Comparative Erythrocyte Glutathione S-Transferase Activity Profile of Non-Malarious Guinea Pigs (*Cavia tschudii*) Administered with Pyrimethamine/Sulphadoxine and Artemether/Lumefantrine Combination Therapies

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**ABSTRACT**

Cellular level of enzyme activity can serve as reliable biomarker in the event of environmental/chemical insults. The present study sought to investigate time-dependent alterations of erythrocyte glutathione S-transferase (Ery-GST) activity of non-malarious guinea pigs (*Cavia tschudii*) administered with pyrimethamine/sulphadoxine and artemether/lumefantrine combination therapies. Eighteen guinea pigs were allotted into three groups of six (n = 6), composed of the control (C1) and two test groups T1; pyrimethamine/sulphadoxine treated group and T2; artemether/lumefantrine treated group. Accordingly, single dose intra-peritoneal injection of pyrimethamine = 3.7 mg/sulphadoxine 0.7 mg mixture and artemether = 0.3 mg/lumefantrine = 1.8 mg mixture per kg of body weight (b.wt.) were administered to the guinea pigs. The animals were fasted for 16 h prior-treatment and blood samples were drawn at time intervals of 3, 6, 9 and 24 h and measured for GST activity using spectrophotometric methods. Ery-GST activity of group C1 (C1<sub>GST</sub>) within 0 h ≤ t ≤ 24 h was fairly constant and did not exhibit significant alterations (p > 0.05), whereas the Ery-GST activity profile of the test groups (T1 and T2) were biphasic. Ery-GST activity of group T1 (T1<sub>GST</sub>) varied within the range of 5.04±0.98-5.60±0.59 IU/gHb with peak enzyme activity at t = 0 h. At t = 9 h, T2<sub>GST</sub> activity was not significantly different (p > 0.05) from T1<sub>GST</sub> activity. The Ery-GST activity profile indicated perturbation of erythrocyte physiochemistry, which could be of relevance from toxicological and therapeutic standpoints.

**Key words:** Artemether, erythrocyte, glutathione S-transferase, lumefantrine, pyrimethamine

**INTRODUCTION**

Combination therapy of sulphadoxine (50 mg) and pyrimethamine (250 mg) exist under the trade mark name of Fansidar™, often administered as prophylaxis and for treatment of certain chloroquine resistant strains of *Plasmodium falciparum* malaria (WHO, 2001). This drug combination effectively inhibits two enzymes involved in the biosynthesis of folic acid within the parasite (Milhous *et al.*, 1985; Nzila, 2006). Sulphadoxine [N-(5, 6-dimethoxy-4-pyrimidinyl) sulphanilamide] is a structural analogue of para-aminobenzoic acid (PABA) that competitively inhibits protozoan dihydropteroate synthetase (DHPS), the enzyme required for incorporation of PABA into dihydropteroic acid and immediate precursor of folic acid. The resultant depletion of folic acid, an essential cofactor in the biosynthesis of nucleic acids, interferes with protozoan nucleic acid and protein biosynthesis (Nzila, 2006). Sensitive malarial parasites are those that must synthesize their own folic acid, whereas those that can utilize preformed folate are not affected.
Pyrimethamine (5-(4-chlorophenyl)-6-ethyl-pyrimidine-2, 4-diamine) is a competitive inhibitor of dihydrofolate reductase (DHFR), the enzyme that reduces dihydrofolate to tetrahydrofolate (FH$_4$), required for one carbon transfer reactions. Because malaria parasites do not incorporate exogenous thymine or thymidine for DNA synthesis, thymidylate must be synthesized de novo in a process that requires active FH$_4$. The inhibition of this pathway effectively interferes with DNA synthesis in the malaria parasite with attendant deleterious consequence.

The combination of artemether and lumefantrine is available from Novartis under the brand name Coartem™. It is the most effective available treatment for malaria in children in areas of Africa where resistance to conventional antimalarial drugs is high (WHO, 2001). Coartem™ is currently the only fixed-dose artemisinin-based combination therapy (ACT) pre-qualified by the World Health Organization (WHO) (Lefèvre et al., 2013). This fixed-dose combination is beneficial to patients as it facilitates treatment compliance and supports optimal clinical effectiveness.

The two active principles of Coartem™ show synergistic anti-protozoan activity against P. falciparum and a 1:6 ratio of artemether and lumefantrine has been described as optimal in vitro (Bakshi et al., 2000; Nzila, 2006; Lefèvre et al., 2013). Artemether therapeutic action depends on its endoperoxide bridge, which interacts with haem iron to cause free radical mediated damage to malaria parasites (Tracy and Webster, 2001; Crespo-Ortiz and Wei, 2012). Lumefantrine, most likely, interferes with haem polymerization, which is an obligatory and critical detoxifying pathway in malaria parasites (Bakshi et al., 2000). Both agents have secondary actions that probably include inhibition of parasite nucleic acid and protein synthesis (MacArthur, 2000). The concept of combination therapy is based on the synergistic or additive potentials of two or more drugs to improve therapeutic efficacy and delay the development of resistance to the individual components of the drug combination (WHO, 2001).

The glutathione S-transferases (GSTs) activity among other functions (Sherratt and Hayes, 2001; Ziglari and Allameh, 2013) catalyzes the transfer of reduced glutathione (GSH) to reactive electrophiles. The GST activity pathways serve to protect cellular macromolecules from the deleterious effects of reactive oxygen and nitrogen species (RONS), which in turn, ensure cellular functionality and structural integrity (Sherratt and Hayes, 2001; Gilliland et al., 2004; Primavera et al., 2008). Several GST isoforms exist in different tissue/organ types (Sherratt and Hayes, 2001; Noce et al., 2012) with remarkable high occurrence in the liver, kidney and intestine (Harvey and Beutler, 1982; Ziglari and Allameh, 2013). Studies have shown that human erythrocyte glutathione S-transferase (Ery-GST) isoforms are immunologically distinct from the human hepatocyte GST isoforms (Awasthi and Singh, 1984). As at present, the physiologic role of Ery-GST is not fully known but there are suggestions that the presence of GST in erythrocytes is ideal for the detoxification and elimination of circulating xenobiotics (Ziglari and Allameh, 2013; Noce et al., 2012). There are also indications that Ery-GST function physiologically as a haemin-binding and/or transport protein in developing erythroid cells (Harvey and Beutler, 1982). Protein binding studies have shown that Ery-GST appear to bind haemin with an affinity equal to, if not greater than that of hepatocyte GST, the so-called ligandins or GST-B. Ligandins that have been posited to be responsible for the transport of haem from mitochondria to cytoplasm (Harvey and Beutler, 1982; Hamza and Dailey, 2012). However, bilirubin appears to be a poor substrate for Ery-GST compared to hepatocyte GST isoforms (Takikawa and Kaplowitz, 1988). These reports affirmed GST binding and transport functions in concurrence with its catalytic actions.
Previous studies have reported the correlation between GST activity and level of exposure of human and animal models to xenobiotics and pathogens (Sherratt and Hayes, 2001; Ismert et al., 2002; Primavera et al., 2008; Ezeji et al., 2012; Zglari and Allameh, 2013). The reproducibility and reliability of GST activity, to serve as a biomarker in the event of environmental/chemical insults, informed the use of Ery-GST activity, to serve as an index to ascertain the level of disturbance in erythrocyte physiochemical status of guinea pigs administered with pyrimethamine/sulphadoxine and artemether/lumefantrine combination therapies. The present study will give an insight into time-dependent alterations of Ery-GST activity of animal models following the administration of the two antimalarial combination therapies as prophylaxis.

MATERIALS AND METHODS
Collection/preparation of anti-malarial drug suspensions: The two antimalarial drugs, Fansidar™ (Swiss (Sipha) Pharmaceuticals Nigeria Ltd) and Coartem™ (Beijing Novartis Pharmaceutical Company Beijing China), were purchased from Cympok Pharmaceuticals, Owerri, Nigeria. Tablets of the two antimalarial drugs, each weighing 2 g, were pulverized separately into a powder form using ceramic mortar and pestle. The two ground drug samples were suspended in separate 50 mL of phosphate buffer saline (PBS) solutions (pH = 7.4) and allowed to stand in a water bath at thermostatically controlled temperature of 25±5°C under continuous agitation for 30 min after which the mixtures were separated using Whatman No. 24 filter paper.

Experimental animals: Healthy non-malarious male guinea pigs (Cavia tschudii) (8-10 weeks old) weighing 550-600 g were generous gift from Professor A.A. Uwakwe of the Department of Biochemistry, University of Port Harcourt, Nigeria. The guinea pigs were maintained at room temperature (25±5°C), 30-55% of relative humidity on a 12 h light/12 h dark cycle, with access to distilled water (DW) and standard commercial feed (SCF) (Ewu Feed Mill, Edo State, Nigeria) ad libitum for 2 weeks acclimatization period.

Ethics: The institutional review board of the Department of Biochemistry, Imo State University, Owerri, granted approval to this study. The handling of the animals was in accordance with the standard principles of laboratory animal care of the United States National Institutes of Health (NIH, 1978).

Study design: The animals were fasted for 16 h before commencement of treatment (control and test experiments) (Ibegbum and Chikezie, 2013). A single dose equivalents of pyrimethamine = 3.7 mg, sulphadoxine 0.7 mg and artemether = 0.3 mg, lumefantrine = 1.8 mg kg⁻¹ b.wt. were prepared from corresponding stock solutions of the drugs and administered by intra-peritoneal injection to the guinea pigs. A total of eighteen guinea pigs were allotted into three groups of six (n = 6) each as follows:

- **Group C1 (Control):** Guinea pigs received only PBS (Vehicle; 2.0 mL kg⁻¹ b.wt., i.p.
- **Group T1:** Guinea pigs received pyrimethamine = 3.7 mg/sulphadoxine 0.7 mg mixture/kg b.wt.; i.p.
- **Group T2:** Guinea pigs received artemether = 0.3 mg/lumefantrine = 1.8 mg mixture/kg b.wt.; i.p.

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Collection of blood and preparation of erythrocyte haemolysate: Blood samples were drawn from the anterior region of the guinea pigs, using hypodermic syringe, at time intervals of 3, 5, 9 and 24 h post-treatment for analyses. The blood volumes obtained were transferred into test tubes containing PBS solution (blood to PBS ratio 1:4; v/v). The blood suspension was subjected to bench centrifugation for 10 min. The pelleted erythrocytes were washed by methods of Tsakiris et al. (2005) as described by Chikezie et al. (2009). Within 2 h of collection of blood specimen, 0.5 mL of harvested erythrocytes were introduced into centrifuge test tubes containing 3.0 mL of buffer solution pH = 7.4: 250 mM tris (hydroxyl methyl) amino ethane-HCl (Tris-HCl)/140 mM NaCl/1.0 mM MgCl₂/10 mM glucose). The erythrocyte suspension was further centrifuged at 1200 g for 10 min and repeated for 3 times. According to Chikezie (2011), the pelleted erythrocytes were re-suspended in 3.0 mL of PBS solution and passed twice through newly packed columns (3.5 cm in a 30 mL syringe) of cellulose-microcrystalline cellulose (ratio 1:1; w/w) to obtain erythrocyte suspension sufficiently devoid of leucocytes and platelets (Kalra et al., 1981). Finally, the erythrocyte suspension was stored at 4°C and lysed by freezing/thawing as described by Galbraith and Watts (1980) and Kamher et al. (1984). The erythrocyte haemolysate was used for the determination of Ery-GST activity.

Erythrocyte haemolysate haemoglobin concentration: The cyanomethaemoglobin reaction modified method of Baure (1980), as described by Chikezie et al. (2009), was used for measurement of haemolysate haemoglobin concentration. A 0.05 mL portion of erythrocyte haemolysate was added to 4.95 mL of Drabkins reagent (100 mg NaCN and 300 mg K₄Fe(CN)₆ per liter). The mixture was left to stand for 10 min at 25±5°C and absorbance measured with a spectrophotometer (Digital Blood Analyzer®; SPECTRONIC 20, Labtech) at λₘₐₓ = 540 nm against a blank. The absorbance was used to evaluate haemolysate haemoglobin concentration by comparing the values with the standards.

Erythrocyte glutathione S-transferase: Ery-GST activity was measured by the method of Habig et al. (1974) as described by Pasupathi et al. (2009) with minor modifications according to Chikezie et al. (2009). The reaction mixture contained 1.0 mL of 0.3 mM phosphate buffer (pH = 6.5), 0.1 mL of 30 mM 1-chloro-2, 4-dinitrobenzene (CDNB) and 1.7 mL of DW. After pre-incubating the reaction mixture at 37°C for 5 min, the reaction was started by the addition of 0.1 mL of erythrocyte haemolysate and 0.1 mL of GSH substrate. The absorbance was measured at time intervals of 30 sec for 5 min at λₘₐₓ = 340 nm. Ery-GST activity was expressed in international unit per gram haemoglobin (IU/gHb) using an extinction coefficient (Σ) of 9.6 mM⁻¹ cm⁻¹ in a reaction mixture in which 1 mole of GSH was oxidized (Eq. 1).

Calculation of Ery-GST activity:

\[
E_A = \frac{100 \times \text{OD/min}}{\Sigma \times \frac{V_C}{V_H}}
\]  

(1)

Where:

- \( E_A \) = Enzyme activity in IU/gHb
- \( \text{Hb} \) = Haemolysate haemoglobin concentration (g dL⁻¹)
- \( \text{OD/min} \) = Change per min in absorbance at 340 nm
- \( V_C \) = Cuvette volume (total assay volume) = 3.0 mL
- \( V_H \) = Volume of haemolysate in the reaction system (0.05 mL)
Calculation of Area Under the Curve (AUC): Cumulative Ery-GST per h (IU/gHb.h) within the experimental time (0 h ≤ t ≤ 24 h) was evaluated using the Simpson’s Rule. Thus:

\[ f(X_1)h_1 + f(X_2)h_2 + ... + f(X_n)h_n \]

Area Under the Curve (AUC) of the plot of IU/gHb versus time (h) is given by:

\[ \text{AUC} = \left( \frac{\text{IU}}{\text{gHb}} \times h \right) = \frac{h}{2} (y_n + 2y_{n-1} + 2y_{n-2} + ... ) \] (2)

Where:
- \( h \) = Time intervals (h)
- \( y \) = Ery-GST activity (IU/gHb) at corresponding time interval

Statistical analyses: The data collected was analyzed by the analysis of variance (ANOVA) procedure while treatment means were separated by the Least Significance Difference (LSD) incorporated in the Statistical Analysis System (SAS) package of 9.1 version.

RESULTS

Table 1 showed that within 0 h ≤ t ≤ 24 h, Ery-GST activity of group C1 (C1\textsubscript{GST}) was fairly constant and did not exhibit significant alterations (p<0.05). Conversely, Ery-GST activity of group T1 (T1\textsubscript{GST}) varied within the range of 5.04±0.98-5.60±0.59 IU/gHb with peak Ery-GST activity at the commencement of the experiment (t = 0 h) (Table 1). In addition, T1\textsubscript{GST} activity showed lower enzyme activity relative to C1\textsubscript{GST} activity. Specifically, at t = 6 h, T1\textsubscript{GST} activity was significantly (p<0.05) lower than C1\textsubscript{GST} activity, whereas no significant difference (p>0.05) was exhibited at t = 3 h. The lowest T1\textsubscript{GST} activity was registered at t = 9 h, which represented 90% relative enzyme activity compared to T1\textsubscript{GST} activity at t = 0 h (Fig. 1).

A cursory look at Table 1 showed that at t = 0 h, T1\textsubscript{GST} and T2\textsubscript{GST} activities were not significantly different (p>0.05) from C1\textsubscript{GST} activity. Similarly, at t = 9 h, T2\textsubscript{GST} activity was not significantly different (p>0.05) from T1\textsubscript{GST} activity. The lowest T2\textsubscript{GST} activity was at t = 3 h, which corresponded

![Fig. 1: Profile of guinea pigs erythrocyte glutathione S-transferase activity](image-url)

Table 1: Guinea pigs erythrocyte glutathione S-transferase activity

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>C1</th>
<th>T1</th>
<th>T2</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.63±0.31</td>
<td>5.60±0.39</td>
<td>5.73±0.37</td>
</tr>
<tr>
<td>3</td>
<td>5.64±0.21</td>
<td>5.38±0.11</td>
<td>4.58±0.73</td>
</tr>
<tr>
<td>6</td>
<td>5.67±0.16</td>
<td>5.15±0.80</td>
<td>4.99±0.27</td>
</tr>
<tr>
<td>9</td>
<td>5.68±0.15</td>
<td>5.04±0.98</td>
<td>5.06±0.26</td>
</tr>
<tr>
<td>24</td>
<td>5.61±0.11</td>
<td>5.25±0.28</td>
<td>5.16±0.29</td>
</tr>
</tbody>
</table>

Results are mean (X)±SD of six (n = 6) determination. Means in the row with the same letter are not significantly different at p>0.05 according to LSD.

Table 2: Area under the curve (AUC_{0,24h}) of guinea pigs erythrocyte glutathione S-transferase activity

<table>
<thead>
<tr>
<th>Parameter</th>
<th>C1</th>
<th>T1</th>
<th>T2</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC (U/gHb·h)</td>
<td>124.29±0.19</td>
<td>113.99±0.66</td>
<td>111.48±0.39</td>
</tr>
</tbody>
</table>

Results are mean (X)±SD of six (n = 6) determination. Means in the row with the same letter are not significantly different at p>0.05 according to LSD.

to 79.93% relative Ery-GST activity post-treatment (Fig. 1). Generally, within 0 h±24 h, Ery-GST activity of the three experimental groups was in the order; C1>T1>T2.

Figure 1 showed that the Ery-GST activity profile of the test groups (T1 and T2) were biphasic, T1_{GST} and T2_{GST} activities were lower compared to C1_{GST} activity. For instance, within the experimental time of 24 h, t<9 h represented the inhibition phase of T1_{GST} activity followed by the enzyme recovery phase, exemplified by relative increase in T1_{GST} activity at t = 9 h by 3.97% compared to T1_{GST} activity at t = 24 h (p<0.05). Likewise, t<3 h represented decreasing T2_{GST} activity, whereas t>3 h showed progressive increase in T2_{GST} activity relative to pre-treatment time at t = 0 h (Fig. 1).

The cumulative Ery-GST activity profile and capacity of the two drugs to alter erythrocyte enzyme activity in the various experimental groups are presented in Table 2. Thus, AUC_{0,24h} showed that the level of alterations in T1_{GST} activity was comparable to T2_{GST} activity and was significantly different (p<0.05) from C1_{GST} activity.

DISCUSSION

Cellular levels of GST activity are of immense application in providing insights into the etiology and diagnosis of diverse pathologic states and for monitoring toxicological and therapeutic events. There are reports on over-expression of GST in erythrocytes of individuals with chronic renal failure with attendant uremia (Romeu et al., 2010) and hyperbilirubinemia (Noce et al., 2012). Also, toxicological studies of human erythrocytes exposed to heavy metals (Goodrich and Basu, 2012) and rainbow trout erythrocytes incubated in aminoglycoside antibiotics in vitro (Comakli et al., 2011) as well as animal models exposed to particulate matters (Gililand et al., 2004), gas substances, aromatic/organic chemicals in situ (Ismert et al., 2002) exhibited decreased levels of Ery-GST activity in a dose/concentration dependent manner. Furthermore, the activities of redox enzymes, Ery-GST activity inclusively serve as reliable biomarkers in environmental and toxicological studies. For instance, Ery-GST activity previously had been employed as a tool for
assessing levels of pesticide contaminations (Domingues et al., 2010) and exposure to pollutants involving two crustacean species; water flea *Daphnia magna*, terrestrial isopod *Porcellio scaber* (Jemec et al., 2010) as well as fish (*Cyprinus carpio* L.) (Valon et al., 2013).

The present study has equally shown that non-malarious guinea pigs administered with pyrimethamine/sulphadoxine and artemether/lumefantrine combination therapies exhibited reduced levels of Ery-GST activity, which was a spinoff effect of the biotransformational pathways of the drugs *in vivo*. The concerted role of cellular GST and cytochrome P450 superfamily in the metabolism of xenobiotics and pathogenic metabolites has been described (Ziglari and Allameh, 2013), which serves to explain the basis for the pattern of Ery-GST activity of present study. Also from the present study, the subsequent time-dependent increasing levels of Ery-GST activity (recovery phases) after initial decay/attenuation of enzyme activity (inhibition phase) was in a manner similar to those earlier reported by Ayalogu et al. (2001). They noted that rats injected with gasoline exhibited early short term low activity of GST, which also affirmed the outcome of Chiapotto et al. (1995) report on inactivation of GST activity by different concentrations of acetaldehyde. Their study further suggested that detoxification process and eventual systemic clearance of the causative metabolites accounted for the subsequent increasing levels in GST activity of rats administered with kerosene and crude oil (bonny light) as the experimental time progressed. Rathore et al. (1998) had reported similar characteristic pattern of GST activity in oxidative stressed rats induced by the administration of isoproterenol. They noted that inactivated GST activity partially recovered 12 h after the administration of isoproterenol in efforts by antioxidant system to counteract and ameliorate oxidative stress. The capability of artemether, lumefantrine and sulphadoxine to generate RONS (Bakshi et al., 2000; MacArthur, 2000; Nzila, 2006; Chikezie et al., 2009; Crespo-Ortiz and Wei, 2012) could be responsible for the time-dependent biphasic Ery-GST activity profile of guinea pigs administered with pyrimethamine/sulphadoxine and artemether/lumefantrine combination therapies.

The initial decreasing levels of Ery-GST activity was in connection with the consumption of GST cofactor-GSH, one of the first lines of antioxidant defense mechanism against xenobiotics, which might have accounted for depletion of intracellular GSH with resultant decreasing levels of relative Ery-GST activity (Sherrick and Hayes, 2001; Primavera et al., 2008; Ezeji et al., 2012). However, increasing levels of Ery-GST activity after the initial inactivation phase, occasioned by low levels of GSH among other factors, could have prompted the positive activation and up-regulation of the redox enzymes in effort to restore homeostasis and prevent cellular damage (Ancisike et al., 1991) consequent upon the propensity of these chemical agents to overwhelm cellular antioxidant system. More recently, reports have shown that xenobiotics promote the expression and activation of transcriptional factors, particularly the nuclear factor erythroid-2 (Nrf2) that has been implicated in up-regulation of antioxidant enzymes (Lodovici and Bigagli, 2011). The processes leading to up-regulation of redox enzymes are to ensure that the cell is equipped with adequate and sustainable quantity of requisite antioxidant enzymes for neutralization of potentially noxious chemical agents.

**CONCLUSION**

The findings of the present study showed that Ery-GST activity of non-malarious guinea pigs administered with pyrimethamine/sulphadoxine and artemether/lumefantrine combination therapies gave a characteristic biphasic profile. The perturbation of erythrocyte physiochemistry of non-malarious guinea pigs administered with pyrimethamine/sulphadoxine and
artemether/lumefantrine combination therapies was typified by the disparities in cumulative Ery-GST activity (IU/gHb.h) among the experimental groups (C1, T1 and T3), which could be of relevance from toxicological and therapeutic standpoints.

REFERENCES


