by two distinct mechanisms.

Quinine interacts with human neutrophil functions. The drug produced depressive effect on neutrophil chemotaxis and oxidative response at concentrations as low as 10 µg/1, which may be achievable in serum during therapeutic use of this compound. (el Benna *et al;* 1990)

Quinine is a substrate for P-glycoprotein. A study of the impact of P-glycoprotein on the distribution of quinine between brain and plasma revealed an enhanced brain concentration in brain tissues deficient in P-glycoprotein. The concentration of quinine and its metabolite 3-hydroxy quinine in plasma were similar in normal and P-glycoprotein deficient mice. (Pussard *et al;* 2007). Investigations into the accumulation of quinine into *Plasmodium falciparum* parasites showed that passive diffusion in accordance with intra-cellular pH gradients and intracellular binding could account for only a small fraction of the amount of quinine accumulated by the parasites investigated. Results of trans–simulation kinetics suggest that high accumulation of quinine is brought about by a carrier-mediated import system. Results of this and other studies (Arndt *et al;* 2001, Sanchez *et al;* 2008) have shown that acidotropic trapping, binding to intracellular sites and carrier–mediated transport, import and export transport systems all contribute to steady-state intracellular quinine accumulation. The weak base quinine passively permeates plasma membrane at physiological pH and the passive uptake increased with increasing pH. At pH 6, over 99% of quinine is positively charged. The substrate site has low affinity for the positively charged quinine.

Study of the disposition of injected quinine in controlled and *Plasmodium berghei*-infected mice showed that whole blood concentration and $AUC0_{o-\infty}$ of quinine increased in a parasitemia dependent manner. The liver and brain concentrations of quinine were similar in control and infected mice but the tissue to plasma free-fraction ratios decreased when the parasitemia rose. This suggests a restrictive uptake of quinine by the liver and brain (Pussard *et al;* 2003)

Administration of quinine to adult Wistar rats for a period of seven days resulted in neuronal degeneration and loss of Nissl substances as evidenced by reduced staining intensity in the Nissl substances of the cerebellar cortex in treated rats. This implies that quinine adversely affects the Nissl substances of the cerebellar cortical layers of the rat model and by extension, may hamper proper coordination of muscular activities and maintenance of posture and equilibrium (Adesina *et al;* 2009). Parenteral quinine administered in therapeutic doses to a pediatric population appears to cause a transient depression in photoreceptor function. No evidence of ocular quinine toxicity was found at the therapeutic doses used (Lochhead *et al;* 2003)

2.3.5. Uses and Therapeutic Dosage

Quinine is used in the chemotherapy of malaria, especially in chloroquine-resistant malaria and cerebral malaria (White & Warrel, 1983; Hall & Peters, 1985). It is also used for nocturnal leg cramps. In patients with myotonia, heroin may be diluted with quinine (Lupovich *et al.*, 1970). Quinine has often been used as an abortifacient. It has also been used as antipyretic and analgesic. Tonic water contains approximately 80 mg/L quinine sulphate as a flavouring agent.

i. Quinine as an Antimalarial

Adults- Oral treatment: 600 to 650 mg three times daily for at least 3 days (USP; Reynolds, 1996), usually as the sulphate, hydrochloride, or dihydrochloride. These 3 salts contain approximately the same amount of quinine and any of them can be used when the dose is cited in terms of "quinine salts" (Reynolds, 1996). The usual dosage of total quinine for malaria is 600 mg three times daily after meals (Webster, 1990).

Intravenous: initial loading dose of 7 mg/kg of quinine dihydrochloride given over 30 minutes followed immediately by maintenance infusions, or an initial dose of 20 mg/kg (up to a maximum of 1.4 g) given over 4 hours with maintenance infusions being started 8 to 12 hours later. Maintenance infusions: 10 mg/kg (up to a maximum of 700 mg) given over 4 hours every

8 to 12 hours. If parenteral therapy is required for more than 48 hours the dose should be reduced by one-third or one-half. Patient should be monitored carefully for signs of cardiotoxicity. Therapy should be changed to oral administration as soon as possible to complete the course. If intravenous infusion is not possible, quinine dihydrochloride may be given intramuscularly where doses, including the loading dose are the same as those used for intravenous administration (Reynolds, 1996).

Children - Oral treatment: 10 mg of quinine salt per kg body-weight 3 times a day (Reynolds, 1996).

ii. Quinine in management of Nocturnal leg cramps

Recumbency leg muscle cramps (night cramps) are reportedly relieved by quinine. The usual quinine sulphate dose is 200 to 300 mg, which may be repeated once before retiring. In some individuals, only a brief period of quinine therapy is required to provide relief; while even large doses of the drug are ineffective in others (Webster, 1990).

Contraindications

- Quinine is contraindicated in patients with a history of hypersensitivity to quinine and other cinchona alkaloids
- In the presence of haemoglobinuria during malaria and in patients with optic neuritis, or tinnitus (Reynolds, 1996).
- It should be used with caution in patients with atrial fibrillation and other myocardial conduction abnormalities.
- Quinine may enhance the effects of anticoagulants.
- Concurrent use with cimetidine, amiodarone, digoxin and other digitalis glycosides
- Therapeutic administration of mefloquine within the preceding 14 days.

- Resistance of Plasmodium falciparum to quinine
- In patients with myasthenia, quinine may cause severe respiratory distress and dysphagia.
- In patients with glucose-6-phosphate deficiency, blackwater fever may be observed when quinine is used (Reynolds, 1996).

2.3.6 Clinical toxicity

Toxicity is observed after overdoses of 2.5 to 4 g in adult (Murray & Jay, 1983). Adverse effects are not dose-related. In children, toxicity is described above 25 mg/kg. Fatalities have occured at the 900 mg dose in children aged 1 to 2 years (Grattan-Smith *et al;* 1987). In a series of overdoses with quinine in adults, 75% of patients were symptomatic, 17% had visual symptoms, and 50% had adverse cardiovascular effects (Dyson *et al;* 1985). In a review of 16 quinine overdose patients, 9 patients had reversible or irreversible visual damage, 3 had cardiac arrhythmias and one death was reported (Bateman *et al;* 1985). The patient may present with stupor, coma, confusion, delirium or have extensive muscle weakness. Convulsions can occur (Wolf *et al;* 1992). The clinical toxicity on intra-venous and intra-muscular is the same as in oral administration but the onset is more rapid.

2.3.7 Chronic poisoning

When ingested, toxicity can be observed in several body systems including central nervous system (CNS), cardiovascular, gastrointestinal (GI), dermatology, hematology and renal. Cinchonism may occur with therapeutic doses (Reynolds, 1996). Progressive visual loss following ingestion of excessive amount of Indian tonic water has been reported (Horgan & Williams, 1995). The major causes of morbidity in quinine overdose include reversible renal

failure, cinchonism, prolonged hearing deficits, and blindness; the skin is often hot and flushed initially then may become cold and pale (Reynolds, 1993; McEvoy, 1994). Death generally follows cardiac disturbances, renal failure, acute haemolytic anaemia and respiratory arrest. In severe poisonings, headache, fever, vomiting, excitement, confusion, delirium, syncope, lowering of the body temperature. Vertigo and ataxia, with presumed involvement of the vestibular nerve may occur (Bateman & Dyson, 1986).

Gastrointestinal effects include nausea, vomiting, abdominal pain, and diarrhea resulting from the local irritant action of quinine. Nausea and vomiting may also be secondary to a CNS mechanism (Goodman & Gilman, 1990). Granulomatous hepatitis has been reported, also cholestatic and hepatocellular toxicity, probably secondary to a hypersensitivity reaction.

Endocrine and reproductive system - Therapeutic doses of quinine may lower blood glucose concentrations by stimulating insulin secretion (Looareesuwan *et al;* 1985). Recurrent hyperinsulinemic hypoglycaemia can be particularly serious when quinine is used during pregnancy or when there is severe infection (Webster, 1999). Quinine has been reported to decrease male reproductive capacity. Use of quinine as an abortifacient can produce poisoning in the fetus with frequent infant deafness

Haematological effects – Quinine-induced agranulocytosis has been reported. It has been confirmed by the inhibition, (in-vitro) of bone-marrow cell cultures by therapeutic concentrations of quinine (Sutherland *et al;* 1977). Other abnormalities include: Coombs' positive haemolytic anaemia, neutropenia, dissiminated intravascular coagulation, hypoprothrombinaemia, and thrombocytopenia (Aster, 1993; Maguire *et al;* 1993). Haemolysis can result when quinine is administered to patients with G6PD deficiency

Local Effects:

Ear: Transient hearing loss, usually a first side effect, occurs a few hours after initiating high-dose therapy (up to 2 g in the treatment of malaria). After prolonged daily courses of 200 to 300

mg, up to 20% of patients can be expected to suffer from hearing loss. The sensorineural hearing loss is typically reversible, bilateral, and symmetric, affecting the high frequencies first (4, 6, and 8 kHz) with a characteristic 4-kHz notch. Ototoxicity of quinine is probably secondary to direct cochlear hair cell damage and vertigo and ataxia secondary to presumed involvement of the vestibular nerve may occur (Bateman & Dyson, 1986).

Eye: as early as 15 to 30 min after a quinine overdose the patient may complain of sight impairment or blindness. The mean time of onset of blindness is 6 hours, while usually gradual, can be sudden. It may pass after 14 to 24 hours, however it may last up to 10 weeks or longer. Generally there is full recovery in 1 to 3 weeks, however the patient may become permanently blind. Ophtalmic findings during the acute phase are dilated pupils and unresponsiveness to light in proportion to the degree of blindness. The ophthalmoscopic appearances have varied considerably from case to case and this has occasioned much dispute concerning the mechanism by which vision is affected. In some cases the retinal vessels have appeared narrowed early. In another group of cases the retina has appeared oedematous and the papilla hyperaemic. Occasionally a red spot in the macula has been noted with oedema. In yet other cases the fundi have appeared normal at an early stage while the patient was already profoundly blind. Pallor of the optic nerve heads has often been noted, developing gradually, generally proportional to the amount of permanent loss of visual field. In the acute stages of loss of vision, the electroretinogram may be normal. Visual loss was observed in a patient who was drinking 4 liters of tonic water each day for 12 months prior to presentation. Retinal vessels were of normal calibre but each macula exhibited fine stippling show by fluorescein angiography (Horgan & Williams, 1995).

Allergic reactions – Some patients are hypersensitive to quinine and even small doses may give rise to symptoms of cinchonism, together with angioneurotic oedema, asthma and other allergic

phenomena (Barr *et al;* 1990). Haemolytic anaemia and thrombocytopenia have been reported. As a test for quinine idiosyncrasy a scratch test may be made with a 1 to 10% solution of a quinine salt in physiological saline: redness, oedema and itching occur within 5 to 15 minutes if the patient is hypersensitive (Reynolds, 1982).

2.3.8 Management of Over-dosage and Toxicity

Admission in an intensive care department is indicated with monitoring of the airway, breathing, and circulation. The use of ipecac should be avoided and gastric lavage used only for recent/large ingestion. Activated charcoal is very effective and should be the primary method of gastric decontamination. Vasopressors and specific treatment of conduction disorders may be required. Antiarrhythmics that depress conduction should be avoided, i.e. quinidine, procainamide. Sodium bicarbonate may be used, while fluids and electrolyte administration may be required. Efficacy of elimination procedures is limited by extensive protein binding and volume of distribution Acidification of urine is not recommended, as it does increase cardiotoxicity. Forced diuresis is not effective. Early resin haemoperfusion may be effective in severe poisoning. In later phases of poisoning, haemoperfusion, haemodialysis or peritoneal dialysis are not effective. Exchange transfusion has not been successful (Bateman *et al;*1985).

2.3.9. Mechanism of action and Pharmacokinetics

Quinine is a potent and fast-acting antimalarial which acts by interfering with protein metabolism in malaria parasite. The antiplasmodial effect is related to the quinine concentration in the parasite. It acts by affecting the parasites intra-erythrocytic mobility and leads to a cessation of haemozoin pigment formation as a result of disturbed or arrested protein metabolism (Martindale 1989). Several studies conducted in adults as well as children in the

malaria endemic zones of Africa indicate that erythrocytes infected by malaria parasites show active uptake of quinine (White and Warrell 1983, Sanche *et al;* 2008).

The pharmacokinetic properties of and therapeutic responses to quinine vary with age, pregnancy, immunity, and disease severity. Bioavailability for quinine increased in approximate proportion to the dose. A linear relationship exists between dose and maximum plasma concentration and peak plasma concentration remains unchanged over dose range (White and Warrell 1983, Babalola *et al;* 1998). The occurrence of adverse effects with oral quinine is also dose and plasma quinine concentration dependent; central nervous system side effects increased as dose and plasma concentrations increased (Orne 1987). In Caucasians with and without malaria, quinine uptake is a time dependent phenomenon and intravenous quinine pharmacokinetics in healthy Caucasians is similar to those reported for Nigerian and Thai subjects (Claessen *et al;* 1998). A population pharmacokinetic analysis of quinine in Ghanaian children with severe malaria was reported by a group of workers (Krishna *et al;* 2001) Pharmacokinetic parameters are consistent with those derived from non-population pharmacokinetic studies (clearance is 0.05 liter/h/kg of body weight; volume of distribution in the central compartment is 0.65 liter/kg; volume of distribution at steady state 1.41 liter/kg; half-life at b phase 19.9 h).

Intramuscular quinine was associated with minor, local toxicity in some patients (12%), and 10% experienced one or more episodes of post admission hypoglycemia. A loading dose of intramuscular quinine results in predictable population pharmacokinetic profiles in children with severe malaria and may be preferred to the intravenous route of administration in some circumstances (Pussard *et al;* 1999).

Quinine is metabolised mainly by the hepatic mixed-function microsomal enzyme cytochrome P_{450} and it is eliminated in healthy volunteers in the urine mainly as unchanged drug and as 3-hydroxyquinine. Glucuronidation is an important pathway for the renal elimination of quinine, mainly as direct conjugation of the drug (Martindale 1989, Orne M. 1987)

Both malnutrition and malaria affect drug disposition and are frequent among children in the tropics. Malaria and malnutrition increase plasma concentrations of quinine and reduced both the volume of distribution and the total plasma clearance. Simultaneously, $alpha_1$-glycoprotein plasma concentrations and protein-bound fraction of the drug is also increased. Similar effective and nontoxic quinine concentration profiles were obtained in malaria with and without malnutrition. Severe global malnutrition and cerebral malaria have a similar effect on quinine pharmacokinetics in children. Moderate malnutrition does not potentiate cerebral malaria-mediated modifications of quinine disposition. These suggest that current parenteral quinine regimens can be used, unmodified, to treat children with both malaria and malnutrition. (Pussard *et al;*1999). Researchers have found that quinine can block a cell's ability to take up the essential amino acid tryptophan, a discovery that may explain many of the adverse side-effects associated with quinine. These findings suggest that dietary tryptophan supplements could be a simple and inexpensive way to improve the performance of this important drug. Avery *et al;* (2009) examined the effects of quinine on a collection of 6000 yeast mutants, each one lacking exactly one of the yeast's 6000 genes. While quite different from humans, yeast is comparable on a cellular level and yeast is frequently and successfully, used as front-line agents in testing chemicals and small molecule drugs. Results showed that strains of yeast that are unable to make tryptophan were extremely susceptible to quinine poisoning, which led them to identify a tryptophan transporter as a key quinine target (yeast that cannot make their own tryptophan have to rely exclusively on external sources, and thus die if tryptophan transport is blocked). Quinine reactions are more severe in malnourished individuals. Unlike yeast, humans cannot make their own tryptophan and thus require dietary tryptophan. Quinine severely reduces tryptophan uptake, therefore malnourished patients especially those with preexisting tryptophan deficiencies would be especially at risk. Tryptophan is important as a precursor for the brain chemical serotonin, so the enhanced tryptophan deficiency induced by quinine could explain why many of quinine's side effects are localized to the head region. Side-effects of quinine

could be averted simply by taking dietary tryptophan supplements in conjunction with quinine treatments. Another group of workers have further shown that transport of [14C] glucose is inhibited by quinine, with kinetic data indicating competitive inhibition. Quinine-induced glucose limitation is a specific response (do Santos *et al;* 2009)

In pregnancy and lactation - Quinine crosses the placental barrier and is also excreted in breast milk in the un-metabolized form. Phillips *et al;* (1986) in a study in pregnant and lactating women found that plasma quinine concentrations fitted closely a single exponential decline after the intravenous infusion with data fitted to a one compartment model. Placental cord plasma quinine concentrations correlated significantly with maternal plasma quinine concentrations. Heart blood from a foetus aborted at term had a plasma quinine concentration of 2.8 mg $l^{-1;}$ simultaneous maternal plasma quinine was 7.1 mg l-1 (ratio 0.39). For lactating mothers, breast milk quinine concentrations and milk to plasma ratios were 0.5 – 3.6 mg l^{-1} and 0.11-0.53 in women who were breast-feeding and had taken oral quinine sulphate for 1-10 days. In women with more serious infections that received intravenous quinine; breast milk quinine concentrations was much higher, ranging between 0.5 and 8.0 mg $l^{-1.}$ The important toxic effect of quinine in late pregnancy is not an oxytocic action but rather it's the capacity to release insulin and produce hypoglyceamia (Looareesuwan *et al;* 1985). The drug should therefore be used with caution in pregnant and lactating women.

In Chronic renal failure - Quinine is often used to prevent muscle cramps in patients with chronic renal failure. A standard dose of 300 mg at bedtime is usually recommended. This class of patients with chronic renal failure has an increase in plasma protein binding and in the clearance of free drug, resulting in lower plasma concentration of free quinine. A study of the pharmacokinetics of quinine in the presence of renal failure indicates that the concentration of α_1-acid glycoprotein (AAG), the major binding protein for quinine, was increased in haemodialysis patients compared with control subjects resulting in decreased free fraction of

quinine (0.024 *vs* 0.063). The half-life of quinine was not prolonged (15 h). Quinine proved to be non-dialyzable. It was shown that this dose of quinine tended to lead to a low level in blood. A normal dose of quinine (2×15 mg/kg per day) is therefore recommended for malaria therapy, even in cases with renal failure requiring haemodialysis, in order to attain the desired plasma level (5 to 15 $mg \cdot l^{-1}$) (Sharma *et al;* 1989). Quinine is a common cause of drug-associated TTP–HUS. It can cause death and chronic renal failure. The U.S. Food and Drug Administration has banned over-the-counter marketing of quinine, but the drug is still available in beverages and from nutrition stores, pharmacies, and Internet sites (Kojouri *et al;* 2001)

Effect of cigarette-smoking - The effect of cigarette smoking on quinine pharmacokinetics or therapeutic response varies in healthy subjects and patients with *falciparum* malaria. Cigarette smoking has been reported to enhance quinine clearance in healthy volunteers. Pukrittayakamee *et al;* (2002) however found no significant differences in clinical responses or cure rates between smokers and non-smokers with uncomplicated falciparum malaria. There were no significant differences in any pharmacokinetic variables for the parent compound or metabolite between the two groups.

2.4. THE SUPPOSITORY AS A DOSAGE FORM

2.4.1. The Suppository

The main form of rectal administration of medication is the suppository. The suppository is a solid dosage form intended for insertion into the rectum, vagina, or urethra. Rectal suppositories are commonly used for local effects and they are more in pediatrics and geriatric management. The suppository dosage form is a useful drug delivery system in many situations. Points advanced for choosing the rectal route for drug administration include

i. The patient is not able to make use of the oral route. Examples include patients who are vomiting, having seizures, who have obstructions of the upper gastrointestinal tract, or when the patient is post-operative. Furthermore, several categories of patients, i.e. the very young, the very old or the mentally disturbed, may more easily use the rectal than the oral route. Drugs that are often compounded as suppositories in the control of nausea include metoclopramide, haloperidol, dexamethasone diphenhydramine, and benztropine. Salbutamol can be administered rectally for long-term control of asthma, and morphine for chronic pain can be administered in suppository form to yield a slow release product (de Blacy & Tukker 2002):

ii. A local effect is desirable: Glycerine suppositories are often administered rectally for their hygroscopic nature in relieving constipation. A local effect is also desirable in the case of pain and itching mostly due to hemorrhoids. Vaginal preparations for which systemic side effects are undesirable may be administered in suppository dosage form. Progesterone vaginal suppositories are commonly requested by physicians (Europe) from retail pharmacists. These are not manufactured commercially, and must be compounded by the pharmacist. Progesterone suppositories are used in the treatment of numerous disorders and symptoms, ranging from premenstrual syndrome to

complications with pregnancy. The vaginal suppository is a desirable delivery system because progesterone is extensively metabolized by the liver. So avoiding the gastrointestinal tract provides a method of drug delivery in which a smaller dose can be used with greater efficacy (Cheymol 1987)

iii. The drug under consideration is less suited for oral administration. This may be the case when the drug is insufficiently stable at the pH of the gastrointestinal tract, or susceptible to enzymatic attack in the G.I. tract or the liver. Examples are drugs which exhibit extensive first-pass metabolism e.g. oral intake may result in gastrointestinal side effects e.g. nausea and vomiting. Drugs with unacceptable taste will also qualify for the rectal route. The formulation into suppositories of certain drugs that are likely candidates for abuse e.g. in suicide may also be considered (de Blacy & Tukker 2002)

In spite of the above listed advantages, the rectal route has many drawbacks. There are strong feelings of aversion to this dosage form in certain countries, e.g. UK and the USA, whereas the suppository is well accepted in Europe. This dosage form is just being introduced in Africa and is more popular in pediatric medications. More scientific reasons include: slow and incomplete absorption and considerable inter and intra subject variation (Marriot 2006). The absorption of a suppository may also be interrupted by defecation. Cases of rectal ulcerations with necrosis and proctitis have been reported following the excessive use of suppositories containing dextro-propoxyphene or ergotamine (Cheymol 1987).

In spite of these drawbacks, rectal administration can be very useful in drug administration especially where there are clear indications that make this route more advantageous to the patient.

2.4.2 Suppository Bases

The characteristics of an ideal suppository base are the same as for an ideal vehicle regardless of route of administration. The base should be non-irritating, have no pharmacological activity, be stable, be compatible with a variety of drugs and be aesthetically pleasing. There are two main classes of bases in use;

- The Glyceride-type Fatty Bases
- The Water – soluble bases

Although the ideal vehicle has not been found, the large varieties of bases which are available provide a good choice for every drug that has to be formulated as a suppository. For anti-malarial drugs, it would be desirable to have a base that does not melt at room temperature, nor in tropical climates. The fatty vehicles that are commonly in use are semi or fully synthetic ones. Cocoa butter is not frequently used because of its well known polymorphic behavior. The general composition of the two types of fatty bases is mixed triglycerides with C12- C18 acids.

The Macrogols are a class of hydrophilic bases that are commonly used. They consist of mixture of polyethylene glycols of different molecular weights. This category however has the disadvantage of being hygroscopic and can therefore attract water from the rectum resulting in a painful sensation. This osmotic effect of the PEGs has been used to increase the osmotic pressure of fluids that are used for cleansing the gastrointestinal tract. It has however been found that the PEGs exert more osmotic effect than would be predictable from its molecular weight. This may reflect interactions between PEG and water molecules that alter the physical chemistry of the solution and sequester water from the solution (Schiller *et al;* 1988). Some substances have been reported as incompatible with PEGs. These are mostly compounds with the phenolic groups. (Silver salts, tannic acid, aminopyrine, **quinine,** ichthamol, aspirin, benzocaine, iodochlorhydroxyquin and sulfonamides. PEGs react with these substances forming precipitates. PEGs also solubilize numerous substances that are sparingly soluble in

water through the formation of loose and reversible complexes. With acetylsalicylic acid, bacitracine and penicillin G, however, the complexes are irreversible and may therefore inactivate the active substance. Solid polyglycols are preferred bases for suppositories. The desired solidity can be adjusted by choosing the molecular weight and suitable ratios. For example, 25% PEG 1000 and 75% PEG 1500 give very soft masses, 25% PEG 4000 and 75% PEG 6000 will give more solid products (Nagarajan 1996).

Other copolymers are also being investigated as suppository bases. The thermally reversible gels of the block copolymer, Synperonic T908 have been evaluated as vehicles for the rectal administration of indomethacin (Miyazaki *et al;* 1995). Various base combinations are also available as commercial products from suppliers of pharmaceutical ingredients.

The following Table (adapted from "The Art, Science, and Technology of Pharmaceutical Compounding" by. Lloyd V. Allen) summarize the release characteristics of drugs in various suppository base formulations.

Table 2 Release characteristics of drugs in various suppository bases

Drug / Base Characteristics	**Approximate Drug Release Rate**
Oil soluble drug; oily base	Slow release; poor escaping tendency.
Water soluble drug; oily base.	Rapid release
Oil soluble drug; water miscible base.	Moderate release
Water miscible drug; water miscible base	Moderate release; based on diffusion, all components are water soluble.

2.4.3 Drug substance related factors.

The following are factors related to the drug substance which are of importance in determining the quality of the suppository:

- Solubility in water and vehicle
- Surface properties of drug substance
- Particle size of drug substance
- Amount of drug substance
- pKa of drug substance

Several studies have been conducted with various rectal drug formulations to test and confirm the effect of these parameters on suppository quality. The drug solubility determines the type of product, a solution suppository or a suspension suppository. Drugs with high affinity for the base will not be easily released into the rectal fluid for absorption. It seems logical therefore that the first choice for a formulation would be a readily water soluble form of the drug dispersed in a fatty base.

The improvement of wetting properties of the drug particle and consequent absorption is the main reason why surfactants are sometimes added in suppository formulations.

2.4.4. SURFACTANTS

A surfactant is a molecule that is soluble in both oil and water due to its possession of both a water miscible group called the hydrophilic head and an oil soluble group known as the hydrophobic tail. The molecules have affinity for both polar and non-polar solvents. The amphiphile may be predominantly hydrophilic or lipophilic or balanced between the two extremes. The determinant is the number and nature of the polar and non-polar groups present within the molecule (Attwood & Florence, 1983).

Surfactants are amphiphilic

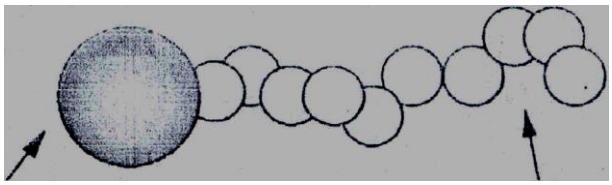

Figure 5: Structure of a surfactant molecule.

(Paunov, V. N.;Introduction to colloids. http://www.hul1.ac.uklscglpaunov/vesko.htm)

2.4.4.1 Classification of Surfactants.

Surfactants have been classified into four groups based on their chemical nature. They are classified as anionic, cationic, and non-ionic or ampholytic according to the charge carried by the surface-active part (polar portion) of the molecule. The hydrophilic part of the most effective soluble surfactant is often an ionic group. Ionic surfactants have a strong affinity for water owing to their electrostatic attraction to water dipoles and are capable of pulling a fairly long hydrocarbon into the solution with them. It is also possible to have a non-ionic hydrophilic group, which also exhibits a strong affinity for water, for instance the monomer units of polyethylene oxide chain each shows a modest affinity.

Surfactants can also be named according to their technological application; hence, names such as detergents', wetting agents, emulsifier and dispersants are also applicable (Aulton 1988).

2.4.4.1.1 Non-Ionic Surfactants

Non-ionic surfactants do not contain a charge on the hydrophilic head hence do not ionize in solution. This advantage results in excellent pH and electrolyte compatibility. Another advantage of this group is that the lengths of both the hydrophilic and hydrophobic groups can be varied. They can accommodate salts of higher salinity than the classic ionic systems. They are generally low in irritation potential, stable and have excellent compatibility. They are effective over pH range of 3-10. Examples of non-ionic surfactants are the Tweens and the Spans.

2.4.4.1.2 Anionic Surfactants

Anionic surfactants contain a negative charge on the hydrophilic head. They are good emulsifiers as well as foaming agents and are therefore applicable as detergents. They are the most widely used of all surfactants on account of their cost and performance. The most commonly used anionic surfactants are those containing carboxylate, sulfonate and sulphate ions. Examples include sodium dodecyl sulphate, alkyl sulphonates and sodium lauryl sulphate (Giles 1981)

2.4.4.1.3 Cationic Surfactants

Cationic surfactants contain a positive charge on the hydrophilic head group. They have biocidal/germicidal activity and conditioning properties. They are effective over pH range 3-7. Examples include the quaternary amines such as Cetrimide.

2.4.4.1.4 Amphoteric Surfactants

Amphoteric surfactants have both negative and positive charges on the hydrophilic group. The major group of molecules in this category are those containing carboxylate or phosphate groups as the anion and amino or quartenary ammonium groups as the cation. They can be used as foam boosters, conditioners, emulsifiers, dispersing agent and thickeners and are stable over a wide range of pH. Examples include alkyl betaines such as N-dodecyl-N, N-dimethylbetaine and the natural phospholipids such as the lecithins and the cephalosporins (Remington, 2000)

Surfactants are added to suppositories for the improvement of wetting properties. They exhibit various degrees of wetting. Non- ionic surfactants and sodium salicylate were investigated as absorption enhancers in in-vitro bioavailability studies of chloroquine suppository in PEG base. Tween 20 was found to be more effective than Brij 35 and Tween 80 in improving drug release. A concentration-dependent effect was observed with Tween 20 which gives an optimal effect with 40% of the surfactant. 25% Sodium salycilate also enhanced drug release (Onyeji 1999). Solutol HS 15, Cremophor RH 40 and Cremophor RH 60 are also examples of formulation enhancers that have been tested in suppository formulations (Berko 2002)

2.5 THE RECTAL ROUTE

2.5.1. Anatomy and Physiology of the Rectal Route

Rectal dosage forms are introduced into the body through the anus and are brought into the lowest portion of the gastro intestinal tract (g.i.t) called the rectum. Anatomically, the rectum is part of the colon, forming the last 150-200mm of the g.i.t. It is separated from the outside world by a circular muscle called the anus. The rectum is a hollow organ with a relatively flat wall surface, without villi and with only three major folds, the rectal valves. The rectal wall is formed by an epithelium which is one cell layer thick, and is composed of cylindrical cells and of goblet cells which secret mucus. The total volume of mucus is estimated as approximately 3ml, spread over a total surface area of approximately 200-400cm^2. The pH of the mucus layer is approximately 7.5. The pH of the rectum will vary with the pH of the drug dissolved in it because of the low buffer capacity (Bottger 1990). The rectum is normally empty and filling provokes defecation reflex. Most rectal suppositories are torpedo-shaped, with the apex or pointed end tapering to the base. Spreading characteristics of rectal formulations may be affected by intraluminal rectal pressure and periodic contractile activity of the rectal wall (Tucker *et al;* 1984)

2.5.2. Absorption Of Drugs From The Rectum

Venous drainage is important for the understanding of drug absorption from the rectum. There are three separate veins. The lower and middle hemorrhoidal veins drain directly into the general circulation, while the upper one drains into the portal vein which flows to the liver. This means that drug molecules absorbed into the lower and middle haemorrhoidal veins can enter the general circulation directly and bye-pass the strongly metabolizing liver. Recent investigations have revealed that avoiding the first passage through the liver is possible. Thus, keeping the drug in the lower part of the rectum is advisable (de Boer 1982). The mechanism of

drug absorption from the rectum is probably not different from that in the upper part of gastrointestinal tract but absorption from suppositories is generally slower and very much dependent on the nature of suppository base, the use of surfactants and other additives and particle size of the active ingredient. Colorectal absorption is a simple diffusion process through the lipoidal membrane. Carrier-mediated mechanisms play no role. Insertion of a suppository into the rectum results in a chain of events which eventually will result in the bioavailability of the drug. Depending on the character of the vehicle, the suppository will either dissolve or melt in the rectal fluid on the mucous layer. The dissolving vehicle attracts water from the rectal region for complete dissolution. This results in a painful sensation for the patient. Suspended drugs will leave the vehicle through concentration gradient and then dissolve in the rectal fluid. The dissolved drug molecules will then diffuse through the mucous layer and then into and through the epithelium of the rectal wall.

Membrane-active adjuvants generally promote absorption more effectively in the colorectal region than in the upper gastrointestinal tract (Muranishi 1984). The rate of release from rectal formulations is however dependent on the nature of the base and the physicochemical properties of the drug involved. It has been reported that in most cases, the rates of absorption increases with increasing solubility and release of drugs from vehicles or adjuvants. Absorption from aqueous and alcoholic solutions may occur very rapidly. This has proved to be of considerable therapeutic value in the rapid suppression of acute convulsive attacks by diazepam suppositories in children (Biggart 2002). The factors involved in the mechanism of availability of drugs from suppositories with lipophilic excipients were studied by using an in-vitro model of the rectal compartment. The solubility in water of the drug substance was found to be the fundamental factor influencing the release rate from suppositories (Realdon *et al;* 2000). Drug concentration in the intrarectal aqueous phase produces the gradient against the large volume of the plasma phase. This gradient regulates the diffusion rate through the barrier. A drug with high water

solubility quickly leaves the excipient, producing a high concentration in the intra-rectal phase which supports a high diffusion rate across the barrier. On the other hand, a drug with low water solubility saturates the intra-rectal phase at low concentration hindering the subsequent dissolution of the drug particles remaining in the melted excipient. The results therefore indicate that drugs with low solubility in water result in low availability, while drugs with good solubility can give an intense and rapid drug supply for a rapid and intense therapeutic response with the dose administered almost completely utilized. With a hydrophilic vehicle, polyethylene glycol (PEG), the drug is released through the progressive dissolution of the PEG into the intra-rectal aqueous phase. The concentration in this small intra-rectal phase produces the gradient against the large volume of the plasma phase. Again drug solubility in water was confirmed as an important factor influencing suppository release rate. Because of the hydrophilicity, PEG influenced in vitro drug availability considerably by increasing both drug solubility and dissolution rate. The osmotic effect of PEG influenced the increase in volume of the aqueous phase. The higher dissolution rate observed with the PEG did not always correspond with a higher diffusion rate. As a matter of fact, drugs less soluble in water showed a greater availability from PEG suppositories while the more soluble drugs were less available (Realdon *et al;* 2001).

Potentially, the rectal route can be said to offer the same absorption possibilities as the oral, but the influence of the formulation seems very critical.

A review of the plasma concentration data following rectal administration of representatives of several classes of drugs reveals that only limited number of cases has actually demonstrated that the rectal route gives plasma concentrations which are comparable to the oral route (de Boer *et al;*1982). The classes of drugs reviewed include anticonvulsants, non- narcotic analgesics, non-steroidal antiinflammatory agents, hypnosedatives and anesthetics, strong analgesics,

theophylline and derivatives, corticosteroids, antibacterial agents, promethazine, progesterone, and ergotamine tartarate.

The avoidance of the first- pass effect has also been assessed by some researchers in Japan using rabbits as experimental animals and lidocaine as drug of study. A comparison of the bioavailability from the rectal route with bioavailability from intra-venous administration indicate that lidocaine was absorbed completely with negligible first-pass effect from intra-venous administration while the avoidance of the first- pass effect through the rectal route was estimated at 60% in spite of complete absorption (Kurosawa 1998).

2.5.3. Antimalarials In Suppository Dosage Forms

2.5.3.1 Chloroquine and Artemisinin Suppositories

A number of anti-malarials have been tested in the suppository form. A suppository formulation of Chloroquine phosphate in polyethylene glycol, PEG 1000 and PEG 6000 (7:3) with 0.5% polysorbate 80 as an absorption enhancer, produced blood levels of chloroquine in children which was considered to be adequate and would be therapeutic in the management of malaria and rheumatoid disease (Onyeji *et al;* 1996, Taylor *et al;* 1995, Antia-Obong *et al;* 1995). The relatively new antimalarial, artemisinin and its derivatives have been subjected to several pharmacokinetic trials in the suppository form. Preliminary studies on artemisinin, artemether and artesunate (Nosten *et al;* 1998, Aceng *et al;* 2005) indicated that the formulations were effective as rectal suppositories with 30 – 50% bioavailability. Doubling or tripling the quantity in the oral formulation might however be necessary to achieve therapeutic blood concentration (Koopmans *et al;* 1999, Krishna *et al;* 2001, Illet *et al;* 2002).

The first detailed pharmacokinetic assessment of intra-rectal artesunate in African children was carried out in Ghana. Intra-rectal artesunate was more rapidly absorbed in low doses than in high doses and parasite clearance kinetics was comparable with intravenous administration. It

was concluded that intrarectal artesunate may be a useful alternative to parenteraly administered artesunate in the management of moderate childhood malaria (Krishna *et al;* 2001). Rectal capsule of Artesunate intended for early intervention in the treatment of severe falciparum malaria has been registered in Nigeria and is currently available in the market (NAFDAC Registered Products, 2009).

2.5.3.2 Intra – Rectal Quinine

Intra-rectal Quinine has been studied extensively in the treatment of children with falciparum malaria. Barennes *et al;* (1995, 1998, 1999, 2001, and 2003), Aceng *et al;* (2007), and Pussard *et al;* (2004) worked extensively on intra-rectal quinine administration in children in some French-speaking African countries (Benin Republic and Niger Republic) and Uganda. Compared to the intramuscular route, Quinimax® intra-rectal solution, applied through an enema pump gave 40% bioavailability. Residual blood quinine concentration at 48 hours was similar, so was mortality and temperature clearance amongst the trial groups. A cream formulation of quinine was prepared as an improvement on the enema with encouraging efficacy profile (Bareness *et al;* 1996). The cream, developed in a French laboratory comes with a syringe-like applicator. An eleven year population pharmacokinetic study to monitor the efficacy of intrarectal quinine by this team of researchers in Uganda (Pussard *et al;* 2004)) revealed very good overall tolerance and no major and/or irreversible complication was observed. Early rejection, intestinal transit problems, and watery stools were the most common problems. In contrast, intramuscular Quinimax® led to residual pain, local inflammation, abscess and possibly lower extremity disability. Babalola *et al;* (1996) tested a quinine suppository formulation in theobroma oil (cocoa butter base) on 12 healthy subjects. The suppository had a poor release profile. Further works therefore needs to be done in the development of quinine suppository formulation to with enhanced release profile.

A number of researchers have tested other forms of quinine suppository formulations. Two quinine rectal gels, designed to produce a sustained release dose were developed and evaluated in the rabbit (Fawaz *et al;* 2008). Both gels exhibit practically similar efficiency of dissolution (ED%) which was however not reflected in the plasma. Bioavailability from the mucoadhesive gel was found to be higher than from the thermosensitive gel. No damage was seen on the rectal mucosa of the rabbit. Another sustained release formulation of "transferring-conjugated solid-lipid-nanoparticles" (SLN) was able to deliver quinine dihydrochloride to the mouse brain. Conjugation with transferring significantly enhanced brain uptake of quinine. A high percentage of the dose was recovered from the brain compared to unconjugated formulation. In comparison, an intravenous quinine dihydrochloride however resulted in much higher concentration of the drug in serum than with SLN (Gupta *et al;* 2007).

The relative ease of administration of the suppository in children with cerebral malaria may account for the interest in this dosage form. A stable and affordable intra-rectal formulation (suppository) of quinine will contribute immensely to the reduction of child mortality in the poor districts of malaria endemic regions. Treatment can be commenced at home since the drug can be administered by care givers. The main objective of these works was to develop and evaluate rectal quinine suppositories to treat acute complicated malaria attack in children. Developed suppository must be able to assure adequate release in the rectum and uptake of the drug in the brain region.

2.6. DISSOLUTION STUDIES

2.6.1. Theory of Dissolution

Dissolution is the process by which a solid of average solubility enters into solution (Remington 12th edition). Noyes and Whitney's (1897) works provides the earliest reference on dissolution. The authors suggested that the rate of dissolution of solid substance is determined by the rate of diffusion of a very thin layer of saturated solution that forms instantaneously around the solid particle (diffusion layer model/film theory model). They developed a mathematical model which is still the basis on which subsequent mathematical analyses of dissolution are based.

$$\frac{dc}{dt} = \frac{DAK}{h}(C_s - C_t);$$

where dc/dt is dissolution rate of the drug , D is diffusion rate constant, A the surface area of the particle, K is the proportionality constant, h is thickness in the stagnant layer, C_s is the saturation concentration (maximum solubility) and C_t is the concentration at time t and $C_s - C_t$ is the concentration gradient. K is also called dissolution constant. Several workers have since worked on further explanations of the different areas of the theory.

In determining the dissolution rate of drugs from solid dosage forms, several physicochemical processes come into play as well as the formulation and the solvent. Temperature of medium and agitation rate also affects rate of dissolution. In-vitro, temperature is kept at 37 ^{0} C and stirring rate is kept constant. An increase in temperature will increase the kinetic energy and the diffusion constant, D while an increase in agitation of the solvent medium will reduce the thickness, h of the stagnant layer, allowing for more rapid dissolution. The effective surface area of the drug may be increased by a reduction in particle size resulting in increase in effective surface area since dissolution takes place at the surface of the solute. Degree of aqueous solubility of the drug also affects rate of dissolution. Ionizable salt of the drug is more water soluble than the free acid or base. Drug in anhydrous state dissolves faster than the hydrous salt. Other factors affecting dissolution rate include; polymorphic state of the drug and formulation

factors. Formulation additives may interfere with dissolution. The nature of the dissolution medium is also very critical. Media commonly used include, water, 0.1N HCL, buffer solutions, water or buffers with surfactants, simulated gastric juice and simulated intestinal juice. The choice depends on the nature of the drug product and the location where the drug is expected to dissolve in the intestinal tract.

The compendia methods for carrying out dissolution tests of drug formulations include:

i. Rotating basket method
ii. Paddle method
iii. Transdermal product testing methods
iv. Enteric coated tablet testing method.

The rotating basket and paddle methods are referred to as 'closed system' methods because a fixed volume of dissolution medium is used

Dissolution data carried out using different methods may not be easy to correlate. Dissolution rates are generally higher with the paddle method than the basket method (Sinko 2006)

2.6.2. In vitro – In vivo correlation of dissolution

Dissolution tests are used for many purposes in the pharmaceutical industry. These include: test of drug release in the development of new products, drug quality control and determination of bioequivalence. For a drug to exert therapeutic action, the active ingredient must get to the site of action in adequate concentration over a definite period of time. Only drug that dissolves and is released from the drug product will be available for absorption into the blood stream. The other critical factor for absorption is the permeability of the drug. This is the rate at which the drug penetrates a biological tissue such as the rectal mucosa and crosses into the blood stream.

With the right dissolution method, rate of dissolution may be correlated to rate of absorption of the drug into the body. This dissolution test therefore becomes a part of the standard quality control procedure for the drug product. If dissolution of drug is rate limiting, faster dissolution

rate may result in faster rate of appearance of drug in the plasma. Absorption time can be correlated to dissolution time. A linear correlation between absorbed drug and dissolved drug may also be obtained if a drug is absorbed completely after dissolution. When different formulations of the same drug are tested, poorly formulated drug will not be completely dissolved and released. This will result in lower plasma drug concentrations. The percentage of drug released at any time interval will be greater for the more available drug product. Recent regulatory developments like the Biopharmaceutics classification Scheme have emphasized the importance of dissolution in the regulation of drug products. The Biopharmaceutics Classification System (BCS) (Amidon *et al;* 1995) classifies drugs using the two important factors of solubility and permeability. BCS has four classes as listed below.

Class I – high solubility and high permeability drugs

Class II- Low solubility, high permeability drugs

Class III- High solubility low permeability

Class IV – Low solubility low permeability

Class I drugs are expected to be well absorbed. The goal of the system is to provide guidance as to when in-vitro studies may be used in lieu of clinical studies to establish bio-equivalence.

Well designed dissolution tests should predict the in-vivo performance of drug products. (*FIP Guidelines 2009, WHO guidelines 2006).* Quinine bisulphate may be regarded as a low solubility drug but the addition of surfactant to the suppository is expected to increase its solubility in the rectal fluid as well as the permeability across the rectal mucosa.

2.7. BRAIN COMPONENTS AND FUNCTIONS

The brain of the mouse is divided into four major sections: the olfactory lobe, cerebrum, cerebellum and the brain stem.

2.7.1 The Olfactory

The olfactory lobe is the most rostral (forward) part of the brain. In humans, however, the olfactory bulb is on the inferior (bottom) side of the brain. The olfactory bulb transmits smell information from the nose to the brain, and is thus necessary for a proper sense of smell. However, the olfactory bulb also receives "top-down" information from such brain areas as the amygdala, neocortex, hippocampus, locus coeruleus, and substantia nigra.

2.7.2. The Cerebrum

The cerebrum, which forms the major portion of the brain, is divided into two major parts: the right and left cerebral hemispheres. The cerebrum is a term often erroneously used to describe the entire brain. The surface of the cerebrum contains billions of neurons and glia that together form the cerebral cortex.

2.7.3. The Cerebellum

The cerebellum is located at the back of the brain beneath the occipital lobes. It is separated from the cerebrum by the tentorium cerebelli. (fold of dura). The cerebellum consist of a cortex and an underlying white matter in which four paired nuclei (fastigial nuclei/ nucleus fastigii, globuse nuclei/ nucleus globosum, dentate nucleus/ nucleus dentatus) can be identified in close relation to the roof of the forth ventricle. The vermis is a part of the cerebellum that is located in

the midline between the two cerebellar hemispheres. The surface of the cerebellum is repeatedly folded in so called folia. The principal functions of cerebellum are to fine tune motor activity or movements e.g. regulate eye movements, e.g. the fine movements of fingers as they perform surgery or paint a picture. It helps one maintain posture, sense of balance or equilibrium, by controlling the tone of muscles and the position of limbs. The cerebellum maintains proper tone of antigravity musculature and provides continuous feedback during volitional movements of the trunk and extremities. Cerebellum is also involved in motor decision-making. There is a somatotopic organisation of the different body parts. The posterior inferior cerebellary artery/a.cerebellaris inferior posterior (PICA), the anterior inferior cerebellary artery/a.cerebellaris inferior anterior (AICA) and the superior cerebellary artery/a.cerebellaris superior (SCA) provides blood supply of cerebellum.

2.7.4. **The Brain Stem**

This is the lower extension of the brain, located behind the cerebellum and connected to the spinal cord. It consists of three structures: the midbrain, pons and medulla oblongata. It serves as a relay station, passing messages back and forth between various parts of the body and the cerebral cortex. This region contains the reticular activating system which is essential for the regulation of sleep, wakefulness and level of arousal as well as for coordination of eye movements. Destruction of these regions of the brain will cause "brain death."

2.7.5. Blood-Brain Barrier

Drugs reach the Central nervous system (CNS) via brain capillaries and cerebro-spinal fluid (CSF). Although the brain receives about one-sixth ($^1/_6$) of cardiac output, distribution of drugs to brain tissue is restricted because the brain's permeability characteristics differ from those of other tissues. Although some lipid-soluble drugs (e.g. thiopental) enter the brain readily, polar compounds do not. The blood-brain barrier (BBB) consists of the endothelium of brain capillaries and the astrocytic sheath. The endothelial cells of brain capillaries, which appear to be more tightly, joined to one another than those of most capillaries, slow the diffusion of water-soluble drugs. The astrocytic sheath consists of a layer of glial connective tissue cells (astrocytes) close to the basement membrane of the capillary endothelium. With aging, the BBB may become less effective, allowing increased passage of compounds into the brain. The blood-brain barrier (BBB) is formed by the brain capillary endothelium and excludes from the brain approximately 100% of large-molecule neurotherapeutics and more than 98% of all small-molecule drugs. The BBB is one of the most challenging barriers in the body. It is created by the way the blood vessels in the brain are organized. Brain capillaries are different from capillaries of other parts of the body in that normal brain endothelia have fewer pinocytic vesicles, more mitochondria, no fenestrations and adjacent cells are maintained in close apposition by tight junctions. The blood-brain barrier (BBB) forms an interface between the circulating blood and the brain and possesses various carrier-mediated transport systems (influx and efflux) for small molecules to support and protect CNS function (Craig 1994). It is important to develop a knowledge base in the fundamental transport properties of the BBB, and the molecular and cellular biology of the brain capillary endothelium. (Pardridge 2005)

The blood-to-brain influx transport systems supply nutrients, such as glucose and amino acids.

Consequently, xenobiotic drugs recognized by influx transporters are expected to have high permeability across the BBB. On the other hand, efflux transporters, including ATP-binding cassette transporters such as P-glycoprotein located at the luminal membrane of endothelial cells, function as clearance systems for metabolites and neurotoxic compounds produced in the brain. Drugs recognized by these transporters are expected to show low BBB permeability and low distribution to the brain. Despite recent progress, the transport mechanisms at the BBB have not been fully clarified yet, especially in humans. Positron emission tomography and proteomic studies have been used to evaluate the activity of human BBB transport systems in vivo. **(**Ohtsuki & Terasaki (2007**)**

BBB transporters also play important physiological roles in maintaining the brain environment. For an energy-storing system, the creatine transporter localized at the brain capillary endothelial cells (BCECs) mediates the supply of creatine from the blood to the brain. The BBB is involved in the brain-to-blood efflux transport of gamma-aminobutyric acid, and GAT2/BGT-1 mediates this transport process. BCECs also express serotonin and norepinephrine transporters. Organic anion transporter 3 (OAT3) and ASCT2 are localized at the abluminal membrane of the BCECs. OAT3 is involved in the brain-to-blood efflux of a dopamine metabolite, a uremic toxin, and thiopurine nucleobase analogues. ASCT2 plays a role in L-isomer-selective aspartic acid efflux transport at the BBB. Dehydroepiandrosterone sulfate and small neutral amino acids undergo brain-to-blood efflux transport mediated by organic anion transporting polypeptide 2 and ATA2, respectively. The BBB transporters are regulated by various factors: ATA2 by osmolarity, taurine transporter by tumor necrosis factor-alpha, and L-cystine/L-glutamic acid exchange transporter by oxidative stress. Clarifying the physiological roles of BBB transport systems should give important information allowing the development of better central nervous system (CNS) drugs and improving our understanding of the relationship between CNS disorders and BBB function

(Ohtsuki S. 2004). The drug penetration rate into CSF, as for other tissue cells, is determined mainly by the extent of protein binding, degree of ionization, and lipid-water partition coefficient of the drug. The penetration rate into the brain is slow for highly protein-bound drugs and nearly nonexistent for the ionized form of weak acids and bases. Because the CNS is so well perfused, the drug distribution rate is determined primarily by permeability. (Goodman and Gilmann 11^{th} edition).

Degree of ionization, and lipid-water partition coefficient (Lipophilicity) is another important factor in drug absorption into the brain. The brain extraction of fifteen C-11-labeled compounds during a single capillary transit was studied. Compounds with log P_{oct} values between 0.9 and 2.5 were found to pass freely across the blood-brain barrier at a cerebral blood flow of 100 $ml \cdot min^{-1} \cdot hg^{-1}$. An apparently decreased extraction of very lipophilic compounds was shown to be related to binding of the tracer to blood components and macromolecules (Dischino *et al;* 1983). A comparison of opioid activity of two dermorphin analogues having an almost identical structure but different structural flexibility showed that the two compounds produced comparable antinociceptive effects in the mouse tail flick test after peripheral administration. This indicates that lipophilicity, rather than side chain flexibility, is the key determinant for blood−CNS barrier penetration (Ballet *et al;* 2008).

CHAPTER THREE

3.0. MATERIALS AND METHODS

3.1. Materials and Equipment

3.1.1 Materials

- The materials used for the study are listed below:
- Cocoa butter
- Fattibase™
- Quinine bisulphate
- Polysorbate 80

3.1.2 Equipment

The equipment used for the study are listed below:

- Digital analytical weighing balance (Denver)
- Stainless steel suppository mould (1g)
- Dissolution Tester USP (Electrolab TDT-08L)
- Ultra-violet/ visible spectrophotometer (Agilent), model 8453
- Vortex mixer (vision scientific 30)
- pH meter (Thermo-Orion, model 420)
- Digital Centrifuge (Centurion K240)
- Micropipette (100 and 200 ml, Ependorf)
- Fluorescence Microscope (Leica DM 1000)
- Light Microscope (Vistavision V169236)
- Hardness tester (Erweka Gmbh, type SBTE. No 51249
- High Pressure Liquid Chromatography Agilent, Model 1100 series

3.2. PRE-FORMULATION STUDIES

3.2.1 Choice of base: It has been reported in literature that water soluble drugs have better release profile from fatty base than from a water soluble base. Two fatty bases were therefore considered for the study: Cocoa butter and Fattibase™. Cocoa butter is available locally and also affordable. When heated, cocoa butter liquefies in 3-7 minutes. Cocoa Butter or Theobroma Oil is an oleaginous base that softens at 30°C and melts at 34°C. It is a mixture of liquid triglycerides entrapped in a network of crystalline solid triglycerides. Palmitic and stearic acids make up about half of the saturated fatty acids and oleic acid makes up the one unsaturated fatty acid. Cocoa Butter contains four different polymorphic forms, α, β, β^1 and γ with melting points of 22°, 34-35°, 28° and 18° C respectively. The β is the most stable and most desired for suppositories. Cocoa Butter will melt to form non-viscous, bland oil. The lower melting point polymorphs will convert to the more stable form over time. When overheated, the suppository will stick to the mould and release will be difficult.

The synthetic Fattibase™ is a tropicalised semi-synthetic base, a pre-blend of triglycerides of edible vegetable oils. FattiBase™ is recommended for use when Cocoa Butter or fatty acid is indicated. The base is a pre-blended suppository base that offers the advantages of a cocoa butter base with few of the drawbacks. It is composed of triglycerides derived from palm, palm kernel and coconut oils with self-emulsifying glyceryl monostearate and polyoxyl stearate used as emulsifying and suspending agents. It is stable with a low irritation profile, needs no special storage conditions, is uniform in composition and has a bland taste and controlled melting range. It exhibits excellent mold release characteristics and does not require mold lubrication. Fattibase™ is a solid with a melting point of 35-37°C, specific gravity of 0.890 at 37°C, is opaque-white and is free of suspended matter.

The Cocoa butter was obtained from Cocoa industries Ltd, Ondo, Nigeria, while the Fattibase™ was obtained from the manufacturer, Paddocks Laboratories, Minneapolis, United States of

America. The surfactant, polyoxyethelene sorbitan monostearate (Polysorbate 80) was obtained from Raymond Lab Chemicals, (U.K) while quinine bisulphate was obtained from BDH, UK

3.2.2. Particle Size Determination – the quinine powder as supplied was crystalline in nature. The particle size of the drug was therefore reduced by communition using a mortar and pestle and the resultant product was passed through sieves of varying sizes; 200, 300 and 500 micron and incorporated into the cocoa butter suppository to determine the particle size that gave the most evenly distributed dispersion in the base.

3.2.3. Characterization and Assays

3.2.3.1 Characterization Studies – these were carried out on the two suppository bases (Cocoa Butter and FattiBase™) using the method in the British Pharmacopeia (BP) 2004 -. The studies carried out are: Acid value (Appendix XB), Iodine value (Appendix XE), Saponification value (Appendix XG) and Melting point (Appendix VA).

Quinine Bisulphate powder and the surface active agent- Polysorbate 80 were assayed to ascertain their purity.

3.2.3.2 Assay of quinine bisulphate. About 0.45g quinine accurately weighed, was dissolved in 15 ml of water. 25 ml of sodium hydroxide (0.1mol/l) was added and extracted with 3 quantities, each of 25 ml of chloroform. The combined chloroform extracts were washed with 20 ml of water. The chloroform extracts were then dried with anhydrous sodium sulfate, evaporated to dryness and the residue dissolved in 50 ml of glacial acetic acid, then titrated with perchloric acid (0.1mol/l). Each ml of perchloric acid (0.1 mol/l) is equivalent to 21.13 mg of $C_{20}H_{24}N_2O_2,H_2SO_4$.

3.2.4. Determination of Displacement Value

The volume of a suppository from a particular mould is uniform but the density of the drug will affect the amount of base that will be required for a particular dose. This allowance for the change in density of the mass due to added medicament is termed 'displacement value'. The displacement value is defined as the number of parts by weight of the drug that displaces one part by weight of the base. The displacement value is to make allowance for each of the additives in the formulation, i.e. quinine bisulphate and polysorbate 80. The displacement value was determined by the procedure reported by Vidras *et al;* (1982). Displacement values of quinine in cocoa butter and FattiBase™ were determined as follows:

The average blank weight per mould was determined for cocoa butter base by preparing six suppositories containing no medicament using the 1g stainless steel suppository mould and the total weight was noted (A). Then six medicated suppositories with 40% $^{w}/_{w}$ quinine bisulphate were prepared and weight noted (B).

The amount of cocoa butter in medicated suppository (C) = $\frac{60}{100} \times B$ Equation 1

Quinine bisulphate content (D) = $\frac{40}{100} \times B$ Equation 2

A – C = weight of cocoa butter displaced by D

The Displacement Value was calculated using the formula below

$$DV = \frac{\text{Quantity of the drug in each suppository}}{\text{Amount of base displaced in each suppository}}$$

$$DV = \frac{D}{A-C} \quad \text{Equation 3}$$

Using this equation, the DV of quinine bisulphate in Cocoa Butter and Fatti Base™ were calculated.

The DVs of Polysorbate 80 in the two bases were also calculated using the same method.

3.2.5. Determination of Quinine Dose in Suppository

Each suppository contained 200mg quinine bisulphate. The WHO recommended dose of quinine for acute complicated malaria are as highlighted:

Oral – 12.5.mg kg^{-1} every 8 hours for 7 days;

Intra muscular dose- 8mg kg^{-1} 8hrly for 3 days,

Intravenous – 7.5mg kg^{-1} four hourly infusion,

The dose in previously tested intra-rectal solution is 12.0 mg kg^{-1} for uncomplicated malaria and 20mg kg^{-1} for complicated malaria. Children aged between 1 – 5 years have a body weight of 10kg to <19kg, the 200mg suppository can therefore be used by a child with 10kg body weight (1year old) and suffering from complicated malaria.

3.3. FORMULATION STUDIES

3.3.1. Preparation of Suppositories

All suppositories were prepared by the fusion method using a stainless steel mould with 12 cavities. The suppository moulds had been previously calibrated with cocoa butter to ascertain the exact weight of the base in each cavity. Each cavity was found to contain 1g. Four batches of suppositories were prepared with each of the bases (Cocoa butter and Fattibase™). The batches of suppositories were prepared with 0 - 5% Polysorbate 80. Eight different formulations were prepared and evaluated.

Table 3 Formula for Prepared Suppositories

Formulation	Cocoa butter (mg)	Fattibase™ (mg)	Quinine bisulphate (mg)	Polysorbate 80 (%)
A	0.8570	-	200.00	0
B	0.847	-	200.00	1
C	0.8373	-	200.00	2
D	0.8077	-	200.00	5
E	-	0. 8776	200.00	0
F	-	0.8699	200.00	1
G	-	0.8625	200.00	2
H	-	0.8393	200.00	5

3.3.2. Characterization of Prepared Suppositories

The following tests were carried out on the quinine suppositories as prescribed in the B.P. (2004)

i. Appearance – the suppositories were examined for uniformity of mix and mottling
ii. Weight variation – 20 suppositories were weighed individually; the mean and variation from mean of each suppository weight were calculated. The standard deviation was also calculated.
iii. Melting range – this was evaluated using a modified method – A Dissolution Tester USP (Electrolab TDT-08L) was filled with 900ml distilled water, heated to 30^{o}C. The suppository was introduced into the medium; heating was continued until commencement of melting. The temperature range of melting was noted. The test was carried out in duplicate and the average determined.

iv. Hardness – an Erweka suppository hardness tester operated at ambient temperature was used for the evaluation. One suppository was placed in the sample holder, known weights in increments of 200g were added to the restraining bar and the weight that crushed the suppository was noted.

v. Chemical assay to ascertain the quantity of quinine bisulphate was carried out using the modified assay method for quinine bisulphate tablet in BP 2004.

Assay method - 20 suppositories were weighed and melted. A quantity of the melted suppositories containing 0.6 g of Quinine Bisulphate was dissolved as completely as possible in 40 ml of acetic anhydride using heat. The mixture was put in the fridge to separate the congealed base. The method I for non-aqueous titration, using crystal violet solution as indicator was carried out (Appendix VIII A). Each ml of 0.1M perchloric acid is equivalent to 54.86 mg of $C_{20}H_{24}N_2O_2,H_2SO_4,7H_2O$.

3.4 RELEASE PROFILE AND STABILITY STUDIES

The Release Profiles (in-vitro release) of quinine from the eight prepared suppositories were evaluated in order to predict the in-vivo release and absorption as well as stability of the drug in the formulation. The stability of the quinine in the formulation was evaluated through the study of the release profile at ambient temperature (20-25^{o}C) and refrigerated conditions (4 ^{0}C) over three months (13 weeks). Other physical parameters were also monitored. The release profile was measured in a pH 8 dissolution media. This represents the pH of the rectum. The pH 8 buffer solution was prepared using KH_2PO4 and NaOH using the formula in the BP 2004 (Appendix 1D A79). The dissolution equipment was an 8 chamber apparatus with a volume of 900ml each. The temperature of the medium was set at 37±1^{o}C and basket rotated at 100 rotations per minute. The basket method was used because the suppositories had low densities and were likely to float in the dissolution medium. 5ml samples were withdrawn from the dissolution medium at time intervals of 5, 10, 15, 30, 45 and 60min. The quinine content was measured using the ultra violet spectrophotometer at 281nm. A calibration curve for quinine was generated by measuring the absorbance of a 0.1 M quinine bisulphate solution in the Ultra – Violet spectrophotometer. Five serial dilutions of the solution were then prepared and absorbance were also measured. The calibration curve was generated by the computer attached to the spectrophotometer (Fig.31). The equation of the curve generated from the calibration curve was used for the calculation of the quinine concentrations. Five samples were tested for each formulation for statistical analysis. The amount of quinine released (percentage) was plotted as a function of time and percentage released from the four cocoa butter formulations were subjected to statistical analysis using the unpaired T-test. The same statistical analysis was carried out on data obtained for the Fattibase™ suppositories. The Fattibase™ + 5% polysorbate was placed on stability at ambient temperature and refrigerator for a 3 – month period. Data obtained was analyzed by ANOVA repeated measure test.

3.5 ANIMAL STUDIES

3.5.1 Preliminary Qualitative Evaluation of Quinine Fluorescence in Water

A preliminary qualitative evaluation of quinine fluorescence in water was carried out to confirm the fluorescence of quinine under the florescent microscope. Various concentrations of quinine bisulphate solution (1, 2.5, 5, 7.5, and 10%) were placed on a slide and observed under the fluorescent microscope.

3.5.2. Monitoring of *Plasmodium berghei* in the brain of mice

The presence of the parasite *Plasmodium berghei* was confirmed in the brain of the infected mice. The four sections of the brains of the mice were removed and microtome cuttings prepared for each section of the brain. The sections were stained with Giemsa stain. The stained sections were then observed under the light microscope and the images were recorded using a Kodak digital camera.

3.5.3. Localization of Quinine Fluorescence in Brain Sections

The murine mice model was used for these studies. The mice were obtained from the pack bred at the Nigerian Institute of Medical Research (NIMR), Yaba, Lagos, Nigeria. The animals were divided into four groups for the studies. Each group was made up of 2 mice which were weighed and the average weight determined.

Group A - This group was made up of healthy mice which served as control. This group also served to detect the presence if any, of naturally fluorescing cells in the brain of the mice. The mice were sacrificed and the brains removed and stored in 1% formalin solution.

Group B – Quinine (dose equivalent to body weight) was administered intra-rectally on two healthy mice. The animals were then sacrificed after 3 hours (C_{max} for quinine) and the brains collected and stored in formalin as in group A.

Group C – This set of animals were infected with *Plasmodium berghei* and sacrificed after 6 days. The brains were removed and stored as in the earlier groups above.

Group D – The last set of animals were also infected with the parasite, *Plasmodium berghei*. Parasite development was allowed to progress for 6 days. Parasite progress was monitored through the animals' PCV. After 6 days, the mice were given a dose of quinine bisulphate suppository corresponding to the body weight of the animals. The brain of the animals were excised and stored in formalin as in the other groups.

The brains were then separated by dissection into the four main segments: olfactory lobe, cerebrum, cerebellum and the medulla oblongata. Microtome sections were prepared from each of the brain segments using the Microtome Cutter in the Morbid Anatomy Department of Lagos University Teaching Hospital (LUTH). The sections from each segment of the brain were observed under the fluorescent microscope. The images were captured using a Kodak digital camera aligned with the eye piece of the microscope. The observed fluorescence was compared for the four groups of animals.

3.5.4. Uptake of Quinine from Suppository in the Brain Sections

The parasitized and non-parasitized murine mice model were used to evaluate the quantity of quinine that gets into the brain within the time frame studied and determine the comparative uptake of quinine in the various brain sections. This set of experiments was also used to compare the uptake in parasitized and non-parasitized mice. The brain of the mice is divided into four main parts: (i) olfactory lobe (ii) Cerebral lobe (iii) Cerebellum and (iv)Medulla oblongata.

The animals for this experiment were also obtained from the pack bred at NIMR. The animals were divided into two groups and kept in cages at the laboratory of the Department of Clinical Pharmacy, Faculty of Pharmacy, University of Lagos. Three groups of animals, Group A B and

C were used for these experiments. Groups A and B were made up of 25 mice each A third group (group C) had 5 mice for measurement of quinine uptake from intra-peritoneal injection. Animals in Group A were divided into five sub groups of five animals each. Each sub-group of healthy mice was administered with a Fattibase™ formulation containing 5% polysorbate 80 and quinine dose equivalent to 20mg/kg body weight of the animals. The average weight of mouse was 25g and the quantity of quinine administered in suppository formulation was 0.5 mg. The suppositories were prepared in a previously calibrated 1ml syringe from which the needle had been removed. The 1ml syringe served as applicator for the administration of the suppository into the anus of the animal. The animals in each group were then sacrificed at the following time intervals:

- Sub-Group AI – 30 min
- Sub-Group AII – 60min
- Sub-Group AIII – 120 min
- Sub-Group AIV – 180 min
- Sub-Group AV – 240 min

The brain for each animal was removed and divided into the four sections that make up the brain. Each brain section for the animals in each sub-group were collected and stored in separate well-labeled bottles containing 1% formalin solution.

The animals in Group B (set of 25 mice) were inoculated with *Plasmodium berghei* (ANKA) (obtained from NIMR) by intra-peritonial injection. The parasites were previously introduced into two animals and kept for two weeks. The PCV and parasite count was checked daily to monitor the proliferation of the parasite in the animals. The two animals were then sacrificed and the blood withdrawn. The blood was diluted with phosphate buffer saline solution (10%) and transferred into the twenty five animals by intra-peritoneal injection. The twenty five

animals were then observed for a period of six days after which quinine suppositories were administered on the infected mice as in group A. The animals in each group were then sacrificed at the same time intervals as in group A and brain sections stored in 1% formalin.

3.5.5. Uptake from Intra-peritoneal administration of Quinine

A third group of five mice (Group C) were infected with *Plasmodium berghei* (Anka) as in group B and quinine bisulphate was administered by intra-peritoneal injection. Dose of quinine administered in intra-peritoneal injection was 0.6mg. By 30min, the animals were very weak. They were therefore sacrificed after 30min and brain sections removed and stored in 1% formalin solution.

3.5.6. Quantification of Quinine in Brain Sections

3.5.6.1 Method of Extraction

The quinine absorbed into the four brain sections of animals in Groups A, B and C was extracted and quantified using High Performance Liquid Chromatography (HPLC). Quinine was quantified in the brain parts using a modified HPLC method previously reported by Krishna *et.al,* (2001) and Babalola *et al;* (1993). This method was chosen because it was sensitive and reproducible. The weighed brain section was macerated in a mortar with the aid of a pestle. 0.5ml pH 7 buffer solution was added to the mixture to moisturize the brain tissue. 10µl of primaquine solution (200µg/ml) was added to the macerated brain section (as internal standard) with thorough mixing. To the mixture was added 60µl ammonia solution with continuous mixing. 230µl of distilled water was then added with further mixing. The content of the mortar was siphoned into a tube and content mixed in a vortex mixer (KMC 1300V). 50 µl of methylene chloride was added with further mixing in the vortex mixer for 45 seconds. The

mixture was centrifuged at 3000g for 10 min. The organic layer was then separated; 200µl of 0.15M hydrochlochloric acid was added and further mixed in the vortex mixer. The mixture was then centrifuged at 3000g for another 45 seconds. The upper aqueous layer was separated and injected into the HPLC. An SMT ELITE column C18, size 4.6 x 250mm was used. Mobile phase was made up of acetonitrile + triethylamine + o-phosphoric acid for adjustment to pH 3. Rate of elution was 1.5ml/min and quinine was eluted at 3.38min

3.5.6.2 Determination of Standard Curve

A standard curve was determined for quinine bisulphate in the HPLC with known concentrations of the drug (50, 100, 250, 500,750 and 1000µg/ml). The area under the curve (AUC) was noted for each concentration using the procedure outlined above and a graph of AUC versus concentration was plotted.

3.5.6.3. Determination of Extraction efficiency

The extraction process was validated by using a known weight (1g) of a whole mice brain, homogenized in a mortar and spiked with varying concentrations of quinine bisulphate solution as used in the determination of the standard curve. The spiked brain parts were taken through the extraction process described above and AUC determined. The AUC of the neat sample was compared with AUC of same concentrations extracted from the spiked brain. Extraction efficiency was calculated as shown in the results.

The area under the curve for each time interval was divided by weight of brain section to get the area per mg of brain section. The average area/mg was multiplied by extraction efficiency to determine actual area. The value was then incorporated into the equation of the graph obtained for neat quinine sample to calculate quantity of quinine extracted. A comparative analysis was

done on the quantities of quinine taken up in the brain of parasitized and non-parasitized mice for the time intervals: 30, 60, 120, 180 and 240 min. The differences in quinine uptake in brain sections of parasitized and uptake in the non-parasitized mice were also analyzed. The uptake from suppository was also compared with uptake from intra-peritoneal injection. The values obtained were subjected to statistical analysis using a 3 way ANOVA,

CHAPTER FOUR

4.0. RESULTS

4.1. Pre-Formulation Studies

4.1.1 Particle size determination - the suppositories prepared using quinine with particle size of 5 micron were smooth and uniform. The 10 and 15 micron particles were not uniformly dispersed in the suppositories. The suppositories had a mottled appearance.

4.1.2 Characterization of Bases

Table 4 Iodine value, acid value, saponification value and melting point of bases

Base	**Iodine value**	**Acid value**	**Saponification value**	**Melting range ^{o}C**
Cocoa butter	**38.75**	4.3	189.34	34.2 – 35.7
Fattibase ™	40.80	4.0	187.45	36.7 – 38.5

The iodine value is a measure of the quantity of iodine that will be taken up by the base while the acid and saponification values measure the quantity of free acids and esters in the base respectively. A good base should have low acid value. The values for the other indicators are as specified in the B.P. The two bases complied with the limits as specified in the B.P. 2005

4.1.3 Displacement value in the bases

The displacement values for quinine and polysorbate 80 in cocoa butter and also in Fattibase™ were calculated and the results are as presented below.

Table 5 Displacement values of quinine and polysorbate 80 in cocoa butter and Fattibase™

	Cocoa butter	Fattibase
Quinine bisulphate	1.39	1.63
Polysorbate 80	1.01	1.30

4.1.4 Assay of Active Ingredients and Excipients

- Assay of quinine bisulphate powder – Mean value = 99.7± 1%
- Assay of Polysorbate 80 – Mean value = 98.80 ± 1 %. The sample of polysorbate 80 complied with the specifications of the British Pharmacopoea (BP) 2005.

4.2. FORMULATION STUDIES

4.2.1. Characterization of suppositories

i. Appearance- Appearance – the cocoa butter suppositories had a light yellow colour which was uniformly distributed. These ingredients were uniformly dispersed with no mottling. The Fattibase^{TM} suppositories were slightly off-white in colour and a uniform appearance

ii. Weight variation – All the suppositories met the acceptable limits of the BP 2005 which states that individual weights of suppositories can vary by 4 ± 0.5% of the average weight of 20 suppositories. The results are presented in Table 6

Table 6 Weight variation of suppositories

Formula	Mean of weight variation of 20 values (%)	Standard Variation (N=20)
A	-2.40	±0.44
B	-2.60	±0.42
C	-1.00	±0.23
D	-2.40	±0.38
E	-1.5	±0.30
F	-1.8	±0.22
G	-2.2	±0.34
H	-2.2	±0.32

iii. Content Uniformity – results are presented in Table 3 of Appendix I

Table 7 Percentage quinine content in suppositories

Formulation	**%quinine content**
A	**90.5**
B	93.2
C	96.50
D	97.80
E	95.40
F	97.60
G	97.80
H	**99.0**

iv. Hardness – see Table 8

Table 8 Hardness of suppositories

Formulation	Hardness (mg)			
	0 month	**1 months**	**2 months**	**3 months**
A	**600**	-	-	-
B	**800**	-	-	-
C	**1000**	-	-	-
D	**1200**	-	-	-
E	**600**	-	-	-
F	**600**	-	-	-
G	**800**	-	-	-
H	**800**	**800**	**1000**	**1000**

v. Chemical assay – assay of quinine in Cocoa butter suppository ranged between 95.5 and 97.8%; while the Fattibase suppository had quinine content of 95.4 – 99.0%. The BP 2005 specifies a limit of 95- 105% for suppositories.

vi. Melting Range of suppositories - All Cocoa butter suppositories melted within the range of 30.5 – 34.5^{o}C while all Fattibase™ suppositories melted within the range of 35.0 -37.5^{o} C

4.3. RELEASE PROFILE OF QUININE FROM SUPPOSITORIES

The cocoa butter-based formulation which contained no polysorbate 80 (formulation A) released 36.5% of the quinine content after 5 minutes in the dissolution medium, rising to 36.86 % after 1 hour. About 73.6 mg of quinine bisulphate was released in 1 hour. Addition of 1% Polysorbate 80 into the formulation (Formulation B) improved release rate slightly with the

release of 75.20 mg quinine bisulphate in 1 hour. The release profile for the formulation containing 2% was erratic while the formulation with 5% released a total quantity at the end of 1 hour which was not significantly higher than the quantity released from Formulation B.

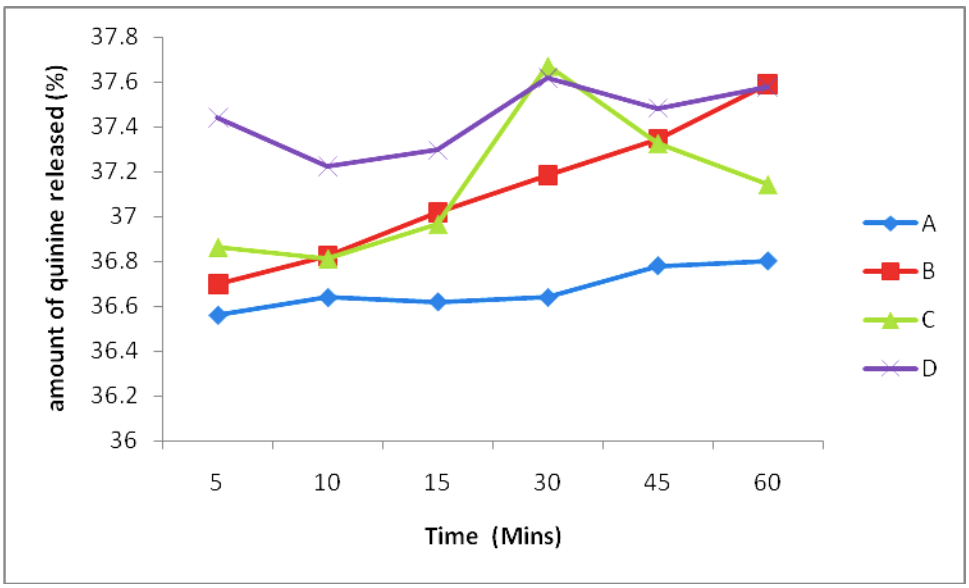

Fig. 6 Amount of quinine released from Cocoa butter suppositories

- A – Cocoa butter + 0% polysorbate 80
- B – Cocoa butter + 1% polysorbate 80
- C – Cocoa butter + 2% polysorbate 80
- D – Cocoa butter + 5% polysorbate 80

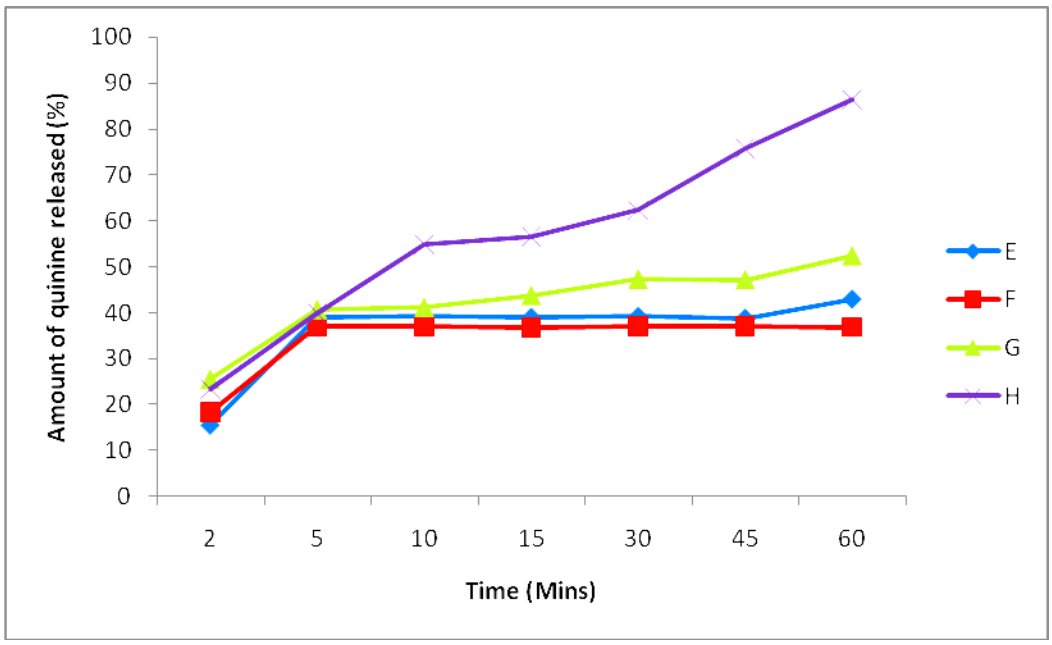

Fig.7 Amount of quinine released from Fattibase^{TM} suppositories

- E – Fattibase^{TM} + 0% polysorbate 80
- F – Fattibase^{TM} + 1% polysorbate 80
- G – Fattibase^{TM} + 2% polysorbate 80
- H – Fattibase^{TM} + 5% polysorbate 80

The best dissolution profile was observed in the Fattibase^{TM} with 5% polysorbate 80 (Formulation H). 85% of the quinine content was released in 60 min. This formulation was therefore placed on stability studies with samples in the refrigerator (4-5^{o}C) and at ambient temperature (20-25^{o}C). The samples were then tested monthly for 3 months

4.4. STABILITY STUDIES

The main purpose of quality assurance is to ensure a high probability that a pharmaceutical formulation will have homogenous characteristics and properties to ensure clinical safety and efficacy. During storage, a pharmaceutical formulation may degrade and the rate of degradation will be determined by the concentration of the drug and the rate constant for the degradation process. The rate constants are temperature dependent. The International Conference for the Harmonization of Technical Requirements for Registration of Pharmaceutical for Human use (ICH) provided a guideline for predicting shelf life from data obtained from a stability testing protocol. Accelerated stability studies at high temperature could not be carried out because the main excipients are fatty bases with melting point at 34-37 ^{0} C.

Physical properties – the suppositories under study were neither disfigured nor melted under ambient temperature and in the refrigerator. The weights of the suppositories also remained relatively constant throughout the study period. Lipophillic bases are not hygroscopic hence they will not easily absorb moisture from the environment. By 3 months, the samples stored at ambient temperature had shown signs of deterioration with mould growth.

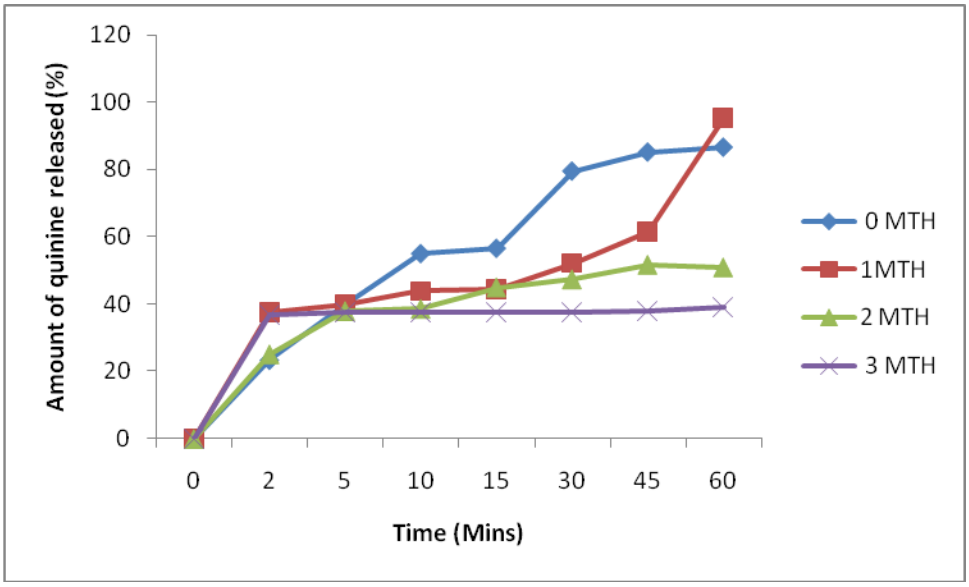

Fig.7 Three-Month Release Profile of quinine in Fattibase™ + 5% polysorbate 80 stored in ambient temperature

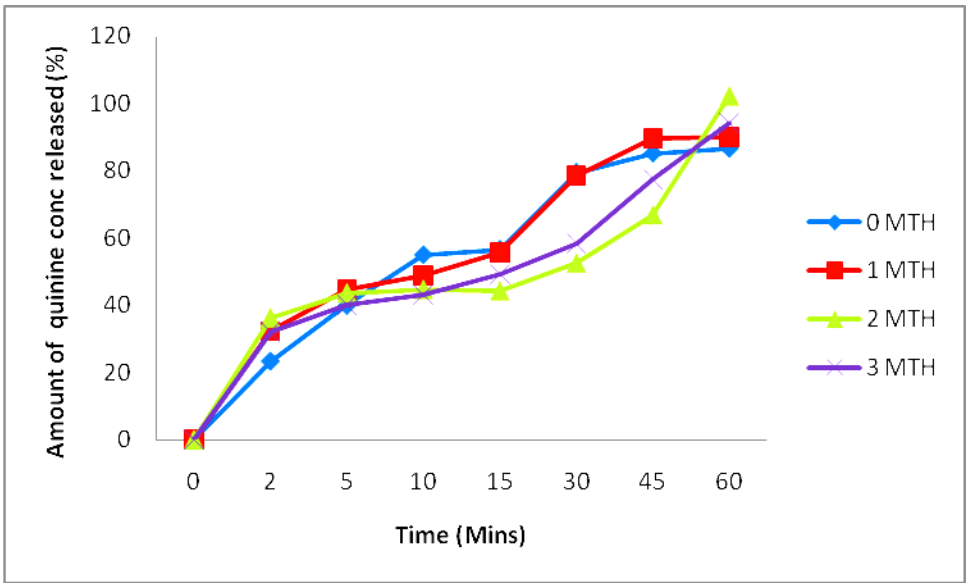

Fig.9 Three-month Release Profile of quinine in Fattibase™ +5% polysorbate 80 stored in refrigerator

The results of release profile show consistent release throughout the 3 months of study for samples stored in the refrigerator (Fig.9). The refrigerator preserved the integrity of the suppositories and they were stable over the period of study. At the end of one month, the release of quinine from samples stored at ambient temperature (20-25°C) was delayed, picking up gradually from 30 min and attaining 80% release by 60 min (Fig. 8). At two months, the sample

released only 45% at 60 min while the 3-month sample released only 40% steadily from 5 min to 60 min.

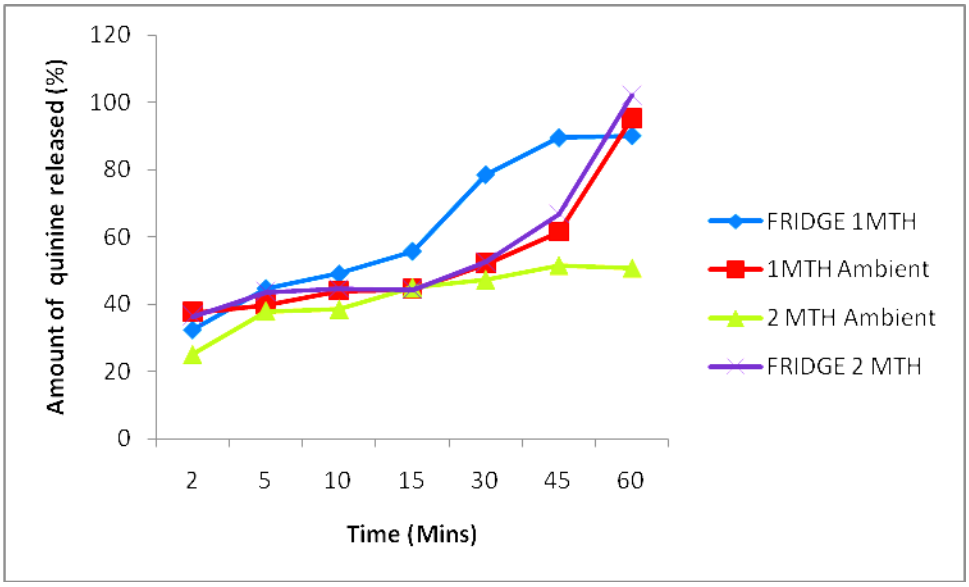

Fig. 10 Comparison of release profile of samples stored in refrigerator and at ambient temperature

The concentration of the quinine released from the refrigerated sample after 1 month storage is much higher than from samples at ambient temperature and did not vary significantly over the 3-month period (Fig. 10). Quantity released from sample at ambient temperature after a one month period was the same as quantity released from samples stored in the fridge for two months. One month storage at ambient temperature had the same effect on drug release as two months of refrigeration.

The Repeated Measure ANOVA Procedure was used to examine the stability of release profile of quinine in Fattibase™ (Appendix 1). The results of release profile show consistent release throughout the 3 months of study for samples stored in the refrigerator. The refrigerator preserved the integrity of the suppositories and they were stable over the period of study. At the end of one month, the release of quinine from samples stored at ambient temperature (20-25^{o}C) was delayed, picking up gradually from 30 min and attaining 80% release by 60 min. At two

months, the sample released only 45% at 60 min while the 3-month sample released only 40% steadily from 5 min to 60 min.

+ 5% polysorbate 80 stored in refrigerator for four different time periods—Month 0, Month 1, Month 2 and Month 3. The months represent four levels of the independent variable.

Table 9 Mean percentage released from refrigerated samples

	Mean	Std. Deviation	N
Fr0mon	60.829033	24.1142957	7
Fr1mon	62.848343	23.1483945	7
Fr2mon	55.795971	22.6095792	7
Fr3mon	56.264286	22.1918407	7

Table 9 displayed the means and standard deviation of each month across time. On average, the release profile after one month is more than the ones for the other months. A 2-way ANOVA was used to analyze the statistical difference in release with month as a variable. There was no significant difference in the release profile across months. This means that there is stability in release profile over the course of study.

The mould growth observed by two months may be due to contamination since the formulations did not contain preservatives and were not packed under sterile conditions.

4.5. ANIMAL STUDIES

4.5.1 Quinine Fluorescence in brain cells

Quinine fluorescence at various concentrations in distilled water was examined and it was found that the fluorescence was highest in the 1% solution. The fluorescence decreased as the concentration increased. Quinine bisulphate is poorly soluble in water therefore dissolution in water reduced as the concentration increased.

The detailed observations in brain section are presented below.

Group A – no fluorescence was observed in brain sections of the healthy mice in the absence of quinine. A few artifacts showed fluorescence on the surface of sections. The fluorescence was of no definite shape (Fig 10).

Group B – cut sections from the four brain portions of healthy mice with quinine all had fluorescence. The fluorescence was seen on the cells and had definite round shapes like dots in every cell. The intensity however varied from one brain part to the other (Fig 11).

Group C – cut sections of the brain of parasitized mice that were not treated with quinine suppository had no green fluorescence. The characteristic green fluorescence with circular dots of quinine was absent in these brain sections (Fig 12).

Group D – sections of the brain of parasitized mice which had been previously treated with quinine suppository had quinine fluorescence. The fluorescence is deeply embedded in the tissue. The fluorescence is more widespread in comparison with the sections from non parasitized mice (Fig 13).

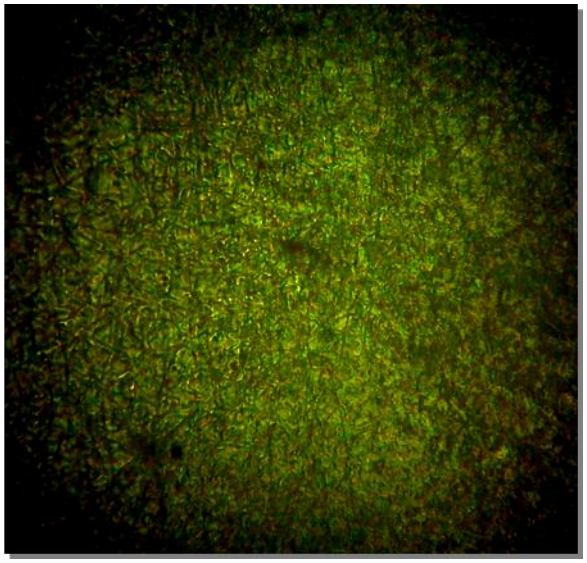

Fig.11 Healthy Brain Tissue of mice
Magnification 63*/0.75

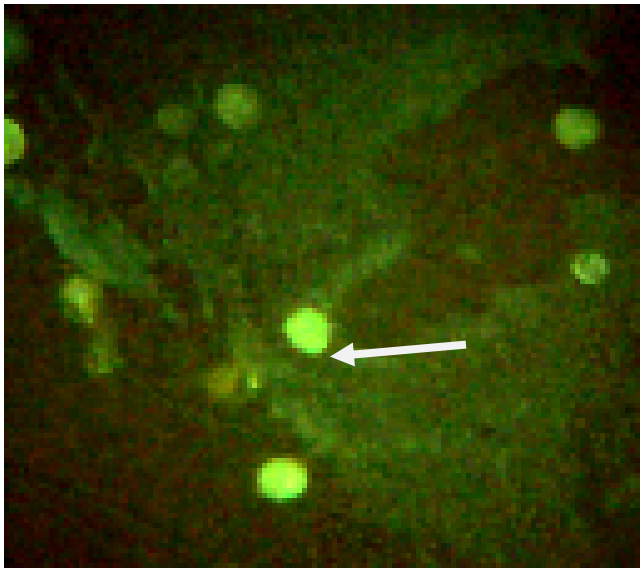

Fig.12 Healthy Brain Tissue with Quinine
Magnification 63 */ 0.75

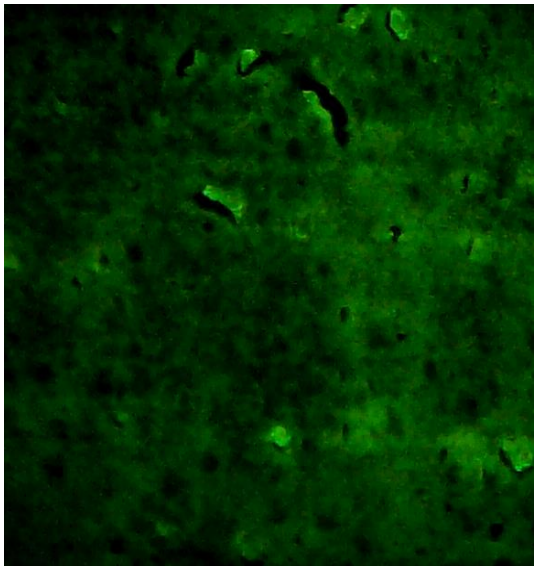

Fig.13. Photomicrograph of Parasitized Mice brain without quinine
Magnification 63*/0.75

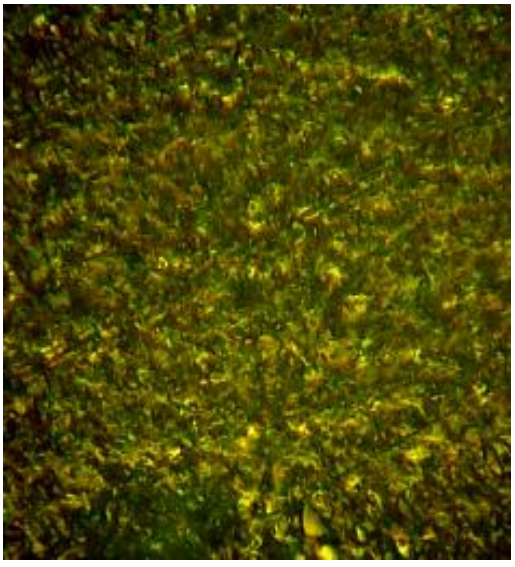

Fig.14 Photomicrograph of Parasitized mice brain with quinine
Magnification 63 */ 0.75

4.5.2 **Parasites in the Four Brain Sections**

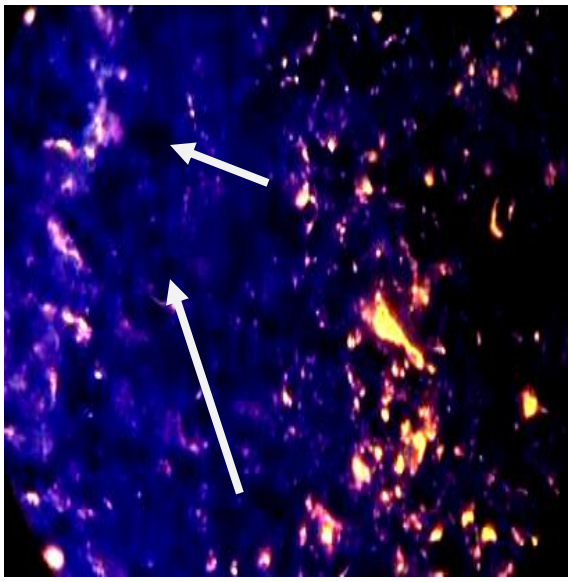

Fig 15 Photomicrograph of Parasites in Olfactory lobe. **Magnification x 100**

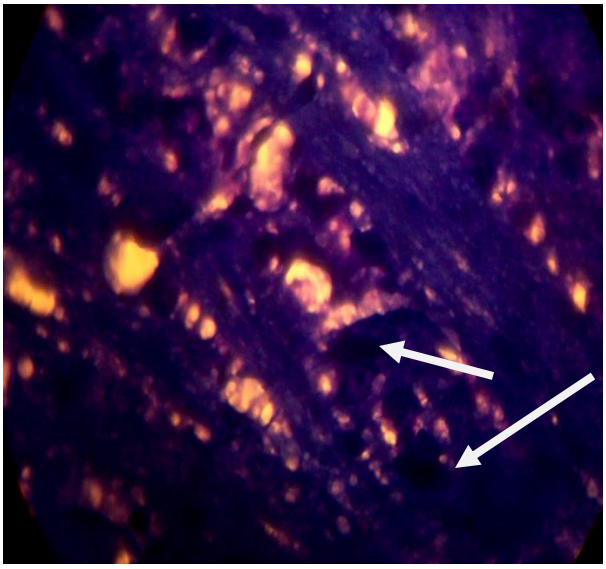

Fig 16. Photomicrograph of parasites in Cerebrum. **Magnification x 100**

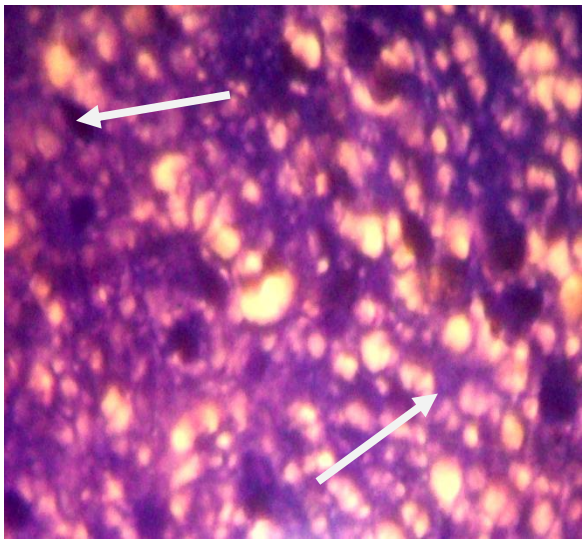

Fig.17.Photomicrograph of Parasites in cerebellum **Magnification x100**

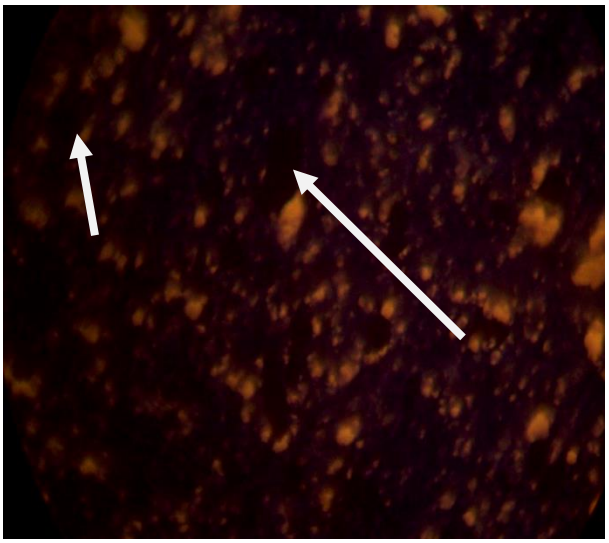

Fig. 18 Photomicrograph parasites in Medulla Oblongata **Magnification x 100**

Arrow indicates ring forms and sequestered PRBC cells

4.5.3 Quantitative Measurements of Quinine in Brain Parts

Extraction of quinine from brain parts and measurement with HPLC

The absorbance of quinine bisulphate was calibrated using the HPLC. The absorbance of known concentrations were measured using a UV detector. Graph of peak area of absorbance versus concentration was plotted. The regression (R^2 =0.9966) and the equation of the graph (Y=3907.8X +2327) was used to calculate concentrations from the area under the curve obtained from the experimental animals (parasitized and non-parasitized mice). (Fig 30)

The validation of extraction procedure is presented in Fig 19 below. The graph was linear with regression R^2 = 0.9925 and Y= 230-170 as the equation of the curve.

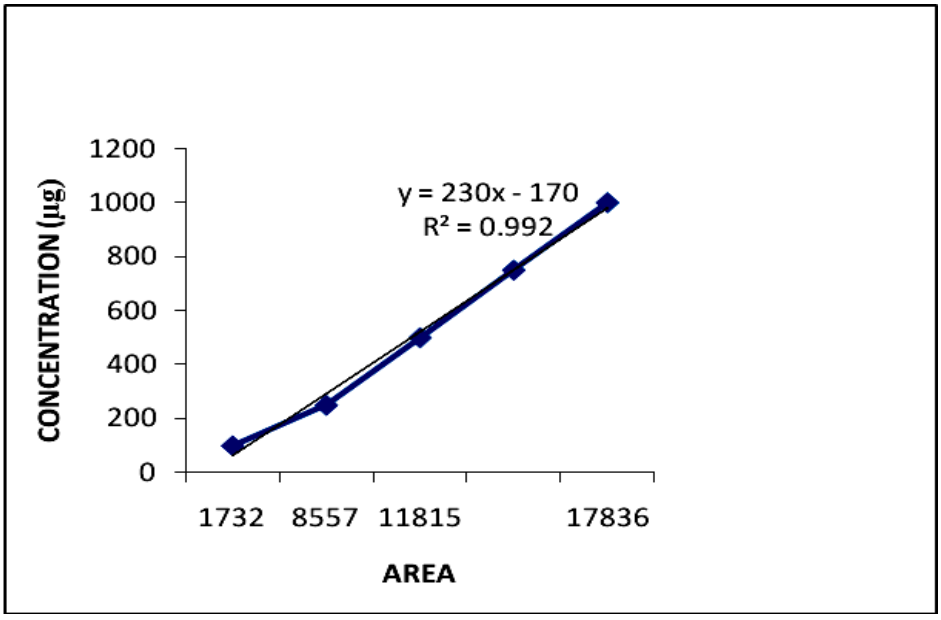

Fig.19 Concentration of spiked quinine vs area of absorption

Table 10 Efficiency Of Extraction Method

Concentration of quinine (µg/ml)	Peak area of absorption (Neat Quinine samples)	Peak area of absorption (extracted sample)	Extraction efficiency (%)
100	1857	1732	93.27
250	5085	4557	89.66
500	13452	11815	87.83
1000	20626	17836	86.47
Average extraction efficiency= 89.29 $\pm$0.03%			

The plot of extracted quantities versus area gave an Extraction Efficiency of 89% $\pm$ 0.03 in relation to the areas observed for neat quinine samples. This implies that the extraction process was quite efficient and the procedure as described in this experiment can be relied upon to produce efficient extraction in subsequent experiments.

4.5.4 QUININE UPTAKE IN PARASITIZED AND NON – PARASITIZED BRAIN SECTIONS OF MICE

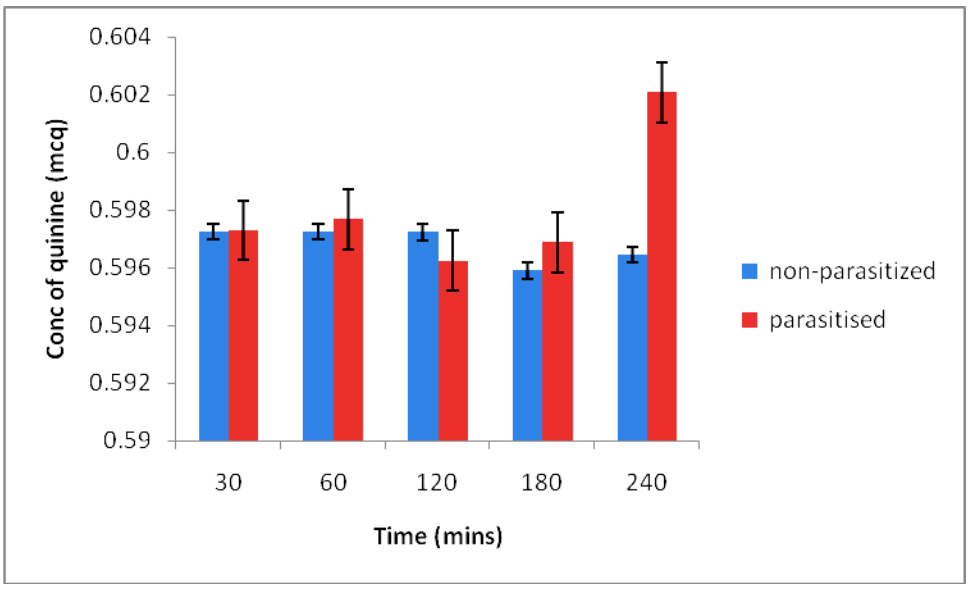

Fig.20 Quinine uptake in olfactory lobe of parasitized and non-parasitized mice

Quinine uptake in the parasitized olfactory is higher than the uptake in the olfactory of non-parasitized mice. Uptake was lowest at 120 min and significantly high at 240min.

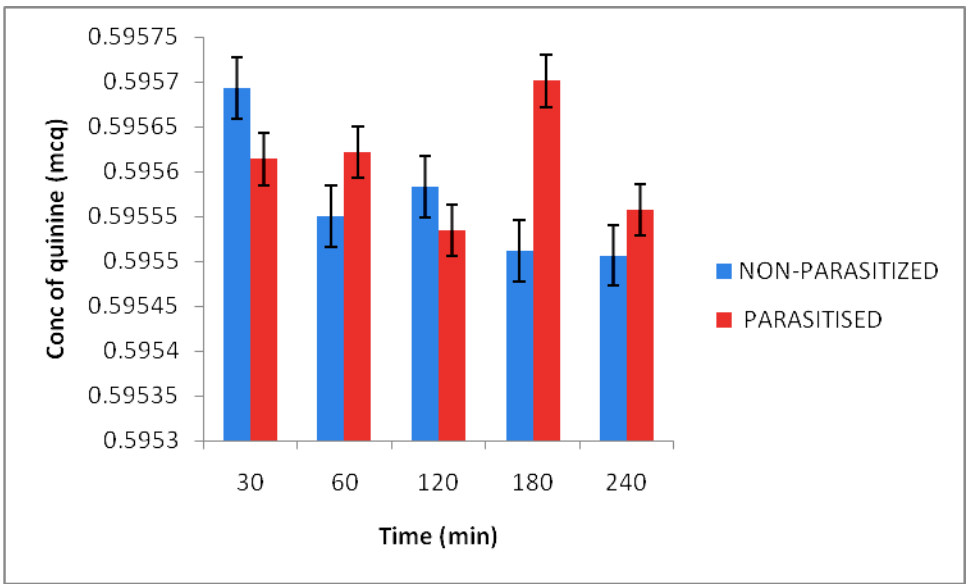

Fig.21 Quinine uptake in the cerebrum of parasitized and non-parasitized mice

Quinine uptake in the cerebrum of the parasitized mice is higher than uptake in non-parasitized animals except for the 30 and 120min period. Uptake is lowest at 120min in parasitised animals while a steady decline in uptake is observed for the non-parasitized. Quinine uptake in parasitized mice seem to be bi-phasic.

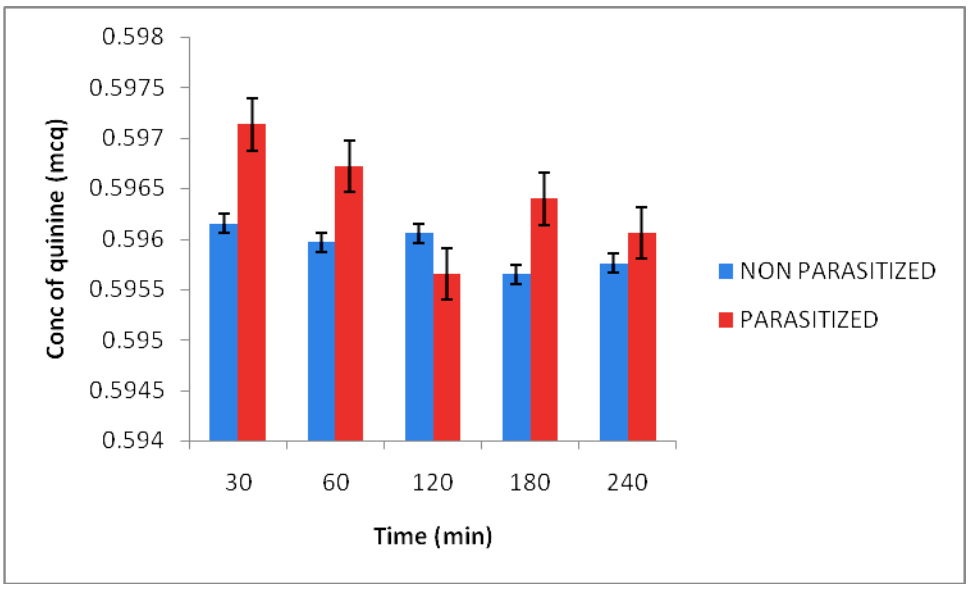

Fig.22 Quinine uptake in the cerebellum of parasitized and non-parasitized mice

Quinine uptake in cerebellum of parasitized resembles the pattern of uptake in the cerebrum. Highest uptake is observed at 30 min.

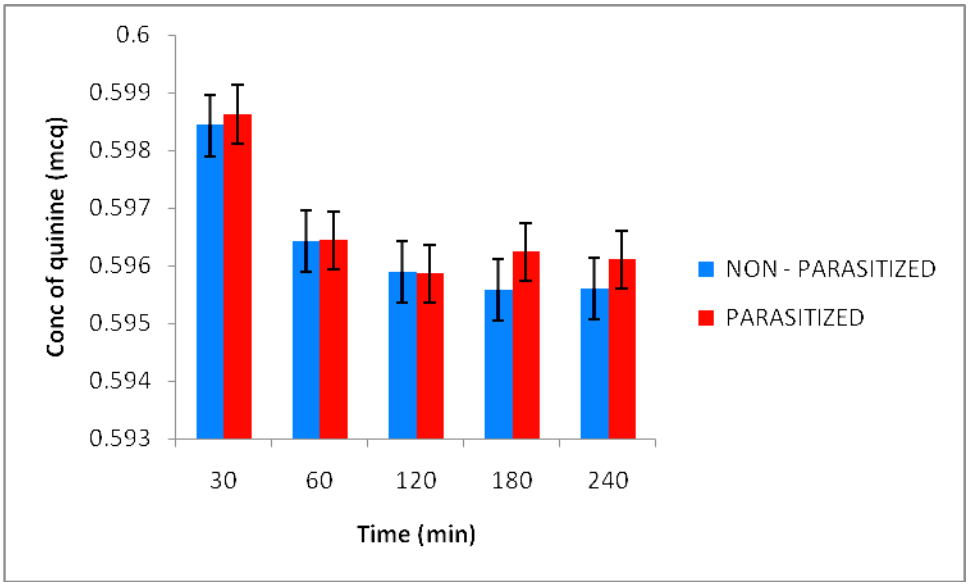

Fig.23 Quinine uptake in Medulla oblongata in parasitized and non-parasitized mice

Quinine uptake is significantly high at 30min in the medulla oblongata of the two groups animals. Lowest uptake is recorded at 120 min. There is a steady decline of quinine upt in the non-parasitized mice

4.5.5 COMPARATIVE UPTAKE OF QUININE IN BRAIN SECTIONS AT VARIOUS TIME INTERVALS

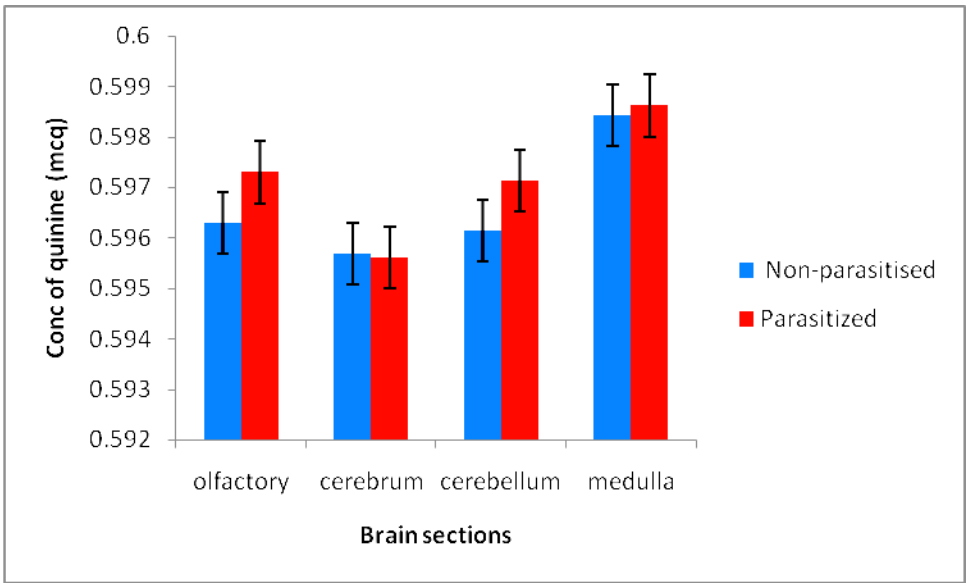

Fig.24 Quinine Uptake in brain sections of mice in 30 Min

Highest uptake at 30 min is observed in the medulla oblongata while the cerebrum had the lowest uptake.

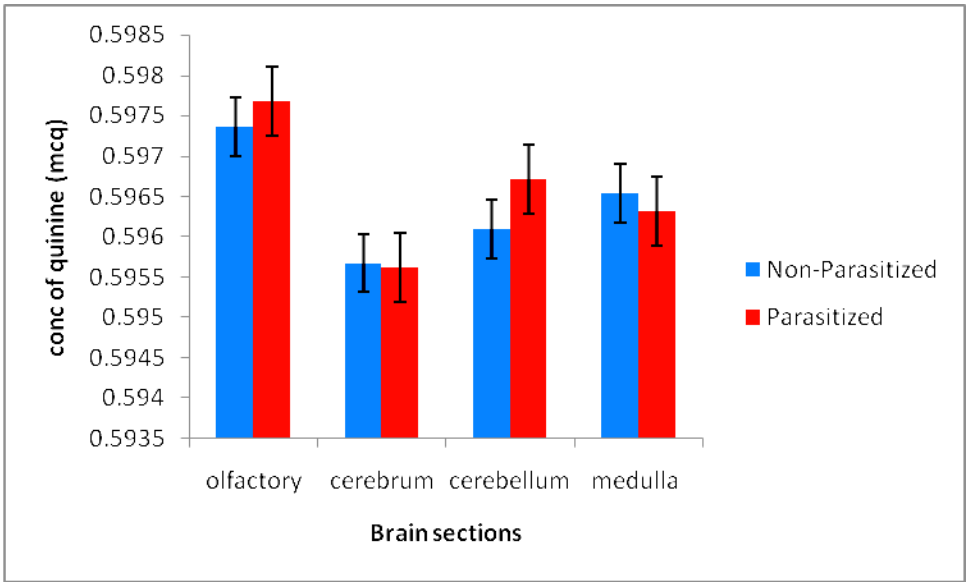

Fig.25. Quinine Uptake in brain sections of mice in 60 Min

Olfactory lobe recorded the highest uptake while lowest uptake is in the cerebrum.

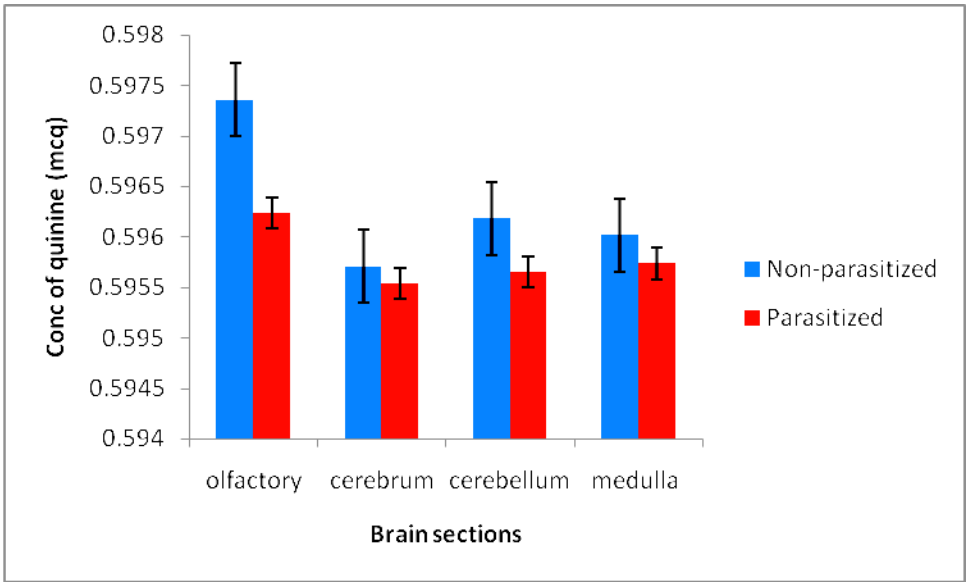

Fig.26. Quinine Uptake in brain sections of mice in 120 Min

Uptake at 120 min is highest in the olfactory lobe. Uptake in non-parasitized is higher in all brain sections with a significantly high uptake observed in the olfactory.

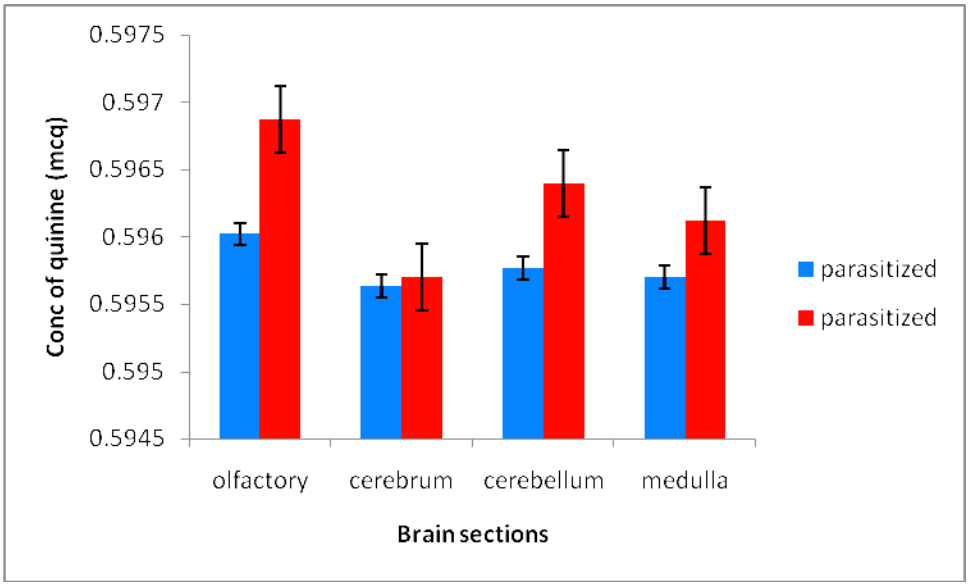

Fig. 27 Quinine Uptake in brain sections of mice in180 min

At 180 min, uptake is highest in the olfactory and lowest in cerebrum.

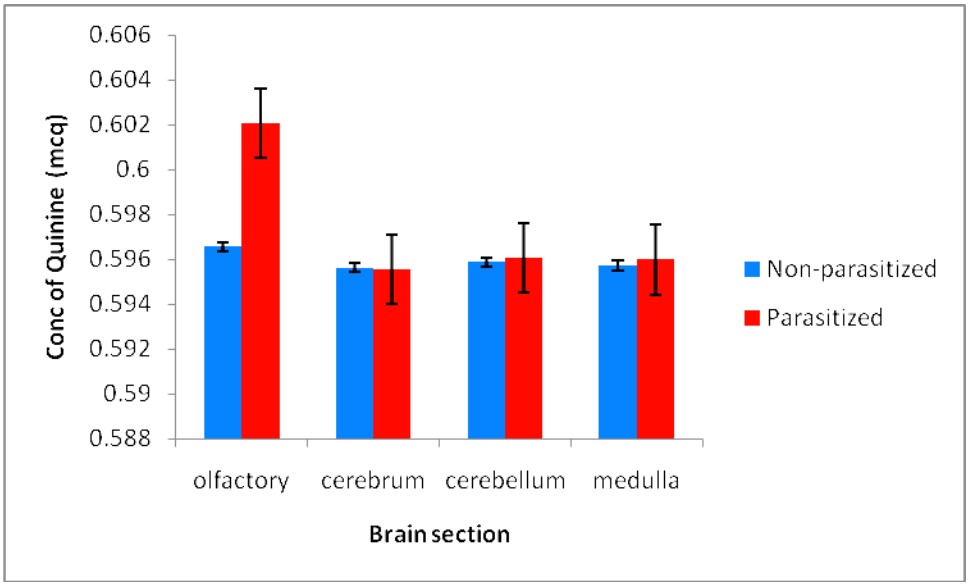

Fig.28. Quinine Uptake in brain sections of mice in 240 Min

At 240 min, uptake is consistently highest in the olfactory lobe but almost of the same quantity in the other brain sections.

Three-way Repeated Measure ANOVA was used to measure the effects of each group (parasitized and non-parasitized) and each time interval on the concentrations of quinine across brain sections while accounting for the effect of brain sections.

The significance values for between-subjects factors, group and time, and within-subjects factor, brain sections were all significant at 0.05 level of significant. For group, the p-value was 0.0000 which was far less than 0.05, $F(1,40)=2292.05$. For time, the p-value was also 0.0000, $F(4,40)=1040.32$. For brain sections, p-value was 0.0000 and $F(3,120)$.

Table 11 Between Factor Group Estimates

Measure: Concentrations

Between Factor 2	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
Parasitized	.596671	.00000795	.596655	.5966869
Nonparasitized	.596132	.00000795	.596116	.5961483

Table 12 Between Factor Time Estimates

Measure: Concentrations

			95% Confidence Interval	
Time	Mean	Std. Error	Lower Bound	Upper Bound
30	.5969232	.0000126	.5968978	.5969487
60	.5964538	.0000126	.5964284	.5964792
120	.5960063	.0000126	.5959809	.5960317
180	.5959872	.0000126	.5959618	.5960126
240	.5966371	.0000126	.5966117	.5966625

	Sum of Squares	df	Mean Square	F	Sig.
Contrast	6.58E-006	4	1.65E-006	1040.320	.000
Error	6.33E-008	40	1.58E-009		

The F tests the effect of Time. This test is based on the linearly independent pairwise comparisons among the estimated marginal means. The table above shows that there was interaction effect between Group and time.

A Pairwise Comparisons was carried out to further check the effect of time. Since all these factors were significant, it is expected that at least one pair of levels of each factor significantly differ in quinine concentrations. For group, there was significant difference between quinine concentrations in parasitized and non-parasitized animals. For time, there was statistical difference between quinine concentrations at 30min and other time intervals, between 60min and other time intervals, between 240min and other time intervals. There was no significant difference between 120min and 180 min. For brain sections, the quinine concentrations significantly differ across pairs of brain sections. See tables in appendix 2.

Profile Plots

The variations in the group and brain sections were as also viewed from profile plots. The first profile plots below compare time with estimated marginal means of concentrations for each brain section. The plots showed that after 30min, olfactory brain section led in quinine concentration. Cerebrum quinine concentration was the most stable. Cerebellum and medulla almost coincide at 120min. Over the course of the study, quinine concentrations were fairly stable from brain section to brain section.

The second plots show the model-estimated means of concentrations for the brain sections for each of the two groups of the study (parasitized and non-parasitized). The plots showed that the quinine concentrations for the parasitized animals were ahead of that of non-parasitized animals at 30min, 60min, 180min and 240min. the concentration for the groups were pretty steady.

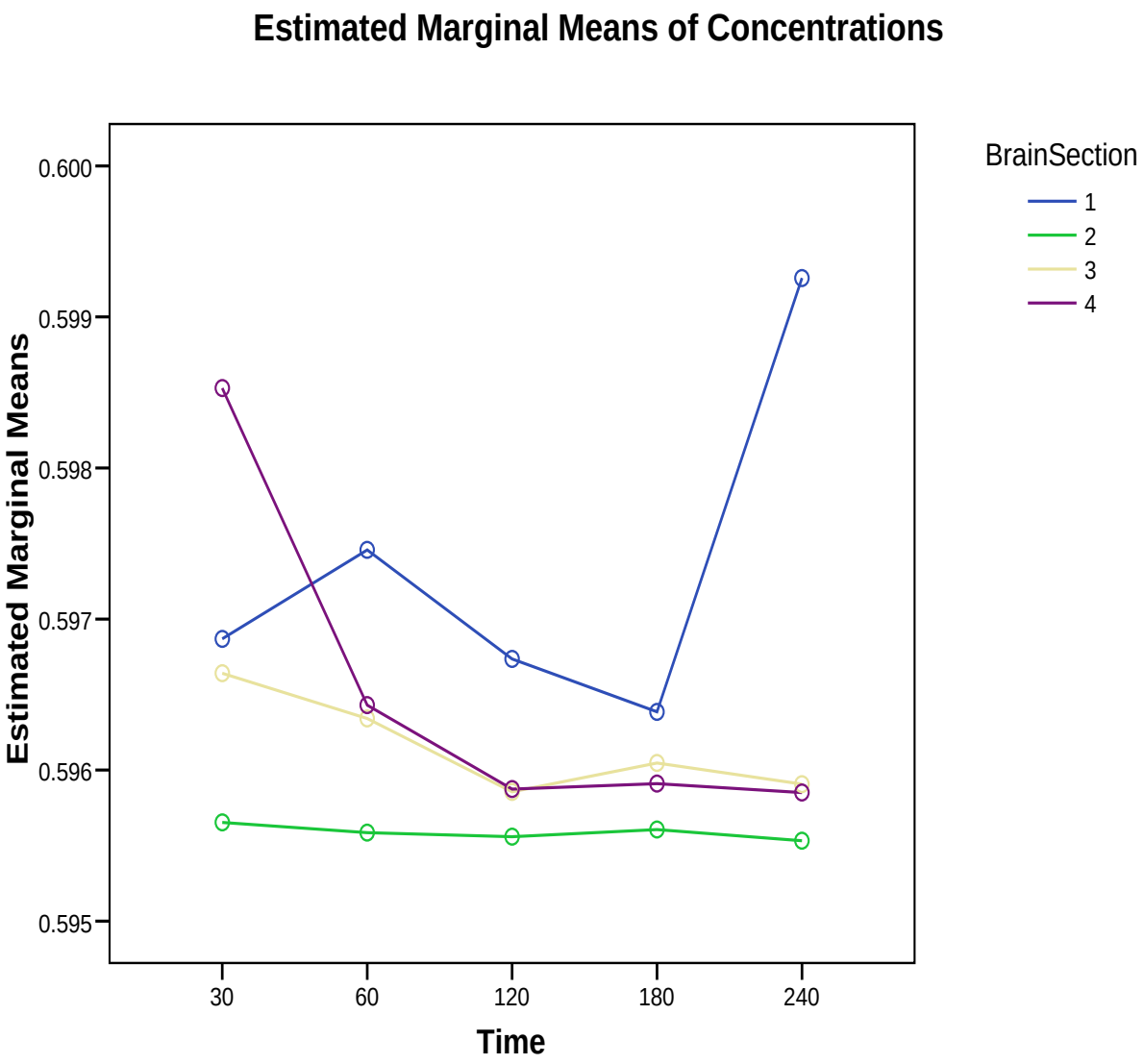

Fig. **29 Estimated marginal means of concentrations vs time for brain sections**

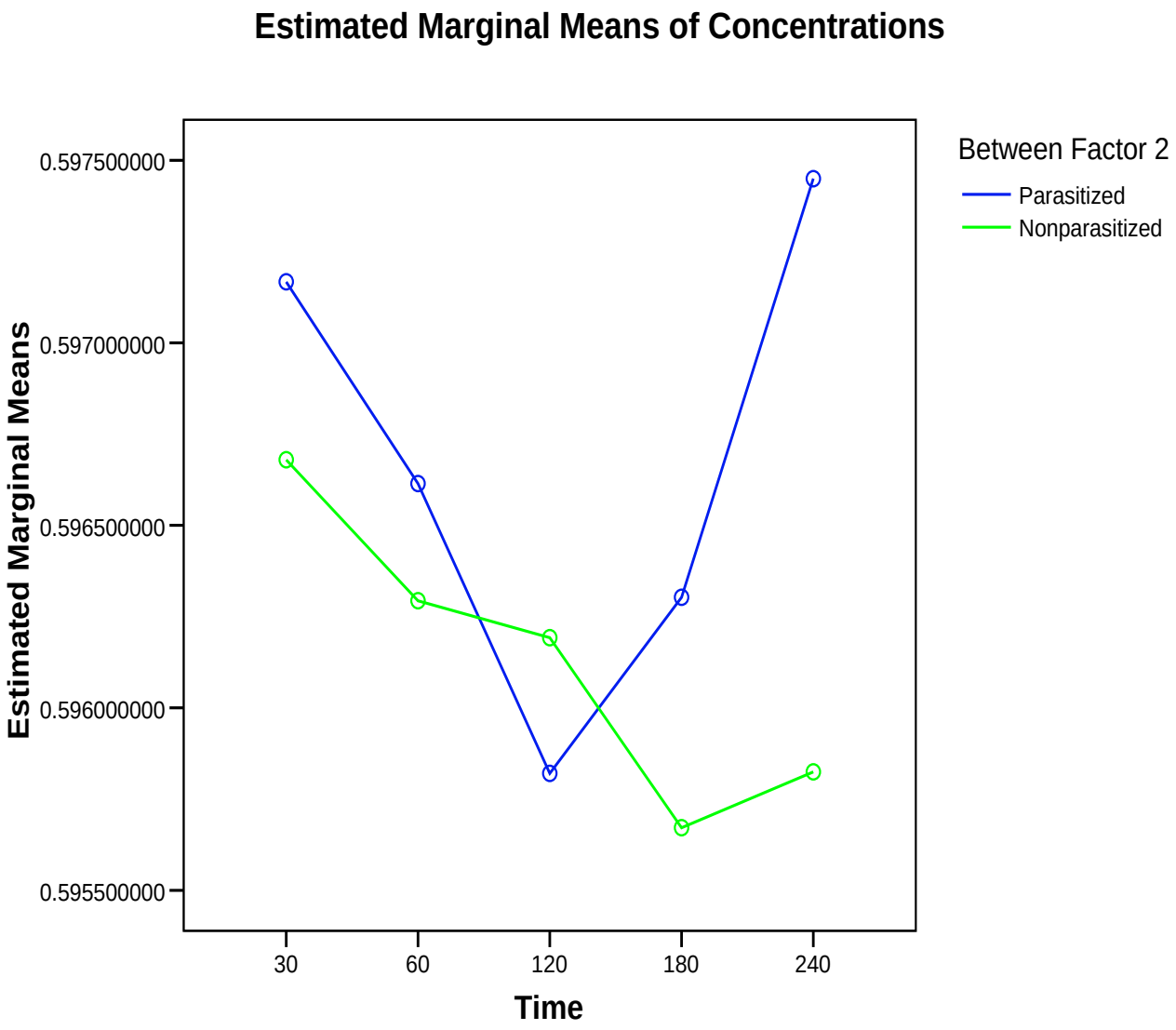

Fig 30 Estimated marginal means of concentrations vs time for parasitized and non-parasitized

4.5.6 UPTAKE IN WHOLE BRAIN

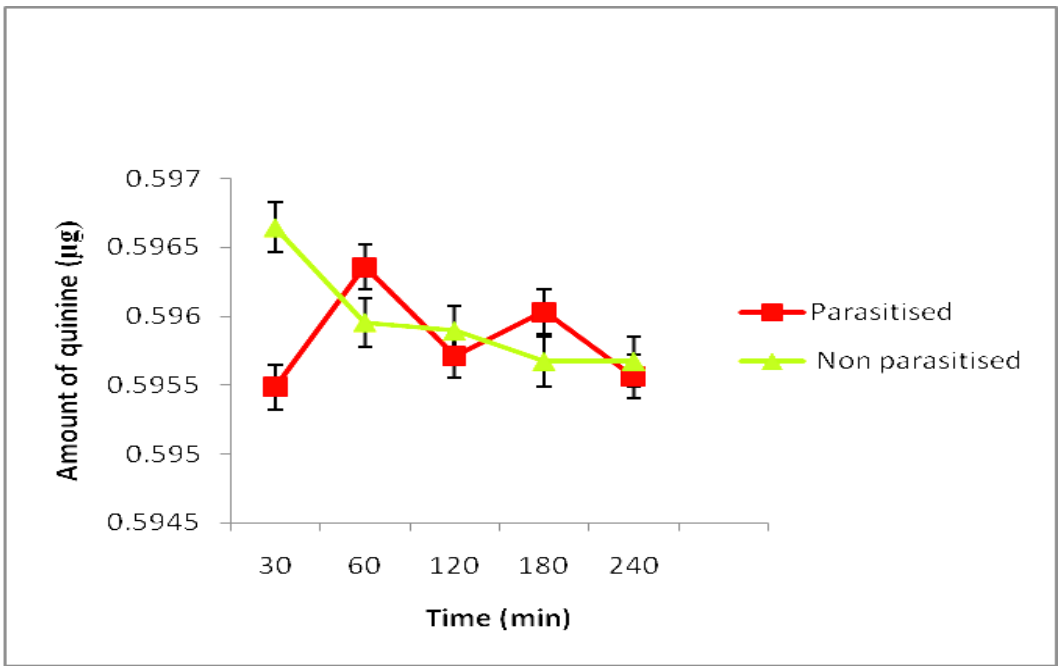

Fig. 31 Quinine uptake per mg of whole brain of mice

The uptake of quinine in the whole brain of the mice is presented in the figure above. The uptake in the parasitized animal is biphasic while the uptake in the non-parasitized animal showed a steady decline from the first sampling time of 30 min. up to 240min. Lowest uptake is observed at 30 min for the parasitized animal while non-parasitized recorded the highest uptake at 30 min.

4.5.7 Uptake from Intra-peritoneal Injection

Table 13 Comparative total uptake of quinine from suppository and intra-peritoneal injection by parasitized and non- parasitized mice

	Suppository		Intra-peritoneal	
	Parasitized	Non-Parasitized	Parasitized	Non-Parasitized
Olfactory	7.26	7.26	7.62	9.95
Cerebrum	182.74	182.77	137.03	332.18
Cerebellum	34.49	34.44	19.37	41.11
Medulla oblongata	20.91	21.01	37.14	114.72
Total uptake (µg)	245.40	245.47	201.16	497.97
Quinine uptake/ mg wt of brain (µg)	0.5955	0.5966	0.5962	0.5956
Administered dose taken up in the brain (%)	48.00	49.10	67	82.99

CHAPTER FIVE

5.0 DISCUSSION

The mechanism of drug absorption from the rectum is probably not different from that in the upper part of gastrointestinal tract but absorption from suppositories is generally slower and very much dependent on the nature of suppository base, the use of surfactants and other additives, and particle size of the active ingredient (de Boer *et al;* 1982). Depending on the character of the vehicle, the suppository will either dissolve in the rectal fluid or melt on the mucous layer. Suspended drugs will leave the vehicle and then dissolve in the rectal fluid. The dissolved drug molecules will diffuse through the mucous layer and the epithelium of the rectal wall. In most cases the rates of absorption of drugs in suppositories increase with increasing solubility and the release of drugs by vehicles or adjuvants (Muranishi, 1984).

The water solubility of the drug substance is the fundamental factor influencing the release rate from suppositories, either with lipophilic or hydrophilic excipients (de Blacy and Tukker 2002). Drug concentration in the intrarectal aqueous phase produces the gradient against the large volume of the plasma phase. A drug with high water solubility quickly leaves the lipophillic excipient, producing a high concentration in the intrarectal phase which supports a high diffusion rate across the barrier. On the other hand, a drug with low water solubility saturates the intrarectal phase at low concentration hindering the subsequent dissolution of the drug particles remaining in the melted excipient. Quinine bisulphate salt is relatively water soluble and the solubility increases with heating. It is therefore expected to have a good release profile from lipophilic bases such as Fattibase^{TM} and Cocoa Butter. The eight formulations from the two bases satisfied the physical and chemical specifications for good suppository formulation. Weight uniformity and content uniformity were within specifications. This is an indication that the two bases gave reproducible

results (Tables 6 - 9). Suppositories from Fattibase™ were however smoother with a better physical appearance.

Release profile - With an appropriate dissolution method, the in-vitro dissolution rate may be correlated to rate of absorption of the drug into the body. If dissolution of drug is rate limiting, faster dissolution rate may result in a faster rate of appearance of drug in the plasma. When different formulations of the same drug are tested, poorly formulated drug will not be completely dissolved and released. This will result in lower plasma drug concentrations. The quantity of drug released at any time interval will be greater for the more available drug product. The in-vitro release profile and availability in the plasma has been correlated for many drug formulations. The specifications for the correlations have been put together by international regulatory organizations (WHO 2006, FIP 2009). A linear correlation between absorbed drug and dissolved drug may be obtained if a drug is absorbed completely after dissolution.

The release of quinine from the suppository formulations was influenced by the addition of the surfactant, polysorbate 80 (Fig 6 & 7). The action of Tween 80, which is a non-ionic surfactant, is due to the increased water incursion into the oily bases by decreasing interfacial tension. Surfactants improve release rate of drugs from suppository formulations (Onyeji *et al;* 1996; Taylor *et al;* 1993). Surfactants may even be the most important adjuvant influencing release rate (Odeniyi & Jaiyeola, 2004). Membrane-active adjuvants generally promote absorption more effectively in the colorectal region than in the upper gastrointestinal tract (Realdon *et al;* 2001). Potentially, the rectal route can be said to offer the same absorption possibilities as the oral, but the influence of formulation factors appear very critical.

The Cocoa Butter based formulation which contained no polysorbate 80 released 36.5% of the quinine content after 5 minutes in the dissolution medium, rising to 36.86 % in 1 hour. This release profile is poor but in the blood, a 36.8% bioavailability level will be therapeutic. 73.6mg of quinine

bisulphate would have been released in 1 hour, a quantity that will be a therapeutic dose in the patient. Addition of 1% Polysorbate 80 into the formulation improved drug release rate slightly with the release of 75.20mg quinine bisulphate in 1 hour. The release profile for the formulation containing 2% and 5% was erratic and the total released at the end of 1 hour was not significantly higher than the concentration released from the formulation with 1% polysorbate 80 (Fig 8). Using an independent T-test to check the significance of effect of surfactant concentration on release from formulation with 0% and 1% polysorbate 80, there was a statistical difference in the concentration of quinine released *(P=0.005)*. There is also a statistical difference between 2% and 5% polysorbate formulation *(P = 0.030)*. The 1% and 2% formulation however did not differ significantly *(P= 0.45)*.

From the descriptive data, the Fattibase™ suppositories had a better release profile in comparison with the Cocoa Butter based formulations. This is expected because Fattibase™ is a pre-blended suppository base that offers the advantages of a cocoa butter base with few of the drawbacks. It is composed of triglycerides with self-emulsifying glyceryl monostearate and polyoxyl stearate used as emulsifying and suspending agents. The incorporation of these emulsifying agents into the base further enhances the release of active ingredients from the suppository formulation. The Fattibase™ formulation without surfactant released 42%, (84mg) in 1hr while the formulation with 1% and 2% polysorbate 80 released 40% and 52% respectively. An addition of 5% polysorbate 80 increased the dissolution in 1hr to 86.5% (173mg). With an oral dose of 10mg/kg body weight, a 10kg child (1yr) will receive an oral dose of 100mg quinine. Testing for statistical significance with a unpaired T-test, no statistical difference was observed in the quantity of quinine released from 0% and 1% polysorbate formulations *(P= 0.33);* while difference in release is statistically different for 1% and 2% formulations *(P=0.034)*. Statistical difference is also observed with the 2% and 5% formulations *(P= 0.05)*. The effect of the surfactant, polysorbate 80 becomes significant at concentrations of 2% and 5%.

The formulations of Cocoa Butter + 1% polysorbate 80, Fattibase™ + 2% and Fattibase +5% polysorbate 80 released therapeutic doses of quinine. These three formulations can therefore be considered in clinical studies to confirm efficacy and bioavailability. An increase in the dose of quinine in the Cocoa Butter or a reformulation with other surfactant combinations may improve release to achieve optimal therapeutic concentration. Cocoa Butter is cheap and locally available hence the Cocoa Butter formulation will be preferred for local manufacture of the suppository.

The results obtained from the study of the release profiles of the quinine suppositories indicate that these formulations have good potential to provide adequate plasma bioavailability of quinine in malaria management. Rectal formulations of antimalarials have been found to yield bioavailability values producing therapeutic effects. Preliminary clinical studies on Artemisinin, Artemether and Artesunate indicated a 30% bioavailabilty and the formulations produced therapeutic efficacy (Nosten *et al*, 1998). Quinimax® intrarectal solution, applied through an enema pump gave 40% bioavailability with residual blood quinine concentration at 48 hours similar with intravenous quinine, so was mortality and temperature clearance amongst the trial group. The cream formulation of quinine containing same dose (200mg) as the Quinimax® intrarectal solution and the studied quinine suppositories (Fattibase™ and Cocoa butter suppositories) also had encouraging efficacy profile (Bareness *et al;* 1996, 2001). Intra-rectal formulations produce very good overall tolerance with no major and/or irreversible complications.

Stability - Stability of a pharmaceutical product is defined as the capability of a particular formulation in a specific container/closure system to remain within its physical, chemical, microbiological, therapeutic and toxicological specifications. It can also be defined as the time from the date of manufacture and packaging of the formulation until its chemical or biological activity is not less than a predetermined level of labeled potency and its physical characteristics have not changed appreciably or deleteriously. The assurance of stability comes from an

accumulation of data on the physical and chemical integrity when stored in its package. 90% of labeled potency is generally recognized as the minimum acceptable potency level. Expiration dating is then defined as the time in which the preparation will remain stable when stored under recommended conditions. The suppository formulation in oily bases cannot be subjected to accelerated studies in high temperatures because high temperature will destroy the integrity of the suppository.

The Fattibase™ suppository with 5% polysorbate 80 has a good stability in the refrigerator (Fig. 8 & 9). The samples had a constant release rate throughout the 3 months storage period. The Repeated Measure ANOVA Procedure was used to examine the stability of release profile of quinine in Fattibase + 5% polysorbate 80 stored in refrigerator for four different time periods – Month 0, Month 1, Month 2 and Month 3. The months represented four levels of the independent variable. On average, the release profile after one month was more than the ones for the other months. A 2-way ANOVA was used to analyze the statistical difference in release with month as a variable. There was no significant difference in the release profile across months. This means that there is stability in release profile over the course of study. The Fattibase™ formulation still released the same quantity of quinine after 3 months in the refrigerator. The formulation stored at ambient temperature (20-25^{o}C) also had a steady and consistent release at one month. From the physical parameters, the suppositories were also physically stable. The mould growth observed by two months may be due to contamination since the formulations did not contain preservatives and were not packed under sterile conditions.

From the melting range FattiBase™ suppository will be stable in the tropic region. It should not require special storage conditions. The Cocoa butter suppository will require refrigeration since the presence of polymorphs in the Cocoa Butter could bring the melting point to as low as 18 ^{0} C, a temperature that is below the ambient temperature of 20-25^{o}C.

The shelf life was extrapolated from the data obtained from the 1 month storage in ambient (20-25^{0}C) and the 3 month storage in refrigerator (4-5^{0}C). The stability in refrigerator can therefore be extrapolated to give an 18 month shelf life for the Fattibase™ suppository.

The three formulations from this study; Cocoa butter + 1% suppository; FattiBase™ +2% suppository and FattiBase™ +5% suppository have demonstrated good release profiles and by extrapolation, potential for adequate plasma concentration. The Fattibase suppository is stable in the refrigerator. Suppositories are relatively easy to administer on children with cerebral malaria. This accounts for the interest in this dosage form (Simoes *et al;* 2003). A stable and affordable quinine suppository will contribute immensely to the reduction of child mortality in Nigeria and other malaria endemic regions especially within the rural and poor districts where access to medical care is low. Treatment can be commenced at home since the drug can be administered by care givers.

Quinine Uptake in the Brain - Eighty percent of malaria related deaths are a result of the complications in the central nervous system, especially in the brain. A drug formulation to be used in the treatment of cerebral malaria must show evidence of availability in the brain. The development of *Plasmodium berghei* was monitored in the mice brain to confirm the presence of infection in the brain of the animals. All the four brain sections had sequestered parasites. Ring form of parasites and non-parasitized erythrocytes were also observed in the cerebellum and the medulla oblongata (Figs.15, 16, 17 and 18). The movement and response to stimuli was also very slow after 6 days. This confirmed the presence of cerebral malaria in the mice used for the studies. Sequestration of parasitized red blood cells (PRBC) in the microvessels results in impairment of microcirculation with organ dysfunction in complicated human *P. falciparum* malaria. In cerebral malaria patients, the percentage of small blood vessels with PRBC

sequestration is higher in the brain than in other organs. The clinical severity of cerebral malaria depends on the level of PRBC sequestration in the brain.

Quinine from suppository formulation was available in the brain of both parasitized and non–parasitized mice. The presence in the brain was first confirmed through observation of brain tissues under the fluorescent microscope. The picture of the non-parasitized mice brain with quinine (Fig.12) shows circular green fluorescence on the surface of the tissues while the parasitized animals had the same green fluorescence radiating out from within the deep tissues (Fig.14). The fluorescence was seen more deeply embedded in the tissue, most likely in the erythrocytes inside the blood microvasculature where the parasites are located. The brain tissues of the non – parasitized and the parasitized mice which were not treated with the quinine suppository did not have the characteristic green fluorescence (Figs. 11 & 13). The formulation therefore shows evidence of quinine bioavailability in the brain.

The bioavailability was confirmed by assessing the quantity of quinine taken up in the brain through extraction and analysis with High Performance Liquid Chromatography. Results of quantitative analysis of quinine in various brain sections confirm quinine uptake in the four sections of the brain of parasitized and non-parasitized mice. Figs 20, 21, 22 and 23 show the quinine uptake in the brain sections of the parasitized and non-parasitized mice while Figs. 24-28 directly compares uptake in brain sections at particular time intervals. In the two groups of animals, quinine was available in the brain within 30min in the four brain sections. Three-way Repeated Measure ANOVA was used to test the effects of each group (parasitized and non-parasitized) and each time interval on the concentrations of quinine present in the four brain sections while accounting for the effect of brain sections. The values for between-subjects factors, group and time, and within-subjects factor, brain sections were all significant at $p < 0.05$. For group (parasitized and non-parasitized), *the p-value was 0.0000* which was less than

0.05, F(1,40)=2292.05. The result from the ANOVA studies was further verified with the Mauchly test of sphericity. The two tests further confirmed the variance of mean. The presence of parasites significantly affected the uptake of quinine into the brain but the uptake was however restrictive. Whole blood concentrations and $AUC0_{o-\infty}$ of quinine increased in a parasitemia dependent manner and clearance correlated with parasitemia in *Plasmodium berghei* - infected and controlled mice (Pussard *et al,* 2003). The presence of parasites affects the pharmacokinetics of absorbed quinine in parasitized plasma. The liver and brain concentrations of quinine were similar in control and infected mice. The tissue to plasma free-fraction ratios decrease when the parasitemia rises suggesting a restrictive uptake of quinine by the liver and brain.

Examining each brain section, quinine uptake in the parasitized olfactory was higher than the uptake in the olfactory of non-parasitized mice. Uptake was lowest at 120 min and significantly high at 240min. Uptake in the cerebrum of the parasitized mice was also higher than uptake in non-parasitized animals except for the 30 and 120min period. Uptake is lowest at 120min in parasitized mice. The pattern of lowest uptake was consistent in all the brain sections. Quinine uptake in parasitized mice seem to be bi-phasic. Measurement of the effect of the various time intervals shows that time has a significant effect on quinine uptake in the brain of the two groups of animals. For time, the p-value was also 0.0000, *F(4,40)=1040.32*. For brain sections, *p-value* was *0.0000* and F(3,120). Time also had a significant effect on quinine concentration in non-parasitized mice brain at 0.05 level of significance. The variation in concentration between 60min and 120min was however not significant. Variations between other time pairs were significant at 0.05 level of significance. See tables in Appendix for detailed statistical analysis.

The variations in the group and brain sections viewed from profile plots (Fig 29) compare time with estimated marginal means of concentrations for each brain section. The plots showed that after 30min, olfactory brain section led in quinine concentration. Cerebrum quinine concentration was the most stable. Cerebellum and medulla almost coincide at 120min. Over the

course of the study, quinine concentrations were fairly stable from brain section to brain section. Fig. 30 shows the model-estimated means of concentrations for the brain sections for each of the two groups studied (parasitized and non-parasitized). The plots showed that the quinine concentrations for the parasitized animals were ahead of that of non-parasitized animals at 30min, 60min, 180min and 240min. The concentrations for the groups were pretty steady. At 120 min, the non-parasitized mice had higher uptake.

The analysis of quinine uptake in the four brain sections per time interval shows that the medulla oblongata has the highest uptake at 30 min while the olfactory consistently recorded the highest uptake for all the other time intervals. The cerebrum was also consistent with lowest quinine uptake for all time intervals. Time effect was evident across brain sections of parasitized animals. For olfactory, cerebellum and medulla, the quinine concentration varied significantly from time to time. Concentration between each time pair was significant. For cerebrum, concentration varied from time to time. Concentration between 30min and 60 min was not significantly different at 0.05 level of significance. Concentrations between other time pairs were also significant.

Quantitative polymerase chain reaction experiment has shown that parasite deposition was greatest in the cerebellum, compared with other areas of the brain, suggesting a correlation between brain parasitemia and loss of control of movement. (Miras-Portugal 2009). In the cerebellum, the percentage of microvessels with PRBC sequestration was higher than that in the cerebrum with a statistical significance. There is a higher degree of vascularity in the cerebellum (7 vessels/mm^2) than in the cerebrum (5 vessels/mm^2), and the difference is also statistically significant ($P < 0.025$). Perivascular hemorrhages also occur more frequently in the cerebellum than in the cerebrum (Sein *et al;* 1993). Differential sequestration of PRBC occurring in the microvessels of the cerebrum and cerebellum explain the varied neurologic manifestations that result from cerebral and cerebellar dysfunction in human cerebral malaria. The cerebrum and the cerebellar have been observed as the most affected parts of the brain in severe malaria. C. T.

scan of brain tissues of patients with cerebral malaria revealed cerebral edema with thalamic and cerebellar white matter hypoattenuation in some patients (Hearn 2000, Tufail *et al;* 2002). Damage to the microvascular supply to these two regions as observed in CM may be responsible for the low quinine uptake. Diffuse hemorrhages have been recorded in the cerebrum and cerebellum at autopsy of CM patients that died. At 120 min, the cerebrum and cerebellum of parasitized animals absorbed even lower concentrations of quinine than the non-parasitized animals.

Quinine uptake in the brain of both parasitized and non-parasitized mice is also weight dependent (Fig.31). The pattern of uptake observed in the cerebrum is reflective of the pattern in the whole brain (Fig.30). The cerebrum makes up over 80% of the total brain mass hence the effect of the cerebrum's mass was reflected in the uptake pattern for the whole brain. This confirms earlier findings that quinine dose is a function of weight (Krishna *et al;* 2001)

The blood-brain barrier (BBB) forms an interface between the circulating blood and the brain and possesses various carrier-mediated transport systems (influx and efflux) for small molecules to support and protect CNS function. P-glycoprotein is expressed on both luminal and abluminal membranes. This location is consistent with its putative role in protecting the neutrophiles from circulating lipophilic molecules. This localization suggests that it acts to exclude P-glycoprotein substrates from the endothelial cells themselves (Stewart *et al;* 1996). Several studies have been carried out on brain uptake of xenobiotics. The concentration of xenobiotics taken up in the brain has been shown to vary among the various sections. Brain uptake of [^{3}H] dexamethasone was increased in the frontal cortex, hippocampus, hypothalamus, and cerebellum of P-gp-deficient mice compared with wild-type controls. Brain uptake of [^{3}H]cortisol was increased in the hypothalamus of P-gp-deficient mice compared with wild-type controls, but no differences were detected in other brain regions (Mason *et al;* 2008). This may imply that the p-glycoprotein which is present in the luminal layer of brain endothelium is not

the only determinant of drug uptake since different distributive uptakes were observed for dexamethasone and cortisol. Degree of ionization, and lipid-water partition coefficient (Lipophilicity) is another important factor in drug absorption into the brain. The brain extraction of fifteen C-11-labeled compounds during a single capillary transit was studied. Compounds with log P_{oct} values between 0.9 and 2.5 were found to pass freely across the blood-brain barrier at a cerebral blood flow of 100 $ml \cdot min^{-1} \cdot hg^{-1}$. (Dischino *et al;* 1983). A comparison of opioid activity of two dermorphin analogues having an almost identical structure but different structural flexibility also showed that the two compounds produced comparable antinociceptive effects in the mouse tail flick test after peripheral administration. This indicates that lipophilicity, rather than side chain flexibility, is the key determinant for blood−CNS barrier penetration (Ballet *et al;* 2008). Quinine has a pKa of 4.1; 8.5 hence will pass freely. The carrier-mediated uptake seems quite significant in the uptake of quinine. Immunogold cytochemistry studies revealed polar (asymmetric) distribution of GLUT-1 in mouse brain microvascular endothelia, which is the anatomic site of the blood–brain barrier (BBB). Subtle differences were revealed in the density of immunolabeling for GLUT-1 in blood microvessels located in four brain regions. The density of immunosignals in the microvessels supplying the cerebral cortex, hippocampus, and cerebellum was essentially similar, whereas in the olfactory bulb it was significantly lower. (Danuta *et al;* 1999). Polar compounds are probably taken up more in the other regions than the olfactory bulb. By extension, the olfactory may be more lipid, possessing higher affinity for lipophillic compounds like quinine.

An assessment of regional differences in cerebral p-glycoprotein (P-gp) distribution through administration of tariquidar to induce changes in distribution volumes (DVs) in 42 brain regions of interest (ROIs) failed to detect significant differences among brain ROIs. Statistical parametric mapping analysis visualized symmetrical bilateral clusters with moderately higher increases in response to tariquidar administration in cerebellum, parahippocampal gyrus,

olfactory gyrus, and middle temporal lobe and cortex, which might reflect moderately decreased P-glycoprotein function and expression in these regions (Bauer 2010). The concentration of metals taken up from environmental contamination showed variation in the different regions of the brain of goats. The highest mean concentration for Fe was found in the olfactory bulb. The levels of Zn and Fe also showed significant differences across the different regions of the brain examined. All the metals analyzed showed relatively high concentrations in the olfactory bulb (Igado *et al;* 2008). The olfactory bulb is the area of highest concentration for the metals. The animals would have ingested the metals from the environment through food, water and environmental gases. This is an indication that xenobiotics ingested through peripheral routes may have different uptake concentration in brain sections.

The uptake of quinine into the brain is regulated by α-acid glycoprotein. Pussard *et al;* (2007) observed a high uptake of quinine into the p-glycoprotein knocked out brain of mice. The uptake in the plasma is not mediated by p-glycoprotein. The concentration of quinine and its metabolite 3-hydroxy quinine in plasma were similar in normal and P-glycoprotein deficient mice. Investigations into the accumulation of quinine into *Plasmodium falciparum* parasites showed that passive diffusion in accordance with intra-cellular pH gradients and intracellular binding could account for a fraction of the amount of quinine accumulated by the parasites investigated. Quinine, a weak base, may move into the brain by passive diffusion. This method of diffusion is concentration-dependent and will account for only a limited uptake of quinine. This may explain the uptake in 30 and 60min. The drop in concentration at 120min may be a result of efflux through the p-glycoprotein transport system or elimination through metabolism. Penet *et al;* (2005) reported an ischemic metabolic profile, reduction of high-energy phosphates and elevated brain lactate in CM. This may therefore lead to slight acidification of the brain. Results of trans–simulation kinetics suggest that high accumulation of quinine is brought about by a carrier-mediated import system.

At pH 6, over 99% of quinine is positively charged and the substrate p-glycoprotein has low affinity for the positively charged quinine. Quinine at pH 6 is cationic and may be transported by cationic transporters. Arndt *et al;* (2001) and Sanchez *et al;* (2008) established that the tissue concentrations of endogenous cations, cationic drugs or xenobiotics are influenced by polyspecific organic cation transporters (OCTs) in kidney, liver, small intestine and brain. These researchers showed that the weak base quinine passively permeates plasma membrane at physiological pH. The combined efflux through the cationic transporters and the binding protein may explain the observed uptake in the second phase. Accumulation of quinine into *Plasmodium falciparum* parasites occurs by passive diffusion in accordance with intra-cellular pH gradients and intracellular binding could account for only a small fraction of the amount of quinine accumulated by the parasites. High accumulation of quinine is brought about by a carrier-mediated import system. (Sanchez *et al;* 2008). P-glycoprotein is an ATP-dependent, efflux membrane transporter with broad substrate specificity for a number of structurally diverse drugs.

Gupta *et al;* and Pussard *et al;* (2007) have monitored the uptake of quinine from intra-rectal and parenteral formulations into the whole brain. Quinine distribution has however not been studied in brain sections. From the brain distribution of other xenobiotics, only moderate differences were observed for p-glycoprotein distribution in brain sections. Liphophilicity of brain region, vascularity and blood flow may be an important factor in the uptake for the same chemical compound as is the case with quinine in this work.

Various strategies have been studied to circumvent the multitude of barriers inhibiting brain penetration by therapeutic agents. These strategies generally fall into one or more of the following three broad categories viz., manipulating drugs, disrupting the Blood brain barrier and exploiting alternative routes for drug delivery (Dwibhashyam *et al;* 2008).

Drug supply into the CNS has been improved by bye-passing the BBB. The neural pathway between the nasal mucosa and the brain provide a unique pathway for noninvasive delivery of therapeutic agents to the CNS (Thorne & Frey 2001, Illum 2000). The olfactory neural pathway provides both intraneuronal and extra neuronal pathways into the brain. The extra neuronal pathway probably relies on bulk transport through perineural channels, which deliver drug directly to the brain parenchymal tissue, to the cerebro spinal fluid (CSF), or to both. This extra neuronal pathway allows therapeutic agents to reach the CNS within minutes. The extraneural pathway also may be involved in rapidly delivering protein therapeutic agents, such as insulin like growth factor-1 to the brain following intranasal administration. Morphine has been transported into the CNS through the olfactory pathway. Location of morphine-derived radioactivity in the rat brain by autoradiography revealed that overall, the levels of morphine in the right olfactory bulbs (ROBs) significantly exceeded those in the left olfactory bulbs (LOBs) and brain tissue samples 15, 60 and 240 min after right-sided nasal administration. The morphine got into the ROB five minutes after nasal administration and reaching one of the ventricles in the brain. After 60 min, radioactivity had reached the peripheral parts of the ROB (Westin *et al;* 2005). Morphine transferred via olfactory pathways to the brain hemispheres contributes to the early high brain concentrations after nasal administration to rats. The olfactory lobe is highly vascularised. Inflammation of the olfactory lobe in humans has resulted in seizures (McEvoy, 2002). Blood supply into the olfactory lobe may be responsible for the high quinine uptake in the mouse brain. Odor stimulation evokes capillary vascular responses that are odorant and glomerulus-specific. These responses consist of increases as well as decreases in RBC flow, both resulting from independent changes in RBC velocity or linear density. In olfactory bulb superficial layers, capillary vascular responses precisely outline regions of synaptic activation (Chaigneau *et al;* 2003).

Various approaches are also being tried to bypass P-gp efflux. Reversal agents such as R-verapamil, PSC 833 (cyclosporine analog), and triqudar, inhibit P-gp mediated drug transport

and increase the influx of therapeutic agents they are co-administered with. Certain non-ionic surfactants like Tween-80 and Cremophor EL have also been found to have the reversal activity. However, most of these agents have been found to be pharmacologically active and elicit significant toxicity at doses required for P-gp inhibition.

Quinine from the suppository formulation crossed the blood brain barrier into the mice brain in the non-parasitized mice. This confirms earlier reports that inflammation is not required for the disruption of the BBB and subsequent transport of quinine into the brain. Quinine transports freely into the brain even in the absence of parasites. Several studies of the involvement of integrity of the blood brain barrier in CM have produced controversial findings (Paco *et al;* 2000, Reed & Weina, 2000, Adams *et al;* 2002). Brown *et al;* (2001) confirmed the disruption of the BBB in cerebral malaria in Malawian children with an activation of endothelial cells and macrophages and the disruption of endothelial intercellular junctions in vessels containing sequestered parasitized erythrocytes but no gross leakage of plasma proteins. This may be indicative of lack of evidence for an inflammatory pathogenesis for human cerebral malaria. Penet *et al;* (2005) found only minimal degree of BBB breakdown in a few cases of CM. Cerebral Malaria seems therefore to focus attention on local events within and around the cerebral microvasculature rather than indicating widespread parenchymal disease.

48% of the administered quinine from the suppository formulation was taken up by the parasitized mouse brain while 49.10% of administered dose was taken up by the non-parasitized mouse brain (Table 13). Uptake from an intra-peritonial injection in the parasitized and non-parasitized mice was 67% and 82.99% respectively. The suppository delivered 70% of the intra-peritonial dose. This further confirms that the suppository formulation may serve as an effective alternative in the management of severe malaria especially in the rural areas where access to health care is poor.

CHAPTER SIX

CONCLUSION AND CONTRIBUTIONS TO KNOWLEDGE

6.1. Conclusion

The two bases, Cocoa butter and FattiBase^{TM} produced suppositories that met all recommended pharmacopeal specifications. The suppository released quinine in a pH 8 buffer medium. This medium simulates the rectal pH. The addition of polysorbate 80 as release enhancer affected the release of quinine from the formulation. Varying concentrations of polysorbate 80 (1%, 2% and 5%) had significant variations in the percentage of quinine released from the eight formulations. The best release profile was obtained with quinine in FattiBase^{TM} + 5% polysorbate 80 with the release of 86.5% (173mg) of the quinine content. The concentration of quinine released from Cocoa Butter + 1% polysorbate 80 (75.20mg) and FattiBase^{TM} + 2% polysorbate 80 (104mg) will also be adequate in the management of malaria. The formulation of Fattibase^{TM} was stable at ambient temperature (25-30^{o}C) for one month and for 3 months in the refrigerator. The formulation stored at ambient temperature still released 80% of the quinine dose in the suppository at the end of the one month storage time. The formulation can therefore be safely stored in homes for one month without adverse effects on the physical appearance, drug content and release of active ingredient, quinine from the suppository.

Quinine from Fattibase^{TM} suppository was available in the brain of the parasitized and non-parasitized mice within 30 minutes of administration. The rapid uptake of quinine into the brain (30min) implies that this suppository formulation will give rapid onset of action against cerebral malaria. Uptake of quinine in the brain was time-dependent and the time-dependence was significant. The uptake was also significantly higher in the parasitized mice than in the non-parasitized mice. The uptake of quinine varied significantly in the four brain sections of infected mice with the olfactory lobe recording the highest uptake. Quinine uptake in the brain appears to be biphasic with the first phase ending by 120min. The second phase commenced at 180 min

and ended by 240 min. The concentration of quinine taken up in the brain increased gradually, reaching a maximum at 60 min followed by a sharp drop by 120 min. The quantity then increased at 180min and is again followed by a sharp drop by 240min. The cerebrum consistently had the lowest concentration of quinine uptake out of the four brain sections. Quinine uptake in the infected mouse brain probably occurs by a combination of passive diffusion, acidotropic trapping, cationic transport and α-acid-glycoprotein transportation. The lipophilicity of the brain section may also be a contributory factor to quinine uptake in brain sections.

6.2. Contributions to knowledge

1. A stable and available formulation of quinine suppository was developed. The suppository released up to 86% quinine, a dose which is quite adequate in the management of cerebral malaria. This formulation can be administered by untrained health care givers thereby promoting early initiation of malaria treatment.
2. Addition of polysorbate 80 significantly affected the release of quinine from both Cocoa butter and Fattibase ^{TM} suppositories.
3. The quinine released from the suppository is available in the brain which is the main area affected by cerebral malaria
4. Quinine crosses the blood brain barrier into the brain even in the absence of malaria confirming that inflammation is not required for quinine uptake in the brain
5. Quinine uptake varies among the four brain sections with highest uptake in the olfactory lobe

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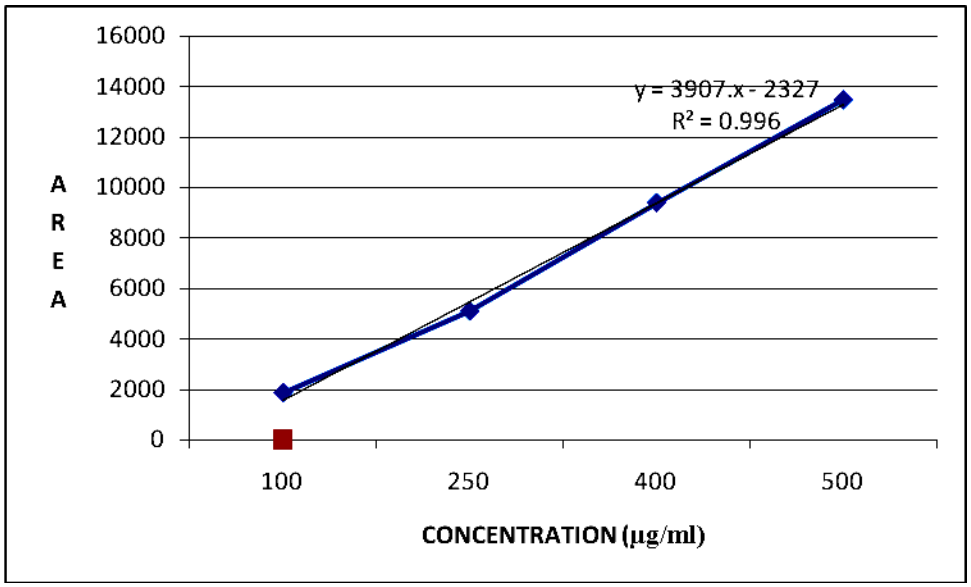
APPENDIX 1

Fig 32 Standard curve of neat quinine sample



Y=3907.8X +2327

R^2 =0.9966

Table 14 Concentration of extracted quinine from spiked brain tissue

	Extracted From Spiked Brain
Concentration Of Quinine Bisulphate (µg/ml)	Area Under the Curve
100	1732
250	8557
500	11815
750	14235
1000	17836

Table 15 Total Quinine Uptake in Whole Brain of Parasitized And Non-Parasitized Mice

	30 min		60min		120min		180min		
	Parasitized	Non-parasitized	Parasitized	Non-parasitized	Parasitized	Non-parasitized	Parasitized	Non-parasitized	Parasiti
Olfactory	7.26	7.26	9.86	9.70	5.61	10.75	4.48	16.45	6.68
Cerebrum	182.74	182.77	48.85	171.07	60.34	170.49	134.36	157.66	156.9
Cerebellum	34.49	34.44	31.93	41.13	14.66	45.25	39.73	32.68	26.23
Medulla oblongata	20.91	21.01	32.21	56.49	4.17	25.86	42.15	39.44	51.68
Total quinine uptake in whole brain (µg)	245.41	245.47	122.85	278.41	84.77	252.36	220.72	246.23	241.5
Administered quinine taken up (%)	49.08	49.10	24.57	55.68	16.95	50.47	44.14	49.24	48.31
total weight of brain (mg)	412.1	411.4	206.0	467.15	142.3	423.5	370.3	413.35	405.6
Ratio of uptake to weight	0.5955	0.5966	0.5965	0.5959	0.5957	0.5959	0.5960	0.5957	0.595

APPENDIX 2

STATISTICAL ANALYSIS

2.1. Stability Studies of Formulation H

The Repeated Measure ANOVA Procedure was used to examine the stability of release profile of quinine in Fattibase + 5% polysorbate 80 stored in refrigerator for four different time periods—Month 0, Month 1, Month 2 and Month 3.The months represent four levels of the independent variable.

Descriptive Statistics

Table 16 Mean and standard Deviation for quinine release profile from Formula H

	Mean	Std. Deviation	N
0 month	60.829033	24.1142957	7
1 month	62.848343	23.1483945	7
2 month	55.795971	22.6095792	7
3 month	56.264286	22.1918407	7

The Descriptive Statistics table displayed the means and standard deviation of each month across time. On average, the release profile after one month is more than the ones for the other months.

Table 17 Tests of Within-Subjects Effects

Measure: MEASURE_1

Source		Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Uptake	Sphericity Assumed	251.215	3.000	83.738	1.434	.266	.193
	Greenhouse-Geisser	251.215	1.173	214.101	1.434	.278	.193
	Huynh-Feldt	251.215	1.287	195.146	1.434	.279	.193
	Lower-bound	251.215	1.000	251.215	1.434	.276	.193
Error(uptake)	Sphericity Assumed	1050.944	18.000	58.386			
	Greenhouse-Geisser	1050.944	7.040	149.280			
	Huynh-Feldt	1050.944	7.724	136.064			
	Lower-bound	1050.944	6.000	175.157			

Table 15 above shows the ANOVA values for within-subjects factor of month. In the table, there is a sum of squares which tells how much of the total variability is explained by the experimental effect (i.e. differences in release profile after each time period). There is also an error term, which is the amount of unexplained variation across the condition of the repeated measures variable.The value of F ratio in the table can be compared against the critical value for 3 and 18 degrees of freedom. This value is inaccurate because sphericity assumption had been violated. (Sphericity assumption simply means that the relationship between pairs of experimental conditions is similar. The level of dependence between pairs of groups is roughly equal).

Mauchly's test statistic is used to examine sphericity. If Mauchly's test statistic is significant (i.e. p-value is less than 0.05), then the condition for sphericity has not been met. The test is significant in the table below, so the degrees of freedom must be adjusted.

Table 18

Mauchly's Test of Sphericity

Measure: MEASURE_1

Within Subjects Effect	Mauchly's W	Approx. Chi-Square	df	Sig.	Epsilon[a]		
					Greenhouse-Geisser	Huynh-Feldt	Lower-bou
uptake	.024	17.587	5	.004	.391	.429	.3

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There are corrections to use in the event of violation. These corrections are Greenhouse-Geisser, Huynh-Feldt and lower bound corrections. All these corrections are not significant at 0.05 level of significance. See table 3 above. In view of this, it can be concluded that there was no significant difference in the release prolife across months. This means that there is stability in release profile over the course of study.

2.2. ANIMAL STUDIES

Three-way Repeated Measure ANOVA

Three-way Repeated Measure ANOVA was used to measure the effects of each group (parasitized and non-parasitized) and each time interval on the concentrations of quinine across brain sections while accounting for the effect of brain sections.

The significance values for between-subjects factors, group and time, and within-subjects factor, brain sections were all significant at 0.05 level of significant. For group, the p-value was 0.0000 which was far less than 0.05, $F(1,40)=2292.05$. For time, the p-value was also 0.0000, $F(4,40)=1040.32$. For brain sections, p-value was 0.0000 and $F(3,120)$. See tables below.

Table 19 Between Factor Group Estimates

Measure: Concentrations

Between Factor 2	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
Parasitized	.596671	.00000795	.596655	.5966869
Nonparasitized	.596132	.00000795	.596116	.5961483

Table 20 Univariate Tests

Measure: Concentrations

	Sum of Squares	df	Mean Square	F value	Sig.
Contrast	3.63E-006	1	3.63E-006	2292.050	.000
Error	6.33E-008	40	1.58E-009		

The F tests the effect of Between Factor 2. This test is based on the linearly independent pairwise comparisons among the estimated marginal means.

Df is degree of freedom

Sig. is significance

Table 21 Between Factor Time Estimates

Measure: Concentrations

			95% Confidence Interval	
Time	Mean	Std. Error	Lower Bound	Upper Bound
30	.5969232	.0000126	.5968978	.5969487
60	.5964538	.0000126	.5964284	.5964792
120	.5960063	.0000126	.5959809	.5960317
180	.5959872	.0000126	.5959618	.5960126
240	.5966371	.0000126	.5966117	.5966625

Table 22 Univariate Tests

Measure: Concentrations

	Sum of Squares	df	Mean Square	F	Sig.
Contrast	6.58E-006	4	1.65E-006	1040.320	.000
Error	6.33E-008	40	1.58E-009		

The F tests the effect of Time. This test is based on the linearly independent pairwise comparisons among the estimated marginal means.

Table 23 Tests of Within-Subjects Effects

Measure: Concentrations

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
BrainSection	Sphericity Assumed	8.09E-005	3	2.70E-005	4327.595	.000
	Greenhouse-Geisser	8.09E-005	2.090	3.87E-005	4327.595	.000
	Huynh-Feldt	8.09E-005	2.704	2.99E-005	4327.595	.000
	Lower-bound	8.09E-005	1.000	8.09E-005	4327.595	.000
BrainSection * BF2	Sphericity Assumed	1.30E-005	3	4.33E-006	693.965	.000
	Greenhouse-Geisser	1.30E-005	2.090	6.21E-006	693.965	.000
	Huynh-Feldt	1.30E-005	2.704	4.80E-006	693.965	.000
	Lower-bound	1.30E-005	1.000	1.30E-005	693.965	.000
BrainSection * Time	Sphericity Assumed	8.28E-005	12	6.90E-006	1106.898	.000
	Greenhouse-Geisser	8.28E-005	8.360	9.91E-006	1106.898	.000
	Huynh-Feldt	8.28E-005	10.815	7.66E-006	1106.898	.000
	Lower-bound	8.28E-005	4.000	2.07E-005	1106.898	.000
BrainSection * BF2 * Time	Sphericity Assumed	4.60E-005	12	3.84E-006	615.550	.000
	Greenhouse-Geisser	4.60E-005	8.360	5.51E-006	615.550	.000
	Huynh-Feldt	4.60E-005	10.815	4.26E-006	615.550	.000
	Lower-bound	4.60E-005	4.000	1.15E-005	615.550	.000
Error(BrainSection)	Sphericity Assumed	7.48E-007	120	6.23E-009		
	Greenhouse-Geisser	7.48E-007	83.597	8.95E-009		
	Huynh-Feldt	7.48E-007	108.147	6.92E-009		
	Lower-bound	7.48E-007	40.000	1.87E-008		

Table 24 Tests of Between-Subjects Effects

Measure: Concentrations

Transformed Variable: Average

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	17.785	1	17.785	11242000 724.910	.0000
BF2	3.63E-006	1	3.63E-006	2292.050	.0000
Time	6.58E-006	4	1.65E-006	1040.320	.0000
BF2 * Time	5.17E-006	4	1.29E-006	817.277	.0000
Error	6.33E-008	40	1.58E-009		

Pairwise Comparisons

Since all these factors were significant, it is expected that at least one pair of levels of each factor significantly differ in quinine concentrations. For group, there was significant difference between quinine concentrations in parasitized and non-parasitized animals. For time, there was statistical difference between quinine concentrations at 30min and other time intervals, between 60min and other time intervals, between 240min and other time intervals. There was no significant difference between 120min and 180 min. For brain sections, the quinine concentrations significantly differ across pairs of brain sections. See tables below.

Table 25 Group Pairwise comparison

Pairwise Comparisons

Measure: Concentrations

(I) Between Factor 2	(J) Between Factor 2	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
Parasitized	Nonparasitized	.001*	.000	.000	.001	.001
Nonparasitized	Parasitized	-.001*	.000	.000	-.001	-.001

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

a. Adjustment for multiple comparisons: Bonferroni.

Table 26 Time- Pairwise comparison

Pairwise Comparisons

Measure: Concentrations

(I) Time	(J) Time	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
30	60	.000*	.000	.000	.000	.001
	120	.001*	.000	.000	.001	.001
	180	.001*	.000	.000	.001	.001
	240	.000*	.000	.000	.000	.000
60	30	.000*	.000	.000	-.001	.000
	120	.000*	.000	.000	.000	.001
	180	.000*	.000	.000	.000	.001
	240	.000*	.000	.000	.000	.000
120	30	-.001*	.000	.000	-.001	-.001
	60	.000*	.000	.000	-.001	.000
	180	1.91E-005	.000	1.000	-3.37E-005	7.20E-005
	240	-.001*	.000	.000	-.001	-.001
180	30	-.001*	.000	.000	-.001	-.001
	60	.000*	.000	.000	-.001	.000
	120	-1.91E-005	.000	1.000	-7.20E-005	3.37E-005
	240	-.001*	.000	.000	-.001	-.001
240	30	.000*	.000	.000	.000	.000
	60	.000*	.000	.000	.000	.000
	120	.001*	.000	.000	.001	.001
	180	.001*	.000	.000	.001	.001

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

a. Adjustment for multiple comparisons: Bonferroni.

Table 27 Brain sections – Pairwise comparison

Pairwise Comparisons

Measure: Concentrations

(I) BrainSection	(J) BrainSection	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
1	2	.002*	.000	.000	.002	.002
	3	.001*	.000	.000	.001	.001
	4	.001*	.000	.000	.001	.001
2	1	-.002*	.000	.000	-.002	-.002
	3	-.001*	.000	.000	-.001	-.001
	4	-.001*	.000	.000	-.001	-.001
3	1	-.001*	.000	.000	-.001	-.001
	2	.001*	.000	.000	.001	.001
	4	.000*	.000	.000	.000	.000
4	1	-.001*	.000	.000	-.001	-.001
	2	.001*	.000	.000	.001	.001
	3	.000*	.000	.000	.000	.000

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

a. Adjustment for multiple comparisons: Bonferroni.