PHARMACOKINETIC PROFILE OF AMODIAQUINE AND ITS ACTIVE METABOLITE DESETHYLAMODIAQUINE IN GHANAIAN PATIENTS WITH UNCOMPLICATED *FALCIPARUM* MALARIA

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ABSTRACT-open

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RATIONALE: The World Health Organization recommends artesunate-amodiaquine for treating uncomplicated malaria. The accurate measurement of antimalarial drug concentrations in key target patient groups is essential to ensure optimal dosing for malaria treatment and to distinguish between inadequate drug exposure and antimalarial drug resistance.

METHODS: A sensitive and selective LC-MS/MS method was developed and validated for the simultaneous determination of amodiaquine and its active metabolite, desethylamodiaquine. This method was used to describe the pharmacokinetics of amodiaquine and desethylamodiaquine in Ghanaian patients with uncomplicated *falciparum* malaria treated with the fixed-dose combination of artesunate-amodiaquine.

RESULTS: The day-28 genotype-adjusted adequate clinical and parasitological response rate in 321 patients was >97% by both intention-to-treat and per-protocol analysis. The pharmacokinetic analysis included 244 patients (13 infants, 131 aged 1-4 years, 100 aged >5 years). A significantly higher amodiaquine exposure was seen in infants ($AUC_{0-\infty}$ 4988ng.h/ml in infants, 3403 ng.h/ml for children aged 1-4 years and 1430 ng.h/ml for ages $\geq$5 years, p=0.0001). Increased median day-3 amodiaquine concentrations were associated with a lower risk of treatment failure [HR 0.87 (95% CI 0.78-0.98), p=0.021]. No significant safety concerns were identified. The ratio of the geometric mean of capillary whole blood and capillary plasma concentrations in 108 matched samples was 2.4 [95% CI 2.3-2.6] for amodiaquine and 3.4 [95% CI 3.2-3.7] for desethylamodiaquine.

CONCLUSIONS: The pharmacokinetic parameters of amodiaquine and desethylamodiaquine were determined in the largest single uncomplicated malaria pharmacokinetic study of fixed dose amodiaquine to date. Efficacy at currently recommended dosage regimens was high across all age groups. Although other widely used antimalarials have been systematically under-dosed in young children, results from this study demonstrate that desethylamodiaquine exposure is remarkably consistent between children aged 1 to 4 years and older children and adults; this is reassuring since the highest *falciparum* malaria burden is carried by children less than 5 years of age. Equally, desethylamodiaquine exposure is not reduced in underweight-for-age young children or those with high parasitaemia, two of the most vulnerable target populations. Further research and pooled analyses are needed to describe the pharmacokinetics and safety of amodiaquine in infants, and to assess whether dose optimization in this vulnerable, understudied population is needed.

DEDICATION

To God be the Honour, Glory and Praise.

Lord, on the day I called for help, you answered me

I will give thanks to you, O Lord with all my heart,
For you have heard the words of my mouth;
In the presence of the angels, I will sing your praise;
I will worship at your Holy temple
And give thanks to your name

Your right-hand saves me
The Lord will complete what he has done for me;
Your kindness O Lord, endures forever;
Forsake not the works of your hands
(CF: Psalm 138:1 & 3)

To the memory of my dear father Mr. Abagna Anyorigiya

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LIST OF ABBREVIATIONS

ACPR ....Adequate Clinical and Parasitological response

AQ…… .....Amodiaquine

AQ LLOQ..... Amodiaquine lower Limit of quantification

AQ LLOQ-2..... Amodiaquine lower Limit of quantification, level 2

ACT ……. .....Artemisinin-based combination therapy

AQ_ISTD……. .....Amodiaquine internal standard

AIC..... Akaike Information Criterion for each model run

ASAQ …….....Artesunate-amodiaquine

$AUC_{0-\infty}$ ….....Area under the concentration-time curve from time 0 to infinity

BIC..... Bayesian Information Criterion for each model run

BLQ ….....Below the limit of quantification

^{o}C.....Degree Centigrade

CHPS .....Community Health Planning and Services

CRF.....Case Report Form

CI.....Confidence Interval

C_{max} …… .....Maximum Observed concentrations

CL/f …….. .....Apparent Clearance

dATP.....deoxyadenosine triphosphate

dCTP.....deoxycytidine triphosphate

DEAQ_ISTD …… .....Desethylamodiaquine internal standard

DEAQ….. .....Desethylamodiaquine

dGTP .....deoxyguanosine triphosphate

DNA.....deoxyribonucleic Acid

DND*i*.....Drugs for Neglected Diseases *initiative*

DTP2.....Diphtheria, Pertussis and Tetanus 2

DTP3.....Diphtheria, Pertussis and Tetanus 3

EPI.....Expanded Programme on Immunization

DTTP.....deoxythymidine Triphosphate

EIR.....Entomological Inoculation Rate

ETF.....Early Treatment Failure

FDA.....Food and Drugs Authority

FDC.....Fixed-Dose Combination

nFDC .....non-Fixed Dose Combination

g/dl..... .....grammes per decilitre

GMR .....Geometric mean ratio

GEE…… Generalized Estimating Equation

GLURP.....Glutamine-Rich Protein

h .....Hour

h^{-1}.....per hour

Hb ……Haemoglobin concentration

hCG.....*Human chorionic gonadotropin*

HPLC..... High Performance Liquid Chromatography

hr .....Hour

INESS.....INDEPTH Effectiveness and Safety Studies

IPT ……Intermittent Preventive Treatment

IPTc…… Intermittent Preventive Treatment in Children

IPTi……Intermittent Preventive Treatment in Infants

IPTp …….Intermittent Preventive Treatment in Pregnancy

IQR.....Inter quartile range

IRS ……..Indoor Residual Spraying

ITT……Intention-to-treat

Ke……. .....Terminal elimination rate constant

kg.....Kilogramme

kg^{-1}.....per kilogramme

KHRC……..Kintampo Health Research Centre

KHDSS.....Kintampo Health **and** Demographic Surveillance System

l..... .....Litre

LC.....Liquid Chromatography

LCF.....Late Clinical Failure

LC-MS/MS.....Liquid Chromatography tandem Mass Spectrometry

-2LL..... -2 Log likelihood

LLE.....Liquid–Liquid Extraction

LLINs..... .....Long- lasting insecticide-treated net

LLME.....Liquid–Liquid Micro-Extraction

LLOQ ….....Lower limit of quantification

LPF.....Late Parasitological Failure

MEPS.....Micro-Extraction by Packed Sorbent

µL .....Microlitre

MIP..... Molecularly Imprinted Polymer

ml..... .....Millilitre

MOH ……Ministry of Health

MSP -1.....Merozoite surface protein-1

MSP-2.....Merozoite surface protein-2

NHRC …… Navrongo Health Research Centre

NHDSS.....Navrongo Health and Demographic Surveillance System

ng…..... .....nanogram

NWMH.....Navrongo War Memorial Hospital

PBS.....Phosphate-buffered saline

PCR..... .....Polymerase Chain reaction

PD.....Pharmacodynamics

PK.....Pharmacokinetics

PLE……..Pressurized Liquid Extraction

pmol.....Picomole

PP……Protein Precipitation

PP.....Per Protocol analysis

QC.....Quality Control

RAM.....Restricted Access Material

RDT……Rapid malaria Diagnostic Test

SMC .....Seasonal Malaria Chemoprevention

SP.....Sulfadoxine-Pyrimethamine

SPME.....Solid Phase Micro-Extraction

STD……Standard

Taq.....Thermus aquaticus

$T_{1/2}$ …….. .....Half-life

T_{max}…….....Time to maximum observed concentrations

UV.....Ultra Violet

Vd/f …….. .....Apparent volume of distribution

WAZ……Weight-for-age z-score

WHO……World Health Organization

WHOPES .....World Health Organization Pesticide Evaluation Scheme

WWARN.....WorldWide Antimalarial Resistance Network

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Clarification of relation between sub-study and parent study

This study was carried out as part of the INDEPTH Effectiveness and Safety Studies (INESS) of the therapeutic efficacy of artesunate-amodiaquine in Ghana. INESS provided the platform on which the Pharmacokinetic sub-study of amodiaquine and desethylamodiaquine was carried out. I acknowledge the technical and logistic support received from the INESS Secretariat. Dr. Bernhards Ogutu provided technical guidance during the conduct of the study. The idea of describing the pharmacokinetic parameters of amodiaquine and its metabolites and to identify the possible factors that may influence these parameters was conceived by me and developed under the guidance of Prof. Karen Barnes. I developed the consent/assent forms, questionnaire, and standard operating procedures and training for the field operations. The standard operating procedures for assays were developed with support and guidance from Drs. Lubbe Wiesner and Sandra Castel.. I also reviewed all case report forms and certified them for data entry. All filter paper blood blots were as part of the parent study, genotyped at the Ifakara Health Institute under the auspices of INESS.

Summary of Key Findings

Assay method development & validation

Although several methods exist for the determination of amodiaquine and its active metabolite desethylamodiaquine, it was necessary to develop a new assay method that requires less invasive techniques for sample collection (i.e. capillary rather than venous blood) and for only small amounts of blood to be collected. Small sample volumes may be necessary for field studies to ease sample collection, particularly in very young and anaemic patients.

A simple liquid-liquid extraction method coupled with LC-MS/MS detection was developed and fully validated for the simultaneous determination of amodiaquine and its active metabolite, desethylamodiaquine with a limit of quantification of 0.781 ng/ml for amodiaquine and 3.91 ng/ml for desethylamodiaquine. This method is specific, sensitive and reproducible and uses a small volume (20 µl) of whole blood or plasma to achieve lower limits of detection with the accuracy of the quality control standards in capillary whole blood (*N*=18; 6 each for high, medium and low QC standards) being between 98.2% and 108.3% for amodiaquine, and 96.1% and 106% for desethylamodiaquine. In capillary plasma samples, the combined accuracy statistics of the quality control standards (*N*=18; high, medium and low) were between 88.2% and 92.4% for amodiaquine, and 98.2% and 99.5% for desethylamodiaquine.

The precision of the assay method was between 4.1% and 5.8% and between 3.2% and 4.4% for amodiaquine and desethylamodiaquine in capillary whole blood respectively. The corresponding values for amodiaquine and desethylamodiaquine in capillary plasma samples were between 4.6% and 11.0% and between 9.3% and 12.6% respectively. This method can readily be used for pharmacokinetic studies and therapeutic drug monitoring in patients, including young children with uncomplicated *falciparum* malaria.

Therapeutic efficacy of fixed dose combination artesunate-amodiaquine treatment of uncomplicated *falciparum* malaria in Ghana

Artesunate-amodiaquine combination treatment is one of five artemisinin-based combination therapies recommended by the World Health Organization for treating uncomplicated falciparum malaria. Since 2005, Ghana has been using artesunate-amodiaquine combination as a first line the treatment for uncomplicated falciparum malaria. Therapeutic efficacy assessments of this combination carried out previously in Ghana report efficacies ranging between 64% (PCR-uncorrected result for unsupervised treatment) and 100% (PCR-corrected). It was therefore imperative to continue to monitor the efficacy of this treatment in order to prevent risk to patients from using the treatment beyond its useful life-span.

It is reassuring to note that the efficacy of fixed dose artesunate-amodiaquine was very high (>97%) at the two study sites. The day 28 PCR-corrected adequate clinical and parasitological response (ACPR) rate by intention-to-treat analysis was 97.7% (260/266) [95% CI 95.2, 99.2] in Navrongo and 98.2% (54/55) [95% CI 90.3, 100.0] in Kintampo, and by per-protocol analysis, the ACPR rate 97.6% [95% CI 94.8- 99.1] in Navrongo and 98.1% [95 % CI 90.1, 100.0] in Kintampo.

Parasite clearance in the current study was fast with over 99% of patients clearing their parasitaemia by day 3 of follow up. The proportion of patients still parasitaemic on day 3 was 0.4% in Navrongo (and not assessed in Kintampo) and falls well below the 5.0% threshold for the initiation of a study to assess whether artemisinin resistance is present (WHO, 2014a) and the stricter 3% threshold previously suggested (Stepniewska et al., 2010). Fever resolution was also fast. Within 48 hours of initiation of treatment, over 98.4% of the patients in Navrongo and 96.4% in Kintampo had cleared their fevers.

The mean haemoglobin concentration increased significantly from enrolment to day 28. At baseline, the mean haemoglobin concentration (± sd) in Kintampo (9.2 ± 1.9 g/dl) was lower than in Navrongo (10.2 ±1.9 g/dl), $p<0.001$. There was a slight decline to a mean haemoglobin of 8.7 ± 2.9 g/dl on day 2 in Kintampo, and to 10.0 (±1.7) g/dl on day 7 in Navrongo. By day 28, the mean haemoglobin concentration had increased significantly from enrolment to 11.6 (±1.4) g/dl in Navrongo and 10.5 ± 1.5 g/dl in Kintampo ($P< 0.001$).

No major safety concerns were identified. The reported adverse events were all considered mild and did not result in the discontinuation of treatment. Adverse events were reported by 71 (23.1%) of the patients from days 1 to 3, although most were consistent with features of

malaria. From days 7 to 28, 68 of the 308 patients (22.1%) reported adverse events with the most common being fever recurrence (4.9%), cough (4.6%) and headache (3.6%).

This study and Abuaku et al., 2016 have shown sustained efficacy of the fixed dose combination of artesunate-amodiaquine in Ghana. However, in order to ensure the sustained efficacy of artesunate-amodiaquine combination, particularly in the light of recent case reports (Lu et al., 2017; Sutherland et al., 2017) of the emergence of indigenous artemisinin-resistant *P. falciparum* in Africa, there is need for continuous monitoring of the therapeutic efficacy of the treatment, ideally every other year.

Matrix effect

Antimalarial drug concentrations are often measured in different matrices. There is need to be able to compare or pool these concentrations across studies. It is therefore imperative that the relationship between the concentrations of these compounds measured in different matrices is accurately established.

The correlation between $\log_e$-transformed amodiaquine concentrations in capillary whole blood and capillary plasma samples was very strong, r_s=0.981, p<0.001. Overall, the ratio of the geometric mean of capillary whole blood to capillary plasma amodiaquine concentrations (amodiaquine ratio) was 2.4 (95% CI 2.3, 2.6). A statistically significant (p<0.001) log-linear relationship between observed capillary whole blood and observed capillary plasma concentrations of amodiaquine was described by the equation:

$$\mathbf{\ln (cAQ_{wb}) = 0.317 + 1.223 * \ln (cAQ_p)},$$

where cAQ_{wb} refers to the capillary whole blood concentrations of amodiaquine and cAQ_p refers to the capillary plasma concentrations of amodiaquine

The concentrations of desethylamodiaquine in capillary whole blood were reasonably strongly correlated with the concentrations of desethylamodiaquine in capillary plasma samples, r_s=0.859; p<0.001. The ratio of the geometric mean of capillary whole blood to capillary plasma desethylamodiaquine concentrations was 3.4 (95% CI 3.2, 3.7), increasing from a median ratio of 2.9 in lowest concentration quintile to 4.5 in the highest concentration quintile. A statistically significant (p<0.001) log-linear relationship between concentrations of desethylamodiaquine in capillary whole blood and plasma was best fitted to the equation:

$$\mathbf{\ln (cDEAQ_{wb}) = 0.959 + 1.051 * \ln (cDEAQ_p)}$$

where $cDEAQ_{wb}$ refers to the concentrations of desethylamodiaquine in whole blood and $cDEAQ_p$ refers to the concentrations of desethylamodiaquine in plasma samples.

Given the variability remaining between the observed and predicted desethylamodiaquine concentrations, population pharmacokinetic modeling of pooled data is suggested to better elucidate this matrix effect.

Amodiaquine and desethylamodiaquine pharmacokinetic parameters

There is need for the accurate measurement of antimalarial drug concentrations in key target patient groups in order to ensure optimal dosing for malaria treatment and to distinguish between inadequate drug exposure and antimalarial drug resistance. Pharmacokinetic parameters of amodiaquine and desethylamodiaquine were determined in the largest single uncomplicated malaria pharmacokinetic study of artesunate-amodiaquine fixed dose combination to date.

The site of sample collection appeared to be an important factor affecting most pharmacokinetic parameters (C_{max}, $AUC_{0-\infty}$, V_d/f and CL/f) of both amodiaquine and desethylamodiaquine despite adjustments for other predefined covariates. This could probably be explained by the different sampling schedules and potential differences in pharmacogenetics or methods for sample collection and storage. It has also been suggested that ethnicity, drug formulation and dosage, and pattern of concomitant medication use (Anderson, 2005; Parikh et al., 2007; Yasuda, Zhang & Huang, 2008; Kerb et al., 2009) are among other factors could account for site effects.

A significantly higher amodiaquine exposure was seen in infants (median $AUC_{0-\infty}$ 4988ng.h/ml in infants age <1 year, 3403 ng.h/ml for children aged 1-4 years and 1430 ng.h/ml for ages ≥5 years, p=0.0001). However, desethylamodiaquine exposure was similar across age groups (median $AUC_{0-\infty}$ 185,043 ng.h/ml in infants age <1 year, 144,841 ng.h/ml for children aged 1-4 years and 121,349 ng.h/ml for ages ≥5 years, p=0.106). The total mg/kg dose administered was associated with a 4% increase in both C_{max} [GMR 1.04 (95% CI 1.02, 1.07), p = 0.003] and $AUC_{0-\infty}$, [GMR 1.04 (95% CI 1.00, 1.07), p= 0.035] of amodiaquine after adjusting for other predefined covariates. Such dose proportional exposure is advantageous for dose optimisation if needed.

In female patients in this study, apparent clearance of amodiaquine was 79% faster than in their male counterparts [GMR 1.79 (95% CI 1.13, 2.85), p=0.014]. After adjusting for other significant covariates, the apparent clearance, CL/f of desethylamodiaquine was about 35% faster [GMR 1.35 (95% CI 1.01, 1.81), p= 0.044] in females than in males.

There was a strong linear correlation between $AUC_{0-\infty}$ and the day 7 DEAQ concentrations; r_s=0.8810, p<0.001. However, the day 7 desethylamodiaquine concentrations, p=0.767, as well as total desethylamodiaquine exposure, $AUC_{0-\infty}$, p=0.363, were similar in patients who achieved adequate clinical and parasitological response and those who failed treatment in this study. This was probably due to the high treatment efficacy rate achieved in this study. The median day 3, 7, 14 and 28 desethylamodiaquine concentrations also did not appear to affect either the duration of gametocyte carriage or treatment outcome in terms of risk of parasite clearance. However, an increase in the median day 3 amodiaquine concentrations was associated with treatment response. The median concentration of amodiaquine on day 3 in patients with parasite recurrence was 4.8 (IQR 3.0 - 8.2) ng/ml compared to 12.5 (IQR 5.9 - 24.2) ng/ml in patients who achieved an adequate clinical and parasitological response, p=0.002. A 1ng/mL increase in day 3 amodiaquine concentration was associated with a 13% reduction in the risk of parasite recurrence, HR = 0.8737 (95% CI 0.7793, 0.9795), p = 0.021

Conclusions

The inclusion of 13 infants aged less than 1 year with uncomplicated falciparum malaria in this study provides preliminary evidence that they may have greater exposure to amodiaquine than older children and adults. Although no safety concerns were identified in this study, further research and pooling of data is needed to assess amodiaquine safety and the need for dose optimization in infants.

The highest *falciparum* malaria burden is carried by children less than 5 years of age. The desethylamodiaquine exposure is remarkably consistent between children aged 1 to 4 years (144,841 (IQR 96,907 – 241,663) ng.h /ml) and older children and adults (121,349 (IQR 78,868 – 198,361) ng.h/m). Equally, desethylamodiaquine exposure is not reduced in underweight-for-age young children or those with high parasitaemia, two of the most vulnerable target populations.

Chapter 1: Rationale, aims and objectives

1. 1 Rationale for the study

In recent years, there have been dramatic increases in political and financial commitments towards efforts to control malaria (Alonso & Tanner, 2013a; Korenromp et al., 2013). To achieve the maximum benefit from this investment, it is important to ensure that patients with malaria receive the best treatment in the correct doses. As a consequence there is widespread deployment of artemisinin-based combination treatments (ACT) as first line treatment of uncomplicated *Plasmodium falciparum* malaria. Artesunate plus amodiaquine (ASAQ) is one of five ACTs currently recommended by the World Health Organisation (WHO, 2010a, 2015a) and is a first-line treatment in Ghana (MOH, 2009, 2014a,b). Available antimalarial pharmacokinetic data are limited for informing optimal dosing due to the small number of patients studied, with even fewer studies conducted in the vulnerable populations that carry the highest disease burden in Africa, i.e. infants, young children and pregnant women. Even fewer data exist that relate antimalarial pharmacokinetic data to therapeutic response and safety (Barnes et al., 2007a; White et al., 2008).

Despite its extensive use for many years both as a monotherapy and currently in combination with artesunate for the treatment of uncomplicated *falciparum* malaria (Olliaro et al., 1996; Olliaro & Mussano, 2003; Olliaro, P., Magnussen, P., & Vaillant, M, 2006; Zwang et al., 2009), there are limited pharmacokinetic data on amodiaquine. There is limited data available on the association between antimalarial treatment responses and drug exposure to amodiaquine and more importantly its active metabolite desethylamodiaquine. Thus, there is need for practical methods for the accurate quantification of amodiaquine and its active metabolite desethylamodiaquine in capillary whole blood for use in routine therapeutic efficacy field studies which are often conducted in remote rural sites with limited infrastructure (WHO, 2011a).

In the evaluation of drug efficacy in clinical trials, treatment failures may be misinterpreted as parasite resistance when they may only be an indication of inadequate drug exposure. It is therefore important that drug concentrations are adequately measured in all key target populations (WHO, 2010a). Sub-optimal blood concentrations of a drug in a patient may result from an inadequate dosing regimen, poor adherence, poor drug quality, interaction with other drugs or malabsorption. It may also be due to vomiting or unusual pharmacokinetic parameters as a results of factors such as poor absorption, an abnormally large volume of distribution or rapid clearance for a particular compound (Barnes, Watkins & White, 2008;

Stepniewska & White, 2008; White et al., 2009; WHO, 2015a). If therapeutic drug levels are not achieved or maintained for a long enough time, treatment failure may result from sub-optimal drug exposure rather than parasite resistance. A correct interpretation of treatment failure is of particular importance in the light of recommendations by the World Health Organization (WHO) that require treatments to be considered reliably effective for use as a treatment policy only if cure rates of at least 95% are achieved in clinical trials in non-immune populations. If the blood concentrations are higher than the minimum inhibitory concentration for long enough to eliminate sensitive parasites, then a strong case for resistance can be made. Blood concentration studies are therefore needed to provide key evidence for determining whether a resistant strain is present (Laufer, Djimdé & Plowe, 2007; WHO, 2010a,b).

Blood concentrations on day 7 (following the start of treatment on Day 0) are a simple measure of parasite exposure to longer-acting antimalarials. A three-day regimen of an artemisinin-based treatment will expose only two 48-hour asexual *P. falciparum* parasite cycles to the rapidly-acting artemisinin component (White, 1997; Hoshen, Stein & Ginsburg, 2002; White, 2004). As a result, residual parasites could still be present in the blood during the fourth post-treatment cycle (i.e. 7 – 8 days following ACT treatment) (Hoshen, Stein & Ginsburg, 2002; White, 2004). Therefore, the blood concentration of an ACT partner drug on day 7 is an important determinant of whether residual parasites will be eliminated and ensure adequate clinical and parasitological response (White, 1997; White et al., 2008). As these day 7 concentrations correlate well with both drug exposure and treatment response, it is postulated that measuring the day-7 antimalarial drug levels would provide a simple tool to help determine whether treatment failures result from low concentrations of partner drugs or from drug resistance (White et al., 2008).

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Transmission intensity affects the incidence of clinical malaria, which determines the frequency of malaria treatments. High re-infection rates lead to high drug consumption which may impact the proportion of individuals with selective drug concentrations (Nyunt & Plowe, 2007; Klein, 2013). However, premunition (partial immunity), acquired after years of repeated infection may result in milder illness and asymptomatic infections in areas of high malaria transmission intensity (Doolan, Dobano & Baird, 2009; Petersen, Eastman & Lanzer, 2011) and may lead to a reduction in drug pressure in such areas (Hastings, 2006; Petersen, Eastman & Lanzer, 2011).

Antimalarial drugs have been developed without necessarily going through stringent selection of doses or dosing regimens based on pharmacokinetic-pharmacodynamic relationships in all the key target populations (Barnes et al., 2007b; Barnes, Watkins & White, 2008). Dose regimens for vulnerable populations such as young children and pregnant women have been extrapolated from studies in non-pregnant adults with uncomplicated malaria and this has often resulted in their sub-optimal dosing (Simpson et al., 2000; Barnes et al., 2006b). Drug resistance is confirmed for all currently available antimalarials, including the pivotal artemisinins (WHO, 2010b; Lu et al., 2017; Sutherland et al., 2017). The development of new drugs for poverty-related and tropical diseases such as malaria tends to be relatively slow in comparison with other compounds; however, the Medicines for Malaria Venture (MMV) and others are starting to address this gap. Therefore there is much to be gained by optimizing dosing of currently available drug therapies for all key target populations in order to prolong their useful therapeutic life until novel antimalarials are available (Simpson et al., 2009; Wells, 2011; Burrows et al., 2013).

In a study conducted in children aged 3 months to 12 years a statistically significant but weak association was established between desethylamodiaquine concentration on day 7 and treatment outcome within one month of follow up. The reason for this weak association was attributed to the small sample size (Hietala et al., 2007). This study included 3 studies: 1) Zanzibar (Kivunge and Micheweni (n=212), 2) Zanzibar(Kivunge (n=12)) in which patients were treated with age-based dosing of amodiaquine plus artesunate combination (Arsucam®, Creapharm, France), and 3) Papua New Guinea (n=20) in which patients aged 1 - 10 years were treated with Infant Camoquin®, (Prawll Laboratories Ltd, India, 100 mg tablet) at a target dose of 30 mg/kg (10 mg/kg daily for 3 days plus a single dose of sulfadoxine-pyrimethamine (25 mg/kg based on sulfadoxine component) on day 7. In total 495 samples (121 for amodiaquine and 374 for desethylamodiaquine) from 117 patients were included in the pharmacokinetic data analysis.

In another study in Burkina Faso, associations between treatment outcome and desethylamodiaquine concentrations on day 7 and 14 following the start of AS-AQ treatment were found. This study compared a fixed-dose combination (n=29) (Arsucam®, Sanofi Aventis, Paris, France) and loose blister pack (n=31) (AS (Arsumax®, Sanofi-Aventis) + amodiaquine (Flavoquine®, Sanofi-Aventis)) artesunate-amodiaquine combination in children 6 months - 5 years. The day 28 PCR-corrected cure rate was 93.7% for the fixed dose artesunate-amodiaquine combination and 93.2% for the loose blister pack tablets (Sirima et al., 2009). Predicted concentrations of desethylamodiaquine were significantly lower in

patients with recurrent parasitaemia. However, the association was less clear for measured desethylamodiaquine concentrations probably due to the small number of samples analysed (n=15). A minimum of 75 ng/ml of desethylamodiaquine on day 7 was estimated as a cut off below which parasite recurrence was more likely to occur (Stepniewska et al., 2009).

An earlier evaluation of the correlation between antimalarial concentrations and treatment outcome in 118 Gabonese children aged 6 months and 10 years treated with amodiaquine (Camoquin, Parke Davis, Dakar, Senegal) monotherapy (10 mg/kg per day for 3 days) established that DEAQ concentrations >135 ng/ml on day 4 were associated with an adequate clinical response (Aubouy et al., 2003). The efficacy of AQ in this study was estimated to be 65.3% (ibid).

Pharmacokinetic information for antimalarial drugs is often determined in plasma but plasma samples are less suitable for field studies and are often impossible to obtain in young children when repeated sampling is required (Schlagenhauf-Lawlor, 2008; WHO, 2011a). Amodiaquine and desethylamodiaquine have been reported to accumulate in white blood cells (lymphocytes) (Laurent et al., 1993) or in neutrophils (Naisbitt et al., 1997). Since whole blood includes the red blood cells (erythrocytes) that are parasitized, which are the site of action of the drug, as well as white blood cells (neutrophils) in which amodiaquine and its main metabolite concentrate, concentrations of amodiaquine and desethylamodiaquine in whole blood may more accurately reflect the concentrations acting on the malaria parasites than plasma. The analysis of whole blood will not only minimize the error associated with drug leaking from cells prior to matrix separation but will be more representative of the effective drug concentration following malaria treatment during field trials (Schlagenhauf-Lawlor, 2008).

The few studies conducted to define the pharmacokinetic parameters of amodiaquine in malaria patients have either used capillary or venous whole blood spotted on filter paper (Hombhanje et al., 2005; Hietala et al., 2007) or venous plasma samples (Winstanley et al., 1990; Adjei, Kristensen, et al., 2008; Stepniewska et al., 2009; Mwesigwa et al., 2010; Jullien et al., 2010). Although several methods have been reported for the simultaneous determination of amodiaquine and desethylamodiaquine concentrations in biological samples, relatively large sample volumes, ranging from 100 µl (Lindegårdh et al., 2002; Minzi et al., 2003) on filter paper to 1 ml (Mount et al., 1986a; Pussard, Verdier & Blayo, 1986; Winstanley et al., 1987a) have been used for these assays. Highly sensitive and selective throughput techniques such as liquid chromatography tandem mass spectroscopy (LC-

MS/MS) are heralded as the gold standard. This will greatly facilitate pharmacokinetic studies as it allows for smaller volumes of samples to ease sample collection from field studies, particularly in young children (Lee & Kerns, 1999a).

The therapeutic efficacy of ASAQ at the time of its introduction as treatment policy in Ghana in 2005 was 100% after 28 days of follow up in children under five years of age (Koram et al., 2005). Subsequently, cure rates ranging from 80% to 95.3% (Koram, Quaye & Abuaku, 2008; Adjei, Kurtzhals, et al., 2008; Oduro et al., 2008; Owusu-Agyei et al., 2008) in various parts of Ghana have been recorded in children 6 months to 10 years, the age group with the largest burden of malaria. In a more recent publication, the day 28 PCR-corrected cure rate of fixed-dose artesunate-amodiaquine combination in 125 patients aged 6 months - 9 years by per protocol analysis was reported to be 100% in two ecological zones in Ghana (Abuaku et al., 2016) (Table 1.1). However, the possibility of differences in drug responses across the country over time may exist.

The prompt and rational use of an effective antimalarial not only reduces the risk of severe disease and death but may also reduce the duration of the illness, malaria transmission and drug resistance. Effective treatment of malaria requires that the dose and frequency of dosing of both antimalarial drugs provides sufficient drug concentrations over time to kill all of the parasites in the body (Simpson et al., 2009). The contribution of inadequate dosing regimens to antimalarial treatment failure and the emergence of resistance have been underappreciated. Sub-therapeutic drug exposures contribute to poorer treatment responses and fuel the spread of antimalarial drug resistance (Barnes, Watkins & White, 2008).

Table 1.1: **Studies of the efficacy of artesunate-amodiaquine combinations in Ghana**

Study period	Study location	Study population age range	Number of patients	Loose / blister pack / fixed dose combination	Manufacturer	Day 28[1]ACPR rate (%)	Reference
June - Aug 2003	Hohoe, Navrongo	6 - 59 months	54	Loose	NS	100	Koram et al., 2005
Sep 2005 - Dec 2006	9 district hospitals	6 - 59 ,months	545	Loose	NS	93.0	Koram et al., 2008
Oct 2004 - Dec 2006	Korle Bu and Mamprobi Polyclinics, Accra	6 months- 14 years	116	Loose	Camoquine® (Pfizer, Dakar, Senegal) Plasmotrim® (Mepha Ltd, Aesch-Basel, Switzerland)	ITT = 91.3 PP= 98.1	Adjei et al., 2008
June 2005 - May 2006	Kintampo	6 months - 10 years	178	Co-blister pack	Arsucam® (Sanofi-Aventis)	93.4	Owusu-Agyei et al., 2008
Nov 2005 - Dec 2006	Navrongo	6 - 120 months	308	Co-blister pack	Ipca Laboratories, Mumbai, India	*Supervised = 80 *Unsupervised = 64	Oduro et al., 2008
Oct 2006 - Sept 2007	Agogo & Agona	6 - 59 months	123	Co-blister pack	Arsucam® (Sanofi-Aventis, Paris, France)	91.7	Kobbe et al., 2008
Jan 2010 - Dec 2011	Korle BuTeaching Hospital, Accra	6months - 12 years	59	Fixed dose	Coarsucam® (Sanofi-Aventis, Paris, France)	*SCD = 96.4 *Non SCD=100	(Adjei et al., 2014)
July 2013- Mar 2014	5 district hospitals	6 months - 9 years	125	Fixed dose	Sanofi-Aventis	100	Abuaku et al., 2016

- **PCR uncorrected ACPR rates; PCR= polymerase chain reaction; [1]ACPR= adequate clinical and parasitological response; NS = Not stated; SCD = Sickle cell disease*

Following more than 5 years since implementation of the ASAQ antimalarial treatment policy in Ghana (MOH, 2009, 2014b), and in the light of reported resistance to the artemisinins in Southeast Asia (Dondorp et al., 2009; Phyo et al., 2012; Ashley et al., 2014; WHO, 2014a), it is prudent to characterize the post-treatment pharmacokinetic and pharmacodynamic (PK/PD) profile of amodiaquine as a partner drug to artesunate. Any substantial pharmacokinetic differences identified between key target populations can be used to inform rationally designed dosing regimens and in so doing maximize cure rates, reduce the emergence of resistance and limit toxicity. This could also prolong the useful therapeutic life of other amodiaquine-based treatments such as seasonal malaria chemoprophylaxis (SMC) in vulnerable populations.

1.2 Aim of study

The main aim of this study was to assess the therapeutic efficacy of the fixed-dose artesunate-amodiaquine combination for the treatment of uncomplicated *falciparum* malaria in Ghana, and to develop and validate a feasible assay for use in routine therapeutic efficacy studies to describe the pharmacokinetic profile of amodiaquine and its active metabolite desethylamodiaquine.

1.3 Primary Objectives

i. To estimate the clinical and parasitological efficacy of artesunate-amodiaquine for the treatment of uncomplicated *falciparum* malaria

ii. To develop a validated simultaneous amodiaquine / desethylamodiaquine assay for the determination of amodiaquine and desethylamodiaquine concentrations from capillary whole blood samples

iii. To describe the pharmacokinetic parameters of amodiaquine and desethylamodiaquine when used as a partner drug to artesunate in the treatment of uncomplicated *falciparum* malaria

1.4 Secondary Objectives

i. Explore factors that influence the pharmacokinetic profile of amodiaquine and desethylamodiaquine in malaria patients, by determining the effects of predefined patient factors [namely site of sample collection, age (age category < 1 year, 1-4 years, 5+ years), sex (male versus female), presence of fever at enrolment (versus only history of fever in past 24 hours), parasite density (≥100,000 versus <100,000 asexual parasites/µL), anaemia (defined as baseline haemoglobin (Hb) <8.0 g/dl versus Hb>8.0 g/dl), weight-adjusted total mg/kg dose and nutritional status (defined for children < 5 years of age as weight-for-age z-score] on the disposition (pharmacokinetic parameters) of amodiaquine and desethylamodiaquine, when amodiaquine is used as a partner drug to artesunate in the treatment of uncomplicated *falciparum* malaria.

ii. To determine whether the desethylamodiaquine area under the curve ($AUC_{0-\infty}$) and concentrations at day 7 post treatment differ between patients who achieved an adequate clinical and parasitological response and patients who failed treatment.

iii. To determine any effect of changes in pharmacokinetic parameters on treatment response in terms of:

   a. Time to parasite recurrence
   b. Time to parasite recrudescence
   c. Duration of gametocyte carriage
   d. Area under the gametocyte density- time curve (AUC)

iv. To compare the matrix effects of capillary whole blood versus capillary plasma samples.

Chapter 2: Literature review

2.1 Burden of malaria

Malaria is a parasitic disease of major global importance. In 2015 there were 91 countries and territories across the world with ongoing malaria transmission (WHO, 2016). About 1.4 billion people are at some risk of being infected with malaria and developing the disease (WHO, 2015b) while 2.57 billion of these people live in regions of the world at risk of *P. falciparum* transmission (Gething et al., 2011). Malaria is caused by the infection of red blood cells with protozoan parasites of the genus *Plasmodium* (P). The parasites are inoculated into the human host by a feeding female Anopheline mosquito. Five *Plasmodium* species infect humans, namely *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae* and recently also the simian malaria parasite, *P. knowlesi* (Singh et al., 2004; WHO, 2012a, 2015b). Of these, *P. falciparum* and *P. vivax* pose the greatest treat. In Africa, *P. falciparum* is the most prevalent parasite species responsible for almost all of the malaria deaths (WHO, 2015b).

Malaria is an important cause of death and morbidity in children and adults, especially in tropical countries. Of the 6.3 million under-5 deaths that occurred worldwide in 2013 (UNICEF, 2013), nearly half were in Sub-Saharan Africa where infectious diseases accounted for 61.5% of these deaths. Of the under-5 mortalities attributed to infectious diseases in Africa in 2013, malaria accounted for 97.0% (Liu et al., 2015). Malaria has killed more young children than any other infectious disease (Fidock, 2013). However, there has been increased attention towards efforts to control malaria (Poore, 2004), and in 2015, malaria ranked as only the fourth highest cause of child deaths in Africa responsible for 10% of all deaths (WHO, 2015b). Despite a drop in malaria mortality by 60% globally and by 66% in Africa between 2000 and 2015, there were an estimated 212 (range 148- 304) million new cases of malaria worldwide resulting in 429 000 deaths in 2015. Most (92%) of the global malaria deaths occurred in the WHO Africa region. About 303,000 (70%) of the global deaths occurred in children under 5 years of age (WHO, 2015b, 2016).

The economic burden of malaria on endemic countries contributes to the cycle of poverty people face in many of these countries. In Africa malaria is estimated to cost at least US$12 billion per year in direct losses such as illness, treatment, premature death, but much more indirectly through lost economic growth (Gallup & Sachs, 2001; RBM, 2014).

In Ghana, malaria is a major cause of morbidity and mortality, particularly among children and pregnant women. The main parasite species responsible for malaria are *P. falciparum* (80 – 90%), *P. malariae* (10 – 20%) and *P. ovale* (0.15%) with parasite prevalence in all age groups ranging from 10 to 70% across the country (MOH, 2013, 2014a). Among children aged 6 - 59 months, the average parasite prevalence rates were estimated to average 27.5% in 2011 (range 4.0% - 51% across the country) and still 27% (range 11.2% - 40.0% across the country) in 2014 (Ghana Statistical Service, 2011, 2015). In 2012, malaria was responsible for 38.9% of all outpatient consultations, 38.8% of all admissions and 33.4% of all deaths in children under five years (MOH, 2014b; President's Malaria Initiative, Ghana, 2014). Malaria infection during pregnancy causes maternal anaemia and placental parasitaemia, leading to miscarriages and low birth weight babies. Among pregnant women, malaria was responsible for 13.8% of all out-patient attendances, 16.8% of all admissions and 3.4% of deaths in 2012 (MOH, 2014a). The economic burden of malaria in Ghana has been estimated to cause the loss of 10.6% Disability Adjusted Life Years (DALYs) which is about the equivalent of 6% of Ghana's gross domestic product (GDP) (MOH, 2009, 2014b).

2.2 Malaria control strategies

In 2005, the 58th World Health Assembly set the target of reducing the global burden of malaria by at least 50% by 2010 and by 75% by 2015 compared to the levels in 2000 (WHO, 2005). These targets and objectives were further updated by the Roll Back Malaria (RBM) Partnership to include the reduction of malaria associated mortality to near zero by 2015 (RBM, 2008, 2011). To achieve these goals, key malaria interventions have been implemented leading to substantial but fragile progress in the fight against malaria (WHO, 2015b,d). In order to ensure sustained fight against malaria, a new set of strategies for the period 2015 - 2030 have been set that place emphasis on the need for universal coverage of core malaria interventions for all populations at risk (WHO, 2015d). The World Health Organization recommends two main strategic integrated

approaches of quality assured malaria prevention and case management (RBM, 2008; WHO, 2010c, 2015d). These two approaches are aimed at ensuring universal access to malaria prevention, prompt diagnosis and effective treatment.

2.2.1 Malaria prevention through vector control

Malaria vector control has two main objectives: to provide protection for individuals against infective malaria bites, and to reduce the longevity, human-vector contact and intensity of the local mosquito vector population and thereby reduce the local malaria transmission at the community level. These objectives are being achieved through the use of long-lasting insecticide-treated nets (LLINs) and indoor residual spraying (IRS) (WHO, 2006a, 2012a, 2015b). There are 12 insecticides belonging to 4 chemical classes: organochlorines, organophosphates, carbamates and the pyrethroids recommended by the World Health Organization Pesticide Evaluation Scheme (WHOPES) for indoor residual spraying (WHO, 2009a, 2012b). The pyrethroids were introduced in the 1970s and account for 75% of the IRS coverage and are the only class of insecticides used on treated bed nets (WHO, 2007a; Davies et al., 2008; Alonso & Tanner, 2013b).

Resistance to insecticides seriously threatens the use of insecticides in the fight against malaria. Resistance to at least one insecticide used for malaria control has been reported in 64 malaria-endemic countries (WHO, 2012b). Despite the progress made to date, the global malaria control programme continues to rely heavily on the pyrethroids and this puts the global malaria control efforts at risk in the face of widespread resistance to pyrethroids and increasing resistance to other available insecticides (Matambo et al., 2007; Enayati & Hemingway, 2010; WHO, 2016).

2.2.2 Malaria control through preventive chemotherapy

Preventive chemotherapy is the intermittent use of complete treatment courses of effective antimalarial medicines for targeted populations at risk of malaria whether they are parasitaemic or not, with the overall aim of maintaining therapeutic antimalarial drug concentrations in the blood throughout the period of greatest malarial risk (WHO, 2015a). This is expected to ensure the clearance of any malaria parasites and prevent

malaria infection, and thereby reducing morbidity and mortality due to malaria. Preventive chemotherapy has now been established as a core component of malaria control in areas of high malaria transmission for groups particularly vulnerable to *P. falciparum* infection (WHO, 2010d, 2011b, 2012c, 2013a,b). The two strategies presently recommended by World Health Organization for the chemo-prevention of malaria are Seasonal Malaria Chemoprevention (SMC) and Intermittent Preventive Treatment (IPT) for pregnant women (IPTp) or infants (IPTi).

2.2.2.1 Seasonal Malaria Chemoprevention

Formerly known as Intermittent preventive treatment in children (IPTc), seasonal malaria chemoprevention (SMC) is the periodic administration of full treatment courses of an antimalarial medicine during the malaria season to prevent malarial illness in children aged between 3 and 59 months with the objective of maintaining therapeutic antimalarial drug concentrations in the blood throughout the period of greatest malarial risk (WHO, 2011b, 2012c). SMC has been shown to be effective, safe and feasible for preventing malaria among children under 5 years of age in areas with highly seasonal malaria transmission (Kweku et al., 2008; Bojang et al., 2010; Dicko et al., 2011; Konaté et al., 2011; WHO, 2011b). As a result, the World Health Organization recommends the use of SMC in areas of highly seasonal malaria transmission across the Sahel sub region of Africa. SMC comprises of a complete treatment course of amodiaquine (AQ) plus sulfadoxine-pyrimethamine (SP) which should be given to children aged 3–59 months at monthly intervals, beginning at the start of the transmission season, up to a maximum of four doses during the malaria transmission season (WHO, 2012c, 2013b). Ten countries had adopted the policy as of 2015 (WHO, 2016). The Ministry of Health of Ghana has revised its antimalarial drug policy to include SP plus AQ for SMC in the Northern savanna belt (MOH, 2014b).

2.2.2.2 Intermittent Preventive Treatment in Pregnancy

A review of trials evaluating the Intermittent Preventive Treatment with sulfadoxine-pyrimethamine for pregnant women (IPTp - SP) showed that three or more doses of SP during pregnancy were associated with a higher mean birth weight and fewer low birth weight (<2500g) deliveries than two doses or less during pregnancy (Kayentao, 2013). IPTp has been adopted as national policy by 36 of the 45 sub-Saharan African countries (WHO, 2013a). It is recommended that the first IPTp-SP dose should be administered as early as possible during the 2nd

trimester of pregnancy and that a dose of SP should then be given at each antenatal visit provided these are at least 1 month apart with the last dose administered up to the time of delivery. Ideally, SP should be administered as a directly observed therapy (DOT) of three tablets SP (each tablet containing 500 mg/25 mg SP), the total recommended adult dosage. The World Health Organization also recommends the administration of a daily dose of 0.4 mg folic acid, which may be safely used in conjunction with SP (WHO, 2013a, 2014b).

2.2.2.3 Intermittent preventive treatment in infants (IPTi)

Intermittent Preventive Treatment in infancy with Sulfadoxine-Pyrimethamine (SP-IPTi) is the administration of a full treatment dose of SP delivered through the Expanded Program on Immunization (EPI) to infants at risk of malaria at defined intervals corresponding to routine vaccination schedules – usually at 10 weeks, 14 weeks, and about 9 months of age (WHO, 2010d). Based on the evidence from several trials (Greenwood, 2006; Aponte et al., 2009), the World Health Organization in 2010 recommended the co-administration of SP-IPTi with DTP2, DTP3 and measles immunization to infants, through routine immunization services in sub-Saharan African countries with moderate to high malaria transmission intensity and where parasite resistance to SP is not high (WHO, 2010d). However, as of 2015, no countries have reported the adoption of IPTi for malaria as a policy (WHO, 2016).

2.2.3 Malaria Case Management

In recent years, there has been a remarkable renewal of political will, increased financial commitments and implementation of programmes to control malaria (WHO, 2012a; Korenromp et al., 2013). In spite of these efforts and increased resource allocation, and notable decreases in the morbidity and mortality resulting from infection with malaria, the global burden (WHO, 2015b, 2016) is still staggering .

In the management of uncomplicated malaria cases, treatment is provided to ensure the rapid and complete clearance of parasites from the body in order to prevent progression to severe disease or death. Importantly, treatment is also provided to reduce malaria transmission by reducing the infectious parasite reservoir from which mosquitoes may infect other persons and to prevent the emergence and spread of resistance to

antimalarial drugs (WHO, 2009b, 2010a, 2015a). In order to achieve these objectives, the World Health Organization recommends, through the Global Malaria Programme's new Test, Treat, Track (T3) initiative, that malaria endemic countries should ensure that every suspected malaria case is promptly tested and confirmed by either microscopy or rapid malaria diagnostic test (RDT) before treatment and that every confirmed uncomplicated case of *P. falciparum* malaria should be treated with a quality-assured artemisinin-based combination treatment (ACT) (WHO, 2012d). The policy also urges malaria-endemic countries to routinely monitor antimalarials for drug quality and therapeutic efficacy, and that individual cases be followed up through registration at the health facility level to ensure all individual malaria cases are tracked (WHO, 2012d).

The goal of treatment for severe malaria is to prevent death. In the treatment of cerebral malaria, the objective is also to prevent neurological deficit (WHO, 2010a). Parenteral artesunate has been shown to significantly reduce mortality from severe malaria mostly in adults from 22.4% to 14.7% compared to quinine in Asia (South East Asian Quinine Artesunate Malaria Trial (SEAQUAMAT) group, 2005). In Africa, parenteral artesunate has equally been shown to significantly reduce the mortality associated with severe malaria in hospitalized children from 10.9% to 8.5% when compared to parenteral quinine (Dondorp et al., 2010). In a Cochrane review published in 2012, parenteral artesunate was found to be associated with a 39% reduction in mortality among adults with severe malaria and about a 24% reduction among children with severe malaria, when compared to quinine. The use of parenteral artesunate was also found to be associated with a small increase in neurological sequelae at the time of hospital discharge but the associated sequelae resolved within 28 days (Sinclair et al., 2012). Consequently, it is recommended that severe *P. falciparum* malaria be treated with intravenous or intramuscular artesunate, followed by a full course of an effective ACT (WHO, 2010a, 2012d, 2015a). In a recent review of the antimalarial drug policy for Ghana, parenteral artesunate has been recommended as the medicine of choice for the management of severe malaria, to be followed by a full course of an ACT when the patient is able to take oral medication. However, parenteral quinine is still the recommended choice of treatment for the first trimester of pregnancy until the patient is able to take oral medication (MOH, 2014b).

2.3 Antimalarial drug resistance

Resistance to antimalarial drugs is a major public health challenge and a threat to efforts to control and eventually eliminate malaria. Antimalarial drug resistance has been documented for *P. falciparum*, *P. malariae* and *P. vivax*. *P. falciparum* resistance has been established to all currently used antimalarial drugs including the pivotal artemisinins (WHO, 2010b). In general, low or sub-therapeutic drug concentrations increase the risk of treatment failure and provide a selection pressure for resistant genotypes (Watkins & Mosobo, 1993; White, 1997; WHO, 2010a). Universal access to antimalarial drugs may have extensive benefits both for the individual and the population at large by decreasing the likelihood of morbidity and mortality and therefore the chances of transmission (Klein, 2013). However, the widespread, irrational and indiscriminate use of antimalarials may exert a selective pressure on malaria parasites leading to the development of high levels of resistance and treatment failures.

Resistance to antimalarial drugs emerges in two discrete phases: *de novo* emergence of resistant parasites, and the survival, expansion and spread of these resistant parasites to other individuals (White & Pongtavornpinyo, 2003; White, 2004; Barnes & White, 2005; Stepniewska & White, 2008). Resistance may emerge *de novo* within an acute infection through spontaneous mutations or gene duplications that are selected for by antimalarial drug concentrations sufficient to clear drug-sensitive strains of the parasite but still allow the mutant parasite sub-population to survive and subsequently multiply. The appearance or emergence of *de novo* resistance to antimalarial drugs as a result of genetic mutations is a rare event and is most likely to arise and subsequently spread from patients with high parasite densities (hyperparasitaemic patients) receiving inadequate treatment (White & Pongtavornpinyo, 2003; White et al., 2009). In order for the mutant resistant parasite population to expand and produce gametocytes sufficient to transmit to biting Anopheline mosquitoes and to spread to other hosts, the mechanism of resistance must not affect their fitness (White, 2004; Barnes & White, 2005). Resistant parasite strains survive because they are associated with increased gametocyte carriage both in the initial and subsequent recrudescent infections. Antimalarial resistance in malaria parasites spreads because it confers a survival advantage in the presence of the antimalarial and therefore results in a greater probability of transmission for resistant than sensitive parasites. Resistant infections are more likely to recrudesce and carry gametocytes, and as resistance worsens, infections with resistant parasites respond more slowly to treatment (White, 2004; Barnes & White, 2005; Barnes et al., 2008).

2.4 Artemisinin-based combination therapy

Antimalarial drug combination therapy is the simultaneous use of two or more blood schizontocidal drugs with independent modes of action and different biochemical targets in the parasite (White, 1999; WHO, 2001). Artemisinin and its derivatives have short half-lives which result in substantial treatment failure rates when used as short-course monotherapy (Woodrow & Krishna, 2006). In order to forestall the catastrophic impact of *P. falciparum* resistance to traditional monotherapies such as chloroquine, sulfadoxine-pyrimethamine, amodiaquine and mefloquine and to improve treatment outcomes, the World Health Organization recommends the use of artemisinin based combination treatments (ACT). In artemisinin based combination therapy (ACT), artemisinin-derivatives namely artesunate, artemether or dihydroartemisinin, which have improved bioavailability compared to artemisinin, are combined with longer acting partner drugs to ensure sustained antimalarial concentrations above the minimum inhibitory concentration (MIC) after the plasma concentrations of the artemisinin-derivatives have fallen below therapeutic levels (White, 1999; Eastman & Fidock, 2009). Provided each component is highly effective, artemisinin-based combinations provide high efficacy, excellent tolerability and mutual protection against resistance and reduce the selective pressure for resistance (White, 1998, 1999; WHO, 2001a). Five ACTs are currently recommended for the treatment of uncomplicated *falciparum* malaria, namely artemether plus lumefantrine, artesunate plus amodiaquine, artesunate plus mefloquine, artesunate plus sulfadoxine-pyrimethamine and dihydroartemisinin plus piperaquine (WHO, 2010a, 2015a). By 2013, ACTs had been adopted by 79 of the 88 *P. falciparum* malaria-endemic countries for use as first line treatments in their national malaria drug policies (WHO, 2014c). Despite these policies, treatment with appropriate antimalarial drugs continues to be inadequate. The median proportion of febrile children aged under 5 years with *P. falciparum* malaria who received an ACT treatment in 2013 - 2015 was estimated to be 14 (IQR 5 - 45)% due mainly to lack of access to ACTs in the informal sector (WHO, 2016). Artesunate-amodiaquine (ASAQ) is being used as either first- or second- line malaria treatment in 27 countries, 25 of which are in Africa. Of the 25 countries in Africa, 23 (10 in West Africa) have adopted artesunate plus amodiaquine as first line treatment (WHO, 2010b, 2015b).

Evidence from five countries in the Greater Mekong sub region point to *P. falciparum* resistance to artemisinin and its derivatives (WHO, 2014a,d, 2016). This manifests mainly as slowed parasite clearance and does not seem to affect the overall efficacy of ACTs provided the

partner drug remains effective (Dondorp et al., 2009; Phyo et al., 2012; Ashley et al., 2014; WHO, 2014a). However, in Cambodia and Thailand, in addition to slowed parasite clearance, resistance to artemisinin partner drugs such as mefloquine and piperaquine has been observed leading to high ACT treatment failure rates (WHO, 2015c).

In accord with recommendations by the World Health Organization for endemic countries to switch treatment to artemisinin based combinations in order to avoid resistance and recurrence of the disease, the Ministry of Health in Ghana changed its antimalarial drug treatment policy in 2004 from chloroquine to the artesunate-amodiaquine combination as the first line treatment, with artemether-lumefantrine or dihydroartemisinin-piperaquine as alternatives for the management of uncomplicated malaria, with the actual roll out of the policy taking place later in 2005 (MOH, 2009, 2014b).

The artesunate-amodiaquine combination is currently available as a fixed-dose formulation (Coarsucam®/ASAQ Winthrop (Sanofi, Maphar Morocco)) with tablets containing 25/67.5 mg, 50/135 mg or 100/270 mg of artesunate and amodiaquine, respectively. Blister packs of separate scored tablets (Arsuamoon®, Guilin Pharmaceutical Co., Ltd., China) containing 50 mg of artesunate and 150 mg amodiaquine (196 mg as hydrochloride), are also available. A target dose of 4 mg/kg body weight per day of artesunate (range 2 to 10 mg/kg) and 10 mg/kg body weight per day of amodiaquine (range 7.5 to 15 mg/kg) once daily for three days is recommended (WHO, 2010a, 2015a).

In an individual patient data analysis of parasite clearance over the period 1999 - 2009 in 44 sites across 20 Sub-Saharan African countries, the proportion of patients treated with artesunate-amodiaquine who were still parasitaemic on day 2 of follow up was estimated to be 8.6% (603/7020, 95% CI 7.9 - 9.3%). The proportion of patients still parasitaemic on day 2 was however, variable and ranged from 1% in Nanoro, Burkina Faso in 2008 and Kindamba, Congo in 2004 to 57.1% in Boende, DRC (Zwang et al., 2014). The main risk factors associated with being parasitaemic on day 2 were identified as higher parasite density prior to treatment and anaemia.

In the same review, the proportion of patients still parasitaemic on day 3 of follow up after treatment with artesunate-amodiaquine was estimated to be 1.5% (116/7550, 95% CI 1.2 - 1.8) and varied from 0% in 17 of 44 study sites across Africa to 55.9% in Boende, DRC. The significant risk

factors for remaining parasitaemic on day 3 following treatment with artesunate-amodiaquine were young age and higher parasite density at enrolment (Zwang et al., 2014). These findings show that most patients treated with artesunate-amodiaquine across Sub-Saharan Africa achieved Plasmodium falciparum parasite clearance by day 3, albeit with variations across sites.

In another systematic review of clinical trials conducted since 1960 with at least one artesunate-amodiaquine arm, the day 28 PCR-adjusted clinical efficacy was estimated to be 98.1% (95% CI: 97.6 - 98.5%) in patients treated with fixed-dose combination and 97.9% (95% CI: 97 - 98.8%) in patients treated with co-blistered non-fixed dose combination. These cure rates were significantly higher than 95.0% (95% CI: 94.1 - 95.9% in patients treated with 30 mg/kg amodiaquine loose non-fixed combination and 93.4% (95% CI: 91.9 - 94.9%) in patients treated with 25 mg/kg loose non-fixed dose combination ($p<0.001$for all comparisons). The day 28 efficacy was found to be lowest at 90.9% (95% CI 85.6 - 96.1%)) in infants (<1 year) treated with 25 mg/kg loose non-fixed dose combination (WorldWide Antimalarial Resistance Network (WWARN) AS-AQ Study Group, 2015)

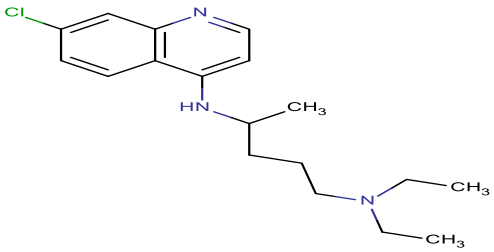
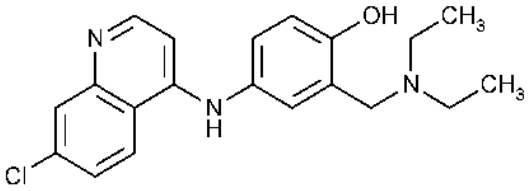
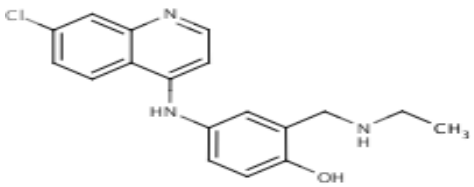
More recently studies with FDC artesunate-amodiaquine in Liberia, Equatorial Guinea and Ghana estimate the efficacies of fixed dose ASAQ to be 97.3% in Equatorial Guinea and Liberia and 100% in Ghana (Charle et al., 2013; Schramm et al., 2013; Abuaku et al., 2016), which are higher than those observed previously with loose tablet combinations.

As the WHO recommended target (range) daily dose is 4 (2-10) for artesunate and 10 (7.5 – 15) mg/kg for amodiaquine are not at a fixed ratio, the loose combination would allow for more dosage precision as the two components are dosed independently. However problems with the loose dose combinations include being less practical and may lead to patient non-adherence (including the risk of single drug for example artesunate monotherapy intake) and consequently reduce drug safety, effectiveness and acceptability (Taylor et al., 2006).

Fixed-dose combinations have higher efficacy compared with loose tablets (WorldWide Antimalarial Resistance Network (WWARN) AS-AQ Study Group, 2015)One hypothesis considered to explain the higher cure rates was the optimal formulation of the fixed dose combination (Taylor et al., 2006) . However, both amodiaquine dose and the area under the concentration-time curve of desethylamodiaquine from time zero

to day 28 (AUC_{0-28}) were found to be significantly lower (~16% lower) among patients treated with fixed-dose compared to those treated with non-fixed dose artesunate-amodiaquine combination, with no difference between the mg/kg dose-adjusted AUCs (Ogutu et al., 2014).

Figure 2.1: Chemical structures of Chloroquine, Amodiaquine and Desethylamodiaquine

<table>
<tr><td>a. Structure of chloroquine
  </td><td>b. Structure of amodiaquine
  </td></tr>
<tr><td>Chemical formula: $C_{18}H_{26}ClN_3$</td><td>Chemical formula: $C_{20}H_{22}ClN_3O$</td></tr>
<tr><td>Chemical name:
7-Chloro-4-((4-(diethylamino)-1-methylbutyl)amino) quinoline</td><td>Chemical name: 4 – [(7- Chloroquinolin – 4 – yl) amino] – 2 – [(diethylamino) methyl] phenol</td></tr>
<tr><td>c. Structure of desethylamodiaquine
  </td><td></td></tr>
<tr><td>Chemical formula: C18-H18-Cl-N3-O</td><td></td></tr>
<tr><td>Chemical name:
4-((7-Chloro-4-quinolinyl) amino)-2-((ethylamino) methyl) phenol</td><td></td></tr>
</table>

2.5 Amodiaquine

Amodiaquine (AQ) is structurally related (phenyl substituted analogue) to chloroquine, both of which are 4-aminoquinoline drugs (Burckhalter et al., 1948). Figures 2.1a and b show the chemical structures of chloroquine and amodiaquine respectively. Amodiaquine has been used widely to treat and prevent malaria for many years. A systematic review of amodiaquine

for the treatment of uncomplicated malaria found that it was as effective as, or more effective than chloroquine, and that its adverse effects were similar to those of chloroquine (Olliaro et al., 1996). Amodiaquine is recommended by the World Health Organization as a partner drug in artemisinin combination therapies (Ridley & Hudson, 1998; WHO, 2010a, 2015a; Olliaro & Mussano, 2003). Its use as a prophylactic however, was associated with high incidences of hepatitis and agranulocytosis (Hatton et al., 1986; Neftel et al., 1986) that led to recommendations for its withdrawal as a malaria prophylactic agent (WHO, 1990)..

2.5.1 Safety and Toxicity of amodiaquine

Amodiaquine is considered to be a prodrug (Churchill et al., 1985) and is rapidly and extensively metabolised through a first pass catalytic oxidation of cytochrome P450 2C8 enzyme to its main active metabolite N-desethylamodiaquine (DEAQ) (Figure 2.1 c), as well as 2-hydroxydesethylamodiaquine and N-bisdesethylamodiaquine. Cytochrome P450 isoform CYP2C8 catalyzes the N-dealkylation of amodiaquine (Churchill et al., 1985; Mount et al., 1986a; White et al., 1987; Li et al., 2002). The formation of N-desethylamodiaquine is fast but its elimination is slow (Winstanley et al., 1987a; Laurent et al., 1993). Amodiaquine and desethylamodiaquine have wide inter-individual variability in their kinetic parameters. This variability may be a reflection of the metabolic capacity of an individual and could result in different therapeutic and toxicological response to amodiaquine (Pussard et al., 1987).

The use of amodiaquine either alone or in combination at a dose of up to 35 mg/kg (Olliaro et al., 1996) for the treatment of uncomplicated malaria has been reported to be safe and tolerable with no serious adverse reactions (Taylor & White, 2004a; Egunsola & Oshikoya, 2013; Dodoo et al., 2014; Pregact Group et al., 2015; Assi et al., 2017). However, when used as a prophylactic, amodiaquine is associated with serious adverse reactions. In areas where malaria is hyperendemic and individuals receive several malaria treatments per year, these treatments tend to resemble prophylaxis. A recent evaluation of the newly introduced Seasonal Malaria Chemoprevention (SMC) in Senegal showed that artesunate-amodiaquine combination is well tolerated in children with no serious adverse events attributable to the intervention detected in a three year high level surveillance among 780,000 documented courses administered (Ndiaye et al., 2016). Additionally, repeated administration of artesunate-amodiaquine and artemether-lumefantrine over a 2 year period did not lead to unexpected safety issues (Yeka et al., 2016).

Adverse events commonly reported among patients treated with amodiaquine, either alone or as a combination include: anorexia, abdominal pain, diarrhoea, nausea, vomiting

necessitating alternative treatment, asthenia, dizziness, insomnia, headache, pruritus and cough (Olliaro et al., 1996; Adjuik et al., 2002; Taylor & White, 2004a).

Case reports of severe adverse events following treatment with amodiaquine or its combination have documented rare neurological or dystonic reactions such as protruding tongue, intention tremor, excessive salivation, dysarthria and bradycardia (Akindele & Odejide, 1976; Kamagaté et al., 2004; Akpalu, Nyame & Dodoo, 2005; Adjei et al., 2009). Some of these events were related to large doses (greater than recommended standard doses) of amodiaquine, while others were related to standard treatment regimens. The cause of these dystonic reactions have been described as idiosyncratic (Akindele & Odejide, 1976). For bradycardia occurring after a standard dose of amodiaquine, an event that coincided with the time of peak concentration of desethylamodiaquine, a direct drug effect has been adduced (Adjei et al., 2009).

Amodiaquine has caused serious and, in some cases, fatal liver and bone marrow toxicity when used as a prophylactic drug (Hatton et al., 1986; Neftel et al., 1986; Taylor & White, 2004b). Agranulocytosis usually developed after between 5 and 14 weeks of prophylaxis and was associated with hepatitis in some travellers (Bepler et al., 1959). The risk of developing agranulocytosis following the prophylactic use of amodiaquine has been estimated as 1 in every 2100 treatments, 1 in 15 650 for serious hepatic reactions and 1 in 30,000 treatments for aplastic anaemia. The total case fatality rate was estimated to be 1 in 31 300 (Hatton et al., 1986; Phillips-Howard & West, 1990).

Amodiaquine and desethylamodiaquine are chemically unstable in aqueous solution and undergo transformation to a protein arylating quinone imine (Maggs et al., 1988). The mechanism underlying the toxicity of amodiaquine is reported to be related to amodiaquine either exerting a direct toxic effect on the liver through the production of a quinone- imine intermediate in bone marrow cell precursors (Winstanley et al., 1990) or to the ability of quinone-imine derivatives of amodiaquine to form immunogenic aryl-protein complexes through IgG anti-amodiaquine antibodies (Maggs et al., 1988; Clarke et al., 1990, 1991). Hepatitis has been reported to occur from as early as 3 weeks, to after as long as 10 months of amodiaquine prophylaxis with reported features of clinical cases ranging from a mild transient elevation of liver enzymes with few symptoms, to fulminant hepatitis resulting in slow recovery of liver function or death (Larrey, 1986).

2.5.2 Pharmacokinetic parameters of amodiaquine and desethylamodiaquine in healthy volunteers and in malaria patients

Table 2.1 presents a summary of some pharmacokinetic parameters of amodiaquine and desethylamodiaquine in healthy individuals. In healthy adult volunteers, amodiaquine is reported to undergo a rapid absorption following the administration of a single oral dose of 600 mg of amodiaquine, reaching a mean ± standard error (se) of the peak concentration (C_{max}) of 32 ± 3 ng/ml at 0.5 ± 0.03 hours in plasma. The mean ± se peak whole blood concentration of amodiaquine was reported to be 60 ± 10 ng/ml at 0.5 ± 0.1 hours in the same study. The mean ± se peak concentration of the active metabolite of amodiaquine, N-desethylamodiaquine was reported as 181 ± 26 ng/ml at 3.4 ± 0.8 hours in plasma and 561 ± 70 ng/ml at 2.2 ± 0.5 hours in venous whole blood (Winstanley et al., 1987a). In adult healthy volunteers dosed with fixed (single dose of 200 mg artesunate plus 540 mg of amodiaquine) or non-fixed (single dose of 200 mg of artesunate plus 612 mg amodiaquine) combinations of artesunate-amodiaquine, the mean peak amodiaquine concentration was 59.4 ± 42.3 ng/ml at 0.89 ± 0.4 hours for the fixed dose and 61.7 ± 24.8 ng/ml at 1.44 ± 1.1 hours for the non-fixed dose. The mean± SD peak concentrations of desethylamodiaquine was 879 ± 634 ng/ml at 1.39 ± 0.8 hours for fixed and 973 ± 511 ng/ml at 1.7 ± 0.77 hours for the non-fixed dose combinations (Navaratnam et al., 2009). Desethylamodiaquine has a longer terminal elimination half-life than amodiaquine with widely reported values ranging from 9 – 31.5 days in healthy adults (Pussard et al., 1987; WHO, 2011a).

Table 2.1: **Some pharmacokinetic parameters of AQ and DEAQ in healthy individuals**

Reference	Dose	C_{max} (ng/ml)		T_{max} (hours)		$AUC_{0-\infty}$ (ng. h /ml)		$T_{½}$ (hours)		Population
		AQ	DEAQ	AQ	DEAQ	AQ	DEAQ	AQ	DEAQ	
Winstanley et al., 1987: Plasma	Single oral dose AQ, 600mg	32±3	181±26	0.5±0.03	3.4±0.8	154±38	8037±1383	5.2±1.7		7 healthy males, 22 - 44 years
Winstanley et al., 1987; Whole blood		60±10	561±70	0.5±0.1	2.2±0.5		20074±3270			
Pussard et al., 1987; Plasma	Single oral dose, AQ, 10mg/kg		203.7±72.6 (104.1 - 258.9)		≤ 1				9 - 18.2 d	4 healthy volunteers, 27 - 50 years
Pussard et al., 1987; Whole blood			1033.3±577.8 (485.2 - 1659.3)		1 - 7				11.9 - 31.5 d	
(Orrell et al., 2008a)	Single dose 4mg/kg AS(Arsumax)+ 10mg/kg AQ (Camoquin)	22.7±9.0 / 29.2±10.9	301.4±166.1 / 268.7±70.8	2.18±1.61 / 2.32±1.16	2.18±1.03 / 3.68±1.85	108.5±56.0 / 162.4±101.4	8,437 ± 4,009 / 12,041 ± 3,480	3.9±1.2 / 5.3±4.1	136.9±83.8/ 240.8±146.9	15 healthy volunteers, 18 – 45 years
(Navaratnam et al., 2009); plasma	Single dose: 200mg AS + 540 mg AQ as FD	59.4±42.3	879±634	0.89±0.4	1.39±0.8	215.6±172.0	63,499±75,732	2.3±1.4	201±119	24 healthy normal volunteer, 21 - 45 years
	Single dose: 200 mg AS + 612 mg AQ as nFD	61.7±24.8	973±511	1.44±1.1	1.7±0.77	226.1±75.2	50,287±25,666	2.2±1.1	224±102	

- *AQ = amodiaquine; DEAQ=desethylamodiaquine; C_{max}= maximum concentration observed; T_{max}=time to maximum concentration; $AUC_{0-\infty}$ = area under the concentration-time curve from time 0 to infinity; $T_{½}$ = half-life; FD = Fixed dose; nFD = non-fixed dose*

Table 2.2 presents a summary of some pharmacokinetic parameters of amodiaquine and desethylamodiaquine in malaria patients. In malaria patients older than 5 years, the elimination half-life of amodiaquine is reported to be in the range of 3.7 – 7.9 hours, but desethylamodiaquine has a much longer but variable half-life ranging from 2.4 – 10.5 days in adult malaria patients (Winstanley et al., 1990; Jullien et al., 2010) (Table 2.2). Potential differences between children and adults have been noted for both amodiaquine and desethylamodiaquine. Available data suggests that the peak amodiaquine concentration in children with malaria is lower (5.2 ng/ml) than in adult malaria patients (21 ng/ml) (Winstanley et al., 1990; Mwesigwa et al., 2010). However, the peak desethylamodiaquine concentration in children with malaria is variable (235 – 1185 ng/ml) (Hietala et al., 2007; Adjei et al., 2008; Stepniewska et al., 2009; Mwesigwa et al., 2010) and higher than in adult malaria patients (161 ng/ml) (Winstanley et al., 1990). Similarly, adult malaria patients appear to achieve peak concentrations of desethylamodiaquine faster (3.9 hours) than in children with malaria (2 – 3 days) (Winstanley et al., 1990; Hombhanje et al., 2005; Adjei et al., 2008).

Table 2.2: **Previously published studies reporting pharmacokinetic parameters of AQ and DEAQ in malaria patients**

Reference	Dose	C_{max}(ng/ml)		T_{max}		AUC $_{0-\infty}$ (ng/ml. h)		$T_{½}$		Population
		AQ	DEAQ	AQ	DEAQ	AQ	DEAQ	AQ	DEAQ	
Whole blood/ filter paper										
Hombhanje et al, 2005	30mg (10mg/kg/day x3) + SP		368.8 (306.6 - 431)		3 days		108,302 (84,415 - 132,190)		10.1 (6.3- 13.9) days	20 malaria patients, 1 - 10 years
Hietala et al, 2007	Arsucam, 10mg/kg/day x 3 days + SP on d7		274 (192 - 369)				32,058 (17,851 - 50,638)		4.9 (3.3 - 8.0) days	212 malaria patients, 3 months - 12 years
(Ntale et al., 2009)	150 mg AQ (Amobin®)+50 mg AS(Arinate®) or 200mg+75mg/day x3	644	1767	3 days						12 malaria patients, 1.5 – 8 years
Plasma										
Winstanley et al., 1990	AQ, 25 mg/kg AQ: 10:5:5:5 mg/kg AQ: 10:10:5mg/kg	21 ±11	161±72	2.0±2.0 h	3.9±1.2 hr			3.7± 1.3 hr		19 malaria patients, 7 – 55 years
Adjei et al, 2008	AQ-10mg/kg alone or + 4mg/kg AS x3 days		537±244 /1185± 432		1.95 ±0.33 / 2.0 ± 0.05 days		40339 ± 16021/ 38516 ± 14138		4.3 (3.3-5.1) / 4.6 (3.3-5.9) days	103 malaria patients, 1 - 14 years
Stepniewska et al, 2009	FDC & non-FDC ASAQ(4mg/kg AS+10mg/kg AQ)						48,700 (39,000-92,300) / 62,500 (24,400 -95,300		9 (7.3 - 11.6) d	60 malaria patients, 6months – 5 years

Mwesigwa et al, 2010	AQ:10mg/kg/day x2, 5mg/kg 3rd day+ 4mg/kg AS bdx3)	5.2	235 (219- 266)			39.3	14,770 (13,914 - 16,226)	3.3 hr	2.5 (2.4- 2.7) d	20 malaria patients, 5 - 13years
Jullien et al, 2010	2 tabs FDC ASAQ (100/270), 4 tabs nFDC AS/AQ (50/153)/day x3 days						27600 ± 3200	7.9 hr	8.5 (7.6-9.4) / 8.9(7.4-10.5) days	54 Adult malaria patients, 18 – 60 years

- *AQ = amodiaquine; DEAQ=desethylamodiaquine; SP= Sulfadoxine-pyrimethamine; ASAQ= artesunate-amodiaquine; AS=artesunate; C_{max}= maximum concentration observed; T_{max}=time to maximum concentration; $AUC_{0-\infty}$ = area under the concentration-time curve from time 0 to infinity; $T_{½}$ = half-life; hr=hour; mg=milligram; mg/kg= milligram per kilogram; FDC= fixed-dose combination; nFDC= non-Fixed dose combination*

2.5.3 Analytical techniques for measuring the concentrations of antimalarial drugs

The choice of analytical technique used to measure the concentrations of antimalarial drugs in biological fluids depends mostly on the concentration range of interest (Bergqvist & Churchill, 1988). The required sensitivity of an assay depends on the matrix and the target concentrations of the drug being investigated.

Several methods for the simultaneous determination of amodiaquine and desethylamodiaquine in biological fluids have been reported (Table 2.3). High performance liquid chromatography (HPLC) and liquid chromatography (LC) are the most widely used analytical techniques for the accurate quantification of antimalarials in body fluids in malarious, resource-poor settings (WHO, 2011a). The principle is based on the partitioning of analytes between a stationary phase and a mobile phase. In recent years, highly sensitive, selective and throughput techniques such as liquid chromatographic tandem mass spectrometry (LC-MS/MS) are heralded as the gold standard for the assay of drugs. This allows for greater sensitivity and smaller volumes of samples for field studies to ease sample collection. This technique lends itself to quicker and more accurate identification and quantification and allows for critical analysis and decision-making to be made earlier in the drug development process (Lee & Kerns, 1999b; Kang, 2012; Roskar & Trdan, 2012).

Table 2.3: Summary of previously published validated methods used in the determination AQ and DEAQ

Reference	Matrix	Volume of sample collected	Sample extraction method	Analytical technique	Limit of quantitation AQ	DEAQ
((Pussard, Verdier & Blayo, 1986)	Plasma	1 ml	Liquid-liquid extraction (LLE)	Reverse phase-HPLC with UV detection	*3.6 ng/ml	*3.3 ng/ml
	Blood					
	Red blood cells					
	Urine	500 µl				
(Mount et al., 1986)	Whole blood	1ml	LLE	Reverse phase -HPLC with oxidative electrochemical detection	*1 ng/ml	*1 ng/ml
(Winstanley et al., 1987a)	Plasma	1 ml	Protein precipitation (PP) + LLE	Reverse phase-HPLC with UV detection at 340 nm	*5 ng/ml	*5 ng/ml
	Whole blood				*12.5 ng/ml	*25 ng/ml
	Red blood cells				*12.5 ng/ml	*25 ng/ml
	Urine				*5 ng/ml	*5 ng/ml
(Lindegårdh et al., 2002)	Capillary blood on Whatman^{TM} 31ET Chr filter paper (DBS)	100µl	Solid phase extraction (SPE)	Reverse phase-HPLC with UV detection at 342nm	100 nmol/l	100 nmol/l
(Minzi et al., 2003)	Whole blood	100 µl	LLE	Reverse-phase HPLC with UV detection	100 nmol/l	100 nmol/l
	Plasma					
	Urine					
(Gitau et al., 2004)	Dried blood spot	200 µl	LLE	Reverse phase-HPLC with UV detection	*5 ng/ml	*10 ng/ml
(Dua et al., 2004)	Plasma	500 µl	LLE	Normal phase-HPLC with UV detection	5 ng/ml	5 ng/ml
	Whole blood+10%					

(Ntale et al., 2007)	Phosphoric acid v/v spotted on filter paper	100 µl	LLE	Reverse phase-HPLC with UV detection	50 nmol/l	50 nmol/l
(Bell et al., 2007)	Whole blood	200 µl	PP + LLE	Reverse phase-HPLC with UV detection at 340nm	100ng/ml	100 ng/ml
(Chen et al., 2007)	Whole blood	200 µl	PP	Ion-pair LC-MS/MS	0.150 ng/ml	1.50 ng/ml
(Lai et al., 2009)	Plasma	500 µl	SPE	Reverse phase-HPLC with electrochemical detector	20 ng/ml	20 ng/ml
(Hodel et al., 2009)	Plasma	200µl	PP	Reverse phase-HPLC-MS	0.3 ng/ml	0.3 ng/ml
(Rathod et al., 2016)	Plasma	250 µL	SPE	Reverse phase- HPLC-MS/MS	0.250 ng/ml	1.5 ng/ml

***** *Limit of detection; AQ= amodiaquine; DEAQ=desethylamodiaquine; ml=milliliter; ng/ml=nanogram per milliliter; nmol/l = nanomole per litre; µl= microlitre; nm= nanometer; LLE=liquid-liquid extraction; PP=protein precipitation; SPE=solid phase extraction; HPLC= high performance liquid chromatography; UV=ultra violet; LC-MS/MS = liquid chromatography coupled mass spectroscopy*

Slowly eliminated antimalarial drugs like amodiaquine necessitate the use of LC-MS/MS with selective preparation techniques to adequately characterize their long terminal elimination phase (WHO, 2011a). For amodiaquine and desethylamodiaquine, liquid chromatography coupled with mass spectrometric (LC-MS) techniques generally have higher sensitivities with lower limits of detection or quantification (Chen et al., 2007; Hodel et al., 2009) than with techniques using UV detectors (Lindegårdh et al., 2002; Gitau et al., 2004; Bell et al., 2007). In line with regulatory requirements, newly developed analytical methods must be validated with respect to their accuracy, precision, selectivity, sensitivity, reproducibility and stability (FDA, 2001).

2.5.4Sample preparation

The success of an analytical method strongly depends on the sample preparation procedure and the extent to which the method has been appropriately tested and validated. The main aim of a sample preparation in an analytical method is to eliminate as many contaminants from the blood or other physiological samples as possible, and to pre-concentrate the analyte of interest in order to simplify chromatographic separation (Ho, Pedersen-Bjergaard & Rasmussen, 2002). Traditionally, sample preparation is carried out by protein precipitation (PP), liquid–liquid extraction (LLE) or by solid-phase extraction (SPE). Conventional sample preparation approaches are highly labour intensive and time-consuming, and involve many steps. Consequently, many new sample preparation techniques have been developed over the years including solid phase micro-extraction (SPME), liquid–liquid micro-extraction (LLME), pressurized liquid extraction (PLE), extraction using restricted access material (RAM), micro-extraction by packed sorbent (MEPS) and molecularly imprinted polymer (MIP) (Nováková & Vlcková, 2009). The method of sample preparation employed is generally based on the analytical technique and the physical characteristics of the analyte being investigated (Watt, Morrison & Evans, 2000).

Protein precipitation (PP) is often the first and sometimes the only sample preparation step performed to eliminate interfering endogenous substances. By PP, soluble proteins are converted to the insoluble state by salting out or by the addition of water miscible precipitation solvent or organic solvent. For this technique to be applicable, the analyte must be freely soluble in the reconstituting solvent. PP results in purification and analyte concentration (Lakshmana & K. Suriyaprakash, 2012). Protein precipitation is typically a cost effective and quick technique to use. The applicability of PP to analytical methods such as

liquid chromatographic (LC) with ultraviolet detection (UV) is however questionable. With increased levels of contamination, this type of extraction is more appropriate for use in combination with LC-MS/MS. However, careful evaluation of matrix effects including the repeat analysis of incurred samples is essential (Hyötyläinen, 2009; Nováková & Vlcková, 2009).

Solid phase extraction (SPE) is used to separate analytes from a mixture on the basis of their physical and chemical properties. SPE reduces the levels of interferences, maximizes the sample sensitivity by minimizing the final sample volume, and provides the analyte fraction in a solvent compatible with the analytical technique. SPE is versatile and amenable to automation. Although SPE is more expensive, it typically produces clean, contaminant-free samples. SPE is never free of co-extracted compounds but is better than PP and similar to LLE (WHO, 2011a; Lakshmana & K. Suriyaprakash, 2012). Liquid-liquid extraction (LLE) is also known as solvent extraction or partitioning. LLE is used to separate analytes based on their relative solubility in two immiscible liquids; an aqueous phase and an immiscible organic solvent. The ability to separate a mixture using LLE is based on the octanol-water partition coefficient which is influenced by the pH of the sample (Nováková & Vlcková, 2009; WHO, 2011a; Lakshmana & K. Suriyaprakash, 2012)

Although PP and SPE have been reported as sample preparation techniques for the determination of amodiaquine and desethylamodiaquine concentrations (Winstanley et al., 1987a; Lindegårdh et al., 2002; Bell et al., 2007; Chen et al., 2007; Hodel et al., 2009; Lai et al., 2009), LLE has also been widely reported (Mount et al., 1986a; Pussard, Verdier & Blayo, 1986; Minzi et al., 2003; Dua et al., 2004; Gitau et al., 2004; Ntale et al., 2007). LLE is simple, rapid, and relatively cost effective with near quantitative recoveries of over 90%. LLE is appropriate for use with both LC-UV and LC-MS systems and provides a balance between efficient sample preparation and cost. LLE produces cleaner samples and can be used to concentrate the analytes. It is thus particularly appropriate for assays requiring high sensitivity (Ji, Todd Reimer & El-Shourbagy, 2004; WHO, 2011a; Lakshmana & K. Suriyaprakash, 2012). Liquid chromatography–mass spectrometry (LC–MS) techniques often suffer from ion suppression which is one form of matrix effect. Irrespective of the sensitivity or selectivity of the mass analyzer used, ion suppression negatively affects the detection capability, precision, and accuracy (Jessome & Volmer, 2006). LLE is manual in nature requiring multiple extraction steps but is preferred over the other extraction methods because it provides cleaner extracts, leading to less ion suppression.

Chapter 3: Study methods

This chapter presents details of the study location and methods used for the therapeutic efficacy study. It includes study design, procedures, and inclusion and exclusion criteria, laboratory procedures, data processing, as well as statistical methods employed in the analysis of both efficacy and pharmacokinetic data and considerations for the ethical conduct of the study.

3.1 Study site

The study was conducted in the Kassena-Nankana and Kintampo districts located in the northern and middle belts of Ghana (Figure 3.1), as a sub-study of the International Network for the Demographic Evaluation of Populations and Their Health in developing countries (INDEPTH) Effectiveness and Safety Studies of antimalarials in Africa (INESS). Therapeutic efficacy testing of the first-line antimalarial drugs combinations was evaluated in Tanzania, Ghana, Burkina Faso and Mozambique. The pharmacokinetic sub-study was added to the therapeutic efficacy trial of artesunate-amodiaquine (ASAQ) in Ghana in order to describe the pharmacokinetic profile of amodiaquine (AQ) and its active metabolite, desethylamodiaquine (DEAQ) when used as a partner drug to artesunate (AS) in uncomplicated malaria patients.

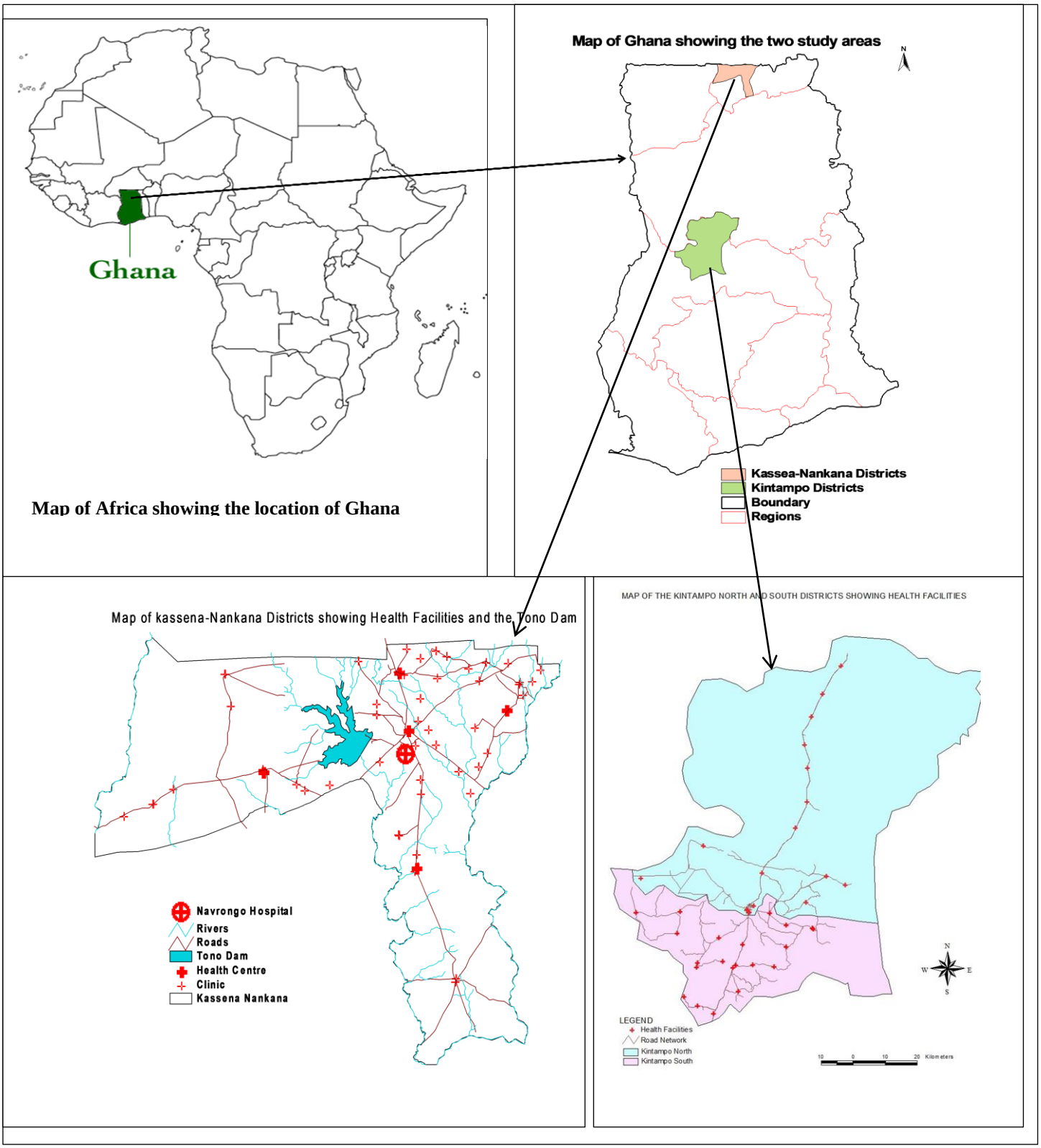
Ethical approved for the parent study was obtained from the Ghana Health Service Ethical Review committee, the Navrongo and Kintampo Health Research Centres' Institutional Review boards. The pharmacokinetic sub-study was approved by the University of Cape Town Faculty of Health Sciences Human Research Ethics committee and the Navrongo Health Research Centre Institutional Review Board.

3.1.1 Kassena-Nankana Districts

The Kassena-Nankana districts (KNDs) comprise of two neighboring rural districts - Kassena-Nankana Municipal Assembly and Kassena-Nankana West district located in the Upper East region of Northern Ghana (Figure 3.1). The two districts together cover an area of 1675 km^2 of Sahelian savannah along the Ghana-Burkina Faso border. Since 1993, the Navrongo Health Research Centre (NHRC) has used the Navrongo Health and Demographic Surveillance System (NHDSS) to monitor the population and health dynamics of both districts. Together, the two districts have a population of about 152,000 people under continuous health and demographic surveillance. About 80% of the population lives in the rural parts of these districts (Oduro et al., 2012). There are two distinct ethno-linguistic

groups: the Kassena who form about 54% of the population and the Nankana who comprise about 42% of the population. There is also a minority Builsa and migrants from other ethnic groups making up 4% of the population. The main occupation in this area is subsistence farming.

Figure 3.1: Map of the study location showing Ghana, Kassena-Nankana and Kintampo Districts, and major health facilities in those districts



The average annual rainfall ranges between 850 – 950 mm, almost all of which falls between May and September. The average monthly temperature ranges from 18 to 45 ^{o}C. There is a large irrigation project (Tono irrigation), which covers 3860 hectares with 42 km of canals within these districts. Additionally, there are nearly 90 small man-made dams, which provide water for the people and livestock during the dry season (Binka et al., 1999).

Malaria transmission in the Kassena-Nankana districts is holoendemic and occurs during most months of the year. However there is a distinct seasonal pattern with the peak of transmission coinciding with the rainy season, and lower rates of malaria infection in the dry season (Binka et al., 1994; Appawu et al., 2004). Between 2001 and 2002 the mean entomological inoculation rate (EIR) for the KNDs was estimated to be 418 infective bites person per year. The main malaria vectors are *Anopheles gambiae* sensu lato (*Anopheles gambiae s.l.)* and *Anopheles funestus* (Appawu et al., 2004; Kasasa et al., 2013). These constitute about 94.3% of the vector population with *Anopheles gambiae sensu stricto (Anopheles gambiae s.s.)* sibling species forming more than 90% of the *anopheles gambiae* complex (Binka et al., 1994; Koram et al., 2003; Appawu et al., 2004).

The PCR-corrected efficacy of loose artesunate/amodiaquine in the Kassena-Nankana districts in 2003, prior to the introduction of the new ASAQ malaria treatment policy in 54 children aged 6 – 59 months was estimated to be 100% (Koram et al., 2005). The day 28 PCR-corrected efficacy of loose artesunate/amodiaquine in 79 children aged 6 – 59 months was estimated to be 98.7% (95% CI 93.1, 100%) in 2006 (Koram et al., 2008). However, another study in the same site reported a PCR-uncorrected efficacy of loose AS/AQ (50 mg AS and 153 mg Amodiaquine hydrochloride tablets, Ipca laboratories, Mumbai) in 154 children aged 6 months -10 years as 80% (Oduro et al., 2008).

There is one main hospital, the Navrongo War Memorial Hospital (NWMH) that serves as a referral hospital for both districts. There are also nine health centres namely Paga, Chiana, Kayoro, Navrongo Central, Biu, Kologo, Sirigu, the Kassena-Nankana East health centres and the St. Jude clinic located across the five geographic zones. In addition, there are nearly 30 community health services and planning compounds (CHPs) (Figure 3.1). These health facilities provide curative and preventive health care, with the latter being mostly in the form of childhood immunization.

3.1.2 Kintampo District

Kintampo is located in the middle belt of Ghana. The main indigenous ethnic groups are the Bono and the Mo. There is however a large immigrant population from the three Northern regions of Ghana. The commonest language spoken is Akan. A large proportion of the population is illiterate (Ghana Statistical Service, 2014). Settlements are mainly concentrated in the Southern part and along the main trunk road linking the Municipal capital to the Northern Region. There are two administrative districts; the Kintampo North Municipal Assembly and the Kintampo South district. These districts together cover a surface area of 7,162 Km^2. The vegetation is mainly of the forest-savannah transition type. There are two rainy seasons: the major rainy season, from March-June and the minor rainy season from July-November. The Kintampo Health Research Centre (KHRC) maintains a health and demographic surveillance system (KHDSS) in the adjoining districts. The KHDSS has a registered population of about 150,615 people as of 2014 with updates on deaths, births and migration every six months (Zandoh, Sulemana & Nettey, 2009; Owusu-Agyei et al., 2014).

There are two district hospitals located in the two district capitals, one of which is a municipal hospital and is better equipped with more up-to-date clinical and laboratory facilities. There are also ten health centres and clinics in the sub-districts which are largely located in the rural areas (Figure 3.1). The municipal hospital serves as a referral point for all the health centres and community clinics, and provides outpatient services and 24-hour Medical and Surgical Emergency care.

Malaria transmission is high throughout the year. The incidence density of malaria among children less than 5 years is about 8.3 malaria episodes per child per year. The entomological inoculation rate (EIR) is estimated to be 269 infective bites per person per year (Owusu-Agyei et al., 2009a).

The PCR-corrected efficacy of loose ASAQ in 37 children aged 6–59 months old in 2006 in Sunyani, the regional capital located about 136 km from Kintampo was estimated to be 97.5% (95% CI 85.8, 99.9) (Koram et al., 2008). However, a similar study conducted in Kintampo in 2006 in 177 children aged 6 months to 10 years estimated the PCR-corrected efficacy of loose AS/AQ to be 93.4% (95% CI 88.5, 96.4)(Owusu-Agyei et al., 2008).

3.2 Study design

The parent study was a one-arm prospective evaluation of clinical and parasitological response to treatment with the fixed-dose combination artesunate-amodiaquine (ASAQ) regimen currently recommended as the first line treatment for uncomplicated *falciparum* malaria in Ghana. All patients presenting at the Navrongo War Memorial and the Kintampo Municipal hospitals during the study period with symptoms compatible with uncomplicated *Plasmodium (P) falciparum* malaria were evaluated and those consenting patients who met the study inclusion criteria were enrolled, given at the manufacturer's recommended weight-based doses of ASAQ (Table 3.1) and monitored for 28 days (Table 3.2), according to the World Health Organization methods for surveillance of antimalarial drug efficacy (WHO, 2009c). In Navrongo all daily doses were observed (Days 0, 1, and 2) while in Kintampo only the first dose was directly observed. Treatment outcomes were classified as Early Treatment Failure (ETF), Late Clinical Failure (LCF), Late Parasitological Failure (LPF) or Adequate Clinical and Parasitological Response (ACPR) as per the World Health Organization guidelines (WHO, 2009c) (Appendix 3.1).

Table 3.1: Dosing chart for ASAQ administration

Weight range (kg)	**Dosage**		
	Day 0 dose	**Number of Tablets**	**Total mg/kg range**
≥ 4.5 to < 9	25 mg AS 67.5 mg AQ	1tab/day x 3 days	8.3 - 16.7 mg/kg AS 22.5 - 45 mg/kg AQ
≥9 to <18	50 mg AS 135 mg AQ	1tab/day x 3 days	8.3 - 16.7 mg/kg AS 22.5 - 45 mg/kg AQ
≥18 to <36	100 mg AS 270 mg AQ	1tab/day x 3 days	8.3 - 16.7 mg/kg AS 22.5 - 45 mg/kg AQ
≥ 36	100 mg AS 270 mg AQ	2tabs/day x 3 days	Max 16.7 mg/kg AS Max 45 mg/kg AQ

AS artesunate; AQ amodiaquine; mg/kg=milligram per kilogram; tab=tablet

Table 3.2: Schedule of activities

	Day							
	0	1*	2	3*	7	14	28	Unscheduled visit
Procedure								
Informed Consent	X							
Clinical assessment	X	X	X	X	X	X	X	(X)
Adverse events (AE)assessment	X	X	X	X	X	X	X	X
Temperature (axillary)	X	X	X	X	X	X	X	(X)
Blood slide (thick and thin smear) for parasite count	X	X	X	X	X	X	X	(X)
Urine sample	X[a]							
Blood for Genotyping	X				X[b]	X[b]	X[b]	X[b]
Haemoglobin	X				X	X	X	(X)
Blood for AQ & DEAQ concentrations	X	X	X	X	X	X	X	(X)
Treatment								
ASAQ	X	X	X					
Rescue treatment		(X)	(X)	(X)	(X)	(X)	(X)	(X)

**Only at the Navrongo site; Parentheses denote conditional or optional activities. X[a] refers to urine for pregnancy test for all females of reproductive age (Ages 12 – 49). X[b] filter paper blood spots were collected if there was microscopic parasite recurrence. Rescue treatment was given on any day, provided the patient met the criteria for treatment failure. Unscheduled visits were any days other than regularly scheduled follow-up days when the patient returned to the facility because of new or recurrence of symptoms. On such days, blood slides were taken routinely*

3.2.1 Inclusion criteria

A patient was enrolled into the parent study if s/he:

1. Was a non-pregnant patient of age greater than 2 months and of weight >4.5 kg as recommended by the manufacturer of the study drug and the national treatment policy;
2. Had a positive blood smear for *P. falciparum* mono-infection and parasitaemia of 1000 - 200,000 asexual forms per microliter (μl) of blood (a modification of criteria for areas of high transmission (WHO, 2009c);
3. Had a fever with axillary temperature ≥37.5^{o}C or history of fever within the preceding 24 hours;
4. Had the ability to swallow oral medication;
5. Had the ability and willingness to comply with the study specific procedures for the duration of the study including continued residence in the study area for the stipulated follow ups; and
6. Provided a written informed consent (written informed consent from adult patients, parental consent for all children under 18 years (Appendix 3.2) in addition to Assent from all children aged 12– 17 years) (Appendix 3.3) prior to enrolment into the study

3.2.2 Exclusion criteria

A potential participant was excluded from the study if s/he:

1. Had general danger signs or severe *falciparum* malaria (Appendix 3.4):
2. Had mixed or mono-infection with *Plasmodium malariae, ovale, vivax or knowlesi* species detected by microscopy;
3. Had febrile conditions due to diseases other than malaria;
4. Had taken medication with antimalarial effects, or drugs which may interfere with amodiaquine pharmacokinetics (Appendix 3.5) within the previous two weeks;
5. Had a history of hypersensitivity reactions or contra-indications to ASAQ, or
6. Was found to be pregnant during the screening process based on a urine hCG pregnancy test (hCG One Step Pregnancy test strip; Lot hCG1010092, expiring: 2013-01, Acon Laboratories Inc., 4108 Sorrento Valley Blvd, San Diego, CA92121, USA).

3.3 Study procedures

Patients of all ages presenting at the Navrongo War Memorial and the Kintampo Municipal hospitals during the study period with symptoms compatible with uncomplicated *P.*

falciparum malaria were evaluated for inclusion in the study. Written informed consent was obtained prior to the performance of study related procedures, such as collection of blood samples for drug concentration measurements, filter paper for genotyping and urine samples for pregnancy tests.

At enrolment (day of first study dose, day 0) a medical history, clinical evaluation of signs and symptoms, measurement of vital signs including body weight and temperature, demographic information and contact details were collected. All patients were examined by a study clinician or physician assistant. All enrolled patients were to be followed up on days 2, 7, 14 and 28 and on any day of unscheduled visit following day of enrolment. Additional follow ups on days 1 and 3 were made at the Navrongo site. During these follow up visits, clinical and laboratory examinations were performed. Adverse events (AEs) were reported according to the **International Conference on Harmonisation** of Technical Requirements for Registration of Pharmaceuticals for Human Use (**ICH**) definition of 'any untoward medical occurrence in a patient or clinical investigation subject administered a pharmaceutical product and that does not necessarily have a causal relationship with this treatment (http://www.ich.org/products/guidelines/efficacy/article/efficacy-guidelines/E2A.html). Adverse events reported following the start of treatment were documented during each of the follow up (and any unscheduled) visits. Symptoms absent at enrolment but which emerged after treatment initiation and those present at enrolment but which resolved and then re-emerged or worsened following the commencement of treatment were considered to be adverse events.
A summary of activities carried out during the study are presented in table 3.2.

3.3.1 Treatment

Patients who satisfied the inclusion criteria were treated with the recommended dose of the World Health Organization prequalified fixed-dose combination of artesunate-amodiaquine (*Coarsucam/ASAQ Winthrop*) developed by Sanofi-Aventis and the Drugs for Neglected Diseases *initiative* (DND*i*), obtained through the Affordable Medicines Facility for malaria (AMFm) programme (Matowe & K'omolo, 2011) and supplied by the Ghana national Malaria Control Programme. Patients were dosed by weight according to a dosing chart (table 3.1) based on recommendations of the manufacturer (Sanofi-Aventis, Maphar Laboratories, Morocco); artesunate, target of 4 mg/kg body weight (range 2 to 10 mg/kg) and amodiaquine target of 10 mg/kg body weight of amodiaquine base (range 7.5 to 15 mg/kg) once daily for 3

days as a fixed dose combination. The details of the administered study drug are as presented in table 3.3. The exact time of each observed dose was captured in the case report form (CRF). Patients were observed for at least 30 minutes after treatment to ensure that they did not vomit. If vomiting occurred within 30 minutes of treatment, the full treatment dose was repeated. Patients who vomited more than once were excluded from the study and referred to the hospital staff for appropriate management. All uncomplicated malaria patients who failed treatment but did not progress to severe malaria were retreated with artemether-lumefantrine. None of the 20 patients who failed treatment within the 28 days follow up period progressed to severe malaria case.

Table 3.3: Manufacturing details of study drug (Coarsucam/ASAQ Winthrop; Sanofi-Aventis, Maphar Laboratories, Morocco)

Batch number	Date manufactured	Expiry date	Tablet strength(ASAQ)	Recommended weight category (kg)
1051	10/2010	10/2013	25mg/67.5mg	4.5 – 8
3067	09/2010	09/2013	50mg/135mg	9 – 17
5248	10/2010	10/2013	100mg/270mg	18 – 35
5243	10/2010	10/2013	200mg/540mg	≥36

AS/AQ=artesunate/amodiaquine; kg=kilogram; mg=milligram

3.3.2 Laboratory procedures

Laboratory tests included 1) examination for malaria parasites using thick and thin blood smears, 2) the measurement of hemoglobin levels, 3) parasite genotyping to differentiate recrudescence from reinfections, 4) capillary blood for drug concentration measurements and 5) urine pregnancy test for females aged ≥12 years using urine hCG pregnancy test (hCG One Step Pregnancy test strip; Acon Laboratories Inc., 4108 Sorrento Valley Blvd, San Diego, CA92121, USA) according to the manufacturer's instruction.

3.3.2.1 Blood smears preparation, staining and reading

Thin and thick blood smears were prepared from finger pricks on days 0, 1, 2, 3, 7, 14 and 28 at the Navrongo site and on days 0, 2, 7, 14 and 28 at the Kintampo site and on any day of an unscheduled visit at both study sites. The smears prepared during screening were made in duplicate. The thin smears were fixed in methanol. The first slides (thick and thin) at screening were stained rapidly with 10% Giemsa for 10 –15 minutes, air-dried and used to count the numbers of asexual parasites and white blood cells in a limited number of microscopic fields. A rapid count of 1 - 100 parasites for every three white blood cells, corresponding to 1000 – 200,000 asexual parasites per microlitre was considered adequate for enrolment (WHO, 2009c). On reaching the count of 1 - 100 parasites for every three white blood cells counted, a particular field was read completely. The second enrolment slides, and all slides prepared during follow up visits were stained more slowly with 3% Giemsa for 45 – 60 minutes and used for parasite identification and quantification against 200 white blood cells (WBCs) (WHO, 2009c). The number of parasites per microlitre of blood was established by assuming a total WBC count of 8000 per microlitre. At least 100 high power fields were read before declaring a slide negative. Two independent microscopists (Reader 1 and Reader 2) separately read each of the slides, and parasite densities were calculated by averaging the two counts. Each microscopist was blinded to the results of the other microscopist. Results were recorded independently in separate logbooks. Blood smears with non-concordant results (differences between the two microscopists in species diagnosis, or differences in parasite density of >50%) were re-examined by a third, independent microscopist, and parasite density calculated by averaging the two most concordant counts. The concordance of the readings between reader 1 and 2 (R1 and R2) was calculated by taking the absolute value of the difference in reading between reader 1 and reader 2 and dividing the value by the mean value of reader 1 and reader 2: {(R1 – R2) / ((R1 + R2)/2)}.Microscopic results were reported as either positive or negative, species present, stage of species present and number of parasites (both sexual and asexual) counted per 200 white blood cells.

3.3.2.2 Haemoglobin measurements

Haemoglobin concentrations were determined on days 0, 7, 14 and 28 at the Navrongo site using Hemocue Hb201+ photometers® (HemoCue AB, SE-262 23 Ängelholm, Sweden) and on days 0, 2 and 28 at the Kintampo site using ABX Micros 60 –OT haematology analyzer (HORIBA Ltd, France).

3.3.2.3 Filter paper blood blots

Capillary blood spots on Whatman 3 mm filter paper were collected on days 0 and on any day of parasite recurrence on or after day 7 from finger prick (capillary) blood, air dried completely at room temperature and genotyped to differentiate between new infections and recrudescences using merozoite surface proteins (MSP) - 1, MSP - 2 and glutamate rich protein (GLURP) as polymorphic markers (WHO, 2007b).

3.3.2.3.1 DNA Extraction from filter paper

P. falciparum deoxyribonucleic acid (DNA) was extracted from dried filter paper blood blots using a modified Chelex method as described previously by Plowe and colleagues (Plowe et al., 1995). Briefly the dried blood spot was excised from the filter paper and solubilized overnight in 1 ml mild detergent (5% Saponin in phosphate-buffered saline (PBS)) at 4^{o}C. The resulting solution was aspirated and the filter paper washed with 1 ml PBS, then boiled with Chelex-100® resin (Bio-Rad Laboratories, USA) to remove contaminants inhibitory to the amplification process. The extracted DNA was stored at -20 °C. To prevent carry-over of DNA from one sample to another or cross-contamination of samples, the scalpels, forceps and scissors used were treated with 5 M Hydrochloric Acid followed by 5 M Sodium Hydroxide between excisions.

3.3.2.3.2 DNA Amplification

The three highly polymorphic *P. falciparum* antigens, MSP -1, MSP-2 and GLURP were used in the analysis to differentiate recrudescences from new infections. The PCR analysis of each gene involved 2 rounds of amplification, using nested primers in the second round. This approach was used to increase the sensitivity of PCR detection and allowed DNA extracted from samples with low parasitaemia to be amplified successfully. Primers and amplification conditions used were adapted from Ranford-Cartwright (Ranford-Cartwright et al., 1997) with modification on cycling parameters as listed in Table 3.4. The amplified PCR products sizes ranged from 150 base pairs for MSP1 to 1200 base pairs for GLURP.

Table 3.4: Primer sequences and cycling conditions for the amplification reactions

Locus and type	Primer names and sequences	Cycle condition
MSP1		
Outer	O1: ACATGAAAGTTATCAAGAACTTGTC	94°C/25s: 50°C/35s:
	O2: GTACGTCTAATTCATTTGCACG	8 °C/150s: 25 cycles
Nested	N1: GCAGTATTGACAGGTTATGG	94 °C /25s: 50 °C /35s:
	N2: GATTGAAAGGTATTTGAC	68 °C /150s: 30 cycles
MSP2		
Outer	S3: GAAGGTAATTAAAACATTGTC	94 °C /25s: 42 °C /60s:
	S2: GAGGGATGTTGCTGCTCCACAG	65 °C /120s: 25 cycles
Nested	S1: GAGTATAAGGAGAAGTATG	94 °C/25s: 50°C/60s:
	S4: CTAGAACCATGCATATGTCC	70°C/120s: 30 cycles
GLURP		
Outer	G4: ACATGCAAGTGTTGATCC	94°C /25s: 45°C /60s:
	G5: GATGGTTTGGGAGTAACG	68 °C /120s: 25 cycles
Nested	Gl: GAATTCGAAGATGTTCACACTGAAC	94 °C /60s:
	G3:TGTAGGTACCACGGGTTCTTGTGG	55 °C /120s:
		70 °C /120s: 30 cycles

MSP1= merozoroite surface protein 1; MSP2= merozoroite surface protein 2; GLURP= glutamine-rich protein; °C= degree Celsius; s= seconds

For each sample, 50 µL reaction mixtures were prepared containing 10x PCR buffer, 200 µM of deoxyadenosine triphosphate (dATP), deoxythymidine triphosphate (dTTP), deoxyguanosine triphosphate (dGTP) and deoxycytidine triphosphate (dCTP) (Roche Diagnostics GmbH, Mannheim, Germany); 10 pmol of each appropriate primer and 1 unit of Taq DNA polymerase (Roche Diagnostics GmbH, Mannheim, Germany). Two microliter (2 µl) of sample DNA was used as template for all the outer PCRs. For all 3 nested amplification reactions, 1 µl of the outer PCR product was transferred as template for nested PCRs in a 20 µl reaction mixture. Following nested primer amplification, 8 µL of the PCR product was loaded on to 1.5% agarose gels pre-stained with ethidium bromide. The pre-treatment (day zero) and post-treatment failure samples (day X) from each patient were run in adjacent lanes for ease of comparison. Following electrophoresis, the sizes of the PCR products were compared between the sample pairs. All unsuccessful amplification reactions were repeated with the starting sample DNA. If a DNA sample failed to be amplified on 3 occasions it was recorded as an unsuccessful amplification.

3.3.2.3.3 Determination of Recrudescence

Parasite recrudescence (Re) was defined as the presence of identical PCR products in the samples from day 0 and the day of parasite recurrence (day X). Such recrudescences were further classified based on the following criteria (Ranford-Cartwright et al., 1997; WHO, 2008):

- Parasites in the day 0 and day X samples which possessed exactly the same alleles at the 3 loci, were considered as recrudescence of single or multiple clone infections.
- Parasites were also considered recrudescent when a subset of the alleles found in the day 0 sample was found in the day X sample. This result could be explained by the original parasites being a mixture of sensitive and resistant parasites, with drug selection removing the sensitive forms before the day of parasite recurrence (day X).

If the parasites in the day 0 and in day X samples differed at all 3 loci, they clearly represented a new population of circulating blood forms (Re-infection, Ni). If they differed at only 2 loci, the frequency of the common allele at the third locus was determined in the parasite population of all the patients sampled.

- If it was high, there could have been reinfection with parasites which by chance carried the same allele as the original infection at this locus only.
- If the day 0 and day X samples were identical at 2 loci, but differed at one, the parasites in the day X sample were classified as recrudescent (Re).
- Samples that contained a subset of identical alleles in day 0 and day X, with new additional alleles, were classified as a mixed infection (Re + Ni).

3.3.2.4 Blood concentrations of amodiaquine and desethylamodiaquine

In order to study the pharmacokinetics of amodiaquine and its active metabolite desethylamodiaquine, about 200 µl of capillary whole blood was collected into lithium heparin tubes (labeled with patient ID, day of follow up, time and date of sample collection) on Days 0, 1, 2, 3, 7, 14 and 28 of treatment. Pharmacokinetic blood samples were collected prior to ASAQ administration on Days 0, 1 and 2.

In order to compare the matrix effects of whole blood and plasma, for patients from whom samples of $\geq$200 µl were obtained, 100 µl samples were aliquoted into a second labeled lithium heparin tube, centrifuged at 3500 rpm for 5 – 10 minutes at 4^{o}C and the plasma

separated into labeled cryotubes. Both samples were stored for about 3 months at -80^{o}C on site until shipment to the University of Cape Town where the assays were performed.

Blood concentrations of amodiaquine and desethylamodiaquine were recovered from 20 µl capillary whole blood and plasma samples by a newly developed and validated liquid chromatography tandem mass spectrometric (LC-MS/MS) method. The method was validated according to FDA guidelines (FDA, 2001). Amodiaquine-D10 and desethylamodiaquine-D5 were used as internal standards. Details of the assay method for the determination of amodiaquine and desethylamodiaquine are provided in Chapter 4.

Statistical analysis

3.4 Sample size

The sample size for the pharmacokinetic sub-study was calculated to compare the exposure of desethylamodiaquine in malaria patients who achieved adequate clinical and parasitological response and those who failed treatment. The null hypothesis, H0 was assumed to be the equality of two parameters, m1=m2, where m1 was assumed to be the median day 7 concentration of desethylamodiaquine in patients who achieved ACPR and m2 was assumed to be the median day 7 concentration for patients who failed treatment. At an alpha of 0.05 (two-sided comparison of medians) and a power of 0.80, with a cure rate of 92.5% and 10% loss to follow up; n1 = 8, n2 =111. Thus a sample size per site of 119 plus 10% for loss to follow up was arrived at. In order to allow for comparisons across sub-populations such as young children, a total of 300 patients were targeted to be enrolled from the two sites.

For the therapeutic efficacy study, an artesunate-amodiaquine treatment cure rate of 92.5% and a loss to follow up of 10% were assumed. At a 95% confidence level with a power of 80% and a precision around the estimate of 3.0, a total of 326 patients were be targeted for enrolment at the two study sites.

.3.5 Data processing

All personal, clinical and laboratory data were captured using structured case report forms and double entered into a computer database using Epidata software (EpiData Data Entry, Data Management and Basic Statistical Analysis System. Odense Denmark, EpiData Association. Http://www.epidata.dk).

Blood concentration measurements were captured using a computer running AB Sciex Analyst version 1.4.2 software (AB Sciex, Ontario, Canada) and exported into Microsoft Excel spread sheets (Microsoft Corporation, USA).

Microsoft Access 2010 (Microsoft Corporation, USA) was used to merge all data files, run consistency checks, identify critical missing variables and to check for outliers. The data was curated according to the WorldWide Antimalarial Resistance Network (WWARN) Pharmacology Module Data Management and Statistical Analysis Plan v1.0. [http://www.wwarn.org/tools-resources/pharmacology-data-management-and-statistical-analysis-plan]. If inconsistencies, unexpected or missing values were identified, these queries were tracked back to source documents for possible validation, explanation or correction. Validated, corrected or additional values were used to update the database used in the final analysis. If corrections could not be made, the inconsistencies and unexpected results were transformed to missing values. The following variables were considered critical and were checked for missing values and a query generated for resolution:

1. Age, weight, parasitaemia and haemoglobin concentration at baseline;
2. Generic treatment name, dose (mg/kg) and day/date and time of each dose administered;
3. Amodiaquine and desethylamodiaquine concentration result, day, date and time;
4. Sample matrix and lowest level of quantification; and
5. Study treatment outcome and day of treatment outcome assessment.

The age-for-weight z-scores were calculated in Stata based on the WHO growth reference for children (Weltgesundheitsorganisation, Onis & Weltgesundheitsorganisation, 2006; WHO Multicentre growth reference study group & Onis, 2007)**.**

Data analysis was done using Stata 12 statistical software package (StataCorp, 4905 Lakeway Drive, College Station, Texas 77845, USA) and Phoenix® WinNonlin® (Pharsight Corporation, 5520 Dillard Drive, Suite 260 Cary, North Carolina 27518).

3.6 Therapeutic efficacy data analysis

Efficacy outcome analysis was carried out using both Kaplan-Meier (intention-to-treat (ITT)) and per protocol (PP) methods. In the ITT analysis, all baseline and follow-up data collected on patients who took at least one ASAQ dose were included. Patients with incomplete follow-up who did not reach the primary outcome of interest were included in the analysis as non-failures, but were censored on the last day of follow-up.

In the per protocol (PP) analysis, patients who did not complete the study as scheduled either through withdrawal, loss to follow up prior to achieving a study endpoint or who were re-infected (based on PCR correction or whose PCR results were indeterminate) were excluded from the analysis. Patients were withdrawn from the study if they failed to attend the mandated scheduled visits during the first three days, missed a dose of the study medication or if they withdrew consent. In addition patients with baseline haemoglobin concentrations < 6 g/dl (lower limit for inclusion) or parasite densities >200,000 (the upper limit for inclusion) parasites per µl were excluded from the PP analysis

Patients were considered lost to follow up if they missed any two (2) consecutively scheduled follow up visits. Statistical significance for all tests was set at 0.05. Proportions and 95% confidence intervals were calculated for baseline characteristics. Comparisons between age groups, by sex and by study site were carried out. In line with the burden of disease in areas of high malaria transmission (Carneiro et al., 2010; WHO, 2016), age was categorized into infants: age less than 1 year, young children aged 1 - 4 years, and older children and adults of 5 years and older.

Gametocyte carriage (microscopic presence of sexual forms of *P. falciparum*) was established by determining the number of patients with gametocytes on days 0, 1, 2, 3, 7, 14 and 28 in Navrongo and on days 0, 2, 7, 14 and 28 in Kintampo. These were plotted as the proportion of patients (percentage (%)) with gametocytes by the day of follow up.

Mean haemoglobin concentrations on days 0, 7, 14 and 28 for the Navrongo site and on days 0, 2 and 28 for the Kintampo site were determined. The mean haemoglobin concentrations were plotted over time to elucidate the trend of mean haemoglobin recoveries over time following ASAQ treatment.

Fever (temperature$\geq$37.5^{o}C) clearance was investigated by determining the proportion of patients with fever on days 0, 1, 2, 3, 7, 14 and 28 in Navrongo and on days 0, 2, 7, 14 and 28 in Kintampo. The proportions of patients with fever at each time point were plotted against days of follow up to describe the trend in fever clearance over time.

Proportions were compared using test of proportions. Student's *t*-test was used to compare normally distributed variables. Data with skewed distributions such as parasite densities were log transformed before being compared using the normal approximation, one-way analysis of variance (ANOVA) with Bonferroni multiple comparison test.

3.7 Pharmacokinetic data analysis

The pharmacokinetic (pk) parameters: maximum observed concentration (C_{max}), time to maximum observed concentration (T_{max}), terminal elimination half-life ($t_{½}$), area under the concentration versus time from zero to infinity ($AUC_{0-\infty}$) and the terminal elimination rate constant ($K_{e)}$ were determined by a non-compartmental analysis using Stata 12 statistical software (StataCorp, 4905 Lakeway Drive, College Station, Texas 77845 USA). The data profiles in the pharmacokinetic data set were hugely varied and diversified. As a result, compartmental modeling was explored using Phoenix® WinNonlin® (Pharsight Corporation, 5520 Dillard Drive, Suite 260 Cary, North Carolina 27518). One- and two- compartmental additive model and one- and two- compartmental multiplicative model-based analyses were carried out in addition to non-compartmental analysis.

The median concentrations, lower and upper quartiles for amodiaquine and desethylamodiaquine at each time point were calculated using Stata. The $AUC_{0-\infty}$ was estimated using the trapezoidal rule with exponential fit. At least 3 data points were required for the estimation of $AUC_{0-\infty}$.

The maximum observed amodiaquine and desethylamodiaquine concentration (C_{max}) and the first time of their occurrences (T_{max}) were obtained directly from the observed concentration-time data

The apparent clearance (CL/f), apparent volume of distribution (Vd/f) and the terminal elimination half-life ($t_{½}$) were calculated using the formulae:

- Area under the concentration-time curve, $AUC_{0-\infty} = AUC_{0-Tmax} + AUC_{Tmax-\infty}$

  or

  $AUC0-\infty = \int_0 \quad Ctdt + \int$

Where $AUC_{0\text{-}tmax}$ refers to AUC from time zero to time of last concentration measurement, $AUC_{Tmax\text{-}\infty}$ = AUC from time of last concentration measurement to infinity, T_{max}= time of last concentration measurement, Ct = concentration at time, t.

- Apparent clearance, CL/f= Total Dose/ $AUC_{0\text{-}\infty}$, with f being the bioavailability

- Apparent volume of distribution, Vd/f = CL/f / K_e

- Terminal elimination half-life = ln(2)/K_e with the terminal elimination rate constant (K_e) was estimated by linear regression of logarithmically transformed concentration versus time data. Only data points judged to describe the terminal exponential decline were used in the regression. A minimum of three data points were used in calculating K_e

For the calculation of typical amodiaquine and desethylamodiaquine concentrations (average profile) at each time point, all amodiaquine and desethylamodiaquine concentrations below the limit of quantification (BLQ) were set to half the lower limits of quantification (LLOQ/2): 0.3905 (0.781/2) ng/ml for amodiaquine and 1.955 (3.91/2) ng/ml for desethylamodiaquine. All BLQ values were set to zero (0) prior to first study drug dose and missing thereafter for the estimation of all pharmacokinetic parameters.

Patients who received treatment with antimalarials, including amodiaquine within two weeks prior to enrolment were excluded from the study. Therefore, even if prior antimalarial treatment was not self-reported, all patients with detectable concentrations of amodiaquine above the lower limit of quantification (LLOQ) prior to first dose of study drug were deemed to have had a recent (within 2 weeks) antimalarial treatment and so were excluded from the analysis. Given the high intensity of malaria transmission, those patients with pre-dose detectable desethylamodiaquine concentrations above LLOQ but with corresponding amodiaquine concentrations below the limit of quantification were included in the analysis (Stepniewska et al., 2009). In addition, data points considered as biologically implausible were excluded from the analysis. A data point was considered biologically implausible or outlier if:

i. In the absorption phase or within the region of C_{max}, a suspected data point is far higher or lower (25 - 30%) than the 2 measured concentrations at adjacent sampling times; and

ii. Once in the terminal elimination phase, any rise or fall (≥50%) in a suspected data point relative to the two measured concentrations at adjacent sampling times.

Kruskal-Wallis tests were used to test any differences in pharmacokinetic parameters with respect to pre-defined variables, namely age [categorized into infants (less than 1 year), 1 – 4 years and ≥5 years]; sex; presence of fever (temperature ≥37.5^{o}C) at presentation; parasitaemia (parasite density ≥100000 or <100000) at presentation; total body weight-adjusted dose (mg/kg) administered; and nutritional status (weight-for-age z-score), anaemia (Hb<8.0 g/dl versus Hb≥8.0 g/dl) and site of sample collection.

Spearman's rank correlation was used to explore the associations between pharmacokinetic parameters and nutritional status (weight-for-age z-score) and weight-adjusted (mg/kg) dose. The Spearman's rank correlation was also used to explore associations between the median day 3, 7, 14 and 28 amodiaquine and desethylamodiaquine concentrations and duration of gametocyte carriage.

To establish whether the pre-defined covariates associated with the differences observed in the amodiaquine and desethylamodiaquine pharmacokinetic parameters identified in the univariate regression analysis persist after adjusting for other covariates, a multivariate linear regression analysis of the log-transformed pharmacokinetic parameters was conducted. This tested for the independent relationships between the pharmacokinetic parameters ($AUC_{0-\infty}$, CL/f, Vd/f, $T_{max,}$ C_{max} and $t_{½}$) and the pre-defined covariates: nutritional status (weight-for-age z-score), age (categorized as infants (<1 year), 1 – 4 years and ≥5 years), sex (males versus females), mg/kg dose, anaemia (Hb>8.0 versus Hb<8.0), fever at enrolment, parasite density (≥100,000 versus <100,000) at enrolment and site of sample collection. The effect or extent of associations of these pre-defined covariates on the pharmacokinetic parameters were established by calculating the geometric mean ratios (GMR) and the 95% confidence intervals for the log-transformed $AUC_{0-\infty}$, C_{max}, CL/f, Vd/f, $t_{½}$,. A GMR of 1.00 indicated no change or difference in pharmacokinetic parameter for the covariate considered. A GMR of 2 was interpreted as a doubling in the pharmacokinetic parameter with respect to the covariate

considered. Where the confidence interval calculated included unity, it was interpreted as not being associated or having a statistically significant effect on the pharmacokinetic parameter under consideration.

Cox-proportional hazard ratios with Breslow correction for ties were used to explore the effects of the days 2, 3, 7, 14 and 28 median concentrations of amodiaquine and desethylamodiaquine on treatment response in terms of parasite clearance times and parasite recurrence. The effect of AUC_{0-3} on treatment response in terms of risk to treatment failure (parasite recurrence) was investigated using Cox regression (proportional hazard model) with Breslow correction for ties. In all cases, a p-value<0.05 was considered statistically significant.

Correlations between concentrations of capillary whole blood and capillary plasma of amodiaquine and between concentrations of capillary whole blood and capillary plasma of desethylamodiaquine were evaluated using Spearman rank correlation. The correlations between the concentrations were considered statistically significant if the rank correlation coefficient, r_s was high and the p-value<0.05. Median concentrations in whole blood and plasma, stratified by day of follow up, were compared using the Wilcoxon rank-sum (Mann-Whitney) test. Concentrations of amodiaquine and desethylamodiaquine have been reported to be higher in whole blood than the concentrations in plasma (Pussard et al., 1987; Winstanley et al., 1987a). Therefore, for the same pairs of samples, plasma concentrations higher than whole blood concentrations were considered to be biologically implausible and due probably to a technical analytical error and so were excluded from the matrix effects analysis. For the same reason, pairs of the same whole blood and plasma samples with one concentration being BLQ and the other >BLQ were also excluded from the analysis.

In order to take into account the within group correlation and to produce a more efficient and unbiased regression estimate, the relationship between capillary whole blood and capillary plasma concentrations of amodiaquine and between capillary whole blood and capillary plasma concentrations of desethylamodiaquine were investigated using the method of generalized estimating equation (GEE) . The intercepts were considered as patient specific random effects. Capillary whole blood concentrations of amodiaquine and of desethylamodiaquine were considered as the dependent variables (y-axis) while capillary plasma concentrations of amodiaquine and of desethylamodiaquine were considered the

independent variables (x-axis). These concentrations were log-transformed to a natural base in order to account for wide and increasing variability at higher concentrations.

3.8 Ethical considerations

The parent study of this sub-study was approved by the Ghana Health Service Ethical Review committee, the Navrongo and Kintampo Health Research Centres' Institutional Review boards. The pharmacokinetic sub-study was approved by the University of Cape Town Faculty of Health Sciences Human Research Ethical committee and the Navrongo Health Research Centre Institutional Review Board. The study was conducted according to the ethical principles of the Declaration of Helsinki (Fortaleza, 2013), Good Clinical Practice (ICH, 1997) and local ethical or regulatory requirements.

Eligible participants were only included in the study and any study-specific procedures performed, after providing written informed consent. Participants were informed about the objectives and practical consequences of participation and asked to sign an informed consent form, which is compliant with GCP and approved by the relevant ethics committee(s). The possibility of withdrawal from the study, at any time and without any declaration of the reason was explained to participants. Given the context of the study, every effort was made to keep the informed consent documents and process as clear and simple as possible. These documents were translated into kasem, nankam and twi, and back translated to ensure that the original meaning is conveyed accurately. A parent or guardian was asked for parental consent of children under 18 years of age (Appendix 3.2). In addition, children aged 12 - 17 years were also asked to provide assent. Once a parent gave consent for enrolment of a child, the study was explained to the child who was then asked to assent to participating in the study (Appendix 3.3). If the parent or guardian is unable to read or write, their thumbprint was used in lieu of a signature, and a signature from an impartial literate witness to the informed consent discussion was obtained.

Participant records were used solely for the purpose of this research project and maintained securely at the Navrongo and Kintampo Health Research Centres. All materials collected in this study were labelled with a personal identification number. The confidentiality of records that can identify participants was protected, respecting the privacy and confidentiality rules in accordance with regulatory requirements.

Participants were reimbursed on each day of follow-up as compensation for the costs incurred in travelling to and from the clinic.

Blood sampling was restricted to the absolute minimum to answer the study questions. The total blood volume that was taken per participant during the entire duration of the study for study specific purposes was at most 5ml.

Chapter 4: Assay method development and validation for determination of amodiaquine and desethylamodiaquine concentrations in whole blood

4.1 Experimental

4.1.1 Materials and Chemicals

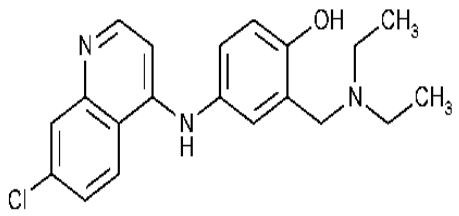
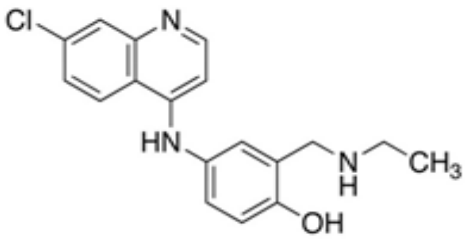
The following materials, reagents and chemicals were used during assay method development and validation: Boric acid (GR for analysis), Acetic acid (GR for analysis), Sodium hydroxide (GC≥99.5%), Acetonitrile (Gradient grade for LC), Formic acid (GR for analysis, 98%), Methanol (Gradient grade for LC), Isopropyl alcohol (propan-2-ol) all purchased from Merck kGaA, Darmstadt, Germany; Phosphoric acid (HPLC grade 85-90%) from Fluka, Ethylacetate (≥98%) from Sigma-Aldrich and Water (prepared in-house using a Synergy S Kit Millipore Water Purification System).

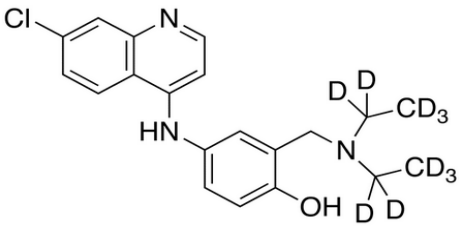
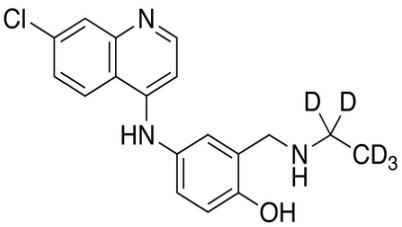
The analytical reference standards, amodiaquine (lot number 5-YM-93-1; 98% purity) and desethylamodiaquine (lot number 4-ABY-144-1; 95% purity) together with the deuterated internal standards amodiaquine-d10 (lot number 4-VKU-20-1) and N-desethylamodiaquine-d5 (lot number 7-BHW-175-2) were purchased from Toronto Research Chemicals (TRC), Ontario, Canada. Drug free lithium heparinized whole blood was obtained from the Western Province Blood Transfusion Services, Cape Town, South Africa. A Phenomenex Luna, PFP (2) 100A, 50mm×2.0mm column (Phenomenex, USA) was used for retaining amodiaquine, desethylamodiaquine and the internal standards.

4.1.2 Chemical structure

The chemical structures of amodiaquine, desethylamodiaquine, amodiaquine-d10 (amodiaquine internal standard) and amodiaquine-d5 (desethylamodiaquine internal standard) are presented in figures 4.1 a, b, c and d respectively.

Figure 4.1a-d: **Chemical structures of amodiaquine, desethylamodiaquine and their internal standards**

a. Structure of amodiaquine (free base)	**b. Structure of Desethylamodiaquine (free base)**
	
Chemical formula: $C_{20}H_{22}ClN_3O$	**Chemical formula**: $C_{18}H_{19}ClN_3O$
Molecular weight: 355.86	**Molecular weight**: 327.27
Chemical name: 4 – [(7- Chloroquinolin – 4 – yl) amino] – 2 – [(diethylamino) methyl] phenol	**Chemical name**: 4-((7-Chloro-4-quinolinyl) amino)-2-((ethylamino) methyl) phenol

c. Structure of deuterated amodiaquine-d10	**d. Structure of deuterated amodiaquine-d5**
	
Chemical formula: $C_{20}H_{12}D_{10}ClN_3O$	**Chemical formula**: $C_{18}H_{13}D_5ClN_3O$
Molecular weight: 365.92	**Molecular weight:** 332.84

4.2 Preparation of solutions

4.2.1 Preparation of amodiaquine and desethylamodiaquine stock Solutions

Stock solutions (SS1) were prepared by weighing a mass of the analyte, amodiaquine (AQ) or metabolite, desethylamodiaquine (DEAQ) into polypropylene tubes labelled amodiaquine SS1 and desethylamodiaquine SS1 respectively and dissolving these in the desired volume of methanol to obtain a concentration of 1000 ng/ml. The desethylamodiaquine was adjusted for salt concentration and purity. All stock solutions were prepared on ice and kept at approximately -80°C until required. These stock solutions were used to prepare the working solutions required for the validation.

4.2.2 Preparation of calibration and quality control standards

Calibration standards and quality control (QC) standards were prepared volumetrically on ice in blank lithium heparinised human whole blood. Eighty microliters (80 µl) of working stock solutions of amodiaquine (SS3 at a concentration of 10 µg/ml) and 80 µl of desethylamodiaquine (SS2 at a concentration of 200 µg/ml) were spiked into 8ml of blank lithium heparinised whole blood to obtain the highest calibration standards (100 ng/ml for amodiaquine and 2000 ng/ml for desethylamodiaquine). The highest calibration standards were vortexed for 2 minutes, incubated on ice for 5 minutes and then vortexed for a further 10 seconds. Serial dilutions with blank whole blood were performed by the same procedure to obtain additional calibration standards at concentrations of 50, 25, 12.5, 6.25, 3.13, 1.56 and 0.781 ng/ml for amodiaquine and 1000, 500, 250, 125, 62.5, 31.3, 15.6, 7.81 and 3.91 ng/ml for DEAQ and labelled as STD 1 - STD 10; STD1 being the highest concentration standard . The calibration standards were aliquoted into individual polypropylene tubes with each tube containing 55 µl and stored at approximately -80 °C.

The same methodology was used for the preparation of the high, medium and low QC standards at concentrations of 75, 37.5 and 2.34 ng/ml for amodiaquine and 1500, 750 and 5.86 ng/ml for desethylamodiaquine. Additional QC standards at the lower limit of quantification (LLOQ) were prepared by the same method to obtain AQ LLOQ-2, AQ LLOQ and DAQ LLOQ-2 at concentrations of 1.56, 0.781 and 3.91 ng/ml respectively. These QC standards were aliquoted into individual polypropylene tubes and stored at approximately -80 °C.

4.2.3 Preparation of Internal Standard Solution

Stock solutions (ISTD_SS1) of the internal standards AQ_ISTD and DEAQ_ISTD were prepared by weighing a mass of each internal standard (AQ-d10 and DAQ-d5) into corresponding labelled polypropylene tubes and dissolving these in the desired volume of methanol to obtain a concentration of 1000 µg/ml. All stock solutions were prepared on ice and kept at approximately -80°C until required. These stock solutions were used to prepare the working solutions of internal standard by spiking 10 µl of the ISTD_SS1 stock solution into 990 µl of methanol to obtain a working solution ISTD-SS2 (10 µg/l). Then 100 µl of the ISTD-SS2 working solution (10 µg/ml) was spiked into 900 µl of methanol to obtain a working solution ISTD-SS4 with a concentration of 1 µg/ml. These working solutions were stored at approximately -80°C until required. They were added to the buffer prior to sample extraction.

4.2.4 Preparation of Britton Robinson universal buffer (pH8.0)

One litre (1l) of solution A was prepared by weighing out 6.005 g of 100% acetic acid, 11.53 g of 85% phosphoric acid and 6.183 g of boric acid into a volumetric flask and making up to the 1l mark with distilled water. The resulting solution contained 0.1M Acetic acid, 0.1M phosphoric acid and 0.1M boric acid.

Solution B was prepared by weighing 20 g of sodium hydroxide into 1 litre of distilled water. About 100 ml of solution A was titrated against Solution B until pH 8.0 was obtained. The buffer was stored at approximately 4^{o}C for a period of one month.

4.2.5 Formic Acid (0.1%)

Formic acid (FA) (0.1%) was prepared by adding 1ml of FA to distilled water, making up to 1L with distilled water. No pH adjustment was made and the solution was stored at room temperature for a period of 2 weeks before it was declared expired.

4.2.6 Autosampler needle wash

The autosampler needle wash solution was made up of acetonitrile, 0.1% formic acid and propan-2-ol (7: 2: 1, v/v/v) respectively. The solution was stored at room temperature in normal light for a period of 1 month before considered expired

4.2.7 Injection solution

The injection solution was prepared from acetonitrile and 0.1% formic acid (1:1, v/v) and was stored at room temperature in normal light for a period of 2 weeks before considered as expired.

4.3 Extraction procedure

The extraction procedure was performed on ice in labelled polypropylene microcentrifuge tubes. The lithium heparinised whole blood samples were thawed on ice and briefly vortexed. Two hundred microliters (200 µl) of a Britton Robinson universal buffer (0.1 M, pH 8.0), containing both deuterated internal standards (AQ-d10 and DAQ-d5) at 1ng/ml each, were transferred into each labelled polypropylene tube. Twenty microliters (20 µl) of the whole blood sample was aliquoted into each tube followed by 600 µl of ethyl acetate as the organic solvent. The samples were vortexed for 1 minute and centrifuged for 5 minutes at 15700 rcf. Five hundred microliters (500 µl) of the organic layer from each tube was transferred to clean corresponding labelled polypropylene tubes and evaporated under a gentle stream of nitrogen at room temperature for 20 – 25 minutes. The dried samples were reconstituted in 100 µl injection solution. The reconstituted samples were vortexed for 30 seconds, transferred to a 96 well polypropylene plate and 2 µl injected onto the HPLC column.

4.4 Instrumentation

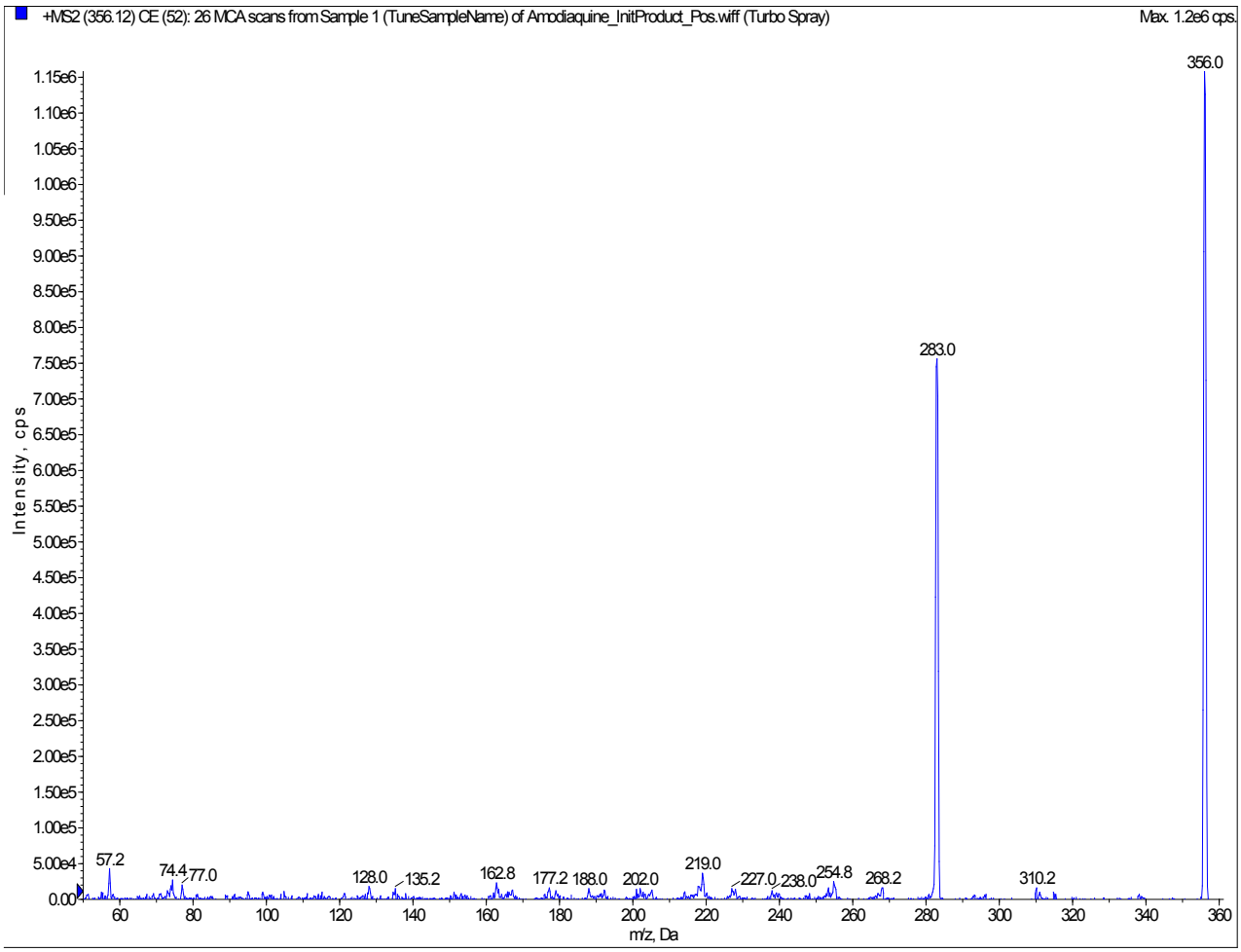
The samples were analysed on an Agilent 1100 High Performance Liquid chromatographic system (Agilent, CA, USA) coupled with an AB Sciex API 4000 mass spectrometer (AB Sciex, Ontario, Canada) fitted with a Turbo V^{TM} ion source as a detector. The mobile phase was delivered with an Agilent 1100 series binary pump and the samples injected with an Agilent 1100 High Performance Autosampler.

4.4.1 Mass spectrometry

Electrospray ionization (ESI) was performed in the positive ionisation mode with nitrogen as the nebulizing turbo spray and curtain gas with the optimum values set at 65, 60 and 30 psi respectively. The heated nebulizer temperature was set at 500°C and the ion spray voltage set at 3500 V. The instrument response was optimized for amodiaquine, desethylamodiaquine and the internal standards by infusing a solution of the compounds dissolved in mobile phase at a constant flow. The pause time was set at 5ms, the dwell time at 150 ms, and the collision gas (N2) was set to medium (arbitrary value). The AB Sciex API 4000 mass spectrometer

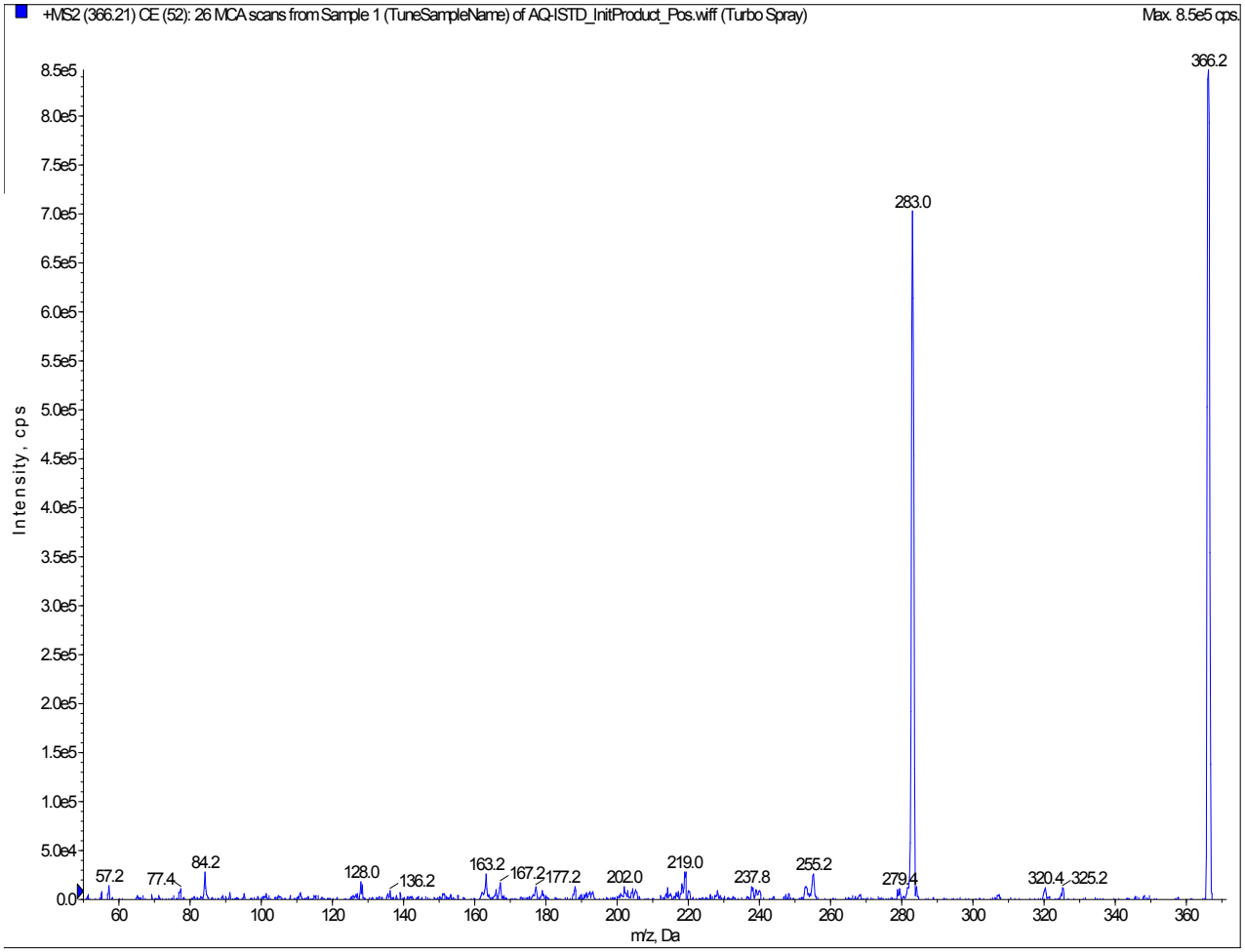
was operated at unit resolution in the multiple reaction monitoring (MRM) mode, monitoring the transition of the protonated molecular ion m/z 356.2 to the product ion at m/z 283.1 for AQ, the protonated molecular ion m/z 328.1 to the product ion at m/z 283.1 for desethylamodiaquine, the protonated molecular ion m/z 366.3 to the product ion at m/z 283.1 for AQ-d10 and the protonated molecular ion m/z 333.3 to the product ions m/z 283.1 for DEAQ-d5. Product ion mass spectra of amodiaquine, desethylamodiaquine, AQ-d10 and DEAQ-d5 are presented in Figures 4.2 a, b. c and d. The instrument was interfaced with a computer running AB Sciex Analyst version 1.4.2 software.

Figure 4.2a: Mass spectrum of analyte, amodiaquine after collision induced dissociation in the fragmentation cell, showing the amodiaquine precursor ion at m/z 356 as well as the product ions



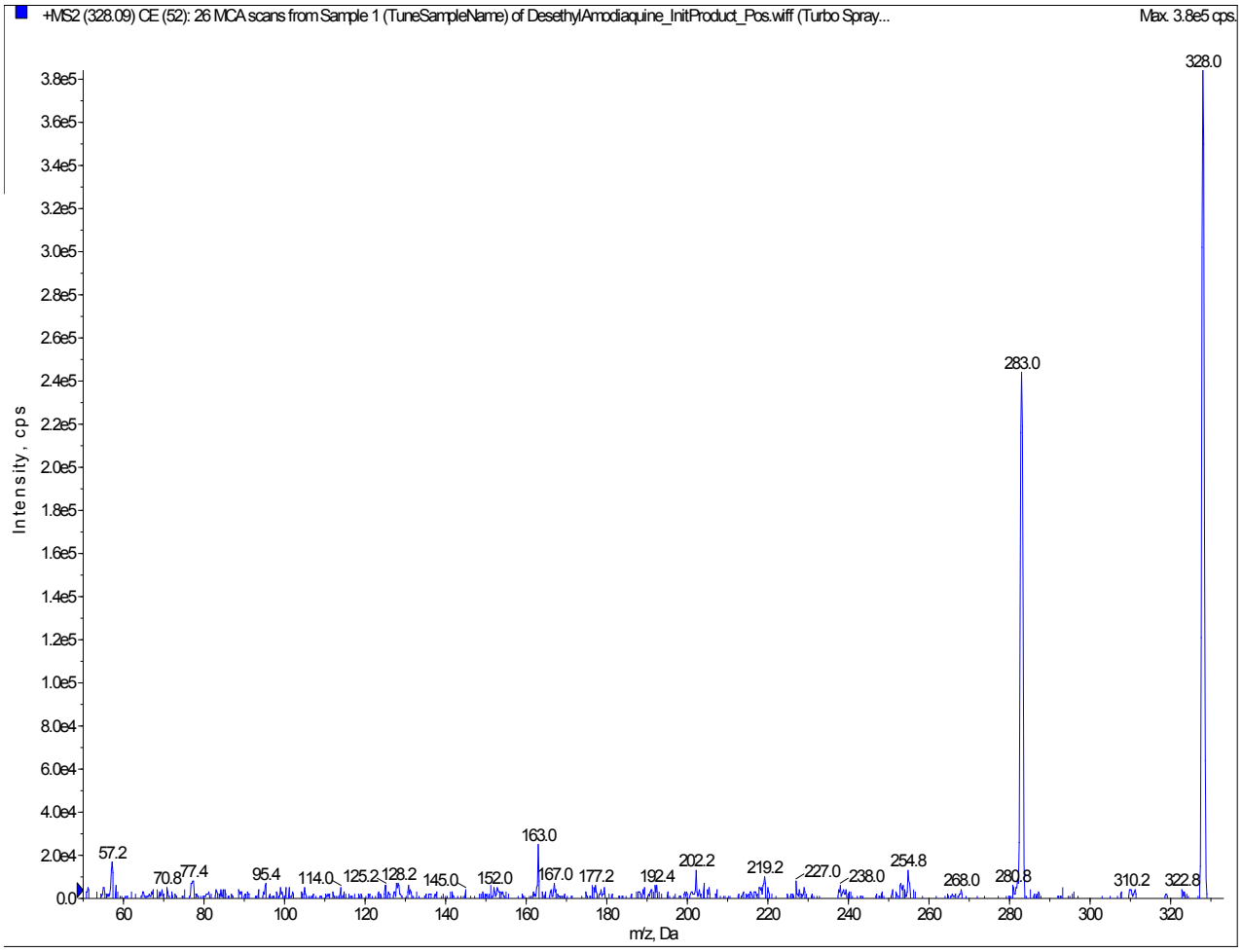
m/z = mass-to-charge ratio; Da= Dalton; cps=counts per second

Figure 4.2b: Mass spectrum of internal standard, AQ-ISTD after collision induced dissociation in the fragmentation cell, showing the AQ-ISTD precursor ion at m/z 366 as well as the product ions



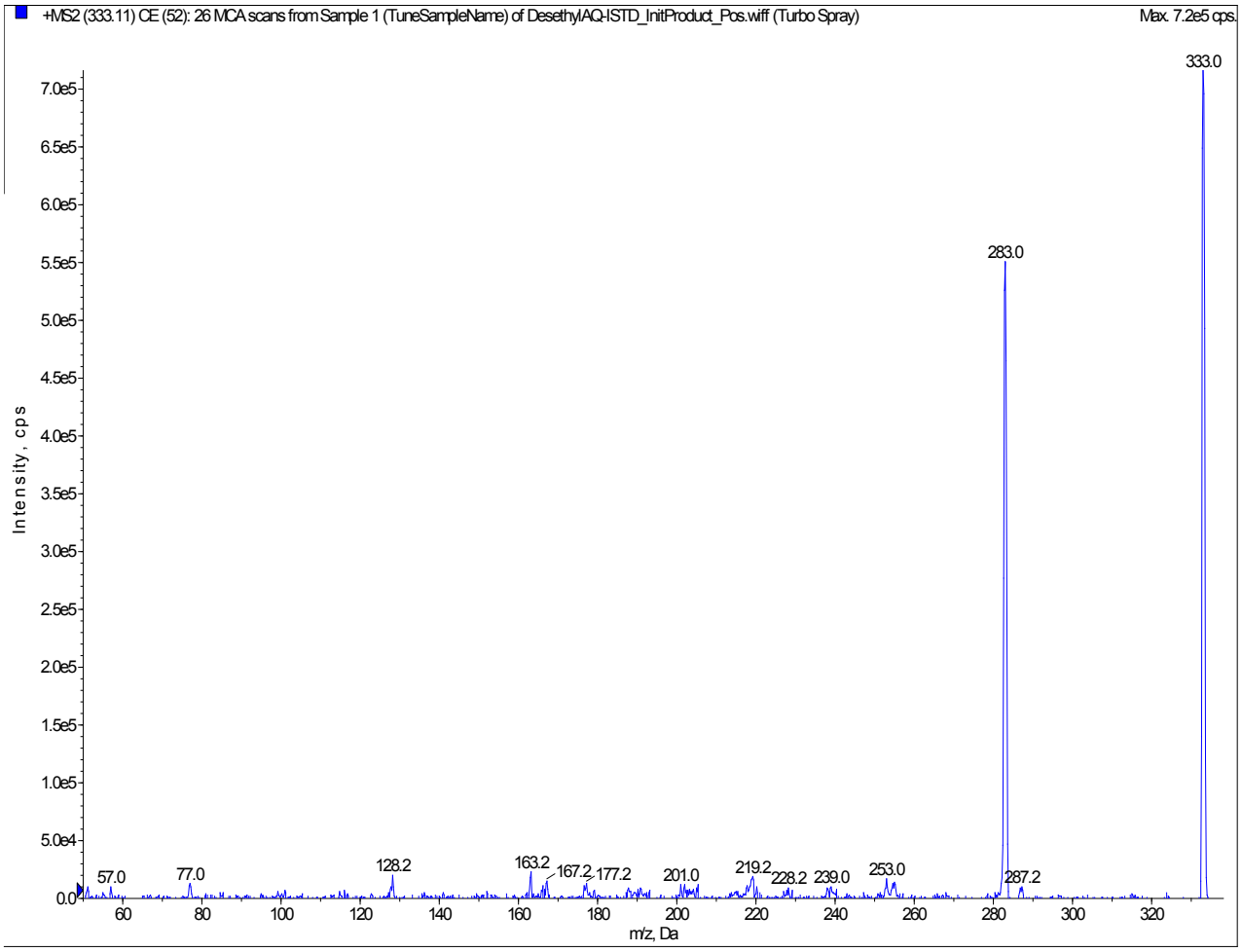
AQ-ISTD = amodiaquine internal standard; m/z= mass-to-charge ratio; Da=Dalton; cps=counts per second

Figure 4.2c: Mass spectrum of metabolite, desethylamodiaquine after collision induced dissociation in the fragmentation cell, showing the desethylamodiaquine precursor ion at m/z 328 as well as the product ions



m/z=mass-to-charge ratio; Da= Dalton; cps=counts per second

Figure 4.2d: Mass spectrum of internal standard, DEAQ-ISTD after collision induced dissociation in the fragmentation cell, showing the DEAQ-ISTD precursor ion at m/z 333 as well as the product ions

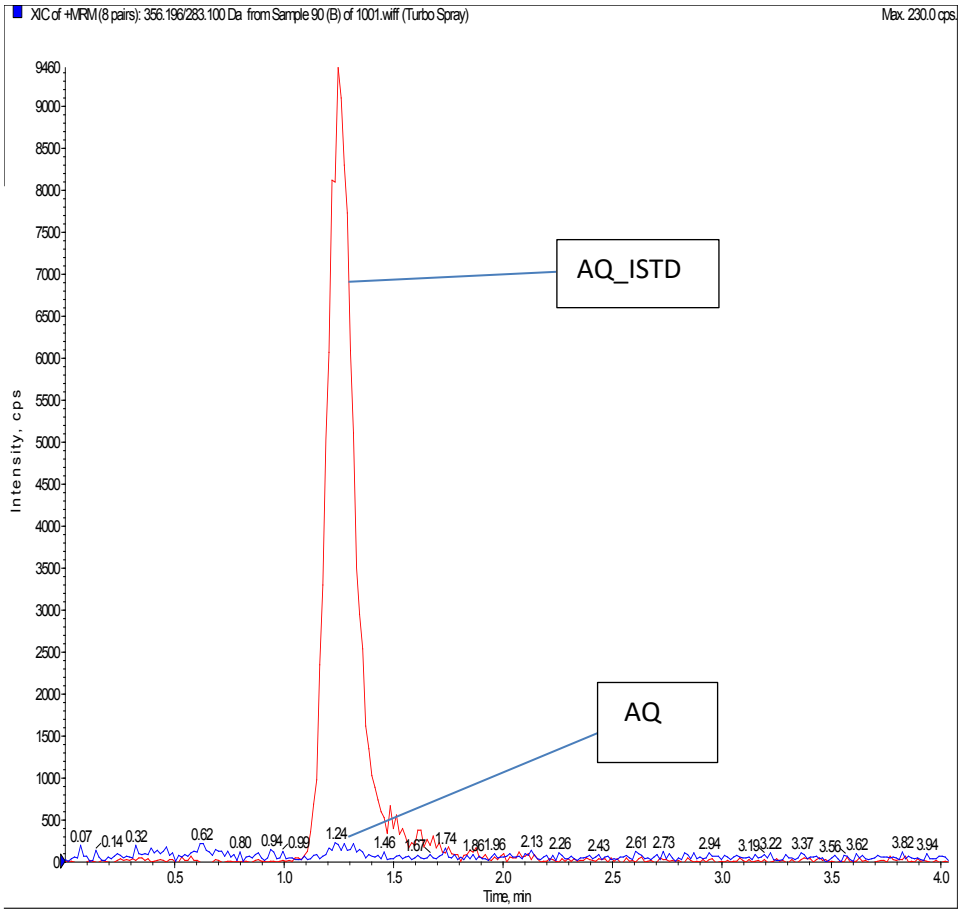


DEAQ-ISTD = desethylamodiaquine internal standard; m/z= mass-to-charge ratio; Da= Dalton; cps= counts per second

4.4.2 Liquid chromatography

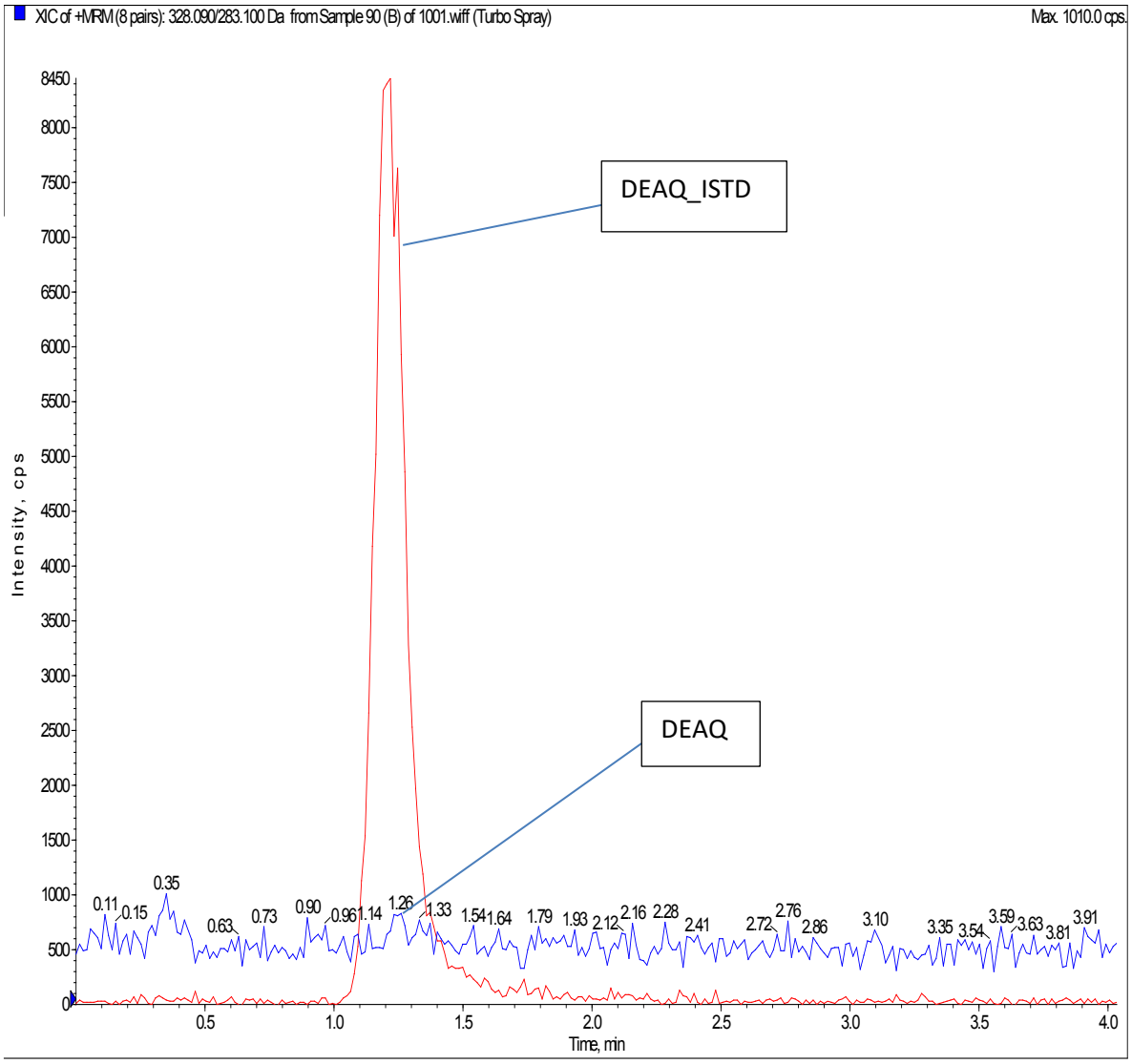
Chromatography was performed on a Phenomenex Luna, Pentafluorophenyl propyl (PFP2) (50mm×2.0mm, 5µm) analytical column. The mobile phase consisted of acetonitrile and water with 0.1% formic acid (86:14, v/v) and was delivered at a constant flow rate of 500 µl/min for 4 minutes. The analytical column was kept in the column compartment at a constant temperature of 30°C. An Agilent 1100 series autosampler injected 2 µl onto the HPLC column. The injection needle was rinsed with the autosampler needle wash solution for 10 seconds using the flush port wash station. The samples were cooled to 8 °C while awaiting injection. Figures 4.3a & b represent raw chromatograms at the lower limit of quantification (LLOQ) for amodiaquine and desethylamodiaquine respectively, and chromatograms for blank samples with internal standards amodiaquine d10 and desethylamodiaquine d5 are presented in figures 4.6a and b respectively.

Figure 4.3a: Chromatogram of a blank whole blood sample with internal standard (amodiaquine)



AQ= amodiaquine; AQ_ISTD= amodiaquine internal standard; min= minutes; cps= counts per second

Figure 4.3b: Chromatogram of a blank whole blood sample with internal standard (desethylamodiaquine)



DEAQ= desethylamodiaquine; DEAQ_ISTD= desethylamodiaquine internal standard; min= minutes; cps= counts per second

4.5 Method validation

4.5.1 Calibration standards and quality controls

The calibration curves for amodiaquine and desethylamodiaquine were validated by analysing whole blood quality control samples in six fold at high (75 ng/ml), medium (37.5 ng/ml) and low (2.34 ng/ml) concentrations for amodiaquine, and 1500, 750 and 5.86 ng/ml for desethylamodiaquine, respectively over a period of 3 days to determine the intra- and inter-day accuracy and precision. The quality control (QC) values were interpolated from the

calibration curve. The calibration curve contained eight different concentrations for amodiaquine spanning a concentration range of 0.781 to 100 ng/ml and ten different concentrations spanning a range of 3.91 to 2000 ng/ml for desethylamodiaquine. Individual calibration curves were constructed using a weighted quadratic regression ($1/concentration^2$) of the peak area ratio of the analyte to its internal standard versus nominal concentration.

4.5.2 Recovery

Recovery was evaluated at high (75 ng/ml), medium (37.5 ng/ml) and low (2.34 ng/ml) QC concentration levels for amodiaquine and 1500, 750 and 5.86 ng/ml QC concentration levels for desethylamodiaquine respectively. Blank whole blood was extracted (with internal standards) and the dried samples reconstituted with mobile phase spiked with both the analyte and the metabolite at the same concentrations as the test samples to generate the reference samples (containing matrix components). The high, medium and low QC standards were used as the test samples. Recoveries were calculated by comparing the peak area ratios (analyte/internal standard) of the extracted samples with those of the reference samples.

4.5.3 Stability tests

4.5.3.1 Stock solution stability

Stock solutions of amodiaquine and desethylamodiaquine were prepared in methanol. A test sample was left on ice for 6 hours and a control sample kept at -20°C for 6 hours and a reference stock solution was kept at approximately -80°C. The reference, test and control samples were diluted with mobile phase (containing the internal standards) at a mid-level concentration of 37.5 ng/ml for amodiaquine and 750 ng/ml for desethylamodiaquine and were analysed as per the method procedure. In addition, reference stocks stored at approximately -80°C for up to 27 days were diluted and compared with freshly prepared stocks to determine stability at approximately -80°C.

4.5.3.2 Freeze and thaw stability

To ascertain freeze-thaw stability, high (75ng/ml for amodiaquine and 1500 ng/ml for desethylamodiaquine) and low (2.34ng/ml for amodiaquine and 5.86 ng/ml for desethylamodiaquine) QC standards were frozen, put through three freeze (approximately -80°C) and thaw (on ice) cycles and analysed against a valid calibration curve.

4.5.3.3 Bench top stability

To establish bench top stability, high (75 ng/ml for amodiaquine and 1500 ng/ml for desethylamodiaquine) and low (2.34 ng/ml for amodiaquine and 5.86 ng/ml for desethylamodiaquine) QC standards were frozen at approximately -80°C, left on ice for 6 hours, and then analysed against a valid calibration curve.

4.5.3.4 On-instrument stability

A 72 hour on-instrument stability evaluation of amodiaquine, desethylamodiaquine and the internal standards was performed. Two medium QC standards were extracted, pooled and analysed over four days. The samples were extracted and analysed on day 1, left on the autosampler and analysed again after 24, 48 and 72 hours.

4.5.4 Matrix effects evaluation

Matrix effects were evaluated both visually and quantitatively. The method described by Matuszewski and co-workers (Matuszewski, Constanzer & Chavez-Eng, 2003; Matuszewski, 2006) was followed to evaluate the influence of co-eluting matrix components on amodiaquine, desethylamodiaquine and the internal standards ionization. Blank whole blood was extracted from eight different sources. The individual dried samples were reconstituted with mobile phase spiked with the analyte and metabolite at a high, medium and low concentration (75, 37.5 and 2.34 ng/ml for amodiaquine and 1500, 750 and 5.86 ng/ml for desethylamodiaquine respectively) and at one concentration of the internal standards. The quantitative assessment of matrix effects was obtained by determining the peak area ratios and assessing the slopes generated for each individual source as a measure of a relative matrix effect on quantitation. Visualization of the matrix effects was achieved by post-column infusion (Bonfiglio et al., 1999). A continuous infusion of amodiaquine, desethylamodiaquine and the internal standards was introduced by a Harvard infusion pump through a T-piece connector to the mass spectrometer while blank whole blood samples were injected. A change in response would indicate ion suppression or enhancement.

4.5.5 Specificity

The high specificity of MS/MS detection prevents the detection of compounds that do not produce the specific parent ion in the Q1 scan and the specific product ion in the Q3 scan. Specificity was assessed by analysing extracts from eight different whole blood sources.

4.5.6 Effect of concomitant medication on the assay

Artemisinin-based combination treatments (ACT) are currently recommended for the treatment of uncomplicated malaria, with artesunate-amodiaquine being one such combination (WHO, 2010b). The potential effect of artesunate on amodiaquine and desethylamodiaquine quantitation was investigated. Test whole blood was prepared by spiking artesunate into blank whole blood at 2000 ng/ml. The effect on analyte and metabolite quantitation was evaluated at a concentration of 100 ng/ml for amodiaquine and 2000 ng/ml for desethylamodiaquine. The peak area ratios (analyte/internal standard) of the test samples were compared with that of the reference samples (not containing artesunate) to calculate the overall accuracy.

4.6 Plasma samples

For capillary plasma, partial validation was performed by replacing capillary whole blood samples with capillary plasma samples and repeating the above processes and procedures for accuracy and precision determinations (FDA, 2001).

4.7 Method validation results and Discussion

The liquid-liquid extraction method for amodiaquine and desethylamodiaquine performed well during validation. The calibration range was validated between 0.781 and 100 ng/ml for amodiaquine and 3.91 and 2000 ng/ml for desethylamodiaquine. A quadratic regression, with peak area ratio (drug/internal standard) against nominal concentration with 1/concentration^2 ($1/x^2$) weighting, was fitted to the calibration curves.

The combined accuracy statistics of the quality control standards (*N*=18; 6 each for high, medium and low QC standards) were between 98.2% and 108.3% for amodiaquine, and 96.1% and 106% for desethylamodiaquine in capillary whole blood. In capillary plasma samples, the combined accuracy statistics of the quality control standards (*N*=18; high, medium and low) were between 88.2% and 92.4% for amodiaquine, and 98.2% and 99.5% for desethylamodiaquine.

The combined precision statistics were between 4.1% and 5.8% and between 3.2% and 4.4% for amodiaquine and desethylamodiaquine in capillary whole blood respectively and between 4.6% and 11.0% and between 9.3% and 12.6% for amodiaquine and desethylamodiaquine in capillary plasma samples respectively.

The percentage recovery was greater than 85% (N = 3) for both amodiaquine and desethylamodiaquine (N = 3), and reproducible over the three levels. The average percentage recovery for amodiaquine across the three levels was 102.4% (CV% = 7.4, N=3) and 86.1% (CV% = 10.3, N=3) for desethylamodiaquine. The CVs were well within internationally accepted criteria (FDA, 2001).

Storage of the amodiaquine and desethylamodiaquine stock solutions in methanol at approximately -80°C for a period of 27 days was examined. The accuracy of the stored stock solution samples compared to freshly prepared samples was 87.4 % (CV% =8.2) for amodiaquine and 85.9% (CV% = 12.7) for desethylamodiaquine (N = 3). Both stock solutions showed acceptable stability after 6 hours on ice and at approximately -20°C. The accuracy of the stock solution test samples compared to the reference samples on ice was 95.7% (CV% = 1.5, N = 3) and at -20°C was 98.8% (CV% = 1.0, N = 3) for amodiaquine. The accuracy for desethylamodiaquine was 99.4% (CV% = 1.1, N = 3) and 98.5% (CV% = 0.5, N = 3) on ice and at -20°C respectively.

Nevertheless, stock solutions stored at -80°C were used within one week for standard and quality control preparation. Freeze-thaw stability was evaluated over three freeze-thaw cycles on consecutive days at the high and low QC levels. The observed concentrations were within 4% of the mean QC concentrations for both amodiaquine and desethylamodiaquine at the high and low levels, demonstrating that the analyte and metabolite are stable through three freeze-thaw cycles. The accuracy of amodiaquine and desethylamodiaquine bench top stability over 6 hours on ice was 95.8% (CV% = 1.0, N = 6) and 96.4% (CV% = 4.9, N = 6) at the high QC level and 93.8% (CV% = 13.7, N = 6) and 92.7% (CV% = 4.6, N = 6) at the low QC level respectively, indicating that both were stable on ice for up to 6 hours (the maximum anticipated time that future study samples will be left thawed until extracted). Sample extracts of amodiaquine, desethylamodiaquine and the internal standards were also stable on-instrument at 8°C for up to 72 hours. The calculated accuracies of the ratios (N = 5) were all well within 15% of the reference (ratios on day 1). After 24 hours (day 2) the accuracy was 99.9% and 96.9%; 99.6% and 98.3% after 48 hours (day 3) and 96.7% and 96.6% after 72 hours (day 4) for amodiaquine and desethylamodiaquine respectively.

The effect of matrix components was evaluated both visually and quantitatively at high, medium and low concentrations. Quantitative assessment was performed using eight different

whole blood sources. The coefficient of variation of the 8 peak area ratios (drug/internal standard) was 1.0% at the high level, 0.7% at the medium level and 7.2% at the low level for AQ and 0.6% (high), 1.5% (medium) and 4.7% (low) for desethylamodiaquine (Table 4.0). The slope variability (CV%) for the eight different whole blood samples encompassing high, medium and low concentration levels is 1.1% for amodiaquine and 0.6% for desethylamodiaquine indicating the reliability and reproducibility of sample analysis from different sources. In addition, no change in response and therefore no significant matrix effects were observed for amodiaquine, desethylamodiaquine or the internal standards in the post-column infusion experiment.

Table 4.0: Matrix effects of eight different sources of matrices

	Amodiaquine			**Desethylamodiaquine**		
	Low 2.34 ng/ml	**Medium 37.5 ng/ml**	**High 75 ng/ml**	**Low 5.86 ng/ml**	**Medium 750 ng/ml**	**High 1500 ng/ml**
Mean ratio of peak areas	0.00656	0.917	1.92	0.107	13.0	25.7
SD	0.000475	0.00604	0.0199	0.00502	0.189	0.152
CV %	7.2	0.7	1.0	4.7	1.5	0.6

Similarly, no significant effect on analyte or metabolite quantitation was observed in the presence of artesunate at 2000 ng/ml. The accuracy of amodiaquine quantitation in the test sample (artesunate present, $N = 3$) relative to a reference sample (artesunate absent, $N = 3$) was 93.2% (CV% = 4.5%) and 99.2% (CV% = 2.9%) for desethylamodiaquine.

Due to the high specificity of MS/MS detection, no interfering or late eluting peaks were found when analysing blank whole blood (figure 4.3a and b). This was confirmed by analysing extracts from eight different whole blood sources. The lower limit of quantification (LLOQ) for the method when injecting 2 µl onto the column was 0.781 ng/ml for AQ and 3.91 ng/ml for desethylamodiaquine (Figures 4.3a & b). The signal to noise ratio at the LLOQ was well above the minimum internationally accepted criteria (S/N > 5). The accuracy for the AQ LLOQ ($N = 18$) was 103.5% (CV% = 4.8) and 101.5% (CV% = 5.0) for the

desethylamodiaquine LLOQ, both well within the acceptable limits of accuracy of within 80 – 120% with precision (total-assay coefficients of variation, CV %) being below 20%.

4.8 Application to clinical pharmacokinetics studies

The assay was applied to measure the concentrations of amodiaquine and desethylamodiaquine in human capillary whole blood in uncomplicated malaria patients administered oral fixed dose combination artesunate-amodiaquine (ASAQ) at 10 mg/kg body weight of amodiaquine plus 4 mg/kg body weight of artesunate once daily for 3 days. The data are presented in subsequent chapters of this thesis. The precision (total-assay coefficients of variation, CV %) during sample analysis in both capillary whole blood and capillary plasma samples was less than 6% at high, medium and low (75, 37.5 and 2.34 ng/ml, respectively) QC levels for amodiaquine and less than 8% at high, medium and low (1500, 750 and 5.86 ng/ml respectively) QC levels for desethylamodiaquine. The LLOQ was 0.781 ng/ml for amodiaquine and 3.91 ng/ml for desethylamodiaquine.

4.9 Conclusion

A simple liquid-liquid extraction method coupled with LC-MS/MS detection has been developed and fully validated for the simultaneous determination of amodiaquine and its active metabolite, desethylamodiaquine with a limit of quantification of 0.781 ng/ml for amodiaquine and 3.91 ng/ml for desethylamodiaquine. This method is specific, sensitive and reproducible and has been successfully used to quantify amodiaquine and desethylamodiaquine in capillary whole blood and capillary plasma samples in malaria patients. This LC-MS/MS method uses a small volume of whole blood or plasma (20 µl) and achieves lower limits of detection and simpler extraction technique, with good sensitivity and reproducibility. Small sample volumes may be necessary for field studies to ease sample collection. This method can readily be used for pharmacokinetic studies and therapeutic drug monitoring in patients, including young children with uncomplicated *falciparum* malaria.

Chapter 5: Therapeutic efficacy of Artesunate-Amodiaquine (ASAQ) for the treatment of uncomplicated falciparum malaria in Ghana

Study methods and procedures are described in chapter 3.

5.1 Trial profile

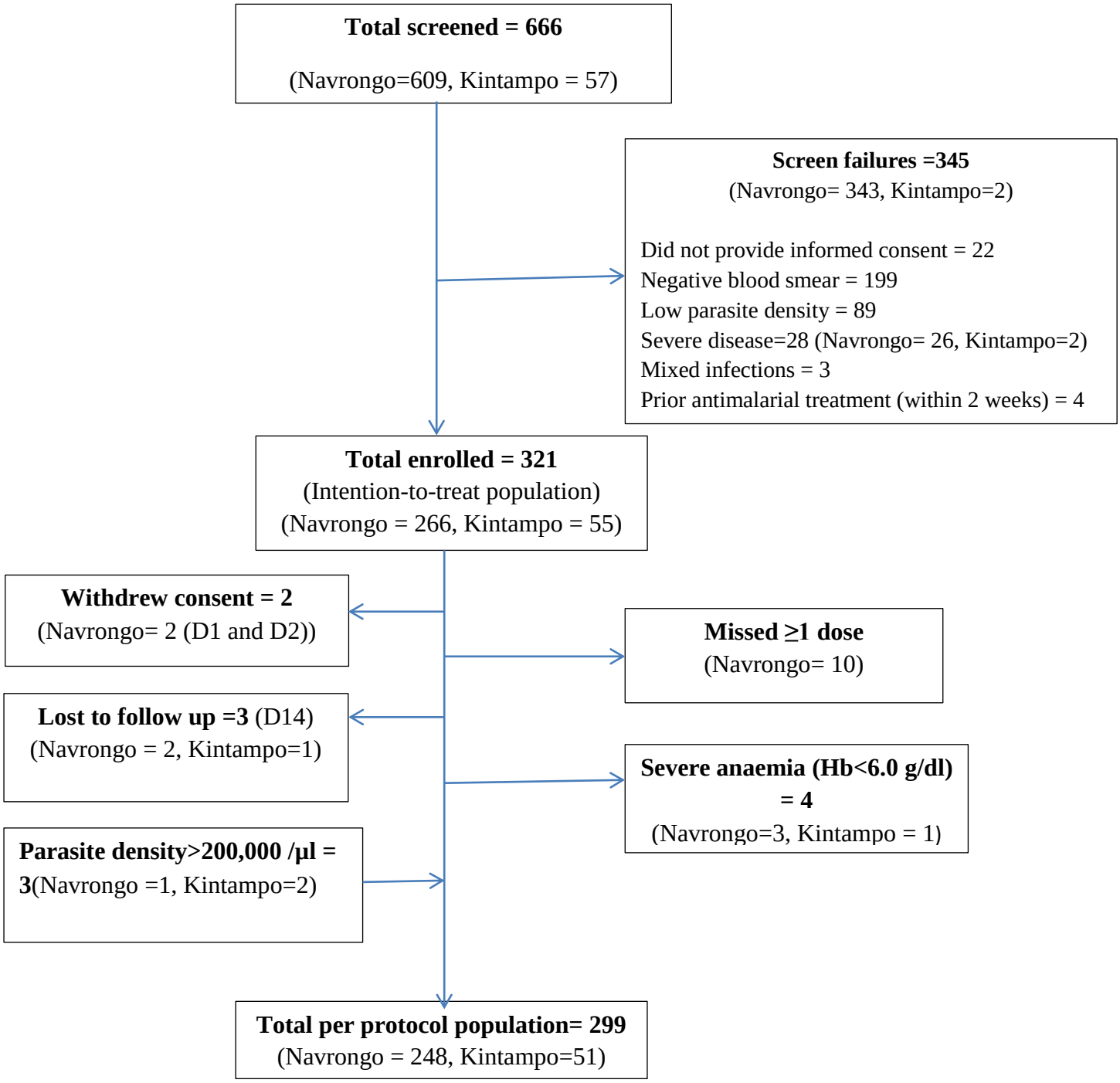
The approved parent protocol was the same for all sites. However, sites were permitted to collect additional relevant data as related to efficacy and effectiveness through amendments at their local institutional review boards. Therefore, there were a few differences in protocol implementation between the two study sites in terms of drug administration (directly observed for all patients and for all doses in Navrongo versus direct observation only for first and third doses in Kintampo), schedule of visits (days 0, 1, 2, 3, 7, 14 and 28 visits in Navrongo compared to days 0, 2, 7, 14 and 28 in Kintampo), and thus frequency and schedule for parasite data collection and haemoglobin concentration measurement. It was therefore not statistically sound to combine the data from the two sites as originally planned and the results had to be presented separately by study site.

A total of 666 potential study participants (509 from Navrongo and 57 from Kintampo) were screened for inclusion into the study from August 2011 to February 2012 in both study sites and then continued from July 2012 to January 2013 in Navrongo (Figure 5.1). Of the total 345 screen failures, 22 did not provide informed consent, 199 tested negative for malaria parasites by microscopy, 89 had parasite densities below the lower limit for inclusion into the study (<1000 /µL) while 3 had mixed infections of *P. falciparum* and *P. malariae.* A further 28 presented with symptoms compatible with severe malaria, while 4 patients had taken antimalarial treatment within two weeks prior to screening.

Overall, 321 patients (266 from Navrongo and 55 from Kintampo) were enrolled. At the Navrongo site, two patients withdrew their consent on days 1 and 2 of follow up respectively. There were two patients in Navrongo who travelled out of the study area by day 14 of follow up and did not return before study completion. One patient in Kintampo missed the scheduled visits on days 7 and 14 after travelling out of the study area. These patients were classified as lost to follow up. Ten enrolled patients, all from the Navrongo site, were withdrawn because they missed either the second dose on day 1 (n=3) and/or the third dose on day 2 (n=7), Figure 5.1. All patients who withdrew their consent, n=2; missed at least a study dose, n=10; had parasite density >200,000 parasites per µL (upper limit for inclusion), n=3, had

haemoglobin concentration<6 g/dl (n=4) or who were lost to follow up (n=3) were excluded from the per protocol analysis but included in the intention-to-treat analysis, up to the point of their exit from the study. Overall 95.3% (306/321) of the patients completed follow up and this was similar at both study sites; 94.7% (252/266) at Navrongo and 98.2% (54/55) at Kintampo (p=0.264).

Figure 5.1: Trial profile



D1 = day 1; D2 = day 2; D14 = day 14; /µl = per microlitre; g/dl = gramme per decilitre

5.2 Baseline characteristics of enrolled patients

The baseline characteristics of the enrolled patients by age category and study site are as presented in Table 5.1. Consistent with areas of high malaria transmission intensities, even though efforts were made to ensure approximately equal numbers of patients were enrolled across age categories, 61.3% of the patients enrolled in Navrongo and 65.5% of the patients enrolled in Kintampo were aged less than 5 years, p=0.559. Young infants aged less than one year constituted only 3.7% (12/321) of the total enrolled participants in the two study sites. There was however a higher proportion of young infants enrolled in Kintampo, 10.9% (6/55) compared to 2.3% (6/266) in Navrongo (p = 0.002). Patients in Navrongo (median age, 4.1 (IQR 2.5 - 7; range 0.5 - 49.7) years) were generally older than patients in Kintampo (median age, 2 (IQR 1 - 5; range 0.5 - 58.7) years). Female patients represented 52.3% and 49.1% of the total patient population in Navrongo and Kintampo respectively, p=0.67.

The median weight of the patients was 15.0 kg (IQR 11.0 – 20.0; range 6.5 – 64.0) in Navrongo and 10.8 kg (IQR 10.0 – 20.0; range 5.5 – 65.3) in Kintampo (Table 5.1). The proportions of patients aged ≤5 years who were underweight-for-age (weight-for-age z-score <-2.0) were similar; 13.9% (23/165) in Navrongo and 16.2% (6/37) in Kintampo (p=0.72). The median weight-for-age z-score for patients who were underweight-for-age was -2.68 (IQR -3.05, -2.34; range -4.69, -2.16) in Navrongo and similar to -2.63 (IQR -3.93, -2.06; range -5.31, -2.04) in Kintampo.

Table 5.1: Baseline characteristics of Intention-to-treat population by Age category and by Study site

Description	Site	Age category			
		total	**< 1**	**1 - 4**	**≥5**
N (%)	Navrongo	266 (100)	6 (2.3)	154 (57.9)	106 (39.8)
	Kintampo	55 (100)	6 (10.9)	30 (54.5)	19 (34.5)
Age (years), median [IQR]	Navrongo	4.1 [2.5 – 7]	*7.5 [6 – 9]	3 [1.9 – 4]	7.8 [6 – 10]
	Kintampo	2 [1 – 5]	*7 [6 – 10]	1 [1 – 2]	8 [5 – 19]
	Navrongo	139/266 (52.3)	5/6 (83.3)	82/154 (53.2)	52/106 (49.1)
Sex, F, n/N (%)	Kintampo	27/55 (49.1)	2/6 (33.3)	13/30 (43.3)	12/19 (63.2)
	Navrongo	15.0 (11.0 – 20.0)	7.5 (7.0 – 8.0)	12.0(10.0 – 15.0)	21.8 (17.1 – 29.0)
Weight (kg), median (IQR);		6.5 – 64.0	6.5 – 8.5	7.0 – 21.0	9.5 – 64.0
Range	Kintampo	10.8 (10.0 – 20.0)	8.0 (8.0 – 10.0)	10.2 (9.6 – 11.0)	20.7 (18.0 – 60.5)
		5.5 – 65.3	8.0 – 11.0	5.5 – 28.0	10.4 – 65.3
Underweight (WAZ<-2.0), n/N (%)	Navrongo	23/162 (14.2)	0/6 (0.0)	22/154 (14.3)	1/2 (50.0)[1]
	Kintampo	6/37 (16.2)	0/6 (0.0)	5/30 (16.7)	1/1 (100)[2]
	Navrongo	33.1 (27.0 - 38.6);	27.1 (25.3 - 28.9);	31.2 (27.0 - 38.6);	33.8 (27.0 – 38.6); 23.0 -
Total dose administered (mg/kg);		23.0 - 45.0	23.8 - 31.2	23.1 – 45.0	45.0
median (IQR), range	Kintampo	39.0 (26.8 - 40.5);	25.3 (25.3 - 36.8);	39.7 (36.8 - 40.5);	33.0 (26.8 - 40.5);
		22.8 - 45.0	25.3 - 40.5	22.8 - 45.0	23.8 - 45.0
Axillary temperature (^{o}C), mean (sd)	Navrongo	38.0 (1.1)	38.0 (0.8)	38.1 (1.0)	37.8 (1.2)
	Kintampo	37.8 (1.2)	38.4 (0.7)	38.1 (1.2)	37.2 (1.1)

Proportion with temperature ≥37.5˚C, n/N (%)	Navrongo	177/266 (66.5)	5 /6 (88.3)	109/154 (70.8)	63/106 (59.4)
	Kintampo	33/55 (60.0)	6/6 (100)	21/30 (70.0)	6/19 (31.6)
Vomited once during drug administration, n /N (%)	Navrongo	16/266 (5.6)	1/6 (16.7)	14/154 (8.3)	1/106 (0.9)
	Kintampo	6/55 (10.9)	1/6 (16.7)	5/30 (16.7)	0/19 (0.0)
Haemoglobin (g/dl), mean (sd)	Navrongo	10.2 (1.9)	8.3 (1.2)	9.7 (1.7)	11.1 (1.8)
	Kintampo	9.2 (1.9)	8.2 (0.7)	8.5 (1.6)	10.7 (1.8)
Proportion with Hb<8.0g/dl, n/N (%)	Navrongo	30/265 (11.3)	2/6 (33.3)	24/154 (15.6)	4/101 (4.0)
	Kintampo	17/55 (30.9)	3/6 (50.0)	13/30 (43.3)	1/19 (5.3)
Geometric mean parasite density (/µl) [95% CI]	Navrongo	28,007 [23921 – 32790]	10,173 [1614 – 64126]	34,323 [28223- 41741]	22,072 [17070 – 28540]
	Kintampo	24,553 [16475 – 36591]	35,640 [10046 – 126434]	36,822 [21967 – 61724]	11,510 [5704 – 23224]
Proportion with parasite density≥100,000 /µl, n/N (%)	Navrongo	35/266 (13.2)	1/6 (16.7)	24/154 (15.6)	10/106 (9.4)
	Kintampo	12/55 (21.8)	2/6 (33.3)	8/30 (26.7)	2/19 (10.5)
Gametocyte carriage, n/N (%)	Navrongo	5/266 (1.9)	0/6 (0.0)	4/154 (2.6)	1/106 (0.9)
	Kintampo	3/55 (5.5)	1/6 (16.7)	1/30 (3.3)	1/19 (5.3)

**Age in months, [1]two patient aged 5 years, [2]One patient aged 5 years; F=female; N= total sample population; n=sub-sample population; IQR=interquartile range; WAZ=weight-for-age z-score; mg/kg=milligramme per kilogramme; ^{o}C = degree Celsius; sd=standard deviation*

Overall, the median total body weight-adjusted dose of amodiaquine administered in Navrongo was 33.1 (IQR 27.0 - 38.6; range 23.0 - 45.0) mg/kg and 39.0 (IQR 26.8 - 40.5; range 22.8 - 45.0) mg/kg in Kintampo, p=0.198. There was a trend towards higher total mg/kg dose administered to patients with age in Navrongo, with young infants aged less than 1 year receiving the lowest dose of amodiaquine (27.1 (IQR 25.3 - 28.9; range 23.8 - 31.2) mg/kg, p=0.076). In Kintampo, there was a difference in the mg/kg dose administered with age, with infants aged less than 1 year receiving a significantly lower dose of 25.3 (IQR 23.3 - 36.8; range 25.3 - 40.5) mg/kg, p=0.032.

Being underweight has been reported as a risk for being either over dosed or under dosed (Taylor et al., 2006; Brasseur et al., 2009). In our study, there was a trend towards higher median total mg/kg dose of 36.8 mg/kg (IQR 32.5 - 40.5; range 24.4 - 45.0) in underweight patients compared to 31.2 mg/kg (IQR 27.0 - 40.1; range 22.8 - 45.0) in patients who were not underweight, p=0.06.

The geometric mean parasite density at enrolment was 28,007 [95% CI 23921, 32790] asexual parasites per microlitre of blood in Navrongo and was similar to the 24,553 [95% CI 16475, 36591] asexual parasites/µl observed in Kintampo. As expected, given their vulnerability due to lower immunity, significantly higher parasite loads of 34,323 (95% CI 28223, 41741), p=0.021 in Navrongo and 36,822 (95% CI 21967, 61724), p=0.019 in Kintampo were recorded in children aged 1 - 4 years compared to older patients. The proportion of patients with parasite density ≥100,000 per µl were similar between the sites, at 13.2% (35/266) in Navrongo and 21.8% (12/55) in Kintampo, p=0.10. There were 3 patients (1 in Navrongo and 2 in Kintampo who were enrolled with parasite densities higher than the upper limit for inclusion of 200,000 parasites/µl. These hyperparasitaemic patients were retained in the intention-to-treat analysis but dropped from the per protocol analysis (Figure 5.1).

At enrolment the prevalence of *P. falciparum* gametocytes was 1.9% (5/266) in patients from Navrongo and 5.5% (3/55) in Kintampo (p = 0.12). However, unlike in Navrongo where most (4 of 5) of the patients carrying gametocytes were aged 1 – 4 years, the 3 cases of gametocytaemia recorded in Kintampo were equally distributed among the 3 age categories.

The mean temperature at presentation was similar at the two study sites, 38.0 ± 1.1^{o}C in Navrongo and 37.8 ± 1.2 ^{o}C in Kintampo (p =0.23). The proportion of patients presenting with a fever (temperature ≥37.5^{o}C) at the time of enrolment was also similar between the two sites, 66.5% (177/266) in Navrongo and 60.0% (33/55) in Kintampo (p=0.35). The remaining patients were enrolled based on a history of fever within the past 24 hours.

The mean haemoglobin concentrations at presentation differed between the two study sites with the Navrongo site recording a higher haemoglobin concentration of 10.2 ± 1.9 g/dl than in Kintampo (9.2 ± 1.9 g/dl) (p<0.001). Similarly, the prevalence of moderate-to- severe anaemia, defined as Hb<8.0 g/dl (Koram et al., 2003; Korenromp et al., 2004), differed between the two sites; 11.3% (30/265) in Navrongo and 30.9% (17/55) in Kintampo (p<0.001). Most of the moderate-to-severe anaemia patients at both study sites were children aged 1 – 4 years, 80% (24/30) in Navrongo and 76.5% (13/17) in Kintampo.

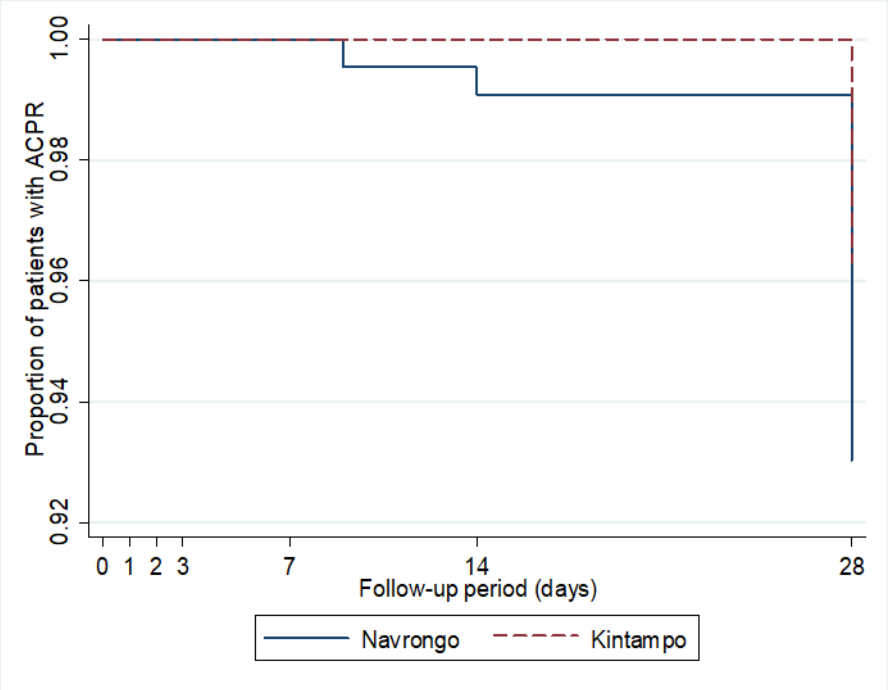
Patients aged <5 years had a lower mean haemoglobin concentration (9.7 ± 1.7 g/dl) than in older patients in Navrongo (11.1 ± 1.8 g/dl, p<0.001). Similarly in Kintampo, patients aged 5 years or older had a higher (10.7 ± 1.8 g/dl) mean haemoglobin than patients aged less than 5 years (8.4 ± 1.5 g/dl), p<0.001. Although haemoglobin concentration <6.0 g/dl was set as an exclusion criterion for enrolment into the study, 4 patients (3 from the Navrongo site and one from Kintampo) aged 1 - 4 years got enrolled in violation of the protocol inclusion criteria. These patients have been retained in the intention-to-treat and safety data analyses but excluded from the per protocol analyses.

Twenty-one patients (6.5%) vomited once within 30 minutes of drug administration on the first day of dosing and had their doses repeated based on the study protocol and were therefore also included in the per protocol analysis. The proportion of patients who vomited was 5.6% (15/266) in Navrongo and 10.9% (6/55) in Kintampo (p=0.15). Per protocol, patients who vomited more than once during drug administration were to be withdrawn. No patient was however withdrawn for this reason. No vomiting was recorded following the second and third doses.

5. 3 Efficacy end point classification

The crude (PCR–unadjusted) day 28 adequate clinical and parasitological response (ACPR) rates by intention-to-treat (ITT) analysis were 93.2% (248/266) [95% CI 89.5, 95.9] in Navrongo and 96.4% (53/55) [95% CI 87.5, 99.6] in Kintampo. The crude day 28 ACPR rate by per protocol (PP) analysis was 93.1% (231/248) [95% CI 89.2, 96.0] in Navrongo and 96.1% (49/51) [95 % CI 86.5, 99.5] in Kintampo. Figure 5.2a presents the intention-to-treat PCR-uncorrected Kaplan-Meier plots for both Navrongo and Kintampo with no difference in cure rates between the two study sites, p-value (log-rank test) =0.79.

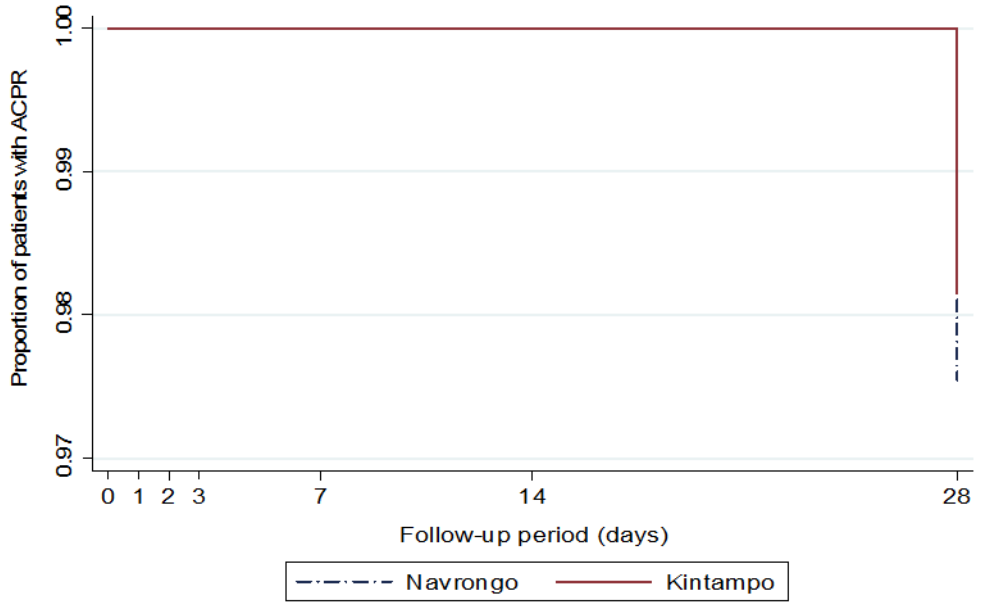
Figure 5.2a: Intention-to-treat Kaplan-Meier plots showing the PCR-uncorrected proportion with an Adequate Clinical and Parasitological Response (ACPR), by study site



PCR: Polymerase Chain Reaction; ACPR: Adequate clinical and parasitological response

The day 28 PCR-adjusted ACPR rate was also similar across the two study sites. By ITT analysis, the overall day 28 PCR (genotype)-adjusted ACPR rate was 97.7% (260/266) [95% CI 95.2, 99.2] in Navrongo and 98.2% (54/55) [95% CI 90.3, 100.0] in Kintampo. The results were also similar by PP analysis; 97.6% [95% CI 94.8- 99.1] in Navrongo and 98.1% [95 % CI 90.1, 100.0] in Kintampo. Figure 5.2b represents intention-to-treat PCR-corrected (i.e. after adjusting for reinfections) Kaplan-Meier plots for both Navrongo and Kintampo; there was no difference in cure rates between the two study sites; p-value (log-rank test) =0.33.

Figure 5.2b: Intention-to-treat Kaplan-Meier plots showing the PCR-corrected proportion with an Adequate Clinical and Parasitological Response (ACPR), by study site



PCR: Polymerase Chain Reaction; ACPR: Adequate Clinical and Parasitological Response

There was no difference in efficacy rates in Navrongo between patients aged less than 5 years and those 5 years and older. By ITT analysis, the day 28 PCR-corrected ACPR rate among patients under 5 years was 98.1% and 97.2% in patients 5 years or older, p=0.63. By PP analysis, the day 28 PCR-corrected ACPR rate in Navrongo was 97.9% in patients less than 5 years and 97.1% in patients 5 years and older, p=0.69 (Table 5.2). Similarly in Kintampo, the cure rates did not differ between patients less than 5 years and those 5 years and older. The day 28 PCR-corrected ACPR rate by ITT analysis was 97.2% among patients less than 5 years and 100% in patients 5 years or older, p=0.49. By PP analysis, the day 28 PCR-corrected ACPR rate was 97.0% in under-fives and 100% in patients 5 years and older, p=0.46 (Table 5.3).

There were no early treatment failures in either study site. Overall, the proportion of ITT patients with parasite recurrence (treatment failure) in Navrongo was 6.8% (18/266) with 2 of these occurring by day 14 (one each on days 9 and 14) and 16 on day 28. In Kintampo 3.6% (2/55) of the ITT patients had parasite recurrence, both of which occurred on day 28. Four of 266 patients (1.5%) were classified as late clinical failures in Navrongo and 1 of 55 patients (1.8%) in Kintampo (p=0.86). Fourteen (5.3%) patients in Navrongo and 1 (1.9%) in Kintampo were classified as late parasitological failures, p=0.27.

Of the 20 treatment failures, there were 7 (35%) recrudescences (6 in Navrongo and 1 in Kintampo), 10 (50%) re-infections (9 in Navrongo and 1 in Kintampo) and 3 (15%) patients with indeterminate or missing PCR results. All recrudescences occurred on day 28 of follow up. Among patients who vomited during drug administration, 95.2% (20/21) achieved adequate clinical and parasitological cure with only 1 recrudescent infection.

Table 5.2: Day 28 Therapeutic efficacy of artesunate-amodiaquine fixed dose combination treatment of uncomplicated falciparum malaria in patients in Navrongo, Ghana, by Intention-to-treat (ITT) and Per Protocol (PP) analysis

Treatment outcome	**Type of analysis**	**Age category (years)**			
		total	**< 1**	**1 - 4**	**≥5**
N	ITT	266	6	154	106
	PP	248	6	140	102
Early treatment failure (ETF), n (%)	ITT	0	0	0	0
	PP	0	0	0	0
Late clinical failure (LCF), n (%)	ITT	4 (1.5)	0 (0.0)	2 (1.3)	2 (1.9)
	PP	3 (1.2)	0 (0.0)	1 (0.7)	2 (2.0)
Late parasitological failure (LPF), n (%)	ITT	14 (5.3)	0 (0.0)	6 (4.0)	8 (7.7)
	PP	14 (5.6)	0 (0.0)	6 (4.2)	8 (7.8)
D28 PCR- uncorrected ACPR, (%)	ITT	**93.2**	**100**	**94.8**	**90.6**
	PP	**93.1**	**100**	**95.1**	**90.2**
Parasite recurrence, n	ITT	18	0	8	10
	PP	17	0	7	10
P. falciparum recrudescence, n	ITT	6	0	3	3
	PP	6	0	3	3
P. falciparum reinfection, n	ITT	9	0	5	4
	PP	8	0	4	4
Indeterminate or missing PCR, n	ITT	3	0	0	3
	PP	3	0	0	3
Day 28 PCR-corrected ACPR, (%)	ITT	**97.7**	**100**	**98.1**	**97.2**
	PP	**97.6**	**100**	**97.9**	**97.0**

***PCR**: Polymerase chain reaction; **ACPR**: Adequate clinical and parasitological response; N= total sample population; n= sub-sample population; ITT= Intention-to-treat; PP= per protocol*

Table 5.3: Day 28 Therapeutic efficacy of artesunate-amodiaquine fixed dose combination treatment of uncomplicated *falciparum* malaria in patients in Kintampo, Ghana, by Intention-to-treat (ITT) and Per Protocol (PP) analysis

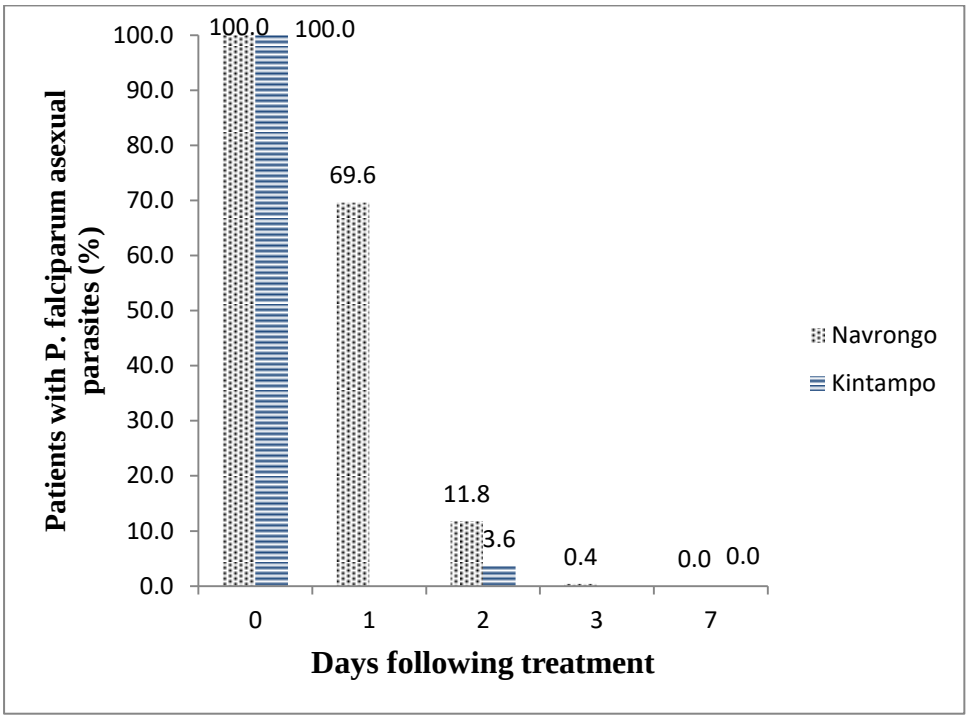
Treatment outcome	Type of analysis	Age category			
		total	**< 1**	**1 - 4**	**≥5**
N	ITT	55	6	30	19
	PP	51	5	28	18
Early treatment failure (ETF), n (%)	ITT	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	PP	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Late clinical failure (LCF), n (%)	ITT	1(1.8)	0 (0.0)	1 (3.3)	0 (0.0)
	PP	1 (1.9)	0 (0.0)	1 (3.6)	0 (0.0)
Late parasitological failure (LPF), n (%)	ITT	1(1.9)	0 (0.0)	1 (3.4)	0 (0.0)
	PP	1 (1.9)	0 (0.0)	1 (3.7)	0 (0.0)
D28 PCR uncorrected ACPR (%)	ITT	**96.4**	**100**	**93.3**	**100**
	PP	**96.1**	**100**	**92.9**	**0**
Parasite recurrence, n (%)	ITT	2 (3.6)	0	2 (6.7)	0
	PP	2 (3.9)	0	2 (7.1)	0
P. falciparum recrudescence, n	ITT	1	0	1	0
	PP	1	0	1	0
P. falciparum reinfection, n	ITT	1	0	1	0
	PP	1	0	1	0
Indeterminate or missing PCR, n	ITT	0	0	0	0
	PP	0	0	0	0
D28 PCR-corrected ACPR, (%)	ITT	**98.2**	**100**	**96.7**	**100**
	PP	**98.0**	**100**	**96.4**	**100**

***PCR**: Polymerase chain reaction; **ACPR**: Adequate clinical and parasitological response; N= total sample population; n= sub-sample population; ITT= Intention-to-treat; PP= per protocol*

5. 4 Parasite clearance

Figure 5.3 presents a plot of the proportion of patients (%) with *P. falciparum* asexual parasitaemia over the first week of follow up. There was rapid parasite clearance at both study sites. On day 2 prior to the administration of the last dose of treatment, the proportion of patients remaining parasitaemic (still carrying *P. falciparum* asexual parasites) was 11.8% (30/255) in Navrongo and 3.6% (2/55) in Kintampo with a trend towards more rapid parasite clearance in Kintampo (p=0.07). By day 3 of follow up, only 0.4% (1/253) of the patients in Navrongo still had parasitaemia. There was no patient visit for patients in Kintampo on day 3. However, all patients were cleared of parasitaemia by day 7 at both study sites.

Figure 5.3: Proportion of patients with *P. falciparum* asexual parasites during follow up by study site (No smears performed on days 1 and 3 in Kintampo)

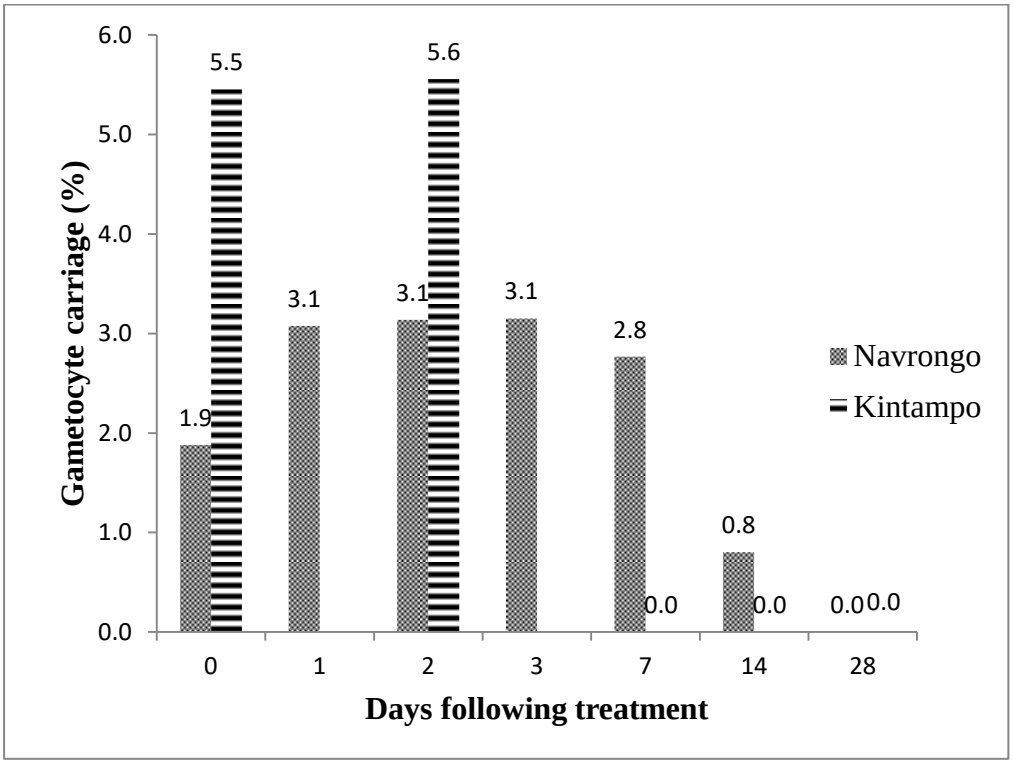


5. 5 Prevalence of gametocytes

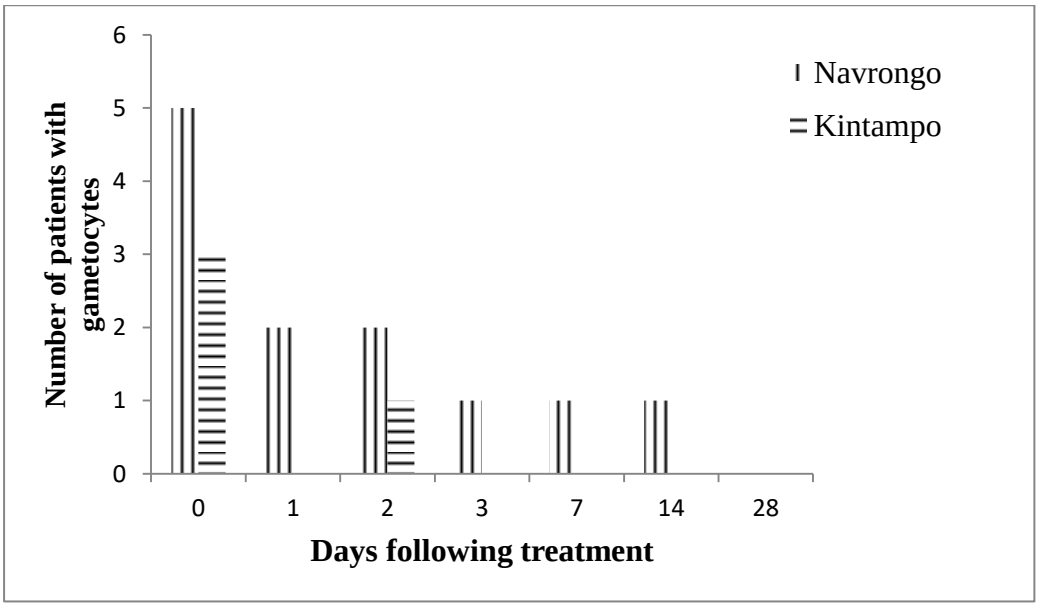
The prevalence of gametocytes was low in both study sites with the proportion of patients carrying gametocytes at enrolment being 1.9% (5/266) (95% CI 0.6%, 4.3%) in Navrongo and 5.5% (3/55) (95% CI 1.1%, 15.1%) in Kintampo (p=0.12). In Navrongo, the number of patients with gametocytes increased to 8 (3.1%) on day 1 and remained 8 on days 2 and 3 but reduced to only 2 patients (0.8%) by day 14. In Kintampo, the prevalence of gametocytes peaked on day 2 when 3/54 (5.6%) of the patients carried gametocytes. All patients were free of gametocytes by day 7 in Kintampo and by day 28 in Navrongo (Figure 5.4a).

The ability of a mosquito to become infective is dependent not only on the density of the sexual parasites but also on the duration of carriage by the host. Among patients with gametocytes at enrolment (5 in Navrongo and 3 in Kintampo), there were 2 patients (0.8%) in Navrongo and 1 (1.8%) in Kintampo that still carried gametocytes by day 2 of follow up (Figure 5.4b). There was no patient with gametocytes on day 7 in Kintampo but gametocytaemia was persistent in one patient in Navrongo until day 14, but on day 28 all gametocytes were cleared.

Figure 5.4a: **Proportion of patients with gametocytes during follow up in Navrongo and Kintampo** (No parasite data for days 1 and 3 in Kintampo)



5.4b: **Gametocyte carriage among patients with gametocytes at enrolment in Navrongo and in Kintampo** (No gametocyte data available for days 1 and 3 in Kintampo)



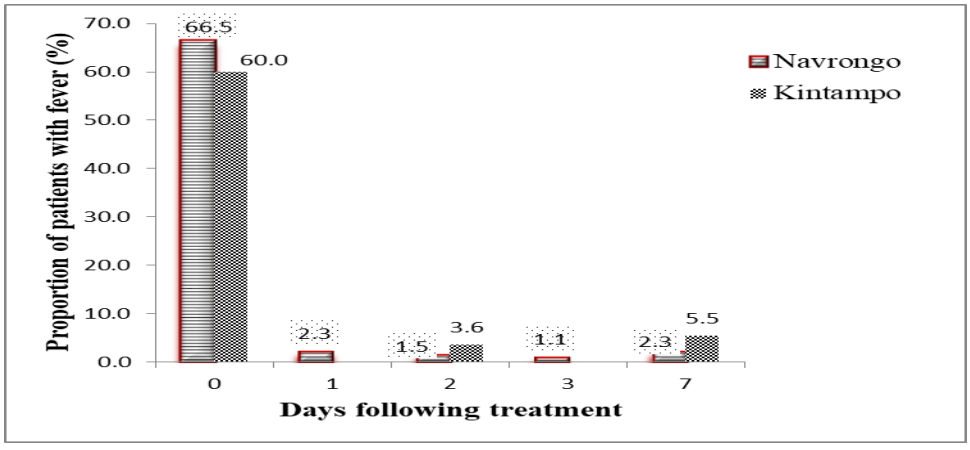
The overall person gametocyte week (PGW) per 1000 person weeks in Kintampo was twice as much as in Navrongo (13.6 and 6.6 PGW/1000 person weeks, respectively). Although the exact reason for this observation is not known, the less frequent sampling in Kintampo could result in this observation. This may also reflect the lower immunity in Kintampo than in Navrongo as typified by the overall younger ages of the patients from Kintampo.

5. 6 Fever clearance

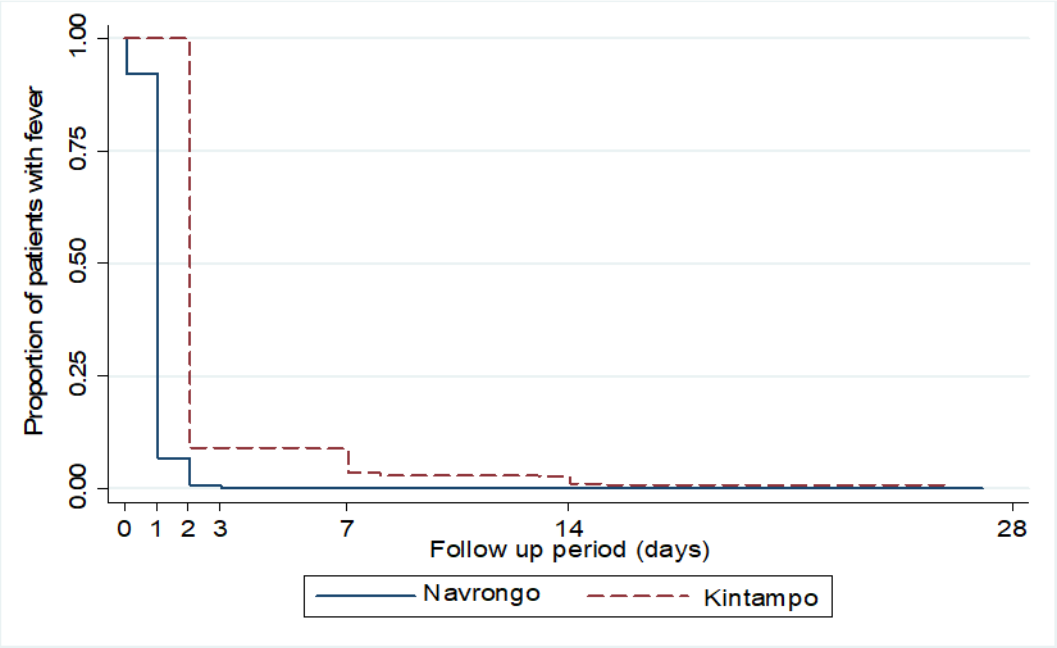
All patients were enrolled into the study based on a measurable fever (axillary temperature≥37.5^{o}C) or a history of fever within 24 hours of being enrolled. In Navrongo, 177 of the 266 (66.5%; 95% CI 60.5%- 72.2%) patients were febrile at presentation while 33 of the 55 patients (60.0%; 95% CI 45.9% - 73.0%) were febrile in Kintampo, p=0.36. Within 48 hours of the commencement of treatment however, those with a fever decreased to only 1.5% (4/266) in Navrongo and 3.6% (2/55) in Kintampo, p=0.29. There was a further reduction in the prevalence of fever to 1.1% (3/266) in Navrongo on day 3 (Figure 5.5a). There was no patient visit for patients in Kintampo on day 3.

The Kaplan-Meier estimate of the fever clearance time (FCT) using the ITT population (Figure 5.5b) showed that it took longer for fever to clear in patients in Navrongo (4.7 days) than in Kintampo (3.7 days), p-value (log-rank test)<0.001. However, as noted earlier, there were no follow ups on days 1 and 3 in Kintampo so this difference may simply reflect the more frequent sampling in Navrongo.

Figure 5.5: **Fever clearance during follow up in Navrongo and Kintampo**
(No fever data available for days 1 and 3 in Kintampo)



5.5b. Kaplan-Meier estimates of fever clearance time (days) in Navrongo and Kintampo



5. 7 Haemoglobin recovery

At baseline, the mean haemoglobin concentration (± sd) in Kintampo (9.2 ± 1.9 g/dl) was lower than in Navrongo (10.2 ±1.9 g/dl), p<0.001. There was a slight decline in mean haemoglobin to 10.0 (±1.7) g/dl by day 7 of follow up in Navrongo. Thereafter, the mean (± sd) haemoglobin concentration in Navrongo increased to 10.7 (±1.5) g/dl by day 14 and further increased significantly to 11.6 (±1.4) g/dl by day 28 compared to the mean haemoglobin at enrolment (P< 0.001).

Similarly, in Kintampo, after a slight decrease to 8.7 ± 2.9 g/dl on day 2 of follow up, there was a significant increase in mean haemoglobin concentration to 10.5 ± 1.5 g/dl on day 28 (p<0.001) (Figure 5.6). The mean haemoglobin concentration in Kintampo on day 28, 10.5 ± 1.5 g/dl was lower than the mean haemoglobin (11.6 (±1.4) g/dl) recorded in Navrongo, p<0.001.

Figure 5.6b presents a boxplot of the mean haemoglobin concentrations on day 28 by age and site. On day 28, haemoglobin concentration was available for only one infant in Navrongo. The mean haemoglobin concentration was lower (11.3 (95% CI 11.0 - 11.6) g/dl) in patients aged 1 - 4 years than in older patients in Navrongo (12.0 g/dl (95% CI 11.7 - 12.3; p<0.001).

In Kintampo, the mean haemoglobin concentration in patients aged 1- 4 years, 9.7 g/dl (95% CI 9.2 - 10.2) was similar to the 10.2 g/dl (95% CI 9.7 - 10.7) measured in infants aged <1 year, p= 0.37. There was however a significantly higher mean haemoglobin (11.8 (95% CI 11.1 - 12.5) g/dl) in patients 5 years or older, than in infants (p<0.01) and in patients aged 1 - 4 years (p<0.001).

Figure 5.6: Mean haemoglobin during follow up in Navrongo and Kintampo

(No haemoglobin data available for days 7 and 14 in Kintampo)

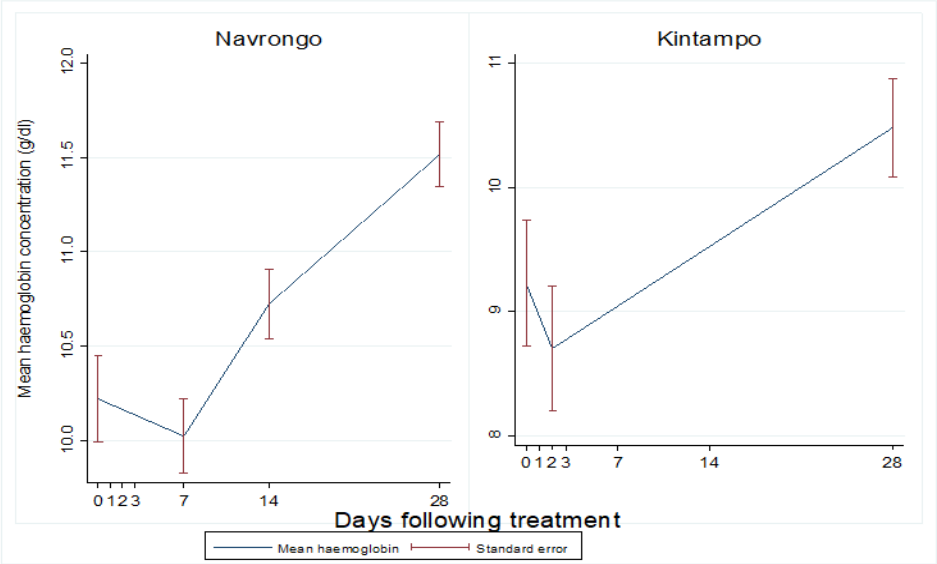
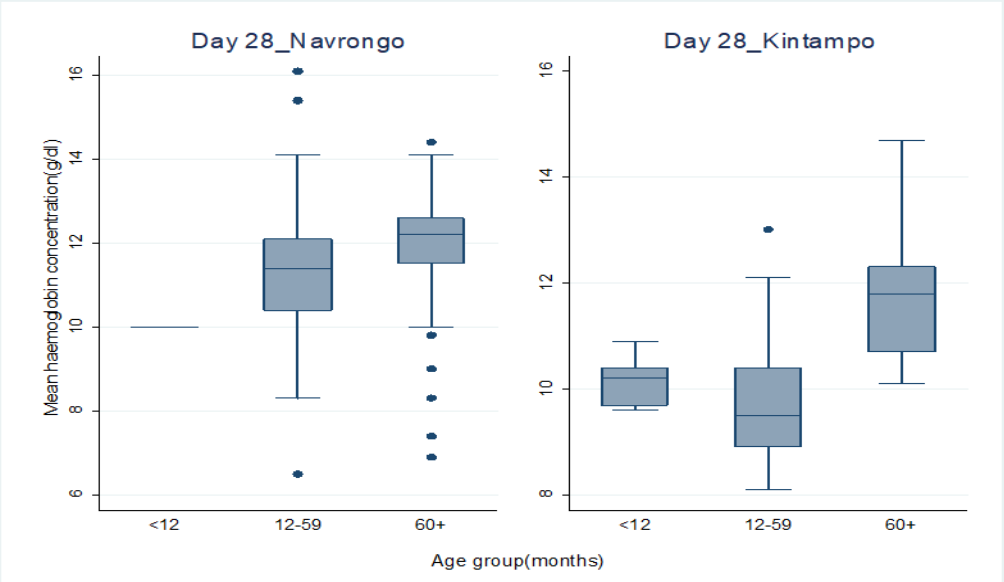


Figure 5.6b: **Box and whisker plots of haemoglobin concentrations at Day 28, by age category in Navrongo and Kintampo study sites**



5. 8 Adverse Events

The reported adverse events (Table 5.4) were all considered mild and did not result in the discontinuation of treatment; there were no severe adverse events at either of the two study sites. Overall, 71 (23.1%) of the patients reported at least one adverse event from days 1 to 3 of follow up, although most were consistent with features of malaria. The most commonly reported adverse events during this period were body weakness (5.2%), abdominal pain and fever (3.3% each), cough (2.3%), diarrhea and headache (1.6% each). From days 7 to 28 of follow up, 68 of the 308 patients (22.1%) reported adverse events with the most common being fever recurrence (4.9%), cough (4.6%) and headache (3.6%). There were other adverse events reported during both periods of follow up at the two study sites but these occurred in less than 1% of the patients (Table 5.4).

Table 5.4: **Adverse events following artesunate-amodiaquine fixed dose combination treatment of uncomplicated *falciparum* malaria, by Intention-to-treat analysis**

	Number of patients, n (%) reporting event	
Adverse event	**Days 1 - 3**	**Days 7 - 28**
Body weakness (Asthenia)	16 (5.2)	0 (0.00)
Abdominal pains	10 (3.3)	3 (0.98)
Fever (Pyrexia)	10 (3.3)	15 (4.9)
Body/joint pains	1 (0.33)	1 (0.33)
Cough	7 (2.3)	14 (4.6)
Headache	5 (1.6)	2 (0.65)
Diarrhoea	5 (1.6)	2 (0.65)
Gastroenteritis	1 (0.33)	2 (0.65
Sepsis	1 (0.33)	2 (0.65)
Itching	3 (0.98)	0 (0.00)
Restlessness	2 (0.65)	0 (0.00)
Vomiting	2 (0.65)	2 (0.65)
Swollen feet/eyes/puffy face	2 (0.65)	1 (0.33)
Sore mouth	2 (0.65)	0 (0.00)
Loss of appetite	2 (0.65)	3 (0.98)
Upper respiratory tract infection (URTI)	0 (0.00)	11 (3.6)
Ear ache/discharge	0 (0.00)	3 (0.98)
Mucoid stool/dysentery	0 (0.00)	2 (0.65)
Slightly distended abdomen	0 (0.00)	1 (0.33)
Boils on head	0 (0.00)	1(0.33)
Anal sore	0 (0.00)	1 (0.33)
Skin rashes	0 (0.00)	1 (0.32)
TOTAL	69 (22.4)	67 (21.8)

5.9 Discussion of results

The World Health Organization recommends that for malaria treatments to be considered effective for policy implementation, a PCR-adjusted adequate clinical and parasitological response (ACPR) rate of at least 95% in clinical trials must be achieved (WHO, 2010a, 2015a) and a change in treatment policy is indicated if recrudescence rates exceed 10% (WHO, 2015b). With day 28 ACPR rates above 97% confirmed in both study sites across all age categories and by both intention-to-treat and per protocol analysis, this study provides reassurance that the efficacy of the fixed dose combination of artesunate-amodiaquine has remained high since its deployment in Ghana in 2005.

In 2005, Ghana introduced ASAQ as loose tablets or co-blister packs to replace chloroquine for the treatment of malaria (MOH, 2009). Prior to this introduction, the PCR-corrected ACPR rate of chloroquine which was the first line drug for the treatment of malaria was estimated to be only 25% with the ACPR rate for ASAQ being 100% after 28 days of follow up (Koram et al., 2005). The introduction of the new treatment policy in Ghana was however, not without controversy. At the inception of the new treatment policy, a fixed dose combination, containing 600 mg AQ and 200 mg AS which had been locally manufactured in Ghana by Kinapharma Ltd, was being prescribed to patients with uncomplicated malaria in the private sector. The use of this combination was reported to be associated with severe adverse effects including deaths (Elorm Hatsu & Asiamah, 2005; WHO, 2006b). This prompted the Ministry of Health in Ghana to call for the withdrawal of this particular formulation from the private sector until additional safety tests were conducted (Ghana News Agency, 2005; Ghanaweb, 2005).

In the current study, the day 28 PCR-adjusted ACPR rate was similar between the two study sites. By intention-to-treat analysis, the day 28 PCR-adjusted ACPR rate for ASAQ for the treatment of uncomplicated f*alciparum* malaria in the two study sites was high, 97.7% (260/266) [95% CI 95.2, 99.2] in Navrongo and 98.2% (54/55) [95% CI 90.3, 100.0) in Kintampo, p=0.82. The PCR-unadjusted ACPR rate of ASAQ in Navrongo and Kintampo were estimated to be 93.2% (248/266) [95% CI 89.5, 95.9] and 96.4% (53/54) [95% CI 87.5, 99.6] respectively.

In 2012, 52.1% of the children under-5 years of age who presented at the out-patient department at health facilities in Ghana were attributable to malaria (NMCP, 2013). The fact

that most of the patients enrolled in this study were aged less than 5 years reflects the heavy burden of malaria in young children in the study area. In spite of the lower host immunity at younger age (Snow & Marsh, 1998; Smith et al., 1999), the ASAQ day 28 PCR-adjusted ACPR rate among these young children was above 95%.

These results are reassuring given that previous studies of co-blister packs of ASAQ (Ipca Laboratories, Mumbai, India) in the same areas put the PCR-unadjusted cure rate at only 80% in children 6 – 120 months in Navrongo (Oduro et al., 2008) and the PCR-adjusted cure rate of ASAQ (Arsucam, Sanofi-Aventis) at 93.4% in children 6 – 59 months in Kintampo (Owusu-Agyei et al., 2008). The current findings are however, similar to an earlier study in 79 children aged 6 – 59 months in 2006 in Navrongo that estimated the PCR-adjusted day 28 efficacy of co-administered ASAQ to be 98.7%. In the same study, the day 28 PCR-adjusted ACPR rate in 37 children aged 6 – 59 months was estimated to be 97.4% in Sunyani, the regional capital of the Brong Ahafo region, the region where Kintampo is located (Koram, Quaye & Abuaku, 2008) (Table 1.1).

The findings in this study are also similar to recently reported studies of fixed dose combination of ASAQ with a day 28 PCR-adjusted ACPR rate of 99.3% [95% CI 95.1, 99.9] in Liberia (Schramm et al., 2013), 97.3% in Equatorial Guinea (Charle et al., 2013) ,98.1% in neighbouring Burkina Faso (Lingani et al., 2013) and 100% in two ecological zones in Ghana (Abuaku et al., 2016). In a large pooled individual patient data analysis of 9,106 patients treated with ASAQ, the PCR-corrected ACPR rate on day 28 of the fixed-dose combination (n =4,138) was 98.1% (95% CI 97.6, 98.5) and was superior to loose tablets of ASAQ (n = 3,711) with ACPR rate of 95.0%; (95% CI 94.1, 95.9); $p<0.001$. The ACPR rate for the fixed-dose combination was similar to the ACPR rate of 97.9% (95% CI 95, 98.8) ($p=0.799$) for the co-blistered ASAQ (n = 1,257) (WorldWide Antimalarial Resistance Network (WWARN) AS-AQ Study Group, 2015)

Parasite clearance in the current study was fast with over 99% of patients clearing their parasitaemia by day 3 of follow up. Only one patient (0.4%) was still parasitaemic on day 3 post-treatment in Navrongo. This trend is in consonance with a recent review of parasite clearance over a period of a decade, from 1999 – 2009 which provides evidence to buttress the observed rapid parasite clearance with ASAQ across Africa (Zwang et al., 2014). Although a recent a priori statistical analysis of pooled data shows that the parasite positivity

rate (PPR) on day 3 for ASAQ (1.3%) was higher than the PPR for artemether-lumefantrine (0.6%) and dihydroartemisinin-piperaquine (0.8%), the differences were not significant and these artemisinin-based combination treatments to date achieve rapid early parasitological clearance in Sub-Saharan Africa (WWARN Artemisinin based Combination Therapy (ACT) Africa Baseline Study Group, 2015). The proportion of patients still parasitaemic on day 3 was 0.4% in Navrongo and falls well below the ≥5.0% recommended as a cut-off for the initiation of a study to assess whether artemisinin resistance is present (WHO, 2014a) and the stricter 3% threshold previously suggested (Stepniewska et al., 2010).

Of the 47 patients presenting with high parasite density (parasite densities ≥100,000/µL of blood), the majority (35/47) were aged under 5 years, (71.4% (25/35) in Navrongo and 83.3% (10/12) in Kintampo). The degree of parasitaemia may be indicative of a patient's premunition (ability to control an infection). A delay in seeking treatment coupled with a lack of host acquired immunity in young children in high transmission areas contribute to their high parasite density. This age group and particularly those with hyperparasitaemia are vulnerable to treatment failure (Dorsey et al., 2004; White et al., 2009; WWARN Artemisinin based Combination Therapy (ACT) Africa Baseline Study Group, 2015).

In general, gametocyte carriage was low at the two study sites with the gametocyte prevalence at enrolment being 1.9% in Navrongo and 5.5% in Kintampo. Most of these gametocytes, 4/5 (80.0 %) in Navrongo and (2/3) 66.7% in Kintampo were found in children aged under 5 years. Gametocytaemia in only 1 of the 5 patients in Navrongo persisted until at least day 14, but all gametocytes were cleared by day 28. However, in Kintampo, all patients were cleared of gametocytes by day 7 of follow up and remained free of gametocytes on days 14 and 28. The time to the clearance of gametocytes following treatment with an ACT has been previously estimated to be 2 days for patients presenting with gametocytes at enrolment and 14 days for patients who develop gametocytes after enrolment (Zwang et al., 2014). Although there were days during follow up (days 1 and 3) in Kintampo when gametocyte data were not available, gametocyte clearance in this study is in contrast to the findings by Zwang and colleagues.

Even though lower in malaria prevalence and entomological inoculation rate, the estimated person gametocyte weeks per 1000 person weeks in this study was higher in Kintampo than in Navrongo (Appawu et al., 2004; Owusu-Agyei et al., 2009b). Although gametocyte

carriage is generally more common in areas of higher malaria transmission intensity (Drakeley et al., 2006; Mabunda et al., 2008), this relationship is complex and there are many examples of similar findings where gametocyte carriage is higher in areas of lower intensity transmission. In Tanzania, gametocytes were prevalent in 17% of malaria asexual parasite positive individuals exposed to 100 infective bites per person per year compared to 24% among those exposed to about 1 infective bites per person per year (Drakeley et al., 2005, 2006). Similarly in Kenya, 18% of parasite carriers exposed to 10 infective bites per person per year carried gametocytes compared to 11% living in an area exposed to 20 - 50 infective bites per person per year (Mwangi et al., 2005; Drakeley et al., 2006). This could be explained by the many other factors associated with gametocyte carriage. Among symptomatic patients presenting at a health facility, the duration of an infection (Price et al., 1999; Robert et al., 2000; Sowunmi et al., 2004), presentation without a fever (von Seidlein et al., 2001; Stepniewska et al., 2008) and recrudescent infections (Price et al., 1999) were found to be more likely to contribute to gametocyte carriage. Recent exposure rather than cumulative exposure to gametocytes is associated with naturally acquired sexual stage-specific immune responses (Bousema et al., 2011; Ouedraogo et al., 2011; Stone et al., 2016) Consistent with other studies of ASAQ (Zwang et al., 2009), fever resolution in this study was rapid and within 48 hours of initiation of treatment, over 98.4% of the patients in Navrongo and 96.4% in Kintampo had their fevers resolved. Antipyretics were provided to all patients who were febrile at enrolment. Therefore the relative contribution of the antipyretic, the known antipyretic effects of the amodiaquine component of the artesunate-amodiaquine combination (Olliaro et al., 1996; WHO, 2001b) and the antimalarial effects of the fixed dose combination to the rapid resolution of the fever cannot be readily established in this study.

Despite an initial decrease in the mean haemoglobin concentration as commonly seen with malaria treatment (Zwang et al., 2009; Cox et al., 2013; Ursing et al., 2016), haemoglobin recovery resulted in significantly higher haemoglobin concentrations on day 28 than day 0 in both study sites. The mean haemoglobin concentrations at the end of the follow up period was significantly higher (11.5 ± 1.4 g/dl) in Navrongo than in Kintampo (10.5±1.5 g/dl) ($p<0.001$). To define the nadir in future studies, haemoglobin should at least be measured on day 3, if not also on days 1 and 2, as the Hb nadir for most patients occurs between days 1 and 3 (Zwang et al., 2017) .Generally, the mean haemoglobin concentration was higher in Navrongo than in Kintampo for each of the follow up days that haemoglobin was measured at

both study sites. In Navrongo, haemoglobin was measured using a Hemocue® photometer. However, an ABX Micros 60-OT haematology analyzer was used in Kintampo. The difference in methodology for measuring haemoglobin could be a source of the inherent differences in absolute haemoglobin concentrations between the two study sites noted in this study. Previous studies have established that haemoglobin concentrations determined by Hemocue® methods are reliable and accurate (Sari et al., 2001; Rechner et al., 2002; Medina Lara et al., 2005) but that automated haematology analyzers have higher precisions than Hemocue® methods (Adam et al., 2012). The correlation between Hemocue® methods and automated haematology analyzers for the determination of haemoglobin is high but Hemocue® methods tend to give consistently higher haemoglobin concentrations (Nkrumah et al., 2011). Other possible explanations for the higher haemoglobin concentrations in Navrongo could include better nutrition or lower helminth prevalences in that area.

Each episode of malaria has been established to be associated with a drop in haematocrit and haemoglobin concentration, regardless of the antimalarial used (Price et al., 2001; Sowunmi et al., 2009). The initial drop in the mean haemoglobin concentrations following treatment in this study may also have been partly attributable to the amodiaquine component of the combination since amodiaquine has been shown to be associated with significant falls in haematocrit compared to artesunate alone (Sowunmi et al., 2009). The use of amodiaquine for chemoprophylaxis has been reported to be associated with fatal adverse drug reactions including neutropenia and aplastic anaemia (Hatton et al., 1986; Neftel et al., 1986; Olliaro et al., 1996). Artemisinin derivatives selectively kill malaria parasites with the spleen removing the dead parasites and leaving the formerly infected red blood cells containing the dead parasites intact (Chotivanich et al., 2000).

Haemoglobin concentrations in Ghana have been reported to be generally lower than those reported and used as reference values, which are based on data primarily from healthy populations in the USA and Europe (Koram et al., 2007, 2014; Dosoo et al., 2012). Differences in haemoglobin values among different population groups have been recognized by WHO (WHO, 2011c). Using the WHO haemoglobin level for diagnosing anaemia, 68.8% of the patients in Navrongo and 85.5% of the patients in Kintampo would be classified as anaemic. Even if limited to the WHO definition of moderate -to severe- anaemia, this remains the majority of study patients, with 50% of the patients in Navrongo and 67% of the patients in Kintampo. Consequently, moderate-to-severe anaemia was based on a previous study at the same site in Ghana and elsewhere in Africa (Koram et al., 2003; Korenromp et al., 2004).

Some of the differences observed between study sites could reflect differences in study design. All three ASAQ doses in Navrongo were administered as directly observed therapy but in Kintampo the second dose was not directly observed. Additionally, while patient follow up were on days 0, 2, 7, 14 and 28 in Kintampo, there were additional follow up on days 1 and 3 in Navrongo. The number of patients enrolled in Kintampo, 55 was less than the target of at least 163 patients. This was because clinical study enrolment started earlier than the pharmacokinetic sub-study could start and ended earlier than expected in the first year in Kintampo.

Although 28 - day follow up is recommended as the minimum duration of follow up for drugs with elimination half-lives of less than 7 days (WHO, 2009c), true recrudescences are frequently reported after day 28 (Stepniewska et al., 2004). Additionally, in areas of high malaria transmission intensity such as the two sites studied, the minimum parasite density recommended for inclusion into an efficacy trial is 2000 per microliter. However, a minimum of 1000 parasites per microliter was the cut off for enrolment into this study in order to allow for the inclusion of older patients (WHO, 2009c). These limitations may result in some level of underestimation of the true ASAQ failure rates, but artesunate-amodiaquine was shown in this study to be efficacious for the treatment of uncomplicated malaria including in young children and those with higher parasite densities. The use of assumed white blood cell (WBC) count for the estimation of malaria parasite density has been reported to be associated with an under-estimation of parasite burden in children under 5 years in Ghana (Adu-Gyasi et al., 2012, 2015) and the over-estimation of parasites in the Brazilian Amazon (Alves-Junior et al., 2014). The use of assumed WBC for the estimation of parasite density in Papua New Guinean children with uncomplicated malaria is reported to be largely robust but it is recommended to use absolute WBC count for estimation of high parasitaemia (Laman et al., 2014). It is also noted that leukopenia could be a cofounder in studies that estimate parasite density using an assumed WBC count of 8000/µl of blood (McKenzie et al., 2005).
In this study however, absolute WBC count was only available for 55 of the 321 (17.1%) enrolled patients. In order to ensure uniformity of data analysis across the sites, it was necessary to use an assumed WBC count of 8000/µl of blood given that most of the patients did not have absolute WBC count.

When used in doses of up to 35 mg/kg, amodiaquine has been reported to be safe and tolerable (Olliaro et al., 1996). Large doses (greater than the recommended standard doses) of

amodiaquine are associated with rare neurological or dystonic reactions such as protruding tongue, intention tremor, excessive salivation and dysarthria. However, some dystonic reactions have also been reported as being idiosyncratic both in Ghana (Akpalu, Nyame & Dodoo, 2005; Adjei et al., 2009; Dodoo et al., 2014) and elsewhere (Akindele & Odejide, 1976; Kamagaté et al., 2004). Bradycardia in a Ghanaian patient treated with a standard dose of amodiaquine was reported to be caused by a direct drug effect (Adjei et al., 2009).

It is therefore worth noting that the initial scare of reported serious adverse effects associated with the use of the fixed dose formulation containing 600 mg amodiaquine and 200 mg artesunate at the inception of the new treatment policy in 2005, and the perception of the populace about the side effects of artesunate-amodiaquine continues to impact on the uptake of artesunate-amodiaquine for the treatment of malaria in Navrongo, particularly among adult patients. Non-adherence to artesunate-amodiaquine treatment was found to be high with 9 out of 15 adult participants interviewed indicating they could not complete their treatments with artesunate-amodiaquine due to its side effects (Chatio et al., 2015). Although 4.3% of the patients screened in Navrongo expressed disinterest in participating in the study at the mention of artesunate-amodiaquine, the medication was generally well tolerated among those completing treatment. The proportion of patients reporting a particular adverse event in this study was lower than previously reported in children in the two study areas (Oduro et al., 2008; Owusu-Agyei et al., 2008). Among the adverse events reported in the two studies cited, pruritus accounted for 18.5% in Navrongo and 7.3% in Kintampo compared with 0.98% in the current study. Similarly, vomiting was quoted as representing 17.5% and 17.4% in Navrongo and Kintampo respectively but only 1.3% in this current study. The reason for these differences is not exactly known but co-blister artesunate-amodiaquine (employed in the two previous studies cited) have been noted to be associated with less accurate dosing and so may lead to more side effects (Brasseur et al., 2009). There is no gold standard method for eliciting adverse events in clinical trials and questioning methods may influence safety data (Allen et al., 2013). The difference in the proportion of patients reporting a particular adverse event in this study and in previous studies in the same study sites may therefore also reflect the methods used in eliciting these events.

It is important to note that the antimalarial drug treatment policy in Ghana has been prudently revised to include two other treatment options, artemether-lumefantrine (AL) and dihydroartemisinin-piperaquine (DHP) as alternative first-line therapies, and for those who do

not tolerate artesunate-amodiaquine for the treatment of uncomplicated *falciparum* malaria (MOH, 2009, 2014b). Although no data exist in Ghana that compares the efficacy of artesunate-amodiaquine head-to-head with DHP and AL in the same study, studies from 12 sites in 7 other African countries show that DHP compares favourably (97.6%) with artesunate-amodiaquine (96.8%) (OR: 0.74, 95% CI: 0.41–1.34) for the treatment of uncomplicated *falciparum* in African children (The Four Artemisinin-Based Combinations (4ABC) Study Group, 2011). In the same studies, artesunate-amodiaquine (97.1%) was found to be non-inferior to AL (94.4%) (OR: 0.50, 95% CI: 0.28–0.92). In a recent study among pregnant women in Ghana, dihydroartemisinin-piperaquine (DHP) was found not to be inferior to artesunate-amodiaquine (ASAQ). The day 28 parasitological efficacy by per protocol analysis, PCR-uncorrected was 91.6% (95% CI: 86.7, 95.1) for patients on DHP compared to 89.3% (95% CI: 83.8, 93.5) for ASAQ patients. The corresponding efficacy on day 42, by per protocol analysis was 89.0% (95% CI: 83.4, 93.0) for DHP and 86.5% (95% CI: 80.6, 91.2) for ASAQ (Osarfo et al., 2017).
In another study in the forest and savannah zones of Ghana, the overall PCR-corrected adequate clinical and parasitological response rate by per protocol analysis was 100% for artesunate-amodiaquine and 97.6 % (95 % CI 93.1, 99.5) for artemether-lumefantrine: 97.2 % (95 % CI 92.0, 99.4) in the forest zone and 100 % in the savannah zone (Abuaku et al., 2016).

For an antimalarial treatment to be reliably effective, it should provide over 95% efficacy in non-immune populations (children under five years in this study area) (WHO, 2010a, 2015a; White, 2013). The overall efficacy of artesunate-amodiaquine for the treatment of uncomplicated *falciparum* malaria was well above 95% in children less than 5 years (98.1% in Navrongo and 97.2% in Kintampo. After more than 10 years of use of artesunate-amodiaquine for the treatment of uncomplicated *falciparum* malaria in Ghana, the evidence from this study provides further justification for the continued use of the fixed-dose combination in the study areas. The combination exacts a fast parasite and fever clearance with high efficacy rate and good haemoglobin recovery. However, there is an ongoing need for vigilance and for therapeutic efficacy studies to be repeated regularly, ideally every two years, in order to ensure the sustained efficacy of ASAQ, particularly in the light of recent case reports (Lu et al., 2017; Sutherland et al., 2017) of the emergence of indigenous artemisinin-resistant *P. falciparum* in Africa.

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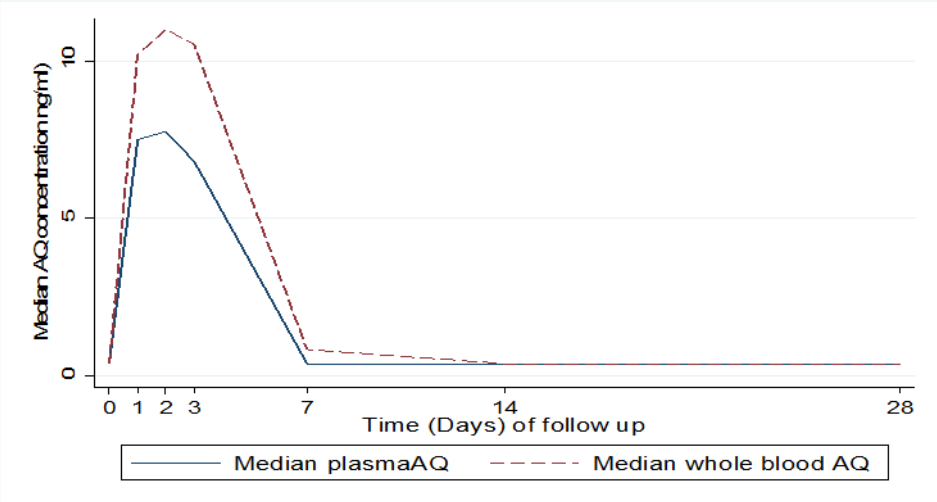
Chapter 6: Comparison of capillary whole blood and capillary plasma amodiaquine and desethylamodiaquine concentrations in Ghanaian patients with uncomplicated *falciparum* malaria

Study methods are described in chapters 3 and 4.
Chapter 6 compares paired capillary whole blood amodiaquine/desethylamodiaquine with capillary plasma amodiaquine/desethylamodiaquine concentrations. Sample extraction and assay used the same steps and procedures. The only difference in the assay process was the matrix use: capillary whole blood versus capillary plasma samples. The assay was validated for both matrices. The lower limit of quantification for amodiaquine was 0.781 ng/ml for both whole blood and plasma samples. The lower limit of quantification for desethylamodiaquine is 3.91 ng/ml. The instrumentation was on HPLC coupled mass spectrophotometer.
Sensitivity, selectivity, reproducibility, accuracy and precision for this assay were all established to be within stated guidelines.
Amodiaquine and desethylamodiaquine have been shown *in vitro* to accumulate in peripheral white cells in vivo (Labro & Babin-Chevaye, 1988). The use of whole blood for the assay is therefore justified. The assay met all the requirements to ensure accurate measurement of drug levels.

6. 1 Results

A total of 110 pairs of capillary whole blood and capillary plasma samples were collected from the malaria patients of all ages from whom at least 200 µl of capillary whole blood could be obtained. Median amodiaquine and desethylamodiaquine concentrations in both matrices are displayed in Figures 6.1 and 6.2, respectively. Tables 6.1 and 6.2 compare the median and the interquartile ranges (IQR) of the concentrations in capillary whole blood and plasma of amodiaquine and desethylamodiaquine respectively, by protocol day of follow up.

Figure 6.1: Plot of median capillary whole blood and plasma amodiaquine (AQ) concentrations by day of follow up.



The distribution of the median capillary whole blood and plasma desethylamodiaquine concentrations are presented in Table 6.2. The median capillary whole blood concentrations of desethylamodiaquine at the various time points were significantly higher than the corresponding capillary plasma desethylamodiaquine concentrations, except when concentrations were below the limit of quantification. Even prior to drug administrations, the median capillary whole blood desethylamodiaquine concentration was 24.9 (IQR 1.955 – 82.4) ng/ml and 7.0 (IQR 1.955 - 11.7) ng/ml in capillary plasma, $p<0.001$. The median amodiaquine concentrations were below the limit of quantification (0.781 ng/ml) in all the patients prior to study drug administration and on days 14 and 28 (Table 6.1). When all concentrations of capillary whole blood amodiaquine were compared with all capillary plasma concentrations, there was a very strong correlation of 0.9806, $p<0.001$. Capillary whole blood and capillary plasma concentrations of amodiaquine were found to be strongly correlated ($r_s > 0.80$) on all follow up days. Overall, the correlation between the capillary whole blood and capillary plasma concentrations of the metabolite, desethylamodiaquine was also strong, $r_s=0.8586$, $p<0.001$. However, the correlations dropped from a high $r_s= 0.9026$, $p<0.001$ on day 0 to weak correlations from Day 3 onwards (Table 6.2).

Figure 6.2: Plot of median capillary whole blood and plasma desethylamodiaquine (DEAQ) concentrations by day of follow up

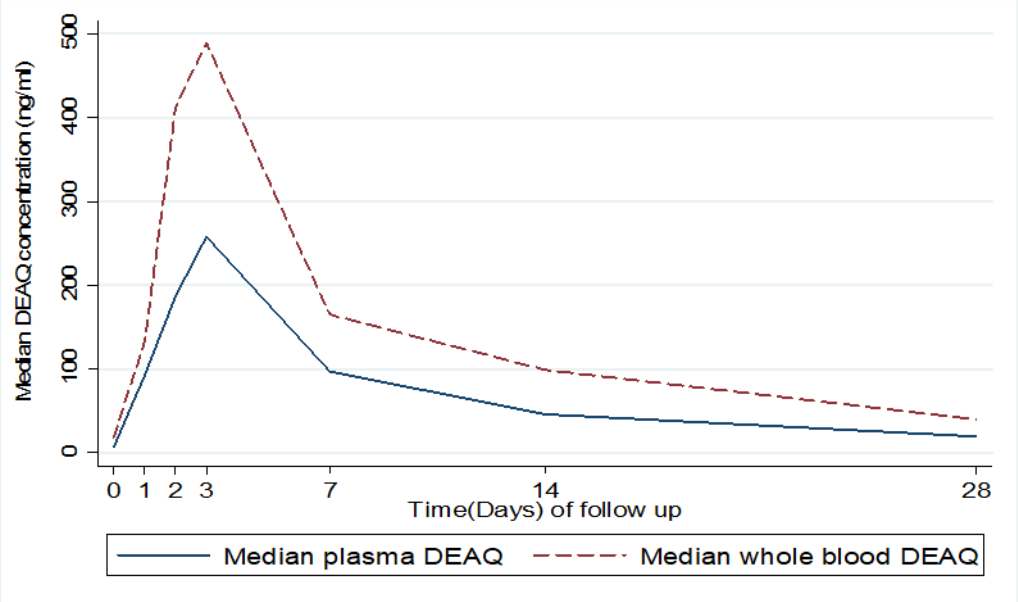


Table 6.1 Median capillary whole blood and capillary plasma amodiaquine (AQ) concentrations and correlation between capillary whole blood and capillary plasma amodiaquine (AQ) concentrations by day of follow up

Follow up (days)	Matrix	Number of samples	Median AQ (ng/ml)	Interquartile range		Kruskal-Wallis p-value	Correlation coefficient (r_s)	Spearman's Rank p-value
0	Whole blood	18	0.3905*	0.3905*	0.3905*	NA	1.0000	<0.001
	Plasma	18	0.3905*	0.3905*	0.3905*			
1	Whole blood	14	12.6	7.3	50.7	<0.001	0.8418	<0.001
	Plasma	14	7.6	3.2	16.9			
2	Whole blood	18	19.9	9.5	45.8	<0.001	0.8372	<0.001
	Plasma	18	7.8	4.0	12.3			
3	Whole blood	15	14.5	7.2	45.3	<0.001	0.8321	<0.001
	Plasma	15	6.5	3.0	20.3			
7	Whole blood	10	2.0	0.3905*	4.7	<0.001	0.9448	<0.001
	Plasma	10	0.745	0.3905*	1.7			
14	Whole blood	14	0.3905*	0.3905*	0.3905*	NA	1.0000	<0.001
	Plasma	14	0.3905*	0.3905*	0.3905*			
28	Whole blood	13	0.3905*	0.3905*	0.3905*	NA	NA	NA
	Plasma	13	0.3905*	0.3905*	0.3905*			

*0.3905 =BLQ (0.781)/2, AQ = amodiaquine, ng/ml = nanogram per millilitre

Table 6.2: Median capillary whole blood and capillary plasma desethylamodiaquine (DEAQ) concentrations and correlation between capillary whole blood and capillary plasma desethylamodiaquine (DEAQ) concentrations by day of follow up

Follow up (days)	Matrix	Number of samples	Median DEAQ (ng/ml)	p-value for medians	Interquartile range		Correlation coefficient (r_s)	p-value
0	Whole blood	17	24.9	<0.001	1.955*	82.4	0.9026	<0.001
	Plasma	17	7.0		1.955*	11.7		
1	Whole blood	14	359	<0.001	149	522	0.7011	0.005
	Plasma	14	81.2		59.6	98.8		
2	Whole blood	18	709	<0.001	419	1181	0.6636	0.003
	Plasma	18	172		154	241		
3	Whole blood	15	531	<0.001	479	1139	0.2143	0.443
	Plasma	15	247		169	268		
7	Whole blood	15	502	<0.001	165	639	0.5536	0.032
	Plasma	15	92.5		76.8	143		
14	Whole blood	15	139	<0.001	94.3	195	-0.0821	0.771
	Plasma	15	44.4		23.0	58.0		
28	Whole blood	14	65.4	<0.001	40.1	119	0.4286	0.126
	Plasma	14	16.1		9.2	32.5		

** 1.955=BLQ (3.91)/2, DEAQ = desethylamodiaquine, ng/ml = nanogram per millilitre*

6.2 Ratio of the geometric mean of capillary whole blood and capillary plasma concentrations of amodiaquine and desethylamodiaquine

A plot of the $\log_e$ observed capillary whole blood versus loge observed capillary plasma amodiaquine and desethylamodiaquine concentrations are presented in Figures 6.3 and 6.4 respectively.

Figure 6.3: Scatter plot of capillary whole blood and plasma amodiaquine (AQ) concentrations plotted as natural logarithms ($\log_e$) and fitted with a regression model

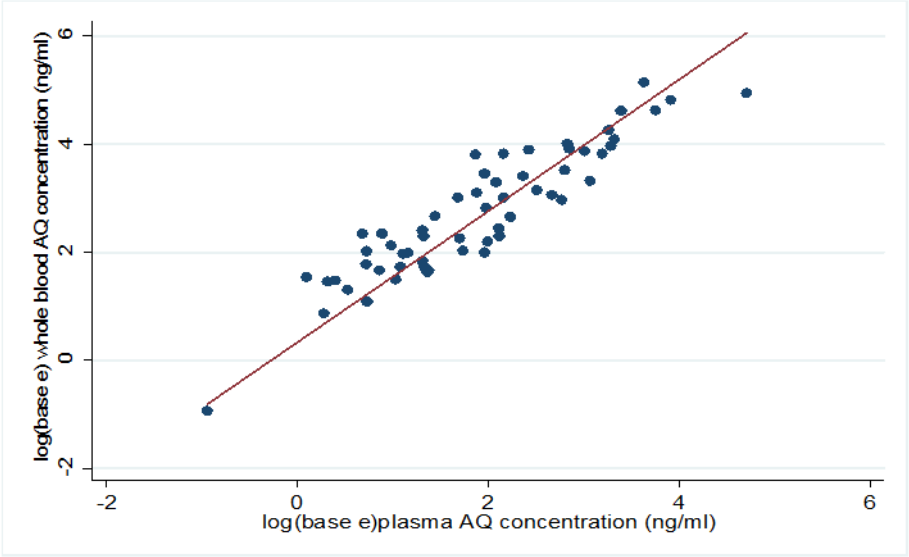
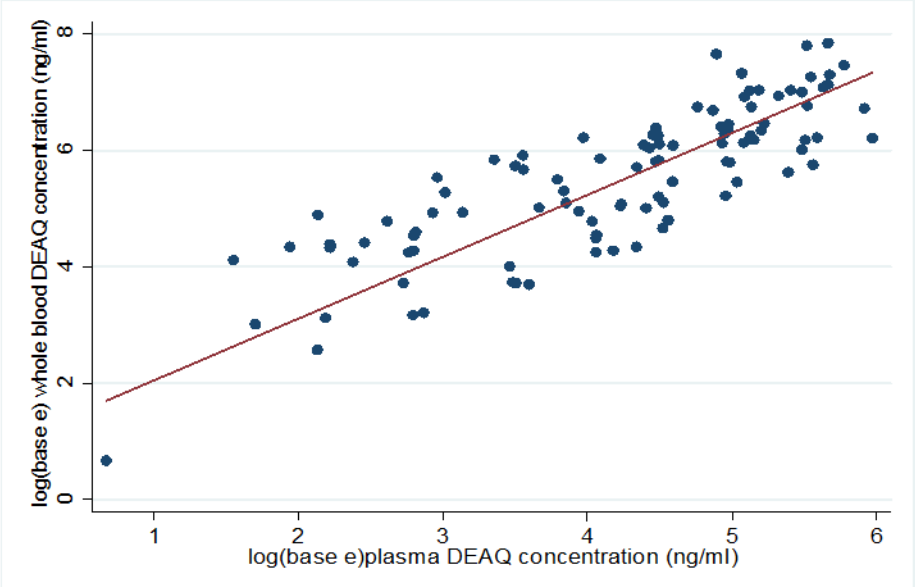


Figure 6.4: Scatter plot of capillary whole blood and plasma desethylamodiaquine (DEAQ) concentrations plotted as natural logarithms ($\log_e$) and fitted with a regression model



Overall, the ratio of the geometric mean of capillary whole blood to capillary plasma amodiaquine concentrations (amodiaquine ratio) was 2.4 (95% CI 2.3, 2.6). In 65.7% (67/102) of the samples, the amodiaquine ratio was between 1 and 2 and was ≥2 in 34.3% (35/102) of the samples, Table 6.3.

Table 6.3 Ratio of capillary whole blood and capillary plasma concentrations of Amodiaquine (AQ) and desethylamodiaquine (DEAQ)

	Paired concentrations in category, n (%)	
Ratio of Whole blood to plasma concentrations	**Amodiaquine**	**Desethylamodiaquine**
1-<2	67 (65.7)	31 (28.7)
2-<3	18 (17.7)	13 (12.0)
3-<5	14 (13.7)	23 (21.3)
≥5	3 (2.9)	41 (38.0)

Overall, the ratio of the geometric mean of capillary whole blood to plasma desethylamodiaquine concentrations was 3.4 (95% CI 3.2, 3.7). The capillary whole blood concentrations of desethylamodiaquine were 2 or more times higher than the capillary plasma desethylamodiaquine concentrations in 71.3% (77/108) of the samples, with 38% (41/108) of the paired samples being > 5-fold higher (Table 6.3).

The ratios of capillary whole blood to plasma amodiaquine concentrations were linear and did not vary with increasing concentrations (Table 6.4). However, for desethylamodiaquine, the mean or median ratio of capillary whole blood to plasma concentrations appeared to increase with concentrations, increasing from a ratio 2.9 for the lowest quintile to 4.5 for the highest quintile (Table 6.5).

Table 6.4 Comparison of capillary whole blood and capillary plasma amodiaquine concentrations by quintiles

Quintile	Percent (%)	Whole Blood		Plasma		Ratio of median AQ WB/plasma
		Number of samples, n (%)	median (IQR)	n (%)	median (IQR)	
1-2	0 - 40	45 (44.1)	0.3905* (0.3905-0.3905)	45 (44.1)	0.3905* (0.3905-0.3905)	1.0
3	40 - 60	17 (16.7)	5.2(4.4 - 5.9)	17 (16.7)	2.1 (1.7 - 2.8)	2.5
4	60 - 80	20 (19.6)	11.3(9.7 - 19.9)	20 (19.6)	6.5 (4.1 - 7.7)	1.7
5	80 - 100	20 (19.6)	50.0 (39.5 - 86.6)	20 (19.6)	21.0 (15.2 -28.8)	2.4

*AQ= amodiaquine; % =percent; n=number of samples in categor***y**

Table 6.5 Comparison of capillary whole blood and capillary plasma desethylamodiaquine concentrations by quintiles

Quintile	Percent (%)	Whole blood		Plasma		Ratio of median DEAQ WB/plasma
		Number of samples, n (%)	median (IQR)	n (%)	median conc (IQR)	
1	0 - 20	22(20.4)	24.3 (1.955 - 55.2)	23 (21.3)	8.4 (1.955* - 13.6)	2.9
2	20 - 40	22 (20.4)	113 (82.4 - 139)	21 (19.4)	33.3 (23.0 - 51.4)	3.4
3	40 - 60	21 (19.4)	276 (201 - 329)	21 (19.4)	81.8 (68.5 - 89.0)	3.4
4	60 - 80	22 (20.4)	500 (450 -531)	22 (20.4)	144 (133 - 161)	3.5
5	80 - 100	21 (19.4)	1134 (869 - 1483)	21 (19.4)	250 (223 - 288)	4.5

*DEAQ= amodiaquine; % =percent; n=number of samples in categor***y**

6.3 Generalized estimating equation (GEE) modeling

The relationships between observed capillary whole blood and capillary plasma concentrations of both amodiaquine and desethylamodiaquine were fitted to log-linear regression models using generalized estimating equation (GEE) modeling. The best fitted model resulted in a statistically significant relationship between observed capillary whole blood and observed capillary plasma concentrations of amodiaquine; Wald χ^2 (chi-square with 1 degree of freedom) = 2485.21, $p<0.001$ as described by the equation:

$$\mathbf{ln\ (cAQ_{wb}) = 0.317 + 1.223 * ln\ (cAQ_p)},$$

where cAQ_{wb} refers to the capillary whole blood concentrations of amodiaquine and cAQ_p refers to the capillary plasma concentrations of amodiaquine

Similarly, concentrations of desethylamodiaquine in capillary whole blood and plasma were best fitted to the equation:

$$\mathbf{ln\ (cDEAQ_{wb}) = 0.959 + 1.051 * ln\ (cDEAQ_p)}$$

where $cDEAQ_{wb}$ refers to the concentrations of desethylamodiaquine in whole blood and $cDEAQ_p$ refers to the concentrations of desethylamodiaquine in plasma samples.

The relationship between concentrations of desethylamodiaquine in capillary whole blood and plasma was also statistically significant; Wald χ^2 (chi-square with 1 degree of freedom) = 540.47, $p<0.001$.

The inclusion of parasite density and haemoglobin concentration did not improve the model fit for both amodiaquine and desethylamodiaquine.

Using the derived equations, capillary whole blood concentrations of amodiaquine and desethylamodiaquine were predicted and plotted as predicted log (base e) versus observed log (base e) capillary whole blood concentrations of amodiaquine (Figure 6.5) and desethylamodiaquine (Figure 6.6) respectively.

Figure 6.5: Scatter plot of observed and predicted log_e whole blood amodiaquine (AQ) concentrations

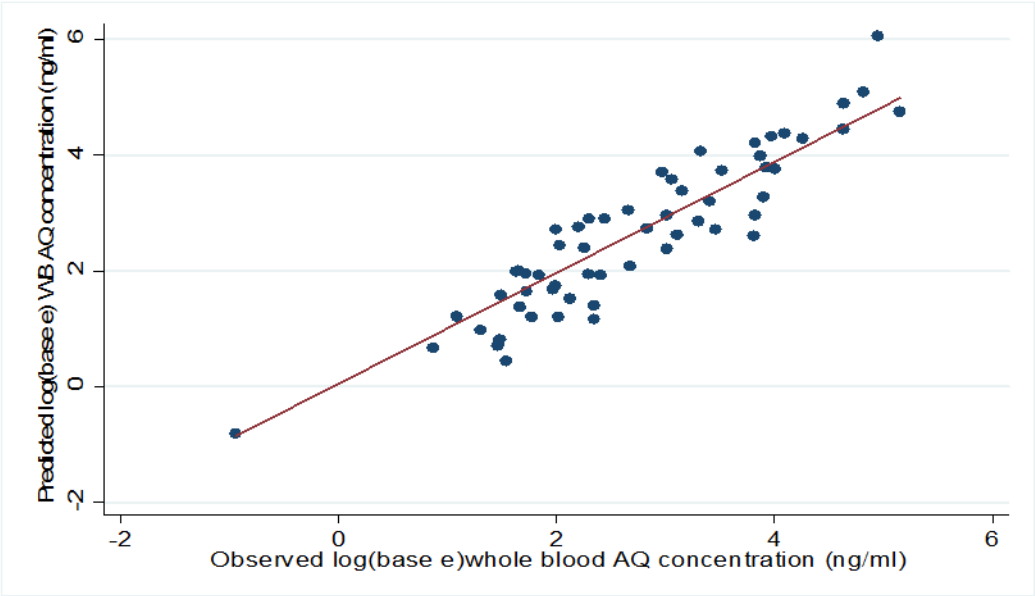
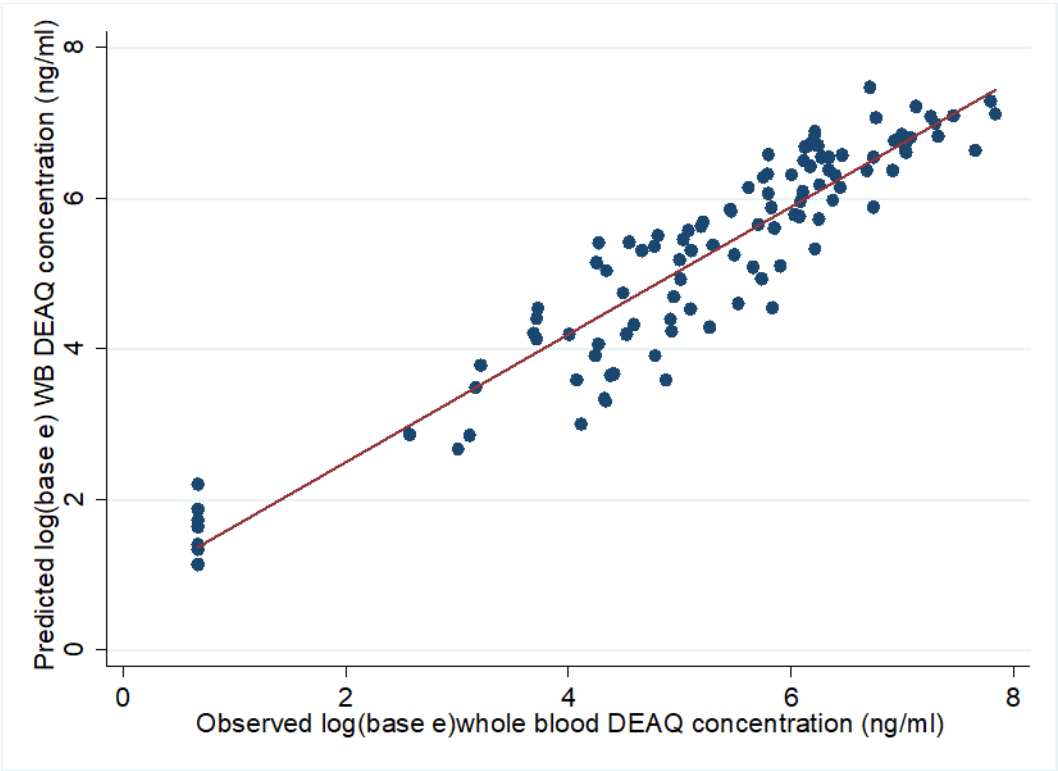


Figure 6.6: Plot of observed and predicted log_e whole blood desethylamodiaquine (DEAQ) concentrations



6.4 Discussion

In order for antimalarial drug concentrations measured in different matrices across studies to be compared or pooled, there is the need for the relationship between the concentrations of the compound measured using different matrices to be accurately established.

In our study in patients with uncomplicated malaria, a log-linear relationship was also established between $\log_e$-transformed amodiaquine concentrations in capillary whole blood and capillary plasma samples and their correlation was very strong, r_s=0.981, p<0.001. The concentrations of desethylamodiaquine in capillary whole blood were reasonably strongly correlated with the concentrations of desethylamodiaquine in capillary plasma samples, r_s=0.859; p<0.001, but with a decrease in correlation over days of follow up.

Overall the ratio of the geometric mean of the concentration of amodiaquine in capillary whole blood to the concentration in capillary plasma in this study was about 2.4, and 3.4 for desethylamodiaquine. However, there were greater variability between capillary whole blood and capillary plasma concentrations of desethylamodiaquine, and these were only significantly correlated between day 0 and day 2.

The concentrations of amodiaquine and desethylamodiaquine in capillary whole blood were generally higher in whole blood compared to plasma and this could be suggestive of the fact that amodiaquine and its active metabolite, desethylamodiaquine concentrate preferentially within blood cells (neutrophils) (Naisbitt et al., 1997). The concentration of desethylamodiaquine in whole blood has been previously reported to be about 3-times as high as the concentration of desethylamodiaquine in plasma (Winstanley et al., 1987a; Laurent et al., 1993) and 4 -6 times higher in whole blood than in plasma in 66 healthy volunteers (Pussard et al., 1987). In these healthy individuals, the relationship between whole blood and plasma concentrations of desethylamodiaquine was reported to be linear and the correlation was strong, r=0.948; p<0.001 (Pussard et al., 1987). No such results have been published previously for amodiaquine, the parent compound.

In malaria patients, the pharmacokinetic properties of antimalarial drugs are altered when compared to healthy individuals (White, 2013). Similarly, the pharmacokinetic properties of antimalarial drugs may be different in key malaria patient subgroups including infants and malnourished children (Barnes, Watkins & White, 2008; WHO, 2015a). In spite of these previously observed differences, the ratio of the concentrations of desethylamodiaquine in whole blood to the concentration in plasma averaged 3.4 and was similar to the mean of 3.1

reported by Winstanley et al., 1987a and Laurent et al., 1993. In 38.0% (41/108) of the paired samples, the ratio of desethylamodiaquine concentrations in whole to plasma was ≥5 which lends credence to the higher ratio reported previously by (Pussard et al., 1987).

There was a statistically significant relationship shown between observed capillary whole blood concentrations and capillary whole blood concentrations predicted from the observed capillary concentrations for both amodiaquine and desethylamodiaquine. However, the greater variability in observed and predicted desethylamodiaquine concentrations would preclude reliable prediction of concentrations from one such matrix to another. The reasons for this greater variability for desethylamodiaquine are not clearly explained. Amodiaquine and its metabolite are highly lipophilic and bind to plasma carriers such as α-acid glycoproteins (Israili & Dayton, 2001; Zsila et al., 2008). Several other factors such as the effects of disease severity, genetic variability in metabolizing enzymes such as cytochrome P450 (Kerb et al., 2009) and interactions with other drugs (Akande et al., 2015) could have influenced the pattern observed in this study. A population pharmacokinetic modeling of pooled data could be used to try and better elucidate this relationship in order to facilitate comparing and pooling of concentrations measured in different matrices.

Chapter 7: Pharmacokinetic profile of amodiaquine and desethylamodiaquine in uncomplicated falciparum malaria patients in Ghana

Study methods are described in chapters 3 and 4.

7.1 Baseline characteristics

Of the 321 patients enrolled for the therapeutic efficacy trial of ASAQ, 308 (96.0%) patients were enrolled for the pharmacokinetic (PK) arm of the study. Reasons for non-inclusion in the PK arm of the study include: missing at least one treatment dose either on day 1 and/or 2 of follow up (10 patients), withdrawal of consent (2 patients) or loss to follow up before day 7 (1 patient). A total of 1900 samples were assayed for amodiaquine and desethylamodiaquine concentrations. However, 64/308 (20.8%) of the patients on the PK arm of the study were found to have concentrations of amodiaquine greater than the lower limit of quantification (LLOQ = 0.781 ng/ml) prior to the first dose of the study drug, suggesting a recent use of amodiaquine and so were retrospectively excluded from the data analysis. Following a visual inspection of the individual concentrations versus time plots, 48 amodiaquine and 66 desethylamodiaquine data points were found to be biologically implausible and so were dropped from the data analysis. Thus a total of 1432 amodiaquine and 1416 desethylamodiaquine concentrations from 244 patients were included in the pharmacokinetic data analysis. The trial profile is as presented in Figure 7.1

Of the 308 patients enrolled into the pharmacokinetic arm of the study, 13 (4.2%) were young infants aged less than 1 year, 176 (57.1%) were children aged 1 – 4 years and 119 (38.6%) were aged 5 years or older. Just over half (52.3%) of the patients were females. The baseline characteristics of the patients are summarised in Table 7.1. At presentation, the mean ± sd temperature was 38.0 ± 1.1^{o}C for all patients. Patients 5 years or older presented with a significantly lower mean temperature at enrolment (37.7 ± 1.2 ^{o}C) compared to 38.1 ± 1.1 ^{o}C in patients aged 1 - 4 years and 38.3 ± 0.8 ^{o}C in infants aged less than 1 year, p=0.017. .

Figure 7.1 Trial profile of the study of amodiaquine (AQ) and desethylamodiaquine (DEAQ) pharmacokinetic parameters (PK)

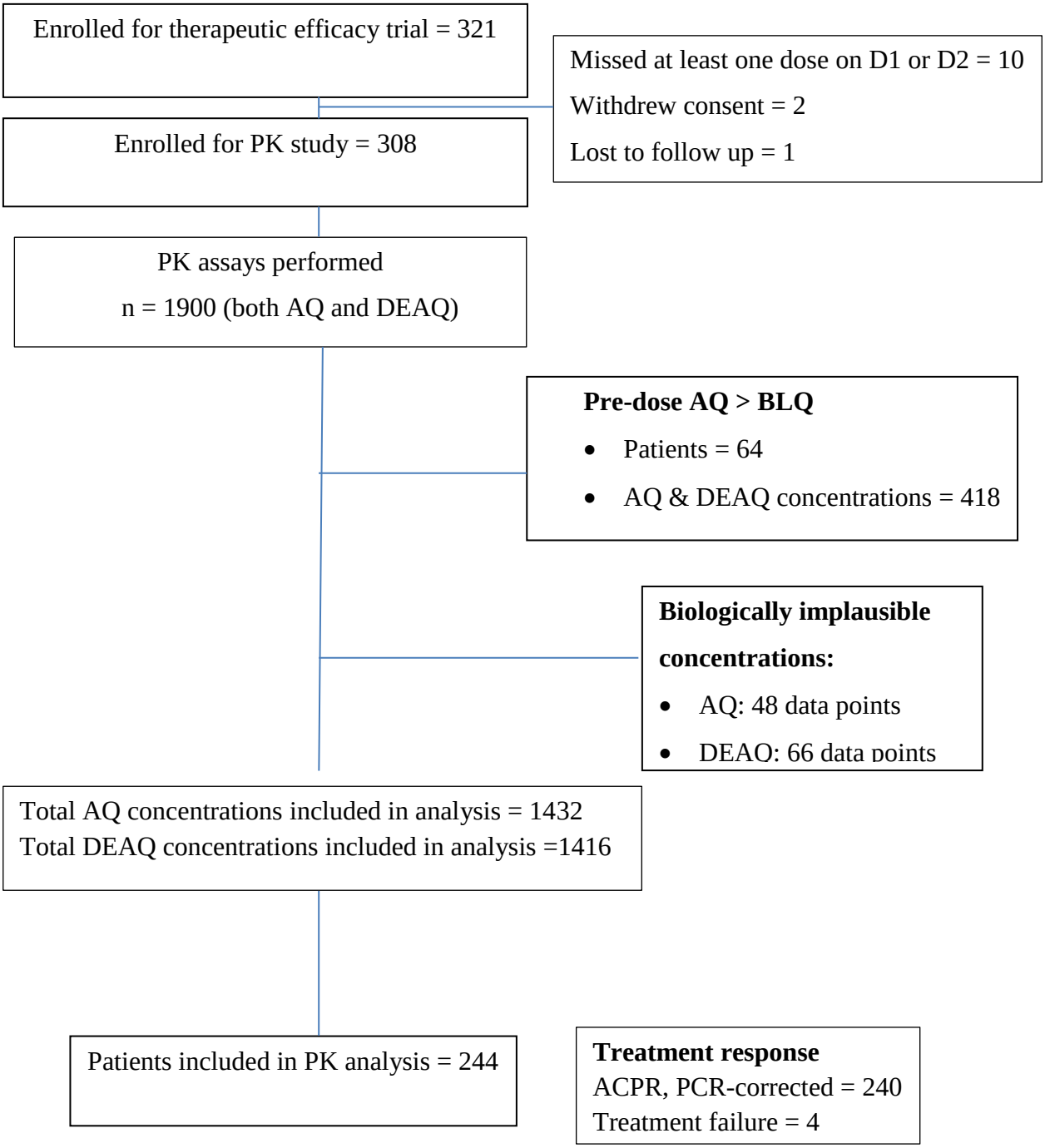


Table 7.1: Baseline characteristics

Description	Age category (years)			
	total	**< 1**	**1 - 4**	**≥5**
N (%)	308 (100)	13 (4.2)	176 (57.1)	119 (38.6)
Sex, F, n (%)	161 (52.3)	8 (61.5)	92 (52.3)	61 (51.3)
Underweight (WAZ<-2.00), n/N (%)	28/189 (14.8)[a]	0 (0.0)	26 (14.8)	
Axillary temperature (^{o}C), mean (sd)	38.0 (1.1)	38.3 (0.8)	38.1 (1.1)	37.7 (1.2)
Haemoglobin (g/dl), mean (sd)	10.0 (1.9)	8.5 (1.3)	9.5 (1.8)	11.0 (1.8)
Moderate- to- severe anaemia patients (Hb<8.0 g/dl), n (%)	47 (15.3)	5 (38.5)	37 (21.0)	5 (4.2)
Severe anaemia (Hb<6.0 g/dl), n (%)	4 (1.3)	0	4 (2.3)	0
Geometric mean parasite density, (95% CI)	27,594 (23,737, 32,079)	15,879 (5871, 42,945)	34,921 (28,916, 42,172)	20,692 (16,184, 26,455)
Proportion with parasite density ≥100,000, n (%)	47 (15.3)	3 (23.1)	32 (18.2)	12 (10.1)
Prevalence of gametocyte, n (%)	10 (3.3)	1 (7.7)	7 (4.0)	2 (1.7)
Total Dose (mg/ kg), median (IQR); range	33.8 (27.0 – 40.5) 23.0 - 45.0	25.3 (25.3 – 28.9) 23.8 - 40.5	33.8 (27.0 – 40.5) 23.1 - 45.0	33.8 (26.8 – 39.5) 23.0 - 45.0
D0 AQ concentration >BLQ, n (%)	64 (20.8)	0	45 (25.6)	19 (16.0)

[a] *The numerator includes two patients aged 5 years.*

N = Sample population, n=number of patients in category, F = Female, WAZ = weight-for-age Z-score, sd = standard deviation, Hb = haemoglobin concentration, CI = confidence interval, mg/kg = milligram per kilogram, IQR = interquartile range, D0= Day 0 pre-dose, AQ = amodiaquine, BLQ = below the limit of quantification

The mean haemoglobin ± sd concentration at presentation was 10.0 ± 1.9 g/dl overall. The mean haemoglobin concentrations increased with age category, and ranged from 8.5 ± 1.3 g/dl in young infants to 9.5 ± 1.8 g/dl in patients aged 1 – 4 years to 11.0 ± 1.8 g/dl in patients 5 years or older, p<0.001. Overall, 47/308 (15.3%) of the patients enrolled were classified as being moderate-to-severely anaemic. Most (37/47, 79%) of the moderate-to-severe anaemic patients were 1 - 4 years old. Four (4) patients IN-EF410 (haemoglobin, 5.6 g/dl), IN-EF442 (haemoglobin 5.6 g/dl), IN-EF468 (haemoglobin 5.8 g/dl) and TEM352 (haemoglobin 5.5 g/dl) in the 1 - 4 years age category were enrolled with haemoglobin <6.0 g/dl in violation of the protocol exclusion criteria. These patients were included in the pharmacokinetic analysis except TEM352 who was excluded on account of having pre-dose amodiaquine concentration greater than the lower limit of quantification (BLQ).

About 13.8% (26/189) of the children <5 years were underweight-for-age (Table 7.1). All the underweight-for-age patients were 1 - 4 years old; none of the infants was underweight.

The geometric mean parasite density overall at enrolment was 27,594 (95% CI 23737, 32079) asexual parasites per microliter of blood. The geometric mean parasite density was lowest in young infants, 15,879 (95% CI 5871, 42945) compared to 34,921 (95% CI 28916, 42172) in children 1 – 4 years and 20,692 (95% CI 16184, 26455) in older patients, p=0.001. The proportion of patients who had parasite density ≥ 100,000 asexual parasites per microlitre was 15.3% (47/308), with most of them, 32/47 (68.1%) being children aged 1 – 4 years. There were 3 patients with parasite densities above 200,000 asexual parasites per microlitre, the upper limit for inclusion into the study. These patients were however included in the pharmacokinetic analysis. There were only 10 patients with gametocytes at enrolment. Most of these gametocytes (7/10) were in patients aged 1 – 4 years old.

Overall, the median total dose in mg/kg administered was 33.8 (IQR 27.0 – 40.5) mg/kg. The total mg/kg dose administered to young infants was significantly lower compared to the dose administered to patients aged 1 - 4 years (p=0.004) or older (p=0.010). However, there was no difference in the total mg/kg dose administered to patients aged 1 - 4 years and those 5 years or older (p=0.698) (Table 7.1). These doses corresponded to a daily amodiaquine dose of 8.4 (IQR 8.4 – 9.6, range 7.9 - 13.5) mg/kg in young infants, compared to 11.3 (IQR 9.0 – 13.5, range 7.7 - 15.0) mg/kg in patients 1 – 4 years and 11.3 (IQR 8.9 – 13.0, range 7.7 - 15.0) mg/kg in patients 5 years or older. All dosages were within the manufacturer's recommended daily dose range of 7.5 to 15 mg/kg.

7.2 Amodiaquine pharmacokinetics

The concentrations of amodiaquine by protocol day of follow up are as presented in table 7.2. Amodiaquine concentrations were above the lower limit of quantification in 107/299 (35.8%) of the patients on day 7. AQ concentrations were above the limit of quantification in only 38/296 (12.8%) on day 14 and in 38/295 (12.9%) on day 28 (Appendix 7.1). The fact that AQ was still quantifiable on days 14 and 28 may be indicative of the slow conversion of AQ to DEAQ in some of the patients and a reflection of the sensitivity of the method employed for the assay. Although 244 of the 308 patients enrolled in the PK sub-study were retained for the PK analysis, there is variation in the number of samples at each time point mainly due to samples being dropped from the analysis due to the presence of biologically implausible concentrations or as a result of patients missing a study follow up schedule. The median whole blood concentrations of amodiaquine by day of follow up are presented in Table 7.2 and displayed in Figure 7.2a as a scatter plot of the individual capillary whole blood amodiaquine concentrations versus time, and Figure 7.2b as the median (IQR) capillary whole blood concentrations versus time profile.

7.2 Median amodiaquine concentrations by day of follow up

Follow up time (days)	No of samples	Median AQ conc (ng/ml)	IQR
0	215	0.3905	0.3905 - 0.3905
1	222	12.4	6.4 - 30.5
2	215	11.3	6.5 - 22.5
3	197	11.6	5.4 - 22.3
7	207	0.789	0.3905 - 2.3
14	193	0.3905	0.3905 - 0.3905
28	183	0.3905	0.3905 - 0.3905

AQ = Amodiaquine, Conc. = concentration, ng/ml = nanogram per millilitre, IQR = Interquartile range, 0.3905=BLQ/2=0.781/2, BLQ = below the limit of quantification

Figures 7.2a & b:

a. **A scatterplot of individual capillary whole blood amodiaquine (AQ) concentrations (ng/ml) versus time (in days)**
b. **The median (IQR) capillary whole blood amodiaquine concentrations (ng/ml) versus time (in days)**

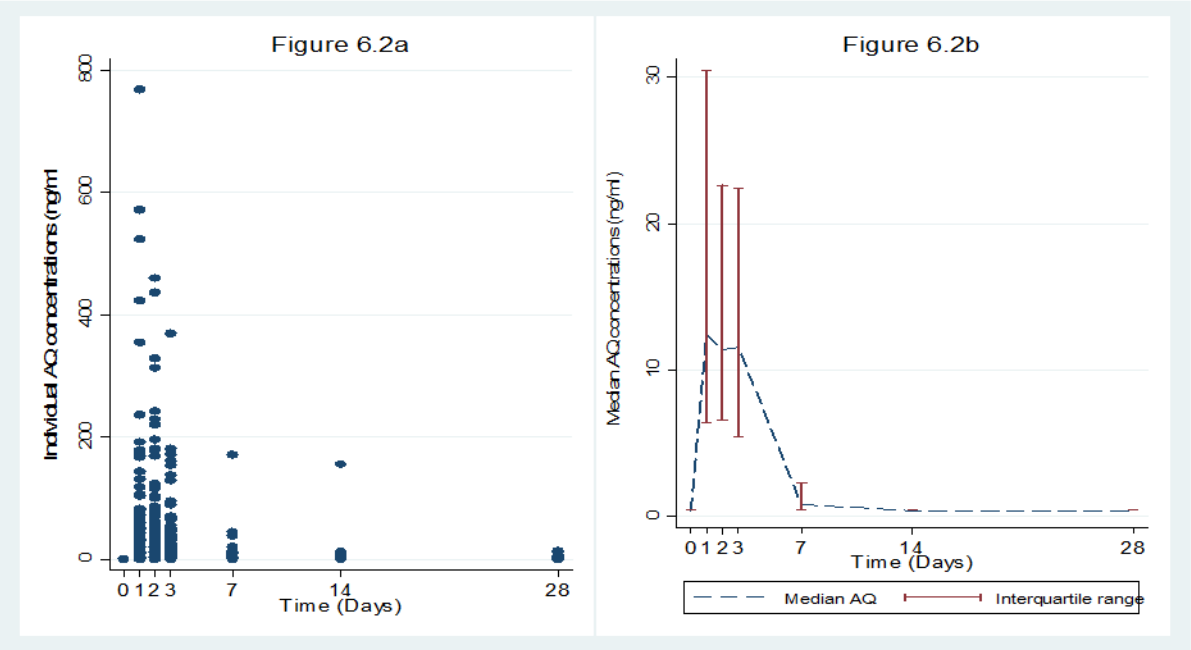


Table 7.3: Pharmacokinetic parameters of amodiaquine, by age category

Parameter	Age category (years)				P-value
	All ages	< 1	1 - 4	≥ 5	
$AUC_{0-\infty}$ (ng.h /ml) (IQR)	2511 (1162- 5426)	4988 (2807-19196)	3403 (1766- 6302)	1430 (997 - 2313)	<0.001
C_{max} (ng/ml) (IQR)	20.4 (11.4 - 52.4)	51.2 (37.2 - 196)	27.7 (14.8 – 70.5)	14.4 (8.1 - 22.5)	<0.001
T_{max} (days) (IQR)	2 (1 – 3)	1.5 (1 – 2)	2 (1 - 3)	2 (1 – 3)	0.528
CL/f ($L.kg^{-1}.h^{-1}$) (IQR)	12.6 (6.8–27.0)	6.0 (1.3 - 11.1)	10.0 (5.2- 22.1)	23.6 (12.6 - 36.1)	<0.001
Vd/f ($L. kg^{-1}$) (IQR)	1052 (437- 1956)	322 (90.9 - 980)	775 (367-1531)	1623 (817 - 2458)	<0.001
$t_{½}$ (days) (IQR)	2.0 (1.4 – 3.0)	2.3 (1.0 – 6.2)	2.0 (1.4–3.7)	1.7 (1.4 -2.3)	0.305
K_e (h^{-1}) (IQR)	0.015 (0.010- 0.021)	0.013 (0.005- 0.028)	0.014 (0.008-0.021)	0.017 (0.012-0.021)	0.305

AQ = amodiaquine, $AUC_{0-\infty}$ = Area under the concentration versus time curve from time zero to infinity, ng.h /ml= nanogram hour per milliliter, IQR = interquartile range, C_{max} = Observed maximum concentration, ng/ml = nanogram per milliliter, T_{max}= time to maximum concentration, CL/f = apparent clearance, $L.kg^{-1}.h^{-1}$= litres per kilogram per hour, Vd/f = apparent volume of distribution, L/kg= Litres/kilogram, $t_{½}$= elimination half-life, K_e=elimination rate constant, h^{-1}= per hour

As a result of the difficulties encountered with obtaining a good fit with the compartmental analysis in Phoenix® WinNonlin®, the pharmacokinetic parameters reported in this thesis are based on non-compartmental analysis (NCA) using Stata. The derived pharmacokinetic parameters of amodiaquine are summarised by age category in Table 7.3. Overall, the median peak amodiaquine concentration, (C_{max}) was 20.4 (IQR 11.4 - 52.4) ng/ml and this was reached (T_{max}) in a median time of 2 (IQR 1 - 3) days. The median capillary whole blood area under the concentration - time curve, $AUC_{0-\infty}$ for all ages was 2511 (IQR 1162 - 5426) ng.h/ml and the median apparent volume of distribution, Vd/f was estimated to be 1052 (IQR 437 - 1956) L/kg. The volume of blood effectively cleared of amodiaquine, CL/f was calculated to be 12.6 (IQR 6.8 - 27.0) $L.kg^{-1}.h^{-1}$ with the median terminal elimination rate constant (K_e) for all ages being 0.015 (IQR 0.010 - 0.021) h^{-1}. The terminal elimination half-life, $t_{½}$ for amodiaquine in this study was estimated to be 2.0 (IQR 1.4 - 3.0) days.

7.2.1 Univariate analysis of the effect of predefined covariates on amodiaquine pharmacokinetic parameters

A univariate linear regression analysis followed by Kruskal-Wallis test was conducted to establish if there were any differences in the amodiaquine pharmacokinetic parameters $AUC_{0-\infty}$, CL/f, V_d/f, $t_{½}$, C_{max}, T_{max} and K_e with respect to pre-defined covariates: age (age category), sex, total (mg/kg) dose administered, nutritional status (weight-for-age z-score), anaemia (Hb<8.0 versus Hb≥8.0), parasite density (parasite density ≥100,000 versus < 100,000) at enrolment, fever (temperature ≥ 37.5^{o}C) at enrolment and site of sample collection.

7.2.1.1 Effect of age on amodiaquine pharmacokinetic parameters

The observed median time to maximum concentration, T_{max} (p=0.528), the elimination half-life, $t_{½}$ (p=0.305) and the terminal elimination rate constant, (K_e (p=0.305) of amodiaquine were similar across the three age categories. However, statistically significant differences in pharmacokinetic parameters of amodiaquine with respect to age were observed for $AUC_{0-\infty}$, C_{max}, V_d/f and $CL_/$f (all with p<0.001) (Table 7.3).

In young infants, despite the lower total mg/kg dose of amodiaquine administered (25.3 mg/kg versus 33.8 mg/kg in both patients aged 1 - 4 years and those aged 5 years or older (p<0.001) (Table 7.1)), the median peak amodiaquine concentration (C_{max}) in young infants (51.2 (IQR 37.2 – 196) ng/ml) was nearly twice as high as the peak concentration in patients

aged 1 - 4 years (27.7 (IQR 14.8 - 70.5) ng/ml), and nearly four times that in patients 5 years or older (14.4 (IQR 8.1 – 22.5) ng/ml; p<0.001 (Table 7.3).

Their higher amodiaquine C_{max} , smaller apparent volume of distribution, Vd/f (p<0.001) and slower clearance (p<0.001), resulted in the highest amodiaquine exposure of 4988 (IQR 2807 – 19196) ng. h /ml in young infants compared to 3403 (IQR 1766 - 6302) ng. h /ml in patients aged 1 - 4 years and 1430 (IQR 997 – 2313) ng. h /ml in patients aged 5 years or older, p<0.001.

The apparent volume of distribution, V_d/f of amodiaquine increased with age. The Vd/f of 322 (IQR 90.9- 980) L/kg in young infants was less than half of the 775 (IQR 367 - 1531) L/kg in patients aged 1 - 4 years and nearly a fifth of the 1623 (IQR 817 - 2458) L/kg in patients 5 years or older, p<0.001, Table 7.3.

The apparent clearance of amodiaquine also increased with age (p< 0.001). The CL/f of 6.0 L/kg/h in young infants was slower than the 10.0 L/kg/h observed in patients aged 1 - 4 years and nearly a quarter of the 23.6 L/kg seen in patients aged 5 years or older, Table 7.3.

7.2.1.2 Effect of sex on Amodiaquine pharmacokinetic parameters

Significant differences in some amodiaquine pharmacokinetic parameters such as $t_{½,}$ T_{max} and K_e were observed between male and female patients. Although a median T_{max} of 2 days was observed in both males and females, their different distributions indicated a longer T_{max} in males (IQR 1 - 3 days) than in females (IQR 1 - 2 days), p=0.039. The median terminal elimination half-life ($t_{½)}$ of amodiaquine of 2.0 (IQR 1.6 - 3.6) days in males was longer than 1.7 (IQR 1.2 - 2.7) days in females, p=0.028. Similarly, the terminal elimination rate constant in males (0.014 (IQR 0.008 - 0.018) h^{-1}) was lower than in females (0.017 (IQR 0.011 - 0.023) h^{-1}), p=0.028 (Table 7.4).

Table 7.4: Pharmacokinetic parameters of amodiaquine and desethylamodiaquine, by sex

Parameter	AQ			DEAQ		
	Male	Female	P-value	Male	Female	P-value
$AUC_{0-\infty}$ (ng.h /ml)	2663	2367	0.436	142,435	125,866	0.364
(IQR)	(1162 - 6063)	(1138 - 4657)		(91,436 - 242,617)	(80,759 - 198,361)	
C_{max} (ng/ml)	19.7	20.4	0.767	592	562	0.348
(IQR)	(11.1- 60.2)	(11.4 - 52.4)		(377- 859)	(270 - 826)	
T_{max} (days)	2	2	0.039	3	3	0.968
(IQR)	(1 – 3)	(1 – 2)		(2 – 3)	(2 – 3)	
CL/f($L.kg^{-1}.h^{-1}$)	12.5	12.7	0.465	0.214	0.238	0.358
(IQR)	(6.2 - 27.0)	(7.8– 27.0)		(0.147 - 0.350)	(0.150 - 0.382)	
Vd/f ($L. kg^{-1}$)	987	1065	0.582	57.6	55.3	0.586
(IQR)	(480 - 2017)	(433 -1757)		(40.7 - 94.7)	(38.4 - 123)	
$t_{½}$ (d)	2.0	1.7	0.028	8.4	7.9	0.924
IQR	(1.6 – 3.6)	(1.2 - 2.7)		(6.3 - 11.4)	(6.4 - 11.5)	
K_e (h^{-1})	0.014	0.017	0.028	0.0035	0.0037	0.924
IQR	0.008 - 0.018	0.011 - 0.023		(0.0025 - 0.0046)	(0.0025 - 0.0045)	

AQ= amodiaquine, DEAQ= desethylamodiaquine, $AUC_{0-\infty}$ = Area under the concentration versus time curve from time zero to infinity, ng.h /ml= nanogram hour per millilitre, IQR = interquartile range, C_{max} = Observed maximum concentration, ng/ml = nanogram per milliliter, T_{max}= time to maximum concentration, CL/f= apparent clearance, $L.kg^{-1}.h^{-1}$= litres per kilogram per hour, Vd/f= apparent volume of distribution, L/kg= Litres/kilogram, $t_{½}$= elimination half-life, K_e=elimination rate constant, h^{-1}= per hour

7.2.1.3. Effect of fever on amodiaquine pharmacokinetic parameters

Being febrile at enrolment resulted in a slower apparent clearance, CL/f, a higher C_{max} and a smaller apparent volume of distribution, Vd/f, compared to patients who only had a history of fever in the previous 24 hours (termed "afebrile patients" here). The median peak amodiaquine concentrations, C_{max} of 24.4 (IQR 13.5 - 52.7) ng/ml in febrile patients was higher than the 15.1 (IQR 8.7 - 49.6) ng/ml observed in afebrile patients, p=0.011. There was a slower apparent clearance, CL/f of amodiaquine from the blood in febrile patients, 11.7 (IQR 6.6 - 23.6) $L.kg.h^{-1}$ compared to 20.2 (IQR 7.7 - 35.8) $L.kg.h^{-1}$ in afebrile patients, p=0.049. The apparent volume of distribution, Vd/f in patients enrolled with fever, 913 (IQR 397 - 1623) was smaller than the 1777 (IQR 593 - 3188) L/kg observed in afebrile patients, p=0.008. There was also a trend towards a higher amodiaquine exposure in patients enrolled with a fever, 2723 (IQR 1430 - 5450) ng.h/ml than in afebrile patients, 1923 (IQR 1010 - 5302) ng.h/ml, p=0.09. The other PK parameters were similar between febrile and afebrile patients (Table 7.5).

7.2.1.4 Effect of parasite density on amodiaquine pharmacokinetic parameters

There was a higher peak amodiaquine concentrations, C_{max} (28 (IQR 17.1 - 76.8) ng/ml) in patients with high parasite densities (≥100,000 (median 129,140 (IQR 112,560 - 151,140)) parasites per microlitre) at enrolment compared to a C_{max} of 18.8 (IQR 11.1 - 50.7) ng/ml in patients with lower parasite densities (median 33,960 (IQR 9,560 - 57,580) parasites per microlitre, p=0.049. Parasite density did not appear to affect the $AUC_{0-\infty}$, T_{max}, CL/f, Vd/f, $t_{½}$ or K_e of amodiaquine (Table 7.6).

7.2.1.5 Effect of total mg/kg dose administered on amodiaquine pharmacokinetic parameters

The total mg/kg dose administered was strongly correlated with the maximum observed amodiaquine concentrations, C_{max} (r_s=0.2460, p=0.014) and total amodiaquine exposure, $AUC_{0-\infty}$ (r_s=0.2591, p=0.010), Table 7.7.

Table 7.5: Pharmacokinetic parameters of amodiaquine and desethylamodiaquine, by presence of fever at enrolment

Parameter	AQ			DEAQ		
	Temp<37.5°C	Temp≥37.5°C	P-value	Temp<37.5°C	Temp≥37.5°C	P-value
$AUC_{0-\infty}$ (ng.h/ml)	1923	2723	0.090	121,525	142,944	0.255
(IQR)	(1010 - 5302)	(1430 - 5450)		(76,824 - 238,088)	(102,218 - 225,757)	
C_{max} (ng/ml)	15.1	24.4	0.011	506	590	0.056
(IQR)	(8.7 - 49.6)	(13.5 - 52.7)		(236 - 804)	(379 - 874)	
T_{max} (days)	2	2	0.566	3	3	0.155
(IQR)	(1 - 3)	(1 - 3)		(2 - 3)	(2 - 3)	
CL/f ($L.kg^{-1}.h^{-1}$)	20.2	11.7	0.049	0.270	0.222	0.366
(IQR)	(7.7 - 35.8)	(6.6 - 23.6)		(0.129 - 0.461)	(0.152 - 0.339)	
Vd/f ($L. kg^{-1}$)	1777	913	0.008	68.4	55.0	0.070
(IQR)	(593 - 3188)	(397 - 1623)		(43.9 - 138)	(38.0 - 90.1)	
$t_{½}$ (d)	1.9	2.0	0.625	8.7	7.9	0.163
IQR	(1.5 - 3.4)	(1.3 - 2.8)		(6.6 - 11.9)	(6.1 - 10.8)	
K_e (h^{-1})	0.0149	0.0146	0.625	0.0033	0.0037	0.163
IQR	(0.0086 - 0.0194)	(0.0102 - 0.0217)		(0.0024 - 0.0043)	(0.0027 - 0.0048)	

AQ= amodiaquine, DEAQ= desethylamodiaquine, Temp = temperature**,** $AUC_{0-\infty}$ = Area under the concentration versus time curve from time zero to infinity, ng.h /ml= nanogram hour per milliliter, IQR = interquartile range, C_{max} = Observed maximum concentration, ng/ml = nanogram per millilitre, T_{max}= time to maximum concentration, CL/f= apparent clearance, $L.kg^{-1}.h^{-1}$= litres per kilogram per hour, Vd/f= apparent volume of distribution, L/kg= Litres/kilogram, $t_{½}$= elimination half-life, K_e=elimination rate constant, h^{-1}= per hour

Table 7.6: Pharmacokinetic parameters of amodiaquine and desethylamodiaquine, by level of parasite density at enrolment

Parameter	AQ			DEAQ		
	<100000/µl	**≥100000/µl**	**P-value**	**<100000/µl**	**≥100000/µl**	**P-value**
$AUC_{0-\infty}$ (ng. h /ml)	2277	2820	0.203	140,228	128,775	0.492
(IQR)	(1131 - 5160)	(2116 - 5678)		(84,422 - 238,088)	(100,044 - 179,264)	
C_{max} (ng/ml)	18.8	28	0.049	563	588	0.848
(IQR)	(11.1 - 50.7)	(17.1 - 76.8)		(349 - 826)	(378 - 835)	
T_{max} (days)	2	2	0.387	3	3	0.139
(IQR)	(1 - 3)	(1 - 2)		(2 - 3)	(3 - 3)	
CL/f($L.kg^{-1}.h^{-1}$)	13.4	11.3	0.194	0.224	0.249	0.540
(IQR)	(6.8 - 28.4)	(6.8 - 13.8)		(0.149 - 0.356)	(0.161 - 0.358)	
Vd/f ($L. kg^{-1}$)	1032	981	0.383	63.9	51.4	0.112
(IQR)	(433 - 1890)	(456 - 1547)		(39.6 - 108)	(40.6 - 74.3)	
$t_{½}$ (d)	1.9	2.1	0.287	8.5	6.7	0.004
IQR	(1.3 - 3.0)	(1.6 - 3.1)		(6.5 - 11.7)	(5.3 - 8.0)	
K_e (h^{-1})	0.0151	0.0136	0.287	0.0034	0.0043	0.004
IQR	(0.0095 - 0.0216)	(0.0094 - 0.0178)		(0.0025 - 0.0044)	(0.0036 - 0.0055)	

AQ= amodiaquine, DEAQ= desethylamodiaquine, /µl = per microliter, $AUC_{0-\infty}$ = Area under the concentration versus time curve from time zero to infinity, ng.h /ml= nanogram hour per milliliter, IQR = interquartile range, C_{max} = Observed maximum concentration, ng/ml = nanogram per milliliter, T_{max}= time to maximum concentration, CL/f= apparent clearance, $L.kg^{-1}.h^{-1}$= litres per kilogram per hour, Vd/f= apparent volume of distribution, $L.kg^{-1}$= Litres per kilogram, $t_{½}$= elimination half-life, K_e=elimination rate constant, h^{-1}= per hour

7.7 Relationship between total mg/kg dose administered and pharmacokinetic parameters of amodiaquine and desethylamodiaquine

Pharmacokinetic parameter	Amodiaquine		Desethylamodiaquine	
	Spearman's rho, r_s	p-value	Spearman's rho, r_s	p-value
$AUC_{0-\infty}$	0.2591	0.010	0.1813	0.038
CL/f	-0.0820	0.420	0.0883	0.316
Vd/f	-0.0111	0.913	0.0739	0.402
$t_{½}$	0.1059	0.297	0.0043	0.962
C_{max}	0.2460	0.014	0.1934	0.027
T_{max}	-0.0667	0.512	-0.0043	0.962
K_e	-0.1059	0.297	-0.0043	0.962

7.2.1.6 Effect of site of sample collection on amodiaquine pharmacokinetic parameters

Differences in amodiaquine pharmacokinetic parameters between the two study sites were observed for $AUC_{0-\infty}$, CL/f, Vd/f, C_{max} and T_{max} (Table 7.8). The apparent clearance of amodiaquine (CL/f) from the blood was about 3-times faster in patients from Navrongo (14.0 L/kg/h) compared to 5.0 L/kg/h in patients from Kintampo, p=0.003. The median apparent volume of distribution, Vd/f was nearly double, 1069 L/kg in patients from Navrongo compared to 568 L/kg in patients from Kintampo, p=0.010. There was a more than four-fold higher C_{max} of 82 ng/ml in patients from Kintampo compared to 18.8 ng/ml in their counterparts from Navrongo, p<0.001. The total amodiaquine exposure ($AUC_{0-\infty}$) in patients from Kintampo was nearly 3 -fold higher, at 6061 ng.h /ml compared to 2244 ng.h /ml in patients from Navrongo, p=0.001. The time to peak amodiaquine concentration for patients in Navrongo (T_{max}= 2 days) was twice the time to peak amodiaquine concentration for patients in Kintampo (T_{max}= 1 day), p=0.0008.

7.2.1.7 Relationship between nutritional status (weight-for-age z-score) and pharmacokinetic parameters of amodiaquine

When children were categorized based on whether or not they were underweight-for-age (WAZ-score <2, Table 7.9a), most pharmacokinetic parameters were similar with a possible trend towards a higher apparent volume of distribution, Vd/f (p=0.11) and a faster apparent clearance, CL/f of amodiaquine (p=0.101). However, when the weight-for-age z-score was analysed as an ordinal variable (Table 7.9b), it was found to be inversely correlated with the apparent clearance, CL/f of amodiaquine (r_s= -0.2542, p= 0.011) and the apparent volume of distribution, Vd/f (r_s=-0.2868, p=0.004) of amodiaquine. The weight-for-age z-score was weakly positively correlated with the maximum observed amodiaquine concentration, C_{max} (r_s=0.2532, p=0.011) and the total amodiaquine exposure, $AUC_{0-\infty}$, (r_s=0.2229, p=0.027). The weight-for-age z-score did not appear to be associated with $t_{½}$, (p=0.405), T_{max} (p=0.451) or K_e, (p=0.405).

7.2.1.8 Effect of anaemia on amodiaquine pharmacokinetic parameters

Patients enrolled with moderate-to-severe anaemia (haemoglobin concentration<8.0 g/dl) had a slower apparent clearance, CL/f (8.6 (IQR 3.8 - 14.3) L/kg/h) and smaller apparent volume of distribution, Vd/f (572 (IQR 247 - 980) L/kg) compared to 13.1 (IQR 7.5 - 27.9) L/kg/h, p=0.044 and 1155 (IQR 454 - 2223) L/kg, p=0.011, respectively in patients who were not

anaemic. Patients who were anaemic on enrolment also had more than double the median maximum observed amodiaquine concentrations, 47.1 (IQR 19.8 - 169) ng/ml compared to 18.8 (IQR 11.0 -48.7) ng/ml, p=0.002 in patients who were not anaemic. Similarly, the total amodiaquine exposure was 2875 (IQR 2137 - 10,710) ng.h/ml in anaemic patients and was higher than the 2311 (IQR 1128 - 4868) ng.h/ml observed in patients who were enrolled without anaemia, p=0.042. Anaemia did not appear to affect $t_{½}$, T_{max} and K_e, Table 7.10.

7.8 Pharmacokinetic parameters of amodiaquine and desethylamodiaquine, by site of sample collection

Parameter	AQ			DEAQ		
	Navrongo	Kintampo	P-value	Navrongo	Kintampo	P-value
$AUC_{0-\infty}$ (ng.h/ml)	2244	6061	0.001	138,742	133,701	0.459
(IQR)	(1128 – 4415)	(2421 – 19,196)		(90,179 – 238,618)	(82,473 – 202,752)	
C_{max} (ng/ml)	18.8	82.0	0.0001	589	466	0.025
(IQR)	(10.8 – 39.8)	(50.7 - 329)		(366 - 893)	(282 - 613)	
T_{max} (days)	2	1	0.0008	3	2	0.014
(IQR)	(1 - 3)	(1 - 2)		(2 - 3)	(2 - 3)	
CL/f($L.kg^{-1}.h^{-1}$)	14.0	5.0	0.003	0.223	0.227	0.174
(IQR)	(8.6 - 28.4)	(2.1 – 13.8)		(0.139 – 0.354)	(0.165 – 0.500)	
Vd/f ($L. kg^{-1}$)	1069	568	0.010	55.2	81.5	0.029
(IQR)	(475 – 1890)	(129 – 1390)		(37.9 – 96.2)	(51.5 – 154)	
$t_{½}$ (d)	1.9	2.1	0.235	7.9	8.8	0.232
(IQR)	(1.3 – 2.8)	(1.6 – 4.2)		(5.8 – 11.5)	(7.5 – 10.5)	
K_e (h^{-1})	0.0148	0.0136	0.235	0.0037	0.0033	0.232
IQR	(0.0102 –0.0217)	(0.0070 – 0.0178)		(0.0025 – 0.0049)	(0.0028 – 0.0039)	

AQ= amodiaquine, DEAQ= desethylamodiaquine, $AUC_{0-\infty}$ = Area under the concentration versus time curve from time zero to infinity, ng.h /ml= nanogram hour per milliliter, IQR = interquartile range, C_{max} = Observed maximum concentration, ng/ml = nanogram per milliliter, T_{max}= time to maximum concentration, CL/f= apparent clearance, $L.kg^{-1}.h^{-1}$= litres per kilogram per hour, Vd/f= apparent volume of distribution, L/kg= Litres/kilogram, $t_{½}$= elimination half-life, K_e=elimination rate constant, h^{-1}= per hour

Table 7.9a: Pharmacokinetic parameters of amodiaquine and desethylamodiaquine, by nutritional status (weight-for-age z-score)

Parameter	AQ			DEAQ		
	WAZ<-2.0	**WAZ -score ≥-2.0**	**P-value**	**WAZ -score<-2.0**	**WAZ -score ≥-2.0**	**P-value**
$AUC_{0-\infty}$ (ng. h /ml)	2849	3840	0.218	112,740	143,453	0.854
(IQR)	(1903- 3647)	(1438 - 8118)		(98,422 - 268,212)	(97,932 - 238,088)	
C_{max} (ng/ml)	29.1	27.4	0.574	603	601	0.787
(IQR)	(16.1 - 39.6)	(13.5 - 79.9)		(468 - 794)	(367 - 937)	
T_{max} (days)	2	2	0.982	3	3	0.642
(IQR)	(1.0 - 2.5)	(1 - 3)		(3 - 3)	(2 - 3)	
CL/f($L.kg^{-1}.h^{-1}$)	13.4	8.2	0.101	0.248	0.214	0.596
(IQR)	(8.6 - 22.5)	(4.2- 19.7)		(0.154 - 0.331)	(0.141 - 0.344)	
Vd/f ($L. kg^{-1}$)	982	748	0.110	50.6	55.3	0.922
(IQR)	(654 - 1889)	(256 - 1577)		(35.9 - 81.5)	(39.3 - 96.3)	
$t_{½}$ (days)	2.1	2.0	0.567	7.1	8.4	0.320
IQR	(1.6 - 3.0)	(1.3 - 4.2)		(6.5 - 10.4)	(6.6 - 11.7)	
K_e (h^{-1})	0.0138	0.0144	0.567	0.0041	0.0035	0.320
IQR	(0.0095 - 0.0177)	(0.0070 - 0.0222)		(0.0028 - 0.0045)	(0.0025 - 0.0044)	

AQ= amodiaquine, DEAQ= desethylamodiaquine, WAZ = Weight-for-age z-score, $AUC_{0-\infty}$ = Area under the concentration versus time curve from time zero to infinity, ng.h /ml= nanogram hour per milliliter, IQR = interquartile range, C_{max} = Observed maximum concentration, ng/ml = nanogram per milliliter, T_{max}= time to maximum concentration, CL/f= apparent clearance, $L.kg^{-1}.h^{-1}$= litres per kilogram per hour, Vd/f= apparent volume of distribution, $L.kg^{-1}$= Litres per kilogram, $t_{½}$= elimination half-life, d=days; K_e=elimination rate constant, h^{-1}= per hour

Table 7.9b: Relationship between nutritional status (weight-for-age z-score<-2) at enrolment and pharmacokinetic parameters of amodiaquine and desethylamodiaquine

Pharmacokinetic parameter	Amodiaquine		Desethylamodiaquine	
	Spearman's rho, r_s	p-value	Spearman's rho, r_s	p-value
$AUC_{0-\infty}$ (ng.h /ml)	0.2229	0.027	0.0330	0.709
CL/f ($L.kg^{-1}.h^{-1}$)	-0.2542	0.011	-0.0526	0.551
Vd/f ($L.kg^{-1}$)	-0.2868	0.004	-0.0485	0.582
$t_{½}$ (days)	-0.0847	0.405	0.0379	0.667
C_{max} (ng/ml)	0.2532	0.011	0.0059	0.947
T_{max} (days)	0.0767	0.451	-0.0573	0.515
K_e (h^{-1})	0.0847	0.405	-0.2516	0.667

r_s = Spearman's rank correlation coefficient, rho; $AUC_{0-\infty}$ = Area under the concentration versus time curve from time zero to infinity, ng.h /ml= nanogram hour per milliliter, IQR = interquartile range, C_{max} = Observed maximum concentration, ng/ml = nanogram per milliliter, T_{max}= time to maximum concentration, CL/f= apparent clearance, $L.kg^{-1}.h^{-1}$= litres per kilogram per hour, Vd/f= apparent volume of distribution, $L.kg^{-1}$= Litres per kilogram, $t_{½}$= elimination half-life, K_e=elimination rate constant, h^{-1}= per hour

Table 7.10: Distribution of amodiaquine and desethylamodiaquine pharmacokinetic parameters, by moderate-to-severe anaemia

Parameter	AQ			DEAQ		
	Hb<8.0 g/dl	**Hb≥8.0 g/dl**	**P-value**	**Hb<8.0 g/dl**	**Hb≥8.0 g/dl**	**P-value**
$AUC_{0-\infty}$ (ng. h /ml) (IQR)	2875 (2137 - 10710)	2311 (1128 - 4868)	0.042	129,115 (107,640 - 272,550)	138,742 (83,196 - 219,924)	0.341
C_{max} (ng/ml) (IQR)	47.1 (19.8 - 169)	18.8 (11.0 - 48.7)	0.002	684 (518 - 927)	547 (342 - 817)	0.054
T_{max} (days) (IQR)	2 (1 - 3)	2 (1 - 3)	0.801	3 (2 - 3)	3 (2 - 3)	0.602
CL/f($L.kg^{-1}.h^{-1}$) (IQR)	8.6 (3.8 - 14.3)	13.1 (7.5 - 27.9)	0.044	0.213 (0.111 - 0.327)	0.232 (0.150 - 0.396)	0.292
Vd/f ($L. kg^{-1}$) (IQR)	572 (247 - 980)	1155 (454 - 2223)	0.011	51.9 (38.4 - 76.8)	62.7 (40.6 - 108)	0.125
$t_{½}$ (d) IQR	2.0 (1.6 - 3.0)	2.0 (1.3 - 3.2)	0.609	7.1 (5.7 - 10.6)	8.4 (6.4 - 11.6)	0.226
K_e (h^{-1}) IQR	0.015 (0.010 - 0.018)	0.015 (0.009 - 0.022)	0.609	0.004 (0.003 - 0.005)	0.003 (0.002 - 0.005)	0.226

AQ= amodiaquine, DEAQ= desethylamodiaquine, Hb = haemoglobin, $AUC_{0-\infty}$ = Area under the concentration versus time curve from time zero to infinity, ng.h /ml= nanogram hour per milliliter, IQR = interquartile range, C_{max} = Observed maximum concentration, ng/ml = nanogram per milliliter, T_{max}= time to maximum concentration, CL/f= apparent clearance, $L.kg^{-1}.h^{-1}$= liters per kilogram per hour, Vd/f= apparent volume of distribution, $L.kg^{-1}$= Liters per kilogram, $t_{½}$= elimination half-life, K_e=elimination rate constant, h^{-1}= per hour

7.2.2. Multivariate analysis of the effect of predefined covariates on amodiaquine pharmacokinetic parameters

Multivariate linear regression analysis to establish the independent relationships between amodiaquine pharmacokinetic parameters and the pre-defined covariates: age (age category), sex (males versus females), nutritional status (weight-for-age z-score), mg/kg total dose, fever at enrolment, parasite density (≥100,000 versus <100,000 asexual parasites per microlitre), anaemia (Hb<8.0 versus Hb≥8.0 g/dL) and site of sample collection were explored. Table 7.11a-e presents details of the final multivariate models for each of the amodiaquine pharmacokinetic parameters ($AUC0_{-\infty}$, C_{max}, CL/f, Vd/f, $t_{½}$). Although baseline parasite density was not significantly associated with any pharmacokinetic parameters it was retained in the model as it improved the model fit, and all covariates were included in each analysis to ensure completeness and consistency.

7.2.2.1 Effect of site

Despite adjusting for the other predefined covariates, site of sample collection persisted as a determinant of some amodiaquine pharmacokinetic parameters: $AUC_{0-\infty}$, C_{max}, $V_{d/}f$ and CL/f. Compared to the Kintampo site, there was a 72% lower peak amodiaquine concentration [GMR 0.28 (95% CI 0.14, 0.53), p<0.001] in patients from Navrongo. There was a 2.5-fold increase in apparent clearance, CL/f of amodiaquine in patients from Navrongo compared to those from Kintampo [GMR 2.57 (95% CI 1.27, 5.18), p=0.009]. The apparent volume of distribution, Vd/f in patients from Navrongo was double that in patients from Kintampo [GMR 2.11 (95% CI 1.04, 4.28), p<0.039].This explains the 65% lower total amodiaquine exposure, $AUC_{0-\infty}$ [GMR 0.35 (95% CI 0.18, 0.70), p= 0.004] in patients from Navrongo compared to patients from Kintampo. Elimination half-life, $t_{½}$ was similar between the study sites.

7.2.2.2 Effect of age category

After adjusting for the other covariates, age group was not associated significantly with any amodiaquine pharmacokinetic parameters, which were similar between children aged 1 to 4 years and those aged 5 years and older. However, there was a trend towards a higher Cmax (GMR 3.65 (95% CI 0.87 – 15.36), p=0.077) and $AUC0_{-\infty}$, (GMR 5.46 (95% CI 0.89 – 33.5), p=0.067), a lower apparent clearance, CL/f (GMR 0.18 (95% CI 0.03 – 1.11), p=0.064) and a longer elimination half-life, $t_{½}$ (GMR 3.60 (95% CI 0.99 – 13.03), p=0.051) in infants than in those aged >5 years.

7.2.2.3 Effect of nutritional status (weight-for-age z-score)

After adjusting for other predefined covariates, the weight-for-age z-score of children did not impact significantly on most of the amodiaquine pharmacokinetic parameters. However, there was a trend towards a 16% increase in the apparent volume of distribution, Vd/f [GMR 1.16 (95% CI 0.74, 1.01), p=0.058] in underweight-for-age children.

7.2.2.4 Effect of total bodyweight adjusted (mg/kg) dose administered

Independent relationships were established between total mg/kg dose administered and the peak amodiaquine concentration, C_{max} and area under the concentration-time curve, $AUC_{0-\infty,}$ after adjusting for other predefined covariates. For each mg/kg increase in total dose administered, there was a 4% increase in both C_{max} [GMR 1.04 (95% CI 1.02, 1.07), p = 0.003] and $AUC_{0-\infty}$, [GMR 1.04 (95% CI 1.00, 1.07), p= 0.035], Table 7.11a-b. As expected, there was no association between total mg/kg dose administered and the primary amodiaquine pharmacokinetic parameters, CL/f and Vd/f, Table 7.11c-d.

7.2.2.5 Effect of sex

After adjusting for other significant predefined covariates, the apparent clearance of amodiaquine (CL/f) of female patients was 79% faster than in their male counterparts [GMR 1.79 (95% CI 1.13, 2.85), p=0.014, Table 7.11c]. There was a trend towards lower amodiaquine exposure, $AUC_{0-\infty}$ [GMR 0.65 (95% CI 0.42, 1.02), p= 0.06] and a shorter amodiaquine elimination half-life, $t_{½}$ [GMR 0.71 (95% CI 0.50, 1.02), p=0.06] in female compared to male patients.

7.2.2.6 Effect of anaemia

After adjusting for other significant predefined covariates, being moderately anaemic (defined as Hb<8.0 g/dl) did not impact on most of the amodiaquine pharmacokinetic parameters. However**,** there was a trend towards an increase in the maximum observed amodiaquine concentration, C_{max} in patients enrolled with moderate-to-severe anaemia when compared with patients who were not anaemic [GMR 1.56 (95% CI 0.95, 2.57), p=0.08, Table 7.11b].

Table 7.11: Multivariate analysis of log-transformed amodiaquine pharmacokinetic parameters versus predefined covariates

Table 7.11a Area under the Amodiaquine concentration time curve ($AUC_{0-\infty}$)

Covariate	GMR	95% CI		P-value
Site				
Kintampo	-			
Navrongo	0.35	0.18	0.70	0.004
Age category				
<1 versus >=5	5.46	0.89	33.50	0.067
1-4 versus >=5	2.10	0.45	9.87	0.344
Presence of Fever (measured) at enrolment				
No fever	-			
Fever	1.13	0.67	1.93	0.639
Nutritional status				
Underweight (WAZ>-2.0)	-			
Normal (WAZ<-2.0)	1.11	0.95	1.28	0.185
Dose, mg/kg	1.04	1.00	1.07	0.035
Sex				
Male	-			
Female	0.65	0.42	1.02	0.060
Parasite density				
<100,000	-			
≥100,000	0.92	0.53	1.60	0.772
Anaemia				
Hb≥8.0	-			
Hb< 8.0	1.30	0.71	2.38	0.387

Table 7.11b Maximum Amodiaquine concentration (Cmax)				
	GMR	**95% CI**		**P-value**
Site				
Kintampo	-			
Navrongo	0.28	0.14	0.53	0.000
Age category				
<1 versus >=5	3.65	0.87	15.36	0.077
1-4 versus >=5	1.74	0.60	5.06	0.309
Presence of fever (measured) at enrolment				
No fever	-			
Fever	1.26	0.82	1.95	0.286
Nutritional status				
Underweight (WAZ>-2.0**)**	-			
Normal (WAZ<-2.0**)**	1.12	0.98	1.28	0.100
Dose, mg/kg	1.04	1.02	1.07	0.003
Sex				
Male	-			
Female	0.81	0.56	1.19	0.283
Parasite density				
<100,000	-			
≥100,000	0.90	0.56	1.46	0.673
Anaemia				
Hb≥8.0	-			
Hb< 8.0	1.56	0.95	2.57	0.080

Table 7.11c Apparent Clearance of Amodiaquine (CL/f)				
	GMR	**95% CI**		**P-value**
Site				
Kintampo	-			
Navrongo	2.57	1.27	5.18	0.009
Age category				
<1 versus >=5	0.18	0.03	1.11	0.064
1-4 versus >=5	0.48	0.10	2.28	0.344
Presence of fever (measured) at enrolment				
No fever	-			
Fever	0.79	0.46	1.37	0.397
Nutritional status				
Underweight (WAZ>-2.0)	-			
Normal (WAZ<-2.0)	0.89	0.77	1.04	0.153
Dose, mg/kg	1.00	0.97	1.03	0.973
Female versus Male				
Male	-			
Female	1.79	1.13	2.85	0.014
Parasite density				
<100000	-			
≥100000	1.28	0.72	2.26	0.396
Anaemia				
Hb≥8.0	-			
Hb< 8.0	0.79	0.43	1.46	0.451

Table 7.11d Apparent Volume of Distribution of Amodiaquine (Vd/f)

	GMR	95% CI		P-value
Site				
Kintampo	-			
Navrongo	2.11	1.04	4.28	0.039
Age category				
<1 versus >=5	1.20	0.25	5.79	0.817
1-4 versus >=5	1.43	0.40	5.06	0.579
Presence of fever (measured) at enrolment				
No fever	-			
Fever	1.39	0.41	1.26	0.247
Nutritional status				
Underweight (WAZ>-2.0**)**	-			
Normal (WAZ<>-2.0**)**	1.16	0.74	1.01	0.058
Dose, mg/kg	1.01	0.98	1.05	0.450
Sex				
Male	-			
Female	1.34	0.84	2.13	0.220
Parasite density				
<100000	-			
≥100000	1.09	0.62	1.91	0.756
Anaemia				
Hb≥8.0	-			
Hb< 8.0	1.29	0.42	1.44	0.415

Table 7.11e Elimination half-life of Amodiaquine ($t_{½}$)

AQ	GMR	95% CI		P-value
Site				
Kintampo	-			
Navrongo	0.87	0.49	1.52	0.620
Age category				
<1 versus >=5	3.60	0.99	13.03	0.051
1-4 versus >=5	1.69	0.60	4.76	0.317
Presence of Fever at enrolment				
No fever	-			
Fever	0.97	0.63	1.49	0.897
Nutritional status				
Underweight for age (WAZ>-2.0)				
Normal (WAZ<-2.0)	1.04	0.90	1.19	0.589
Dose, mg/kg	1.01	0.99	1.04	0.283
Sex	-			
Male	0.71	0.50	1.02	0.060
Female				
Parasite density	-			
<100000	0.82	0.53	1.26	0.359
≥100000				
Anaemia	-			
Hb≥8.0	0.86	0.53	1.40	0.546
Hb<8.0	-			

AUC_AQ = the area under the concentration versus time curve of amodiaquine, Cmax_AQ = the maximum observed amodiaquine concentration, CL/f_AQ = the clearance of amodiaquine, $t_{½}$_AQ = the elimination half-life of amodiaquine, CI = confidence interval, ZWEI = weight-for-age z-score, Hb = haemoglobin concentration, DEAQ = desethylamodiaquine, GMR=geometric mean ratio, CI= confidence interval

7.3 Desethylamodiaquine pharmacokinetic parameters

The median capillary whole blood concentrations of desethylamodiaquine by protocol day of follow up are summarised in table 7.12 and also displayed as a scatterplot of the individual capillary whole blood desethylamodiaquine concentrations (ng/ml) versus time (in days) (Figure 7.3a) and the median capillary whole blood desethylamodiaquine concentrations (ng/ml) versus time (in days) profile in figure 7.3b. Desethylamodiaquine concentrations were measurable in all patients throughout the follow up period of 28 days. This may suggest that longer follow up would be required to detect all failures, as recrudescent parasitaemia may be being suppressed by these >LLOQ concentrations.

7.12 Median desethylamodiaquine concentrations by day of follow up

Follow up time (days)	No of samples	Median DEAQ conc (ng/ml)	IQR
0	217	1.955	1.955 - 21.3
1	222	201	130 - 357
2	210	433	266 - 661
3	194	562	331 - 854
7	193	281	171 - 485
14	199	115	68 - 182
28	181	51.1	24.8 - 93.6

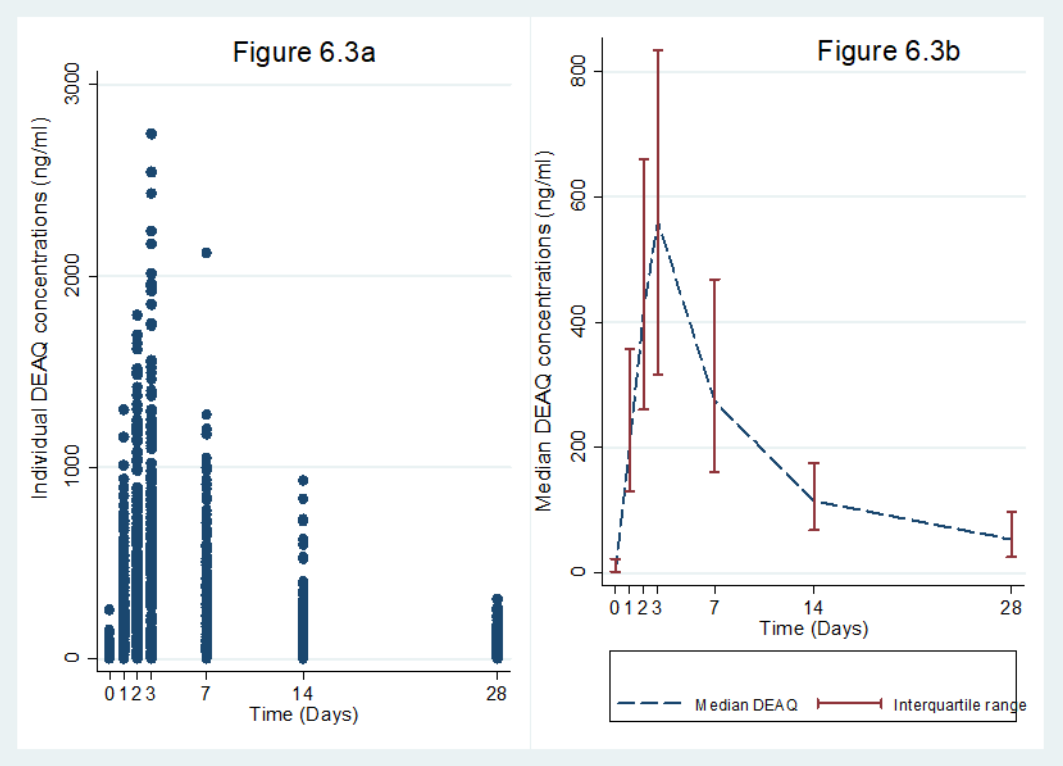
DEAQ = Desethylamodiaquine, Conc. = concentration, ng/ml = nanogram per milliliter, IQR = Interquartile range, 1.955=BLQ/2=3.91/2, BLQ = below the limit of quantification

Prior to the administration of the first study drug dose, desethylamodiaquine concentrations greater that the lower limit of quantification (LLOQ=3.91 ng/ml) were measured in 141 patients. Of these patients, 48 (34%) also had amodiaquine concentrations greater than the lower limit of quantification (LLOQ>0.781) and were thus considered to have taken a recent (within two weeks, an exclusion criterion) treatment with amodiaquine and were retrospectively excluded from the PK analysis. The remaining desethylamodiaquine concentrations greater than the LLOQ (93 patients) (but with amodiaquine concentrations <LLOQ) were included in the analysis. The median desethylamodiaquine concentration prior

to first study drug administration was 1.955 (IQR 1.955 - 21.3), which is only about 1% of the median desethylamodiaquine concentration on day 1.

Figures 7.3a & b:

a. **A scatterplot of the individual capillary whole blood desethylamodiaquine (DEAQ) concentrations (ng/ml) versus time (in days)**
b. **The median capillary whole blood desethylamodiaquine (DEAQ) concentrations (ng/ml) versus time (in days) profile**



Although 244 of the 308 patients enrolled in the PK sub-study were retained for the PK analysis, there were differences in the number of samples at each time point included in the pharmacokinetic analysis. This was due to the retrospective removal of certain data points from the analysis due to the presence of desethylamodiaquine concentrations that were considered biologically implausible or as a result of patients missing a follow up schedule.

As a result of the relatively poor model fit, inconsistent results and the difficulty in establishing a good fit with the compartmental analysis in Phoenix® WinNonlin®, the pharmacokinetic parameters reported in this thesis are based on non-compartmental analysis (NCA) using Stata. However, a summary of the Phoenix® WinNonlin® compartmentally modelled pharmacokinetic parameters estimated and model comparisons are included as Appendix 7.2a, b and c. One and two compartment disposition models with first-order

absorption were compared, with additive, multiplicative, additive plus multiplicative error models evaluated. A 1-compartment model provided the best fit for the desethylamodiaquine data. Based on the Bayesian Information Criteria (BIC), the one-compartmental multiplicative model appeared better fitted to the data. However, examination of the diagnostics suggested that a one- compartmental additive model was a better fit overall.

The non-compartmental analysis of the pharmacokinetic parameters of desethylamodiaquine by age category is presented in table 7.13. As expected, when compared with amodiaquine, pharmacokinetic parameters of desethylamodiaquine exposure, $AUC_{0-\infty}$ and the peak desethylamodiaquine were very much larger than the amodiaquine $AUC_{0-\infty}$ and C_{max}. The median time to reach the peak desethylamodiaquine, T_{max} concentration was 3 days compared to 2 days for amodiaquine. The apparent volume of distribution, Vd/f was larger and apparent clearance, CL/f slower for desethylamodiaquine than amodiaquine, explaining the median $t_{½}$ of desethylamodiaquine being four-fold longer than the $t_{½}$ of amodiaquine.

Table 7.13: Pharmacokinetic parameters of desethylamodiaquine, by age category

Parameter	Age category (years)				P-value
	All ages	**< 1**	**1 - 4**	**≥ 5**	
$AUC_{0-\infty}$ (ng.h /ml) (IQR)	138,746 (88,663 -230,122)	185,043 (111,693 - 294,793)	144,841 (96,907 - 241,663)	121,349 (78,868- 198,361)	0.106
C_{max} (ng/ml) (IQR)	569 (357 – 831)	480 (357 – 917)	604 (379 – 937)	528 (260 – 720)	0.057
T_{max} (days) (IQR)	3 (2 – 3)	2 (2 – 3)	3 (2 – 3)	3 (2 – 3)	0.351
CL/f($L.kg^{-1}.h^{-1}$) (IQR)	0.227 (0.149- 0.357)	0.168 (0.086 - 0.227)	0.208 (0.146 - 0.348)	0.262 (0.151 - 0.403)	0.056
Vd/f ($L. kg^{-1}$) (IQR)	56.8 (39.6.- 96.3)	50.2 (34.7 - 77.8)	54.8 (38.4– 94.9)	66.2 (43.5 - 120)	0.187
$t_{½}$ (d) (IQR)	8.1 (6.4 – 11.4)	9.9 (8.5 – 11.7)	7.8 (6.5 - 11.4)	8.3 (5.7 – 11.2)	0.590
K_e (h^{-1}) (IQR)	0.0036 (0.0025-0.0045)	0.0029 (0.0025 - 0.0034)	0.0037 (0.0025 - 0.0045)	0.0035 (0.0026 - 0.0050)	0.590

DEAQ= desethylamodiaquine, $AUC_{0-\infty}$ = Area under the concentration versus time curve from time zero to infinity, ng.h /ml= nanogram hour per milliliter, IQR = interquartile range, C_{max} = Observed maximum concentration, ng/ml = nanogram per milliliter, T_{max}= time to maximum concentration, CL/f= apparent clearance, $L.kg^{-1}.h^{-1}$= liters per kilogram per hour, Vd/f= apparent volume of distribution, L/kg= Liters/kilogram, $t_{½}$= elimination half-life, K_e=elimination rate constant, h^{-1}= per hour

7.3.1 Univariate analysis of the effect of predefined covariate on desethylamodiaquine pharmacokinetic parameters

A univariate analysis was conducted to establish if there were any significant differences in the pharmacokinetic parameters ($AUC_{0-\infty}$, CL/f, V_d/f, $t_{½}$, C_{max}, T_{max} and K_e) with respect to the pre-defined covariates: age (age category), sex, total (mg/kg) dose administered, nutritional status (weight-for-age z-score), anaemia (Hb<8.0 versus Hb>8.0 g/dL), baseline parasite density (parasite density ≥100,000 versus < 100,000 asexual parasites per microlitre), fever (temperature ≥ 37.5^{o}C) at enrolment and site of sample collection.

7.3.1.1 Observed age differences in desethylamodiaquine pharmacokinetic parameters

The overall median peak desethylamodiaquine concentration, C_{max} was 569 (IQR 357 – 831) ng/ml and was reached at a median maximum time (T_{max}) of 3 (IQR 2 – 3) days. There was a trend towards a lower C_{max} in infants (480 ng/ml) versus 603 ng/ml in patients aged 1 - 4 years and 528 ng/ml in patients 5 years and older, p=0.070 (Table 7.13).

The overall median area under the capillary whole blood desethylamodiaquine concentrations versus time curve ($AUC_{0-\infty)}$ was 138,746 (IQR 88,663 – 230,122) ng.h /ml. The $AUC_{0-\infty}$ by age category possibly suggested a lower $AUC_{0-\infty}$ in older patients although the difference was not significant (p=0.106). There was a trend towards faster clearance of desethylamodiaquine (CL/f) with age (p=0.056), increasing from 0.168 L/kg/h in infants to 0.262 L/kg/h in patients aged 5 years and older. The overall median terminal elimination half-life ($t_{½}$) was 8.1 (IQR 6.4 – 11.4) days and was similar across age categories, p=0.57. The median apparent volume of distribution, V_d/f (p=0.19), time to peak desethylamodiaquine concentration, T_{max} (p=0.35) and the terminal elimination rate constant, K_e (p=0.59) were all similar across the three age categories (Table 7.13).

7.3.1.2 Association of total mg/kg dose administered with desethylamodiaquine pharmacokinetic parameters

Figures 7.4a & b present a scatter plot between total dose in mg/kg administered, and the maximum observed desethylamodiaquine concentrations, C_{max} and the area under desethylamodiaquine concentration-time curve, $AUC_{0-\infty}$, respectively. There was a correlation between total mg/kg dose administered and both the peak desethylamodiaquine concentration, C_{max} (r_s = 0.1934, p= 0.027) and the area under the desethylamodiaquine concentrations versus time curve, $AUC_{0-\infty}$ (r_s = 0.1813, p= 0.038), but none of the other pharmacokinetic parameters (Table 7.7).

Figure 7.4a: Scatter plot of desethylamodiaquine (DEAQ) area under the concentration-time curve (AUC) (ng.h/ml) versus total dose administered (mg/kg)

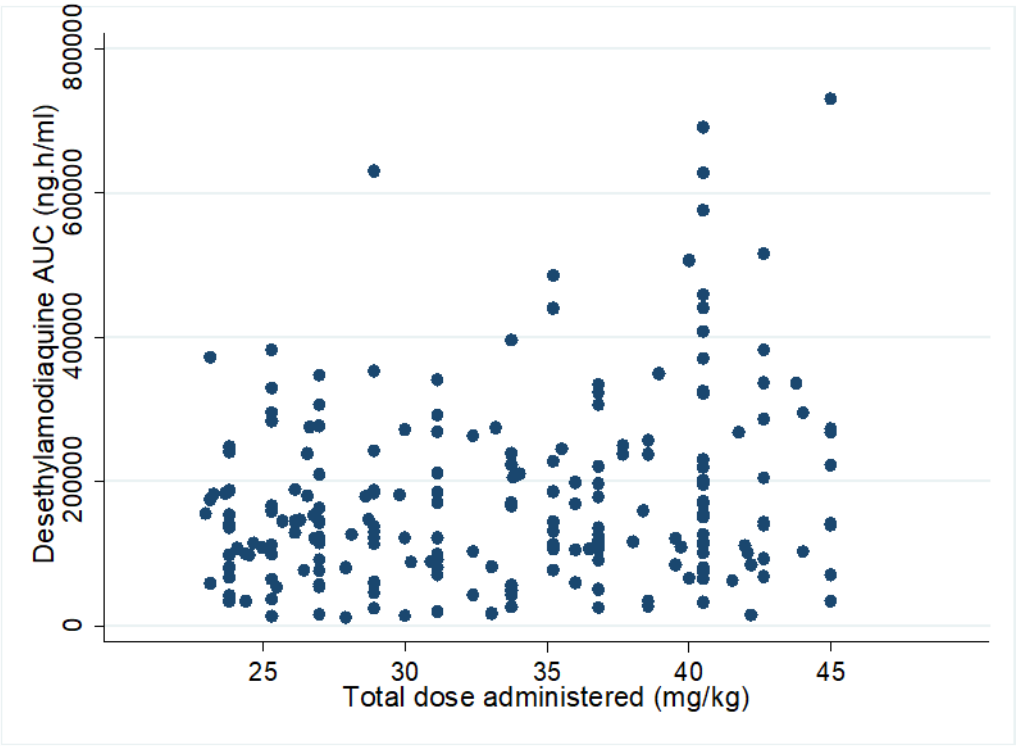
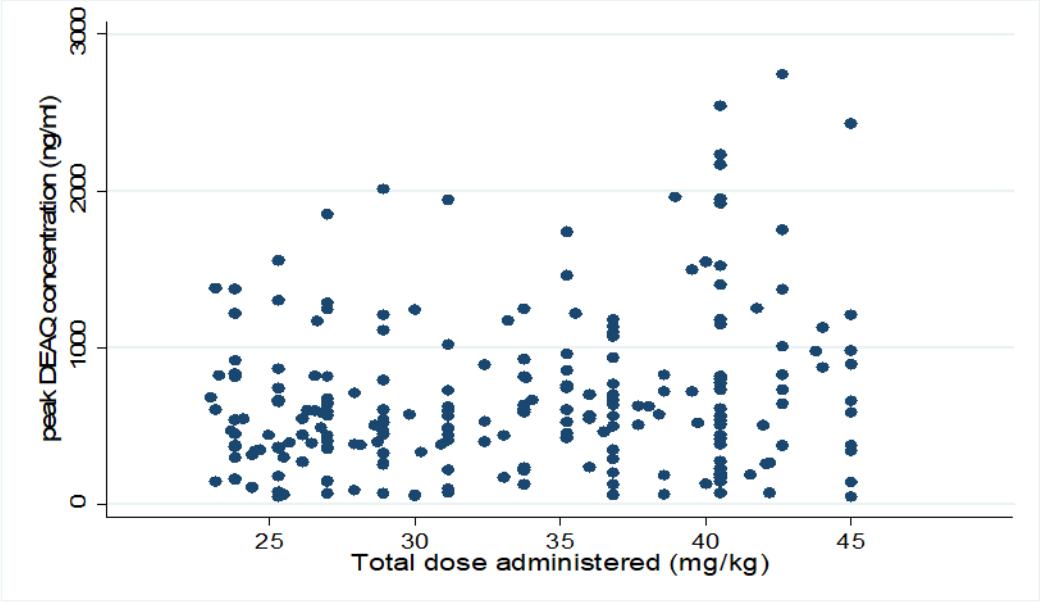


Figure 7.4b: Scatter plot of peak desethylamodiaquine (DEAQ) concentrations (ng/ml) versus total dose administered (mg/kg)



7.3.1.3 Differences in desethylamodiaquine pharmacokinetic parameters by sex, nutritional status and by presence or absence of fever at enrolment

All desethylamodiaquine pharmacokinetic parameters $AUC_{0-\infty}$ (p=0.36), V_d/f (0.59), $t_{½}$ (p=0.92), C_{max} (p=0.35), CL/f (p=0.36), K_e (0.92) and T_{max} (p=0.97) were all similar between male and female patients (Table 7.4).

The PK parameters of desethylamodiaquine were similar between children who were underweight-for-age (z-score<-2.0) and those who were not underweight (z-score >-2.0) (Table 7.9a). There was no correlation between desethylamodiaquine PK parameters and weight-for-age z-score (Table 7.9b)

There was a trend towards higher C_{max} (p=0.056) and a lower apparent volume of distribution, Vd/f (p=0.070) among patients enrolled with a fever compared to those with only a history of fever within the previous 24 hours (Table 7.5).

7.3.1.4 Desethylamodiaquine pharmacokinetic differences in terms of parasite density at enrolment

Statistically significant differences in desethylamodiaquine terminal elimination half-life, $t_{½}$ and the elimination rate constant, K_e were observed for patients who presented with parasite densities of at least 100,000 [median 129140 (IQR 112560 - 151140) per microlitre of blood when compared to those with parasite densities <100,000 [median 33960 (IQR 9560 - 57580)] per microlitre of blood. Patients with high parasitaemia had shorter $t_{½}$ of 6.7 (IQR 5.3 - 8.0) days compared to 8.5 (IQR 6.5 - 11.7) days in patients with parasite densities <100,000, p=0.004 (Table 7.6). Parasite density (as a continuous variable) was also positively correlated with $t_{½}$, r_s = 0.2902, p= 0.001 and inversely correlated with the terminal elimination rate constant, K_e, r_s=-0.2902, p=0.001.

7.3.1.5 Observed desethylamodiaquine pharmacokinetic differences in terms of site of sample collection

By site of sample collection, pharmacokinetic parameters of desethylamodiaquine were found to be similar except for the peak desethylamodiaquine concentration, C_{max}, the time to the maximum observed concentration, T_{max} and the apparent volume of distribution, Vd/f. Patients from Navrongo had a higher median C_{max} of 589 (IQR 366 - 893) ng/ml, p=0.025 and took a longer median time (T_{max}) of 3 (IQR 2 -3) days, p=0.014 to reach the maximum

observed desethylamodiaquine concentrations compared to patients from Kintampo (who were not routinely assessed on day 1 or 3). The apparent volume of distribution, Vd/f in patients from Navrongo, 55.2 (IQR 37.9 - 96.2) L/kg was smaller than in patients from Kintampo, 81.3 (IQR 51.5 - 154) L/kg, p=0.029, Table 7.8.

7.3.1.6 Effect of anaemia on desethylamodiaquine pharmacokinetic parameters

The PK parameters for desethylamodiaquine were similar between patients who were enrolled with moderate-to-severe anaemia (Hb<8.0 g/dl) and those who were not anaemic (Hb≥8.0g/dl) except for the maximum observed desethylamodiaquine concentrations. There was a trend towards a higher median C_{max} of 684 (IQR 518 - 927) ng/ml in patients who were anaemic compared to 547 (IQR 342 - 817) ng/ml in patients who were not anaemic, p=0.054 Table 7.10).

7.3.2. Multivariate analysis of the effects of predefined covariates on desethylamodiaquine pharmacokinetic parameters

Table 7.14a-e presents details of the final multivariable model of the effects of predefined covariates (age category, sex, nutritional status (weight-for-age z-score), mg/kg total dose, fever at enrolment, parasite density (≥100,000 versus <100,000 asexual parasites per microlitre), anaemia (Hb<8.0 versus Hb≥8.0 g/dL) and site of sample collection) on each of the desethylamodiaquine pharmacokinetic parameters ($AUC0_{-\infty}$, Cmax, CL/f, Vd/f, $t_{½}$). Although age category, presence of fever at enrolment, nutritional status and anaemia were not significantly associated with any pharmacokinetic parameters they were retained in the model as they improved the model fit, and all covariates were included in each analysis to ensure completeness and consistency.

7.3.2.1 Effect of site of sample collection

After adjusting for other covariates, there was a more than two-fold increase in the peak desethylamodiaquine concentration, C_{max} in patients from Navrongo [GMR 2.36 (95% CI 1.49, 3.75), p<0.001, Table 7.14b] and a near doubling in the area under desethylamodiaquine concentration-time curve, $AUC_{0-\infty}$ [GMR 1.98 (95% CI 1.23, 3.19), p=0.005, Table 7.14a]when compared to patients from Kintampo. The apparent clearance, CL/f of desethylamodiaquine was 49% lower in patients from Navrongo compared to those from Kintampo [GMR 0.51 (95% CI 0.32, 0.81), p= 0.005, Table 7.14c]. The apparent volume of distribution of desethylamodiaquine, Vd/f was about 54% smaller in patients from

Navrongo compared to their counterparts from Kintampo [GMR 0.46 (95% CI 0.26, 0.81), p=0.008, Table 7.14d]. Elimination half-life was similar at both sites (p=0.525, Table 7.14e).

7.3.2.2 Effect of age category, fever at enrolment, nutritional status, and anaemia

In contrast to the trend towards a higher C_{max} and $AUC_{0-\infty}$, and lower apparent clearance and a longer elimination half-life in infants than in those aged ≥5 years found for amodiaquine after adjusting for other covariates, no independent effect of age category was observed for desethylamodiaquine. Neither was nutritional status, anaemia, nor having a fever at enrolment independently associated with any desethylamodiaquine pharmacokinetic parameters (Table 7.14a-e).

7.3.2.3 Effect of total mg/kg dose administered

After adjusting for other significant covariates, a 1 mg/kg increase in the total mg/kg dose administered was associated with a 3% increase in the desethylamodiaquine area under the concentration-time curve, $AUC_{0-\infty}$ [GMR 1.03 (95% CI 1.00 - 1.05), p=0.019, Figure 7.4a, Table 7.14a]. A similar effect of mg/kg dose on C_{max} was not observed (Figure 7.4b, Table 7.14b).

7.3.2.4. Effect of Sex

Sex did not appear to impact on most of the desethylamodiaquine pharmacokinetic parameters besides its independent effect on the apparent clearance and the maximum observed concentration. After adjusting for other significant covariates, the apparent clearance, CL/f of desethylamodiaquine was about 35% faster [GMR 1.35 (95% CI 1.01, 1.81), p= 0.044, Table 7.14c] and there was a 25% lower maximum observed desethylamodiaquine concentration, C_{max} in female malaria patients compared to males [GMR 0.75 (95% CI 0.57, 0.99), p=0.045, Table 7.14b].

7.3.2.5 Effect of level of parasite density at enrolment

The terminal elimination half-life of desethylamodiaquine, $t_{½}$ appeared to be affected by the density of parasitaemia a patient presented with. After adjusting for other significant predefined covariates, high parasitaemia was associated with a 40% reduction in the terminal elimination half-life ($t_{½}$) of desethylamodiaquine when compared with patients with parasite densities less than 100,000 [GMR 1.40 (95% CI 1.08, 1.80), p=0.011, Table 7.14e] but not with total exposure [Table 7.14a].

Table 7.14: Multivariate analysis of the log-transformed DEAQ pk parameters versus covariates

Table 7.14a: Area under the desethylamodiaquine concentration time curve ($AUC_{0-\infty}$)

	GMR	95% CI		P-value
Site				
Kintampo	-			
Navrongo	1.98	1.23	3.19	0.005
Age category				
<1 versus >=5	1.93	0.76	4.92	0.168
1-4 versus >=5	1.10	0.56	2.17	0.775
Presence of Fever (measured) at enrolment				
No fever	-			
Fever	0.88	0.64	1.22	0.455
Nutritional status				
Underweight for age (WAZ>-2.0	-			
Normal (WAZ<-2.0)	1.05	0.95	1.17	0.310
Dose, mg/kg	1.03	1.00	1.05	0.019
Sex				
Male	-			
Female	0.80	0.60	1.07	0.136
Parasite density				
<100,000	-			
≥100,000	1.06	0.73	1.54	0.744
Anaemia				
Hb≥8.0	-			
Hb<8.0	1.21	0.83	1.76	0.314
Day 0 DEAQ conc>1/10 (Day1 median DEAQ conc)				
Day 0 DEAQ<20	-			
Day 0 DEAQ>20	1.21	0.89	1.64	0.225

Table 7.14b: Maximum desethylamodiaquine concentration (Cmax)

	GMR	95% CI		P-value
Site				
Kintampo	-			
Navrongo	2.36	1.49	3.75	0.000
Age category (years)				
<1 vs. ≥5	1.45	0.59	3.57	0.420
1 - 4 vs. ≥5	1.18	0.61	2.26	0.625
Presence of Fever (measured) at enrolment				
No fever	-			
Fever	0.93	0.68	1.27	0.658
Nutritional status				
Underweight for age (WAZ>-2.0	-			
Normal (WAZ<-2.0)	1.06	0.96	1.17	0.269
Dose, mg/kg	1.01	0.99	1.04	0.165
Sex				
Male	-			
Female	0.75	0.57	0.99	0.045
Parasite density				
<100,000	-			
≥100,000	0.83	0.58	1.18	0.287
Anaemia				
Hb≥8.0	-			
Hb<8.0	1.29	0.90	1.85	0.160
Day 0 DEAQ conc>1/10 (Day1 median DEAQ conc)				
Day 0 DEAQ<20	-			
Day 0 DEAQ>20	1.21	0.90	1.62	0.204

Table 7.14c: Apparent Clearance of Desethylamodiaquine (Cl/f)

	GMR	95% CI		P-value
Site				
Kintampo	-			
Navrongo	0.51	0.32	0.81	0.005
Age category (years)				
<1 vs. ≥5	0.48	0.19	1.20	0.115
1 - 4 vs. ≥5	0.86	0.44	1.67	0.647
Presence of Fever (measured) at enrolment				
No fever	-			
Fever	1.04	0.75	1.44	0.801
Nutritional status				
Underweight for age (WAZ>-2.0	-			
Normal (WAZ<-2.0)	0.95	0.85	1.05	0.289
Dose, mg/kg	1.01	0.99	1.03	0.510
Sex				
Male	-			
Female	1.35	1.01	1.81	0.044
Parasite density				
<100,000	-			
≥100,000	1.00	0.69	1.44	0.990
Anaemia				
Hb≥8.0	-			
Hb<8.0	0.93	0.64	1.35	0.702
Day 0 DEAQ conc>1/10 (Day1 median DEAQ conc)				
Day 0 DEAQ<20	-			
Day 0 DEAQ>20	0.86	0.64	1.17	0.342

Table 7.14d: Apparent Volume of distribution of Desethylamodiaquine (Vd/f)

	GMR	95% CI		P-value
Site				
Kintampo	-			
Navrongo	0.46	0.26	0.81	0.008
Age category (years)				
<1 versus >=5	0.55	0.18	1.65	0.282
1-4 versus. >=5	0.82	0.37	1.81	0.622
Presence of Fever (measured) at enrolment				
No fever	-			
Fever	1.09	0.74	1.60	0.664
Nutritional status				
Underweight for age (WAZ>-2.0	-			
Normal (WAZ<-2.0)	0.97	0.86	1.10	0.637
Dose, mg/kg	1.01	0.99	1.04	0.342
Sex				
Male	-			
Female	1.21	0.86	1.71	0.278
Parasite density				
<100,000	-			
≥100,000	1.16	0.75	1.79	0.491
Anaemia	-			
Hb≥8.0	0.81	0.52	1.26	0.338
Hb<8.0				
Day 0 DEAQ conc>1/10 (Day1 median DEAQ conc)	-			
Day 0 DEAQ<20	0.91	0.63	1.30	0.588
Day 0 DEAQ>20	-			

Table 7.14e: Elimination half-life of desethylamodiaquine ($t_{½}$)

	GMR	95% CI		P-value
Site				
Kintampo	-			
Navrongo	0.90	0.64	1.26	0.525
Age category (years)				
<1 versus >=5	1.07	0.55	2.07	0.851
1-4 versus >=5	0.88	0.54	1.42	0.591
Presence of Fever (measured) at enrolment				
No fever	-			
Fever	0.93	0.74	1.17	0.546
Nutritional status				
Underweight for age (WAZ>-2.0	-			
Normal (WAZ<-2.0)	1.01	0.94	1.09	0.810
Dose, ng/kg	1.01	0.99	1.02	0.241
Sex				
Male	-			
Female	1.00	0.81	1.22	0.971
Parasite density				
<100,000	-			
≥100,000	1.40	1.08	1.80	0.011
Anaemia				
Hb≥8.0				
Hb<8.0	0.98	0.75	1.28	0.885
Day 0 DEAQ conc>1/10 (Day1 median DEAQ conc)				
Day 0 DEAQ<20	-			
Day 0 DEAQ>20	1.09	0.88	1.35	0.418

AUC_DEAQ = the area under the concentration versus time curve of desethylamodiaquine, Cmax_DEAQ = the maximum observed desethylamodiaquine concentration, CL/f_DEAQ = the apparent clearance of desethylamodiaquine, Vd/f = apparent volume of distribution, $t_{½}$_DEAQ = the elimination half-life of desethylamodiaquine, CI = confidence interval, ZWEI = weight-for-age z-score, Hb = haemoglobin concentration, DEAQ = desethylamodiaquine, Patients with Day 0 DEAQ concentrations greater than a tenth of the day 1 median concentration, GMR=geometric mean ratio, CI= confidence interval

7.4 Pharmacokinetic-Pharmacodynamic relationship of amodiaquine and desethylamodiaquine

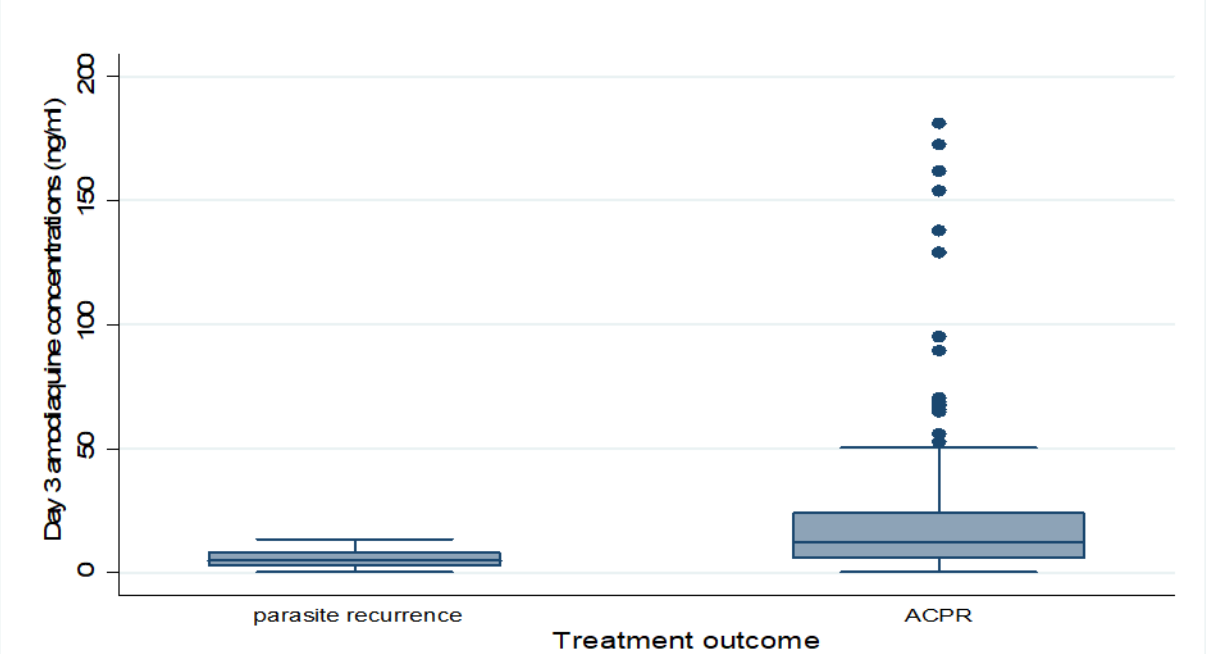
7.4.1 Effect of desethylamodiaquine concentrations at day 7 and the area under the concentration-time curve (AUC) on treatment outcome

Treatment efficacy was very high in this study with a PCR-adjusted adequate clinical and parasitological response rate of over 97% in both study sites and by both intention to treat and per protocol analysis. The study was therefore underpowered to show any pharmacokinetic differences between patients who achieved an adequate clinical and parasitological response and those who failed treatment. Given the few treatment failures, treatment response categories were simplified to either an adequate clinical and parasitological response (ACPR) or treatment failure (i.e. parasite recurrence, including both recrudescences and re-infections and any indeterminate PCR results). The day 7 desethylamodiaquine concentrations, p=0.767, as well as total desethylamodiaquine exposure, $AUC_{0-\infty}$, p=0.363, were similar in patients who achieved adequate clinical and parasitological response and those who failed treatment.

7.4.2 Effect of median Day 2 and day 3 amodiaquine concentrations on parasite clearance time and parasite recurrence

The median day 2 amodiaquine concentration was not significantly associated with parasite clearance time [HR = 0.942 (95% CI 0.8708, 1.019), p = 0.135] and there was no difference in the day 2 median amodiaquine concentrations between patients who achieved adequate clinical and parasitological cure, 11.5 (IQR 6.7 - 23.9) and those who had parasite recurrence, 8.8 (IQR 5.5 - 11.5), p=0.113. However, the median day 3 amodiaquine concentrations were associated with a significant 13% reduction in the risk of parasite recurrence, HR = 0.8737 (95% CI 0.7793, 0.9795), p = 0.021. The median concentration of amodiaquine on day 3 in patients with parasite recurrence was 4.8 (IQR 3.0 - 8.2) ng/ml and was significantly lower than the median day 3 amodiaquine concentrations of 12.5 (IQR 5.9 - 24.2) ng/ml in patients who achieved adequate clinical and parasitological cure rate, p=0.002 (Figure 7.5).

Figure 7.5 Box and whisker plots of Day 3 amodiaquine concentrations by treatment response.



ACPR: Adequate Clinical and Parasitological Response

7.4.3 Effect of median Day 3, 7, 14 and 28 desethylamodiaquine concentrations on response to treatment in terms of duration of gametocyte carriage

The median day 3 (r_s=0.0854, p=0.624), day 7 (r_s =0.0461, p=0.242), day 14 (r_s =0.0176, p=0.519) and day 28 (p=0.817) desethylamodiaquine concentrations were not associated with the duration of gametocyte carriage.

Neither did the median day 3 [HR=0.9995 (95% CI 0.9982, 1.0008), p=0.45], day 7 [HE= 0.9992 (95% CI 0.9968, 1.0016), p=0.52], day 14 [HR=0.9993 (95% CI 0.9951, 1.0035), p=0.75] and day 28 [HR= 1.0021 (95% CI 0.9924, 1.0120), p= 0.67) appear to affect treatment outcome in terms of the risk of parasite recurrence.

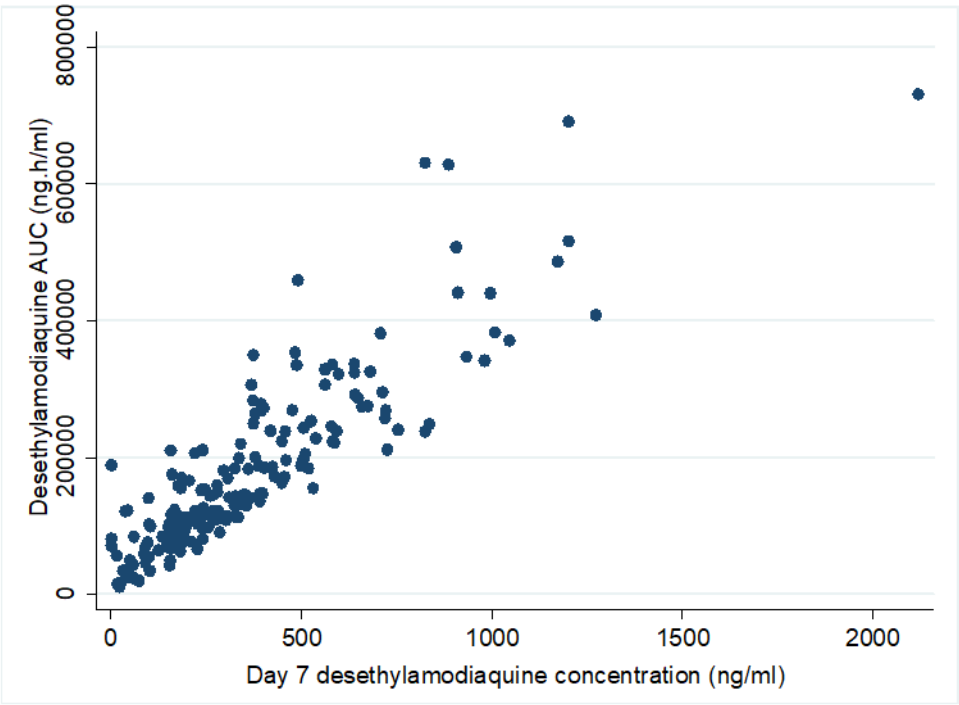
7.4.4 Effect of desethylamodiaquine AUC_{0-3} on parasite recurrence and on parasite clearance time

The desethylamodiaquine AUC_{0-3} was not associated with treatment response in terms of parasite recurrence; HR =1.000 (95% CI 1.000, 1.000), p=0.903 or parasite clearance time; HR=1.00 (95% CI 1.000, 1.000), p= 0.284**.**

7.4.5 Correlation of $AUC_{0-\infty}$ and day 7 desethylamodiaquine concentrations

The relationship between area under the capillary whole blood desethylamodiaquine concentration-time curve, $AUC_{0-\infty}$ and the day 7 desethylamodiaquine concentrations is displayed in Figure 7.6. There was a strong linear correlation between $AUC_{0-\infty}$ and the day 7 DEAQ concentrations; r_s=0.8810, p<0.001.

Fig 7.6: A scatter plot showing the correlation between the area under the capillary whole blood concentration-time curve of desethylamodiaquine ($AUC_{0-\infty}$) and the day 7 desethylamodiaquine (DEAQ) concentrations



7.5 Discussion of Results

A non-compartmental analysis (NCA) was used to characterize the kinetics of amodiaquine (AQ) and its active metabolite desethylamodiaquine (DEAQ) in malaria patients from Ghana. Concentrations of amodiaquine and desethylamodiaquine were measured using a newly developed and validated liquid chromatographic tandem mass spectrometric (LC-MS/MS) method that employed 20 µl of whole blood and liquid-liquid extraction for the assay.

7.5.1 Discussion of amodiaquine pharmacokinetic parameters

Earlier reports suggest that the formation of desethylamodiaquine from amodiaquine is rapid with very little of the parent drug, amodiaquine being detectable in plasma beyond the third day post-dose (Churchill et al., 1985; Winstanley et al., 1987a; Hombhanje et al., 2005). Unlike these earlier observations however, and similar to the work of Ntale et al., 2009, amodiaquine concentrations were still quantifiable in 107 (44%) patients on day 7 of follow up and in 13% of patients on days 14 and 28 post treatment. We observed a trend towards a higher maximum observed concentration and total exposure, a slower clearance and a longer elimination half-life of amodiaquine in the 13 infants studied. Infants comprised only 5% of the patients included in the PK analysis and accounted for only 1.9 (2/107) of the patients with quantifiable amodiaquine concentrations on day 28.

Desethylamodiaquine, the main active metabolite of amodiaquine was quantifiable throughout the period of follow up. The attrition in the number of patients with quantifiable amodiaquine from days 7 to 28 reflects the conversion of amodiaquine to its active metabolite desethylamodiaquine. Patients with CYP2C8*2 have been noted to have impaired amodiaquine metabolism (Röwer et al., 2005) with poor or slow metabolizers experiencing a longer amodiaquine elimination half-life (Parikh S, et al., 2007). The prevalence of CYP2C8 in Ghana ranges from 16.8 - 17.9% (Röwer et al., 2005, Adjei et al., 2008, Kudzi et al., 2009). Therefore the role played by CYP2C8*2 resulting in amodiaquine still being quantifiable between day 7 and 28 of follow up cannot be discounted.

In endemic areas like Ghana, repeated use of antimalarials is common (Omole & Onademuren, 2010). This is reflected by the relatively large number of the patients enrolled in this study, 64/308 (20.8%) who were found to have quantifiable amodiaquine and desethylamodiaquine concentrations, despite the exclusion criterion of antimalarial treatment in the previous two weeks. However, repeated and indiscriminate use leads to increased drug

pressure and exposure of malaria parasites to low concentrations of the drug that may lead to the development of resistance (White & Pongtavornpinyo, 2003; White, 2004), particularly in the wake of the appearance of resistance to ACTs in multiple locations in Southeast Asia (Ashley et al., 2014; Tun et al., 2015) and recent case reports in Africa (Lu et al., 2017; Sutherland et al., 2017). In addition, repeated exposure to amodiaquine over long periods of time may mimic amodiaquine prophylaxis and could lead to immunoglobulin G immune-mediated liver and neutrophil toxicity(Clarke et al., 1991; Orrell, Taylor & Olliaro, 2001; Taylor & White, 2004b) Infants may be at higher risk given their trend towards higher and prolonged amodiaquine exposure, yet they have been systematically under-studied to date. This could also have consequences for the uptake of the recently recommended Seasonal Malaria Chemoprophylaxis (SMC) (WHO, 2012c, 2013b), which has been adopted in Ghana for the northern Sahel regions of the country that includes Navrongo (MOH, 2014b).

Maximum observed amodiaquine concentration and time to peak concentration

There was wide variability in the maximum observed amodiaquine concentrations. The pharmacokinetic properties of antimalarial drugs differ between healthy individuals and malaria patients (White, 2013). The relatively high maximum observed capillary whole blood amodiaquine concentration, C_{max} of 51.2 (IQR 37.2 - 196) ng/ml in infants aged less than 1 year in this study is similar to (60 ± 10 ng/ml) reported after a single oral dose of 600 mg amodiaquine to 7 healthy males aged 22 – 44 years (Winstanley et al., 1987a). The maximum observed amodiaquine concentration, C_{max} of 27.7 (IQR 14.8 - 70.5) ng/ml in patients aged 1 - 4 years and 14. 4 (IQR 8.1 - 22.5) ng/ml in patients 5 years or older is lower than those reported after a single oral dose of 600 mg to adult healthy males (Winstanley et al., 1987a) but similar to the 21± 11 ng/ml observed in Zambian and Nigerian malaria patients aged 7 – 55 years dosed with 25 mg/kg amodiaquine in two different dosing schedules (Winstanley et al., 1990). The median capillary whole blood C_{max} obtained in this study is however lower than those reported from venous whole blood samples spotted on filter paper obtained from malaria patients aged 1.5 – 8 years treated with amodiaquine plus artesunate over 3 days based on age group in Uganda (Ntale et al., 2009).

The time to the maximum observed amodiaquine concentration, T_{max} in this study is similar to those in the Ugandan study (Ntale et al., 2009) and those obtained from malaria patients by Winstanley and colleagues (Winstanley et al., 1990) but higher than those obtained from healthy individuals after a single dose of 600 mg amodiaquine (Winstanley et al., 1987a).

Sample times in this study were not optimal for accurately defining absorption as concentrations were only measured on day 0,1,2,3, 7, 14 and 28.

Amodiaquine area under the concentration-time curve

There was a significant decrease in the amodiaquine exposure, $AUC_{0-\infty}$ from young infants (<1 year) to older patients in this study. The median area under the whole blood amodiaquine concentration versus time curve ($AUC_{0-\infty}$) for all patients was estimated to be 2511 (IQR 1162 – 5426) ng.h/ml and varied from 4988 (IQR 2807 – 19,196) ng.h/ml in infants to 3403 (IQR 1766 - 6302) ng.h /ml h in children 1 – 4 years to 1430 (IQR 997 – 2313) ng.h/ml in patients 5 years or older. The amodiaquine $AUC_{0-\infty}$ in patients aged <1 year was 3.5 times the $AUC_{0-\infty}$ of amodiaquine in patients 5 years or older. Similarly, the $AUC_{0-\infty}$ of amodiaquine in patients 1 - 4 years was 2.4 times the $AUC_{0-\infty}$ of amodiaquine in patients 5 years or older. Age-dependent changes in body composition or maturational effects on drug absorption and metabolism may have accounted for these differences (Bartelink et al., 2006). Literature on whole blood amodiaquine $AUC_{0-\infty}$ is rather scarce. However, the whole blood $AUC_{0-\infty}$ in patients aged 5 years or more (1430 ng.h/ml) in this study is substantially higher than the few published $AUC_{0-\infty}$ values reported, all of which were measured in plasma. In children 5 – 13 years treated with amodiaquine at 10 mg/kg on first two days and 5 mg/kg on the third day plus artesunate, 4 mg/kg twice a day for 3 days (Mwesigwa et al., 2010), the estimated $AUC_{0-\infty}$ was 39 ng.h/ml. The plasma $AUC_{0-\infty}$ of amodiaquine in healthy volunteers after a single oral dose of 600 mg of amodiaquine was estimated to be 154 ng. h/ml (Winstanley et al., 1987a) and 108.5 ng.h/ml in healthy adult volunteers aged 18 – 45 years administered with a single dose of 4 mg/kg artesunate and 10 mg/kg amodiaquine (Orrell et al., 2008b) which were lower than recorded in this study. The 2.5-fold increase in whole blood concentrations compared to plasma concentrations [GMR 2.5 (95% CI 2.4, 2.8)] described in Chapter 6 does not fully explain the much higher amodiaquine $AUC_{0-\infty}$ observed in our Ghanaian patients. Another contributory factor could be the three-fold higher $AUC_{0-\infty}$ Kintampo compared to Navrongo, which reflect the fewer sampling times in Navrongo and potential pharmacogenetic differences between these sites. The relatively high prevalence of CYP2C8*2 in Ghana (Röwer et al., 2005, Adjei et al., 2008, Kudzi et al., 2009) could further explain the much higher amodiaquine $AUC_{0-\infty}$ observed in our Ghanaian patients.

Amodiaquine terminal elimination half-life

The median capillary whole blood terminal elimination half-life, $t_{½}$ of amodiaquine was estimated to be 2. 0 (IQR 1.4 – 3.0) days for all ages. The estimated capillary whole blood $t_{½}$ of amodiaquine in this study is far longer than the plasma $t_{½}$ of 5.2 hours reported after a single oral dose of 600 mg in healthy individuals (Winstanley et al., 1987a), 3.7 hours in Nigerian and Zambian malaria patients aged 7 - 55 years treated with 25 mg/kg (10:5:5:5 or 10:10:5 mg/kg) (Winstanley et al., 1990), 3.3 hours in malaria children aged 5 - 13 years in Uganda treated with artesunate-amodiaquine (200 mg amodiaquine+50mg of artesunate) (Mwesigwa et al., 2010) and 7.9 hours in Kenyan adult malaria patients treated with fixed dose artesunate-amodiaquine (Jullien et al., 2010). Pharmacogenetic differences and the inclusion of infants in our study could have contributed to the substantially longer terminal elimination half-life that we observed.

The activities of amodiaquine and its metabolite are said to be synergistic with trace amodiaquine concentrations enhancing the effect of desethylamodiaquine during the first 3 days of treatment and beyond (Winstanley et al., 1990; Laurent et al., 1993; Minzi et al., 2003). During a standard amodiaquine treatment regimen, putative residual concentrations of amodiaquine are likely to complement and improve the efficacy of desethylamodiaquine for up to 7 days during which time a radical cure is capable of being exacted (Mariga et al., 2004).

Apparent volume of distribution of amodiaquine

Overall, the apparent volume of distribution, Vd/f of amodiaquine was estimated in this study to be 1052 (IQR 437 – 1956) L/Kg. The Vd/f of amodiaquine was 322 (IQR 90.9 - 980) in infants aged < 1 year and increased with age to 1623 (IQR 817 – 2458) L/kg in patients 5 years or older. The Vd/f of amodiaquine in patients 5 years or older in this study were higher than the plasma Vd/f of 39,200 L (664 L/kg, normalised for a median weight of 59 kg as published) estimated for Kenyan adult malaria patients aged 18 – 60 years treated with artesunate-amodiaquine either as a fixed dose or co-packaged over three days (Jullien et al., 2010). This difference may in part reflect the younger population we studied in Ghana, with only 13/244 (4.9%) aged over 18 years.

Apparent clearance of amodiaquine

The overall oral apparent clearance, CL/f of amodiaquine in this study was estimated to be 12.6 (IQR 6.8 – 27.0) L/kg/h with the slowest clearance being in infants, 6.0 (IQR 1.3 – 11.1) L/kg/h. In patients aged 1 – 4 years, CL/f was 10.0 (IQR 5.2 – 22.1) L/kg/h which is similar to the CL/f of (14 l/kg/h) previously estimated for malaria patients aged 3 months – 12 years (Hietala et al., 2007). In patients aged 5 years or older in this study, the CL/f of amodiaquine was estimated to be 23.6 (IQR 12.6 – 36.1) l/kg/h, which is much slower than the plasma CL/f of 3410 l/h (60.9 L/kg/h normalised for median weight of 59 kg as published) estimated for adult malaria patients in Kenya (Jullien et al., 2010). These findings may reflect age and pharmacogenetic differences between patients in Ghana and Kenya.

Determinants of amodiaquine pharmacokinetic parameters

Fever (temperature $\geq 37.5^{o}C$) may be considered as a surrogate marker of disease severity in malaria (Jullien et al., 2010) and has been associated with variability in certain mefloquine pharmacokinetic parameters in malaria patients (Simpson et al., 2000; Ashley et al., 2006). A study of the effect of fever on the disposition of quinine and quinidine in rats showed that the presence of fever resulted in an increase in clearance and in the volume of distribution but a reduction in the half-life of quinine; for quinidine, there was an increase in the clearance but with a much reduced half-life (Mansor et al., 1991). In our current study, although being enrolled with a fever was associated with a higher peak concentration, a reduced apparent clearance and a larger total exposure to amodiaquine, $AUC_{0-\infty}$ in a univariate analysis, these associations were not found after adjusting for pre-defined covariates. Being enrolled with a fever rather than only a history of fever was not independently associated with any of the pharmacokinetic parameters of amodiaquine in this study.

Patients with high parasite burdens have been observed to be an important source of de novo resistance to antimalarial drugs (White et al., 2009). In the univariate analysis, high parasite density (parasite density ≥100,000 parasites per microlitre of blood) was associated with a much higher amodiaquine C_{max} in this study. The effect of high parasitaemia was however not apparent after adjusting for predefined covariates.

In Sub-Saharan Africa, malaria and malnutrition often co-exist and are important public health conditions (Nubé M & Sonneveld BG., 2005). Malaria and malnutrition have been observed to increase the plasma concentration of quinine and reduce its total plasma

clearance and volume of distribution (Pussard et al., 1999). The weight-for-age z-score in the current study was inversely correlated with the systemic clearance and the volume of distribution of amodiaquine but positively correlated with amodiaquine exposure, $AUC_{0-\infty}$ and the maximum observed amodiaquine concentrations. After adjustment for predefined factors, the weight-for-age z-score for underweight-for-age children was marginally associated with a 16% reduction in the apparent volume of distribution of amodiaquine.

In the presence of other predefined covariates, a unit increase in the total mg/kg dose administered was associated with a 4% increase in the maximum observed amodiaquine concentration and a 4% increase in the total amodiaquine exposure. Dose linear drug exposure can facilitate dose optimization.

Gender-related differences including body size and muscle mass may lead to differences in pharmacokinetic parameters (Beierle, Meibohm & Derendorf, 1999; Meibohm, Beierle & Derendorf, 2002). Being a female in this study was associated with 79% faster total hepatic clearance of amodiaquine, a 35% less amodiaquine exposure and a shorter terminal elimination half-life compared to their male counterparts.

Differences in blood concentration data, higher values for C_{max} and T_{max} have been previously observed between malaria children in Papua New Guinea (Hombhanje et al., 2005) when compared to those from Zambia and Nigeria (Winstanley et al., 1990). The site of sample collection appeared to be an important factor affecting C_{max}, $AUC_{0-\infty,}$ V_d/f and CL/f of amodiaquine despite adjustments for other predefined covariates. Ethnic or regional differences have been noted to contribute to differences in response (Yasuda, Zhang & Huang, 2008). While the exact reason for the disparities observed in this study by site of sample collection is not known, these differences may be consistent with higher bioavailability in Kintampo than Navrongo. This could also be explained by potential other differences in pharmacogenetics or methods for sample collection and storage. It has been suggested that ethnicity, drug formulation and dosage, and pattern of concomitant medication use (Anderson, 2005; Parikh et al., 2007; Yasuda, Zhang & Huang, 2008; Kerb et al., 2009) are among other factors that may be account site effects.

7.5.2 Discussion of desethylamodiaquine pharmacokinetics

The median capillary whole blood concentrations of desethylamodiaquine, the main active metabolite of amodiaquine, as expected were much higher than those of the parent compound with wide inter-individual variability (White et al., 1987; Winstanley et al., 1990; White, 2013).

Maximum observed amodiaquine concentration and time to peak concentration

Overall, the median peak capillary whole blood concentration of desethylamodiaquine was 569 (IQR 357 – 831) ng/ml at a median time to peak concentration of 3 (IQR 2 – 3) days. Young infants had the lowest median C_{max} of 480 (IQR 357 – 917) ng/ml at a T_{max} of 2.0 (IQR 2.0 – 3.0) days. The highest C_{max} of 604 (IQR 379 – 937) ng/ml was observed in children aged 1 – 4 years. Although measured in capillary whole blood, the C_{max} obtained in this study is similar to the plasma C_{max} value of 537± 244 ng/ml in malaria patients aged 1 – 14 years in Ghana (Adjei, Kristensen, et al., 2008) and the 561 ± 70 ng/ml estimated after a single oral dose of 600 mg amodiaquine in healthy adult volunteers (Winstanley et al., 1987a). The C_{max} values in this study are slightly higher than the whole blood C_{max} values of 368.8 ng/ml (95% CI 306.6, 431.0) in malaria patients aged 1 – 10 in Papua New guinea (Hombhanje et al., 2005); 273.6 (range 192.3 – 368.7) in malaria patients aged 3 months – 12 years (Hietala et al., 2007) and 301± 166 ng/ml in healthy adult volunteers after a single oral dose of 10 mg/kg AQ (camoquin) plus 4mg/kg AS (Arsumax)(Orrell et al., 2008a). The C_{max} values obtained in this study are however lower than the 1016.4± 568.3 ng/ml reported after a single oral dose of 10 mg/kg of amodiaquine in healthy adult volunteers (Pussard et al., 1987) and much lower than 1766.8 ng/ml in another study conducted in Northern Uganda in patients aged 1.5 – 8 years (Ntale et al., 2009). Such differences are consistent with differences in study populations, dosage regimens, matrix effects and sampling times. The time to peak desethylamodiaquine concentrations, T_{max} recorded in the literature is rather variable, ranging from 3 days (Hombhanje et al., 2005), 2 days (Adjei, Kristensen, et al., 2008) to about 2.2 hours after a single oral dose in healthy adult volunteers (Winstanley et al., 1987a).

Desethylamodiaquine area under the concentration-time curve

The overall median capillary whole blood $AUC_{0-\infty}$ of desethylamodiaquine was estimated to be 138,746 (IQR 88,663 – 230,122) ng.h/ml and decreased with age, with a high of 185,043

(IQR 111,693 – 294,793) ng.h/ml in infants, 144,841 (IQR 96,907 – 241,663) ng.h /ml in children aged 1 – 4 years to a low of 121,349 (IQR 78,868 – 198,361) ng.h/ml in patients 5 years or older although these age related differences were not statistically significant (p=0.106). The $AUC_{0-\infty}$ of desethylamodiaquine in infants < 1 year was 1.5 times the $AUC_{0-\infty}$ of patients aged 5 years or older. The $AUC_{0-\infty}$ of desethylamodiaquine in patients 1 - 4 years was 1.2 times the $AUC_{0-\infty}$ of desethylamodiaquine in patients 5 years or older. The median capillary whole blood $AUC_{0-\infty}$ in this study is similar to those obtained in malaria patients aged 1 - 10 years in whole blood blots Papua New Guinea (mean: 4512.6 µg.d/l (108302.4 ng.h/ml)) (Hombhanje et al., 2005), but about 3-times higher than 40,339 ng.h/ml reported in plasma samples in Ghanaian malaria patients aged 1 – 14 years (Adjei, Kristensen, et al., 2008).

Overall, the ratio of the $AUC_{0-\infty}$ of desethylamodiaquine to the $AUC_{0-\infty}$ of amodiaquine was 55 and ranged from 37 in infants through 43 in patients 1 - 4 years to 85 in patients 5 years or older.

Desethylamodiaquine terminal elimination half-life

The terminal elimination half-life ($t_{½}$) of desethylamodiaquine is longer than that for the parent compound amodiaquine with widely variable reported values ranging from 9 - 31 days (Winstanley et al., 1987b; Pussard et al., 1987; Hombhanje et al., 2005; Stepniewska et al., 2009).

The overall terminal elimination $t_{½}$ in this study was estimated to be 8.1 (IQR 6.4 – 11.4) days. The half-life was longer in young infants, 9.9 (IQR 8.5 - 11.7) days and shortest in patients aged 1 - 4 years, 7.8 (IQR 6.5 - 11.4) days. These values are comparable to 9.0 days (range 7.3 - 11.6) in malaria patients from Burkina Faso (Stepniewska et al., 2009), 10.1 days (90% CI 6.3, 13.9) in malaria patients aged 1 - 10 years from Papua New Guinea (Hombhanje et al., 2005) but much longer than 4.6 ± 1.3 (mean ± sd) days in Ghanaian malaria patients aged 1- 14 years (Adjei, Kristensen, et al., 2008).

Apparent volume of distribution of desethylamodiaquine

The apparent volume of distribution, Vd/f of desethylamodiaquine was estimated to be 56.8 (IQR 39.6 – 96.3) $L.kg^{-1}$ in all patients, and increased with age from 50.2 (IQR 34.7 – 77.8) $L.kg^{-1}$ in infants to 66.2 (IQR 43.5 - 120) $L.kg^{-1}$ in patients 5 years or older. The apparent volume of distribution estimated in this study is similar to the volume of the peripheral

compartment of 62.4 Lkg^{-1}(Hietala et al., 2007) and 87.9 Lkg^{-1}(Stepniewska et al., 2009), who estimated the volume of the central compartment, $V_{central}/F$ as 12.8 $Lkg^{-1}$and 35.4 Lkg^{-1}, respectively.

Apparent clearance of desethylamodiaquine

The oral apparent clearance, CL/f was 0.227 (IQR 0.149 – 0.357) $Lkg^{-1}h^{-1}$ and increased from 0.168 (IQR 0.086 – 0.227) $Lkg^{-1}h^{-1}$ in infants, to 0.208 (IQR 0.146 – 0.348) $Lkg^{-1}h^{-1}$ in 1 – 4 year olds and to 0.262 (IQR 0.151 – 0.403) $Lkg^{-1}h^{-1}$ in patients 5 years or older. These values are nearly a quarter of the 0.861 $Lkg^{-1}h^{-1}$ (Adjei, Kristensen, et al., 2008) reported in malaria patients from Ghana and about one third of the 0.67 $Lkg^{-1}h^{-1}$ (Hietala et al., 2007) and 0.610 $Lkg^{-1}h^{-1}$ (Stepniewska et al., 2009) reported previously from Papua New Guinea and Burkina Faso.

Clearance increases with age for both amodiaquine and desethylamodiaquine, but the terminal elimination rate constant does not change with age. The terminal elimination rate constant (K_e) is calculated as the Clearance (CL) divided by the Volume of distribution (Vd). These findings suggest that the age-related changes in the Volume of distribution (Vd), and the additional variability in CL result in the changes in K_e with age being constant.

Determinants of desethylamodiaquine pharmacokinetic parameters

After adjusting for other predefined covariates, the clearance of desethylamodiaquine from the blood was much faster in female patients compared to their male counterparts. Females generally have lower body weight and organ sizes, higher percentage of body fat, lower glomerular filtration rate and different gastric motility compared with men. These have resulted in pharmacokinetic differences by gender. However, the clinical significance of these gender-based differences is yet to be proven (Beierle, Meibohm & Derendorf, 1999; Meibohm, Beierle & Derendorf, 2002).

The effect of site of sample collection persisted with respect to C_{max}, $AUC_{0-\infty}$, CL/f and Vd/f despite adjustments for other significant predefined covariates. The C_{max} in patients from Kintampo was almost double the Cmax in patients from Navrongo. Patients in Kintampo appeared to clear desethylamodiaquine from the blood at a slower rate and had a smaller Vd/f compared to patients from Navrongo. The $AUC_{0-\infty}$ was thus much higher in patients from Kintampo compared to their Navrongo counterparts. As discussed above, this could probably be explained by potential differences in pharmacogenetics or methods for sample collection

and storage. It has also been suggested that ethnicity, drug formulation and dosage, and pattern of concomitant medication use (Anderson, 2005; Parikh et al., 2007; Yasuda, Zhang & Huang, 2008; Kerb et al., 2009) are among the other factors accounting for site effects.

7.5.3 Discussion of amodiaquine and desethylamodiaquine pharmacokinetic-pharmacodynamic relationships

The extent and duration of parasite exposure following a treatment is defined by the area under the concentration-time curve, AUC. Antimalarial drug concentrations on day 7 have been suggested as correlates of AUC (Barnes et al., 2006a; White et al., 2008). Concentrations of antimalarials on day-7 post treatment represent parasite exposure in the fourth asexual cycle after the start of treatment and therefore reflect the concentrations occurring in the presence of relatively few residual parasites. Blood levels of long-acting partner drugs are therefore important determinants of cure, since residual parasites at this time point must be eliminated (White, 1997; White et al., 2008). In this study however, with its high cure rates, there was no difference in day 7 desethylamodiaquine concentrations between patients who achieved adequate clinical and parasitological response and those who failed treatment. The predictive value of the day 7 concentrations may need to be enhanced through data pooling since efficacy rates are high and most field trials may not be powered enough to achieve this goal. It has also been argued that the analysis of the correlation of the day 7 concentration with treatment outcome should be done using receiver operator characteristic (ROC) curves (Kay, Hodel & Hastings, 2014). Similarly, the study was underpowered to show any effect of desethylamodiaquine exposure, $AUC_{0-\infty}$ on treatment outcomes. As expected, $AUC_{0-\infty}$ and day 7 concentrations were strongly correlated.

The efficacy of artesunate-amodiaquine was very high, >97% as expected of currently used artemisinin-based combination treatments (WHO, 2010b,a). In the light of this evidence, a much larger study would be required to provide the statistical power to discriminate or correlate treatment outcomes with pharmacokinetic parameters (White, 2013).

Clinically, the antimalarial activity of amodiaquine is reported to be exerted mainly through its metabolite, desethylamodiaquine (Winstanley et al., 1988) and there have only been reports of a breakpoint concentration of desethylamodiaquine on day 3 being associated with treatment outcomes (Aubouy et al., 2003). In this study, an increase in the median day 3 capillary whole blood concentrations of amodiaquine were found to be significantly

associated with the rate of parasite recurrence [HR = 0.8745 (95% CI 0.7802, 0.9803), p = 0.021]. This is probably the first time that concentrations of the parent compound, amodiaquine rather than the metabolite, desethylamodiaquine are being associated with treatment outcome in terms of parasite recurrence and parasite clearance time.

In addition, an increase in the median day 2 capillary whole blood amodiaquine concentration was found to be associated with a reduction in the rate of parasite clearance time in this study; HR = 0.994 (95% CI 0.99078, 0.99813), p = 0.003. The clinical significance of this relatively small effect is unclear. It is important to note that the impact of the artemisinin component with respect to the effects of amodiaquine on days 2 and 3 could not be established in this study that did not determine artesunate pharmacokinetics, or any correlation with amodiaquine exposure. This early period coincides with the peak activity of the artemisinin component.

7.6 Conclusion

The efficacy of artesunate-amodiaquine in this study was very high at 97%. This high cure rate may partly reflect the higher amodiaquine and desethylamodiaquine exposure, and slower clearance in Ghana found in this study and in a previous study (Adjei, Kristensen, et al., 2008), when compared to studies in other countries. Given the few treatment failures, any association of day 7 desethylamodiaquine concentrations or the desethylamodiaquine area under the concentration-time curve with treatment outcome in terms adequate clinical and parasitological response could not be established. However, an increase in the day 3 concentrations of amodiaquine were found to be associated with a reduction in the risk of parasite recurrence and an increase in the day 2 concentrations of amodiaquine were found to be associated with a reduction in parasite clearance time. No such effect was however established for the metabolite which has been previously reported to be the main metabolite through which amodiaquine exerts its antimalarial activity.

This is the largest pharmacokinetic study on amodiaquine published to date. The results demonstrate that desethylamodiaquine exposure is remarkably consistent across all age groups, which is reassuring since the highest *falciparum* malaria burden is carried by children under 5 years of age. This is in contrast to findings for a number of other widely used antimalarials including lumefantrine, piperaquine, and sulfadoxine-pyrimethamine that have been systematically under-dosed in young children (Hung et al., 2003; Barnes et al., 2006a;

Checchi et al., 2006; Price et al., 2007). Equally reassuring is that desethylamodiaquine exposure is not reduced in underweight-for-age young children or those with high parasitaemias, two of the most vulnerable target populations.

The inclusion of 13 infants with uncomplicated malaria in this study provides preliminary evidence that they may have greater exposure to amodiaquine than older children and adults. Although no safety concerns were identified in this study, there is the potential for more adverse events in infants, particularly with increasing exposure to seasonal malaria chemoprophylaxis (SMC). There is therefore the need for pharmacovigilance and further research to assess amodiaquine safety and possibly dose optimization in infants.

The WorldWide Antimalarial Resistance Network (WWARN) platform is well placed to further examine the findings of this study since no single pharmacokinetic study may be large enough to further explore the key findings and associations identified herein.

The pharmacokinetic data analysis was carried out using non-compartmental approach. This analytical approach requires intensive sampling, assumes linear pharmacokinetics; is model independent and is not useful for predictive work. Barring these limitations, this analytical technique was employed to great effect to the analysis of this complex and heterogeneous data set.

The findings on the pharmacokinetic parameters of amodiaquine and its active metabolite desethylamodiaquine in this study are based on when amodiaquine was administered in a fixed dose combination with artesunate. These findings may therefore not be generalisable to studies where amodiaquine is administered alone or with another antimalarial other than artesunate.

A statistically significant decrease in dihydroartemisinin (DHA), the main active metabolite of artesunate, occurs with concomitant use of artesunate and amodiaquine (C_{max} decreased 47%, $AUC_{0-\infty}$ decreased 17%) (WHOPAR, 2011). Fortin and colleagues also showed that the plasma concentrations of artesunate and DHA in healthy volunteers were substantially lower (47–56% for C_{max} and 24–27% for AUC) following the administration of the fixed drug combination compared to the non-fixed dose combination (Fortin, Verbeeck & Jansen, 2011).

The maximum plasma desethylamodiaquine concentration was higher ($P < 0.001$) in the amodiaquine-only group than in the artesunate-plus-amodiaquine group, although their AUCs were similar (Adjei, Kristensen, et al., 2008).

In vitro, amodiaquine showed marked synergism when combined with the artemisinins (Mariga et al., 2004).

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Appendix 3.1: Classification of treatment outcomes

Early treatment failure

- danger signs or severe malaria on day 1, 2 or 3 in the presence of parasitaemia;
- parasitaemia on day 2 higher than on day 0, irrespective of axillary temperature;
- parasitaemia on day 3 with axillary temperature ≥ 37.5 ºC;
- parasitaemia on day 3 ≥ 25% of count on day 0

Late treatment failure

Late clinical failure

- danger signs or severe malaria in the presence of parasitaemia on any day between day 4 and day 28 in patients who did not previously meet any of the criteria of early treatment failure;
- presence of parasitaemia on any day between day 4 and day 28 with axillary temperature ≥ 37.5 ºC in patients who did not previously meet any of the criteria of early treatment failure.

Late parasitological failure

- presence of parasitaemia on any day between day 7 and day 28 and axillary temperature < 37.5 ºC in patients who did not previously meet any of the criteria of early treatment failure or late clinical failure.

Adequate clinical and parasitological response

- absence of parasitaemia on day 28, irrespective of axillary temperature, in patients who did not previously meet any of the criteria of early treatment failure, late clinical failure or late parasitological failure.

Appendix 3.2: Informed consent form

Informed consent form for participation in the pharmacokinetic arm of therapeutic efficacy study of Artesunate-amodiaquine

Information sheet

We are from the [Navrongo/ Kintampo] Health Research Centre. We are doing a study on the treatment of malaria. Malaria is a dangerous disease; however, it can be treated with medicine. The purpose of this study is to confirm that the medicine called Artesunate-Amodiaquine is still effective for treating malaria. We understand that you have agreed (for your child/ward) to take part in this study.

We are also inviting all malaria patients aged 2 months or more (or those with weight 4.5kg or more) living in this area to take part in another aspect of this study. Taking part in this portion of the study is completely voluntary. If you choose (not to allow your child/ward) not to participate, all the services you (your child/ward) receive(s) at this clinic will continue as usual. Even if you agree now but decide to change your mind and withdraw later, the services you (your child/ward) receive (s) at this clinic will continue.

If you agree (for your child/ward) to take part, we will like to test your (his/her) blood for the amount of the drug in your (his/her) blood at different time points so that when the drug is not working, we will be able to tell whether the amount of the drug in your (his/her) body is not enough or that it is because the drug is not able to kill the malaria germs. These tests will be done after you (s/he) have (has) completed the study. If you agree we will take this blood today and at six other different time points on days 1, 2, 3, 7, 14 and 28. We will not use your (his/her) name in any dissemination of the test results. You (your child/ward) may experience a bit of pain or fear when the blood is being collected. This may cause discomfort and may carry a small risk of bruising, bleeding or infection. However, this will be carried out by well-trained staff, using only new and clean materials.

Today, we will take a small amount of your (his/her) blood, 200 µl (about 4 drops) to test for the amount of the drug in your (his/her) blood.

On the

- 2nd visit (day 1): We will take a small amount of your (his/her) blood, 200 µl (about 4 drops) to test for the amount of the drug in your (his/her) blood.
- 3rd visit (day 2): We will take a small amount of your (his/her) blood, 200 µl (about 4 drops) to test for the amount of the drug in your (his/her) blood.
- 4^{th} (day 7), 5^{th} (day 14), and 6^{th} (day 28) visits, we will take a small amount of your (his/her) blood, 200 µl (about 4 drops) to test for the amount of the drug in your (his/her) blood.

Certificate of consent

I (My child/ward) have/has been invited to provide some blood on seven different occasions for them to test for the amount of malaria drug (amodiaquine) in my (his/her) blood. If the drug is not working, they should then be able tell whether the amount of the drug in my (his/her) body was not enough or that the malaria germs are resistant to this treatment.

☐ I have read the above information, or it has been read to me.

☐ I have had the opportunity to ask questions, and any questions that I have asked have been answered to my satisfaction.

☐ I know that I (my child/ward) can refuse to participate in this aspect of the study. I understand that if I agree or allow my child/ward to participate, I can withdraw my consent at any time without losing any benefits or services to which I am entitled.

☐ I understand that any information collected will be treated confidentially.

☐ I agree voluntarily (to allow my child/ward) to participate in this aspect of the study. After signing below, I will receive a copy this agreement form.

Name of participant (parent/guardian): …….

Signature or Thumb Print participant (parent/guardian): ……

Date: -----/-----/-----

(dd/mm/yyyy)

Witness' signature: (A witness' signature and the participant's (parent/guardian's) thumbprint are required only if the patient is illiterate. In this case, an independent literate witness must sign. If possible, this person should be selected by the participant (parent/guardian) and should have no connection with the study team.)

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

Name of witness: ……

Signature of witness: ---------- and thumbprint of participant (parent/guardian)

Date: -----/-----/--------

(dd/mm/yyyy)

Investigator's signature:

I have accurately informed the participant (parent/guardian) (or witnessed the participant (parent/guardian) being informed) of the purpose, procedures, potential risks and benefits of this aspect of the study. The participant (parent/guardian) has had the opportunity to ask questions and I have answered all questions to the best of my ability. I confirm that the participant (parent/guardian) has given the consent freely.

Name of study personnel: ……..

Signature: ……

Date: -------/------/------

(dd/mm/yyyy)

A copy of this document has been provided to the participant. _____ (initials of the principal investigator or designee).

Appendix 3.3: Assent form

Assent form for participation in the pharmacokinetic arm of therapeutic efficacy study of Artesunate-amodiaquine (for patients aged 12 – 17 years)

We are from the [Navrongo/ Kintampo] Health Research Centre. We are doing a study on the treatment of malaria. Malaria is a dangerous disease; however, it can be treated with medicine. The purpose of this study is to confirm that the medicine called Artesunate-Amodiaquine is still effective for treating malaria. We understand that you and your parent/guardian have agreed for you to take part in this study

We are also inviting all malaria patients aged 2 months or more (or those with weight 4.5kg or more) living in this area to take part in another aspect of this study. We have discussed this aspect with your parent/guardian, and they know that we are also asking you for your agreement.

You can choose whether you want to participate. If you decide to take part in this part of the study, your parent/ guardian also has to agree. If you do not wish to take part in this aspect of the study, you do not have to, even if your parents have agreed. It is your choice. If you decide not to participate, nothing will change. Even if you say 'Yes' now, you can change your mind later and it will still be okay. Before you decide whether to participate, you may discuss it with your parents or friends or anyone else you feel comfortable talking to. There may be some things that you want me to explain more because you are interested or concerned. Please ask me to stop at any time, and I will take time to explain.

☐ **Interviewer:** I have checked with the child, and he or she understands that participation is voluntary. _____ (initials)

If you agree to take part, we will like to test your blood for the amount of the drug in your blood at different time points so that when the drug is not working, we will be able to tell whether the amount of the drug in your body is not enough or that it is because the drug is not able to kill the malaria germs. This test will be done after you have completed the study. If you agree we will take this blood today and at six other different time points on days 1, 2, 3, 7, 14 and 28.

☐ **Interviewer:** I have checked with the child, and he or she understands the procedures. _____ (initials)

You may experience a bit of pain or fear when the blood is being collected. This may cause discomfort and may carry a small risk of bruising, bleeding or infection. However, this will be carried out by well-trained staff, using only new and clean materials.

☐ **Interviewer:** I have checked with the child, and he or she understands the risks and discomforts. _____ (initials)

There is no direct benefit for you from participating in this part of the study. Your participation will help us to make sure the medicine is still working. It will also help us to ensure that malaria patients receive the best treatment in the correct dose and this will benefit society.

☐ **Interviewer:** I have checked with the child, and he or she understands the benefits. _____ (initials)

We will not tell other people that you are taking part in this aspect of the study, and we will not share information about you with anyone who does not work in the study. We will not use your name when we are sharing the test results with other people.

☐ **Interviewer:** I have checked with the child, and he or she understands the confidentiality of the study results. _____ (initials)

You can ask me questions now or later. If you want to talk to someone else whom you know, like your teacher, doctor or auntie, that is okay too.

Certificate of assent

☐ I have been invited to provide some blood on seven different occasions for them to test for the amount of amodiaquine in my blood. If the drug is not working, they will then be able tell whether the amount of the drug in my body was not enough or that the malaria germs are resistant to this treatment.

☐ I have read the above information, or it has been read to me and I understand it. I have had my questions answered and know that I can ask questions later if I have them.

☐ I agree to take part in this aspect of the study. _____ (initials)

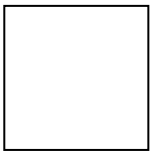
Or

☐ I do not wish to take part in this aspect of the study and I have not signed the assent below. _____ (initials)

Child's signature (only if the child assents)

Name of child:…….

Signature or Thumb Print of child: ……



Date: -----/-----/-----

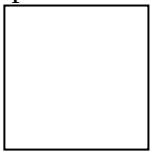
(dd/mm/yyyy)

Witness' signature: (A witness' signature and the child's thumbprint are required only if the child is illiterate. In this case, an independent literate witness must sign. If possible, this person should be selected by the participant and should have no connection with the study team.)

I have witnessed the accurate reading of the assent form to the potential participant, who has had the opportunity to ask questions. I confirm that the participant has given assent freely.

Name of witness: ……

Signature of witness: ---------- and thumbprint of child



Date: -----/-----/--------

(dd/mm/yyyy)

Investigator's signature

I have accurately informed the participant (or witnessed the participant being informed) of the purpose, procedures, potential risks and benefits of this study. The participant has had the opportunity to ask questions and I have answered all questions to the best of my ability. I confirm that the participant has given the assent freely.

Name of study personnel: ……..

Signature: ……

Date: -------/------/------

(dd/mm/yyyy)

A copy of this informed assent form has been provided to the participant. _____ (initials of the principal investigator or designee).

Appendix 3.4: Signs of Severe falciparum malaria

In a patient with *P. falciparum* asexual parasitaemia and no other obvious cause of symptoms, the presence of one or more of the following clinical or laboratory features classifies the patient as suffering from severe malaria:

Clinical features*:*

– Impaired consciousness or unrousable coma

– Prostration, i.e. generalized weakness so that the patient is unable walk or sit up without assistance

– Multiple convulsions – more than two episodes in 24 h

– Deep breathing, respiratory distress (metabolic acidosis)

– Circulatory collapse or shock, systolic blood pressure < 70 mmHg in adults and < 50 mmHg in children

– Clinical jaundice plus evidence of other vital organ dysfunction

– Haemoglobinuria

– Abnormal spontaneous bleeding

– Pulmonary oedema (radiological)

Laboratory findings:

– Hypoglycaemia (blood glucose < 2.2 mmol/l or < 40 mg/dl)

– Metabolic acidosis (plasma bicarbonate < 15 mmol/l)

– Severe anaemia (Hb < 5 g/dl, haematocrit < 15%)

– Hyperparasitaemia (> 2% or 100 000 /µl in low intensity transmission areas or > 5% or 250 000 /µl in areas of high stable malaria transmission intensity)

– Hyperlactataemia (venous lactate > 5 mmol/l)

– Renal impairment (serum creatinine above normal ranger for age)

Appendix 3.5: List of medications with antimalarial activity or that are likely to interfere with the pharmacokinetics of amodiaquine

- Chloroquine,
- Quinine, Quinidine
- Mefloquine, Halofantrine, Lumefantrine
- Artemisinin and its derivatives (Artemether, Arteether, Artesunate, Dihydroartemisinin)
- Proguanil, Chlorproguanil, Pyrimethamine
- Sulfadoxine, Sulfalene, Sulfamethoxazole, Dapsone
- Primaquine
- Atovaquone
- Antibiotics: Tetracycline, Doxycycline, Erythromycin, Azithromycin, Clindamycin, Rifampicin, Trimethoprim;
- Pentamidine

Drugs likely to interfere with Amodiaquine pharmacokinetics

a. Drugs known to inhibit CYP 2A6 or CYP2C8
   - Trimethoprim, Ketoconazole, Ritonavir, Saquinavir, Lopinavir, Gemfibrozil, Montelukast
   - Methoxsalen, Pilocarpine, Tranylcypromine
   - Efavirenz

Appendix 7.1: Patients with amodiaquine concentrations greater than lower limit of quantification on days 14 and 28

PATIENT ID	DAY OF FOLLOW UP	AQ (ng/ml)
IN-EF069	14	12.7
IN-EF070	14	1.46
IN-EF071	14	0.804
IN-EF083	14	1.18
IN-EF093	14	2.89
IN-EF1016	14	0.805
IN-EF1017	14	6.48
IN-EF103	14	0.913
IN-EF1059	14	2.38
IN-EF109	14	0.892
IN-EF110	14	0.88
IN-EF1114	14	2.1318
IN-EF1122	14	2.6928
IN-EF1124	14	4.0953
IN-EF113	14	0.942
IN-EF117	14	4.08
IN-EF136	14	2.07
IN-EF138	14	1.36
IN-EF146	14	6.68
IN-EF168	14	0.946
IN-EF192	14	4.96
IN-EF193	14	2.95
IN-EF228	14	1.81
IN-EF245	14	5.82
IN-EF246	14	0.815
IN-EF352	14	0.828
IN-EF354	14	11.8
IN-EF457	14	1.1
IN-EF714	14	1.57828
IN-EF788	14	4.301
IN-EF912	14	155.397
IN-EF938	14	1.04
TEM295	14	1.21
TEM299	14	0.82
TEM316	14	0.991
TEM318	14	1.54
TEM319	14	2.31
TEM331	14	2.98

PATIENT ID	DAY OF FOLLOW UP	AQ (ng/ml)
IN-EF068	28	0.843
IN-EF079	28	2.4
IN-EF080	28	2.14
IN-EF083	28	0.923
IN-EF101	28	0.88
IN-EF1017	28	6.23
IN-EF103	28	1.42
IN-EF107	28	1.02
IN-EF110	28	2.69
IN-EF1122	28	2.1505
IN-EF113	28	0.86
IN-EF114	28	1.24
IN-EF136	28	1.54
IN-EF139	28	5.75
IN-EF245	28	1.4
IN-EF253	28	1.59
IN-EF354	28	3.23
IN-EF492	28	1.14
IN-EF665	28	4.8246
IN-EF709	28	2.5806
IN-EF744	28	1.72601
IN-EF745	28	1.65121
IN-EF788	28	1.48291
IN-EF797	28	2.8611
IN-EF862	28	1.23
IN-EF913	28	2.5806
IN-EF978	28	1.51
TEM298	28	0.853
TEM308	28	1.63
TEM310	28	4.74
TEM313	28	3.05
TEM317	28	12.7
TEM319	28	5.45
TEM331	28	1.97
TEM333	28	13.6
TEM338	28	2.2
TEM349	28	0.789
TEM350	28	0.826

Appendix 7.2a: Compartmental models parameters

Median (IQR) desethylamodiaquine pharmacokinetic parameter estimates from various compartmental models tested in Phoenix® WinNonlin®

Parameter	1 compartment Additive model, n=225	2 compartment Additive model, n=220	1 compartment multiplicative model, n=225	1 compartment additive + multiplicative model, n=225	Non-compartmental model (WinNonlin NCA), n=138
AUC (ng.h/ml)	37257 (24,837 - 63,859)	36,959 (24, 825 - 63,448)	20,880 (3276 - 50, 306)	44,847 (28,277-70902)	133,673 (92,523-220,265)
C_{max} (ng/ml)	226 (146 - 379)	234 (149 - 393)	85.3 (14.2 - 211)	208 (132- 342)	564 (349 - 827)
K_e (h^{-1})	0.0068 (0.0047 - 0.0107)	0.007 (0.005 - 0.011)	0.004 (0.003 - 0.0056)	0.0049 (0.0038-0.0071)	0.0034 (0.0025-0.0043)
$t_{½}$ (days)	4.3 (2.7- 6.2)	4.14 (2.68 - 6.02)	6.6 (5.2 - 8.0)	5.9 (4.0- 7.7)	8.5 (6.8 - 11.6)
T_{max} (days)	0.40 (0.25 - 1.65)	0.39 (0.24 - 1.59)	0.33 (0.27 - 1.11)	0.46 (0.29 -1.07)	3 (2 - 30
CL ($L.kg^{-1}.h^{-1}$)	4.25 (2.37 - 7.60)	4.24 (2.34 - 7.66)	9.46 (3.28- 53.95)	3.69 (2.17-6.39)	1.20 (0.64 - 2.02)
CL2 ($L.kg^{-1}.h^{-1}$)		1.00 (0.9999 - 1.0002)			
K_a (h^{-1})	0.376 (0.048 - 0.825)	0.451 (0.04 -0.822)	0.656 (0.124-0.826)	0.398 (0.101-0.690)	
V ($L.\ kg^{-1}$)	581 (300 - 1067)	559 (275 - 1004)	2139 (666- 9218)	709 (340 - 1141)	354 (210 - 608)
V2 ($L.\ kg^{-1}$)		0.9998 (0.9994 - 1.0002)			

Appendix 7.2b: Model comparison for desethylamodiaquine compartmental analysis: Additive: Multiplicative: additive+ multiplicative

1 compartmental (additive) versus 1 compartmental (multiplicative) versus 1 compartmental (additive+ multiplicative) model

Goodness of fit criterion	**-2LL**			**AIC**			**BIC**		
Model Source	Additive + Multiplicative	Additive	Multiplicative	Additive + Multiplicative	Additive	Multiplicative	Additive + Multiplicative	Additive	Multiplicative
Mean	-280.2697	59.7586	59.3615	-270.2697	67.7586	67.3615	-292.4184	71.4954	71.0683
Min	-698.4457	-57.2408	-63.6588	-688.4457	-49.2408	-55.6588	-690.3985	-51.6956	-58.1136
Max	91.205	97.2304	110.1301	101.205	105.2304	118.1301	100.9345	105.014	117.9137
Median	0	67.67	63.2522	10	75.67	71.2522	-602.8101	76.3166	73.8151
25%	-638.5268	47.9929	45.2517	-628.5268	55.9929	53.2517	-631.8168	60.8417	57.1779
75%	66.8176	78.5405	82.8011	76.8176	86.5405	90.8011	77.5053	86.6764	92.4651

-2LL =-2 Loglikelyhood ratio; AIC= Akaike Information Criterion; BIC = Bayesian Information Criterion

Appendix 7.2c: Model comparison for desethylamodiaquine compartmental analyses

1 compartmental (additive) versus 2 compartmental (additive) versus 1 compartmental (additive+ multiplicative) models

Goodness of fit criterion	**-2LL**			**AIC**			**BIC**		
Model Source	1-compartment Additive	2-compartment Additive	1-compartment Additive+ Multiplicative	1-compartment Additive	2-compartment Additive	1-compartment Additive+ Multiplicative	1-compartment Additive	2-compartment Additive	1-compartment Additive+ Multiplicative
Mean	59.7586	60.1198	-280.3623	67.7586	72.1198	-270.3623	71.4954	75.4948	-292.518
Min	-57.2408	-57.5127	-697.4824	-49.2408	-45.5127	-687.4824	-51.6956	-49.1949	-689.4353
Max	97.2304	97.2304	91.205	105.2304	109.2304	101.205	105.014	108.9058	100.9345
Median	67.67	67.67	0	75.67	79.67	10	76.3166	80.4166	-604.5061
25%	47.9929	47.9929	-638.196	55.9929	59.9929	-628.196	60.8417	64.2409	-631.9559
75%	78.5405	78.5405	66.8176	86.5405	90.5405	76.8176	86.6764	90.5683	77.5053

-2LL =-2 Loglikelyhood ratio; AIC= Akaike Information Criterion; BIC = Bayesian Information Criterion