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Full Length Research Paper

Comparative methaemoglobin concentrations of three erythrocyte genotypes (HbAA, HbAS and HbSS) of male participants administered with five antimalarial drugs

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In the present *in-vivo* study, the capacities of five antimalarial drugs (Fansidar, Halfan, Quinine, Coartem and Chloroquine phosphate) to alter/distort methaemoglobin concentrations of three human erythrocyte genotypes (HbAA, HbAS and HbSS) was investigated. Spectrophotometric method was used to ascertain this erythrocyte parameter. The male participants enrolled for this study were grouped according to their genotypes, pathologic status, (that is, non-malarious and malarious individuals). Determination of erythrocyte methaemoglobin concentration was carried out before (control; t = 0 h) and after (tests; that is, at t = 3, 6 and 18 h) the five (5) antimalarial drugs were administered to various corresponding groups of participants. The results showed that methaemoglobin concentrations of these individuals ranged between 1.45+/-0.13 and 2.50+/-0.43%; 8.27+/-2.41 and 14.78+/-2.45%, for non-malarious and malarious male individuals respectively. There was no significant difference ($p > 0.05$) between methaemoglobin concentrations of HbAA and HbAS erythrocyte of non-malarious participants. The doses of the five antimalarial drugs administered to non-malarious individuals did not cause toxic methaemoglobinemia. Under the same experimental conditions, erythrocytes obtained from persons of HbSS genotype exhibited significant ($p < 0.05$) elevation of methaemoglobin concentration. Relatively high levels of methaemoglobin concentration of parasitized red blood cells decreased in a time dependent manner after administration of the five antimalarial drugs. Therefore, erythrocyte methaemoglobin evaluation is a reliable biochemical marker and rational for diagnostic and therapeutic potential in malaria. Furthermore, moderate increases of erythrocyte methaemoglobin in HbSS individuals served as point of caution when administering these drugs to this category of human subjects.

Key words: Antimalarials, erythrocyte, malaria, genotype, methaemoglobin.

INTRODUCTION

Concisely, methaemoglobin is formed when the iron of deoxyhaemoglobin is oxidized from its ferrous (Fe^{2+}) to the ferric state (Fe^{3+}) (Murray et al., 2006). The oxidation of haemoglobin to methaemoglobin can arise from auto oxidation engendered by the activities of pro-oxidants and oxidizing substances (Callister, 2003), deficiency or impaired activity of methaemoglobin reductase enzymes (Yubisui and Takashita, 1980; Board, 1981,) and hereditary abnormal haemoglobin, haemoglobin M (Prchal and Jenkin, 2001). Methaemoglobin will not bind reversibly with oxygen. Under normal physiologic condition, methaemoglobin is continually formed in red blood cells (Tietz, 1976; Callister, 2003).

Malaria parasites are particularly vulnerable to oxidative stress during the erythrocytic life stages (Muller et al., 2003; Becker et al., 2004). This is not surprising as the parasites live in a pro-oxidant environment that contains oxygen and iron, the key prerequisite for the formation of reactive oxygen species (ROS) via the Fenton reaction.

The present study seeks to ascertain whether erythrocyte methaemoglobin concentration could serve as a reliable biochemical parameter for diagnosis and monitoring of therapeutic events in malarial disease. Furthermore, these parameters may give insight into the role, influence of these antimalarials on some functional status of the red blood cells, and unfold hitherto, unknown toxic/benefi-

Table 1. The participants administered with a single dose of each of five antimalarial drugs.

Male participants	Drugs/doses administered
1).Non-malarious	Fansidar: (pyrimethamine) = 14.9 mg/kg; (sulphadoxine)= 2.9mg/kg Halfan: (Halofantrine base) = 13.9 mg/kg Quinine = 5.9 mg/kg) Coartem:(artemether) = 1.2 mg/kg (lumefantrine)= 7.2mg/kg Chloroquine Phosphate = 14.9 mg/kg
2). Malarious	Fansidar: (pyrimethamine) = 14.5 mg/kg; (sulphadoxine)= 2.9 mg/kg Halfan: (Halofantrine base) =13.5 mg/kg Quinine = 5.8 mg/kg) Coartem: (artemether) = 1.2 mg/kg (lumefantrine) = 7.0 mg/kg Chloroquine Phosphate = 14.5 mg/kg

cial aspects of these drugs to humans who express the three red cell genotypes, for better-informed prescription and use.

MATERIALS AND METHODS

Anti-malarial drugs

Five (5) antimalarial drugs were used in this study: Fansidar™ {Swiss (Swipha) Pharmaceuticals Nigeria Ltd}, Coartem™, (Beijing Norvatis Pharmaceutical Company, Beijing, China) Chloroquine phosphate (May and Baker, Pharmaceutical Company, Nigeria Plc), Halfan™ (Smithkline Beecham Laboratories Pharmaceutical Company, France) and Quinine (BDH, UK).

Selection of volunteers/experimental design

Forty-three (43) non-malarious male (61-73 kg) participants of confirmed HbAA (15), HbAS (15) and HbSS (13) genotypes between the ages of 20-28years enrolled for this study. The malarious group consisted of forty-five (45) male (59 - 79 kg) participants – HbAA (15), HbAS (15) and HbSS (15). They aged between 21 - 34 years.

The participants administered with a single dose of each of five antimalarial drugs, were grouped according to their individual genotype and malarial status. The doses were administered in the following specifications: **Table** 1:

Blood samples were withdrawn from these participants at time intervals of 3, 6 and 18 h after dosage and analyses were carried out to ascertain for erythrocyte methaemoglobin concentration. The determinations of the red blood cell parameter prior to the administration of the five antimalarial drugs to participants constituted the control analysis.

Ethics

The institutional review board of the Department of Biochemistry, University of Port Harcourt, Port Harcourt, Nigeria, granted approval for this study and all participants involved signed an informed consent form. This conducted study was in accordance with the ethical principles that have their origins in the Declaration of Helsinki. Individuals drawn were from Imo State University, Owerri, Nigeria and environs. The research protocols were in collaboration with registered and specialized clinics and medical laboratories.

Collection of blood samples/preparation of erythrocyte haemolysate

Five milliliters (5.0 ml) of human venous blood of HbAA, HbAS, and HbSS genotypes obtained from participants by venipuncture was stored in EDTA anticoagulant tubes. Blood of HbSS genotype and malarious blood samples were from patients attending clinics at the Federal Medical Center (FMC), Imo State University Teaching Hospital (IMSUTH), Orlu, St. John Clinic/Medical Diagnostic Laboratories, Avigram Medical Diagnostic Laboratories, and Qualitech Medical Diagnostic Laboratories. These centers are located in Owerri, Imo State, Nigeria.

The erythrocytes were washed by methods as described by Tsakiris et al. (2005). Within 2 h of collection of blood samples, portions of 1.0 ml of the samples were introduced into centrifuge test tubes containing 3.0 ml of buffer solution pH = 7.4: 250 mM tris-HCl (Tris-HCl)/140 mMNaCl/I.0 mMMgCl_2/10 mMg lucose). The erythrocytes were separated from plasma by centrifugation at 1200 g for 10 min, washed three times by three similar centrifugations with the buffer solution. The erythrocytes re-suspended in 1.0 ml of this buffer were stored at 4℃. The washed erythrocytes were lysed by freezing/thawing as described by Galbraith and Watts, (1980) and Kamber et al., (1984). The erythrocyte haemolysate was used for the determination of methaemoglobin concentration.

Determination of methaemoglobin concentration of erythrocyte lysate

Determination of methaemoglobin content of red cell lysate was by modification of the method of Evelyn and Malloy, (1938), as described by Akomopong et al. (2000). A total of 400 µl of 0.5 M Phosphate buffer (pH 6.5) was added to 600 µl of the cell lysate, and the mixture was centrifuged at 16,000 g for 5 min to sediment debris. A total of 700 µl of the supernatant fraction was used to measure the absorbance at λmax = 630 nm (the absorbance maximum for methaemoglobin), and the reading was recorded as SI. A total of 50 µl of 10gpercentageKCN was added, and after 5 min at room temperature (24℃), a second reading (S2) was recorded. KCN converts methaemoglobin to cyanomethaemoglobin, which does not absorb at 630 nm; hence, the difference between absorbance readings S1 and S2 represents the absorbance due to methaemoglobin.

To measure total hemoglobin levels, all of the hemoglobin was converted to methaemoglobin, the absorbance of the sample at λmax = 630 nm was recorded, and then KCN was added to form cyanomethaemoglobin. Specifically, 70 µl of the supernatant fraction was diluted 10-fold into 600 µl of 0.1 M phosphate buffer (pH 6.5). Next, 30 µl of freshly prepared 20g% $K_3Fe(CN)_6$ was added and incubated for 5minutes at room temperature (24℃) and an initial reading (T1) was recorded. A total of 50 µl of 10% KCN was subsequently added, and a second reading (T2) was recorded. The percent methaemoglobin in the sample was calculated as [100(S1-S2)] / [10(T1-T2)].

Statistical analyses

The experiments were designed in a completely randomized method and data collected were analyzed by the analysis of variance

Table 2. Methaemoglobin Concentration (Met. Hb %) of Male Erythrocyte Haemolysate.

Genotype	(Met. Hb %) (X±S.D)	
	Non-malarious	Malarious
1). HbAA ($n=15^{NM}; 15^{M}$)	1.48±0.14[a]	14.07±2.56[b]
2). HbAS ($n=15^{NM;}15^{M}$)	1.45±0.13[a]	8.27±2.41[a]
3). HbSS ($n=13^{NM};15^{M}$)	2.50±0.43[b]	14.78±2.45[b]

M and NM = number of malarious and non-malarious blood samples respectively. Means in the column with the same letter are not significantly different at $p < 0.05$ according to LSD.

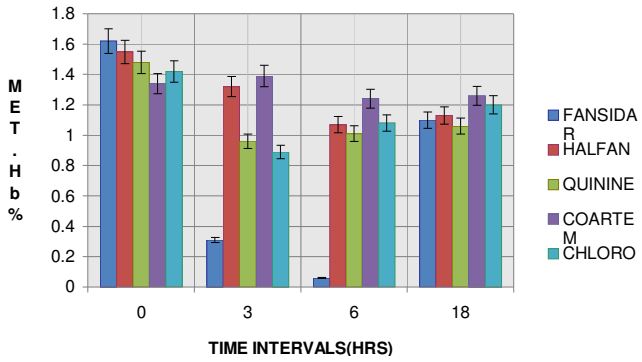

Figure 1. Comparative *in vivo* methaemoglobin concentrations of HBAA erythrocyte haemolysate of non-malarious male participants administered with five antimalarial drugs

procedure while treatment means were separated by the Least Significance Difference (LSD) incorporated in the Statistical Analysis System (SAS) package of 9.1 versions (2006).

RESULTS

The mean (+/-S.D) methaemoglobin concentration, expressed as percentage (Met.Hb%) of total haemoglobin concentration of three erythrocyte genotypes (HbAA, HbAS and HbSS) of blood samples obtained from non-malarious and malarious male participants, before being administered with the corresponding five antimalarial drugs (control values) is presented in Table 2.

A cursory look at Table 2 showed erythrocyte obtained from blood sample of malarious male participant exhibited significantly ($p < 0.05$) higher levels of methaemoglobin concentrations than those of non-malarious individuals, irrespective of their genotype. There was no significant difference ($p > 0.05$) between methaemoglobin concentrations of HbAA and HbAS erythrocyte of non-malarious participants. An overview of the results showed that methaemoglobin concentrations of these individuals ranged between 1.45±0.13 and 2.50±0.43%; 8.27±2.41 and 14.78±2.45%, for non-malarious and malarious male

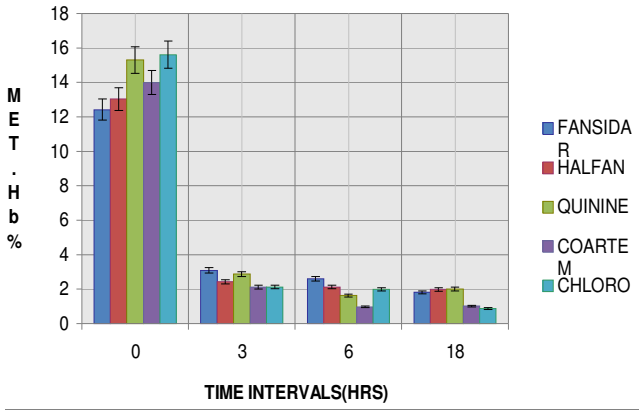

Figure 2. Comparative *in vivo* methaemoglobin concentrations of HBAA erythrocyte haemolysate of malarious male participants administered with five antimalarial drugs.

individuals respectively.

Non-malarious participants (Figure 1)

An overview of the results showed the five antimalarial drugs caused a time dependent decrease in erythrocyte methaemoglobin concentrations within approximately 6 h after administration. This followed a paradoxical increase in methaemoglobin concentration as the experimental time progressed.

Administered Fansidar elicited the lowest methaemoglobin concentration at the 6th h (Met. Hbpercentage = 0.06±0.02%). However, erythrocyte methaemoglobin concentration reached 1.18±0.47% at the 18th h. The fall in methaemoglobin concentration in the presence of the drug between the 3rd and 6th hours was not significantly different ($p > 0.05$).

Although decreased level of erythrocyte methaemoglobin concentration occurred 3 h after Halfan was administered, it was not significantly different ($p > 0.05$) compared to the control/reference values. Furthermore, there was no significant difference ($p > 0.05$) in subsequent rise and fall in methaemoglobin concentration within the experimental period.

Coartem caused no significant difference ($p > 0.05$) in the levels of erythrocyte methaemoglobin throughout the period of the experiment.

Malarious participants (Figure 2)

The relatively high levels of methaemoglobin concentration of parasitized red blood cells decreased in a time dependent manner after administration of the five antimalarial drugs. It is worthwhile to note that the rate of decrease in erythrocyte methaemoglobin concentration of these individuals was by far more rapid in the first 3 h. In addition, methaemoglobin content of the red blood cells showed no significant difference ($p > 0.05$) after the 3rd,

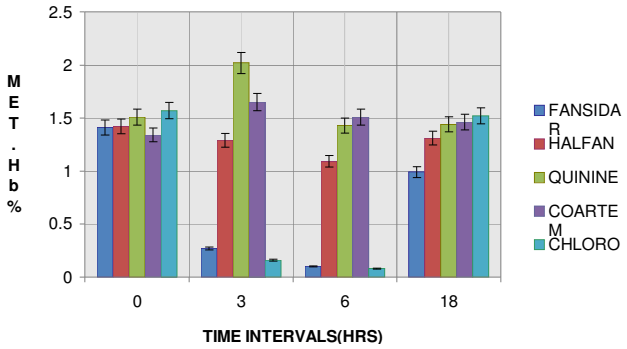

Figure 3. Comparative *in vivo* methaemoglobin concentrations of HbAS erythrocyte haemolysate of non-malarious male participants administered with five antimalarial drugs.

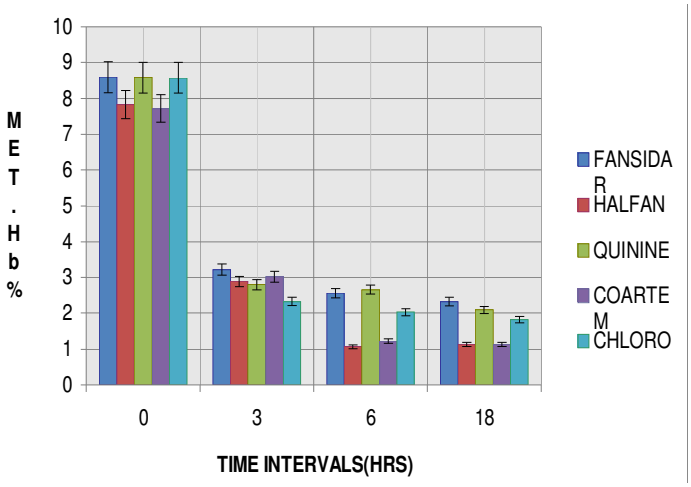

Figure 4. Comparative *in vivo* methaemoglobin concentrations of HbAS erythrocyte haemolysate of malarious male participants administered with five antimalarial drugs.

6^{th} and 18^{th} h, after the drugs were administered.

Non-malarious participants (Figure 3)

Three antimalarial drugs, Halfan, Chloroquine Phosphate and Fansidar promoted declining red blood cell methaemoglobin concentration within 6 h. However, no significant difference ($p < 0.05$) in methaemoglobin concentration was exhibited after 6 h of administration of Chloroquine Phosphate, Quinine, Coartem and Halfan to these individuals.

Conversely, Quinine and Coartem caused the elevation of methaemoglobin concentration. Specifically, administered Quinine engendered erythrocyte methaemoglobin concentration to increase from 1.51±0.09% to a peak value of 2.02±0.15% at zero and 3 h respectively.

Malarious participants (Figure 4)

Generally, there was a time dependent decline in methaemoglobin concentration in the same manner as earlier described in malarious male participants of HbAA genotype. An overview of the results showed no significant ($p > 0.05$) corresponding capacities of the five antimalarial drugs to elicit declining methaemoglobin concentration with time (that is, at $t > 3$ h).

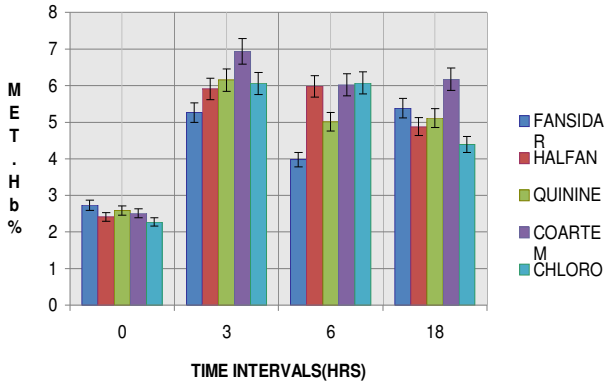

Figure 5. Comparative *in vivo* methaemoglobin concentrations of HbSS erythrocyte haemolysate of non-malarious male participants administered with five antimalarial drugs.

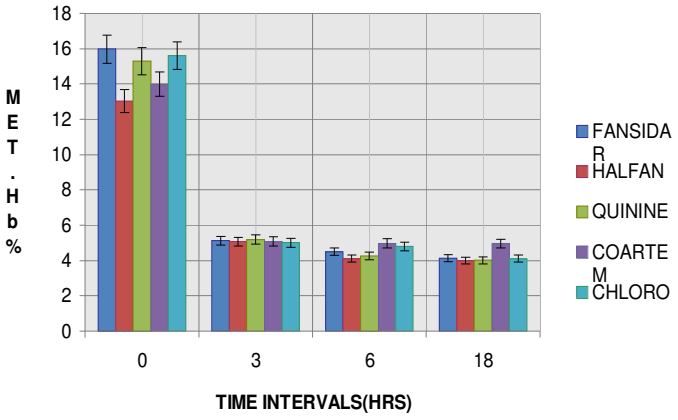

Figure 6. Comparative *in vivo* methaemoglobin concentrations of HbSS erythrocyte haemolysate of malarious male participants administered with five antimalarial drugs.

Non-malarious participants (Figure 5)

Elevation of erythrocyte methaemoglobin concentration occurred in these individuals approximately 3hours after dosing these participants with the five antimalarial drugs. Specifically, Coartem caused attainment of a peak value of 6.94±0.61% methaemoglobin concentration at the third hour. Further declining concentrations of methaemoglobin concentration was not significantly different ($p > 0.05$) after 3, 6 and 18 h after dosage.

Malarious participants (Figure 6)

In this experimental group, all corresponding erythrocyte

methaemoglobin concentrations, 3 – 18 h after the five antimalarial drugs were administered, were significantly different ($p < 0.05$) compared to the control/reference values (that is, at t = 0 h). Similar to non-malarious male individuals, further declining concentrations of methaemoglobin concentration was not significantly different ($p > 0.05$) after 3, 6 and 18 h of dosage.

DISCUSSION

The determination of erythrocyte methaemoglobin concentration as a toxic endpoint in chemical poisoning and pathologic conditions showed reliability and reproducibility in clinical diagnostic procedures (Hopkins, 2000; Bradberry, 2003; Uko et al., 2007). This present study reported significant ($p < 0.05$) elevation of methaemoglobin concentrations of human participants infected with malaria, irrespective of their genotype (Table 1). This observation was in agreement with the reports of Uko et al., (2007) who demonstrated high level of methaemoglobin in subjects with severe malaria parasitaemia and suggested routine estimation of methaemoglobin in malaria for clinical evaluation of patients. Furthermore, Anstey et al. (1996) documented elevated methaemoglobin concentration of Tanzanian children with severe and uncomplicated malaria. In the same vein, the present report was in concordance with observations of Friedman et al. (1979). These authors showed that cultures with high levels of parasitaemia contained between 3 to 10 times more methaemoglobin than those with low levels of parasitemia. Akompong et al. (1999) found isolated malarial parasites contained between 20 - 42% of methaemoglobin concentration. In contrast, uninfected red blood cells presented between 0.5 - 1.0% of methaemoglobin. Therefore, these reports suggest malaria parasite induced raised levels of erythrocyte methaemoglobin. In addition, these authors noted that increased methaemoglobin in malarial infection was a reflection of rapid oxidation of haemoglobin ingested by the parasites. It worthwhile to recall that raised oxidant levels compounded by compromised activity of erythrocyte redox enzymes further exacerbate the tendency towards spontaneous oxidation of haemoglobin molecule in parasitized red blood cells. De Rosa et al. (2003), used methaemoglobin levels as a marker molecule for the presence of nitrogen oxide generated by parasite as consequence of their metabolism (Clark et al., 1991). All these were pointers to the fact that parasitized erythrocytes exhibited raised levels of methaemoglobin. Similar to the reports of Neupane et al. (2008), the decreasing methaemoglobin concentrations with time after administration of the five antimalarial drugs suggest decreasing levels of ROS due to the killing of the parasites. Relationship exists between levels of parasitaemia and levels of ROS production (Friedman et al., 1979; Uko et al., 2007; Neupane et al., 2008).

The pattern of variability of basal erythrocyte methaemoglobin concentrations amongst non-malarious human participants showed that the dysfunctional red cell of HbSS genotype was significantly ($p < 0.05$) higher than the normal one, HbAA (Table 1). This result conformed to earlier reports by Tamer et al. (2000). They noted that the primary reason for the relatively raised concentration of oxidized haemoglobin (methaemoglobin) in HbSS erythrocytes was higher production of superoxide ion by these erythrocytes compared to those of HbAA and HbAS erythrocytes. Furthermore, erythrocyte endogenous oxidant (haemin) showed higher levels in HbSS than HbAA erythrocytes. Haemin has a profound capacity to activate certain erythrocyte redox enzymes e.g. NADH methaemoglobin reductase (Uwakwe, 1991) and its presence at high concentration was attributable to the high level of haemolytic phenomenon peculiar to this haemoglobin variant cell (Orjih et al., 1985). There is also the case of certain methaemoglobinopathies found in association with HbSS erythrocytes. These are $HbM_{Boston,}$ HbM_{Iwate}, $HbM_{Hydepark}$ and $HbM_{Hammersmith}$, noted for tendency towards spontaneous oxidation *in vivo* and resistant to enzymatic reduction.

Early studies have noted that certain xenobiotics were capable of eliciting elevation of erythrocyte methaemoglobin concentration, thereby distorting normal plasma haemoglobin (Fe^{2+})/methaemoglobin (Fe^{3+}) ratio. Callister, (2003) reported the nitrates and anilines as the most common cause of methaemoglobin toxicity in man. This physiologic dysfunctional state (methaemoglobinemia), presented in form of clinical cyanosis occur when plasma methaemoglobin concentration exceeds 15% (Hopkins, 1998). Methaemoglobin concentrations of HbAA and HbAS erythrocytes obtained from non-malarious human participants administered with the five antimalarial drugs fell within the normal physiologic range proposed by Tietz, (1976). Therefore, from methaemoglobin toxicity standpoint, these drugs did not induce methaemoglobinemia at the experimental doses while the investigation lasted. Similarly, Chikezie and Enemor, (2005), reported that guinea pigs administered with quinine did not present plasma methaemoglobin concentration that was diagnostic of toxic methaemoglobinemia. These authors averred that the capacity of the drug to oxidize ferrous (Fe^{2+}) haemoglobin did not overwhelm the physiologic systems responsible to maintain erythrocyte methaemoglobin concentration below the critical value of 15% that is diagnostic of toxic methaemoglobinemia. Furthermore, it is probable the redox potentials of these drugs and its metabolites were not high enough to engender the oxidation of considerable population of ferrous (Fe^{2+}) state haemoglobin. However, moderate increases of erythrocyte methaemoglobin in HbSS individuals (Figures 5 and 6) served as point of caution when administering these drugs to this category of human subjects. Decreasing erythrocyte methaemoglobin concentrations of malarious human participants of HbAA and HbAS genotypes administered with corresponding antimalarials confirmed the suitability of this parameter for evaluation of level of parasitaemia and drug efficacy (Alloueche et al., 2003; Uko et al., 2007).

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