Pharmacology of Artemether in Children with Protein Energy Malnutrition in The Gambia

Thesis submitted in accordance with the requirements of the University of Liverpool for the Degree of Doctor of Philosophy

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Abstract

Malaria and malnutrition are causes of high morbidity and mortality in developing countries especially in sub-Saharan Africa and Asia. Malnourished children are at higher risk of developing malaria, a problem compounded by the fact that malnutrition affects the metabolism of different antimalarials including chloroquine and quinine. Emerging resistance to chloroquine, which was the drug of choice, has led to the widespread use of artemisinin combination therapy in the population including children. To date, no studies have been undertaken on the pharmacokinetics of artemisinin compounds in malnourished children. This thesis aims to fill this evidence gap by studying patients with uncomplicated malaria of different nutritional status in The Gambia.

Analysis of the nutritional status of 97 children in The Gambia with uncomplicated malaria showed that 30% were both underweight and wasting, while 28% were categorised into stunting. This was much higher than the national average which has been estimated to be 17.4%, 9.5% and 23% for underweight, wasting and stunting, respectively, demonstrating a potential relationship between malaria and Protein energy malnutrition.

In-vitro studies showed that although pre-treatment of HL-60 cells with the iron chelator (DFO) did not affect the bioactivation of artesunate, there was a 20% increase in cell viability with IC_{50} increasing from 7.0 ± 4.3 to 33.3 ± 2.9. This is believed to be as a result of DFO chelating the toxic iron generated as a result of artesunate bioactivation which increased from 0.32 ± 0.6 ng/mol in the control incubations to 0.84 ± 0.1ng/mol at 100 μmol artesunate concentration. In light of the fact that iron was important in the mechanism of action of these compounds, and the fact that iron deficiency is commonly in malnourished children, the effect of both PEM and anaemia on plasma drug levels of artemether and DHA was also studied.

LC-MS/MS method was optimised and validated for the simultaneous analysis of artemether and DHA in plasma with ≥80% precision and accuracy. Plasma artemether and DHA concentration analysed 2h post first dose was 138.4 ± 80.9 ng/ml and 58.8 ± 43.7 ng/ml respectively. Severely wasted and wasted children had the highest artemether (156.5 ±69.6 ng/ml) and DHA (84.1 ±62.6) plasma concentrations respectively but values were not statistically significant. Anaemic status of children did not have an influence on drug plasma concentration with anaemic children having artemether and DHA plasma concentration of 138.5 ± 73.7 and 57.9 ± 36.6, respectively, and 138.43 ± 85.3 and 59.3 ± 46.8 for non-anaemic children respectively. However, conclusive results were limited by sample size.

In conclusion, this thesis has demonstrated a relationship between malaria and Protein energy malnutrition, and highlighted the possible effects pathophysiological changes as a result of protein energy malnutrition can have on drug pharmacology and therapeutic effects in these children. There is a need for further studies in larger cohorts of children with protein energy malnutrition to determine whether therapeutic efficacy of artemisinin combination therapy is affected in an adverse manner, and whether there is a need for changes in dosing recommendations.

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Thesis declaration

I hereby declare that this thesis is my own work and contains no material that has been presented previously, in whole or in part, for the award of any other academic degree. The use of other source of information which has been used has been acknowledged.

Signature…….

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Abbreviations

ACT	Artemisinin combination therapy
BCA	Bicinchoninic acid
C_L	Clearance
CYP	Cytochrome
DCFH	Dichlorofluoroescein
DCFH-DA	Dichlorofluoroescein diacetate
DFO	Deferoxamine
DFO-HES	Hydroxyethyl starch deferoxamine
DFP	Deferiprone
DHA	Dihydroartemisinin
DHFR	Dihydro-folate reductase
DHPS	Dihydropteroate synthetase
DMSO	Dimethylsulphoxide
ECD	Electrochemical detection
FPPIX	Ferriprotopophyrine IX
FV	Food vacoule
H_2O	Water
H_2SO_4	Sulphuric acid
HAZ	Height-for-age z-score
HBSS	Hank's balanced salt solution
HCL	Hydrocholric acid
HPLC	High performance liquid chromatography
I.S	Internal standard
IgG	Immunoglobulin
K	Kwashiorkor
kDa	kilodalton
$KMnO_4$	Potassium permanganate
LC-MS	Liquid chromatography mass spectrometry
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LLE	Liquid liquid extraction
LLOD	Lower limit of detection
LLOQ	Lower limit of quantification
LPME	Liquid phase micro extraction
M	Marasmus
MDA	Malondialdehyde
MeCN	Acetonitrile
MICS	Multiple Indicator Survey
MRC	Medical Research Council
MRM	multiple reaction monitoring
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide
MUAC	Mid-upper arm circumference
N	Normal
NCHS	National Centre for Health Statistics
NH_4FA	Ammonium formate
OFC	Occipito-frontal circumference

PBS	Phosphate buffered
PCR	Polymerase chain reaction
PEM	Protein energy malnutrition
PK	Pharmacokinetics
QBC	Quantitative buffy coat
RDT	Rapid diagnostic test
RIPA	Radioimmunoprecipitation
ROS	Reactive oxygen species
ROS	Reactive oxygen species
SA	Succinylacetone
SDS	Sodium dodecyl sulphate
SM	Severe malnutrition
SPE	Solid phase extraction
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid-reactive substances
TBS(T)	Tris Buffered Saline Tween 20
Tf	Transferrin
TfR	Transferrin receptor
THF	Tetrahydrofuran
TMRE	Tetramethylrhodamine ethyl ester
U	Undifined
UNICEF	United Nations Children's Fund
Vd	Volume of distribution
WAZ	Weight-for-height z-score
WHO	World Health Organisation
WHZ	Weight-for-age z-score

I General Introduction

1.1 Malaria

The word malaria comes from the Latin word “mal aria” meaning “bad air”. This was because people living around the Roman swamps would come down with recurrent and debilitating fever. They thought the disease emanated from the gases from the swamp. The first clinical description of malaria was made by Hippocrates in 400 BC, but it was not until the 1700s that malaria was discovered to be caused by a protozoan infection of the blood (Carter and Mendis, 2002).

Among the two billion people at risk of malarial infection in 2008, WHO estimated 243 million cases of malaria resulting in 863,000 deaths. 85% of the cases were in Africa followed by South-East Asia with 10%. 89% of the deaths occurred in Africa and 85% of all deaths were in children under 5 years of age (WHO, 2009). Table 1.1 shows the number of malaria case and deaths by region in 2008.

Malaria is an infectious disease of the blood caused by a protozoan parasite of the genus *Plasmodium*, and is transmitted from one human to another by the female anopheles mosquito. Although there are more than 400 species of *plasmodium*, only four species cause malaria infection in humans: *Plasmodium vivax, falciparum, ovale and malaria. P vivax* and *P falciparum* are the most commonly encountered malaria parasites (Tangpukdee et al., 2009).

Table 1.1 Estimated number of malaria cases, 2008.

WHO Region	Cases			Deaths		
	Estimates			Estimates		
	Number	%	P.falciparum (%)	Number	%	Under 5 %
Africa	208	85	98	768	89	88
Americas	1	0.4	32	1	0.1	30
Eastern Mediterranean	9	3.7	75	52	6	77
Europe	0	0	4	0	0	3
South-East Asia	24	10	56	40	4.6	34
Western Pacific	2	0.8	79	3	0.3	41
Total	**243**	**100**	**93**	**863**	**100**	**85**

Estimated number of malaria cases (in millions) and deaths (in thousands) by WHO region in 2008.
Adapted from WHO World Malaria Report, 2009.

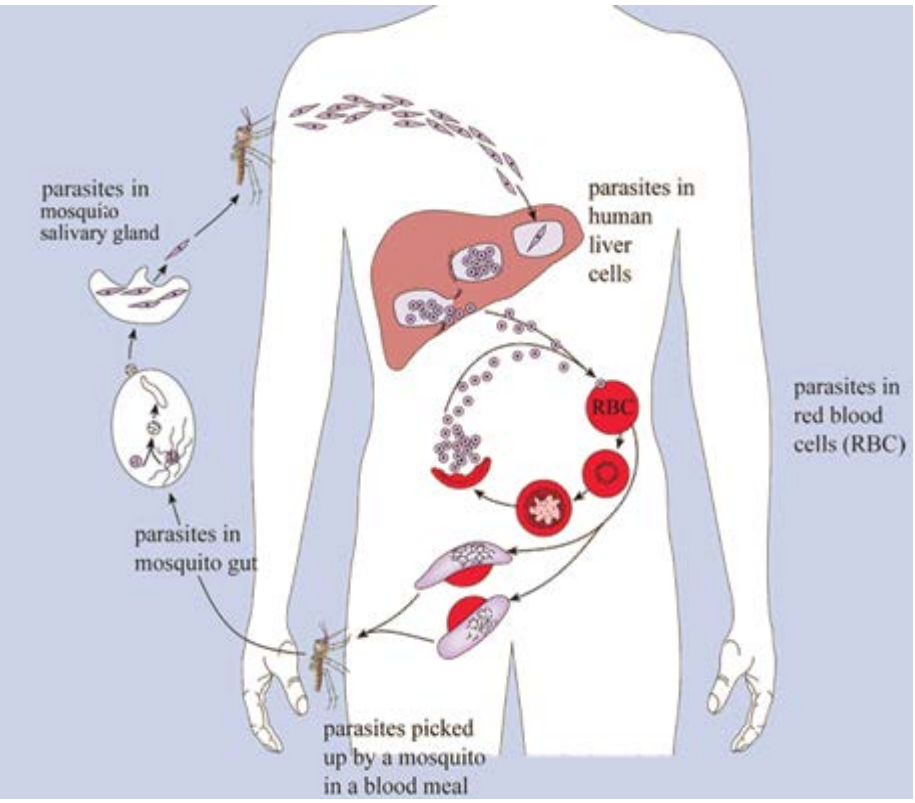
P falciparum and *P vivax* account for 95% of all malaria infections, but almost all severe and deadly cases are caused by *P falciparum* (Schlitzer , 2007). *P falciparum* is the most prevalent and is normally found in tropical, subtropical and warm temperate regions whilst *P vivax* is more prevalent in Asia and the Latin American subcontinents and account for 10% of malaria cases in Africa (Carter and Mendis, 2002).

1.1.1 Life Cycle of Malaria Parasite

Transmission of the malaria parasite from mosquitoes to the human body takes place in the form of a cycle (Figure 1.1) through the bite of a female *Anopheles* mosquito. It comprises two phases, the asexual phase which takes place in humans, and the sexual phase which takes place in the body of the mosquito. The asexual

stage (human stage) of the life cycle begins with the exo-erythrocytic stage and ends with the formation of non-multiplying sexual forms or gametocytes called the erythrocytic phase (Suh et al., 2004).

Figure 1.1 Life cycle of a malaria parasite



Schematic diagram of the life cycle of a malaria parasite in the human host and mosquito vector (Adapted from labspace, The Open University)

The infected female anopheles mosquito injects the parasites (sporozoites) in the bloodstream whilst taking a "blood meal". Parasites invade and develop in the liver cells where they begin a phase of asexual reproduction called the hepatic or pre-erythrocytic phase, resulting in the formation of schizonts (Beeson and Brown, 2002). The mature schizonts rupture to release the merozoites which then enter the blood stream and invade red blood cells, thus starting the erythrocytic phase of the life cycle. In the red blood cells, merozoites mature to form trophozoites which then

again develop to schizonts. As the parasites mature and replicate during the erythrocytic phase of malaria infection, it ingests a huge amount of haemoglobin for its own protein production. The heme is digested in the parasites food vacuole (FV) releasing toxic heme moiety which the parasite has the ability to detoxify. Protein synthesis and nuclear division of the parasites causes red blood cell distortion and rupture releasing more merozoites and continuing the cycle of infection (Elliot et al., 2007).

1.1.2 Clinical manifestation

The severity of the symptoms of malaria depends largely on the nature and load of the parasite, and the immune status of an individual towards malaria. Partial immunity against malaria is progressively acquired in adults living in malaria endemic areas usually due to repeated infections (Doolan et al, 2009).

Symptoms are generally more severe in children, pregnant women and non-immune individuals. The most common symptoms of uncomplicated malaria are non-specific and include prodromal symptoms like headache, nausea, fever, general body pain and vomiting. Fever, chills and headache can come in bursts which correspond with the release of the merozoites and toxins from the red blood cells to the blood stream. Complicated malaria is said to occur in the presence of life threatening conditions (Suh et al., 2004). Table 1.2 summarises the signs and symptoms of complicated malaria.

Table 1.2 Signs and Symptoms of complicated malaria

- Impaired consciousness
- Unrousable coma not associated to any other cause - Glasgow coma scale ≤ 9
- Repeated generalised convulsion - ≥ 3 episodes in 24 H
- Prostration or weakness
- Acute respiratory distress syndrome
- Pulmonary oedema (radiological)
- Abnormal spontaneous bleeding or disseminated intravascular coagulation
- Metabolic acidosis - arterial pH < 7.25 or plasma
- bicarbonate < 15 mmol/l
- Circulatory collapse/shock
- Hyperpyrexia - Core body temperature > 40
- Hyperparasetaemia - >5 % parasitised erythrocytes or > 250000 parasites/µl
- Severe anaemia - Haemoglobin < 50g/dl in the presence of parasite count > 10000/µl
- Hypoglycaemia - Blood glucose concentration < 2.2 mmol/l
- Renal failure - Urine output < 400 ml/24 hour in adults (< 12 ml/kg/24 hour in children), serum creatinine > 265 µmol/l (> 3.0 mg/dl) despite adequate volume repletion
- Hyperbilirubinaemia - Total bilirubin > 43 µmol/l (2.5 mg/dl)
- Macroscopic haemoglobinuria - Haemolysis not secondary to glucose-6-phosphate dehydrogenase deficiency

1.1.3 Diagnosis of malaria

Rapid and correct diagnosis is important in malaria treatment as delays in diagnosis can lead to complicated malaria, and inevitably be fatal. In areas of high malaria transmission, clinical diagnosis is very common and is based on the signs and symptoms of the patient. This form of diagnosis might not be reliable due to the

non-specific symptoms of malaria leading to high number of false positives and mistreatment (Tangpukdee et al., 2009).

Light microscopy by Giemsa stain of thick and thin blood smears remains the most commonly used method for the diagnosis of malaria (Moody and Chiodini, 2000). Thick smears are sensitive for screening for plasmodium parasites whilst thin smears allow for species identification, parasite quantification and the different developmental stage of the parasite (schizonts and gametocytes) (Trampuz et al., 2003). Alternative diagnostic methods have been developed to overcome the limitations of microscopy. These include polymerase chain reaction, rapid diagnostic test (RDT), automated malaria pigment detection, quantitative buffy coat (QBC) (Bhandari et al., 2008), OptiMal, Para HIT-f, ParaScreen, SD Bioline and Paracheck. With the exception of RDTs, these methods are relatively more expensive and need specialised equipment. RDT on the other hand detects malaria antigen in blood and is quick, simple, accurate and cost effective for *Plasmodium* detection (Tangpukdee et al., 2009).

1.1.4 Treatment for malaria

1.1.4.1 Drugs used in the treatment of malaria

Antimalarials used in the treatment of malaria include 7 drug classes namely; 4-aminoquinolones, 8-aminoquinolones, arylaminoalcohols, antifolates, inhibitors of the mitochondrial respiratory chain, antibiotics and artemisinins (Schlitzer, 2008). Table 1.3 lists the different drugs in each class and summarises the mode of action of the different classes of antimalarials.

Chloroquine and sulphadoxine-pyrimethamine have been used for many decades as the drug of choice for the treatment of uncomplicated malaria but the emergence of parasite resistance to these traditional antimalarials saw a rise in malaria morbidity and mortality especially in children. In response to this serious situation, steps were taken to deliver more effective interventions. This included drug combinations with an artemisinin derivative and anti-vector measures (Greenwood et al., 2008).

Artemisinin (1) and its derivatives are a class of antimalarial drugs which may meet the challenges posed by drug-resistant parasites and the rapid progression of malarial illness. They are the most rapidly acting and potent of all the antimalarial drugs and have been used successfully in malaria treatment. With the rising resistance to malaria, the use of the drug has become important (White, 2005).

1.1.4.1.1 Artemisinins

Artemisinins are derived from a plant called sweet worm (or sweet Annie; Artemisinin annua) in China where they were first discovered. “Ginghae” extracts were reported to have antipyretic properties more than 1500 years ago. A co-ordinated programme was started by the Chinese government in 1967 to discover antimalarial principles in various medical herbs including “qinghao” (Woodrow et al., 2005).

Table 1.3 Drugs used in the treatment of malaria and their mode of action

Drug class	Drug(s)	Mode of action	Active against
4-Aminoquinolones	Amodiaquine, chloroquine, hydroxychloroquine	Form complexes with ferriprotopophyrin IX (FPPIX) to prevent polymerisation to non-toxic hemozoin	trophozoytes
8-Aminoquinolones	Piperaquine	Disrupts mitochondrial internal structure	Different developmental stages including hypnozoites and sexual stages
Arylaminoalcohols	Halofantrine, lumefantrine, mefloquine, quinine	Believed to interfere with heme digestion	Different developmental stages
Antifolates	Chloroproguanil[1], dapsone[2], pyramethamine[1], sulphadoxine[2]	Inhibition of tetrahydrofolate biosynthesis by inhibiting either dihydro-folate reductase[1] (DHFR) or dihydropteroate synthetase[2] (DHPS)	Erythrocytic stage
Antibiotics	Clindamycin, doxycycline	Interacts with protein biosynthesis of the mitochondria	
Inhibitors of the respiratory chain	Atovaquone	Inhibits the mitochondrial transport chain resulting in rapid breakdown of mitochondrial memebrane potential	Hepatic and erythrocytic stages
Artemisinins	Artemether, artesunate, dihydroartemisinin	Inhibition of PfATPase6, inhibition of hemozoin formation	Early and late ring stages

Artemisinin is a phyto-constituent obtained from aerial portions of the herb. The antimalarial activity was re-discovered in China in 1971, when low temperature ethyl ether extraction of the plant produced encouraging results in mice infected with the malaria *Plasmodium berghie* (Medhi et al., 2009).

Since artemisinin is poorly soluble in water and oil, semi synthetic derivatives of artemisinin were developed to circumvent the problem of parenteral administration due to its low water and oil solubility (Meshnick et al., 1996). These derivatives include a water soluble hemisuccinate artesunate (3) and artelinate, and oil soluble ethers, artemether (2) and arteether. They have a greater antimalarial activity than their parent compound. The most widely used derivatives in clinical practice are artemether and artesunate, and though they have different physicochemical properties they are both pro-drugs of dihydroartemisinin (4) (Hien et al., 2004).

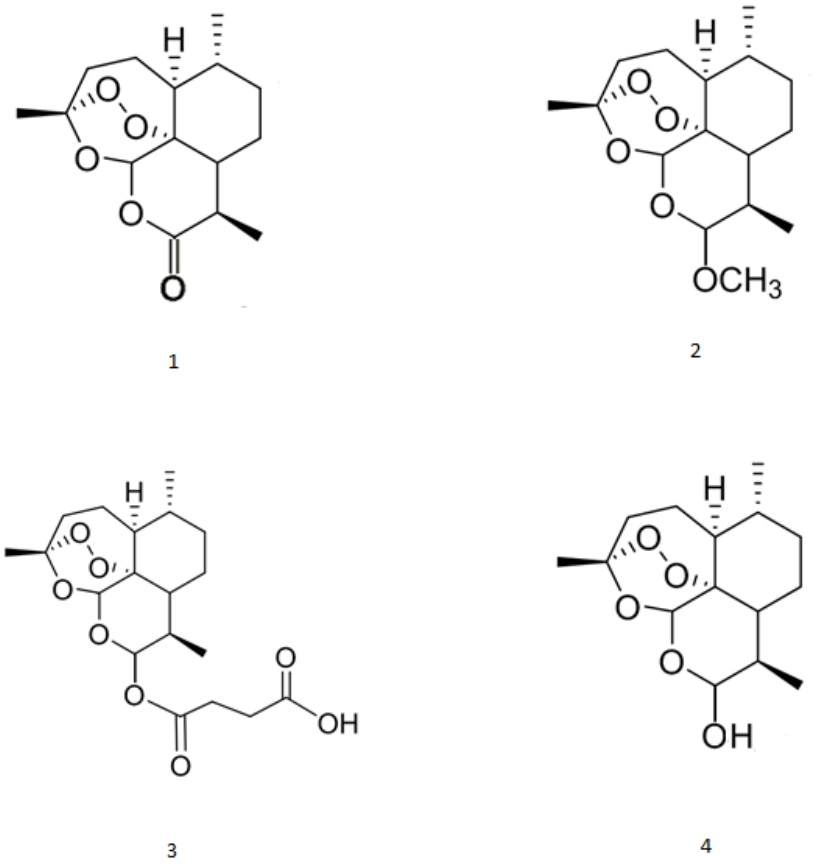
1.1.4.1.2 Mechanism of action of the artemisinin compounds

Its antimalarial mechanism of action depends on its endoperoxide bridge (Navaratnam et al., 2000). Although the exact mode of action is still debatable, and a couple of theories have been proposed, it is believed that haem iron within the parasite, a bi-product of haemoglobin digestion, catalyses the cleavage of the endoperoxide bridge.

The parasite digests and detoxifies host haemoglobin in its food vacuole through polymerisation to form hemozoin, which is a rich source of Fe^{2}-heam

believed to be responsible for the activation of artemisinin compounds in the parasite (Niles et al., 2009). The Fe2-heam rich environment is believed to be an important factor for the selective toxicity of artemisinin toxicity towards the malaria infected red blood cells compared to non-infected red blood cells (Meshnick et al., 1996, Meshnick et al., 1991).

Figure 1.2 Structures of the artemisinin compounds



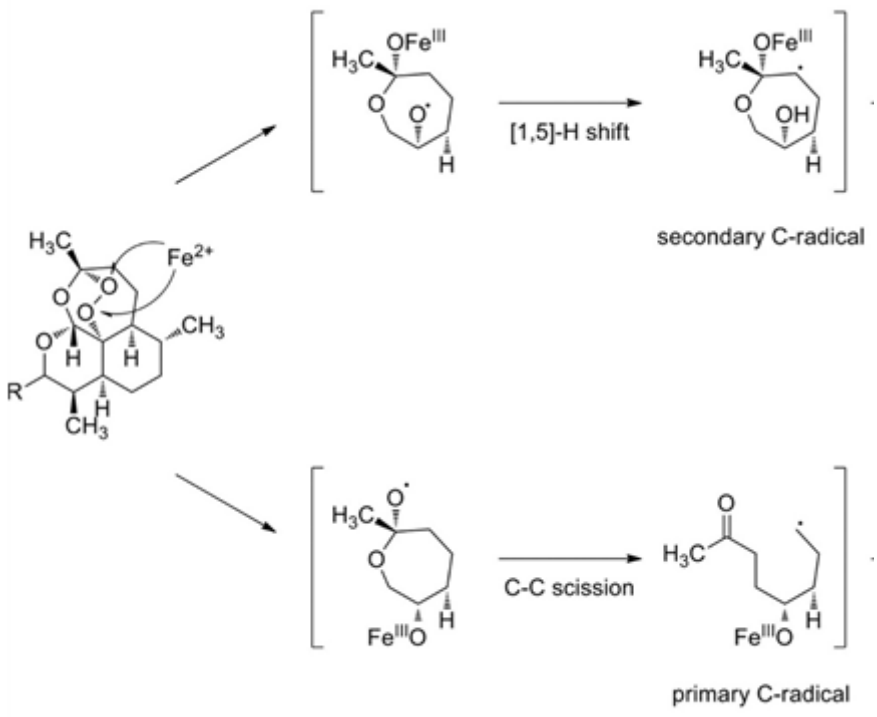
The structures of artemisinin (1), artemether (2), artesunate (3) and dihydroartemisinin (4).

This is followed by the formation of highly reactive free radicals that rearrange to form more stable carbon cantered-radicals. It has been suggested that

these centred-radicals alkylate and damages macromolecules in the parasite (Medhi et al., 2009).

The inhibition of the enzyme sarcoplasmic-endoplasmic reticulum ATPase (PfATP6), critical for parasite survival, has been widely accepted (Woodrow et al., 2005, Haynes et al., 2007) The PfATP6 enzyme is the only SERCA-type calcium-ATPase responsible for maintaining calcium ion concentration critical in calcium-mediated signalling and post-translational processing of proteins (Golenser et al., 2006). Eckstein-Ludwig (2003) has demonstrated the inhibition of PfATP6 outside the food vacuole following artemisinin activation (Eckstein-Ludwig et al., 2003).

Figure 1.3 Proposed artemisinin mechanism of action



The proposed mechanism of action of the endoperoxide compounds

The interaction of P *falciparum* histidine-rich protein with artemisinin-derived radicals to inhibit hemozoin formation has also been suggested (Pandey et al., 1998, Kumar et al., 2007) but other findings have contradicted this method (Meshnick 1996, Haynes et al., 2003).

1.1.4.1.3 Artemisinin pharmacokinetics

Whilst artemisinin can only be administered intravenously due to its water solubility, artemisinin, artemether and artesunate can all be administered orally, intramuscularly and rectally (Silamut et al., 2003). Although artemisinin is thought to pass through the gut membrane fairly easily, there is low bioavailability resulting from high first-pass metabolism of the compound. Mean oral bioavailability of artesunate is relatively lower (15%) with a high variability when compared to intravenous administration (82%) (Batty et al., 1998). This was also observed in artemether, with intramuscular and intra rectal bioavailabilities of 25% and 35% respectively in relation to oral administration in healthy volunteers (Teja-Isavadharm et al., 1996). This further suggests the involvement of high first pass metabolism in both the gut and liver (Karbwang et al. 1997, Teja-Isavadharm et al., 1996).

Peak plasma levels are reached within minutes after oral administration of artesunate (Olliaro et al., 2001), whilst artemether peaks at about 2 hours with a half-life of 1-3 hours (White et al., 1999). Because red blood cells are host cells for the malaria parasite, levels of drug concentration in them would have an important therapeutic effect (Asawamahasakda et al., 1994). In-vitro studies have demonstrated higher uptake of artemisinin and its derivative by malaria infected red

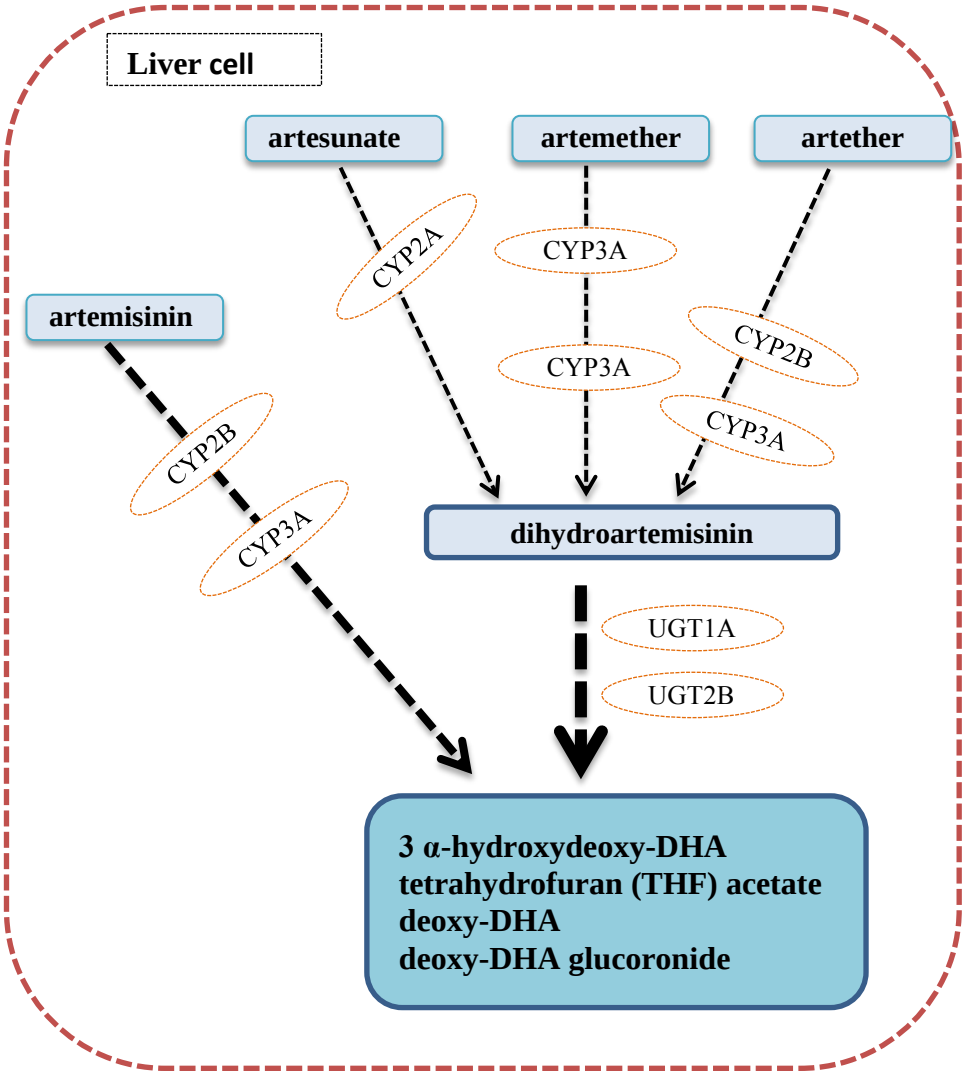
blood cells when compared to non-infected red blood cells (Shah et al., 2009, Vyas et al., 2002). Membranes of parasitised red blood cells are modified as a result of the malaria parasite invasion causing structural and functional changes (Pasvol et al., 1992), resulting in increased permeability to higher molecular weight solutes thus causing passive but facilitated uptake drug uptake (Shah et al., 2009). Saturation levels of artemisinin and selective derivatives have shown saturation levels in parasite infected red blood cells compared to between 33 – 43% partitioning in non-parasite infected red blood cells (Shah et al., 2009, Vyas et al., 2002)

Tissue distribution of the artemisinin compounds in rats showed that whilst artemether was seen to cause the highest concentration in rat brain, both artemisinin and artesunate also cross the blood brain barrier. Artesunate levels were highest in rat intestines followed by brain, liver and kidney (Navaratnam et al., 2000).

Dihydroartemisinin is the active metabolite of the artemisinin derivatives (De Vries and Deen, 1996). Metabolism of artemisinin in liver microsomes is mediated mainly by CYP2B6 with contributions from CYP2A6 and CYP3A4. Figure 1.4 illustrates the cytochrome (CYP) enzymes involved in the metabolism of artesunate and its derivatives.

The CYP enzyme family plays an important role in phase-1 metabolism of many drugs. Clinical case reports or studies usually provide the first evidence of interaction between drugs. Central to this point is an understanding of the catalytic importance of individual CYP isoenzymes in particular metabolic pathways (Badyal et al., 2001).

Figure 1.4 Metabolism pathways of artemisinin and its derivatives



Proposed metabolic pathway of artemisinin, artesunate, artemether, arteether and dihydroartemisinin.

A study done by Elsherbiny et al. (2008) showed that the production rate of CYP2B6 was increased by 79.9% by artemisinin, 61.5% by arteether, 76.1% by artemether, 19.9% and 16.9% by dihydroartemisinin and artesunate respectively. The production rate of CYP2C19 was increased 51.2% by artemisinin, 14.8% by arteether and 24.9% by artemether. These different inductive capacities among the artemisinin drugs are important in selecting drugs in combination therapies to minimise drug-drug interactions (Elsherbiny et al., 2008).

Four metabolites (Figure 1.5) namely 3 α-hydroxydeoxy-DHA (5), tetrahydrofuran (THF) acetate (6), deoxy-DHA (7) and deoxy-DHA glucoronide (8) have been identified in human urine following oral administration of artemisinin. Studies using rat liver microsomes have shown the conversion of DHA to other inactive metabolites (figure 1.7) including 3 α-hydroxydesoxy-DHA glucoronide (5), the glucoronide of tetrahydrofuran acetate isomer of DHA (6) deoxy-DHA (7) and its glucoronide (8) (Maggs et al., 1997, 2000).

α-DHA-β-glucoronide (α-DHA-G) and the tetrahydrofuran isomer of α-DHA-G were the metabolites identified in human urine with DHA-G being the only liver metabolite (Ilett et al., 2002). This process is catalysed by UDP-glucoronosyltransferase, in particular UGT1A9 and UGT2B7 in both rats and humans (Maggs et al., 1997, Ilett et al., 2002).

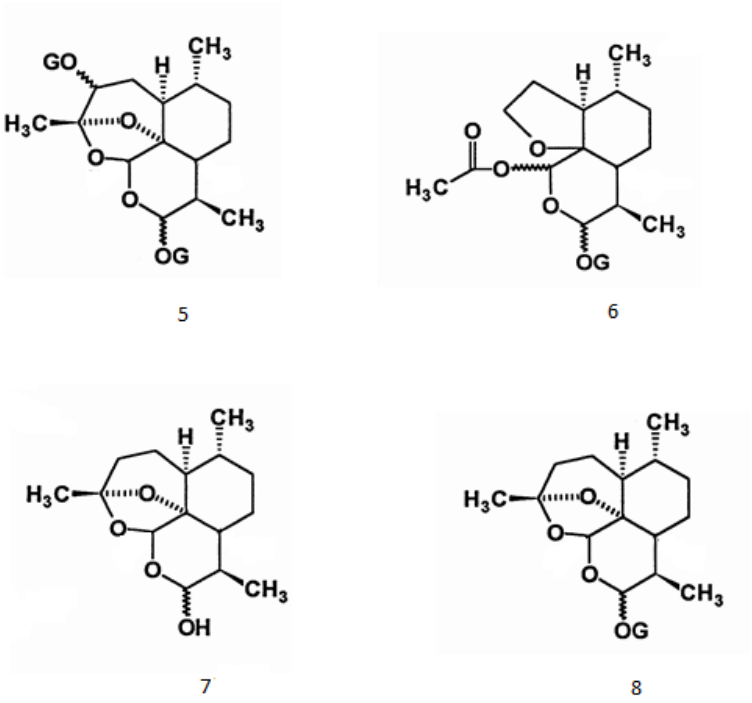
1.1.4.1.4 Artemisinin toxicity

Although the safety profiles of these drugs are thought to be excellent in humans (Nontprasert et al., 2002), concern about their possible neurotoxicity and embryotoxicity remains based on studies confirming embryotoxic effects in animals, including primates, with risk being confined to a defined period of gestation (Dellicour et al., 2007).

Fetal resorption was observed in rats and rabbits exposed to relatively low doses of artemisinin during early pregnancy (Boareto et al., 2008). In vivo studies have shown artesunate, dihydroartemisinin, artemether and arteether ten day post conception caused embryolethal and teratogenic effects (White et al., 2006). The

signs of toxicity of artemisinin family of compounds in laboratory animals, and lack of such findings in human subjects, can be explained by persistent drug concentrations after repeated intramuscular concentrations using oil based vehicles in animals. Another possible explanation may be the relatively high doses used in pre-clinical experiments (Clark et al., 2006).

Figure 1.5 Structures of artemisinin metabolites



The structures of 3 α-hydroxydesoxy-DHA (5), tetrahydrofuran (THF) acetate (6), deoxy-DHA (7) and Deoxy-DHA glucoronide (8).

Several clinical trials indicate that artesunate is far less toxic than the quinolones with common adverse effects observed are nausea, vomiting, and diarrhoea all of which are also characteristics of acute malaria (Clark 2009). Large scale human studies with artemisinin compounds have not shown any neurotoxic side effects but there are isolated case reports of possible neurological dysfunction after

administration of associated artemisinin compounds (Li et al., 1998). Because of limited safety data, WHO recommended that they should only be used in 2nd and 3rd trimester of pregnancy, but should only be used in the 1st trimester only if it is the only effective treatment (WHO, 2006).

1.1.5 Artemisinin combination therapy

The main advantage of the artemisinin compounds against the other antimalarials is the rapid mode of action against all stages of the plasmodium and excellent tolerability in humans (White, 2005). However, the main limitation of this class of drugs is its short half-life requiring frequent administration. When used as monotherapy, optimum cure rates are observed with 7-day treatment regiments. However compliance with 7-day treatment is very low and 5 and 3-day treatment regimens showing low cure rates and high failure rates respectively (Yeung et al., 2004).

Table 1.4 Drug combinations in artemisinin combination therapy (ACT)

- Artemether/lumefantrine
- Artesunate plus amodiaquine - in areas where the cure rate of amodiaquine monotherapy is greater than 80%
- Artesunate plus mefloquine - insufficient safety data to recommend its use in Africa
- Artesunate plus sulfadoxine/pyrimethamine - in areas where the cure rate of sulfadoxine/pyrimethamine is greater than 80%
- Dihydroartemisinin plus piparaquine

In order to both improve cure rates and reduce the development of drug resistance, WHO recommended the use of artemisinin combination therapy as the first line treatment for uncomplicated malaria. artemisinin derivatives are combined with another longer-acting antimalarial drug which eliminates the residual malaria parasite. A combination of artemisinin derivative with a longer acting drug, a shorter course of treatment of 3 days provides effective therapeutic outcomes (WHO, 2006). The current artemisinin combination therapies (ACT) recommended by WHO are listed in table 1.4.

1.5.1 Artemisinin Combination Therapy (ACT) in Children

Artemisinin derivatives are safe and well tolerated in children and ACT is currently the recommended treatment for uncomplicated malaria in children > 6 months/ >5kg. Although the differences in the pharmacokinetics of drugs between children and adults have been recognised, dosing guidelines have been deduced from adult-based regiments adjusted for body weight (WHO, 200. Few pharmacokinetic studies of ACT have been carried out in children with some of them suggesting that children may be receiving suboptimal doses of ACT (Sidhu et al., 1998). Sidhu et al. (1998) investigated potential differences between adults and children and reported that adults had slightly lower C_L rates than children, which may explain the reported longer half-life of artemisinin in adults (2.6 h) compared to children (1.8 h).

Physiological processes during childhood development which control the pharmacology, absorption, metabolism, distribution and excretion of drugs differ between infants and young children. The exact effect of the developmental

differences is drug specific and depends on the lipophilicity, solubility, protein-binding capacity and route of administration. Drug absorption is generally slower in children due to increased gastric pH, slow intestinal motility and decreased gastric emptying (Tetelbaum et al., 2005, Kearns et al., 2003).

Rapidly absorbed water soluble drugs like artesunate may be reduced in children leading to sub-optimal plasma drug concentration. In addition, changes in total body water composition and body fat content in children of different ages might affect the volume of distribution (V_d) impacting differently on drugs depending on their physiochemical properties and route of administration (Johnson et al., 2006, Kearns et al., 2003).

Metabolism of drugs, including the CYP450 enzymatic system is thought to be age dependent. CYP3A4 and CYP2A6, which are involved in the metabolism of ACT, are reported to have decreased activity in neonates and young children, increasing with age (Edginton and Willmann, 2006, Benedette and Bates, 2003). Full CYP1A2 and CYP2B6 enzymatic maturation are not reached until around puberty (Johnson et al., 2006).

In addition to age related and maturational differences in children, malnutrition affects the absorption, distribution, metabolism and excretion of drugs (Murphy et al., 2002, Oshikoya and Senbenjo, 2009). The effect of malnutrition on the pharmacokinetics of ACT is important due to the prevalence of malnutrition in many areas worst affected by malaria, with a strong correlation between malaria and malnutrition (Müller et al., 2003).

1.2 Protein energy malnutrition (PEM)

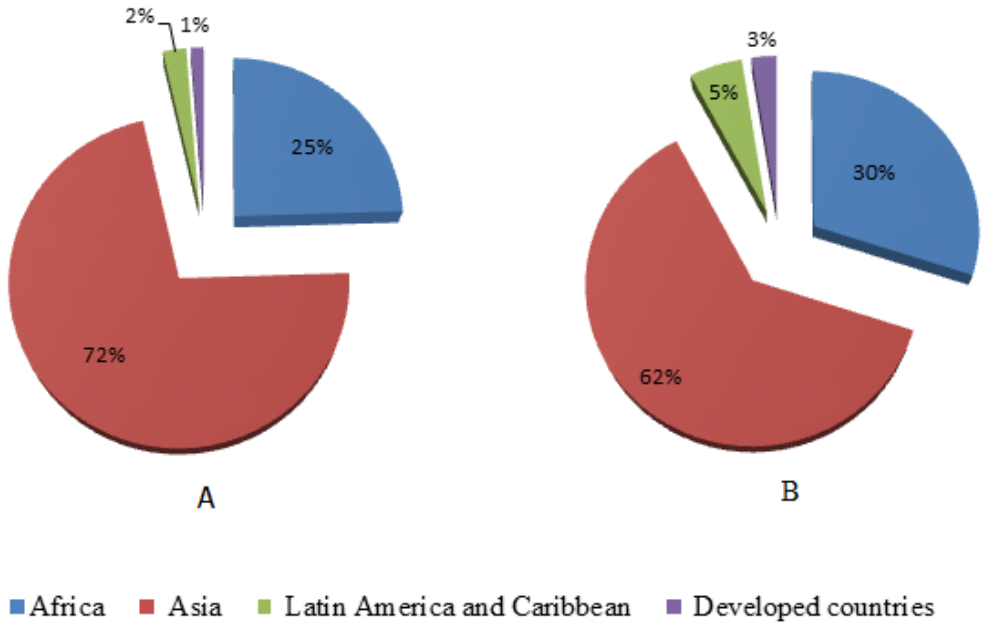
Protein-energy malnutrition (PEM) refers to a nutritional problem resulting from different degrees of protein and calorie insufficiency, most commonly seen in infants and young children (Mora, 1999, Oshikoya and Senbanjo, 2009). It encompasses a range of nutritional disorders ranging from mild moderate to severe. The mild forms can be hidden in that they are subclinical and can usually be detected only thorough anthropometric and or biochemical tests. It is likely to be undiagnosed and as a result can lead to long-term problems (Ahmed et al., 2009). The interchangeable term of PEM and malnutrition will be used throughout the thesis but will mean the same thing.

Malnutrition accounts for about 54% of the 10.8 million deaths per year and contributes directly or indirectly to about half of the deaths associated with infectious diseases among children under 5 years of age in developing countries (Schaible and Kaufmann, 2007, Caulfield et al., 2004). Although the relative risk of mortality is highest for severe malnutrition with 8.4 compared to moderate and mild nutrition which are 4.6 and 2.5 respectively, the majority of deaths occur in children with mild and moderate malnutrition. This is due to the fact that most of the children with malnutrition are classified either mild or moderate (Grover and Ee, 2009, Pelletier and Frongillo, 2003, Pelletier et al., 1995).

In 2005, the global estimate of wasting (weight for height z-score< -2) in children below the age of 5 years was 10% with south-central Asia having the highest prevalence estimated at 16%. 20% of children below the age of 5 years in

low and middle income countries were estimated to be underweight (weight for age z-score < -2) and 32% of children in developing countries were estimated to be stunted (height for age z-score < -2). The prevalence of underweight was highest in south central Asia (33%) and eastern Africa (28%), whilst the highest prevalence of stunting was in central Africa and south central Asia with 50% and 41% respectively (WHO, 2006, Grover and Ee, 2009, Black et al., 2008).

Figure 1.6 Global prevalence of protein energy malnutrition, 2005.



Estimates of prevalence of underweight (A) and stunting (B) by region in children under five years of age in 2005

1.2.2 Classification of Protein Energy Malnutrition

There are different classifications of malnutrition (Table 1.3). WHO classifies acute malnutrition as low weight for height below -2 Standard deviation of the median WHO growth standard. Other methods of protein-energy malnutrition classification have been used in clinical studies. The Gomez, Waterlow and Wellcome Trust classifications of protein-energy malnutrition are more widely used according to previous studies evaluating drug disposition in children with protein-energy malnutrition (Oshikoya and Senbanjo, 2009).

1.2.2.1 Gomez Classification

Gomez was a Mexican paediatrician who classified malnutrition into degrees based on the percentage body weight of children. First degree malnutrition occurs when under-nutrition is moderate, or for a short period, and the body weight for age of the patient ranges between 76-90 %. As under feeding becomes progressive, the body weight for age drops (61-75%) resulting in second degree malnutrition. Third degree malnutrition occurs when the body weight for age becomes less than 61% (Gomez et al., 1956).

Although the main use of the Gomez classification was to standardise reference values and allow meaningful comparison between and within populations at different times, it did not take into consideration children whose birth weight was low, children who were born prematurely or small children from small parents (Gueri et al., 1980).

The percentage weight for age of the patient is calculated by using the equation

$$\frac{a}{b} \times 100$$

where a is the weight of the patient

b is the expected weight of a normal child of the same age

1.2.2.2 The Wellcome classification

Because the Gomez classification did not take into account the clinical features associated with malnutrition, the Wellcome working party in 1969, classified malnutrition based on the most clinically obvious forms of malnutrition found in an undernourished population. It evaluates the child for the presence or absence of oedema, combined with the Gomez classification system (Waterlow, 1972).

1.2.2.3 The Waterlow Classification

The Waterlow classification concentrates on the much larger group of children described as underweight in the welcome classification. It is based on height for age and weight for height and concentrates on the determination of stunting and wasting (Waterlow et al., 1977, Waterlow, 1972).

Stunting is said to occur when a child is below the median height for age of the reference population and is determined by using the equation

$$\frac{c}{d} \times 100$$

where c is the height of the patient

d is the expected height of normal child of the same age

Wasting occurs when a child is below the median weight for height of the reference population. Wasting is determined using the equation

$$\frac{e}{f} \times 100$$

where e is the weight of the patient

f is the expected weight of a normal child of the same height

Alternative methods have been proposed in assessing malnutrition. Mid upper arm circumference (MUAC) and head circumference can be used in the place of weight and height respectively where these measurements are not possible. The degree of malnutrition in this case is calculated by dividing MUAC by head circumference (Grover and Ee, 2009).

Table 1.5 Classification summary of protein energy malnutrition

Classification	Definition	Grading	Change
Gomez	% body weight for Age	Normal Grade I: mild Grade II: moderate Grade III: severe	90% – 110% WA 75% - 89% WA 60% - 74% WA < 60% WA
Waterlow	Z-score (SD) weight for height	Normal Mild Moderate Severe	> 90% WH 80% - 90% WH 70% - 80% WH < 70% WH
	Z-score (SD) height for age	Normal Mild Moderate Severe	> 95% HA 90% - 95% HA 85% - 90% HA < 85% HA
Wellcome	Weight for age with edema	Kwashiorkor Marasmic-kwashiorkor	60% - 80% WA <60% WA
	Weight for age without edema	Undernutrition Marasmus	60% -80% WA < 60% WA
WHO	Z-score (SD) weight for height	Moderate Severe	-3 ≤ Z-score <-2 WH Z-score <-3 WH
	Z-score (SD) height for age	Moderate Severe	-3 ≤ Z-score <-2 HA Z-score <-3 HA
	MUAC divided by OHC	Mild Moderate Severe	< 0.31 < 0.28 < 0.25

WA, weight for age; WH, weight for height: WA weight for age; SD, standard deviation; WHO, World Health Organisation; MUAC, mid upper arm circumference; OFC, occipito-frontal circumference.

1.2.3 Clinical Syndromes

The clinical signs and symptoms (Table 1.6) present in protein energy malnutrition are believed to be linked to its pathogenesis and can be defined as

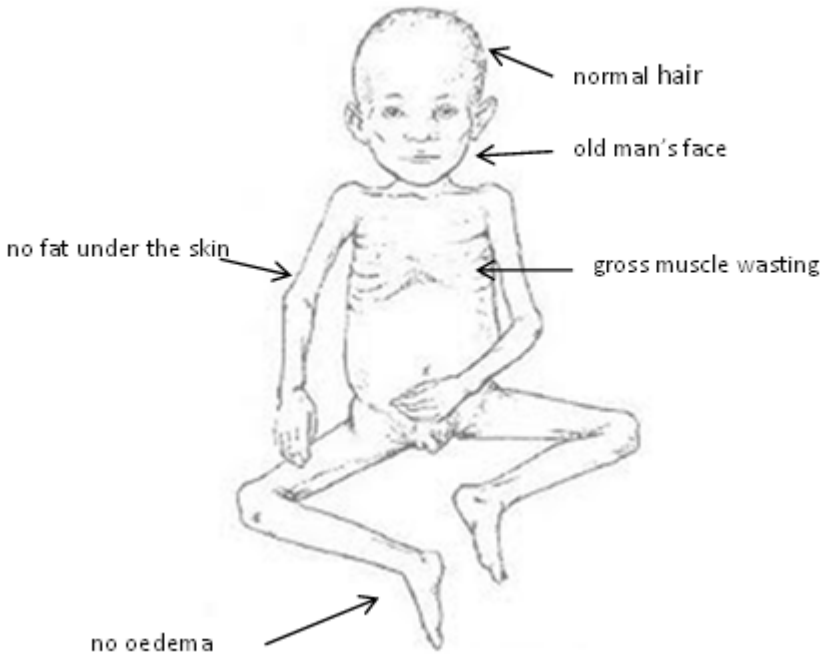
marasmus or kwashiorkor based on specific signs and symptoms at presentation (Balint, 1998, Jahoor et al., 2008).

Table 1.6 Clinical features of protein energy malnutrition

Syndrome	Clinical features
Marasmus	- loss of subcutaneous fat - Muscle wasting - Wrinkled skin - Hypotonia - Subnormal body temperature - Decreased basal metabolic rate - Alert and irritability - Constipation/diarrhea and dehydration
Kwashiorkor	Constant features - Oedema - Muscle wasting - Growth retardation - Psychomotor changes Usual features - Moon face - Hair changes - Skin depigmentation - Diarrhoea - Anaemia Occasional features - Dehydration - Dermatitis - Hepatomegaly - Cardiomyopathy - Signs of vitamin deficiency - Signs of infection

Marasmus is the most common syndrome and occurs when there is primarily energy deficiency as a result of inadequate intake of both protein and calories in a diet (Tatli et al., 2000). This leads to wasting as a result of the body's adaptation to starvation (Ece et al., 2007).

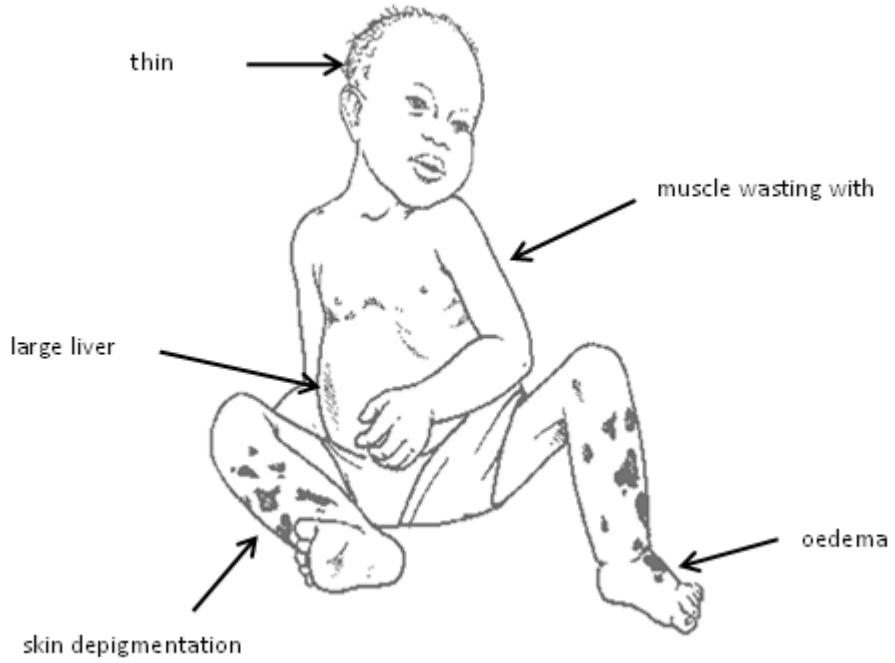
Figure 1.7 Distinct features of a child with marasmus



Adapted from WHO pocket book of hospital care for children 2005, pp. 364.

Kwashiorkor on the other hand is the opposite spectrum of the disease and occurs due to protein deficiency. There is usually adequate calorie intake but marked protein deficiency often aggravated by superimposed infection. The decreased protein intake leads to decreased protein turnover, increased free fatty acid, fatty liver (Franco et al., 1999) and increased oxidative stress (Manary et al., 2000, Golden and Ramdath, 1987).

Figure 1.8 Distinct features of a child with kwashiokor



Adapted from WHO pocket book of hospital care for children 2005, pp. 364

The milder forms of malnutrition may present as a mixed picture of marasmus and kwashiorkor. Marasmic-Kwashiokor manifests as concurrent gross wasting and edema with mild hair and skin changes. Milder forms are particularly likely to be undiagnosed and as a result can lead to long-term problems (Golden and Ramdath, 1987).

1.2.4 Pathophysiological changes in protein energy malnutrition

Insufficient intake of protein and energy leads to physiological adaptations which include growth restriction, loss of muscle, fat and visceral mass, reduced basal metabolism and total energy expenditure. It has been suggested that marasmus

represents the adaptive process of low energy intake whilst the features seen in kwashiorkor represents a failure in adaptation (Grover and Ee, 2009).

The most conspicuous pathological changes are seen in children with kwashiorkor which includes oedema and fatty infiltration of the liver (Doherty et al., 1992). Atrophy of the pancreas and the mucosa of the small intestines is also a common feature in kwashiorkor. This leads to the malabsorption of fat resulting in steatorrhea and fatty infiltration of the liver. Decreased dietary proteins in combination with malabsorption causes decreased plasma protein concentrations leading to fluid retention and oedema (Oshikoya and Senbanjo, 2009, Krishnaswamy et al., 1989).

1.2.5 Biochemical changes in protein energy malnutrition

A number of biochemical changes have been observed in protein energy malnutrition. Malabsorption of different fat fractions leads to decreased plasma levels of cholesterol, triglycerides and fat soluble vitamins and hypernatremia. Serum albumin level is one of the most useful indicators of protein energy malnutrition as it is relatively easy to measure and can be an indicator for the effectiveness of treatment. It might be normal or slightly reduced in marasmus but markedly reduced in kwashiorkor (Shaaban et al., 2005). Low blood glucose level is a common feature in protein energy malnutrition. It is mostly seen in kwashiorkor (Wharton, 1970, Buchanan et al., 1976) but can also occur in marasmus (Das et al., 1998, Kerpel-Fronius and Kaiser, 1967), commonly attributed to decreased glucose absorption,

increased glucose clearance or impaired hepatic endogenous glucose production (EGP) (Das et al., 1998).

Increased intracellular fluid with water and electrolyte imbalance, present in children with PEM, can lead to potassium and magnesium deficiencies. This often leads to reduced serum sodium and osmolarity resulting in simultaneous presence of oedema and signs of malnutrition (Akuyam, 2007).

1.2.6 Micronutrient Deficiencies in Protein Energy Malnutrition

The most common micronutrient deficiencies in children with PEM include iron, vitamin A, zinc and iodine (Grover and Ee, 2009, Bhutta, 2008, Pollitt, 1995, Macdougall et al., 1982). Anaemia is a common feature in PEM with several studies showing high incidence of iron deficiency anaemia in these children (Chen et al., 2009, Van Nhien et al., 2008, Castejon et al., 2004)

1.2.7 Management of protein energy malnutrition

Management of PEM depends on the type and severity. Children with mild and moderate malnutrition can successfully be treated at home or in an outpatient health facility (Groove and Ee, 2009). Cases of severe acute malnutrition present with life threatening conditions and are usually associated with high fatality rates, making a clinical inpatient approach crucial. The most common life threatening conditions include infections, hypoglycaemia and hypothermia, accounting for 26.3, 3.6 and 2.4% respectively (Bernal et al, 2008).

Table 1.7 Guidelines for the treatment of severely malnourished children

Activity	Initial treatment		Rehabilitation	Follow-up
	Day 1-2	Day 3-7	Wk 2-6	Wk 7-26
Treat or prevent - Hypoglycaemia	→			
- Hypothermia	→			
- Dehydration	→			
Correct electrolyte imbalance	→			
Treat infection		→		
Correct micronutrient deficiency	without iron →		with iron →	
Begin cautious feeding	→			
Increase feeding to catch up			→	
Growth Emotional and sensory	→			
Stimulation Prepare for discharge and			→	
Follow-up				

The ten step guidelines for the inpatient treatment of severely malnourished children

WHO (1999) has developed guidelines to help improve the quality of care given to malnourished children in health facilities in a three phase management approach with a recommended time frame of ten essential steps (table 1.9). Quality of care and case fatality rates improved with the use of WHO guidelines in Africa (Ashworth et al., 2004, Deen et al., 2003), Bangladesh (Ahmed et al., 1999) and South America (Bernal et al., 2008, Cavalcante et al.,1998).

1.2.9 Protein Energy Malnutrition and Infection / Immunity

Various studies have demonstrated the association between malnutrition and increased risk of infection and death (Berkley et al., 2005, Norton et al., 2004). Chronic PEM during childhood affects the development of the thymus which reduces peripheral lymphocytes compromising immunity. In addition, there is a decrease in T cell function, cytokine production and the ability of lymphocytes to respond appropriately to cytokines. This affects both innate and acquired immunity and can lead to most children with PEM having opportunistic and asymptomatic infection (Schaible et al., 2007).

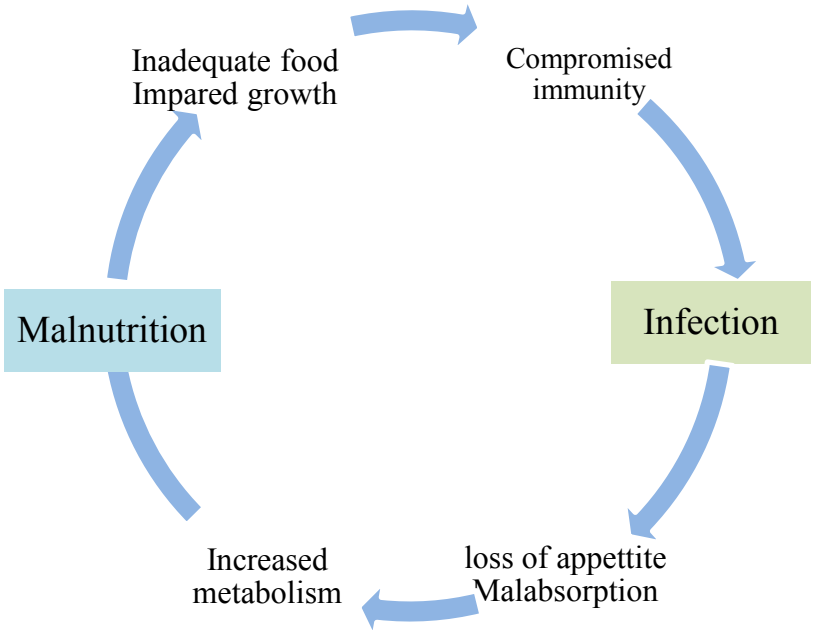
The relationship of PEM and infection is complicated by the effect of infections on nutrition causing a vicious cycle of events (Figure 1.9) Infection can lead to decrease food intake, diarrhoea, malabsorption and diversion of nutrients for immune response leading to impair growth further contributing to malnutrition. In addition, the metabolic demands of repeated infection increase catabolic state leading to further weight loss (Katona and Katona-Apte, 2008, Schaible and Kaufmann, 2007).

More than half of the five leading causes of infectious diseases in children under five years of age, which include pneumonia, diarrheal diseases and malaria are associated with some form of PEM (Katona and Katona-Apte, 2008). A study by Man et al. (1998) in a large population of hospitalised Gambian children showed lower mean admission weights compared to the control population. Malaria was the

leading cause of admission and mortality with death rates for infectious diseases rising with decreasing admission weights (Man et al., 1998).

Although studies have proposed malnutrition to provide protective factor against malarial infection (Genton et al., 1998,) or have seen no association between the two (Ghosh et al., 1995, Snow et al., 1991), more recent studies have demonstrated that chronically malnourished children may be at a higher risk for developing malarial episodes (Deen et al., 2002, Danquah et al., 2009). In addition, children with PEM have double the risk of dying from malarial infection compared to their normally nourished counterpart (Danquah et al., 2009, Muller et al., 2003).

Figure 1.9 Vicious cycle of malnutrition and infection



1.2.10 Effect of PEM on Pharmacokinetics of Drugs in Children

Most of the pathophysiological changes seen in protein energy malnutrition either directly or indirectly affect drug disposition. Various studies have analysed the pharmacokinetics of drugs in children with PEM. Results of the different pharmacokinetic parameters obtained in these studies have been summarised in tables 1.7, 1.8 and 1.9. Various studies have indicated major pharmacokinetic differences in some drugs as a result of PEM (Pussard et al., 1999, Bolme et al., 1995, Ashton et al., 1993, Salako et al., 1989., Eriksson et al., 1983.).

In general, PEM can affect drug absorption, distribution, metabolism and elimination (Oshikoya and Senbanjo, 2009). In addition to vomiting and diarrhoea, malnutrition is also associated with villous atrophy of the jejunal mucosa (Brewster, 2006) which might lead to impaired drug absorption. The oral absorption of chloroquine (Walker et al., 1987) and carbamazepine (Bano et al., 1986) has been reported to decrease significantly in children with PEM compared to normally nourished children which is attributed to the morphological changes in the jejunum (Oshikoya and Senbanjo, 2009).

Total body water increases in proportion to the degree of malnutrition so a significant reduction in adipose mass, often observed in marasmus and marasmic-kwashiorkor, can alter the volume of distribution of drugs (Hansen et al., 1965). Drugs that are protein bound might also be affected by PEM as a result of reduced serum albumen and glycoproteins. As a result, there is an increase in plasma free

drug resulting in either variable drug response or increased risk of drug toxicity. (Krishnaswamy, 1989).

In addition, the CYP450 enzymes are heme-containing proteins and iron deficiency can directly or indirectly affect CYP450 (Pai et al., 2007). As a result of iron deficiency, malnourished children may display altered metabolism due to altered intestinal and hepatic drug biotransformation via the cytochrome P450 enzymes. In a rodent model, PEM reduced total hepatic CYP by 55% with individual CYP enzymes affected by different amounts (Cho et al., 1999). Glucuronidation, which is a major pathway in the metabolism of DHA, is also inhibited in PEM due to a lack of glucose which is critical in the synthesis of glucuronic acid (Hamberg et al., 1990, Aw and Jones, 1984).

Table 1.8 Pharmacokinetic parameter of drugs in children with PEM

Source	Age (months)	Degree of malnutrition	Drug	Dose and Route	Mean CL (ml/min/kg)		Mean $t_{1/2}$ (h)	
					Controls	PEM	Controls	PEM
Pussad et al (1999)	24-72	M	Quinine	6mg/kg IV	4.0±2.1	1.7±1.5*	5.1±2.6	7.2±5.9*
Treluyer et al (1996)	9-60	M, M-K	Quinine	16mg/kg IM	2.3±1.4	4.4±3.6*	10.1±3.4	6.3±1.8*
Bolme et al (1995)	7-78	U	Penicillin	20mg/kg oral			0.65±0.1	0.7±0.1
Bolme et al (1995)	7-78	M	Penicillin	20mg/kg oral				0.9±0.04
	7-78	K	Penicillin	20mg/kg				2.3±0.8
Bolme et al (1995)		U	Penicillin	30mg/kg IV	22.2±0.9	15.1±0.9**	0.7±0.1	1.1±0.55
		M	Penicillin	30mg/kg IV		14.0±0.5**		0.8±0.1
		K	Penicillin	30mg/kg IV		16.9±1.0**		0.8±0.2
Ashton et al (1993)	9-120	U	Chloramphenicol	25mg/kg IV		5.9±1.6		2.4±0.9
Ashton et al (1993)	9-120	M	Chloramphenicol			4.8±1.5		3.7±1.7
		K				2.9±1.5***		4.9±4.6

N – normal, SM – severe malnutrition, M – marasmus, K – kwashiorkor, U – undefined

Source	Age	Degree of malnutrition	Drug	Dose and Route	Mean CL (ml/min/kg)		Mean $t_{1/2}$ (h)	
					Controls	PEM	Controls	PEM
Blome et al (1998)	6-144	U	Streptomycin	20mg/kg IM	79±2	63±20	1.69±0.4	2.4±0.2
		M				83±18		1.77±0.2
		K				51±7		5.36±2.5**
		U	Streptomycin	30mg/kg IM	67±15	74±8	2.6±0.4	2.1±0.3
		M				91±14		2.3±0.2
		K				49±7		9.7±2.6**
Salako et al. (1989)	17-36	K	Quinine	10mg/kg oral	108.5±34.8	31.5±8.5***	8.0±1.3	15.0±4.4***
Eriksson et al (1983)	6-72	M	chloramphenicol	25mg/kg IV	7.5±1.1	8.2±2.3	2.9±0.3	2.9±0.5
		K				4.2±0.7**		3.8±0.4*
		M	chloramphenicol	25mg/kg oral			2.07±0.4	3.2±0.5
		K						3.97±0.9

N – normal, SM – severe malnutrition, M – marasmus, K – kwashiorkor, U - undefined

Table 1.9 Pharmacokinetic parameter of drugs in children with PEM

Source	Age (months)	Degree of malnutrition	Drug	Dose and Route	C_{max} (mg/L)		AUC_{0-24} (mg/h/L)	
					Controls	PEM	Controls	PEM
Pussard et al. (1999)	24 – 72	M	Quinine	6mg/kg / IV	6.6	7.7	40.9	43.0
Lares-Asseff (1997)		U	Cyclosporine	3/mg/kg	387.5	136.1	856.0	1481.4
Tréluyer et al. (1996)	9 - 60	M, M-K	Quinine	16mg/kg	10.7	9.9		
Bolme et al. (1995)		U	Penicillin	20mg/kg / oral			423.4	396.0
		M-K						323.2
		K						319.5
		U	Penicillin	30mg/kg / IV			1351	1993.3
		M						2138.2
		K						1769.2
Ashton et al. (1993)	9 – 120	M	Chloramphenicol	25mg/kg/ IV		11.1	20.4	
		K					39.9	
Lares-Assef et al. (1992)	4 – 43	M	Metronidazole	30mg/kg / oral		9.9	153.7	

N – normal, SM – severe malnutrition, M – marasmus, K – kwashiorkor, U - undefined

Table 1.10 Pharmacokinetic parameter of drugs in children with PEM

Source	Age	Degree of malnutrition	Drug	Dose and Route	Volume of distribution	
					Controls	PEM
Pussad et al (1999)	24-72	M	Quinine	6mg/kg IV	1.6±1.05	0.6±0.3*
Treluyer et al (1996)	9-60	M, MK	Quinine	16mg/kg IM	1.7±0.4	2.0±0.8
Bolme et al (1995)	7-78	U	Penicillin	20mg/kg[a] oral		
Bolme et al (1995)	7-78	M	Penicillin	20mg/kg[a] oral		
Bolme et al (1995)	7-78	K	Penicillin	20mg/kg[a]		
		U	Penicillin	30mg/kg IV	1.39±0.2	1.47±0.6
		M	Penicillin	30mg/kg IV	1.39±0.2	0.91±0.1
		K	Penicillin	30mg/kg IV	1.39±0.2	1.18±0.2
Ashton et al (1993)	9-120	U	Chloramphenicol	25mg/kg IV		1.2±0.5
Ashton et al (1993)	9-120	M	Chloramphenicol			1.3±0.4
		K				0.7±0.3**
Lares-Assef et al (1992)	4-43	SM	metronidazole	30mg/kg oral	1.6±1.0	1.5±0.8
Blome et al (1998)	6-144	U	streptomycin	20mg/kg IM	0.3±0.03	0.3±0.1
		M				0.3±0.02
		K				0.4±0.02*
		U	Streptomycin	30mg/kg IM	0.3±0.02	0.2±0.02

		M				0.3±0.02
		K				0.7±0.1**
Eriksson et al (1983)	6-72	M	chloramphenicol	25mg/kg IV	1.9±0.2	2.1±0.5
						1.33±0.2

N – normal, SM – severe malnutrition, M – marasmus, K – kwashiorkor, U -undefined

1.3 Aims

Artemisinin combination therapy is now the standard treatment for malaria however little is known regarding the pharmacokinetics of the artemisinin compounds in children. With over 45 countries having adopted ACT as the first line therapy, it is expected that millions of doses of these drugs will be administered in the coming years. Despite this massive deployment, relatively limited data exists regarding the PK of these drugs in the most vulnerable population, young children. Studies have shown that children may exhibit distinct PK characteristics (Pussard et al., 1999, Bolme et al., 1995, Ashton et al., 1993, Salako et al., 1989., Eriksson et al., 1983.). that must be considered for optimum dosing to preserve these valuable classes of antimalarial drugs. Accurate dosing strategies are necessary to achieve optimum drug exposure which will minimise risk of treatment failure, drug toxicity and the development of drug resistance.

The effects of malnutrition in children have the potential to greatly affect the pharmacokinetic properties and, therefore, efficacy and safety of ACTs. However, to date, this has not been studied and the complex drug-dependent inter-play among physiological changes in absorption, metabolism and V_d has not been addressed. This is part of a general trend that, despite the high morbidity and mortality of patients with PEM, pharmacokinetic studies of essential drugs used to treat these children have dramatically declined since the 1970 (Cohen-Kohler, 2007). Given the high incidence of malnutrition and PEM in the areas most severely affected by malaria with, therefore, high deployment levels of ACTs, and the susceptibility of

malnourished children to contracting malaria, it is essential that the consequences of malnutrition and specifically PEM on the pharmacological and toxicological activity of the artemisinin drugs are investigated further.

In addition there are almost 20 dietary minerals and trace elements essential for proper functioning of the body among which are iron, zinc and iodine (Stein, 2009). Of particular interest is the low level of iron stores seen in children with PEM predisposing them to a higher risk of developing iron deficiency anaemia (Macdougall et al., 1982). As described, the activation of the endoperoxide group in ARTs by an iron (II) source is essential for both pharmacological and toxicological activity and is also involved in systemic activation by red blood cells. Therefore, the reduction of physiological iron levels could have several pharmacological and toxicological consequences including a decrease in pharmacological activity in infected cells, a decrease in direct cytotoxicity against susceptible cells or a decrease in extracellular detoxification which may in turn lead to increased plasma concentrations that may alter the benefit: risk ratio.

1.3.1 Primary hypothesis

The primary hypothesise was that the pharmacokinetics of artemisinin might differ in malnourished children when compared with children with no evidence of malnutrition.

1.3.2 Secondary hypothesis

The secondary hypothesis was that deferoxamine (DFO), an iron chelator, decreases artesunate toxicity

2 The Mechanism Underlying Protection Against Artesunate Toxicity by The Iron Chelator Deferoxamine

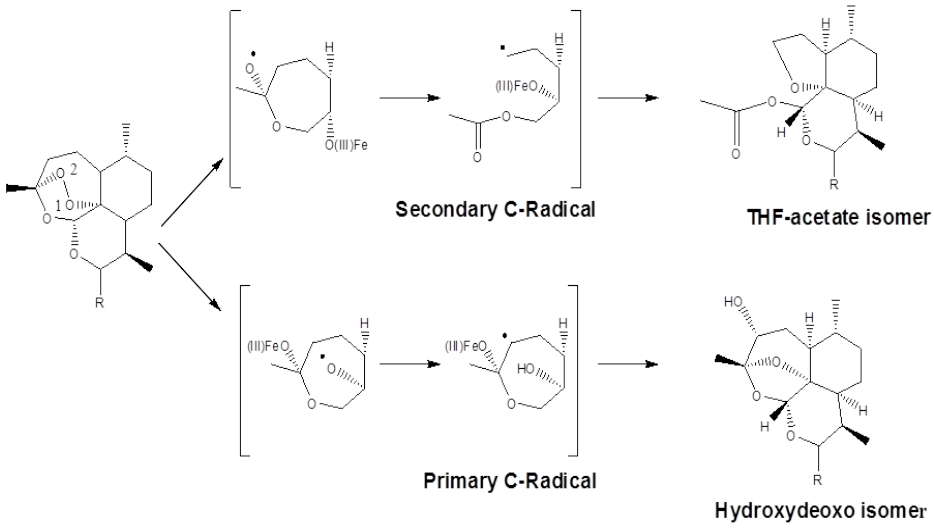
2.1 Introduction

The rapid emergence of resistance to the traditional antimalarials prompted WHO to recommend the use of artemisinin combination therapy as the first line of treatment for uncomplicated malaria (Suh et al. 2004). In addition to its effectiveness in the treatment of drug resistant malaria, it offers rapid malaria parasite clearance and has a high tolerability. Despite no reported serious adverse effects in humans, concerns remain due to reported neurotoxicity and embryotoxicity in animal studies (Boareto et al. 2008; Clark 2009).

Its characteristic endoperoxide bridge is essential for its antimalarial activity as well as its toxicity (Navaratnam et al. 2000). This is reinforced by the fact that analogues without a peroxide oxygen atom do not have antimalarial activity (Meshnick et al, 1996, Beekman et al 1997). The mechanism of action of artemisinin and that of its analogues is still a matter of intense debate but it is widely believed that it is iron (II) or heme mediated, generating reactive oxygen species (Stocks, et al. 2007; Mercer 2009).

Artemisinin binds to low valent transition irons (ferrous heme/non heme, exogenous Fe^{2+}) leading to electron transfer induced reductive scission of the peroxide bridge to produce oxygen centred radicals (Figure 2.1) (Messori et al. 2006). These oxygen centred radicals rearrange to form carbon centred radicals. Because of the asymmetrical nature of the endoperoxide bridge, iron interacts with peroxide in different ways to produce either a primary or secondary carbon centred radicals. The generation of free radicals by the artemisinin compounds may be critical for killing the parasite as is consistent with the importance of the endoperoxide bridge for drug efficacy (Krishna et al., 2008).

Figure 2.1 Proposed mechanism of action of the endoperoxides



A proposed mechanism of action in malaria parasites include the interference with protein export pathways of the malaria tubulovesicular network inhibiting the parasite endocytic pathway thus interfering with mitochondrial membrane potential (Hoppe et al., 2004, Li et al., 2005). The reactive oxygen carbon species may target

essential parasite organelles such as the mitochondria, endoplasmic reticulum and food vacuole causing cell death. Some molecular targets may include inhibition of the parasites sarco/endoplasmic reticulum calcium ATPase known as PfATP6 (SERCA/PfATP6). This was based on the antagonistic nature of thapisgargin towards artemisinin inhibition of PfATP6 (Eckstein-Ludwig 2003, Krishna et al 2010). This alternative mechanism has been challenged due to the fact that insensitivity to artemisinin was observed in purified PfATP6 (Cardi et al, 2010).

The idea that iron is required for the activation of the artemisinin compounds appears to rely in large measure on reports from earlier studies that iron chelators such as deferroxamine B (DFO) antagonize its antimalarial activities in vitro (Meshnick et al., 1993, Wei and Sadrzadeh 1994). The protective role of DFO in the cytotoxicity against the endoperoxides was determined using an MTT assay which measured the activity of cellular dehydrogenase enzyme (Mosmann 1983).

Meshnick et al (1993) showed that iron chelators decrease the toxic effects of artemisinin in mice and that those that were dosed with artemisinin and iron chelator DFO-HES lived considerably longer than those who received equal doses of only artemisinin, especially high dose artemisinin (Meshnick et al., 1993). Stocks et al have shown that both DFO and DFP (both selective for ferric iron) caused antagonism towards the artemisinin compounds suggesting that either bioactivation of the endoperoxide bridge is mediated by a ferric iron source in combination with an endogenous reducing agent or that these chelators alter the equilibrium between iron stores within the parasite (Stocks et al,. 2007). DFO also inhibits parasite protein targets in parasite infected erythrocytes with abrogation of artemisinin induced

inhibition of PfATP6. This resulted in reduction of antimalarial activity by the endoperoxide compounds (Haynes et al. 2007, Uhlemann et al. 2007).

The selectivity of artemisinin compounds to parasites and certain cancer cells is rationalised by their high iron content. The malaria parasite is rich in heme-iron, derived from the proteolysis of host cell haemoglobin. Cancer cells, like malaria parasites, also contain high levels of iron and have demonstrated increased toxicity against their normal cell counterparts (Singh and Lai, 2001, Chen et al., 2009, Hou et al., 2008). Iron is essential for ATP production and DNA synthesis and its uptake by cells from transferrin (Tf) is controlled by transferrin receptor (TfR), which is controlled by intracellular iron levels. TfR levels are normally low in most normal human cells (Nadadur et al., 2008).

The role of heme in the bioactivation of artemisinin compounds has been investigated and has been shown that inhibition of heme synthesis caused a reduction in toxicity with decreased activation of the endoperoxide to the THF acetate biomarker (fig 2.1) whilst an addition of a heme precursor increased toxicity and bioactivation (Mercer 2011, Zhang and Gerhad 2009). THF acetate isomer is a stable metabolite of PFDHA that can be used as a biomarker for bioactivation (Maggs et al., 1997). This has reinforced the fact that free or protein bound heme is responsible for the intracellular activation of the endoperoxides stimulated by the electron transport chain in the mitochondria, leading to a cascade of processes which include the generation of reaction oxygen species, mitochondrial membrane depolarisation, caspase-3 and -7 activation which finally leads to DNA degradation resulting in cell death (Figure 2.2) (Mercer et al, 2011 Wang et al., 2010,). Mercer et al., observed

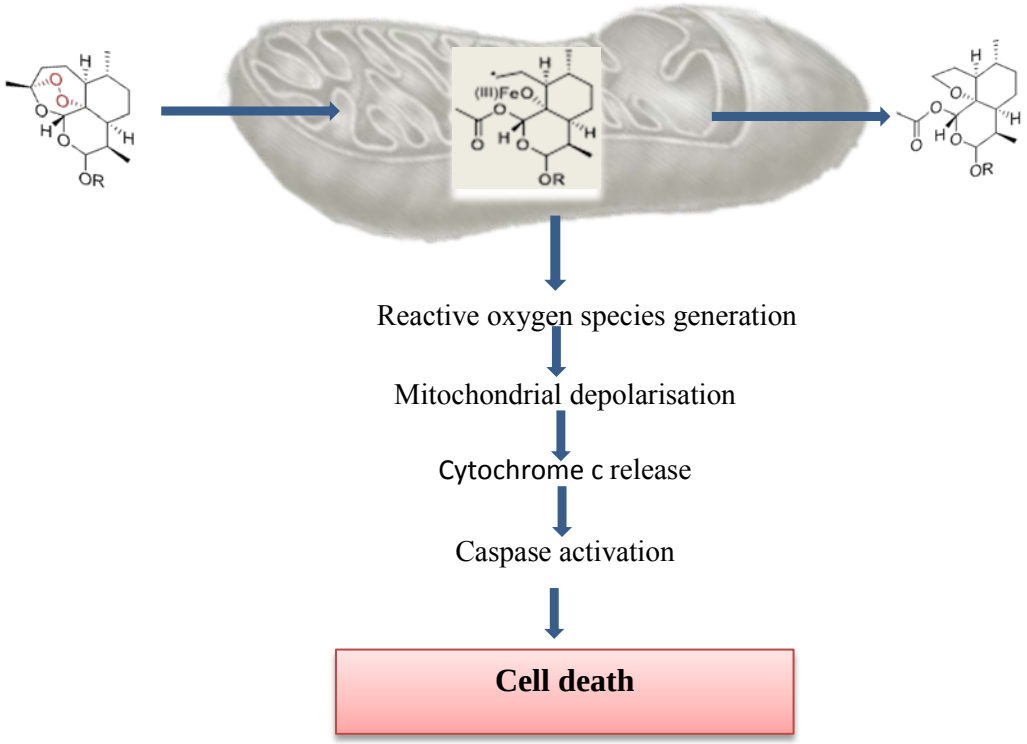
that although the addition of extrinsic iron did not induce cytotoxicity, it did cause bioactivation of endoperoxides suggesting that cytotoxicity can only occur when bioactivation is heme or heme-containing protein mediated (Mercer et al., 2011).

It is the intended aim of this study to identify the position on the artemisinin activation pathway where DFO inhibits. The study will develop an understanding of the mechanism underlying protective effects of iron chelators against artemisinin induced cytotoxicity and thus provide further information on the mechanism of action of the artemisinin compounds. In order to achieve this, artesunate was used in HL60 cells. HL-60 cells are human leukemic cell lines known to be high in iron content and show cytotoxicity with the endoperoxides (Mercer et al., 2007). Artesunate, which is a water soluble semi-synthetic derivative of artemisinin, was used. Deferoxamine, the iron chelator used in this study, is a siderophore with high affinity for ferric (Fe^{3+}) and stored iron in cells. DFO binds to Fe^{3+} and forms a stable complex which is redox inactive thus preventing the generation of reactive oxygen species (Dayani et al., 2004).

Fe (II)-heme is freely oxidized to Fe (III)-heme by deoxygenation. The Fe (III) produced is readily reduced to Fe (II) generating reduced oxygen species in a catalytic fashion (kalinowski and Richardson, 2005). A significant production of ROS might change membrane potential and the electron transport chain of the mitochondria in the parasite (Meunier and Robert, 2010). DFO prevents the formation of reaction oxygen species by inhibiting the catalytic role of iron in the fenton reaction (Arora and Gores, 1996). ROS can react with lipids to induce lipid peroxidation process, resulting in the formation of malondialdehyde, a by-product of

lipid peroxidation. In-vitro studies have shown that DFO inhibits the production or reactive oxygen species.

Figure 2.2 Proposed mechanism of cell death of the artemisinin compounds



A pool of chelatable iron can accumulate in the mitochondria which can cause increased ROS exacerbating oxygen damage (Thomas et al., 2009). It will be imperative to investigate the effect of artesunate treatment on intra-cellular heme and iron content in HL-60 cells. This will be done by measuring intra-cellular heme and iron content in HL-60 cells treated with artesunate and pre-treatment with DFO.

Apoptosis is known to precede necrosis via a controlled biochemical pathway. Mitochondrial depolarisation is an early occurrence in the intrinsic apoptotic pathway whereas activation of the caspase cascade occurs later in the pathway and plays a vital role in the apoptotic process. The mitochondrial membrane potential will be measured using Tetramethylrhodamine ethyl ester (TMRE). Western blot will be used to investigate the activation of caspase-3 and whether pre-treatment with DFO will have any effect. It is one of the caspases that activate destructive enzymes and is activated by both the extrinsic and intrinsic pathways (Slee et al., 2001).

2.2 Materials and Methods

2.2.1 Materials

The human cell lines HL-60 cells were obtained from the European Collection of Cell Cultures (Salisbury, UK). RPMI-1640 culture media, L-glutamine, Hank's balanced salt solution (HBSS), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethylsulphoxide (DMSO) and trypan blue solution (0.4 %) solution, Tween-20 and non-fat dried milk were all purchased from Sigma Aldrich (UK). Foetal bovine serum was from Bio Whittaker. Lymphoprep was from Nycomed (Birmingham, UK). Polyacrylamide gel was purchased from National Diagnostics (Yorkshire, UK). Hybond-C extra nitrocellulose membrane, Hyperfilm ECL photographic film and ECL detection reagents were purchased from Amersham (Bucks, UK). Caspase-3 and caspase-7 rabbit polyclonal antibody were kindly donated by Prof. G. Cohen (MRC Toxicology Unit, Leicester). Goat anti-

rabbit IgG secondary antibody conjugated to HRP was purchased from DakoCytomation (Cambs, UK). BCA reagent was purchased from Sigma. Artesunate was kindly donated by Dafra Pharma International (Belgium). PFDHA and the THF-acetate isomer of PFDHA was synthesised in the Department of Chemistry (University of Liverpool).

2.2.2 Cell culture

HL-60 cells were maintained in RPMI 1640 medium which was supplemented with FBS (10% v/v) and L-glutamine (1% w/v). On reaching a density of 1 x 10^6 cells/ml in a 75 cm^2 flask, cells (2 x 10^6) were seeded in 30 ml of freshly supplemented media. The cells were incubated at 37 °C under humidified air containing 5% CO_2. Cell growth was maintained below 1 x 10^6 cells/ml. This was done to ensure exponential growth and avoid differentiation of the cells.

Cell viability was maintained above 95 % for all experiments. The viable cell count was based on trypan blue exclusion from the cells performed in a haemocytometer using a light microscope (x 10; Zeiss Axioskop, Welwyn Garden City, UK). To 90µl of HL-60 cells, 10µl of trypan blue 0.4% solution was added and an aliquot was counted. Stock solutions of the drugs were made up in DMSO and the final solvent concentration was below 0.5% (v/v) for each incubation. Every concentration in each experiment was carried out in triplicate and the experiments were all repeated on at least three separate occasions.

2.2.3 Measurement of Cytotoxicity Using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium Bromide (MTT) Assay

The MTT assay is based on the ability of dehydrogenase enzymes within viable cell lines to form an insoluble formazan salt (Mosmann, 1983). The amount of formazan present is directly proportional to the number of viable cells. HL60 cells (5 x 10^5) were plated in triplicate, in flat-bottomed 96-well plates and exposed to 0.01 μM to 100 μM of artesunate and incubated for 24 h. After the 24 h incubation, 20 μL of MTT solution (5 mg/ml in HBSS) was added to each well. The plate was incubated for 2 h at 37 °C. 100 μL of lysis buffer (20 % w/v sodium dodecyl sulphate; 50 % v/v n-dimethylformamide) was added to each well, to dissolve the formazan crystals, and was incubated overnight. The absorbance of the wells was read using a test wavelength of 570 nm and a reference wavelength of 590 nm with a plate reader (MRX, Dynatech Laboratories). The results were expressed as a percentage of vehicle-only treated cells. The IC_{50} was estimated from individual inhibition curves plotted by GraFit software (Erithacus, West Sussex, UK).

2.2.4 Determination of the protein content of cellular samples

The protein content of the cellular samples was assayed using BCA protein assay. The BCA Protein Assay combines the protein-induced biuret reaction with the highly sensitive and selective colorimetric detection of the resulting cuprous cation (Cu^{1+}) by bicinchoninic acid (BCA). A standard curve of 0 – 18 μg BCA was prepared using a stock solution of 2mg/ml BCA. Samples were analysed by spectrophotometric analysis (MRX microplate reader, Dynatech Laboratories)

2.2.5 Analysis by Flow Cytometer

A flow cytometer (Canto, BD Biosciences Beckman Coulter, Oxford, U.K) was used to analyse the cells. Parameters were set using control cells. The forward scatter (FS) and side scatter (SS) were adjusted to obtain a population of viable cells with a voltage threshold set to exclude cell debris. FS determines cell size and SS determines granularity. At least 5000 cells were analysed in all experiments.

2.2.6 LC-MS/MS Analysis and Quantification of Intracellular Endoperoxide Bioactivation

Intracellular activation of the endoperoxides was monitored by LC-MS/MS. The instrument was an API 2000 triple-quadrupole mass spectrometer (AB Sciex, Warrington, UK) interfaced to a PerkinElmer Series 200 autosampler and a PerkinElmer pump. The data were collected and analysed by the Analyst 1.3 software (AB Sciex). Cells (4 ml of 1×10^6 HL-60 cells/ml) were either incubated with PFDHA alone or pretreated with DFO (10μM) at 37 °C. Following 24 h incubation, artesunate (1nmol) was added as an internal standard before the samples were prepared.

Cells were extracted with chloroform (4ml, three times). Anhydrous magnesium sulphate was added to the extracts to dry. The mixture was then filtered through a glass funnel to remove the magnesium sulphate and cell debris. The organic phase was dried under nitrogen gas at 37°C. The residue was dissolved with 50% methanol immediately before analysis by LC-MS/MS multiple reaction monitoring (MRM). Chromatographic separation was achieved on an Agilent

ZORBAX Eclipse XDB-C8 column (150 × 3.9 mm inner diameter, 5 μm; Agilent Technologies, Santa Clara, CA). The mobile phase consisted of methanol with 10 mM aqueous ammonium acetate (70:30, v/v) delivered at a flow rate of 0.4 ml/min. The mass spectrometer was operated in positive ion mode. The operating parameters were optimized via the quantitative optimization facility in Analyst software as follows: ion spray voltage of +5.0 kV, back pressures for the collision gas of 2 p.s.i., curtain gas of 20 p.s.i., nebulizer gas (GS1) of 30 p.s.i., and turbo gas (GS2) of 65 p.s.i.; the turbo gas temperature was 300 °C. Analyte-specific parameters and fragmentation transitions are detailed in table 2.1. All of the gases used were nitrogen. Calibration curves of peak area versus analyte mass (5–5000 pmol) were generated from solutions of synthetic PFDHA, PFDHA THF acetate, and artesunate in methanol, and the limit of quantification was calculated to be 50 pmol using the method of least squares line fit. The efficiency of PFDHA and PFDHA THF acetate recovery was corrected for by the quantification of the internal standard, artesunate.

Table 2.1 Analyte specific parameters and precursor ions for artesunate, PFDHA and PFDHA THF acetate

	DP	EP	CE	CxP
PFDHA, 396.0/163.0	6	8	17	10
PFDHA THF-acetate, 396/266.7	21	5	30	14
Artesunate, 401.1/163.1	21	12	30	26

DP is declustering potential, EP is entrance potential, CE is collision energy, CxP is collision exit potential

2.2.7 Determination of oxidative stress in HL60 cells

In order to measure artesunate induced ROS production in HL-60 cells, 2',7'-dichlorodihydrofluorescein diactetate (DCFH-DA) was used. DCFH-DA is a cell permeable flurogenic dye. It diffuses into cells and is converted to a non-fluorescent dye (DCFH) by cellular esterases. DCFH is then rapidly oxidised to a highly fluorescent 2',7'-dichlorodihydrofluorescein (DCF) by intracellular ROS and other peroxides. The fluorescence intensity is proportional to the ROS levels in the cells. DCFH-DA is not specific but is sensitive to various ROS and reactive nitrogen species (RNS) (Rastogi et al., 2010). HL60 cells (0.5×10^6) were first washed in serum free media before DCFH-DA (5 μM) was added and incubated for 45 min. Cells were then washed again and treated with artesunate (0 – 100 μM) alone or pre-treated with DFO (10 μM, 10 min). After the desired incubation time, cells were washed and resuspended in HBSS. Duplicate samples were prepared without the addition of DCFH-DA for all incubations to correct for the intrinsic fluorescence of the drugs. Samples were analysed by flow cytometry (Section 2.2.6)

2.2.8 Lipid peroxidation assay

Following incubation of HL-60 cells (10×10^6 cells) with artesunate alone or pre-treated with DFO (10 μM, 10 min), samples were prepared for lipid peroxidation assay. Cells were resuspended in ice cold HBSS (200μl) and sonicated to disrupt the cell membrane and release the cellular components. Ice cold TCA (200 μl, 10% w/v) was added to the cell lysates, vortexed and incubated for (5 min) on ice. Samples were then centrifuged (14,000 rpm, 5 min) and the supernatant were used for the

assay. Standards were made from 500 nM MDA stock solution (0 – 1.5 μM) and treated the same as the samples. TBA solution (200 μl, 0.67% w/v) was added to both samples and standards and incubated (100 °C). After 60 min incubation, samples (100 μl) were loaded on a black plate in duplicate and read on a plate reader (530 nm excitation and 550 nm emission). Values were normalised to protein content, determined by the BCA assay.

2.2.9 Western Blot analysis Caspases-3 processing

Cell lysate samples were prepared by washing incubated cells (2 x 10^6) with PBS (4 mls) and resuspended in RIPA buffer (30 μl) and vortexed to lyse the cells. The samples were than assayed for protein content using a BCA protein assay. The protein (20 μg in SDS loading buffer) was denatured at 95 °C for 3 min. It was then loaded on to 14 % SDS polyacrylamide gels. The gel was run initially at a voltage of 70V for 10 min (until samples pass stacking gel) and then 170V for 1 h.

Following separation of the protein complexes, the gel was placed onto a filter paper and nitrocellulose membrane was placed on top of it followed by another filter paper. They were then sandwiched between sponges and loaded into a transfer cassette (Bio-rad, U.K.). The samples were transferred onto the nitrocellulose membrane (230amps, 1 h in transfer buffer). Following protein transfer, the membrane was stained with Ponceau red to ensure equal protein loading and transfer of cellular extracts, and briefly washed with TBS(T). Membranes were then blocked (10 % w/v non-fat dried milk in TBS(T) for 1 h) and washed (TBS(T), 5 min) before incubating with rabbit serum antibody (1: 1000 dilution, 2 % milk in TBS(T)) and

left overnight to recognise the caspase-3 proform (p32) and the p19 subunits. Following this, the membranes were washed with TBS(T) and protein detection was attained using anti-rabbit antibody (secondary antibody) (1:5000 dilution, 2% milk in TBS-T). The membrane was blocked in mouse antibody (1:10,000 dilution, 2% milk in TBS(T)) for ten minutes and then in anti-mouse antibody (1:10,000 dilution,2% milk in TBS(T)) for 1 h. This was followed by two quick washes of both membranes and three consecutive washes (TBS-T, 5 min x 3). Blotting was also done for actin. The protein-antibody conjugate was visualised using ECL Western blotting detection reagents. This was followed by the exposure of membranes to photographic film and band volumes quantified by UVI band software (UVI Tech, Cambridge, U.K.)

2.2.10 Determination of Mitochondrial Depolarisation with TMRE

TMRE (tetramethylrhodamine, ethyl ester) is a fluorescent compound that accumulates in the mitochondrial matrix of live cells. The amount of fluorescence can be measured by flow cytometry and the accumulation of TMRE is proportional to the mitochondrial membrane potential of cells (Scaduto et al., 1999). Cells (5 x 10^5 cells) were treated with desired drugs. After 24 h incubation, they were centrifuged (1400 rpm, 5 min). The cell pellets were washed in HBSS and resuspended in 500 µl of TMRE solution (50 nM in HBSS) and incubated in a 37 °C water bath for 10 min. A minimum of 5000 cells were analysed by flow cytometry (section 2.2.6). TMRE fluorescence was measured on fluorescence channel, FL-3. The data was analysed using cyflogic.

2.2.11 Determination of Cellular Heme Content

The method was based on the protocol of Sassa (1976). An aqueous solution of oxalic acid (2 M, 500 µL) was added to a pellet of cells (1×10^5) treated with artesunate (1 – 100 µM) only or pre-treated with DFO (10 µM, 10 min) for 24h. The samples were shaken before heating (100 °C, 30 min). Standard solutions of hemin (0.01–10 mM) were prepared (water/methanol, MeOH 1:1, v/v containing 1% bovine serum albumin) and heated with oxalic acid, as above. The samples and standard solutions (200 µl) were plated onto a white 96-well plate, and the fluorescence of the deferrated heme was measured (excitation, 400 nm; emission, 662 nm). The results were corrected for non-heme endogenous porphyrins by preparing cell blanks in oxalic acid without heating.

2.2.12 Cellular iron content assay

The ferrozine based colorimetric assay by Reiner et al. (2004) was validated for HL-60 cells. The cells (2×10^6) were incubated with artesunate alone or pre-treated with DFO (10 µM, 10 min) for 24 h. Cell pellets were lysed with NaOH (50 mM, 200 µl) and shaken in a humidified atmosphere (2 h, room temperature). HCL (10 mM, 100 µl) was added to cell lysates (100 µl). HCL (10 mM) solution was also used as the iron standard $FeCl_3$ solvent (0 – 300 µM). Iron releasing agent (freshly made solution of 1.4M HCL and 4.5 % (w/v) $KMnO_4$ in H_2O, 100 µl,) was added to both sample and standard solutions. The mixtures were incubated in a fume hood (2 h, 60 °C). The HCL/$KMnO_4$ pre-treatment releases iron from iron-storage protein and heme proteins (Riemer et al., 2004). The mixtures were then cooled at room

temperature and incubated with iron detection reagent (6.5mM ferrozine, 6.5mM neocuproine, 2.5M ammonium acetate and 1M ascorbic acid dissolved in water, 30 µl). After incubation (30 min), the solution (280 µl) was transferred in 96 well plates and read with a microplate reader at 550nm absorbance. The iron content of the cells were calculated from standard concentrations and normalised for protein content.

2.2.13 Statistical analysis

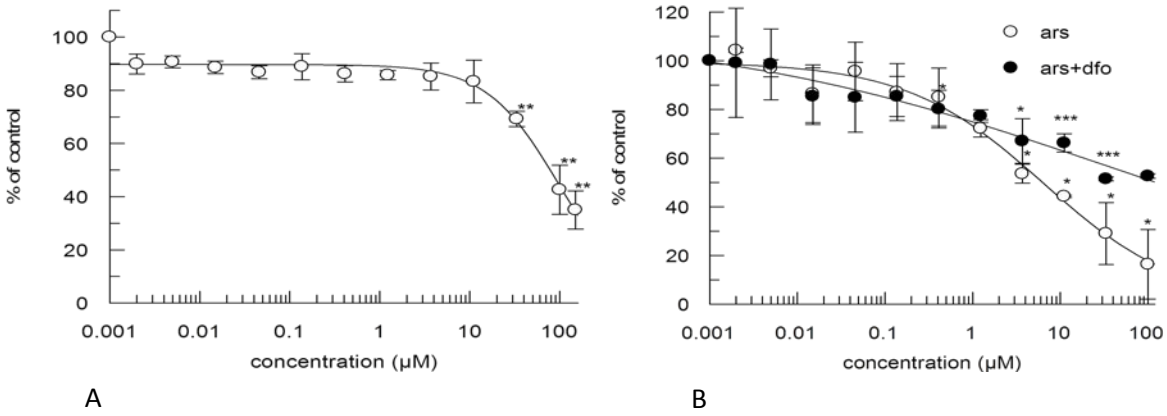
Values are expressed as a mean ± standard deviation (SD), represented as error bars on graphs. Data were analysed for non-normality using a Shapiro-Wilk test. Student's t-test was used when data were normally distributed. A Mann-Whitney U test was used for non-parametric data. All calculations were performed using Stats Direct statistical software. Results were considered to be significant when two-sided P-values were less than 0.05. Significance is indicated as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

2.3 Results

2.3.1 DFO pre-treatment decreases artesunate induced toxicity

The effect of DFO on HL 60 cell viability was first assessed. This was to ensure that the right concentration of DFO was used in order to avoid any cytotoxic effect on the cells when incubated with artesunate (Figure 2.3A). It was observed that DFO on its own did not influence HL 60 cell viability at low concentrations but reduces cell viability at high concentration (100 µM) with an IC_{50} of 96.6 ± 5.3 (Table 2.2). 10 µM of DFO was chosen as the concentration for the experiments.

Figure 2.3 Dose response curve of HL 60 cells treated with artesunate and DFO



24 h A: DFO. B: Artesunate and Artesunate with DFO. Results are the mean ± SD of three independent sets of experiment. * P < 0.05, ** P < 0.01, *** P < 0.001, significance of treated HL 60 data compared to blank. ++ P < 0.01, significance of artesunate alone compared to artesunate with DFO data tested by Mann – Whitney U test

Table 2.2 IC_{50} values of artesunate

Drugs	IC50 (μM)
DFO	96.61 ± 5.3
ART	6.96 ± 4.3
ART+DFO	33.35 ± 2.9

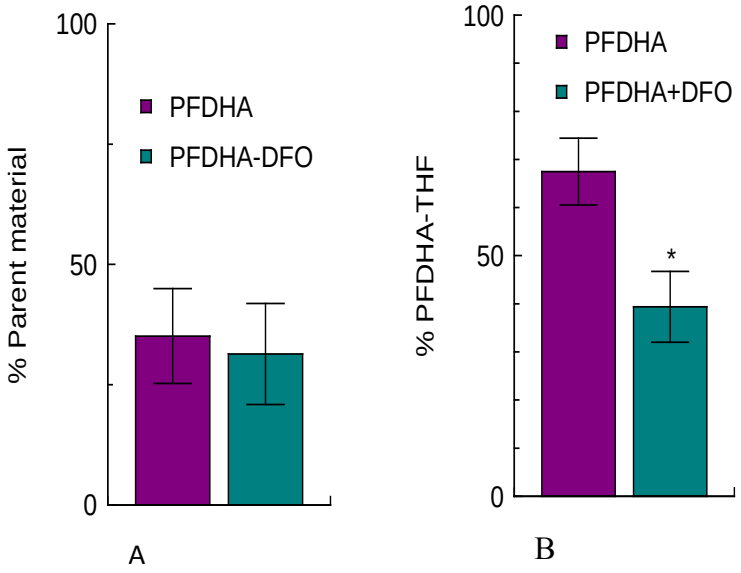
IC_{50} values IC_{50} values ± S.D, n = 3. Results are the mean of three independent sets of experiments

HL60 cells were incubated with artesunate (0 – 100 μM) for 24hr. The result obtained showed significant reduction in HL 60 cell viability (Figure 2.3B). This was shown to be dose dependent manner (IC_{50} – 6.96 ± 4.3 uM). The cytotoxic effects were blocked by the addition of DFO (10 μM) with a 20 % increase in cell viability (Table 3.2). DFO had little effect on cell viability at lower concentrations of artesunate but enhanced cell survival at high concentrations of artesunate. The

difference was significant at higher concentrations of artesunate (10 – 100 μM) $p<0.001$.

2.3.2 Effect of DFO on endoperoxide bioactivation

Figure 2.4 Effect of DFO on PFDHA bioactivation



%	Parent Cmp	THF
PFDHA	35.12	67.46
PFDHA+DFO	31.40	39.38 (p = 0.01)

A. PFDHA B THF acetate. Results are the mean ± SD of three independent sets of experiments. * P < 0.01significance of data tested by Mann – Whitney U test.

The effect of DFO on the intracellular bioactivation of the endoperoxide bridge in HL 60 cells was measured using PFDHA (1 μM) and DFO (10 μM) pre-treatment. PFDHA underwent bioactivation (Figure 2.4A) which is shown by a significant decrease in the parent compound (64.8 ± 3.54 %) and the formation of the THF biomarker (fig 2.4B) (67.5 ± 5.62 %). The addition of DFO (Figure 2.4B) did

not have an impact on the bioactivation of the parent compound (68.6 ± 3.59 %) but significantly decreased the THF acetate biomarker (39.4 ± 3.79 %).

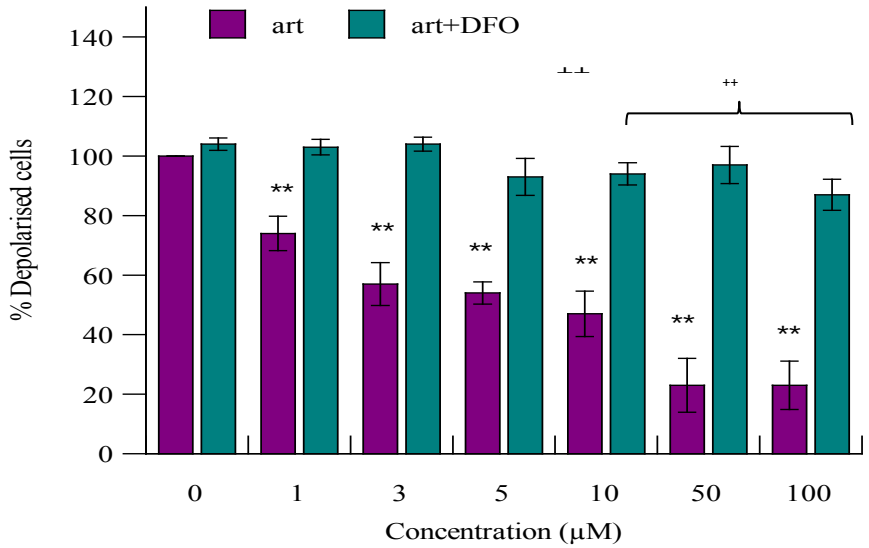
2.3.3 Mitochondrial membrane depolarisation

The role of mitochondria and the effect of DFO were investigated by examining the mitochondrial membrane depolarization potential which was measured by flow cytometry (Figure 2.5). Tetramethylrhodamine ethyl ester (TMRE) was used to label cells with increased mitochondrial membrane potential. Artesunate induced dose-dependent mitochondrial membrane depolarisation in HL 60 cells reaching a maximum of 77 ± 8.1 (100 μM) of cells with depolarised MMP. The addition of DFO (10 μM) was seen to significantly reduce MMP reaching a maximum of only 22 ± 9.3 % cells with depolarised mitochondria at 100 uM artesunate.

2.3.4 The measurement of Caspase-3 activation

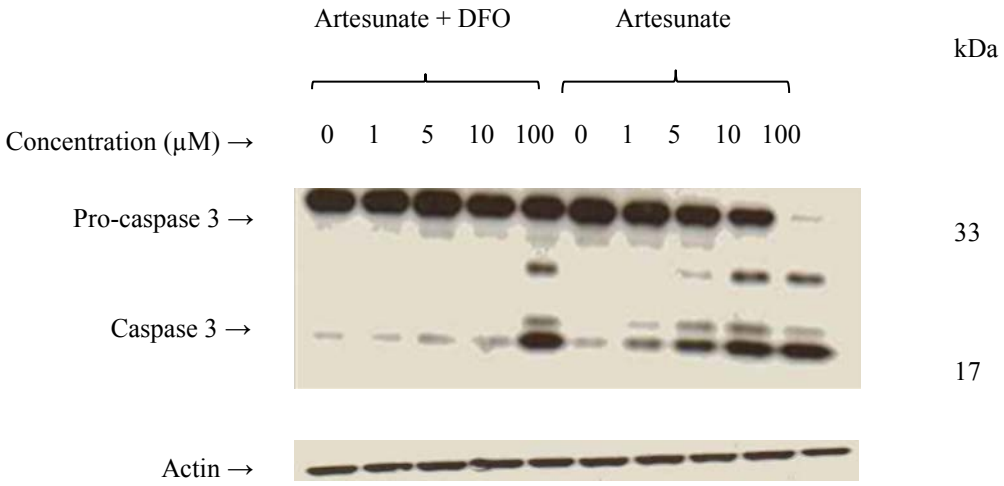
Western Blot was used to measure the effect of DFO upon caspase-3 processing of artesunate (0 – 100 μM) in HL 60 cells. Representative gel showing concentration dependent caspase-3 processing is shown in Figure 2.6. The activation of caspase-3 resulted in a decrease in the amount of intact 33 kDa preform (p33) and the formation of the catalytically active subunit of 17kDa fragment (p17) (Anuradha et al., 2000). Artesunate induced caspase 3 processing in a dose-dependent manner. Pre-treatment with DFO (10 μM) has shown to reduce the processing of caspase 3. The formation of the active form (p17) was inhibited by DFO but was only seen in DFO pre-treated cells at high artesunate concentration (100 μM)

Figure 2.5 Effect of DFO on artesunate induced mitochondrial depolarisation



HL 60 cells treated with artesunate and DFO (10 μM) and the amount of depolarisation assessed with TMRE. Results are the mean ± S.D of three independent sets of experiment. ** $P < 0.01$ significance of data compared to the control. ++ $P < 0.01$ significance of artesunate alone compared to artesunate with DFO data tested by Mann – Whitney U test

Figure 2.6 Western blot of caspase-3 processing in HL 60 cells

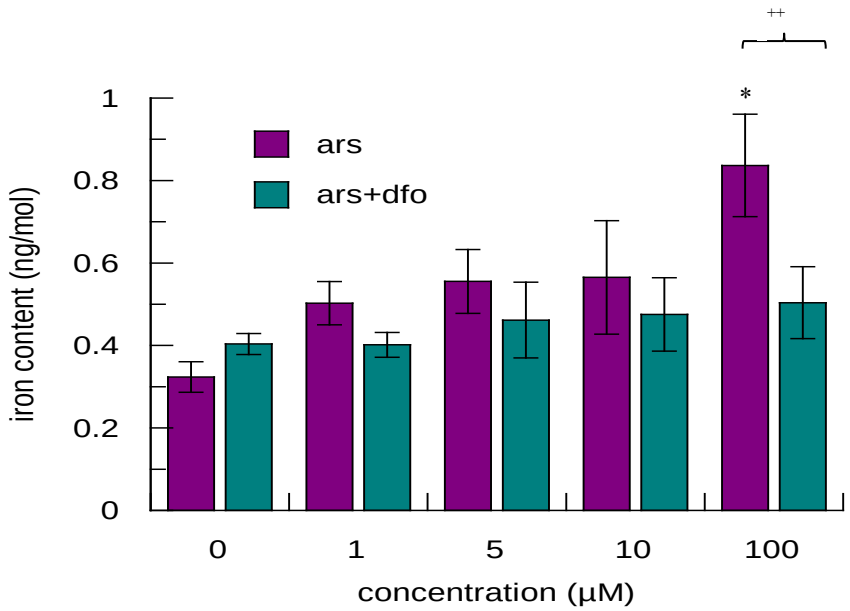


Representative of a western blot gel of dose dependent caspase-3 processing. HL 60 cells were treated with artesunate and pre-treated with DFO (10 μM).

2.3.5 The effects of artesunate on intracellular iron content

The colorimetric feroxine based assay measures all cellular iron including protein bound iron (Reiner et al., 2004). This study demonstrated that artesunate caused a significant dose dependent increase in intracellular iron especially at high concentrations showing almost a 3 fold increase at 100 µM compared to the control. Intracellular iron content increased at concentrations as low as 1 µM. With the addition of DFO, the increase in intracellular iron content induced by artesunate was reduced significantly (Figure 2.7). HL 60 cells pre-treated with DFO (10 µM) did not show a dose-dependent increase in iron-content with increasing concentrations of artesunate (0.4 – 0.5 ng/mol)

Figure 2.7 Effect of artesunate and DFO on intracellular iron content



Results are the mean ± S.D of three independent sets of experiment. * $P < 0.05$, ** $P < 0.01$, significance of data compared to the control. ++ $P < 0.0$, significance of artesunate alone compared to artesunate with DFO data tested by Mann – Whitney U test.

2.3.6 Effect of DFO on intracellular heme content of artesunate treated HL-60 cells

The role of heme in the bioactivation of artemisinin compounds has been strongly suggested. The effect of artesunate in the intracellular heme content in HL 60 cells was analysed by flurometric detection (Table 2.3). Artesunate treatment (0 – 100 μM) did not have any effect on intracellular heme in HL 60 cells. Succinylacetone (SA) was added as a positive control to confirm the validity of the test. SA is a potent permanent inhibitor of heme biosynthesis (Bourque et al., 2010). When HL 60 cells were treated with DFO (10 μM), a decrease in heme content was observed at high concentrations of artesunate (10 – 100 μM) although it was not significant.

Table 2.3 Intracellular heme content in HL 60 cells

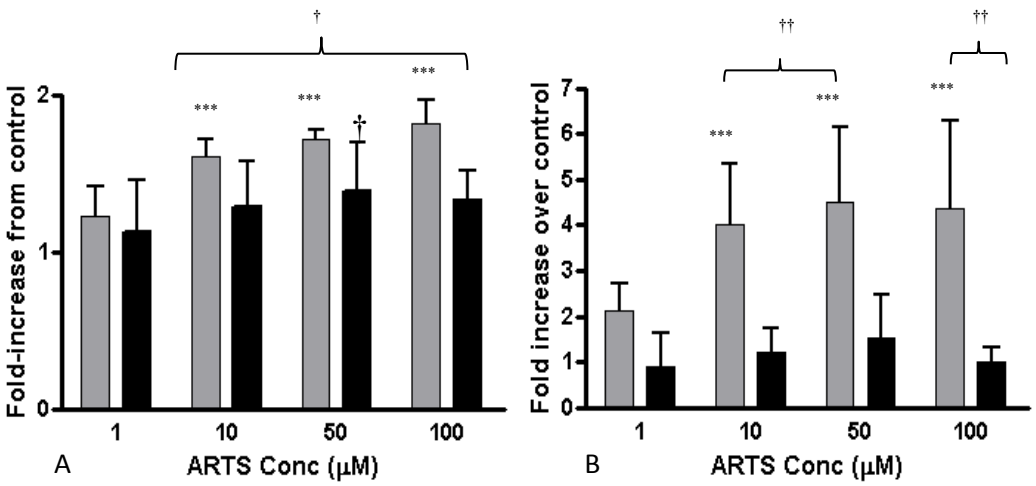
	Artesunate		**DFO**	
Conc. μmol	Heme content ($ng/10^6$ well)	P value	Heme content ($ng/10^6$ well)	P value
0	14.83±6.6		14.04 ±0.95	0.1
3	14.43 ±2.77	0.8	14.61 ±0.6	0.6
10	14.82 ±1.01	0.2	11.56 ±2.4	0.1
100	14.32 ±1.7	0.7	11.84 ±0.9	0.3
SA	0.6± 3.16	0.008^{**}		

HL 60 cells treated with artesunate and DFO (10 μM) Results are the mean ± S.D of three independent sets of experiments. ** P <0.01 significance of the positive control compared to the vehicle control. SA used as +ve control.

2.3.7 Measurement of levels of reactive oxygen species (ROS)

2.3.7.1 Artesunate induced the formation of ROS measured by DCFH-DA

Figure 2.8 Effect of DFO on artesunate induced reactive oxygen species



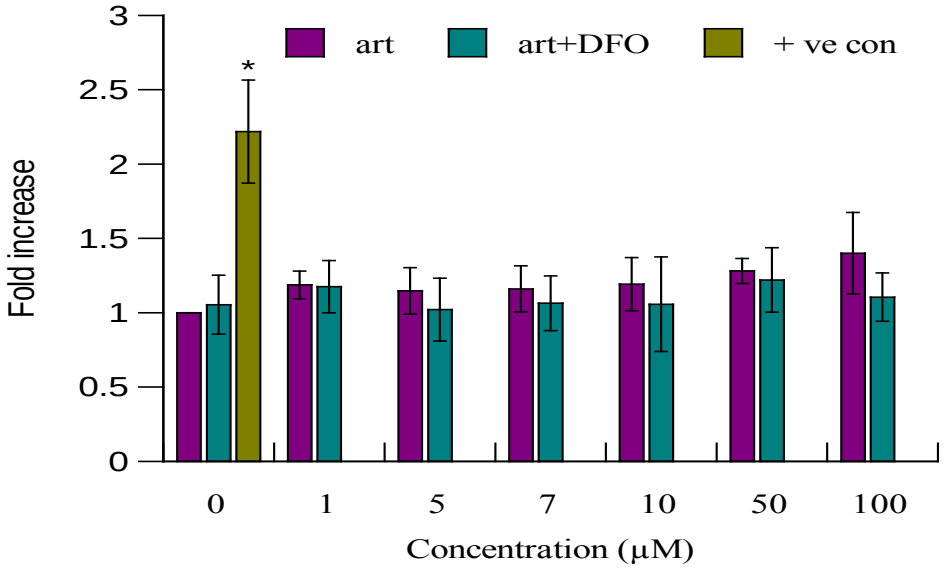
A: 16 hr**.** B: 24 hr. HL-60 cells treated with artesunate alone or pre-treated with DFO (10μM). Results are mean ± S.D of three independent sets of experiments. *** $P < 0.001$ significance of data compared to control. ++ $P < 0.01$, significance of artesunate alone compared to artesunate with DFO data tested by Mann – Whitney U test.

The generation of reactive oxygen species is one of the mechanisms proposed in artemisinin toxicity. DCFH-DA dye was used to measure the amount of ROS generated by artesunate. ROS production started at 16 h artesunate incubation in a dose dependent manner (Figure 2.8A), reaching 1.98 ± 0.52 fold increase at 100 μM concentration. ROS production peaked at 50 μM (24 h) artesunate concentration (2.6 ± 0.47 fold increase) and started decreasing at higher concentrations (100 μM). ROS generation was reduced when HL-60 cells were pre-treated with DFO (10 μM). Reduction of ROS by DFO was significant at 16 h incubation but more significant

reduction levels were observed at 24 h incubation starting at from 10 μM artesunate concentration (Figure 28B).

2.3.8 Lipid peroxidation assay

Figure 2.9 Effect of DFO on artesunate induced lipid peroxidation



HL 60 cells treated alone or pre-treated with DFO (10 μM).Results are mean ± S.D of three sets of independent experiments.

To determine the extent of oxidative damage in HL-60 cells caused by artesunate, lipid peroxidation assay was performed. This assay measures malondialdehyde, a by-product of lipid peroxidation. The results obtained showed no significant increase in malondialdehyde in HL-60 cells after 16 h incubation with artesunate (Figure 2.9). There was slight increase seen at 100 μM artesunate concentration but it was not statistically significant (0.4 fold increase). The addition of DFO did not significantly alter the levels of malondialdehyde product in HL-60

cells. These results demonstrate that artesunate might not have an impact on lipid peroxidation.

2.4 Discussion

The cytotoxic mechanism of action of the artemisinin antimalarials has been controversial but the involvement of heme has been demonstrated in previous studies (Mercer et al., 2011, Zang et al., 2009, Messori et al., 2006). Iron chelators such as DFO have been used both *in vivo* and *in vitro* to both probe the involvement of iron in artemisinin activation and toxicity.

It is hypothesised that DFO functions to chelate iron needed for the bioactivation of the artemisinin compounds thus inhibiting their bioactivation and subsequent toxicity (Meshnick et al., 1993), however the chemistry underlying this hypothesis has not been measured. The aim of this research was to define the mechanisms underlying the protective mechanism against artemisinin induced toxicity by the iron chelator, DFO. Pre-treatment with DFO protected HL-60 cells against artesunate toxicity when analysed by cell the viability assay. The IC_{50} value increased from 6.96 to 33.35.

The ability of DFO to decrease artemisinin toxicity has also been documented in various studies (Uhlemann et al., 2007, Haynes et al., 2007, Stocks et al., 2007, Meshnick et al., 1993). These results are in line with other studies involving the endoperoxides in HL-60 cells (Lu et al, 2008) and other cell lines including parasite infected human red blood cells (Stocks et al., 2007). Although these results are

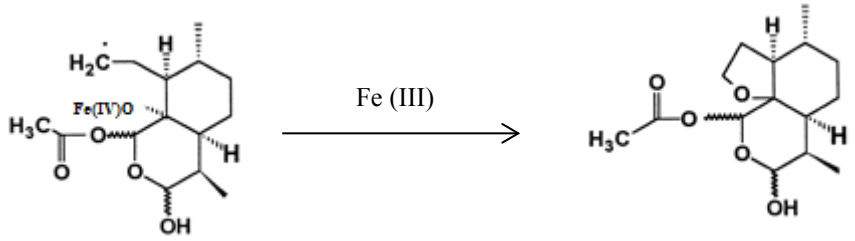
important, they have not defined whether DFO actually inhibits the bioactivation of the artemisinin antimalarials or inhibits cytotoxicity through another mechanism.

Due to the proposed iron-dependent bioactivation by the Meshnick group, it was believed that DFO might inhibit artemisinin induced toxicity by chelating iron needed to initiate the bioactivation process. With this in mind, quantitative LC-MS/MS analyses of PFDHA and heme content assay were performed. PFDHA undergoes bioactivation to form THF acetate which is a stable biomarker of carbon-centred radical production. THF-acetate is formed when c-centred radicals arrange in the absence of biomolecular targets (Mercer, 2009). Bioactivation of the endoperoxide group was studied by Mercer et al using different modulators of heme synthesis. They demonstrated that cellular heme was important for the bioactivation of the endoperoxides. The heme biosynthesis inhibitor, succinyl acetate (SA), decreased cellular heme content resulting in decreased bioactivation of the endoperoxide and reduced toxicity. The addition of a heme precursor (PPIX) had the opposite effect and showed an increase in cellular heme content with increased endoperoxide bioactivation and cytotoxicity. The addition of HTF, which is an extrinsic source of iron, caused a significant increase in the bioactivation of PFDHA but did not necessarily translate to increased toxicity (Mercer et al., 2011).

In this study, we have seen that the addition of DFO did not have much effect on the bioactivation of the endoperoxide but it is important to note the significant decrease in the THF acetate biomarker. The absence of an effect on bioactivation might be due to the fact that the chelating activity of DFO is almost limited to ferric iron (Fe^{3+}), and not ferrous iron (Fe^{2+}), which is essential for the activation of

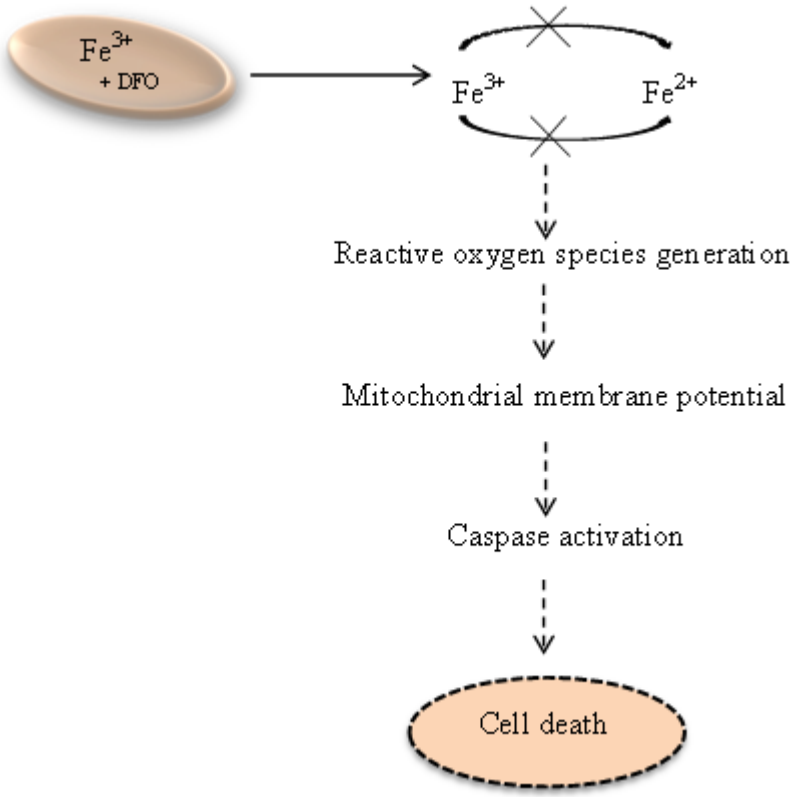
artemisinin and their compounds (Efferth et al., 2004). The reduction of THF acetate isomer might be explained by the chemical rearrangement which can be affected by iron chelatable by deferroxamine. The proposed mechanism of the formation of THF-acetate (figure 2.10) involves the presence of Fe (III) (Maggs et al., 1997), explaining the reduction of THF-acetate formation with DFO treatment.

Figure 2.10 Proposed metabolism of THF formation



Mercer et al. (2009) which demonstrated that bioactivation of the endoperoxides, which results to cytotoxicity, is heme or a heme-containing protein mediated. Exogenous iron, HTF, did increase bioactivation but there was no increase in cytotoxicity. This study showed no effect of artesunate on intracellular heme content with slight decrease in heme content at high concentrations of artesunate. On the other hand, this study shows that artesunate produced a significant dose dependent increase in cellular iron producing more than two and a half fold increase at high concentration (100µM) when compared to the control.

Figure 2.11 Proposed mechanism underlying DFO protection against artesunate toxicity



Pre-treatment of cells with DFO significantly reduced artesunate induced iron accumulation. The artemisinin compounds induce an iron dependent vesicle clustering at the perinuclear region of lysosomes (Hamacher-Brady et al., 2011). Iron is usually stored in a stable form as transferrin and ferritin but lysosomal iron is in a labile state. The large amount of redox active iron only stays in the lysosomes temporarily for further transit to places that require iron but that also makes it an important place for Fenton-type reaction, resulting in the formation of reductive hydroxyl radicals (Kurz et al., 2007).

Being a hydrophilic compound, DFO does not cross through cell membranes but enters cells by endocytosis ending up in the lysosome. It chelates iron present in the lysosomes and in the absence of excess iron starves cells of iron resulting in cell death. DFO protects against early and late lysosomal rupture (Kurz et al.,2006).

Lysosomal iron has been shown to be a toxic source of artemisinin generated ROS (Crespo-ortiz et al., 2011) and oxidative damage has been implicated in the mechanism of action of artemisinin and is an important factor in artemisinin-induced cell death (Efferth et al., 2007, Bedelle et al., 2011).

ROS production by artesunate was seen from 16 h incubation showing a dose dependent increase by 24 h and DFO significantly reduced artemisinin induced ROS production. Since the generation of reactive oxygen species is an important aspect in artemisinin induced toxicity, chelating the toxic iron by DFO prevents ROS formation by inhibiting the catalytic role of iron in the fenton reaction (Molina-Jijon et al 2012). Treatment with DFO in the absence of iron overload can itself cause oxidative damage (Reeder and Wilson, 2005, Reeder et al., 2008) as evident in these investigations but when artesunate was added, its effect was negated. This might be due to the fact that the treatment of cells with artesunate caused significant iron production thereby giving DFO the iron to chelate rather than the iron the cells need to function.

Lipid peroxidation does not seem to be affected by artesunate treatment and thus might not contribute to artesunate induced ROS formation. This is in line with Berman and Adams (1997) who demonstrated that artemisinin treatment had no

effect in basal malondialdehyde production in human erythrocyte membranes but importantly, addition of heme with artemisinin caused a spike in MDA formation (Berman and Adams, 1997).

The dose dependent loss of mitochondrial membrane potential confirms the role of mitochondria in artemisinin induced cell death. The generation of reactive oxygen species causes mitochondrial membrane potential loss and disrupts normal mitochondrial function eventually leading to cell death (Wang et al., 2010). The inhibition of mitochondrial membrane potential loss through pre-treatment with DFO, at an early time point before the induction of apoptosis (16 h) further reinforces the contribution of ROS in the cascade of events leading to artesunate induced cell death. The inhibition of artesunate induced ROS formation protects mitochondria from damage and in turn inhibits caspase activation that leads to cell death. The activation of the caspase cascade is regarded as the hallmark of apoptotic cell death (Elmore, 2007). The endoperoxides have previously shown to induce cleavage of pro-caspase-3 (32kDa) to the active subunit (17kDa) (Mercer et al., 2007).

The work presented in this chapter demonstrates that artemisinin induced cytotoxicity can be inhibited by the iron chelator DFO. The bioactivation of the artemisinin compounds, which is believed to be Fe^{2+}/iron mediated was not inhibited by DFO but the subsequent mechanisms involved in the apoptotic process of the artemisinin compounds were inhibited. The results from this study seem to suggest that DFO chelates artemisinin induced accumulation of iron in cells probably in the lysosome. Reactive oxygen species formation is catalysed by artesunate induced iron

accumulation which in turn triggers a downstream cascade of mitochondrial damage resulting in caspase formation and cell death. This work has identified the mechanism of action of the artemisinin compound where DFO blocks the cascade of events leading to cell death. The results obtained are in line with Hamacher-Brady and group (2011) who identified artemisinin induced lysosomal iron as causing ROS formation. The ROS formation was blocked by DFO, a lysosomal iron chelator.

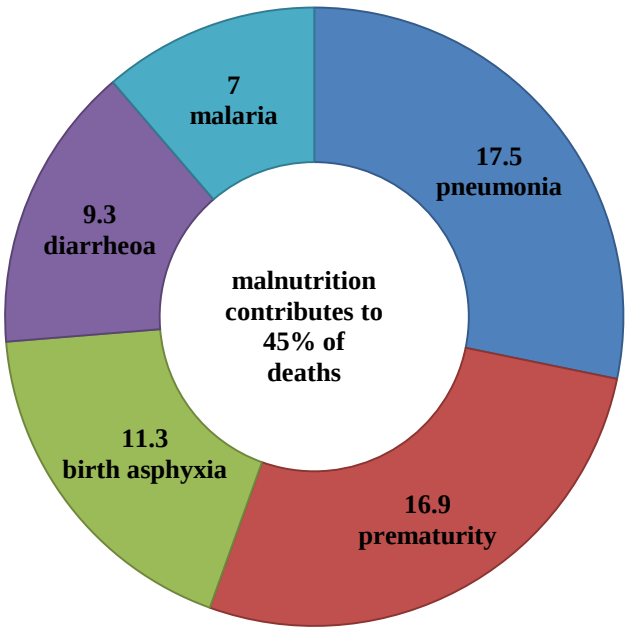
Although the safety profile of the artemisinin compounds is thought to be excellent in human, a definitive statement on their safety cannot be made due to reported toxicity in animal studies (Efferth and Kaines 2010). This chapter has demonstrated and identified the process by which DFO inhibits artemisinin compound toxicity in cells, and with reports that DFO reduces artemisinin induced toxicity in animals (Meshnick et al., 1993), there might be its potential use in clinical practice if human toxicity was to be identified. However, further studies need to be carried out. With the knowledge that malnutrition can alter drug safety profile, the rest of the chapters of this thesis will be to identify potential effect of malnutrition on artemether safety.

3. Incidence of Protein Energy Malnutrition in Children With Uncomplicated Malaria in The Gambia

3.1 Introduction

Infectious diseases account for more than 65% of deaths in children under five years of age in Sub-Saharan Africa. Pneumonia, diarrheal disease and malaria are responsible for more 50% of those mortalities, with malnutrition believed to be directly or indirectly responsible for around half of the mortalities (Black et al., 2010). In 2011, malaria alone accounted for 7 % of total mortality from infectious diseases (WHO, 2012).

Figure 3.1 Global causes of mortality in children under five years of age, 2011



Malnutrition was a contributing factor to almost half of all infant mortality in children under five years of age in 2011.

There is substantial evidence that malnutrition causes decreased immunity, including cell mediated immune responses, immunoglobulins, antibodies, cytokine production and phagocyte function. This causes significant alteration to immunity and predisposes to infection. Conversely, infectious diseases can precipitate childhood malnutrition (Schaible and Kaufman, 2007).

Plasmodium falciparum malaria and malnutrition are both a major health problem in many tropical countries and are thought to be sometimes associated (Fillol et al., 2009). They both cause high infant mortality and morbidity especially in rural sub-Saharan Africa. Control clinical trials of Vitamin A and zinc supplementations in under nutrition have shown to significantly reduce malaria incidence (Owusu-Agyei et al., 2013, Zeba et al., 2008), further supporting the association between malnutrition and malaria.

Malnutrition has been shown to influence susceptibility and manifestation of malarial infection and the association and impact of this combination and interaction is still contradictory (Nyakeriga et al., 2004). However, some have found an increased risk of malaria in malnourished children (Friedman et al., 2005, Deen et al., 2002, Verhoef et al., 2002, Man et al., 1998). Studies looking into the relationship between malaria, malnutrition and specific immunity have different results regarding antibody (Ab) responses to *P. falciparum* (Genton et al., 1998, Blair et al., 2003, Fillol et al, 2008). Blair et al (2003) showed no impact of malnutrition on antibody response to malaria infection. In contrast to this, other authors have

reported lower specific antibody levels in children with malnutrition compared to their normally nourished counterpart (Fillol et al., 2008, Genton et al., 1998).

Table 3.1 summarises the relationship between protein energy malnutrition and malaria from selected studies. Stunting, a measure of prolonged malnutrition, has been associated more with malarial infection than either wasting or underweight. A longitudinal study of rural children in The Gambia reported no association between malaria and wasting but discovered increased risk of malarial episodes in stunted children (Deen et al., 2002). The same findings were also reported in Kenya were a cross sectional studies shown stunting to be associated with concurrent malaria, high parasitemia and malarial anaemia. On the contrary, other studies in Kenya and Congo have shown stunting to protect children from malaria (Mitangala et al. 2013, Mitangala et al., 2008, Genton et al., 1998). Fillol et al (2009) on the other hand observed wasting to be protective against clinical malaria whilst stunting and underweight had little association with malarial episodes (Fillol et at, 2009).

A couple of studies have highlighted no association between PEM and the incidence of malaria (Deribew et al., 2010, Danquah et al., 2010, Muller et al., 2010), but discovered that malnourished children had more than double the risk of dying from malaria than their normally nourished counterpart (Danquah et al., 2009, Muller et al., 2003). Intermittent preventive treatment (IPT) in malnourished children offered less immunity to malarial episodes compared to the normally nourished children (Danquah et al., 2009).

Anaemia is also associated with malaria and malnutrition, with underlying anaemia contributing to the severity of malaria and malnutrition, leading to increased mortality. Red blood cell destruction and reduced production, associated with the pathophysiology of malaria, causes a reduction of haemoglobin levels leading to anaemia (Menendez et al., 2000). Continued malaria infection and further destruction can result in severe malarial anaemia (Haldar and Mohandas, 2009). Severe malarial anaemia is defined as Hb concentrations <5 g/dl in the presence of any density anaemia (WHO, severe falciparum malaria, 2000) and is one of the leading causes of malaria related mortalities (Menendez et al., 2000).

This chapter will evaluate the prevalence of malnutrition in children under five years of age with malaria in The Gambia. Malaria and malnutrition are major public health problems in The Gambia. According to The Gambia's Multiple Indicator Survey (MICS) 2010, stunting was 23.4%, wasting 9.5% and underweight estimated to be 17.4% in children under five years of age (MICS, 2010). Although the incidence of malaria is declining in The Gambia, it is still a major cause of morbidity and mortality among children under five years of age (Ceesay et al., 2010). Malaria is the highest cause of death in children in The Gambia accounting for 60% of all under five years of age mortality, followed by pneumonia, malnutrition and diarrhoea (National Health Policy, The Gambia).

It is the primary aim of this chapter to investigate the prevalence of malnutrition in children under five years of age presenting with uncomplicated malaria compared to the prevalence of malnutrition in children under five years of age in The Gambia. The prevalence will be measured as the percentage of children

with malnutrition according to the WHO classification against the total number of children with uncomplicated malaria.

Table 3.1 Association between malaria and protein energy malnutrition

Source	Country	Number Age	Indicator for malnutrition	Interpretation
Danquah et al. 2009	Ghana	N = 1200 2 – 24 months	WHZ, WAZ, HAZ,	Protective efficacy of IPT halved in malnourished children No malarial association between nutritional status
Fillol et al. 2009	Senegal	N = 874 2 - 59 months	WHZ, WAZ, HAZ	Wasting associated with clinical malaria
Friedman et al. 2005	Kenya	N = 1862 0 – 36 months	WHZ, WAZ, HAZ	Stunted children had more parasitaemia, increased incidence of clinical malaria and severe anaemia
Muller et al. 2003	Burkina Faso	N = 686 0 – 30 months	WHZ, WAZ, HAZ	No incidence in malaria incidence
Deen et al 2002	Gambia	N = 487 < 5 years	WHZ, WAZ, HAZ	Stunted children had higher risk of malaria incidence
Genton et al. 1998	Papua New Guinea	N = 136 10 months – 10 years	WHZ, HAZ	Stunted children at lower risk of malaria incidence
Tshikuka et al 1997	Zaire	N = 558 4 months – 10 years	WHZ, HAZ	Stunting and wasting had higher risk of malaria infection
Snow et al. 1991	Gambia	N = 138 1 – 4 years	WHZ, WAZ, HAZ	No malarial association between nutritional status

WHZ – weight-for age Z score (underweight), WAZ – weight for height z score (wasting), HAZ – height-for-age z score (stunting), IPT – intermittent preventive treatment.

The generation of free radicals have been associated with the pathogenesis of PEM. The major sources of anti-oxidants are dietary intake and nutritional supplements (Ece et al., 2007), thus malnourished children are believed to be in a state of oxidative stress (Granot et al., 2004). Studies of malnourished children have demonstrated increased pro-oxidant and decreased antioxidant (perampalli et al., 2010, Catal et al., 2007, Ece et al., 2007, Shabaan et al., 2002, Reid et al., 2000,), putting them is a state of oxidative stress. It is believed that the higher pro oxidants found in malnourished children are responsible for the pathological changes such as edema, fatty liver and skin lesions (Golden et al., 1990). Plasma malondialdehyde (MDA), an end-product of lipid peroxidation, is a one of the most reported biomarkes of oxidative stress. The secondary aim of this chapter will be to measure MDA levels in the study population to ascertain their oxidative status.

3.2 Materials and methods

3.2.1 Materials

Sulphuric acid (H_2SO_4), Phosphotungstic acid, thiobarbituric acid (TBA), glacial acetic acid and n-butanol were all purchased from Sigma Aldrich (UK). Distilled water was obtained from the laboratory distilled water taps. All the chemicals were HPLC grade.

3.2.2 Study Area

Research participants were recruited from Serrekunda Hospital and Brikama Health centre in The Gambia. The Gambia is located on the coast of West Africa and stretches about 350km from the coast to the inland, with 20% considered as wetland (Sallah and Williams, 2011). According to the 2003 census, the population of the Gambia was about 1.3 million people with a projected population of over 1.7 million in 2009. The population is heavily concentrated around Banjul, Kanifing municipality and Brikama. Brikama has the highest population with 28% followed by Kanifing Municipality with 24%.

Serrekunda hospital is situated in a relatively modern area and the population there are relatively well off. On the other hand, Brikama Major Health Centre covers a wide catchment area for many villages where villagers go to the health centre for first line treatment. The health centre also serves as a referral place for minor health centres and health posts around the catchment area. The patients going to the health centre are economically and socially diverse.

The study was conducted between October and December 2010. Malaria transmission peaks between the months of August and November. At the time of the studies, Artemether/Lumefantrine was the first line of treatment for uncomplicated malaria. Local ethics approval was obtained from the Gambia Government/MRC Joint Ethics Committee. Approval was also obtained from the management of Serrekunda health centre, and the divisional health committee to conduct the study at Brikama health centre.

3.2.3 Participants

Parents of children diagnosed with uncomplicated malaria from the out-patient department were approached for inclusion. Parent information leaflets were given to the adult accompanying the identified child. The information on the leaflet was explained in detail to parents that were not literate in English. Questions and enquiries were answered by study leaders. Consent was obtained from the parents/guardians who accepted for their children to be part of the study. Those who could sign were given a consent form to sign and those that could not sign put their thumb print on the signature area.

3.2.4 Inclusion criteria

- Diagnosis of uncomplicated malaria
- Age between 12 – 59 months
- Weight ≥ 5kg
- Blood film of *Plasmodium falciparum* infection of 1,000 parasites/μL
- No medication containing artemisinin 4 weeks prior
- Willing to give informed consent

3.2.5 Exclusion criteria

- Diagnosis of complicated malaria
- Age < 12 and ≥ 60 months
- Weight < 5kg

- Medication containing artemisinin within 4 weeks
- Unwilling/ unable to give informed consent

3.2.6 Ethical Approval

Ethical approval of the study was approved by The Gambia Government/MRC Joint Ethics Committee. This approval covered the recruitment of participants in health facilities in The Gambia.

3.2.7 Informed Consent

Parent information leaflets and patient information leaflets for children ≥ 48 month of age were written in English. A proportion of people in The Gambia are illiterate (WHO, world health statistics 2012) so verbal translation to a local language they understand was done in the presence of an independent member of staff. Agreement was documented by signature for those that were literate in English or a thumbprint for those who were not.

3.2.8 Measurement of clinical parameters

Anthropometric measurements (weight, height) were taken. Weight was measure on a digital scale. Length was taken for children under the age of two years on a platform with a sliding headboard. Standing height was measured for children above two years with a height scale. Weight-for-height *z* scores (WHZ), weight-for-age *z* scores (WAZ) and height-for-age *z* scores (HAZ) were calculated on the basis of the National Centre for Health Statistics (NCHS/WHO) reference data set, version

3.2.2 software (WHO 2011) Z scores < -2 are suggestive of wasting (WAZ), underweight (WHZ) and stunting (HAZ). Scores < -3 show severe malnutrition. Age, sex and axillary temperature was recorded for every child.

3.2.9 Malondialdehyde Analysis

MDA concentration was measured by thiobarbituric acid-reactive substances (TBARS) method (Yagi, 1998). The assay was performed in glass tubes. To 50µl of plasma was added 12N H_2SO_4 (4ml) and mixed gently. 10% phosphotungstic acid (0.5ml) was added to the mixture, left to stand at room temperature (5 min) and then centrifuge (3000 rpm, 10 min). 12N H_2SO_4 (2ml) and 10 % phosphotungstic acid (0.3 ml) was added to the sediment, vortexed, and centrifuged again (3000 rpm, 10min). 0.5nmol tetramethoxypropane was used as the standard solution. The standard solution and sediment were suspended in distilled water (4 ml) and TBA reagent (a mixture of equal volumes of 0.67 % TBA aqueous solution and glacial acitic acid, 1 ml). The mixture was heat in a heating block (95°C, 60 min) and immediately cooled under running tap water. Butanol (5 ml), was added to the cooled mixture and shaken vigorously. The mixture was centrifuged (3000 rpm) and the top layer was taken for fluorescent measurement at 550nm with excitation at 530nm. Plasma peroxide levels was measured using the formula

$$0.5 * \frac{f}{F} * \frac{1.0}{0.05} = \frac{f}{F} * 25 \text{ (nmol/ml of plasma)}$$

where f is the results of samples

F is the results of the standard

3.2.10 Statistical Analysis

Data was entered in excel and statistical analysis was performed using statistical package, SPSS. Nutritional status was assessed using WHO Standardised Z-scores used as per WHO guidelines: weight-for-age z-score (WAZ) for underweight, weight-for-height z-score (WHZ) for wasting and height-for-age z-score (HAZ) for stunting. Measures of malnutrition were categorised as z-score >1, <-1, <-2, <-3 for normally nourished, mild, moderate and severely malnourished respectively. SPSS statistic for windows, version 20.0 was used. Normality was tested using t test and chi-square test. ANOVA was used to test for significance of data in different nutritional status. Interaction between potential confounders was examined by analysis of covariance. Post-hoc analysis was carried out where significance was observed using independent sample t-test.

3.3 Results

350 parents/guardians of children diagnosed with uncomplicated malaria were approached during the period of the study. Consent was obtained for 105 children to be part of the study. Five children developed complicated malaria and had to be withdrawn and a further three parents withdrew their children from the studies. A total of 97 participants (age range; 12-59 months) were recruited, of whom 49 were boys (50.5%) and 48 (49.5%) were girls. The mean age was 33.5 months (SD – 15.2) and the mean axillary temperature was 37.2°C (± 0.27).

Table 3.2 Demography of study participants

Age (months)	No. (%)	Gender	No. (%)	Hb (g/dl)	No. (%)
12-17	15 (15.5)	Male	49 (50.5)	≥ 11	3 (3.1)
18-23	18 (18.6)	Female	48 (49.5)	10.9–10	66 (68.0)
24-29	13 (13.4)			9.9-7	28 (28.9)
30-35	6 (6.2)			<7	0 (0)
36-41	11 (11.3)				
42-47	9 (9.3)				
48-53	12 (12.4)				
54-59	13 (13.4)				

Demographic summary of study participants according to age groups, gender and haemoglobin (Hb) level.

Table 3.2 summarises the participants in relation to age groups, gender and haemoglobin levels. Malaria was highest in the younger age groups with 18-23 months age group accounting for 18.6% (n=18) followed by 12-17 months age group (15.5%). The least malaria incidence was seen in the 30-35 months age group (6.2%). The mean haemoglobin (Hb g/dl) level was 9.5g/dl (± 0.92) with 96.9% of the children classified as anaemic according to the WHO definition. 28.9% of children were mildly anaemic (10-10.9 g/dl) and 68% of children were moderately anaemic (7-9-9g/dl).

The mean Z-scores (SD) for weight-for-age, weight-for-height and height-for-age of the study population were -1.483 (1.602), -1.178 (1.573) and -1.297 (1.602) respectively. Using the WHO classification of malnutrition, 30.9% of children in the study were malnourished.

The prevalence of underweight (WAZ) and wasting (WHZ) were both 30.9%, and stunting (HAZ) was 28.9% (Table 3.3). Their corresponding severe nutritional condition (Z score < -3) was observed in 13.4%, 12.4% and 9.3% in children respectively. There was no statistical difference in malnourished status between boys and girls however, there were more malnourished boys compared to girls. Tables 3.4 summarises the type of PEM in relation to age groups in months.

Table 3.3 Summary of Nutritional Indices (Z score)

	Weight-for-age (WAZ)	**Weight-for-height (WHZ)**	**Height-for-age (HAZ)**
No. below <-2 (%)	30 (30.9)	30 (30.9)	28 (28.9)
Sex			
No. of girls <-2 (%)	12 (25)	13 (27.1)	11 (22.9)
No. of boys <-2 (%)	18 (36.7)	17 (34.7)	17 (34.7)

Characteristics of the study population in relation to nutritional indicators using z-score. Malnutrition is categorised as weight-for-age (WAZ), weight-for-height (WHZ) and height-for-age (HAZ).

Table 3.4 Category of protein energy malnutrition according to age groups

	Category of PEM		
Age (months)	**WHZ**	**WAZ**	**HAZ**
12–17 (%)	6 (20)	8 (26.7)	6 (21.4)
18-23 (%)	4(13.3)	4 (13.3)	6 (21.4)
24-29 (%)	2 (6.7)	3 (10)	7 (25)
30-35 (%)	1(3.3)	2 (6.7)	1 (3.6)
36-41 (%)	2 (6.7)	2 (6.7)	2 (7.1)
42-47 (%)	3 (10)	2 (6.7)	0 (0)
48-53 (%)	5(16.7)	4 (13.3)	3 (10.7)
54-59 (%)	7 (23.3)	5 (16.7)	3 (10.7)

Prevalence of wasting **(**WHZ), underweight (WAZ) and stunting (HAZ) according to age groups in months.

Underweight

Underweight is indicated by weight-for-age less than 2 SD of the population using the NCHS/WHO reference data set and severe underweight is indicated by weight-for-age less than 3 SD. Table 3.5 shows the demographics of underweight in relation to age groups, gender and Hb levels. The prevalence of underweight among children with uncomplicated malaria was 30.9% with the severe form being 13.4%. There was no significant difference between gender (p =0.56).

Underweight was highest in the youngest age group (12-17 months) accounting for 8.2% of the study population. This also constituted more than half (53.3%) of the population of that age group. 14.4% of the total study group were severely underweight. The highest percentages seen in the two youngest age groups (12-17, 18-23) and the eldest age group (54-59) in equal proportion (21.4%).

Underweight was least between 30 – 47 months and there was no observed trend of underweight in relation to age group.

Table 3.5 Demographics of weight-for-age category

	Z score value No.				
Age groups (months)	**Normal**	**< -1**	**< -2**	**< -3**	**p value**
12–17 (%)	5 (16.7)	2 (5.4)	5 (31.2)	3 (21.4)	NA
18-23 (%)	7 (23.3)	7 (18.9)	1 (6.2)	3 (21.4)	
24-29 (%)	2 (6.7)	8 (21.6)	2 (12.5)	1 (7.1)	
30-35 (%)	2 (6.7)	2 (5.4)	2 (12.5)	0 (0)	
36-41 (%)	5 (16.7)	4 (10.8)	1 (6.2)	1 (7.1)	
42-47 (%)	4 (13.3)	3 (8.1)	0 (0)	2 (4.3)	
48-53 (%)	3 (10.0)	5 (13.5)	3 (18.8)	1 (7.1)	
54-59 (%)	2 (6.7)	6 (16.2)	2 (12.5)	3 (21.4)	
Gender No. (%)					0.56[5]
Male	15 (50.0)	16 (43.2)	9 (56.2)	9 (64.3)	
Female	15 (50.0)	21 (56.8)	7 (43.8)	5 (35.7)	
Weight	13.05±2.63	11.22±2.15	9.98±2.32	8.67±2.10	< 0.001[6]
Height	90.36±12.10	90.08±2.15	84.94±12.07	85.59±14.15	0.32[6]
Age continuous	31.93±14.20	35.57±14.52	32.25±17.12	33.07±17.92	0.28[6]
Hb[1] mean	10.29±0.66	9.65±0.50	8.90±0.65	8.19±0.52	<0.001[7]

Prevalence of the degree of underweight in children by age, gender and haemoglobin levels.
[1]including weight as confounding factor
[5]Chi-square test
[6]ANOVA
[7]Analysis of covariance

Wasting

Wasting is indicated by weight-for-height less than 2 SD of the population using the NCHS/WHO reference data set and severe wasting is indicated by weight-for-height less than 3 SD. Table 3.6 shows the demographics of wasting in relation to age groups, gender and Hb levels. The prevalence of wasting was 30.9% with no significant gender difference ($p = 0.59$). Wasting was highest in the older age group (54-59), accounting for 7.2% of the overall study group. More than half (53.8%) of children in this age group were wasted. Severe wasting accounted for 12.4% of the study population and was highest in the 18-23 months and 54-59 months age group. Wasting was least observed in the 30-35 age group (3.3%) but also low between 23 – 41 months.

Stunting

Stunting and severe stunting were indicated by height-for-age less than 2 SD and 3 SD respectively, of the population using the NCHS/WHO reference data set. Table 3.7 shows the demographics of stunting in relation to age groups, gender and Hb levels. The prevalence of stunting was 28.9% with no significant difference ($p = 0.58$) in relation to gender. 24-29 month age group accounted for the highest percentage of stunting (25%). Severe stunting was 9.3% of the study population with the highest percentage seen in the 24-29 age group. It accounted for just under half (44.4%) of total severely stunted children, followed in equal numbers by 12-17 months and 18-23 months age groups (7.1%).

Table 3.6 Demographics of weight-for-height category

	Z score value No.				
Age groups (months)	**Normal**	**< -1**	**< -2**	**< -3**	**p value**
12–17 (%)	8 (14.5)	1 (8.3)	5 (27.8)	1 (8.3)	NA
18-23 (%)	14 (25.5)	0 (0)	1 (5.6)	3 (25.0)	
24-29 (%)	9 (16.4)	2 (16.7)	1 (5.6)	1 (8.3)	
30-35 (%)	4 (7.3)	1 (8.3)	1 (5.6)	0 (0)	
36-41 (%)	6 (10.9)	3 (25.0)	1 (5.6)	1 (8.3)	
42-47 (%)	5 (9.1)	1 (8.3)	1 (5.6)	2 (16.6)	
48-53 (%)	6 (10.9)	1 (8.3)	4 (22.2)	1 (8.3)	
54-59 (%)	3 (5.5)	3 (25.0)	4 (22.2)	3 (25.0)	
Gender No. (%)					
Male	28 (50.9)	4 (33.3)	10 (55.6)	7 (58.3)	
Female	27 (49.1)	8 (66.7)	8 (44.4)	5 (41.7)	0.59[5]
Weight	11.89±2.57	12.14±2.30	11.02±3.07	9.01±2.03	< 0.006[6]
Height	86.32±10.58	93.67±11.48	91.68±14.98	89.93±13.03	0.14[6]
Age continuous	30.49±13.38	40.25±14.10	36.33±18.18	36.58±17.34	0.28[6]
Hb[1]	9.95±0.76	9.51±0.76	9.05±0.53	8.18±0.53	<0.001[7]

Prevalence of the different degrees of wasting in children by age, gender and Haemoglobin levels.
Results are expressed as mean±SD
[1]including weight as confounding factor
[5]Chi-square test
[6]ANOVA
[7]Analysis of covariance

Although boys were more underweight (P = 0.56), wasted (P = 0.59) and stunted (P = 0.58) compared to the girls, the difference was not statistically significant. Half of the underweight boys were severely underweight accounting for

18.4% of boys whilst 10.4% of girls were severely underweight. Severe wasting and stunting was also higher in boys accounting for 14.3% and 12.2% respectively compared to 10.4% and 6.3% in girls.

Table 3.7 Demographics of height-for-age category

	Z score value No.				
Age groups (months)	**Normal**	**< -1**	**< -2**	**< -3**	**p value**
12–17 (%)	4 (11.8)	5 (14.3)	4 (21.1)	2 (22.2)	
18-23 (%)	3 (8.8)	9 (25.7)	6 (31.6)	0 (0)	
24-29 (%)	3 (8.8)	3 (8.6)	3 (15.8)	4 (44.4)	
30-35 (%)	1 (2.9)	4 11.4)	0 (0)	1 (11.1)	NA
36-41 (%)	7 (20.9)	2 (5.7)	0 (0)	2 (22.2)	
42-47 (%)	5 (14.7)	4 (11.4)	0 (0)	0 (0)	
48-53 (%)	5 (14.7)	4 (11.4)	3 (15.8)	0 (0)	
54-59 (%)	6 (17.6)	4 (11.4)	3 (15.8)	0 (0)	
Gender No. (%)					
Male	15 (44.1)	17 (48.6)	11 (57.9)	6 (66.7)	
Female	19 (55.9)	18 (51.4)	8 (42.1)	3 (33.3)	0.58[5]
weight	13.16±2.51	10.87±2.43	10.38±2.25	9.02±2.28	< 0.001[6]
Height	97.24±9.96	86.61±10.14	82.62±11.17	77.07±7.04	0.001[6]
Age continuous	38.32±14.41	32.37±15.18	30.53±17.35	26.33±9.34	0.10[6]
Hb[2]	9.92±0.86	9.45±0.92	9.36±0.65	8.52±0.81	<0.001[7]

Prevalence of the different degrees of stunting in children by age, gender and haemoglobin levels. Results are expressed as mean±SD
[2]including weight and height as confounding actors
[5]Chi-square test
[6] ANOVA
[7]Analysis of covariance

Haemoglobin levels

Hb levels showed significant difference in relation to nutritional status. Hb levels of malnourished children were significantly lower ($p < 0.001$) than the normally malnourished children in all three categories of malnutrition (Table 3.8). Hb levels progressively decreased with the severity of malnutrition. The mean difference (95% CI) for underweight and wasting were 1.39 (0.90, 1.02) and 0.90 (0.39, 1.41). Significant difference of Hb levels in the stunting group was only observed in children with severe stunting. There was a mean difference (95% CI) of 1.40 (0.55, 2.25) for severe stunting and 2.10 (1.59, 2.01) and 1.77 (1.17, 2.37) for severe underweight and wasting respectively.

Table 3.8 Analysis of haemoglobin levels in relation to nutritional status

Nutritional Status	Individual differences (Z score)		Mean difference	95% Confidence Interval[8]
WAZ	Normal	< -1	0.64	0.25, 1.02
	Normal	<-2	1.39	0.90, 1.87
	Normal	<-3	2.10	1.59, 2.61
	< -1	< -2	0.75	0.28, 1.22
	< -1	< -3	1.46	0.97, 1.96
	< -2	<-3	0.71	0.14, 1.29
WHZ	normal	< -2	0.90	0.39, 1.41
	normal	< -3	1.77	1.17, 2.37
	< -1	<-3	1.33	0.56, 2.09
	< -2	< -3	0.87	0.17, 1.57
HAZ	normal	< -3	1.40	0.55, 2.25

Post-hoc analysis for haemoglobin levels testing each pair of PEM category with haemoglobin concentration
[8]Independent sample t-test

Malondialdehyde (MDA) levels

In order to assess the relationship between pro-oxidants and PEM, lipid peroxide levels expressed in terms of MDA were measured. Plasma MDA levels in malnourished children of all three categories were significantly higher ($p < 0.001$) compared to the normally nourished children. The mean difference of MDA levels in relation to each PEM category is summarised in Table 3.9 and Table 3.10. The increase in MDA levels was in relation to the degree of severity of malnutrition with the severe forms showing the highest.

Table 3.9 Malondialdehyde levels in children with different nutritional status

MDA (nmol/ml)	normal	< -1	< -2	< -3	p value
WAZ[1]	0.59±0.25	0.73±0.18	1.05±0.32	1.67±0.71	<0.001
WHZ[1]	0.67±24	0.84±0.50	0.99±0.28	1.69±0.73	<0.001
HAZ[2]	0.69±0.27	0.87±0.44	0.93±0.36	1.49±0.97	<0.001

Malondialdehyde levels in different degrees of underweight (WAZ), wasting (WHZ) and stunting (HAZ). Results are expressed as mean±SD
[1] including weight as a confounding factor
[2] Including weight and height as a confounding factor

The MDA levels of both wasting and underweight (0.99±0.28 and 1.05±0.23 nmol/ml), with their severe forms (1.69±0.73 and 1.67±0.71), were significantly higher compared to levels in normally nourished children (0.67±24 and 0.59±0.25). The mean difference (95% CI) for wasting and severe wasting were -0.32 (-0.59, -0.04) and -1.02 (-1.34, -0.69) respectively, and underweight and severely underweight were -0.45 (-0.74, -0.17) and -1.07 (-1.37, -0.77) respectively, compared to normally nourished children. Stunting on the other hand, was only significantly

higher in the severe category with a mean difference (95% CI) of -0.80 (-1.25, -0.34) when compared to the normally nourished children. The MDA levels in children with normal height-for-age, stunting and severe stunting was 0.69±0.27, 0.93±0.36, 1.49±0.97 nmol/ml respectively.

Table 3.10 Analysis of malondialdehyde

Nutritional Status	Individual differences (Z score)		Mean difference	95% Confidence Interval[8]
WAZ	Normal	< -2	-0.45	-0.74, -0.17
	Normal	<-3	-1.07	-1.37, -0.77
	< -1	< -2	-0.31	-0.59, -0.03
	< -1	< -3	-0.93	-1.23, -0.64
	< -2	<-3	-0.62	-0.96, -0.28
WHZ	normal	< -2	-0.32	-0.359, -0.04
	normal	< -3	-1.02	-1.34,- 0.69
	< -1	<-3	-0.85	-1.26, -0.43
	< -2	< -3	-0.70	-1.08, -0.32
HAZ	normal	< -3	-0.80	-1.25, -0.34
	< -1	< -3	-0.62	-1.07, -0.17
	< -2	< -3	-0.56	-1.05, -0.07

Post-hoc analysis for malondialdehyde levels. testing each pair of PEM category with malondialdehyde levels
[8]Independent sample t-test

3.4 Discussion

Malaria is endemic in The Gambia with cases occurring throughout the year, but peaks during the months of September to November. 39% of infant visits to the health facilities are due to malaria infection (GMSP, 2009). Protein energy malnutrition affects about 20% of the population in the developing world (FAO,

2000). It is mostly seen in children, with 70% from Asia, and Africa and Latin America accounting for 26% and 4% respectively (WHO, 2006). 54% of the 10.8 million deaths per year is attributed to PEM and is directly or indirectly associated with infectious diseases among children under 5 years of age in developing countries (Schaible et al., 2007). Malaria and malnutrition are sometimes associated in African children and both cause high morbidity and mortality (Danquah et al., 2009, Deen et al., 2002).

It was the aim of this chapter to assess the prevalence of malnutrition in children with malaria in The Gambia. Using the WHO classification of malnutrition, it was observed that 31.6% of children with uncomplicated malaria were malnourished. Underweight, wasting and stunting accounted for 30.9%, 30.9% and 28% respectively. According to The Gambia's 2010 Multiple Indicator Cluster Survey (MICS4), stunting in The Gambia is 23.4%, wasting 9.5% and underweight estimated to be 17.4% 16% (MICS4, 2012). The prevalence of stunting has decreased when compared to the 2005/2006 MICS3 where stunting was 28% (MICS3, 2006). However, wasting and underweight has increased by 2.5% and 1.4% respectively.

Stunting is a measure of chronic malnutrition indicating long term food deficiency. Wasting on the other hand is mainly caused by acute or short term food deficiency and severe infections and is mostly reversible (Khatab, 2010). Infectious diseases can directly or indirectly affect nutritional status of children as is observed in children with HIV infection. Krawinkel et al (2012) reported a higher incidence of malnutrition in HIV infected children (Krawinkel et al., 2012). The demands of

immune responds to infection increase the metabolic demand of the body thus increasing anabolic metabolism. Infection can aggravate symptoms like diarrhoea, malabsorption and loss of appetite altered metabolism (Katona and Katona-Apte, 2008).

The higher prevalence of wasting obtained in this study, compared to the national data, might be due to the effects of malarial infection. Children presenting to the health facilities usually present late allowing the progression of the infection and the initiation of the cascade of malnutrition. Fever, a common symptom of malaria, increases the metabolic demands on the body. Other symptoms of malaria like diarrhoea and vomiting can cause acute nutrient loss and coupled with loss of appetite, might contribute to acute weight loss.

On the other hand, the higher prevalence of wasting might be associated with general food shortage during the malaria season in The Gambia. Both malarial infection and food shortage are usually higher during the rainy season, which runs from June to October, when food stocks in the rural arears are running low and harvest season has not yet started (Jawara et al., 2008, Moore et al., 2001). Food is most abundant in The Gambia in November when crop harvest has started (Deen et al., 2001), which was in the middle of the study period. Although harvest started during most of the study period, the effect of food shortage cannot be excluded as a cause of the high prevalence of acute malnutrition.

There was no significant difference in the prevalence of malnutrition between genders. The same was reported by Ubesie et al (2012) in a review covering a period

of ten years. Wasting was more prevalent in the older age groups but underweight and stunting was higher in the younger age groups (Ubesie et al., 2012). The non-significant gender difference is in line with WHO figures (WHO). This study observed no significant gender difference and there was no age trend in regards to the different categories of malnutrition. This might suggest that gender might not be a risk factor for developing malnutrition.

Iron plays a critical role in the oxygen transportation and cellular processes of growth division throughout the body. Iron deficiency leads to a decrease in haemoglobin concentration, generally known as anaemia. Anaemia is also cause or aggravated by infections especially malaria, further increasing mortality risk (Bhargava et al., 2001). 96.9% (n=94) of children in the study group were considered anaemic with an Hb level <11g/dl. This is significantly higher than results obtained from other studies where the prevalence of anaemia was seen in about half of the children infected with the malaria parasite (Oladeinde et al., 2012, Bouyou-Akotet et al., 2009).

It would be important to note that anaemia is multifactorial and these children might have other underlying comorbidities and malnutrition is one of the most important factors. Iron deficiency is the most prevalent micronutrient deficiency causing anaemia in 45% of under five years of age (Black et al., 2008). A study in Brazil discovered that 88% of children with malnutrition were anaemic with 30% having Hb levels < 7g/dl (Caminha et al., 2011). Hb levels were significantly lower ($p < 0.001$) in malnourished children regardless of category, and progressively decreased with severity of malnutrition.

The safety of iron supplementation has also been controversial especially in severe malnourished children. Whilst some studies reported an increase predisposition of malarial infection with iron supplementation, others have seen no harmful effects. Studies have suggested that iron deficiency anaemia might be a protective factor for malarial infection and iron supplements might increase morbidity and mortality of malaria. A study in Zanzibar, Tanzania prompted the WHO and UNICEF in a joint statement recommending that iron should only be given to children with anaemia and not as a preventative measure in children in malaria-endemic areas (Harding and Neufeld, 2012). It was discovered during the process of the investigations that iron supplementation in children led to higher hospitalisation or deaths (Sazawal et al., 2006). However, a recent study discovered that use of micronutrient powder with iron supplementation was not a risk factor for increased malaria incidence in malaria endemic countries with the use of insecticide treated bed nets with appropriate malaria treatment (Zlotkin et al., 2013).

Even though the specificity of using TBARS assay has been controversial, it is still a popular method for the assessment of MDA levels in serum and plasma (Catal et al., 2007). Plasma malondialdehyde levels were significantly higher in malnourished children in all three categories ($p < 0.001$) compared to normally nourished children. These results might suggest increased lipid peroxidation and oxidative damage in children with PEM. It is believed that the higher pro oxidants found in malnourished children is responsible for the pathological changes such as edema, fatty liver and skin lesions (Golden et al., 1990). Although a number of studies show children with kwashiorkor to have higher biomarkers of oxidative stress when compared to marasmic children (Fuchs et al., 2005, Manary et al., 2000), Catal

et al (2010) found high levels of plasma malondialdehyde in marasmic children. Jain et al (2008) on the other hand, showed a progressive increase of plasma malondialdehyde with severity of PEM regardless of which spectrum of PEM. The severe malnourished group in his study were composed of both marasmic and kwashiorkor children as well as those who were marasmic-kwashiorkor. The same trend was observed in this study where plasma malondialdehyde levels were progressively higher with the severity of PEM. This provides evidence that malnourished children have less anti-oxidant defence system and increase oxidative damage, further predisposing them to infections and other co-morbidities.

The data presented in this chapter has shown a high level of wasting and underweight in children presenting with uncomplicated malaria, when compared to the local data. Progressive increase in plasma malondialdehyde levels with PEM severity is a biochemical confirmation of the presence of PEM. Various studies have confirmed PEM as a major cause of risk factor in the morbidity and mortality of infectious diseases in children, including malaria. The aetiology of PEM however, is multifactorial and follows a vicious cycle with infection. The high level of PEM observed might be as a result of the combination of the pathophysiology of malaria infection, confirming infection as a risk factor for developing PEM. Both malaria and malnutrition are known to cause anaemia, thus the high level of anaemia in the study population might be multifactorial.

4. A High Performance Liquid Chromatography/Tandem Mass Spectrometry Method for the Simultaneous Determination of Artemether and Dihydroartemisinin in Human Plasma

4.1 Introduction

Over the past decades, a number of methods have been developed for the analysis of artemisinin and its derivatives in biological samples (Edwards, 1994). Developing a sensitive and accurate quantification method had for some time posed problems because of their lack of ultraviolet or fluorescent chromophores. They are also thermally labile and do not contain functional groups for derivatisation (Tija-Isavadharm et al., 2004).

Methods previously used included high performance liquid chromatography (HPLC) with UV detection (Batty et al., 1996, Chimanuka et al., 2002, Navaratnam et al., 1995,), HPLC with electrochemical detection (Karbwang et al., 1997, Melendez et al., 1991), gas chromatography mass spectrometry (Sipahimalani et al., 1991, Mohamed et al., 1999) and the most recent and sensitive method, liquid chromatography with mass spectrometry (Wiesner et al., 2011, Magalhaes et al., 2010, Huang et al., 2009, Hanpithakpong et al., 2009, Souppart et al., 2002).

The need to define and evaluate pharmacokinetic and pharmacodynamics (Lindegardh et al., 2011) as well as monitoring plasma drug levels of artemisinin for the optimisation of artemether dose treatment (Thuy et al., 2008) has become even more important since the publication of a report showing possible drug resistance (Yeung et al., 2009). Thus a highly sensitive and specific analytical method for the quantification of artemisinin and its derivative is necessary.

Artemether/Lumefantrine combination was the first fixed dose of Artemisinin combination therapy (ACT) and is one of the most widely used therapies (Abdulla et al., 2010, Kokwaro et al., 2007). It is a formulated tablet combination comprising 20 mg artemether and 120 mg lumefantrine (Lefevre et al., 2001). Artemether is a fat soluble semisynthetic derivative of artemisinin. Like other artemisinin compounds, it is rapidly absorbed, reaching a maximum plasma concentration at about 2 hours. It is extensively metabolised to its active form, dihydroartemisinin, by cytochrome P450 (CYP) enzymes CYP3A4 with secondary contributions from CYP2B6 AND CYP1A2 (). Both artemether and DHA exhibit short elimination half-lives between 2 to 3 hours (Kokwaro et al., 2007).

4.1.1 HPLC methods

Previously, HPLC with UV detection (HPLC-UV) was used for the quantification of artemisinin derivatives and its metabolite. Acid or base hydrolysis of the compound was necessary to produce UV chromophores prior to HPLC analysis (Lindegardh et al., 2011, Gu et al., 2008). Another HPLC-UV method reported LLOQ levels of 10 – 30 ng/ml of artemisinin in plasma. These values were

not deemed to be low enough to qualify for bioanalytic quantification according to the committee for medicinal products for human use (Gordi et al. 2000).

The use of electrochemical detection (HPLC-ECD) has become the most used technique over the past decades for the determination of these metabolites. This detection technique takes advantage of the presence of a peroxide bridge in their structure for reductive electrochemical detection (Mohamed et al., 1999), which uses electronic capture detection and was shown to enhance sensitivity with limits of detection as low as 3ng/mL (Sandrenen et al., 1997, Navaratnam et al., 1997, Karbwang et al., 1997). Although the HPLC-ECD method is sensitive and selective, it has its limitations**.** Large sample volumes are needed which might not always be possible. The maintenance and cleaning of the electrochemical detector, which is needed to keep the sensitivity of the equipment, can be difficult, challenging, expensive and time consuming (Megalhaes et al., 2010, Gu et al., 2008, Hanpithakpong et al., 2008). HPLC-ECD was the technique of choice until the introduction of more sensitive and improved liquid chromatography mass spectrometric techniques.

4.1.2 Mass spectrometry

Recent more sensitive methods include the use of gas and liquid mass spectrometry. Liquid chromatography mass spectrometry (LC-MS) and liquid chromatography tandem mass spectrometry (LC-MS/MS) are now considered to be the preferred quantification method for the analysis of most drugs including artemisinin and its derivatives in biological fluid (Grebe and Singh, 2011). It is

proven to be more sensitive and selective whilst using less volume of plasma for quantification when compared to HPLC-ECD. It offers the capability of simultaneous identification and quantification of several compounds by using their mass-to-charge ratio.

A triple quadrupole mass spectrometer is the instrument of choice for quantitative analysis. It offers different scan types such as neutral loss, precursor ion, product ion and multiple reaction monitoring (MRM). The latter is employed for quantification purposes and it involves the use of electrospray ionisation followed by two stages of mass selection. The first stage (MS 1) selects the mass of the parent ion and the second stage (MS 2) selects a specific fragment of the parent (called product or daughter ion) after collision with inert gas atoms (Pitt, 2009). Several LC-MS/MS methods have been developed and validated for the quantification of artemether and its metabolite, dihydroartemisinin.

A range of LC-MS/MS instruments have been validated successfully for the simultaneous analysis of artemether and DHA in human plasma (Wiesner et al., 2011, Magalhaes et al., 2010, Hunag et al., 2009, Shi et al., 2006, Souppart et al., 2002), using different extraction techniques. Mass spectrometers used included AB Sciex API 4000 (Weisner et al., 2011), PE Sciex API 2000 triple quadrupole (Huang et al., 2009), Finnigan Quanum Discovery system (Shi et al, 2006) and a TSQ AP II spectrometer equipped with an atmospheric pressure APCI interphase (Souppart et al., 2000).

Although these studies all reported acceptable precision and accuracy, LLOQ obtained ranged from 2 ng/mL (Wiesner et al., 2011, Huang et al., 2009) to 5 ng/mL (Shi et al., 2006, Souppart et al., 2002) using different plasma volumes. Results from both Haung et al. (2009) and Weisner et al. (2011) showed higher instrument sensitivity but Huang et al., concentrated the plasma sample preparation by approximately 5 fold. Weisner et al. (2011) on the other hand obtained the same values without concentrating plasma samples. This might indicate the superiority of the API 4000 over other mass spectrometers

4.1.3 Sample preparation

A number of methods exist for preparing plasma samples for analysis by mass spectrometry and they depend on the chemical characteristics of the analytes to be measured. Blood and blood products contain relatively low analyte concentrations, due to *in vivo* metabolism. This combined with sample matrix effects influences the choice of sample preparation (Strathmann and Hoofnagle, 2011). Sample preparation methods for artemether analysis include liquid-liquid extraction (LLE) (Wiesner et al., 2011, Souppart et al., 2002), solid phase extraction (SPE) (Huang et al., 2009), and liquid phase micro extraction (LPME) (Magalhaes et al., 2010).These methods have been used by different studies in determining artemether and DHA in plasma samples.

LLE is also known as solvent extraction. It is used to separate compounds, based on their relative solubility, into aqueous and organic solvents. The two

solvents have to be immiscible for separation to occur (Zang et al., 2008). It is simple, cheap and is compatible with most analytical systems (Raikos et al., 2009)

SPE is an affinity-based method used in separating analytes from unwanted matrix components. Unlike liquid-liquid extraction which uses two immiscible liquid phases, it involves separating unwanted matrix components from the analytes between a liquid and a solid phase. The analytes, which have high affinity with the solid phase interact with and are absorbed by it, whilst the matrix components have the opposite effects. (Zwir-Ferenc and Biziuk, 2006).

LPME is a modified form of LLE that uses microliters of solvents for the extraction process. The development of LPME was brought about to overcome the limitations observed with LLE. These included the tedious multistep technique which offered a possible source of contamination or analyte loss with a possible effect on measurement outcomes and the use of large amounts of toxic organic solvents (Lucena et al., 2009). LPME method is based on diffusion, in which a high partition coefficient is used for extraction. Several factors can influence the extraction outcome and they include the volume ratio of extractant (acceptor) and sample (donor) phases, pH of the phases, ionic concentration of the donor phase, extraction time, organic solvent properties and sample agitation. The chemical nature of some analytes can give poor partition coefficient thus preventing them from being extracted. This has led to the development of various techniques like the supported liquid membrane to overcome the problem (Xu et al., 2009).

Authors have reported success, though some liquid-liquid extraction methods have generated low recoveries prompting many to try solid phase reaction. The problem identified in liquid-liquid extraction is the interactions that occur when organic solvents are added during sample processing, causing analyte and metabolite degradation. Compounds are relatively stable in haemolysed plasma (in the presence of iron products) but degrade rapidly when they come into contact with organic solvents (Lindergardh et al., 2008). Despite these concerns, the use of liquid-liquid extraction is still being used and has proven to be effective (Wiesner et al., 2011)

4.1.4 Criteria for validation/ Method validation parameters

Specificity and selectivity

Specificity is considered as the ability to determine unambiguously the analyte of interest in the presence of other compounds. On the other hand selectivity refers to the ability to differentiate and measure the analyte in the presence of other compounds.

Linearity

The linearity is the ability to test the goodness-of-fit of the model. In other words, it is the ability to obtain results that are directly proportional to the concentration of analyte in the sample. It is important in validation for the exploration of the calibration range for providing an adequate calibration model which includes the concentration range within which the method is likely to comply with the acceptance requirements.

Precision and accuracy

The precision is the closeness of agreement between a series of measurements obtained from multiple samples taken from the same homogenate sample batch. The accuracy is defined as the closeness of test results to the true or reference value. It is sometimes referred as bias and it is often measured as a percent deviation from the accepted reference value.

LLOQ and LLOD

LLOQ corresponds to the lowest concentration that can be quantified within acceptable precision and /or accuracy requirements. LLOD is the lowest amount of analyte that can be detected.

Matrix effects

It is described as the result of co-eluted matrix components that affect the detection capability, precision, or accuracy for the analytes of interest. The main manifestation of matrix effect in LC-MS analysis is ion suppression which is associated influencing the extent of analyte ionisation. This influence is often observed as a loss in response thus the term ion suppression. However, depending upon the type of sample it also can be observed as an increase in the response of the desired analyte.

Recovery

Recovery is related to the extraction efficiency of an analytical method. The recovery of a desire analyte compares the detector response obtained from the extracted analyte which is spiked into the biological matrix, and the detector response obtained for the true concentration of the pure reference standard range

The assay range is defined as the interval between the lowest and the highest sample concentration for which the quality of precision and accuracy of the method is acceptable (Chandramouli et al., 2010)

4.1.5 Aims

- To optimise and validate a liquid chromatography mass tandem spectrometry to quantify artemether and its active metabolite dihydroartemisinin in human plasma.

- To optimise and validate liquid chromatography mass tandem spectrometry that can be used to analyse patient samples

4.2 Materials and Methods

4.2.1 Reagents and materials

Artemether (ARM), DHA and artemisinin (internal standard, IS) (Fig.1), acetonitrile (MeCN), formic acid, methanol, ammonium formate (NH_4FA) and water were obtained from Sigma Aldrich. All chemicals and water were LC-MS grade. Human plasma was obtained from volunteers.

4.2.2 Instrument and analytic conditions

4.2.2.1 HPLC equipment

HPLC analysis was performed with Dionex Ultimate 3000 micro pump system (Thermo Scientific, Warrington, UK). Chromatographic separation was performed on C_{18} XDB analytical column (150mm x 2.1mm, 5µm particle size) equipped with its compatible guard column purchased from Agilent.

4.2.2.2 Chromatographic conditions

Chromatographic separation was performed according to conditions described by Huang et al. (2009) which was modified to improve peak shape for accurate quantification. The reported mobile phase used consisted of: solvent A, an aqueous 10mM Ammonium formate at a pH of 4.1 and solvent B, Acetonitrile with 0.1% formic acid. LC elution was accomplished with 20% solvent A and 80% solvent B in isocratic mode at a flow rate of 1mL/min for 6mins and the injection volume was 50µL. The modified method consisted of the same gradient but solvent B was replaced with methanol with 0.1% formic acid at a flow rate of 200µL/min for 15mins. The pH of NH_4FA was adjusted by titration with formic acid. The injection volume was 20µL.

4.2.3 Optimisation and validation of mass spectrometric conditions

4.2.3.1 MS instrumentation

A hybrid triple quadupole/ion trap mass spectrometer (4000 QTRAP) from ABSciex (Manchester, UK) was operated in positive mode using the TurbolonSpray source.

4.2.3.3 Tuning and optimisation of source- and compound-dependent parameters

Source dependent parameters were optimised using flow injection analysis (FIA). Three different values were selected for each of the following parameters: Turbo gas (TEM), curtain gas (CUR), nebulizer gas (GS1), auxiliary gas (GS2) and the ionspray voltage (IS). Compound dependent parameters defined by the manufacturers trademark as declustering potential (DP) and collision energy (CE) were optimised by direct infusion. Automatic and manual optimisation was done by infusing the mixture of artemether, DHA and the I.S (1µg) at 200 µl/min. Ammonium adduct $[M+NH_4]^+$ ion pairs were selected from Q1. The MRM ion pair was *m/z* 316→267 for artemether, m/z 302→267 for DHA and m/z 300→209 for artemisinin (I.S). The optimised acquisition parameters were as follows: Turbo (Heater) set at 250°C; Curtain gas (CUR), 40psi (99.9% nitrogen); Nebulizer Gas (Gas 1), 60 psi (nitrogen); Auxillary (turbo) Gas (Gas 2), 20 psi (nitrogen); Collision-Activated Dissociation (CAD) Gas:4: IonSpray Voltage (IS), 5000 v. The optimised parameters are summarised in Table 4.1. The scan time was set at 100 ms for each transition. Data was processed with analyst software.

4.2.3.4 Preparation of calibration standards

Primary stock solution of artemether, DHA and artemisinin were each prepared in methanol at 1 mg/mL. Artemether, DHA and I.S. solutions were diluted with 50% methanol in water to prepare working stock solutions (1µg/mL) and working solutions. Calibration standards were prepared in concentrations ranging from 0.5 - 50 ng/mL. They were prepared in blank human plasma. The I.S. concentration was 5 ng/mL. All solutions were kept at -80 °C.

4.2.3.5 Extraction procedure

4.2.3.5.1 Method 1

Analyte extraction was performed according to the liquid-liquid extraction method mentioned in Huang et al. (2009). Taking into account the volume of samples, 50 µl of plasma was added to 900 µL of methyl t-butyl ether in a glass tube instead of the 100 µL.. The mixture was vortexed for 1 min and then placed on a tube rotator for 30 min. The extraction tubes were then placed on dry ice until the plasma became frozen (~30 sec). The liquid organic phase was then transferred to a clean tube and left at room temperature in a vented fume hood overnight to dry. The residue was then reconstituted in methanol water (50:50 (v/v) and vortexed for 30 s. The samples were then transferred into an auto sampler vial and 20 µl was injected into the LC-MS/MS system.

4.2.3.5.2 Method 2

An alternative method for artemether and DHA extraction [17] was employed for comparison (Wiesner et al., 2011). The procedure was carried out on ice. 50µl of plasma was added to 200µl of Britton Robinson universal buffer (0.1 M, pH 10). 2 ml ethyl acetate was then added vortexed for 1 min and centrifuged at 16,000 rpm for 5 min. The organic phase was then transferred to tubes and evaporated under nitrogen gas at 30 °C. The residue was reconstituted in methanol water (50:50 (v/v) and vortexed for 30 s. The eluents were filtered with 0.25 µm 96 well plate and transferred into an auto sampler vial and 20µl was injected into the LC-MS/MS system.

4.2.4 Method validation

4.2.4.1 Recovery and matrix effects

The recoveries of the extraction method were prepared by spiking blank plasma with known amounts of Artemether, DHA and I.S. Six replicates were carried out at three different levels of concentrations, low 5 ng/mL, medium 15 ng/mL and high 50 ng/mL. Recovery was determined by comparing the peak areas of the spiked compounds in plasma against their corresponding solutions.

The recovery of each of the compounds was calculated using the formula below

$$\text{Recovery} = \frac{A}{B} \times 100$$

Where set A was the mean peak area (n=6) of the compound spiked in plasma prior to extraction. Set B was the mean peak area (n=6) of neat solution of the compounds.

For the matrix effects, six replicates of plasma were post extraction. The matrix effects was determined using the formula below

$$\text{Matrix effects} = \left(\frac{C}{B} - 1\right) \times 100$$

Where set A and B is the same as above and set C the mean peak area (n=6) of spiked plasma after extraction.

4.2.5.2 Calibration curve, reproducibility and precision

Calibration curves were obtained by linear regression of the peak area ratio of the analyte to the internal standard (Y-axis) versus the nominal analyte concentrations (X-axis). The plots showed linearity ($R^2 \geq 0.99$). Calibration curves in plasma were constructed by spiking blank plasma with standard solutions of Artemether and DHA to produce concentrations of 0.5 – 50 ng/mL. I.S was added to make 5 ng/mL. Eight point calibration curves were constructed for each run.

Reproducibility and precision of the method was assessed by determining the-inter and intra-day variability of the compounds in plasma. Intra-assay (within–day) precision and accuracy was determined by analysing six replicated of three concentrations (low, medium and high) 5, 15 and 50 ng/mL, of each analyte in the same day and run. The inter assay (day to day) variability was assessed by analysing

3 replicates of the three concentrations (5, 15 and 50 ng/mL) of artemether and DHA on three different days. The relative standard deviation (RSD) was then calculated.

The accuracy of the method was assessed by comparing values from the spiked plasma at low, middle and high concentrations and comparing them with values from the neat solutions using the matrix matched calibration curves.

4.2.5.3 Lower limit of quantification (LLOQ) and Lower limit of detection (LLOD)

LLOQ corresponds to the lowest concentration that was quantified within acceptable precision and/or accuracy requirements. LLOD is the lowest amount of the analyte that can be detected. LLOD were found by injecting 20 µl of spiked plasma and solution mixture at decreasing concentrations using signal-to-noise approach of 3:1 for LLOD and using the lowest drug concentration that could be determined with an accuracy of 80 – 100 % and a precision of ≤ 20 % for LLOQ.

4.3 Results and discussion

4.3.1 LC-MS/MS optimisation

Simultaneous determination of artemether and DHA in human plasma have been analysed using a range of different analytical methodologies, but the most recent methods employed are liquid chromatography mass spectrometry (Wiesner et al., 2011, Magalhaes et al., 2010, Huang et al., 2009). Initially, a 2000API ABSciex mass spectrometer was employed for method optimisation. However, this instrument proved to be inadequate for the required sensitivity.

The 4000 QTRAP mass spectrometer is characterised for its enhanced sensitivity and selectivity compared to previous generations of triple quadrupole instruments. MS/MS scans were performed in MRM mode. Reversed-phase separation on a C-18 column was achieved for artemether, DHA and the internal standard quantitative and qualitative analysis (Huang et al., 2009, Souppart et al., 2002). The MRM optimisation procedure involves a sequence of experiments where the voltages of the various ion optics parameters (i.e DP and CE) are ramped to determine the maximum signal intensity for each ion (Grebe and Singh, 2011).

Table 4.1 MS/MS product ion spectra of precursor ions of artemether, DHA and I.S

Name, m/z	DP	EP	CE	CxP
ARM, 316/267	30	3	10	8
DHA, 302/267	30	6	15	10
Artemisinin 300/209	23	12	15	12

MS/MS product ion spectra of the precursor ions of artemether, DHA and artemisinin (I.S). DP is declustering potential, EP is entrance potential, CE is collision energy, CxP is collision exit potential

The values were first scanned automatically by using an application of the software (Analyst. software version 1.5 (AB Sciex)) that operated the instrument. Manual optimisation of compound dependent parameters were also performed to verify those values obtained automatically. The selected parameters are shown in the Table 4.1. Ammonium adducts $[M+NH_4]^+$ were selected for the detection of the

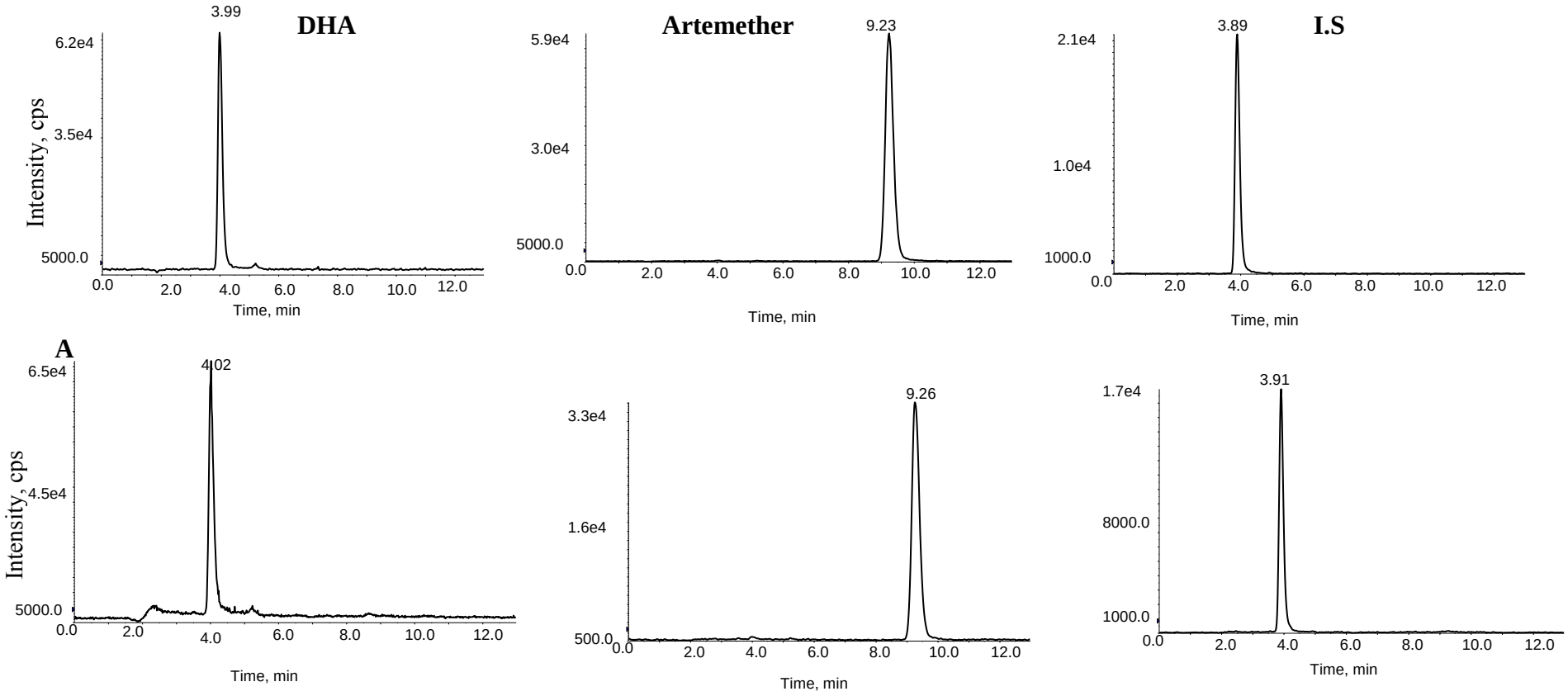
analytes. The precursor-product ion pairs monitored in MRM mode were 316/269 for Artemether, 302/267 for DHA and 300/209 for the internal standard Artemisinin.

The ion pairs obtained were consistent with reports from Huang et al (2009) although they used API 2000. The optimised acquisition parameters derived were as follows: Curtain gas (CUR) – 40 psi, IonSpray (I.S) voltage – 5000V, Temp - 250°C gas 1 (GS1) – 60 psi and gas 2 (GS2) – 20 psi. These parameters were consistent with those reported by Weisner et al (2011) who also used QTrap 4000. The organic mobile phase was changed from acetonitrile to methanol to obtain better and sharper peaks. The run time was then increased 5 min to 13 min because artemether took longer to elute in methanol compared to acetonitrile. The average eluting times for artemether and DHA were 9.5 and 4.1 min respectively, and 3.9 min for artemisinin as shown in figure 4.1.

4.3.2 Sample preparation

The extraction procedure of choice in clinical samples is critical because it needs to be robust and sensitive (Strathman and Hoofnagle, 2011). Some reports have suggested the degradation of compounds when samples came into contact with organic solvents during sample preparation especially when employing liquid-liquid extraction, which is thought to be minimised in solid phase extraction. Another aspect that is critical in sample preparation is the drying step as compound loss might occur when using Nitrogen gas (Huang et al., 2009). Nevertheless, several studies have shown good recovery levels with liquid-liquid extraction using different organic solvents (Wiesner et al., 2011, Malgahaes et al., 2010, Souppart et al., 2002).

Figure 4.1 Chromatographs of DHA, artemether and I.S



Representative chromatographs at 20 ng concentration (I.S – 5 ng/ml) of DHA, artemether and artemisinin in drug solution and spiked plasma.

Two liquid-liquid extraction methods were compared. These methods were selected on the basis of their low cost, ease of setting up and reproducibility. The method mentioned by Huang et al (2009) showed recoveries of more than 80% on extraction of samples. A second method by Weisner et al. (2011) was also employed for comparison purposes. Both methods were adapted to use less plasma (50 µl) than the volume reported.

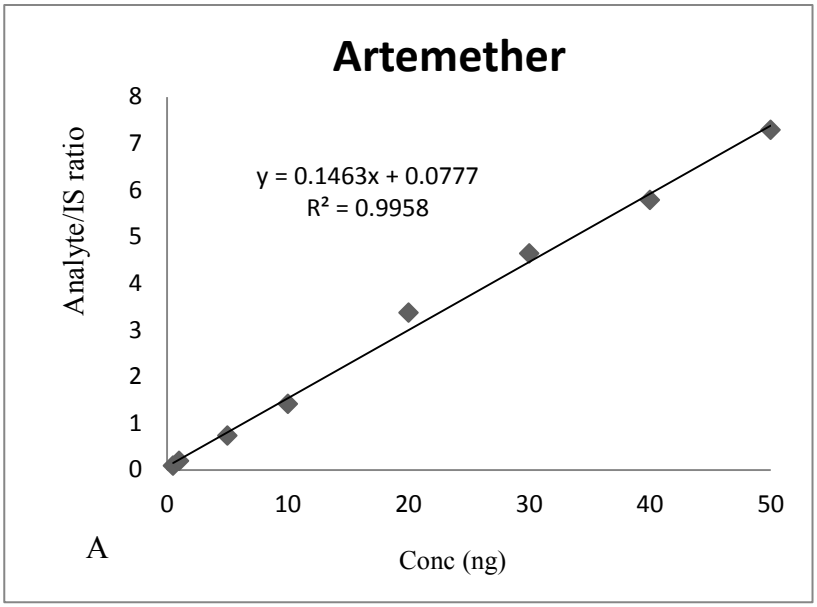
4.3.3 Method validation

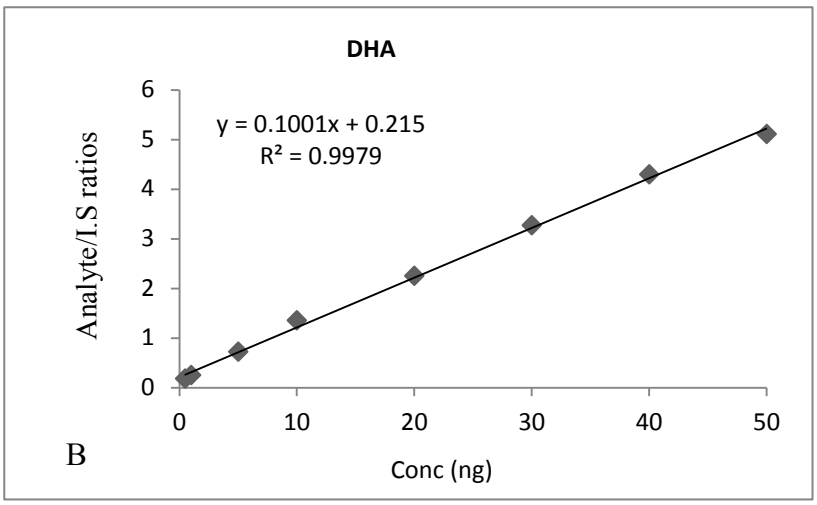
4.3.3.1 LLOD and LLOQ

The LLOD for artemether and DHA were 0.1 ng/ml and 0.4 ng/ml, respectively. The LLOQ were determined to be 0.3 for artemether and 1ng/ml for DHA. Other published data have showed LLOQ to be 2 ng/mL (Wiesner et al., 2011, Huang et al., 2009) and 5ng (Souppart et al., 2002, Mohamed et al., 19990 for both artemether and DHA. Huang et al. reported having LLOQ of 1ng/ml with liquid-liquid extraction but was not reproducible prompting them to use solid phase extraction. None of the literature reviewed (Wiesner et al., 2011, Huang et al., 2009, Souppart et al., 2002) mentioned their LLOD except one (Mohamed et al., 1999)that reported a LLOD of 2ng/ml. The present LC-MS/MS method showed better sensitivity compared to previously reported values for LLOD and LLOQ for the simultaneous determination of artemether and DHA in clinical samples even using lower sample volumes.

4.3.3 Linearity

Figure 4.2 Standard calibration graphs for artemether and DHA





Representative graph of standard calibration lines for artemether (A) and DHA (B)

An appropriate calibration model is essential in obtaining reliable analyte quantification. The relationship between analyte concentration in the samples and detection response was investigated by using internal standardisation. Standard curves were evaluated and found to be linear in the range between 0.5 to 50 ng/mL. Linearity was lost after 50 ng/mL. Representation of calibration lines for artemether and DHA are shown in Figure 4.2. Linear regression on the concentration data showed that both analytes were linear for the same range of concentrations (R^2 $\geq$0.99).

4.3.4 Matrix effects and use of internal standard

Internal standardisation was selected to obtain accurate quantification of the analytes. Artemisinin was used as an internal standard because it has similar chemical composition as artemether and DHA, and is relatively stable. Artemisinin also provides a good intra and inter-assay precision and provides compensation for unforeseen matrix effects (Lindegardh et al., 2009) and has also been used previously as an internal standard (Huang et al., 2009, Classen et al., 1999) hence its use in this study.

The determination of matrix effect is important for precision, accuracy and robustness of a bioanalytical method (Chambers et al., 2007). Matrix effects of the compounds were evaluated during the validation of the LC-MS-MS method. Dried extracted plasma samples were reconstituted with 50% methanol and spiked with artemether, DHA and the I.S. at high (50 ng/mL), middle (15 ng/mL) and low (5 ng/mL) concentrations. The mean response of each analyte spiked post extraction

was compared to the same analyte concentration in solution. The matrix effect for artemether, DHA and the I.S are shown in Table 4.2.The results show that the analytes and internal standard were not significantly affected by ion suppression. The matrix effect for DHA showed 4, 7 and 2 % enhanced for 5, 15 and 50 ng/ml concentration and artemether showed 2 % suppression for 5 ng/mL and 0 % and 2 % enhancement for 15 and 50 ng/ml.

4.3.5 Precision and accuracy

Plasma samples were extracted by using Method 1, described earlier. Artemether and DHA spiked drug free plasma samples were prepared at low, medium and high concentrations. Six replicates of extracted spiked samples of both analytes were run in sequence on three different days. This was performed in order to obtain intra and inter-day precision and accuracy. Recovery was calculated by comparing the response of plasma samples spiked with Artemether and DHA standards prior to extraction, with corresponding standard solution concentrations at low, medium and high concentrations. The recoveries for Artemether, DHA and the I.S are shown in Table 4.3. Percentage recoveries for artemether (n=6) at low and middle concentrations were 82 % and high concentration at 80 %.

DHA showed recoveries of 102, 97 and 84% for low, middle and high concentration and the I.S had recoveries between 73 to 76%. Recoveries by published data have shown percentages ranging from 73 to 95% for artemether and 76 to 99% for DHA (Wiesner et al., 2011,Huang et al, 2009 Souppart et al., 2002, Mohamed et al., 1999). Souppart et al. (2002) and Wiesner et al. (2011) employed

liquid-liquid extraction and obtained recoveries of 76 and 84% and 80 and 77% and 86 and 76% for low and high concentrations of artemether and DHA respectively. These results are consistent with those obtained in this study.

Table 4.2 Recovery values of artemether, DHA and I.S

	Conc (ng/mL)	% Recovery±SD (n=6)	%CV
ARM	5	82±5.9	7
	15	82±2.3	3
	50	80±1.1	1
DHA	5	102±14.6	14
	15	97±8.4	9
	50	84±2.1	3
I.S	5	73±4.2	6
	15	76±2.8	4
	50	73±0.7	1

Recovery expressed in % ± SD. Precision is expressed in %CV

Studies that employed solid phase extraction showed recoveries of 95% (Mohamed et al., 1999) and 81 and 73% for high concentrations of artemether and 92% and 99 and 90% for DHA (Huang et al., 2009). Lindegardh et al. (2008) discussed in detail the problems that occur during analyte analysis highlighting that sample processing is the most critical step during analysis.

It has been previously mentioned that organic solvent added to plasma during sample preparation may cause significant analyte degradation and a solid phase extraction method was proposed as an alternative solution (Lindegardh et al., 2008). However, a report showed that it might not be superior to liquid-liquid extraction for

artemether analysis. Huang et al. (2009) on the other hand obtained significantly higher recoveries, from 40-60 % to around 84 %, when the organic solvent was left to dry at room temperature. This highlights that low recoveries obtained from liquid-liquid extraction might not necessarily be due to the addition of organic solvents but the drying process.

Table 4.3 Intra-assay variation of artemether and DHA in plasma

	Conc (ng/mL)	Mean±SD (ng)	%CV	Accuracy
ARM	5	4.5±0.2	4.4	90
	15	14.8±0.7	4.7	99
	50	49.5±2.9	5.8	99
DHA	5	4.2±0.5	11.9	84
	15	14.6±1.1	7.5	97
	50	46.1±4.4	9.5	92

Recovery is expressed in % ±SD n=6. Accuracy is expressed as %. Precision is expressed in %CV

Table 4.4 Intra-assay variation of artemether and DHA in plasma

	Conc (ng/mL)	Mean±SD (ng)	%CV	Accuracy
ARM	5	4.7±0.4	8.5	94
	15	14.4±0.3	2.1	96
	50	47.5±1.7	3.6	95
DHA	5	4.5±0.7	8.8	90
	15	12.4±1.1	8.9	83
	50	42.7±4.3	10.1	85

Recovery is expressed in % ±SD n = 6. Accuracy is expressed as %. Precision is expressed in %CV

The results of intra-day accuracy for the analytes ranged between 92 to 99% from low to high concentrations for artemether and 84 to 97% for DHA (Table 4.4). Inter-day accuracy ranged between 94 to 96% and 83 to 90% for artemether and DHA (Table 4.5). Intra-day precision ranged from 4.4 to 5.8 % for artemether and 7.5 to 11.9 % for DHA and inter-day precision ranged from 2.1 to 8.5 % for artemether and 8.8 to 10.1 % for DHA.

The percent coefficient of variance (% CV) value for both analytes at low, middle and high concentrations were all <15% which is in accordance with the suggested guidelines for quantitative bioanalysis provided by the FDA (Viswanathan et al., 2007).

4.4 Conclusions

A sensitive and robust LC-MS/MS was developed for the simultaneous analysis of artemether and DHA. This method has shown that liquid-liquid extraction method can provide good recoveries using relatively low sample volumes and showing good accuracy and precision in accordance with the FDA guidelines. The extraction method is relatively cheap and simple which are important considerations in drug analysis and monitoring in developing countries with limited resources. It makes it a reliable methodology for the simultaneous quantification of artemether and DHA in plasma in vulnerable population such as children and pregnant women where sample collection volume might be limited.

5 Plasma level of Artemether and Dihydroartemisinin (DHA) in malnourished children

5.1 Introduction

In response to widespread resistance of the malaria parasite to traditional antimalarials, the WHO recommended the use of artemisinin-based combination therapy (ACT) for the treatment of uncomplicated malaria. ACT is a combination of a short acting artemisinin derivative (artemether and artesunate) with a longer acting drug. This is designed to impede the emergence of parasite resistance and avoid the high recrudescence rate observed in artemisinin monotherapy (WHO, 2001).

ACTs are known to be efficacious and safe in children and are recommended for children from > 6 months and 5kg. Although there has been a decline in the incidence of malaria by 17% between 2000 and 2010, children under the age of 5 years still account for 85% of the deaths (Mwesigwa et al., 2010, Stepniewska et al., 2009).

Of the ACT's Artemether/lumefantrine combination was the first pre-qualified fixed dose recommended by the WHO and is the most widely adopted ACT in Africa. The two drugs show synergistic action against *P. falciparum* and act at different stages of the parasite life. A tablet contains 80mg of artemether and 120 mg of lumefantrine (Premji, 2009). Table 5.1 shows the dosage regimen per kg body weight.

Table 5.1 Artemether/Lumefantrine Dosage Regimen

Weight (Kg)	Number of tablets (0, 8h, 24h, 36h, 48h, & 60h)	Artemether/lumefantrine
5 – 14	1	20 mg A + 120 mg L
15 – 24	2	40 mg A + 240 mg L
25 – 34	3	60 mg A + 360 mg L
>34	4	80 mg A + 480 mg L

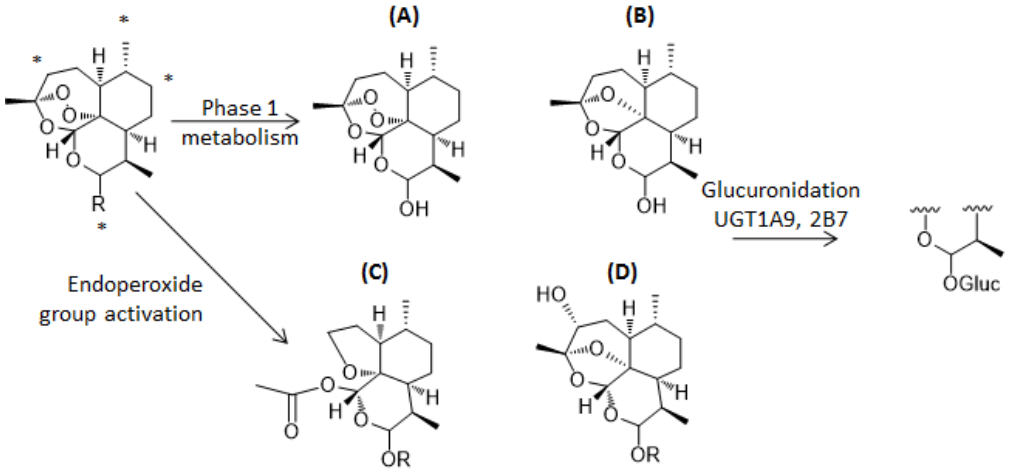
Artemether (A) and lumefantrine (L) combination tablets per dose in milligrams (mg) per kilogram (KG) bodyweight. Each tablet contains 20 mg of artemether and 120 mg of lumefantrine.

Artemether is a lipid soluble derivative of artemisinin and is relatively stable in biological fluids. Although the exact mechanism of action of the artemisinin derivatives in the malaria parasite is still under debate, the involvement of iron for its activation is widely accepted (Stocks et al., 2007, Haynes et al., 1996, Meshnick et al., 1991). Artemether has extensive first pass metabolism which contributes to its incomplete oral bioavailability (Kokwaro et al., 2007). Food has a significant effect on artemether bioavailability. There is a two fold increase in artemether in individuals who have consumed a high fat meal compared to fasted individuals (White et al., 1999). Artemether is readily absorbed and peaks in plasma in 1.5 to 2 h (White et al., 1999, Ezzet et al., 1998). It is highly bound to plasma proteins (92 to 98 %) primarily to α_1- acid glycoprotein (33%), albumin (17%), high density lipoproteins (12%) and low density lipoproteins (9.3%) (Aweeka and German, 2008, White et al., 1991).

Artemether hydrolyses in vivo to its active metabolite dihydroartemisinin (DHA) (A), predominantly via the activity of the cytochrome P450 (CYP) enzymes

CYP3A4 and CYP3A5. DHA is intrinsically more active as an antimalarial, (Van Agtmael et al., 1999, Lefévre et al., 1999). Figure 5.1 shows a chemical illustration of the metabolic pathway of artemisinin, the parent compound of artemether The role of intestinal CYP3A4 was established with studies where grapefruit juice, an inhibitor of CYP3A4, caused the plasma concentration of artemether to double (Van Agtmael et al., 1999).

Figure 5.1 Metabolic pathway of the artemisinin compounds



Schimatic diagram of the major and minor metabolic pathway of the artemisinin compound.
* Sites of CYP-mediated hydroxylation, Gluc: A glucoronide group

DHA reaches plasma peak concentrations around 2 h after dosing and is converted to inactive metabolites (B) and rapidly cleared by phase II glucoronidation, via the uridine 5'-diphosphate-glucuronosyltransferases (UGT) family of enzymes, UTG1A1, 1A8/9 and 2B7 (Maggs et al, 2000, Batty et al., 1998). In addition, chemical activation of the endoperoxide group can occur in the systemic circulation leading to the formation of two minor metabolites, the THF acetate isomer (C), and hydroxydeoxo isomer (D) (Figure 5.1) and are excreted as their glucoronide counterparts (Maggs et al., 1997).

Although ACT is the recommended treatment of uncomplicated malaria in children > 6 months/ > 5kg, published pharmacokinetic studies are mostly limited to adults with little detailed information for children. Paediatric dosing is mainly derived from adult-based regiments adjusted for body weight (Mercer and Sarr Sallah, 2011). Table 5.2 summarises the findings of pharmacokinetic studies in children. Pharmacokinetics of rectally administered artesunate is the most well-studied dosing regimen in children (Keranjeewaet al., 2004, Halpap et al., 1998, Sirivichayakul et al., 2007, Sabchareon et al., 1998, Krishna et al., 2001, WHO, 2002).

Differences in study design and blood sampling schedules, in addition to the small groups of subject studies, can introduce errors and skew pharmacokinetic values. However, despite this, similar values of DHA were reported for each paediatric studies. Similar C_{max} values were also reported when studies of similar designs were compared between adult and paediatric groups but on the contrary,

AUC values was higher and t_{max} values almost double in adults when compared to the paediatric population (WHO, 2002).

Pharmacokinetic properties of several orally administered ACT has also been studied in children (Mwesigwa et al., 2010, Abdulla et al, 2008, Sabchareon et al., 1998, Ramharter et al, 2007, Sidhu et al., 1998, Ramharter et al., 2008), with artesunate being the most common ACT. Sidhu et al (1998) investigated potential differences between adults and children. It was reported that adults clearance rate (C_L) in adults were slightly lower compared to children which may explain the reported longer half-life in of artemisinin in adults (2.6 h) than children (1.8 h). This is comparable to data from intra rectal studies demonstrating that adults may be exposed to higher amounts of drugs due to decreased clearance.

Physiological parameters of development and nutrition in children with malaria, in combination with the variable physiochemical properties of the ACT, can affect drug absorption, metabolism and excretion and hence determine individual drug exposure levels (Van den Anker, 2010, Mahmood, 2007). Malnutrition and malaria cause high morbidity and mortality and are sometimes associated, especially in Sub-Saharan Africa (Fillol et al., 2009). Morbidity and mortality of malaria is increased in children with PEM with the protective efficacy of intermittent preventive treatment observed to be 50% compared to normally nourished children (Danquah et al., 2009, Deen et al., 2002).

Table 5.2 Summary of pharmacokinetic studies in paediatric patients treated with ACT

Source	Country	Dose mg/kg	Population	Study Design	Cmax DHA (µM)	Tmax DHA (h)	$AUC_{0-\infty}$ ($\mu mol.h.L^{-1}$)
Rectal Administration (Artesunate Montherapy)							
Halpaap et al (1998)	Gabon	1.8 (median)	7 - 12 years 12 patients (*u.m.*)	Blood samples collected at 0.5, 1, 3 h following first dose and 0.5 h following second dose at 4 h.	0.6 (55)	1.1 (51)	N.D.
Sabchareon et al (1998)	Thailand	15 (median) (+ mefloquine)	5 – 12 years 9 patients (*u.m.*)	Blood samples collected at 0, 0.5, 1, 1.5, 2, 3, 4, 6, 12, 24, 30, 36, 48, 54, 60, 72 h following administration.	2.4 (87) (range 0.3 -5.7)	N.D.	*6 (0.2 – 8.7)
Karunajeewa et al (2004)	Papua New Guinea	13	5 – 10 years 47 patients (*u.m.*)	Blood samples collected at 0, 1, 2, 3, 4, 6, 8 ,12 h following first dose and at 2, 4, 8, 12 h following second dose at 12 h.	2.5 (range not stated)	2.3 (range not stated)	N.D.
Krishna et al (2001)	Ghana	10 20	2 – 7 years 26 patients (*m.s.m*)	Blood samples collected at 0, 0.25, 0.5, 1, 1.5, 2, 4, 8, 12 h following dose.	2.4 (0.8 – 5.8) 3.1(0.7 – 6.8)	1.7 (0.9 – 3.2) 1.8 (0.6 – 3.3)	9.8 (1.4 – 28.2) 13.2 (2.9 – 26.2)
Sirivichayakul et al (2007)	Thailand	10 20	5 – 10 years 16 patients (*u.m.*)	Blood samples collected at 0, 0.5, 1, 1.5, 2, 3, 4, 6, 8 h following dose.	3.2 (1.2 -6.6) 5.4 (0.7-16.1)	1.5(1.0-3.0) 2.0 (1.0 -4.0)	8.5(0.1 – 50.1) 19.8 (4.4 – 63.0)

WHO (2002)	Ghana	10	2 – 7 years	Blood samples collected	2.8 (50)	1.8 (49)	9.8 (72)
		20	36 patients	at predose to 12 h post	4.0 (75)	2.2(53)	18.6 (130)
			Adults	dose.			
	Thailand	10	96 patients		3.8 (68)	2.8 (61)	15.1 (106)
		20		Abbreviated sampling	7.1 (80)	4.2 (50)	40.1 (104)
	South		27 Patients	predose to 8 h post dose			
	Africa	10	(*m.s.m.*)	.	3.0 (79)	4.4 (52)	14.3 (76)
Oral Administration (ACTs)							
Bethell et al (1997)	Vietnam	3	6 – 15 years	Blood samples collected	2.4 (CI 95% 1.3 –	1.7 (CI 95%	4.5 (CI 95%
			10 patients	at 0, 0.25, 0.5, 1, 2, 3, 4,	3.3; range 0.6 –	0.8 – 2.6;	3.1 –6.0;
			(*m.s.m*)	6, 8, 10, 12, 24 h	4.9)	range 0.3 –	range 2.1 –
		Artesunate		following		4.0)	8.0)
				administration			
Ramharter et al (2007)	Gabon	4 (granules)	10 - 20 kg	Blood samples collected	2.9 (CI 90% 2.4 –	1.5 (1.1 – 6.1	10.1 (CI
				at 0, 0.5, 1, 1.5, 2, 3, 6 h	5.9; range 0.5 –		90% 7.7 –
				following	11.9)		14.5; range
				administration			4.9 – 23.5)
		8 (co-blister)			3.1 (CI 90% 2.6 –	1.5 (0.4 –	
		Artesunate	20 – 40 kg		5.0; range 1.0 –	4.1)	9.4 (CI 90%
		(+	12 patients		7.7)		7.3 –14.8;
		mefloquine)	(*u.m.*)				range 3.7 –
							27.4)

Ramharter et al (2008)	Gabon	6:2 (A), 9:3 (B), 12:4 (C) ratio (pyronaridine: artesunate) Tablets (fixed-dose combination)	2 -14 years 10 – 40 kg 60 patients (*u.m.*)	Blood samples collected at 0, 0.25, 0.5, 1.0, 1.5, 2.5, 4.0, 8.0, and 12.0 h following administration of first drug.	A – 1.7 ± 1.1 B - 3.3 ± 1.9 C – 4.2 ± 1.3	A – 1.4 ± 0.7 B – 1.7 ± 1.9 C – 1.7 ± 0.6	A – 3.7 ± 1.6 B – 7.0 ± 3.8 C – 10.4 ± 3.4
Sabchareon et al (1998)	Thailand	6 Artesunate (+ mefloquine)	5 – 12 years 10 patients (*u.m.*)	Blood samples collected at 0, 0.5, 1, 1.5, 2, 3, 4, 6, 12, 24, 30, 36, 48, 54, 60, 72 h following administration.	7.0 (71) (range 1.1 – 16.0)	N.D.	*13.2 (61) (range 2.4 – 27.1)
Mwesigwa et al (2010)	Uganda	4 Artesunate (+ amodiaquine) 2 Artemether (+ lumefantrine)	5 – 13 years 45 patients (*u.m.*)	Blood samples collected at 0, 2. 4, 8, 24, 120 h following administration	1.5 (1.5 – 2.2) 0.4 (0.4 – 0.5) Artemether: 0.1 (0.1 – 0.2)	N.D.	4.9 (4.6 – 6.1) 1.3 (1.2 – 1.5)

Abdulla et al (2008)	Benin, Kenya, Mali, Mozambique, Tanzania	1.5 – 4 (based on number of tablets per weight group) Crushed tablets Dispersible Artemether (+ Lumefantrine)	0 – 12 years 5 – 35 kg (*u.m.*) 91 patients 93 patients	Blood samples taken at 1 and 2 h following administration	0.2 (90) Artemether: 0.6 (96) 0.2 (102) Artemether: 0.6 (88)	N.D.	N.D.
Sidhu et al (1998) ***Values reported for artemisinin.***	Vietnam	10 Artemisinin	2 – 12 years 23 patients Adults 16 – 45 years) 31 patients *(u.m.)*	Blood samples collected at 0, 2.5 or 4, 8 h in children and 0, 2.5 or 4, 10 h in adults following administration	Clearance (CL/F) 13.2 (8) $L.h^{-1}kg^{-1}$ 402 (5) $L.h^{-1}$ (9.3 $L.h^{-1}kg^{-1}$ based on median weight 46.5 kg)	Distribution Volume (V/F) 36.7 (9) $L.kg^{-1}$ 1504 (6) L. (34.4 $L.kg^{-1}$ based on median weight 46.5 kg)	

*AUC_{0-12}

ACT: Artemisinin combination therapy; DHA: Dihydreoartemisnin; MSM: Moderately severe malaria; UM: Uncomplicated malaria

Despite the high morbidity and mortality caused by PEM in children, pharmacokinetic studies of essential drugs have declined since the 1970 (Oshikoya et al., 2010), with no known published studies in the artemisinin compounds. The current recommendation for the treatment of uncomplicated malaria for normally nourished children is the same as children with PEM (WHO, 2010).

It is important to consider the potential pathophysiological effect of malnutrition on absorption, distribution, metabolism and excretion of the artemisinin compounds. The absorption of lipids and fats are specifically reduced which may affect the more lipid soluble drugs (Murphy et al., 2002) like artemether.

Artemether is primarily bound to α_1- acid glycoprotein (AAG) (Aweeka and German, 2008, White et al., 1991), in a reversible state of equilibrium which is generally maintained between bound and unbound drug fractions. Only free (unbound) drug fractions can distribute into tissue compartments thus any changes in their concentration might have an effect in artemether distribution. AAG concentration is decreased in disease state and malnutrition, which substantially increase plasma free drug in plasma. As a result, there may be greater amount of free-drug fraction of artemether in plasma and children with malnutrition may experience variations in their response to drug treatment or be at risk of increased drug toxicity (Oshikoya and Senbanjo, 2009).

In addition to decreased drug absorption, physiological effects of PEM can result in reduced drug-binding capacity, altered volume of distribution (V_d), increased half-life ($t_{1/2}$), altered drug biotransformation and reduced elimination and

clearance (C_L). These changes might have potential efficacy and safety issues in children with PEM, and thus might require drug-dosage adjustment (Oshikoya and Senbanjo, 2009, Oshikoya et al., 2010).

PEM can significantly impact on the degree of hepatic metabolism with animal studies showing a 55% reduction of total hepatic CYP activity (Cho et al., 1999). It also affects glucuronidation, a major DHA metabolism pathway, due to decreased blood glucose which is essential in the synthesis of glucuronic acid (Hamberg et al., 1990).

A review done by Mercer and Sarr Sallah (2011) has highlighted the potential effects of PEM on the pharmacokinetic properties of ACT, which might have an impact on efficacy and safety of these drugs on children with PEM. The review discussed the effect of PEM on the absorption, metabolism and excretion of the ACT compounds with the extent of the effects dependent on the type and severity of PEM. The effects of PEM may lead to an increased total body weight, delayed or decreased absorption of lipid soluble ACT, reduced protein binding capacity, altered volume of distribution (V_d), increased half-life ($t_{1/2}$), altered biotransformation and reduced elimination and clearance (C_L), with a potential of altering the efficacy and safety of the ACT in children with PEM (Mercer and Sarr Sallah, 2011).

Iron deficiency anaemia is a common condition associated with PEM as a result of low iron levels from poor nutrition (Macdougall et al., 1982). Another important factor highlighted in the review was the possible importance of iron deficiency. Due to the importance of iron in the bioactivation of the artemisinin

compounds, it was suggested that low levels could alter the pharmacological and toxicological profile leading to a decrease in the pharmacological activity in infected red blood cells or decreased extracellular detoxification resulting in the alteration of the benefit:risk ratio (Mercer and Sarr Sallah, 2011).

The use of ACT is increasing, especially in children under five years of age, who suffer the highest morbidity and mortality from malaria. Currently, the paediatric dosage is derived from adult based dosing regimen with no consideration regarding nutritional status. Protein energy malnutrition may have an impact on the absorption, distribution metabolism and detoxification of artemisinin drugs. Due to the prevalence of PEM in many malaria endemic countries, it is important to study the pharmacokinetic properties of these compounds in children with PEM.

The primary aim of this chapter was to investigate whether the current dose of artemether/lumefantrine (AL) provides adequate plasma drug concentrations for maximal drug efficacy in children with PEM. Artemether and DHA plasma concentrations in Gambian children were assayed, after the first dose of artemether/lumefantrine combination, using liquid chromatography-tandem mass spectrometry (LC-MS/MS).

The secondary aim of the study is to assess the effect of haemoglobin on the plasma concentration of artemether and its active metabolite, DHA. Artemether and DHA plasma concentrations were analysed against the haemoglobin levels of children.

5.2 Materials and methods

5.2.1 Study design and population

The study area and participants were the same as that described in Chapter 3, section 3.21 and 3.22. Briefly, children diagnosed with uncomplicated malaria and taking artemether/lumefantrine treatment were approached. Confirmation of a positive blood malaria smear, and haemoglobin concentration were available from the laboratory reports.

5.2.1.1 Inclusion criteria

- Diagnosis of uncomplicated malaria
- Age between 12 – 59 months
- Weight ≥ 5kg
- Blood film of *Plasmodium falciparum* infection of 1,000 parasites/μL
- Haemoglobin concentration of ≥ 7 g/dl
- No medication containing artemisinin 4 weeks prior to recruitment
- Willing to give informed consent

5.2.1.2 Exclusion criteria

- Diagnosis of complicated malaria
- Age < 12 and ≥ 60 months
- Weight < 5kg
- Medication containing artemisinin within 4 weeks

- Unwilling/ unable to give informed consent
- Haemoglobin concentration of < 8 g/dl

5.2.2. Ethical approval and Informed consent

The study received ethical approval from the University of Liverpool ethics committee (Ref. RETH000395) and The Gambia government-MRC joint committee (Ref. R10031). Parents/guardians and subjects were informed about the study in detail and questions and queries answered. Written consent was obtained in agreement for the study to be conducted.

5.2.3. Measurements of clinical parameters

Anthropometric measurements (weight, height) were taken. Weight was measured on a digital scale. Length was taken for children under the age of two years on a platform with a sliding headboard. Standing height was measured for children above two years with a height scale. Weight-for-height *z* scores (WHZ), weight-for-age *z* scores (WAZ) and height-for-age *z* scores (HAZ) were calculated on the basis of the National Centre for Health Statistics (NCHS/WHO) reference data set, version 3.2.2 software (WHO 2011). Z scores < -2 are suggestive of wasting (WAZ), underweight (WHZ) and stunting (HAZ). Scores < -3 suggest severe malnutrition. Age, sex and axillary temperature was recorded for every child.

5.2.4. Drug administration

The study drug (artemether/lumefantrine) was dispensed to the patients by the local health centre pharmacies based on body weight according to WHO guidelines (table 1). The first dose was administered under supervision with flavoured full fat milk, to enhance artemether absorption. For children who could not swallow the tablet(s) whole, they were crushed and given to them followed by 150ml of milk. Tablets were administered whole to children who were able to swallow them with 150 ml of milk. The dose was repeated if vomiting occurred within 30 min of administration.

5.2.5 Blood sample collection and plasma separation

An area on the forearm was identified and local anaesthetic applied. An intravenous cannula was inserted and a pre dose blood sample collected. The arm with the cannula was immobilised to maintain the cannula for a post dose blood collection. Venous blood samples were taken just prior to administering the first dose and at 120 min post dose. Samples were collected in sodium heparinised tubes and immediately placed on ice. They were centrifuged at 1,500 g for 15 min as soon as possible, within the hour. The plasma was stored at -80°C until shipment on dry ice to the University of Liverpool laboratory department. The maximum duration of sample storage was 20 months at -80°C.

5.2.6 Plasma sample analysis

Plasma concentrations of artemether and DHA were determined by liquid phase extraction and liquid chromatography-tandem mass spectrometry. The optimised method described in chapter 4 was used.

5.2.6.1 Instrumentation and chromatographic conditions

A hybrid triple quadupole/ion trap mass spectrometer (4000 QTRAP) from AB Sciex (Manchester, UK) was operated in positive mode using the Turbolon Spray source. Chromatographic separation was achieved on C_{18} XDB analytical column (150 mm x 2.1 mm, 5 μm particle size). Retention times for artemether and DHA were 9.5 and 4.1 min, respectively, and 3.9 min for artemisinin. LC elution was accomplished with 20% v/v aqueous 10mM ammonium formate at a pH of 4.1 (solvent A) and 80% v/v acetonitrile with 0.1% v/v formic acid (solvent B) in isocratic mode at a flow rate of 1mL/min for 6mins and the injection volume was 50μL.

The MRM ion pair *m/z* 316→267 for artemether, m/z 302→267 for DHA and m/z 300→209 for artemisinin which was used as an internal standard (I.S) were used. The optimised acquisition parameters were as follows: Turbo (Heater) set at 250°C; curtain gas (CUR), 40psi (99.9% nitrogen); nebulizer Gas (Gas 1), 60 psi (nitrogen); auxillary (turbo) Gas (Gas 2), 20 psi (nitrogen); collision-activated dissociation (CAD) Gas:4: IonSpray Voltage (IS), 5000 v. Calibration standards were prepared in concentrations ranging from 0.5 - 50 ng/ml and the standard curves were linear ($r^2 \geq 0.99$). No matrix effect was observed during method validation.

Average percentage recoveries for artemether, DHA and the internal standard were 81, 94 and 75 % and inter and intra-day variation accuracy were assessed to be above 80%. The lower limits of detection and quantification were 0.1 and 0.3 ng/ml respectively for artemether and 0.4 and 1 ng/ml for DHA.

5.2.6.2 Sample preparation

Liquid-liquid extraction was carried out as validated in chapter 4. Briefly, plasma (50 µl) was added to methyl t-butyl ether in a glass tube (900 µl). The mixture was vortexed, put on a tube rotator (30 min) and then on dry ice until the plasma was frozen. The liquid organic phase was extracted and left in a fume hood overnight at room temperature to dry. The residue was then reconstituted in methanol water (50:50 (v/v)) and 20 µl was injected into the LC-MS/MS system.

5.2.7 Data analysis

The chromatographic data (the peak area ratios of DHA to artemisinin and artemether to artemisinin) were analysed using Analyst® software version 1.5 (AB Sciex). Maximal plasma concentrations of artemether and DHA were obtained from the peak area ratio of the analytes to the peak area ratio of the internal standard (artemisinin). Statistical analysis was conducted using SPSS for windows, version 20.0. Normality was tested using t test and chi-squared test. ANOVA was used to test for significance of drug concentration with different degrees of malnutrition and haemoglobin.

5.3 Results

A total of fifty-one children were enrolled for the study. Three children vomited within 30 minutes of receiving the first dose, and dosing was repeated 15 min after vomiting without any event. Table 5.3 summarises the demographics and anthropometric measurements of the study group. The mean ± SD for age, weight and height of the participants were 33.8 ± 15.26 months, 11.49 ± 2.89 kg and 90.14 ± 12.79 cm respectively. The mean ±SD haemoglobin concentration was 9.59 ± 0.88 g/dl.

Table 5.3 Demography of the study population.

Variable		Value
Total number		51
Gender	Male (%)	19 (37.3)
	Female (%)	32 (62.7)
Age (months)		33.8 ±15.26
Weight (kg)		11.49 ±2.89
Height (cm)		90.14 ±12.79
Haemoglobin (g/dl		9.59 ± 0.88

Study population demographics. Age, weight, height and haemoglobin concentration are mean ± SD values.

Mean ± SD z-score for weight-for-height (WHZ), weight-for-age (WAZ) and height-for-age (HAZ) were -1.38 ± 1.61, -1.50 ± 1.36 and -1.07 ± 1.93 respectively (Table 5.4). Children were said to be malnourished and severely malnourished when the z-scores for WHZ, WAZ and HAZ were < -2 and < -3 respectively below the median of the WHO child growth standard. 21.6% (n=11) of the study population

were considered to be underweight with wasting and stunting accounting for 29.4% (n=15) and 21.6% (n=11) respectively.

Table 5.4 Nutritional status of the study population

No. (%)	Z score value No. (%)				p value
	Normal	< -1	< -2	< -3	
WHZ	30 (58.9)	6 (11.8)	8 (15.6)	7 (13.7)	NA
WAZ	16 (31.4)	24 (47.1)	5 (9.8)	6 (11.8)	
HAZ	20 (39.2)	20 (39.2)	8 (15.6)	3 (5.9)	
Weight (kg)					
WHZ	11.50±2.27	13.53±1.75	12.19±2.62	9.14±1.82	0.007*
WAZ	12.85±2.18	11.57±2.17	10.00±2.55	9.05±1.98	0.003*
HAZ	12.92±2.13	10.83±2.15	10.89±2.44	8.73±2.19	0.003*
Height (cm)					
WHZ	85.45±9.32	99.83±6.79	97.65±12.	91.74±11.61	0.002*
HAZ	98.64±8.19	84.96±9.38	84.69±10.65	78.87±2.31	0.001*
Age continuous WHZ	29.20±12.68	44.17±12.06	44.50±15.98	36.26±16.46	0.01[6]

Prevalence of different z scores of weight-for-height (WHZ), weight-for-age (WAZ) and height-for-age (HAZ). Weight, height and age continuous are expressed as mean±SD

* ANOVA

5.3.1 The effects of nutrition on pharmacokinetics

Drug plasma concentrations of artemether and DHA in different nutritional categories are shown in a scatter plot (Figure 5.2) and summarised in Table 5.5. The overall mean ± SD C_{max} for artemether and DHA were 138.44 ± 80.89 ng/ml (median; 157.3 ng/ml) and 58.84 ± 43.73(median; 48.79 ng/ml) ng/ml respectively, with observed high inter-patient variability. Mean ±SD C_{max} values for artemether and DHA in wasted children were 124.46 ±84.26 ng/ml and 84.13 ± 62.59 ng/ml respectively.

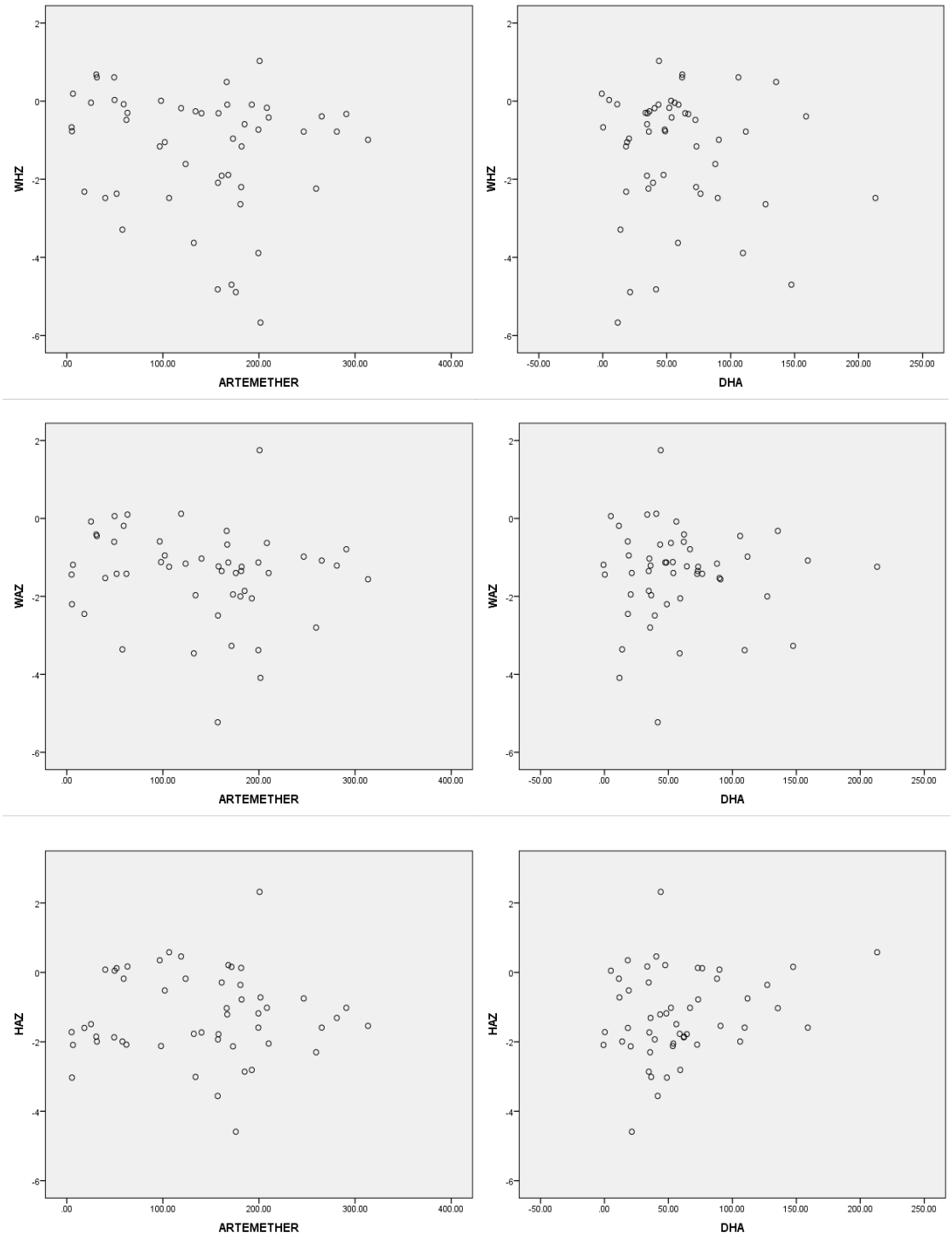
Plasma artemether concentrations were highest in the severely wasted children category while DHA was highest in the moderately wasted category. However, the differences were not considered significant when the values were adjusted for age, height and weight. Children with moderate wasting (Z-score < -2) had the lowest artemether concentration (124.46 ±84.26) and the highest DHA concentration (84.13 ±62.59) in the weight-for-height category, but again this was not significant. Plasma DHA concentrations in stunted (41.14 ± 23.52) and severely stunted children (42.32 ± 6.15) were lower compared to their normally nourished children (64.34 ± 53.82) but the difference was not statistically significant.

Unlike the mildly wasted and stunted children who had plasma artemether concentration values close to their normally nourished counterparts, mildly underweight children, though not statistically significant, had higher mean ± SD artemether concentration (150.09 ±79.63) compared to their normally nourished counterpart (119.12 ± 84.22).

5.3.2 The effects of anaemia on Artemether and DHA plasma levels

Table 5.6 summarises the anaemic status of the study group. Overall, 96.1 % of children in the study group were classified as anaemic (Hb < 11 g/dl) using the WHO classification, with 66.7 % (n = 34) having moderate anaemia (Hb 10.9 – 10g/dl). Mean artemether and DHA plasma concentrations were not affected by the anaemic status of the children (Table 5.7 and Figure 5.3).There were no differences in artemether and DHA concentrations in patients with different degrees of anaemia.

Figure 5.2 Scatter plot of Artemether and DHA concentration



Scatter plot of artemether (ng/dl) in relation to wasting (A), underweight (B) and stunting (C) and DHA (ng/dl) in relation to wasting (D), underweight (E) and stunting (F). WHZ-wasting, WAZ – underweight, HAZ - stunting

Table 5.5 Artemether and DHA plasma concentration 2 h post dosage

	Z score value No.				
	Normal	**< -1**	**< -2**	**< -3**	**p value**
WAZ	N=16	N=24	N=5	N=6	
Artemether	119.12±84.22	150.09±79.63	126.60±111.09	153.25±53.55	0.65^{*}, $0.73^{\ddagger,\alpha}$
DHA	54.23±37.02	64.54±48.64	40.27±15.31	63.84±54.45	0.67^{*}, $0.67^{\ddagger,}$
WHZ	N=30	N=6	N=8	N=7	NA
Artemether	137.87±93.27	138.93±36.25	124.46±84.26	156.49±49.62	0.91^{*}, $0.15^{\infty,\alpha}$
DHA	54.77±36.81	46.75±28.76	84.13±62.59	57.77±52.24	0.33^{*}, $0.34^{\infty,\alpha}$
HAZ	N=20	N=20	N=8	N=3	
Artemether	134.07±60.50	144.82±99.13	148.32±84.66	98.81±81.72	0.81^{*}, $0.63^{\neq,\alpha}$
DHA	64.34±53.82	62.90±40.05	41.14±23.52	42.32±6.15	0.536^{*}, $0.63^{\neq,\alpha}$

Plasma levels of artemether and DHA, in children under 5 years 2 h post dose of artemether/lumefantrine. Values are expressed as mean ± SD

*ANOVA

$^{\alpha}$Analysis of covariance

‡ including weight as a confounding factor

$^{\infty}$ including age, height and weight as a confounding factor

$^{\neq}$ including height and age as a confounding factor

Table 5.6 Anaemic status of children

WHO classification	No. (%)	Hb (g/dl)
≥ 11 (g/dl)	2 (3.9)	11.4 ±0.14
10.9 – 10 (g/dl)	15 (29.4)	10.4 ±0.31
9.9 – 7 (g/dl)	34 (66.7)	9.1 ±.65

Anaemic status of the patients according to WHO classification. Results are expressed as mean ±SD.

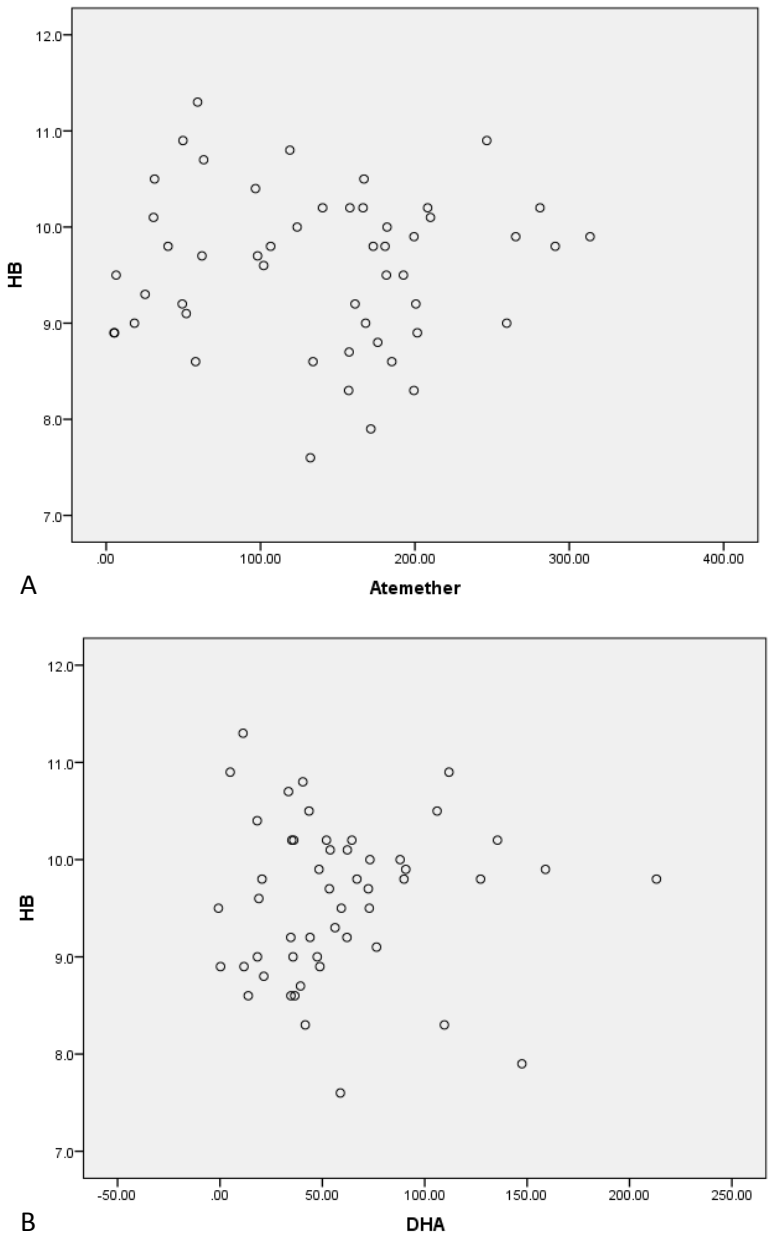
Table 5.7 Artemether and DHA plasma concentration in relation to anaemia

	No (%)	Hb (g/dl)	Artemether (ng/ml)	P value	DHA (ng/ml)	P value
≥ 10 g/dl	17 (33.3)	10.51±0.45	138.47 ±73.74	0.92	57.90 ±36.65	0.89
≥ 9.9 g/dl	34 (66.7)	9.13 ±0.65	138.43 ±85.31		59.31 ±46.78	

Plasma levels of artemether and its active metabolite, DHA in relation to haemoglobin level in children under 5 years 2 h post dose of artemether/lumefantrine combination. Results are expressed as mean ± SD

Table 5.8 shows the results of artemether and DHA plasma concentrations in relation to Hb levels reclassified in ranges of 1 g/dl. Children with Hb levels ≥ 11 g/dl had mean plasma artemether concentration level of 55.56 ±5.19 ng/ml. This was was observed to be lower however, only two children were in that category. Children with Hb levels between 7.9 – 7g/dl showed the highest mean artemether concentration (162.90 ±27.41). There was no significant difference observed in the mean DHA plasma concentration.

Figure 5.3 Scatter plot Hb against Artemether and DHA



Scatter plot of haemoglobin levels (g/dl) in relation to artemether (A) (r = 0.04) and DHA (B) (r = 0.10) plasma concentration (ng/dl)

Table 5.8 Artemether and DHA concentration in relation to Hb levels

	No. (%)	Hb (g/dl)	Artemether (ng/ml)	P value	DHA (ng/ml)	P value
≥ 11	2 (3.9)	11.40 ±0.14	55.56 ±5.19		43.86 ±46.07	
≥ 10	15 (29.4)	10.4 ±0.31	149.52 ±71.4		59.77 ±36.76	
≥ 9	22 (43.1)	9.5 ±0.33	142.02 ±93.81	0.69	66.02 ±48.65	0.87
≥ 8	9 (17.6)	8.67 ±0.24	121.50 ±78.44		35.89 ±32.00	
≥ 7	3 (5.9)	7.70 ±0.17	162.90 ±27.41		46.97 ±12.08	

Artemether and DHA concentration (ng/ml) in relation to haemoglobin levels (g/dl). Results are expressed as mean ± SD

5.3 Discussion

Mortality and the severity of malarial infection in children have been linked to the comorbidity of PEM. The pathophysiological changes in PEM are known to alter drug pharmacokinetics resulting in impaired drug metabolism with possible toxicity, and altered response to treatment (Oshikoya and Senbanja, 2009). In this study, peak plasma concentration (C_{max}) of artemether and DHA were analysed in children less than five years of age with different nutritional status. Children with malaria generally eat little during the acute phase of malaria and since dietary fat is known to enhance artemether bioavailability (White et al., 1999), full fat flavoured milk was given with the first dose of artemether/lumefantrine.

The aim of the initial research proposal was to build a pharmacokinetic model of artemether and DHA in normally nourished children and malnourished children in order to compare the pharmacokinetic values in normally nourished children and children with different degrees of malnutrition. 100 children, who met the inclusion

criteria, were to be admitted in a health facility to perform serial venous blood sampling starting before the first dose and at 15mins, 30mins, 1hr, 2 hrs, 12hrs and 24 hrs after the first dose. However, the research was conducted at the peak of the malaria season resulting in heavy patient population and admissions, limiting space in health facilities. The local ethics committee was contacted in order to revise the protocol for a more practical approach. Sample size, blood sample times and number were modified with the consent of the local ethics committee.

As a result of the limitation the sample size was reduced (51). Although blood collection in children is limited by age, size and disease status (Reed, 1999), sparse sampling was adopted mainly due to heavy patient population and limited space in the health facilities. Taking into account the average t_{max} of artemether and DHA, 2 h post dose samples were used to analyse the C_{max} of both artemether and DHA. Djimde et al (2011) also used a sparse sampling method and considered t_{max} of artemether to be between 1 and 2 h, talking the highest value from the two time points as the Cmax.

The values obtained in this study for artemether and DHA were 138.44 ± 80.89 ng/ml and 58.84 ± 43.73 ng/ml respectively. These results were comparable to a multi-centre study across Africa covering six countries with the same study population. They reported an artemether and DHA C_{max} of 190±168 and 63.7±65.0ng/ml respectively following oral administration of crushed artemether/lumefantrine tablets (Djimdé et al., 2011). The length of sample storage (20 months) prior to analysis is unlikely to have affected the levels obtained as

artemether and DHA are reported to be stable for at least 2 years when stored at -80°C (Ali et al., 2010).

Other studies have published data on the pharmacokinetics of artemether and DHA in children with uncomplicated malaria (Hietala et al., 2010, Mwesigwa et al., 2010) but the study design and sampling time's makes comparing their data and the one obtained in this study difficult. Following intense pharmacokinetic sampling beginning just before the administration of the last dose of artemether/lumefantrine Mwesigwa et al. (2010) reported C_{max} and $AUC_{0-\infty}$ of artemether values of 34 ng/ml and 168 ng/ml respectively with DHA values 119 ng/ml and 382 ng/ml respectively. Whilst C_{max} values of artemether and DHA in children reported by Djimdé et al. (2011) were similar to those earlier reported in adults (Lefèvre et al., 2001), Mwesigwa (2010) reported a 2 to 3 fold increase of C_{max} and $AUC_{0-\infty}$ values of artemether in children compared to adults (German et al., 2009, Lefèvre et al., 2002).

In order to obtain full pharmacokinetic parameters in children, population pharmacokinetics (PPK) modelling is been increasingly developed and used (Jamsen et al., 2012, Ogungbenro et al., 2009). Pharmacokinetic information obtained through PPK studies in children is also used in paediatric drug development to obtain clinical efficacy and safety of drugs, which often lack in paediatric drug development De Cook et al., 2011).

Despite differences in drug responses in children compared to adults, the paediatric dosing for majority of drugs are derived from adult dosing regimens based on bodyweight. With an estimation that ≥ 50% of drugs used have never been tested

for efficacy and safety in this population (Ogungbenro et al., 2009). This is as a result of both ethical and practical challenges. Unlike adults, research in healthy children is considered unethical, thus all paediatric pharmacokinetic studies are performed in children suffering from a disease. In addition to the limited availability of the number of samples in this vulnerable population, there are limitations on the volume and number of samples that can be collected per child, resulting in sparse sampling (Jamsen et al., 2012, Cock et al., 2011). Table 5.9 summarises traditional pharmacokinetic studies versus population pharmacokinetic studies.

Table 5.9 Summary of traditional pharmacokinetics versus population pharmacokinetics

	pharmacokinetics	**Population pharmacokinetics**
Population	Health volunteers Patients	Target patient population - paediatric - elderly Specific disease state
Sampling data	Dense	Sparse
Relationships of PK/PD	Limited	Extensive - make predictions on steady state concentrations - dosage guidelines - determine therapeutic window
Inter-individual variability	minimised	Demographics Pathophysiological Concomitant medication

In Addition, pharmacokinetic data obtained through PKK modelling can help in detecting differences in efficacy and safety in sub-groups within the studies age

interval, helping to identify subgroups in which exposure differs from the overall study population (White, 2013).

PPK approach offers the possibility of achieving information on pharmacokinetic from relatively sparse data, allowing information to be borrowed across individuals to obtain parameter estimates. It also allows the analysis of data from a variety of unbalanced designs, including data from paediatric and elderly patients (Hennig et al, 2006, Shoemaker and Cohen., 1996). The two common PPK analysis methods used are; the two-stage approach and the nonlinear mixed-effects modelling approach.

The nonlinear mixed-effects modelling approach is often used in PPK where there are limited sparse sampling. This involves the simultaneous evaluation of data from all individuals in a population. It results in less biased estimates where residual error is present. It can be used in sparse data situations where traditional two-stage approach is not applicable. It considers population study samples and uses individual data of observational type which may be sparse, unbalanced and fragmentary (Mould and Upton, 2013).

PEM continues to be a major health problem but pharmacokinetic studies of drugs in that population has declined over the decades (Oshikoya and Sammons 2010). Pharmacokinetic studies of antimalarial drugs in children with PEM are limited despite the high prevalence of children with PEM especially in malaria endemic countries. The effect of nutritional status on the efficacy of ACT in children showed that artemether/lumefantrine therapy was an effective antimalarial for

children with chronic malnutrition showing 99% parasite clearance by the 3rd day (Verret et al., 2011). However, this is the first pharmacokinetic study of artemether in children with PEM.

Results obtained showed that the Cmax of both artemether and DHA were not influenced by PEM nonetheless further studies involving more pharmacological parameters such as t_{max}, AUC and clearance in combination with bigger sample size are needed for better evaluation. Peak plasma concentration and AUC of chloroquine in children with kwashiorkor have shown to be significantly lower when compared to their normally nourished counterpart (Walker et al., 1987). Given the fact that chloroquine, like artemether is also metabolised by hepatic CYP 3A4/5 (Kim et al., 2003), extreme spectrums of PEM should be considered in future pharmacokinetic studies of artemether and DHA.

Another aim of this study was to evaluate the effect of haemoglobin on artemether plasma concentration. Anaemia is a common accompaniment of PEM with iron deficiency being the commonest cause seen in more than 85% of anaemic children with PEM (Ejaz and Latif, 2010). Considering the fact that iron is important in the activation of the artemisinin compounds, haemoglobin levels were analysed against the Cmax values of both artemether and DHA. The results obtained showed no correlation between the level of haemoglobin and plasma artemether concentrations. Although children with Hb $\geq$ 11 g/dl showed lower plasma artemether concentration, they only constituted 3.9% of the study population limiting the statistical interpretation. The lower level of Cmax might be multifactorial and because samples were obtained from only one time point, C_{max} might have been

missed. One explanation might be that children with higher haemoglobin levels have higher iron contents in their red blood cells thus exhibiting quicker bioactivation resulting in lower t_{max} values. Although it might be challenging to obtain a study group of children with normal haemoglobin levels due to the common association of malaria infection and anaemia (Menendez et al., 2000), a large study group of children with normal haemoglobin levels will be imperative for better analysis.

Studies evaluating the association between iron status and malaria have discovered that iron deficiency is a protective factor for malaria, showing a decreased malarial incidence with decreasing levels of stored iron (Sazawal et al., 2006, Nyakeriga et al., 2004). Although the protection offered by iron deficiency against malaria has been explained arise via an immune-mediated mechanism (Nyakeriag et al. 2004), it would be important to note the importance of host haemoglobin in the development of the malaria parasite. Iron is an important component in haemoglobin formation. The parasite degrades and detoxifies host haemoglobin (Medhi et al., 2009). In the presence of low haemoglobin, the amount of iron present might have an influence on parasite maturity, but most importantly on artemisinin activation. Selectivity of the artemisinin compounds towards parasite infected red blood cells over red blood cells is provided by the higher iron content in infected cells.

Finally, a number of limitations have to be highlighted. Although the sample size enabled the research to be conducted within a short time frame, it has its limitations. It might not be representative of the population under study and thus cannot offer a conclusive result. The study should be conducted in a larger sample size to ensure a better representative of the population and more accurate statistical

analysis. The sparse sampling adopted in this study has limited the degree of pharmacokinetic analysis thus cannot offer a more conclusive result. The C_{max} values were obtained from only one time point and may not represent an accurate and true C_{max} value. Analysing samples at different time points will enable a broader pharmacokinetic analysis including t_{max}, volume of distribution and clearance, to name a few. The inclusion of severely anaemic children and children with extreme spectrums of PEM (kwashiorkor and marasmus) would give a wider understanding of other important pharmacokinetic properties.

The findings in this study have shown that PEM and anaemia might not have a significant effect on the peak plasma concentration of artemether and DHA. However, to enable more definitive results and a better pharmacokinetic profile evaluation, the study should be conducted in a larger sample size and across several study sites in different countries.

6 Final Discussion

Malaria is one of the leading causes of death in children under five years of age accounting for 7% of infant mortality. The use of artemisinin combination therapy (ACT) was proposed by the WHO in 2006 to address the resistance of *Plasmodium falciparum* to traditional antimalarials and improve treatment outcomes (WHO, 2006). ACT is now the recommended drug of choice for uncomplicated malaria in children ≥ 5 kg and or 6 months of age (WHO, 2010).

The few pharmacokinetic of ACT carried out in children have suggested that children might be receiving sub optimal levels of ACT (WHO, 2002, Sidhu et al., 1998), thus might experience lower cure rate (Li et al., 2004, Looareeswan et al., 1998) due to the physiological and developmental differences in children (Kearns et al., 2003, Johnson et al., 2003). In addition, protein energy malnutrition (PEM), a common co-morbidity with malaria in African children (Fillol et al., 2009), may also have an effect on the pharmacokinetics of ACT.

PEM has been linked to around half of all infant mortality including malaria (Black et al., 2010). With PEM linked to the morbidity of malaria in children (Ehrhardt et al., 2006, Friedman et al., 2005, Verhoef et al., 2002, Deen et al., 2002), a huge proportion of children with PEM will be taking ACT. Among the sample of children with uncomplicated malaria studied in The Gambia, a high level of malnutrition (31.6%) was noticed when compared with the prevalence of PEM in children within the general population (17.4%).

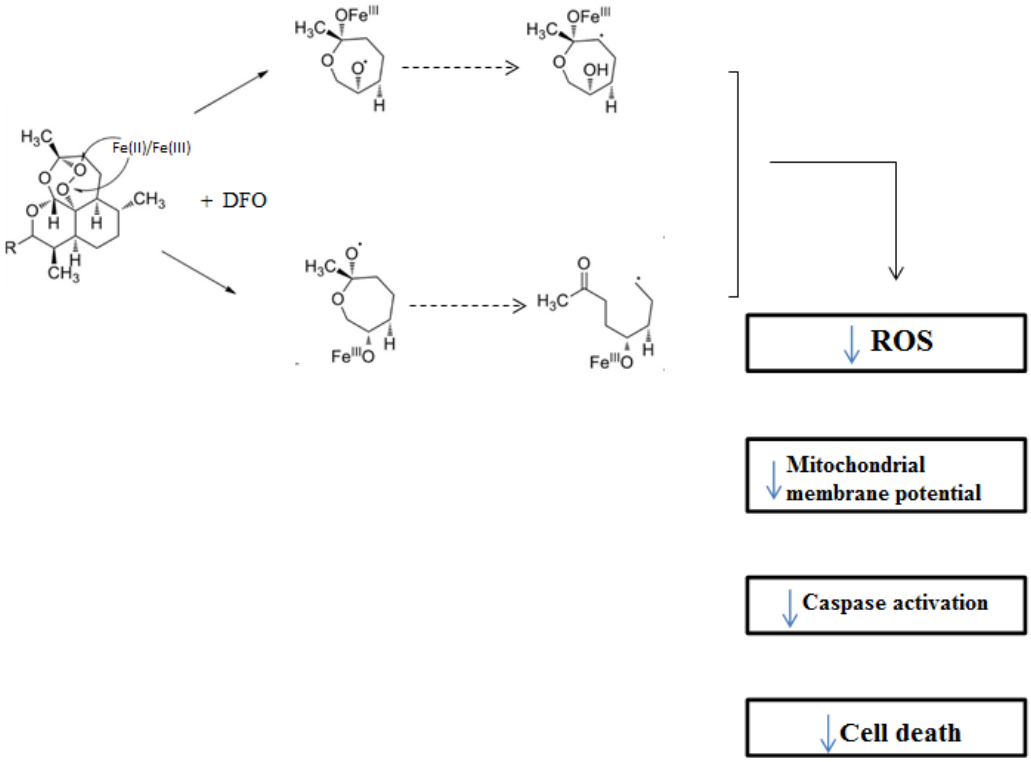
However, the study did not examine the relationship between the two co-morbidities. Just because the relationship between malaria and malnutrition was not analysed, it should not overshadow the importance of high prevalence of PEM in children with malaria. Though the cause and effect of the two co-morbidities has not been confirmed, the results obtained in this study reaffirms and strengthens the presence of PEM in children with malaria. The physiological changes that occur in children with PEM directly or indirectly affect the disposition of drugs.

PEM is associated with deficiencies of various mineral including iron, which is of significance in the bioactivation of the artemisinin compounds. Though the exact mechanism of action of the artemisinin compounds is still a matter of debate, the role of iron is well established. It is known that the compounds are activated in the presence of iron from haem to cause highly reactive free radicals rearranging and forming carbon centred radicals, which target and inhibit specific parasite molecules resulting in parasite death (Meshnick et al, 1996, Posner et al., 1995, Meshnick et al., 1991).

The effect of decreased iron was initially studied in vitro. Several studies have used iron chelators to demonstrate the involvement of iron with the observation that they either inhibit bioactivation or iron-related oxidative stress (Meshnick et al., 1993, Wei and Sadrzadeh 1994, Stocks et al., 2007). In order to further understand the mechanism by which iron chelators inhibit artemisinin toxicity, the effect of artesunate was studied in HL-60 cells in the presence of the iron chelator, deferoxamine (DFO). Although the toxicity of artesunate was inhibited, DFO had no

effect on bioactivation, as evidenced by the decrease in the parent compound (Chapter 2). However it is important to note the significant decrease in the metabolite biomarker.

Figure 6.1 Proposed mechanism of DFO on the artemisinin compounds



DFO inhibits the formation of reactive oxygen species with subsequent inhibition of cell death

From this, it would be safe to conclude that chelatable iron might not make a significant contribution to the bioactivation of the artemisinin compounds, but plays an important role in inhibiting the cascade of events leading to cell death. Studying the different proposed mechanism of action of the compounds, the presence of DFO was shown to inhibit the effect of artesunate in all subsequent steps, which include

ROS formation, mitochondrial membrane potential depolarisation, caspase activation and cell death.These results appear to be in line with the idea that artemisinin bioactivation causes an accumulation of iron which in turn causes the formation of ROS in the lysosomes in cells, which is analogous to the parasite food vacoule (Hamacher-Brady et al., 2011). Lysosomes are an important source of redox active free iron that is sensitive to artemisinin generated ROS forming reactive hydroxyl radical in a fenton reaction (Kurz et al. 2008, Uchiyama et al., 2008). The removal of toxic iron in cells inhibits the fenton reaction thus preventing the formation of reactive oxygen species, and inhibiting the cascade of reactions that result in cell death.

With the in vitro study showing that chelating iron reduces artesunate toxicity, it would be important to assess the effects of iron deficiency anaemia on the artemisinin compound. Anaemia is one of the commonest micronutrient deficiencies associated with PEM (Ejaz et al., 2010), with iron deficiency being the commonest cause of anaemia. Iron deficiency has been associated with decreased parasitaemia and malaria associated morbidity and mortality (Gwamaka et al., 2012, Jonker et al., 2012, Nyakeriga et al., 2004). This has been explained to be as a result of specific defect in the production on particular immunoglobins, it would be important to also look at the possible low haemoglobin in red blood cells as another factor. Iron deficiency might lead to decreased haem derived from haemozoin formed when the *plasmodium* parasite digests haemoglobin in the red blood cells, and with in-vitro studies showing the chelation of iron leading to decreased cell death, it would be important to assess the effect of iron deficiency anaemia on the artemisinin compounds.

As one of the determinants of treatment response, optimal plasma drug concentrations are essential for effective treatment outcome in malaria (Price et al., 2007). In order to analyse artemether and DHA plasma concentrations, plasma samples were analysed in 50 children. Plasma concentrations of artemether and DHA were measured using a sensitive LC-MS/MS method developed and optimised for the simultaneous analysis of both artemether and DHA in small plasma volumes (Chapter 4). Although over the years, different methods have been developed to analyse drugs in plasma, mass spectrometry is now considered the preferred method as it offers better sensitivity and selectivity with simultaneous analysis of multiple compounds (Grebe and Singh, 2011). Our method was validated according to established recommendations (Chandramouli et al., 2010) which included assessment of precision and accuracy, matrix effect and recovery.

Artemether and DHA plasma concentration was not influenced by the anaemic status of the children. Even though this study showed no association between the degree of anaemia with drug plasma concentrations, the importance of iron deficiency in children should not be overlooked. Iron is essential in the activation of the artemisinin compounds in red blood cell and low levels could have a significant influence in the pharmacological profile of the drugs. This can lead to a decrease in toxicity of the malaria parasite or a decrease in extracellular detoxification which might ultimately lead to an increase in plasma concentration thus altering the benefit: risk ratio. The alteration of the benefit: risk balance might lead the revision of the ACT treatment protocol in children with malnutrition.

Artemether and DHA plasma concentrations measured in children with uncomplicated malaria in Gambian children were also analysed in relation to their nutritional status as defined by WHO. PEM is associated with villous atrophy which might influence drug absorption, and has been associated with altered plasma drug concentrations in relation to other antimalarial drugs. The plasma concentrations of antimalarial drugs are much lower in malnourished children compared to normally nourished children (Salako et al., 1996, Tréluyer et al., 1996). In addition, PEM has a significant impact on the cytochrome (CYP) enzymes including CYP3A4 and CYP3A5 (Mao et al., 2006), which are important in the pharmacokinetics of artemether and DHA. The nutritional status of the children did not have any significant influence on the plasma concentrations of artemether and DHA.

The lack of a significant difference in drug plasma concentrations in this study between the normally nourished and children with PEM cannot be used as conclusive evidence that PEM does not affect artemether drug metabolism. Volume of distribution (Vd), an important factor in determining the peak concentration of drugs, and is affected differently by marasmus, kwashiorkor and marasmic-kwashiorkor. Whilst kwashiorkor causes a decrease in Vd in some drugs, marasmus might have the opposite effect (Oshikoya et al., 2010). Although some of the children included fit the classification of severe malnutrition, none of them were classified as either marasmus or kwashiorkor.

6.2 Future research arrears

Based on the findings reported in this thesis, there are several key areas which warrant further research.

6.2.1. Explore the relationship between PEM and malaria

An important finding in this thesis was the higher prevalence of underweight and wasted in children with uncomplicated malaria. Although the association between malaria and PEM is still controversial, and whilst recent studies have observed an increased risk of malaria related morbidity (Ehrhardt et al., 2006, Friedman et al., 2005, Verhoef et al., 2002, Deen et al., 2002) and mortality (Müller et al., 2003, Rice et al., 2000) in children with PEM, the contribution of malaria as a causal factor for PEM has also been suggested (Nyakeriga et al., 2004). Understanding the effect of PEM on malaria and vice versa in malaria endemic areas may help in addressing public health interventions. Thus further studies on the relationship between malnutrition and malaria are needed for better understanding of any association.

6.2.2 Detailed pharmacokinetic studies of artemisinin compounds in children with Protein energy malnutrition

PEM is shown to affect pharmacokinetics of drugs in children and despite it being a major contributor to infant mortality and co-morbidity, such studies have decreased over recent years (Oshikoya et al., 2010). With the strong association between PEM and malaria in children, a huge proportion of children taking ACT as

the first line of malaria treatment will suffer from some aspect of PEM. Very few studies have looked at the pharmacokinetics of antimalarial drugs in this vulnerable population and to the end of writing this thesis; no data has been published on the pharmacokinetics of the artemisinin compounds in children with PEM. There is a need to not only study the plasma levels of these drugs in a larger sample size, but also assess the rate of absorption, distribution, clearance and half-life, as they all affect drug response outcomes. It should also be noted that PEM is a spectrum of diseases with marasmus at one end and kwashiorkor at the other, with intermediate states of varying degrees. The type and severity of PEM impacts differently on pathophysiological changes in children. This in turn results in varying effects on drug pharmacokinetic parameters. It would be important to evaluate the pharmacokinetics of the artemisinin compounds at the two ends of the spectrum to gain better understanding of their differential effects and help formulate appropriate dosing schedules in children with different degrees of PEM.

6.2.3 Effect of protein energy malnutrition on treatment response to the artemisinin compounds

Artemisinin compounds are well tolerated with no reported toxicity in children with studies in general reporting good clinical outcomes (Djimdé et al., 2011, Hietal et al., 2010, Abdullah et al., 2008).The endoperoxide bond, which is responsible for its antimalarial activity, is activated by cellular iron. There is high rate of iron deficiency anaemia in children with PEM. With red blood cells being the target for the artemisinin compounds, it would be important to study the effect of low haemoglobin on the efficacy of the drug. Early and late analysis of the parasite

density in red blood cells will help gain a better understanding of the importance of *in-vivo* activation of the compounds by iron. It will also help to assess drug sensitivity and therapeutic index in the presence of low iron. Obtaining this data will help to further bridge the gap between *in-vitro* and *in-vivo* work on these compounds.

6.3 Conclusion

Although the in-vitro studies in this thesis (Chapter 2) have demonstrated that iron chelators inhibit artemisinin toxicity, the effect was possibly due to the chelation of toxic iron resulting from rather than inhibiting the bioactivation of the compounds. There is a high prevalence of PEM in children with uncomplicated malaria in The Gambia (Chapter3). Coupled with the fact that PEM alters drug metabolism, it was important to assess the pharmacokinetics of artemether in this vulnerable group. Thus the analysis of artemether and DHA plasma levels in children with differing degrees of malnutrition and anaemic status was undertaken (Chapter 5) together with the development of a sensitive LC-MS/MS method (Chapter 4).Even though no artemether and DHA plasma concentration differences between different nutritional or anaemic states was observed, the study was limited by sample size, and needs to be undertaken in a larger number of patients in the future. Overall, the thesis has highlighted the importance of treatment with the artemisinin compounds in children, and in particular, shown the high prevalence of PEM in these children, which should be further investigated in order to ensure that dosing regimens can be optimised as much as possible to maximise efficacy in this vulnerable patient population.

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8 Appendices

8.1 BCA Protein Assay

Standards Preparation of different concentrations

Vial	Concentration (mg/ml)	Volume of H_2O (µl)	Volume BSA (µl)	Protein (µl/µg)
A	2	0	200	18
B	1.5	66	200	13.5
C	1	100	100 (A)	9
D	0.75	100	100 (B)	6.75
E	0.5	100	100 (C)	4.5
F	0.25	100	100 (E)	2.25
G	0.125	100	100 (F)	1.125
H	0	100	0	0

9 µl of each standard are added in a 96-well plate in duplicates

1.5 µl of samples are added to appropriate wells in duplicates

Mix BCA assay reagent A & B (50:1)

200 µl of BCA reagent is added to wells in both standard and samples

Incubate plate at 37ºC for 30 min

96-well plate is read at 570 emission and 590 absorption

8.2 Consent Form

I have read the information sheet/ The information sheet has been read to me. I have had the opportunity to ask questions about it and any questions that I have asked have been answered to my satisfaction. I consent voluntarily for my child/ward to be a participant in this research.

Name of Participant _____

Signature of Participant _____ Date _____
Day/month/year

If illiterate

I have witnessed the accurate reading of the consent form to the parent/guardian to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Name of Witness _____

Signature of Witness _____ Date _____
Day/month/year

Name of Participant _____-

Date _____
Day/month/year

Thumb print

I have accurately read out the information sheet to the potential participant, and to the best of my ability made sure that the participant understands that the following will be done:

1. The child/ward will be admitted for 24 hours

2. The pills will be given under supervision while on admission and with milk

3. An indwelling cannulla will be inserted in the child/ward's arm for serial blood withdrawal

I confirm that the participant was given an opportunity to ask questions about the study, and all the questions asked by the participant have been answered correctly and to the best of my

ability. I confirm that the individual has not been coerced into giving consent, and the consent has been given freely and voluntarily.

A copy of the parent information sheet has been provided to the parent/guardian.

Name of Researcher/person taking the consent_____

Signature of Researcher /person taking the consent_____

Date _____

Day/month/year

8.3 Parent information leaflet

PHARMACOKINETICS OF ARTEMETHER/LUMEFANTRINE (COARETEM) IN MALNOURISHED CHILDREN

PARENT INFORMATION LEAFLET

INTRODUCTION

Malaria is one of the most common diseases in The Gambia and also one of the leading causes of childhood death. Many of the children who have malaria also suffer from malnutrition. Childhood deaths in Africa climbed due to parasite resistance to chloroquine, the then first line therapy, which prompted the introduction of artemisinin combination therapy (ACT), as the first line therapy in many parts of Africa. Although the artemisinin compounds are well tolerated and rapidly clears the parasite from the blood, it is associated with high relapse due to its short action. In order to counter this, WHO recommended that it should be used with a longer acting antimalarial drug to mop up and remaining parasites.

WHAT IS THE STUDY ABOUT?

Little is known regarding the effect of these drugs in children and whether optimum drug exposure is achieved with current dosing regimens. Optimum dosing will minimise the risk for treatment failure, drug toxicity and drug resistance. Paediatric doses were formulated from adults based regimens adjusted for body weight with no regards to the effect of malnutrition. It is expected that a lot of these drugs will be administered in the coming years especially in children as they are more susceptible to having malaria. The study will compare the effectiveness of coartem between normally nourished and malnourished children and to see if the current dose regimen is as effective in malnourished children as it is in normally

nourished children. This will help formulate proper dose regimens to increase the effectiveness of the drug and reduce the harmful effects.

WHY HAS MY CHILD BEEN CHOOSEN?

Your child/ward has been diagnosed with malaria and as a result will be given coartem. The study requires children between the ages 6 months up and including 5 years who are taking coartem.

WHAT WILL HAPPEN IF WE AGREE TO TAKE PART?

After making sure that you understand what the study entails, we will ask you to sign or thumb print a consent form. Taking part will involve admitting your child/ward for twenty-four hours. His/her weight, height, mid upper arm circumference, temperature, respiratory rate, blood pressure and pulse will be taken. After admission, a little amount of blood will be collected just before he/she drinks the medicine, which will be taken with milk to help absorb the drug better. If the medicine is vomited, within an hour, he/she will have to repeat the dose. Blood samples will be collected at various intervals (6 times) within the 24 hours. The blood collected will be used to study the level of drug in their boold and how long it stays there and also to see if different genes will cause the different levels.

HOW WILL BLOOD BE COLLECTED

A needle (cannula) will be inserted in his/her arm but before that a cream will be applied on the site to minimise the pain caused by he needle prick. Small amounts will be collected (less than half a teaspoon) just before they take the medicine and 6 different times (15mins, 30mins, 1hr, 2 hrs, 12hrs and 24 hrs after the first dose) in various tubes. The blood collected will be minimal and cause no harm to your child/ward.

WHAT WILL HAPPEN TO THE SAMPLES COLLECTED?

The samples will be stored in a freezer in Royal Victoria Teaching Hospital until the study is finish and will be transported to the Department of Pharmacology at the University of Liverpool where they will be kept in a locked freezer. The samples will only be identified by a code, for increased security. Once this study has been completed the samples will made anonymous, so it cannot be traced back to your child. We might wish to use this anonymous sample in the future for other research and we would ask an ethics committee for permission to do this.

ARE THERE ANY RISKS OR BENEFITS TO TAKING PART?

There are no anticipated risks in taking part in the studies. There might not be direct benefits for your child/ward but information obtained from the study will help in the better management for other children in the future.

- **DOES MY CHILD/WARD HAVE TO TAKE PART?**

No. Your child/ward's participation is voluntary. If you do not wish to participate, this will not affect your child's right to proper management in the hospital. If you are not sure then we can arrange for someone to talk to you and help you make up your mind.

- **WHAT IF I CHANGE MY MIND?**

This is not a problem. If you/your child change your mind, you can withdraw from the study at any point.

- **WHO IS ORGANISING THIS RESEARCH?**

The research is being organised by the Department of Pharmacology at the University of Liverpool in collaboration with the Royal Victoria Teaching Hospital and it is also part of the PhD programme I am doing.

- **HAS THE STUDY BEEN CHECKED?**

Yes. All studies involving patients must be approved by the ethics committee in The Gambia before it starts. The committee is satisfied that your child's rights will be respected, that any risks have been reduced to a minimum and balanced against possible benefits, and that you have been given sufficient information on which to make an informed decision to take part or not. The Liverpool Paediatric Research Ethics Committee has also reviewed and approved this study.

- **HOW DO I GET MORE INFORMATION?**

The person who gave you this information can discuss the study with you more. You can also contact

Dr Mariama A Sarr Sallah

Telephone number – 9925301

Email – mariama@liv.ac.uk

8.4 Participant questionnaire

PARTICIPANT DATA QUESTIONNAIRE

Date

1 Serial Number _____

2 Child Initial _____

3 Parent/guardian Initial _____

4 DOB/Age ☐

5 Sex a. Male ☐ b. Female ☐

6 Symptoms Duration

	Numbers of Days			
	One	Two	Three	Four or more
a. Fever	☐	☐	☐	☐
b. Vomiting	☐	☐	☐	☐
c. Headache	☐	☐	☐	☐
d. Diarrhoea	☐	☐	☐	☐
e. Abdominal pain	☐	☐	☐	☐
f. Weakness/Lethargic	☐	☐	☐	☐
g. Other	☐	☐	☐	☐

7 Weight ☐

☐

8 Height

9 Z score

10 Mid upper arm circumference

11 Temperature

12 Pulse

13 Respiratory rate

14 Malaria parasite count _____

15 Haemoglobin _____

16 Full blood count _____

8.4 Consent Form

UNIVERSITY OF THE GAMBIA

SCHOOL OF MEDICINE & ALLIED HEALTH SCIENCES
RESEARCH AND PUBLICATION COMMITTEE (RePublIC)

30th August 2010

Dr. Mariama A. Sarr-Sallah
Department of Pharmacology & Therapeutics
University of Liverpool
Ashton Street
Sherrington Building
L69 3GE

Dear Dr. Sarr-Sallah,

RE: #R10 031 "PHARMACOKINETICS OF COARTEM IN MALNOURISHED CHILDREN"

Thank you for submitting the above project.

I appreciated your clear description of what you plan to do, the procedures and materials to be used during the project. The information sheet and consent forms are appropriate to the task.

I am happy to give Chair's approval, given the imminent start of data collection during the current malaria season.

I will forward the proposal documents to the Gambia Government/Medical Research Council Joint Ethics Committee for final clearance.

Thank you.

Best wishes,

.....
Dr. Ousman Nyan
Chair

Cc: File

P.O. Box 1646 Banjul; Tel: 00220 4201407; Mob: 00220 9934848; Fax: 4201407; Email: onyan@utg.edu.gm

RECRUITMENT ADVERTISEMENT

STUDY ON COARTEM

Malaria is a serious disease that affects mostly children, especially during the rainy season. Continuous studies are always been done to help minimise the burden of the disease.

The resistance of malaria to chloroquine led to the introduction of coartem (the pills in the packet) which are given in hospitals and health centres for the treatment of malaria.

These pills are effective and make you feel better within a day or two. The pills are known to be safe in children but studies are still going on to improve its safety and effectiveness.

In order to help achieve this, children between 6 months up to and including 5 years with malaria are needed to take part in the study. They will be admitted for 24 hours and will receive their normal pills, which will be taken with milk under supervision.

Their weight, height and mid upper arm circumference will be measured to assess how well nourished they are. A cream will be applied on an identified place on their arm were a needle will be inserted. The cream will minimise the pain of the needle. Blood will be taken at various times from this needle (7 times) during the course of the admission.

The children will be checked regularly by a nurse and will be given additional treatment if needed. Food will also be provided to both children and parent during the course of admission.
For further enquires, a member of the team will always be around to answer any question you have

or contact Fama Jaye,
Tel: 7676210

Mariama Sarr Sallah
Tel: 9925301/7925301