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Research Article

Efficacy, Safety and Pharmacokinetics of a Triple Combination of Artemether-Lumefantrine and Amodiaquine in Laboratory Rodents

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ABSTRACT

Presently artemisinin-based combination therapies (ACT) are now widely recommended as first-line treatment of uncomplicated malaria, however there are some reports and evidence of treatment failure despite adequate drug concentrations. Addition of a second long-acting partner drug to the existing single partner artemisinin-based combination therapy may delay the development of resistance. The objective of the present study is to determine the efficacy, of a triple combination of artemether-lumefantrine and amodiaquine in laboratory rodents. The blood schizonticidal activity of the proposed triple combination of artemether-lumefantrine (AL) and amodiaquine (AQ) was evaluated in a rodent model of *Plasmodium berghei*. Animals were treated orally with standard doses of artemether-lumefantrine (AL), amodiaquine (AQ) or the triple combination (ALAQ). Parasitological activity and survival of the animals were assessed over 24 days. Safety and plasma concentrations of artemether, amodiaquine and lumefantrine were determined both in the standalone and in the triple combination treatment groups using uninfected but treated albino rats. There was a progressive significant decline in parasitemia in all therapeutic groups with the triple combination (ALAQ) achieving a 100% suppression of parasites by day 16. ALAQ resulted in significant elevations in total white blood cell counts, platelet counts, alanine transaminase and urea levels. There were significant reductions in blood pressure and heart rate. Compared to artemether-lumefantrine administered alone the triple combination (ALAQ) showed lower plasma artemether levels and area under the curve (AUC) values at 72hours with low day 7 lumefantrine plasma levels in the triple combination (ALAQ). These preliminary results showed that the triple therapy's efficacy, safety and pharmacokinetics are quite encouraging. Human studies are required to confirm the efficacy, safety and pharmacokinetic findings in this study.

Keywords: *Uncomplicated malaria, Artemisinin-Based Combination Therapy, Amodiaquine, Artemether-Lumefantrine Plasmodium berghei..*

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INTRODUCTION

Despite the scale up of antimalarial interventions and the resultant reduction in malaria transmission in some areas, the morbidity and mortality due to malaria disease is still high in other endemic areas of sub-Saharan Africa (Prudhomme *et al*., 2010). The emergence of antimalarial drug resistance has led to many countries in sub-Saharan Africa to adopt the World Health Organization (WHO) recommended artemisinin-based combination therapy for treatment of uncomplicated P. falciparum malaria (World Health Organization, 2006). Artemisinin-based combination therapies (ACT) have a fast-acting artemisinin derivative with rapid effects on parasite clearance, and a long-acting drug to prevent recrudescence and development of resistance (World Health Organization, 2006; World Health Organization, 2010). Artemeter-Lumefantrine and artesunate-amodiaquine are both recommended by the WHO and have shown good efficacy and safety outcomes which has resulted in a reduction of malaria morbidity and mortality in different populations (Van Vugt *et al*., 2000 ; Mutabingwa *et al*., 2005 ; Makanga *et al*., 2006; Pecoul *et al*., 2008).

Delayed clearance times suggest emergence of resistance and there are reports of artemisinin resistance in P falciparum malaria in western Cambodia, Thai-Burmese border and the Greater Mekong region (Noedl *et al*.,2008; Mizuno *et al*., 2009 ; Dondorp *et al*., 2009; Phyo *et al*., 2012; Carrara *et al*.,

2013; Ariey *et al*., 2014). Mutations associated with artemisinin resistance on chromosome 13 have been identified and treatment failures with artemisinin-based combination therapies have also been reported in non-immune Europeans and travelers after visiting endemic regions (Jackson *et al*., 2006 ; Repetto *et al*., 2011; Färnert *et al*., 2012). Failure of artemisinin efficacy would render partner drugs susceptible to greater selection pressure for the development of resistance, compromising the value of the combination (White and Pongtavornpinyo , 2003; Kay and Hastings ,2015).

In endemic regions with high malaria transmission the use of one long acting partner drug with a very short acting artemisinin may expose parasites to the emergence of resistance because the single partner drug exposes remaining and new parasites to sub therapeutic drug levels over a long time. A perfect drug combination is one with an excellent overlap in the exposure of the drug components. It has been postulated that addition of a second long acting partner drug to the present artemisinin-based combination therapy will strengthen the combination and prevent the parasites from developing resistance to artemisinins. Addition of a third partner drug with a longer half-life than lumefantrine such as amodiaquine (2 weeks), mefloquine (2–3 weeks) or piperaquine (4-5weeks) will strengthen the present combination., and delay development of resistance. A third partner drug with a different mechanism of action and longer half-lives will further reduce the time window in which remaining and new parasites can be selected by offering better therapeutic drug levels over a long time(White ,1998 ; Dondorp *et al*., 2010). There are unpublished reports of the use of triple combination therapy for the treatment of uncomplicated P. falciparum malaria in Nigeria. In such situations, a third schizonticidal drug usually a monotherapy is administered to a patient either together or after a presumed treatment failure of an artemisinin-based combination therapy. The present study evaluated the efficacy, safety and pharmacokinetics of a triple combination of artemeter-lumefantrine and amodiaquine administered together in laboratory rodents. A major objective is to assess if the simultaneous administration of artemeter-lumefantrine with amodiaquine will have better cure rates, be safe and will not affect the bioavailability of the individual drugs when compared with existing one partner artemisinin-based combination.

MATERIALS AND METHODS

Animals: Adult albino mice of weight range 20-25g (average age 7 weeks) were obtained from the laboratory animal center, College of Medicine, University of Lagos, Lagos Nigeria. The animals were housed in well ventilated cages in 4 groups of 5 animals per cage at a regular 12 h light-dark cycle. The animals were acclimatized for a minimum period of three days prior to the experiment and allowed free access to drinking water and standard pellets diet (Pfizer standard rodent pellet diet). All experimental protocols maintenance and care of experimental animals complied with research guidelines for the use and care of laboratory animals (American Physiological Society, 2002; National Research Council, 1985; National Academy of Science, 2011).

Administration of drugs: Treatment was carried out twice daily for artemether-lumefantrine and once daily for amodiaquine for a period of 3 days. Artemether-lumefantrine 20mg/120mg (AL) was sourced from Novartis Pharma, Lagos Nigeria while Amodiaquine syrup 60ml (AQ) was sourced from Pfizer, Lagos Nigeria. The amodiaquine syrup and the AL tablets dissolved in distilled water were administered to the mice orally using an oral cannula as follows;

Group 1: Control group. Each mouse received distilled water for 7 days

Group 2: This group was treated with amodiaquine (0.02ml) 10g/kg orally (p.o.) once daily, for 3 days starting from the fourth day post infection

Group 3: This group was treated with artemether and lumefantrine.(0.025ml) [artemether 2mg/kg and lumefantrine 12mg/kg orally (p.o.) twice daily, for 3 days starting from the fourth day post infection

Group 4: This group was treated with artemether and lumefantrine(AL) twice daily plus amodiaquine. Each mouse was administered 0.025ml of AL and 0.02 ml of amodiaquine orally once daily for 3 days starting from the fourth day post infection

Inoculation of mice with malaria parasites. : Evaluation of schizonticidal activity on established infection was done by the curative test (Madara *et al*., 2010; Akinyede *et al*., 2013). The rodent parasite chloroquine sensitive Plasmodium berghei NK 65 was sourced from National Institute for Medical Research (NIMR), Lagos, Nigeria. Animals were infected with parasitized blood obtained from the tail veins of infected experimental mice. Approximately 0.1 mL of infected blood (3–4 drops) was diluted in freshly prepared phosphate buffer solution (PBS). Mice were inoculated intraperitoneally with 0.1 mL of parasitized saline suspension containing approximately 1 x107 parasites on the first day. On the 4th day the mice were divided into four groups of five mice each and a smear was taken to confirm presence of P.berghei in the mice before the start of treatment (White, 1997; Shida *et al*., 1989).

Parasite counts in infected mice and assessment of efficacy: Blood smears were collected daily and examined microscopically to monitor the parasitaemia level until the parasites were cleared. Peripheral blood smears were prepared by using blood obtained from the tail veins of infected experimental mice. The thin films were fixed in methanol (3 min) and then stained with May-Grunewald Giemsa. Blood smears were examined at a magnification of X 100 by oil immersion light microscopy. Parasitemia was determined by counting 30 or 100 fields of view for >0.5% and <0.5% infected erythrocytes, respectively ensuring an acceptable standard error of 22% at 0.1% parasitemia and a limit of detection on the order of 0.002% parasitemia. The total number of erythrocyte and the parasitized erythrocyte in the four experimental groups were counted and recorded. Infected erythrocytes were counted by using the formula;

Malaria parasite density/mL of blood

= No. parasites counted +8,000/ 200 leukocytes.

(Shida *et al*., 1989; World Health Organization, 2009).

Mean parasite counts mice in each treatment group was determined and compared on days 4, 8, 12, 16, 20 and 24.

Mice were euthanized by sodium pentobarbitone injection (50 to 100 mg/kg i.p.) (Fish *et al*., 2008).

Assessment of safety: We assessed the haematological, biochemical and cardiovascular safety of the proposed triple artemisinin-based combination therapy. Normotensive albino rats, of either sex weighing 220-250g not infected with malaria parasites were used for the assessment. The rats were divided into four groups of control, amodiaquine alone, artemether-lumefantrine alone and artemether-lumefantrine co-administered with amodiaquine for 3 days. Blood samples were collected from the ocular veins with capillary tubes and put into EDTA and heparinized sample bottles to assess the haematological (haemoglobin level, leukocyte count), and biochemical parameters (serum alanine aminotransferase, aspartate aminotransferase, total bilirubin, glycaemia, urea and creatinine) respectively (Osonuga *et al*., 2012; Olayemi *et al*., 2012; Kotepui *et al*., 2014). The cardiovascular safety of the triple combination therapy was assessed by determination of blood pressure and heart rate (Feng, *et al*., 2015). The rats were anaesthetized with intra-peritoneal injection of 0.5 ml of 25% urethane (1000mg/kg) (Fish *et al*., 2008). The anaesthetized rat was fixed in a supine position on a dissecting table and the temperature of the animal was maintained at 37°C by the use of an overhead lamp. A longitudinal midtracheal incision approximately 2 cm long was made in order to expose the trachea, the right jugular vein and left carotid artery. After tracheotomy, the trachea was cannulated with polyethylene tube 92.75 mm diameter to maintain a free airway for spontaneous respiration .The right jugular vein was cannulated with polyethylene tube for flushing with 0.9% NaCl (normal saline). The cannulation of the carotid artery was performed in the same manner as the cannulation of jugular vein and the polyethylene tube (1 mm diameter) filled with heparin sodium in saline solution was used. The arterial blood pressure was measured from the left carotid arterial cannula connected to a research grade blood pressure transducer (Harvard, 60-3003) which was connected to an oscillograph (Harvard) for recording. The animal was allowed to equilibrate for at least 30 min. before recording. The mean arterial Blood pressure was calculated using the following formula MABP = DP + 1/3 (SP-DP); Where, DP= diastolic pressure; SP= systolic pressure. The response of mean arterial blood pressure (MABP) was expressed as percent change from the control group measurement (Adeboye *et al*., 1999; Lahlou *et al*., 2002; Feng *et al*., 2015).

Pharmacokinetic study: Simultaneous determination of artemether, lumefantrine and amodiaquine, plasma concentrations was done in treated non-infected albino rats by high-performance liquid chromatography with UV detector (HPLC-UV) at the Central Research Laboratories, College of Medicine,University of Lagos. Artemether , lumefantrine ,amodiaquine concentrations and the internal standard (Halofantrine) were measured using high performance liquid chromatography methodologies adapted from previous studies (Virendra *et al*., 2004; Huang *et al* ., (2010); Maddela *et al* ., 2015) .The area under the blood concentration-time curve (AUC) of artemether , amodiaquine and lumefantrine were determined using a non-compartmental model in WinNonLin Professional pharmacokinetic software version 2.1 (Pharsight Corporation, Mountain View, USA). The concentrations and the AUCs were then compared between treatment arms.

Blood samples for pharmacokinetic analysis were collected from the ocular veins with capillary tubes and put into EDTA and heparinized sample bottles for assessment of artemether , amodiaquine, and lumefantrine levels . I ml of blood was drawn at pre-determined time peaks from each treated albino rat at the following times 24hrs, 48hrs, 72hrs and on day7 for lumefantrine levels. The Blood samples were centrifuged at a speed of 4000rpm for 10mins with a centrifuge.(5702 by Eppendorf).The plasma was stored at -70 degrees centigrade.

All chemicals (Methanol (MeOH), KH2PO4 (potassium dihydrogen orthophosphate, acetonitrile) were of HPLC grade and purchased from Merck (Darmstadt, Germany). Reference standards of artemether , amodiaqine , lumefantrine, and halofantrine (Internal Standard) were sourced from Ipca Laboratories Ltd.(Mumbai, India). Blank plasma was collected from untreated rats. Water was distilled water,

The HPLC system consisted of Agilent 1100 series with UV detector. Separation was achieved on a Zorbax Eclipse XDB RP C8 HPLC column (150 X 4.6mm, 5 µm, Agilent). All chromatographic experiments were carried out in the isocratic mode at room temperature. The mobile phase was vacuum degassed before use. The mobile phase consisted of a mixture of Acetonitrile: Potassium dihydrogen orthophosphate (H25mMKH 2PO4) ; (70:30) %. Injection volume was 20µl at a flow rate of 1.0ml /min and wavelength 216 nm at ambient temp and pH 3.9.

The powdered drugs were weighed and dissolved in in MeOH (methanol) and water (1:1) at room temperature to prepare working stock solutions of 1.0 mg/ml and working solutions. Serial dilutions of the stock solutions were appropriately diluted to lower concentration for spiking the calibration standards. Spiked mixed standards of Amodiaquine (1.25) + lumefantrine (46.875)+Artemether 7.812+ halofantrine (Internal Standard) (10) µg/mL were added to drug-free human plasma and mixed properly by vortexing. Working solutions were made by diluting with appropriate volume of mobile phase. Calibration standards and QC samples were prepared from separately weighted stock solutions. The stock solutions, standards, QC samples, and the I.S. working solution (100 g/mL) were stored at −70 0 C between uses.

A Liquid-liquid extraction method was followed for extraction of artemether, lumefantrine and amodiaquine from rat plasma. An aliquot of 1 mL of plasma spiked with 0.05ml of internal standard (3µg/ml) and extraction solvent (2 ml of chilled acetonitrile) was dispensed into 5 mL plain polypropylene sample bottle, vortex mixed for 1 minute and sonicated for 10 minutes using ultrasonic bath. The resultant precipitant was centrifuged at 4000 rpm for 10 minutes. The supernatant was aspirated using disposable Pasteur pipette and filtered using 0.45 µm syringe filter and this was subjected to HPLC analysis. Extraction recoveries of artemether ,lumefantrine and amodiaquine were satisfactory (75-80%) .

A complete validation was carried out as per US FDA (US DHHS, FDA and CDER.(2001) and EMEA guidelines (European Medicines Agency (2011). The validation

parameters tested include specificity, linearity, precision and accuracy, limit of detection (LOD) and limit of quantification (LOQ), recovery and stability

Statistical Analysis: Results were expressed as mean ± standard error of mean. Descriptive summary statistics was presented for values and concentrations of artemether,amodiaquine and lumefantrine. The results of the effects of artemether-lumefantrine alone, amodiaquine alone, was compared to the effects of the triple combination of the three drugs administered together. The two way ANOVA test Bonferonni Post Test was used to analyse and compare the results at a 95% confidence level. Values of $p < 0.05$ and <0.001 were considered significant. Graph pad prism version 5.1 was used for statistical analysis.

RESULTS

Efficacy (Parasite clearance): There was a progressive significant decline in parasite counts in all three therapeutic groups. In the group that received amodiaquine or artemether–lumefantrine as separate medications there were residual parasitaemia up till the 20th day of observation. In the animal group that received a combination of artemether lumefantrine and amodiaquine administered together (ALAQ), there were no parasites detected after day 12. In this group there was 100% clearance of parasites on days 16 and 20. In the control group that received distilled water there was a progressive increase in parasite counts with all mice dying after the 16th day. Table 1 below shows the effect of amodiaquine alone, artemether-lumefantrine alone and the combination of artemether-lumefantrine and amodiaquine on parasite counts in P. berghei infection in albino mice.

Safety:
The effect of artemether-lumefantrine alone (AL), amodiaquine alone (AQ), and the combination of artemether-lumefantrine and amodiaquine (ALAQ) on haematological ,hepatic , renal and cardiovascular parameters were evaluated and shown in Tables 2, 3 and 4 respectively. The triple combination of artemether-lumefantrine and amodiaquine (ALAQ) showed significant elevations in total white blood cell counts, red blood cells platelet counts, alanine transaminase and urea levels compared to the control, amodiaquine alone and artermether-lumefantrine alone groups. On the cardiovascular function, the triple combination showed significant reductions in both systolic and diastolic blood pressure , mean arterial blood pressure and heart rate compared to the control and artemether-lumefantrine alone groups. However, the amodiaquine alone group showed lower values in these cardiovascular parameters compared to the triple combination therapy.

Pharmacokinetics: The plasma concentrations of artemether lumefantrine and amodiaquine were measured up to 72hours with day 7 concentrations for lumefantrine after oral administration. Total-assay coefficients of variation (CVs) for artemether during analysis was <5% at all quality-control levels. The lower limit of quantification (LLOQ) was set to 1.4 ng/mL. for lumefantrine with an LLOQ of 50 ng/ml, a calibration range of 50 to 10,000 ng/ml, and a CV range of 1.1 to 6.7%

Table1:
Effect of artemether-lumefantrine alone , amodiaquine alone and the combination of artemether- lumefantrine with amodiaquine on parasite counts in *Plasmodium berghei* infection in albino mice

Experimental animal groups	Day 4 parasite count/µL	Day 8 parasite count/µL	Day 12 parasite count/µL	Day16 Parasite count/µL	Day 20 parasite count/µL	Day 24 parasite count/µL
Control	24.64±0.49	30.04±0.19	60.64±2.46	85.97 ±2.46	All animals died	All animals died
Artemether-lumefantrine (AL)	15.50±0.32	12.82±0.30^{c}	10.44 ± 0.19	8.31±0.27 ^{cY***}	1.83±0.13 ^{cY***}	0
Amodiaquine(AQ)	16.26±0.31	11.52±0.57 ^{c}	7.52±0.16 ^{cY}	6.26±0.24 ^{cY*}	0.13±0.12 ^{cY***}	0
Artemether-lumefantrine + amodiaquine (ALAQ)	14.60±0.08	10.06±0.09 ^{c}	5.89±0.18 ^{cY}	0.00±0.00	0.00±0.00 ^{cY***}	0

*Values are Mean ± SEM; (n=5); $^{c}P<0.001$ vs Day 0, $^{Y}P<0.001$ vs Day 1; **P<0.01, *P<0.05, ***P<0.001 vs Day 2 (Two-Way NOVA followed by Bonferonni Post Test). Day 0 refers to 1St day of therapy.*

Table 2:
Effect of artemether-lumefantrine alone (AL), amodiaquine alone (AQ), and the combination of artemether-lumefantrine and amodiaquine (ALAQ) on haematological values in albino rat

Parameter	Control	Artemether-lumefantrine (AL)	Amodiaquine (AQ)	Artemether-lumefantrine + Amodiaquine (ALAQ)
White blood cell (x10^{3}/µL)	8.70±1.06	11.45±0.40*	11.02±0.92*	13.77±0.59*≠α
Haemoglobin (g/dl)	10.66±0.72	12.35± 0.36*	11.42±0.79	11.32±0.22≠
Red Blood Cell (x10^{6}/µL)	5.20±0.22	6.06±0.18*	6.13±0.18*	6.02±0.10*
Mean cell volume (MCV /fl)	61.27±0.75	56.47±1.48*	55.92±1.66*	55.05±1.51*
Mean cell haemoglobin (MCH)(pg/cell)	20.00±0.47	20.77±0.52	17.65±1.13*≠	18.72±0.31*≠
Mean cell haemoglobin conc.(MCHC) (g/dl)	33.22±0.46	31.70±1.51	31.92±1.00*	32.60±0.98
Platelet count (x10^{3}/µL)	255.28±118.8	605.25±2.17*	530.25±48.58*≠	682.50±42.73*≠α

=p<0.05 when triple combination group is compared with the control group,#=p<0.05 when compared with the artemether-lumefantrine group and *# α =p<0.05 when compared with the amodiaquine group

The within-assay and between-assay coefficients of variations for amodiquine were always <10% at the limits of quantification. In the three treatment groups there were no significant changes to amodiaquine levels. Compared to artemether-lumefantrine administered alone the triple combination (ALAQ) showed significant lower artemether levels and a lower area under the curve at 72hours. The lumefantrine levels were similar in all treatment groups with a lower area under the curve for day 7 lumefantrine levels. (Tables 5, 6 and 7). Figure 1 shows a representative chromatogram for amodiaquine, lumefantrine, artemether and halofantrine (Internal standard) at 72 hours with 1.451, 4.646, 5.542 and 2.756 minutes retention time respectively

Table3:
Effect of amodiaquine alone (AQ), artemether-lumefantrine alone (AL) and the tripple combination of artemether-lumefantrine and amodiaquine (AL+ AQ) on hepatic and renal parameters in albino rat.

Parameter	Control	Artemether-lumefantrine (AL)	Amodiaquine (AQ)	AL + AQ
Aspartate transferase (AST) U/L	95.25±5.96	235.72±5.84*≠	191.40±6.56*	214.75±3.59*≠α
Alanine transaminase (ALT) U/L	32.37±0.44	22.02 ± 4.41 *≠	94.80±4.57*	69.07±6.72*≠α
Alkaline phosphatase (U/L)	164.40±4.80	260.75 ± 36.77*	221.92±13.67*	295.55±17.83*≠
Total Protein (g/dl)	0.82±0.02	1.57 ± 0.52 *	1.62±0.35*	0.62±0.20≠α
Urea (mmol/L)	1.62 ±0.21	2.92 ± 1.47	4.27±0.21*	5.27±0.16*≠α
Creatinine (µmol/l)	31.60±1.16	42.09 ± 5.35	27.83±0.95	33.82±3.58

**=p<0.05 when combination group is compared with the control group,*≠=p<0.05 when compared with the artemether-lumefantrine group and *≠ α =p<0.05 when compared with the amodiaquine group*

Table 4:
Effect of amodiaquine alone, artemether-lumefantrine alone and the combination of artemether- lumefantrine plus amodiaquine on cardiovascular parameters in rat

Cardiovascular Parameters	Control	Artemether lumefantrine	Amodiaquine (AQ)	ALAQ
Systolic blood pressure (mmHg)	99.32±2.12	83.55±3.09*	59.00±1.77*≠	66.72±1.79*≠α
Diastolic blood pressure (mmHg)	79.00±0.57	63.62±3.13*	38.20±1.95*≠	45.77±4.56*≠α
Pulse pressure (mmHg)	20.32±1.76	19.92±1.80	20.80±1.51	19.95±3.15
The mean arterial blood pressure (mmHg)	85.77± 1.01	70.26 ±3.00*	45.13±1.75*≠	52.42±3.57*≠α
HR (beats /min)	495.00±45.00	450.00±17.32	330.00 ±17.32*≠	375.00±28.72*≠α

=p<0.05 when triple combination group is compared with the control group, ≠=p<0.05 when compared with the artemether lumefantrine group and *≠ α =p<0.05 when compared with the amodiaquine group

Table 5:
Plasma concentrations and area under the curve of artemether in albino rat administered with artemether-lumefantrine (AL) alone and the triple combination of artemether-lumefantrine plus amodiaquine (AL+AQ)

Parameter	Artemether conc. in AL Alone	Artemether conc. in AL +AQ	P-value
Cmax (ng/ml)	1.38 (0.68 – 2.74)	0.26 (0.14 – 0.51)	0.12
24 hour conc. (ng/ml)	0.255 (0.218 -0.280)	0.230 (0.150 -0.264)	0.287
48hour conc. (ng/ml)	0.270 (0.255 – 0.289)	0.250 (0.238-0.273)	0.086
72 hour conc. (ng/ml)	0.289 (0.272 -0.315)	0.260 (0.249 -0.271)	0.006*
$AUC^{0-\infty}$ µgh/ml	17.558 (11.259 -24.263)	11.778 (6.021 – 17.897)	0.003**

**P<0.05*

Table 6:
Plasma concentrations and area under the curve of amodiaquine in albino rat administered with amodiaquine (AQ), and the combination of artemether- lumefantrine and amodiaquine (AL+AQ)

Parameter	Amodiaquine conc. in AQ alone	Amodiaquine conc. in AL +AQ	P-value
Cmax (ng/ml)	59.0(29.0 -118.0)	62.6 (31.1 – 131.5)	0.215
24 hour conc. (ng/ml)	58.66 (38.50 -62.79)	55.83 (43.15 - 59.87)	0.902
48hour conc. (ng/ml)	59.01 (28.55 -64.08)	57.40 (46.66 - 62.23)	0.496
72 hour conc. (ng/ml)	57.80 (38.50 – 60.17)	62.61 (51. 39 - 69.85)	0.127
$AUC^{0-\infty}$ µgh/ml	2703.995 (1291.995-4105.664)	2606.700 (1355.393 – 4046.900)	0.052

**P<0.05*

Table 7:
Plasma concentrations and area under the curve of lumefantrine in albino rat administered with artemether-lumefantrine (AL) and the combination of artemether- lumefantrine and amodiaquine . (AL+AQ)

Parameter	Lumefantrine conc. in AL	Lumefantrine conc. in ALAQ	P-value
Cmax (ng/ml)	1924 (984 -3865)	2189 (1094 -4339)	0.115
24 hour conc. (ng/ml)	1928 (1653 -2493)	1951 (1786 -2106)	0.64
48hour conc. (ng/ml)	1768 (1587 -1955)	1603 (1564 -1894)	0.331
72 hour conc. (ng/ml)	1912 (1776- 2294)	2189 (1973 – 2383)	0.212
Day 7 conc. (ng/ml)	1670 (1153 -21187)	1683 (774-2591)	0.992
$AUC^{0-\infty}$ µgh/ml	84921.17 (41830.25-127814.60)	88347.50 (45699.50 – 133851.50)	0.031*

**P<0.05*

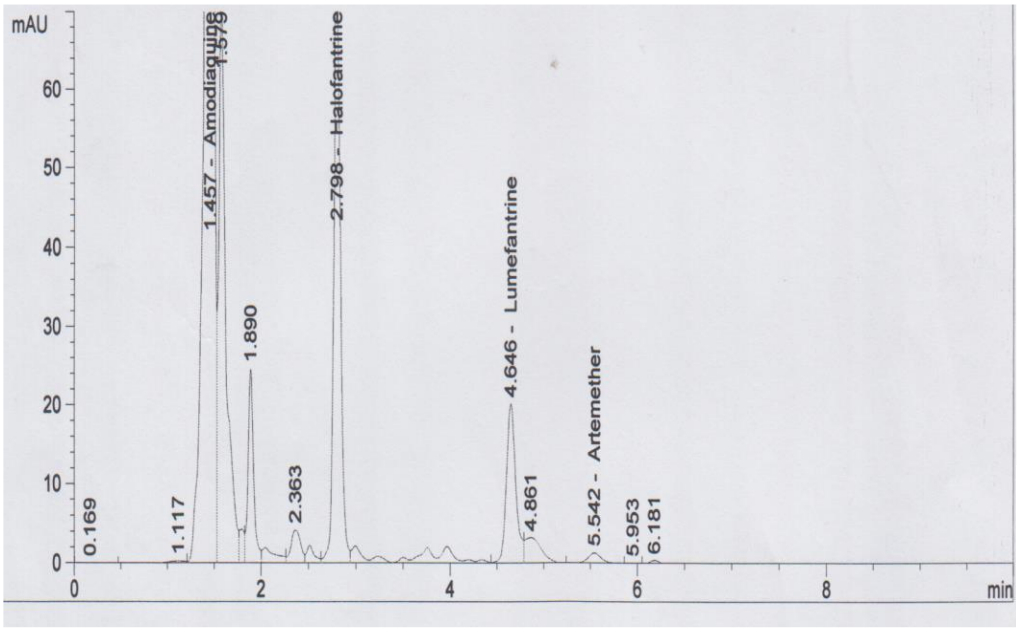

Figure 1:
Representative chromatograms for amodiaquine and artemether, lumefantrine and halofantrine (internal standard) at 72 hours.

DISCUSSION

Deployment of a triple artemiisinin based combinations as a standard regimen is one of the options to delay the development of resistance to artemisinin and its derivatives (Dondorp *et al*., 2010). In the present study, the proposed triple combination of artemether-lumefantrine plus amodiaquine was compared to the conventional artemisinin-based combination therapy. The triple combination therapy was very effective with a rapid blood schizonticidal action. Strengthening the conventional ACT with a second long acting partner drug will make the emergence of mutant parasites unlikely as the parasites will be exposed to three drugs instead of two and the drugs are all schizonticidal and have independent mechanisms and sites of action. This complete parasite clearance is expected to have positive implications for prevention of recrudescence because the combination of artemether, which rapidly reduces parasite biomass, with longer-acting lumefantrine that is capable of eliminating residual parasites and amodiaquine with its active metabolites that have a long half-life will achieve parasitologic cure and offer protection against recrudescent infection after therapy (White ,1999a) . A rapid and complete clearance time will reduce the probability of the parasite developing resistance because delayed clearance times are associated with development of resistance (Noedl *et al*., 2008). The parasites would need to develop mutations at three resistance loci at the same time and this appears very unlikely and may further delay development of resistance (Meshnick, 1998)

Both artemether lumefantrine and amodiaquine are safe and well tolerated (Makanga *et al*., 2006; Olliaro and Mussano ,2003; Faye *et al*., 2007). Haematological changes during malaria infection, such as thrombocytopenia and leucocytosis or leucopenia have been extensively documented (Maina *et al*., 2010 ; Kotepui *et al*., 2014). Amodiaquine prophylaxis has been associated with agranulocytosis , neutropenia and hepatitis (Thomas *et al* ., 2004). In this study the triple combination of amodiaquine and artemether-lumefantrine showed significant elevations in total white blood cell counts,red blood cell and platelet counts. These findings suggest that the triple combination may have a good effect on post-treatment haematological recovery. Previous studies have documented anaemia of persistent parasitaemia and showed that repeated exposure to artemether-lumefantrine improved anaemia and thrombocytopaenia (Okafor and Nwaiwu, 2001; Bakshi *et al*., 2000). The observation of significant elevations in alanine aminotransferase and urea calls for more human safety evaluations.

Assessment of cardiovascular safety is important because delay in ventricular depolarisation resulting in prolongation of the QRS complex and worsening malaria-associated orthostatic hypotension have been documented with some antimalarial drugs as well as malaria disease itself (White, 2007; von Seiddlein *et al*., 1998). In this study we observed reductions in heart rate, and blood pressure and this should be explored with ECG studies in humans.

In the present study we evaluated the effect of the triple combination on the plasma concentrations of artemether, lumefantrine and amodiaquine. Co-administration of amodiaquine had no significant effect on plasma concentration of artemether and lumefantrine in the first 48 hours. However, the proposed triple combination showed significant lower artemether levels and the area under the curve at 72hours. The area under the concentration-time curve (AUC) is a measure of drug exposure and the routine measurement of drug concentration at day 7 as part of anti-malarial treatment is recommended (White *et al*., 2008). .The lumefantrine levels in this study were similar in all treatment groups, however we observed a lower area under the curve at day 7. The observed low AUC of lumefantrine could raise concerns that the triple therapy may put the patients at risk of treatment failure because day 7 lumefantrine concentration is the principal determinant of artemether-lumefantrine anti-malarial activity which drives the 28-day cure rate(Singh *et al*., 2011 ; Ezzet *et al*., 2000). Previous studies show lumefantrine displays similar pharmacokinetics in the rat as in humans with a long terminal elimination half-life, and therefore a higher post-treatment prophylactic effect (Singh *et al*., 2011). Lumefantrine, is mainly metabolized by cytochrome P450 3A4 (CYP3A4), to desbutyl lumefantrine (Ezzet *et al*., 2000) which has a higher in-vitro antiparasitic effect and could reduce the impact of low day 7 plasma concentrations observed.

There were some limitations in this study and the results should be interpreted within the limits of the study design and context which is to determine preliminary data on efficacy, safety and kinetics of the proposed triple artemisinin combination therapy. The efficacy, safety and pharmacokinetics of antimalarials have been documented in infected and uninfected rodents (Olayemi *et al*, 2012; Osonuga *et al*., 2012) and humans (Van Vugt *et al*., 2000; Makanga *et al*., 2006; White *et al*., 1999b; Jullien *et al* 2010). We looked at the hematological profile in uninfected rats to limit blood sampling and improve survival rates in the infected mice. The duration of pharmacokinetic sampling was too short (72hrs). However we determined up to day 7 lumefantrine concentration which is the principal determinant of artemether-lumefantrine anti-malarial activity and drives the 28-day cure rate (Singh *et al*, 2011; Ezzet *et al*., 2000). Furthermore chromatographic separation and simultaneous quantification of artemether and lumefantrine is better with reversed-phase HPLC with MS/MS detection. Artemether and the biologically active metabolite dihydroartemisinin are difficult to measure in body fluids. These compounds lack UV or fluorescent chromaphores, are thermally labile, and lack functional groups for reliable derivatisation. Post-column derivatisation methods in which artemether and metabolite are converted to a UV-detectable product have yielded variable results, often with poor reproducibility (Melendez *et al*., 1991; White, 1994; Navaratnam *et al*., 1995). HPLC with MS/MS detection was not available and the use of HPLC with UV detector could explain the low artemether levels observed (Sandhya *et al*., 2015).

In conclusion, the present study in rodents, suggest that the proposed triple artemisinin-based combination will improve efficacy and further delay the emergence of resistance to artemisinins as there was a complete clearance of parasites with the combination of artemether-lumefantrine and amodiaquine after day 12. The lower area under the curve at 72hours with lower artemether and lumefantrine levels observed with the proposed triple artemisinin-based combination (ALAQ) will need further evaluation. We recommend randomized controlled human studies to further evaluate efficacy and safety and pharmacokinetics.

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