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In Vitro Studies of Human Erythrocyte Glutathione-S Transferase Activity in the Presence of Five Antimalarial Drugs.

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ABSTRACT: *In-vitro* effect of the five antimalarials(Fansidar, Halfan, Quinine, Coartem and Chloroquine phosphate) on levels of erythrocyte glutathione-S transferase (GST) activity was ascertained in the presence of separate increasing concentrations (0.2, 0.4, 0.6, and 0.8mg% w/v) of the five drugs. Erythrocyte GST activity was determined spectrophotometrically at 340nm with the standard substrate (1-chloro-2,4-Dinitrobenzene) and co-substrate (reduced glutathione). The control/reference values ranged between 3.27+/-0.13 and 3.40+/-0.05iu/gHb. The introduction of increasing concentrations of Fansidar, Quinine, Coartem, and Chloroquine Phosphate engendered decreasing levels of GST activity in a concentration dependent manner, which was in the order: Chloroquine Phosphate> Coartem> Quinine> Fansidar. Specifically, 0.8mgpercentage of Chloroquine Phosphate showed the highest capacity to cause decreased GST activity from values of 3.41+/-0.06 to 2.18+/-0.09 iu/gHb. This represented 33.8% relative inhibition. In contrast, concentrations of Halfan between 0.2 to 0.6mgpercent caused elevation of GST activity above the values of the control samples. However, these increased values of GST activity was not significantly different (p>0.05) compared to the control samples. The five antimalarials caused disturbances in erythrocyte GST activity. This was an indication these drugs perturbed the oxidative potentials of human erythrocytes.

KEYWORDS: Antimalarials, 1-chloro-2,4-dinitrobenzene, erythrocytes, glutathione S-transferase (GST) activity, *Invitro*.

INTRODUCTION

The ubiquitous enzyme glutathione *S*-transferase (GST; EC 2.5.1.18) is primarily involved in the neutralization of harmful exogenous or endogenous compounds by enzymatic conjugation with the scavenger peptide glutathione (GSH) and/or by direct binding of non-substrate ligands (Habig *et al*., 1974; Backett and Hayes, 1993). Other functions of GST include protection against oxidative damage to lipids and nucleic acids and participation in the metabolism of some steroids and leukotrienes (Backett and Hayes, 1993). This enzyme is represented by a family of cytosolic proteins bearing a dimeric structure, the polymorphic expression of which has been widely studied in human tissues and in a wide series of cells (Backett and Hayes, 1993; Corrigail and Kirsch, 1988). In human erythrocytes, GST is present in large amounts (Marcus *et al.,* 1978) and in two forms: a highly cationic enzyme (designated with the Greek letter rho), which accounts for <5% of the total GST activity; and the main anionic enzyme corresponding to the P form (rho) (Awasthi and Singh, 1984; Fazi *et al.,* 1991). Because of its abundance and overlapping substrate specificity with the rho form, the GST P1-1 dimer is often considered the sole GST enzyme in the erythrocyte (Beckett and Hayes, 1993; Fazi *et al.,* 1991).

Chemotherapeutic agents interact with red cell constituents, specifically, haemoglobin, diverse enzymes and membrane architectural components. Therefore, this present study intend to ascertain the capacity of five antimalarial drugs (Fansidar™, Halfan™, Quinine, Coartem™, and Chloroquine phosphate) to interfere with erythrocyte GST activity. One of the various enzymes is required for cellular integrity and functionality.

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).

Collection of Blood Samples/Preparation of Erythrocyte Haemolysate:

Twenty-five (25) blood samples of HbAA genotype from clinically confirmed healthy non-malarious male volunteers qualified for this study. Five milliliters (5.0ml) of venous blood obtained from the volunteers by venipuncture was stored in EDTA anticoagulant tubes. The erythrocytes were washed by methods as described by Tsakiris *et al.,*(2005).Within 2hours of collection of blood samples, portions of 1.0ml of the samples were introduced into centrifuge test tubes containing 3.0ml of buffer solution pH=7.4: 250mM tris (hydroxyl methyl) amino ethane HCl (Tris - HCl)/140mMNaCl/I.0mMMgCl_2/10mMglucose). The erythrocytes were separated from plasma by centrifugation at 1200g for 10minutes, washed three times by three similar centrifugations with the buffer solution. The erythrocytes re-suspended in 1.0ml of this buffer were stored at 4^0C. 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 glutathione-S transferase activity.

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Determination of Erythrocytes Haemolysate Haemoglobin Concentration:

A modified method (Baure, 1980), based on cyanomethaemoglobin reaction was used for the determination of haemolysate haemoglobin concentration. The expressed values were in grams per deciliter (g/dl). A 0.05ml portion of human red blood cell haemolysate was added to 4.95ml of Drabkin reagent. The mixture was left to stand for 10minutes and absorbance read at λmax=540nm against a blank (Drabkin reagent only). The absorbance was used to evaluate for haemolysate haemoglobin concentration by comparing the values with the standards.

Determination of Erythrocyte Haemolysate Glutathione S-transferase Activity:

Glutathione-S transferase activity was assayed spectrophotometrically by monitoring the conjugation of 1-chloro-2,4dinitro benzene (CDNB) with glutathione (GSH) at λmax=340nm at 37°C. (Habig *et al*., 1974).

CDNB + GSH ⟶ CDNB- S- glutathione.

The enzyme assay was according to methods of Habig *et al.,* (1974) with minor modifications (Anosike *et al*.,1991).The 1.0ml in 2% ethanol enzyme assay mixture contained 0.5mMCDNB (0.02ml),1.0mMGSH(0.05ml),0.68ml of distilled water and 100mMPhosphate buffer (K_2HPO_4/KH_2PO_4; pH=6.5) (0.2ml).

The CDNB was pre-mixed with the Phosphate buffer before use. The Phosphate buffer-CDNB mixture was pre-incubated for 10 minutes at 37°C and the reaction started by adding GSH, followed immediately by an aliquot (0.05ml) of the haemolysate. To the control samples, the rate of increase in absorbance at λmax = 340nm was measured for 10minutes at 37°C against a blank solution containing the reaction mixture, in which; the haemolysate was substituted with distilled water.

To the test samples, 0.68ml of varied concentrations (0.2, 0.4, 0.6 and 0.8mg% w/v) of the five (5) separate antimalarial drugs were added to corresponding enzyme assay test tubes. In the control experiment, the various drug concentrations were substituted with distilled water.

Statistical Analyses:

The experiments were designed in a completely randomized method and data collected were analyzed by the analysis of variance procedure while treatment means were separated by the Least Significance Difference (LSD) incorporated in the Statistical Analysis System (SAS) package of 9.1 version, (2006).

Calculation of Enzyme Activity:

The expression below was used to evaluate erythrocyte glutathione-S transferase activity in international unit per gram haemoglobin (iu/gHb).

$$E_A = \frac{100}{Hb} \times \frac{0.D/min}{E} \times \frac{V_C}{V_H}$$

Where,

E_A = Enzyme activity in iu/gHb

Hb = Haemolysate haemoglobin concentration (g/dl)

0. D/min = Change per minute in absorbance at 340nm.

E = Millimolar extinction coefficient = 9.6, in reaction in which 1mole of glutathione (GSH) is oxidized or reduced.

V_C = Cuvette volume (total assay volume) = 1.0ml.

V_H = Volume of haemolysate in the reaction system (0.05ml).

RESULTS:

The mean (+/-S.D) of erythrocyte haemolysate GST activity and the corresponding relative enzyme activity in the presence of increasing experimental concentrations of the five antimalarial drugs is presented in Table1below.

The control/reference values ranged between 3.27+/-0.13 and 3.40+/-0.05iu/gHb. Introduction of increasing concentrations of Fansidar, Quinine, Coartem, and Chloroquine Phosphate engendered decreasing levels of GST activity in a concentration dependent manner, which was in the order: Chloroquine Phosphate> Coartem> Quinine> Fansidar. Specifically, 0.8mgpercentage of Chloroquine Phosphate showed the highest capacity to cause decreased GST activity from values of 3.41+/-0.06 to 2.18+/-0.09 iu/gHb. This represented 33.8% relative inhibition.

In contrast, concentrations of Halfan between 0.2 to 0.6mgpercent caused elevation of GST activity above the values of the control samples. However, these increased values of GST activity was not significantly different (p>0.05) compared to the control samples.

DISCUSSION

In the present *in vitro* study, the most likely explanation for the drop in erythrocyte GST activity was because of the alternative role the enzyme in detoxification- the irreversible binding of xenobiotics to the enzyme. In concordance with the present findings, Ayalogu *et al.*, (2000) reported that rat erythrocyte enzyme (GST) was found to be inhibited *in vitro*, by the antimalarials, alkaloid drugs, chloroquine and Fansidar (sulfadoxine + pyrimethamine); an effect that was interpreted to mean a possibility of these drugs being capable of raising the oxidant stress of the erythrocytes. In a different study, *in vitro* incubation of rat liver GST with a variety of chlorophenoxyalkyl acid herbicides (CPAs) resulted in a dose dependent inhibition of GST activity (Dierickx, 1983) that was in concordance with the present investigations

.Incubation of GST from three aquatic species with CPAs, quinones and *o*-chloranil also resulted in inhibition (Dierickx, 1984). Similarly, haloacetonitriles also inhibited the enzyme activity *in vitro* and *in vivo* after 18 hours (Lin and Guion, 1989). Most importantly, Davies, (1985) demonstrated that chlorothalonil could bind to GST at low glutathione concentrations.

Table 1.0: *In Vitro* Glutathione S-transferase Activity of HbAA Erythrocyte Haemolysate in the Presence of Five Antimalarial Drugs:

[DRUG]mg%	GST Activity (iu/gHb)	Relative Activity(%)
1).FANSIDAR		
0.0	3.41+/-0.06[a]	100.0
0.2	3.39+/-0.13[a]	99.4
0.4	3.35+/-0.06[a,b]	98.2
0.6	3.22+/-0.05[b,c]	94.4
0.8	3.09+/-0.16[c]	90.6
2).HALFAN		
0.0	3.37+/-0.08[a]	100.0
0.2	3.39+/-0.10[a]	100.6
0.4	3.41+/-0.10[a]	101.2
0.6	3.40+/-0.10[a]	100.9
0.8	3.34+/-0.15[a]	99.1
3).QUININE		
0.0	3.27+/-0.27[a]	100.0
0.2	3.04+/-0.07[b]	93.0
0.4	2.43+/-0.07[c]	74.3
0.6	2.41+/-0.09[c]	73.7)
0.8	2.39+/-0.07[c]	73.1)
4).COARTEM		
0.0	3.37+/-0.07[a]	100.0
0.2	2.46+/-0.08[b]	73.0
0.4	2.47+/-0.12[b]	73.3
0.6	2.39+/-0.07[b,c]	70.9
0.8	2.30+/-0.08[c]	68.2

[DRUG]mg%	GST Activity (iu/gHb)	Relative Activity(%)
5).CHLOROQUINE (P)		
0.0	3.29+/-0.09[a]	100.0
0.2	2.44+/-0.06[b]	74.2
0.4	2.29+/-0.09[c]	69.6
0.6	2.19+/-0.04[c,d]	66.6
0.8	2.18+/-0.05[d]	66.3

The binding was irreversible, probably covalent, and inhibited the enzyme activity. Since the production of enzyme is energetically expensive, it seems logical that this defensive mechanism only came into play when enzymatic detoxification had been overwhelmed. It is possible that the decreased glutathione concentration represented an overwhelming of the antioxidant defense, leaving GST opened to attack. Replenishment of glutathione levels followed recovery and increasing GST activity with time.

It is important to mention that GSTs also have a high affinity for endogenous compounds such as bile acids, haemin, bilirubin, fatty acids and steroids (Hayes and Pulford, 1995; Hiller *et al.,* 2005), but the GSTs involved do not form glutathione conjugates with their substrates (Litwack *et al.,* 1971; Hayes and Pulford, 1995). Therefore, these GSTs have been termed "ligandins" and the non-enzymatic substrates referred to as "non-substrate ligands" (Mannervik and Danielson, 1988). This interaction most often compromise GST activity (Hiller *et al.,* 2005). These findings are been exploited to achieve therapeutic benefits in malaria disease. Ahmad and Srivastava, (2007), averred that selective inhibition of this enzyme from malarial parasites by various classes of inhibitors could be viewed as a potential chemotherapeutic strategy to combat malaria. These authors showed that purified GST from *Plasmodium yoelii* was inhibited by compounds like protoporphyrin IX, cibacron blue, as well as by the GSH depleting agent menadione. Therefore, the selective inhibition of parasitic GST in relation to the host cell by these agents showed their promising therapeutic benefits in malaria chemotherapy.

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