



Original Research Article

## Impact of Abacavir Sulfate and Lamivudine on Rat Erythrocyte Methaemoglobin Concentration and NADH Methaemoglobin Reductase Activity

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### ABSTRACT

The effects of two nucleoside analogue class of antiretroviral drugs, abacavir sulfate and lamivudine on methaemoglobin concentration and methaemoglobin reductase activity in the erythrocytes of wistar rats were investigated. Drugs at dosage 0.2, 0.4, 0.6 and 0.8mg/100g body weight of lamivudine; and 0.6, 0.8, 1.0 and 1.2mg/100g body weight of abacavir sulfate were administered orally by intubation. Blood samples were collected from the experimental animals by cardiac puncture. Spectrophotometric methods were used to ascertain the methemoglobin concentrations and the activity of NADH methaemoglobin reductase (NADH MR). The results revealed that both drugs increased methaemoglobin concentrations. The increase from  $1.53 \pm 0.11$  to  $3.53 \pm 0.03$  in the presence of abacavir sulfate was significant ( $p < 0.05$ ), while lamivudine cause a non-significant ( $p > 0.05$ ) increase from  $1.52 \pm 0.11$  to  $1.61 \pm 0.03$ . Methaemoglobin reductase activity decreased significantly ( $p < 0.05$ ) from  $5.50 \pm 0.13$  to  $3.74 \pm 0.01$  in the presence of abacavir sulfate, while the decrease from  $5.21 \pm 0.09$  to  $4.90 \pm 0.02$  due to lamivudine was not significant ( $p < 0.05$ ). To an extent both drugs adversely affected erythrocytes metabolism, thus compromising its important role of maintaining haemoglobin in its reduced state for oxygen transport.

**Keywords:** Antiretroviral drugs Abacavir Sulfate, Lamivudine, Methaemoglobin concentration, Methaemoglobin Reductase, Erythrocyte.

### INTRODUCTION

Methemoglobin (MetHb) is a form of the oxygen-carrying metalloprotein hemoglobin, in which the iron in the heme group is in ferric ( $\text{Fe}^{3+}$ ) state, not the ferrous ( $\text{Fe}^{2+}$ ) of normal haemoglobin. In this state it is unable to bind oxygen. MetHb is continuously formed in the normal red blood cells by the process of autoxidation and also MetHb is rapidly reduced to hemoglobin by intraerythrocytic MetHb reductase after sampling (Shihana *et al.*, 2011). Methaemoglobin is continuously being reduced by specific mechanisms so

that concentration at any given time is very small less than 1- 2% of the total pigment. (Wright *et al.*, 1999; Trapp and Will 2010). The NADH-Methaemoglobin Reductase (NADHMR) enzyme reduces MetHb to hemoglobin in the body. Methemoglobinemia results due to either inadequate activity of this enzyme or due to much MetHb production.

NADHMR (EC: 1.6.2.2) is an enzyme localized in the endoplasmic reticulum of all cells (Dekker *et al.*, 2001). It transfers electrons from NADH to cytochrome b5 via its flavin adenine

dinucleotide (FAD) prosthetic group (Yubisui and Takeshita, 1980). Under normal conditions, it is the only system within the erythrocyte responsible for maintaining hemoglobin in its oxygen-carrying reduced state (Rockwood *et al.*, 2003). The second form of this enzyme is NADPH dependent, is less active in mammals in converting MHb back to hemoglobin than the NADH dependent form (Smith, 1991). In general, cellular activity of NADH-Met HbR reflects an organism's capacity to reduce MHb. Thus, it is an important factor in evaluating MHb formation and etiology of methaemoglobinemia. It is the rate-limiting enzyme controlling the toxicokinetics of the reduction of MHbR. The activity of NADH-MetHbR is generally reduced in erythrocyte of patients with recessive hereditary methaemoglobinemia (Borgese *et al.*, 1987). Activity of this enzyme has been measured in a variety of non-human species (Whittington *et al.*, 1995) and these studies have reported that species with lower NADH-MetHbR activities convert MHb back to haemoglobin at slower rates than the species with higher NADH-MetHbR activities (Smith, 1991).

Abacavir sulfate and lamivudine are nucleoside analogue reverse transcriptase inhibitor class of antiretroviral drugs. Abacavir sulfate is (1S,cis)-4-[2-amino-6-(cyclopropylamino) - 9H-purin-9-yl]-2-cyclopentene-1-methanol sulfate, an enantiomer with 1S, 4R absolute configuration on the cyclopentene ring (Lachlam *et al.*, 2013), while lamivudine is (2R,cis)-4-amino-1-(2-hydroxymethyl-1, 3-oxathiolan-5-yl) - (1H) - pyrimidin-2-one. Lamivudine is the (-) enantiomer of a dideoxy analogue of cytidine, also referred to as (-) 2', 3'-dideoxy, 3 thiacytidine (DAD, 2008).

Being substrate analogue, CBV-TP inhibits reverse transcriptase enzyme both by competing with the natural substrate, dGTP and by its incorporation into the viral DNA (Lachlam *et al.*, 2013). The principal mode of action of 3TC-TP is the inhibition

of the RNA- and DNA-dependent polymerase activities of HBV reverse transcriptase via DNA chain termination after incorporation of the nucleotide analogue into viral DNA (DAD, 2008). This research intends to ascertain the capacity of the antiretroviral drugs namely, lamivudine and abacavir sulphate, to alter the methaemoglobin concentration and NADH methaemoglobin reductase activity as well as its ability to reduce methaemoglobin to the oxygen carrying haemoglobin in the erythrocytes.

## MATERIALS AND METHODS

### Drug preparation

The drugs were crushed into fine powder using a clean mortar and pestle. The crushed drugs were then mixed with distilled water into different concentrations. The concentrations were calculated from the normal dosage in a 70kg man.

### Experimental design

Thirty rats with average body weight 100 grams were allowed to acclimatize for two weeks and were distributed into two groups. Each group was divided into five subgroups. The first in each subgroup in each group served as control while the other four groups served as tests to which four different dosages (0.2, 0.4, 0.6 and 0.8mg/100g body weight of lamivudine; and 0.6, 0.8, 1.0 and 1.2mg/100g body weight of abacavir sulfate) of the drugs were administered to the rats by intubation. Blood samples were collected from the rats by cardiac puncture each week for analysis.

### Sample collection and preparation

Blood samples obtained from the rats by cardiac puncture were stored in EDTA anticoagulant tubes. The erythrocytes were washed by methods as described by Tsakiris *et al.*, (2004). The method: 1.0 ml portion of the sample was introduced into centrifuge test tubes containing 3.0 ml of buffer solution (pH 7.4): 250mM tris (hydroxyl methyl) amino ethane-HCl (Tris-HCl)/140m MNaCl/1.0m MMgCl<sub>2</sub>/ 10mMglucose). The erythrocytes were separated from plasma by

centrifugation at 1200g for 10 minutes, washed and centrifuged thrice with the buffer solution. The erythrocytes were resuspended in 1.0 ml of this buffer and stored at 4°C. The washed erythrocytes were lysed by freezing and thawing as described by Galbraith and Watts (1980). The erythrocyte haemolysate was used to determine the erythrocyte enzyme activity.

#### **Determination of Methaemoglobin Concentration**

Method used in the determination of methaemoglobin content of erythrocyte haemolysate was a modified form of the method of Evelyn and Malloy, (1938) as described by Chikezie et al., (2009). 400µl of 0.5M Phosphate buffer (pH 6.5) was added to 600µl of the cell haemolysate and was centrifuged at 16,000g for 5 minutes to sediment debris. 700µl of the supernatant was used to measure the absorbance at 630nm and the reading was recorded as S1. 50 µl of 10g% KCN was added and after 5minutes at room temperature the second reading S2 was recorded. KCN converts methaemoglobin to cyanomethaemoglobin, which does not absorb at 630nm. The difference between absorbance readings of 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 630nm was recorded and then KCN was added to form cyanomethaemoglobin. 70 µl of the supernatant fraction was diluted 10-fold into 600 µl of 0.1M phosphate buffer (pH=6.5). Then, 30µl of freshly prepared 20g% K<sub>3</sub>Fe (CN)<sub>6</sub> was added, and then incubated for 5minutes at room temperature and an initial reading (T1) was taken. After that 50 µl of 10% KCN was subsequently added, and the second reading (T2) was recorded. The percent methaemoglobin in the sample was calculated by the formula  $[100(S1-S2)] / [10(T1-T2)]$ .

#### **Determination of Haemolysate NADH-Methaemoglobin Reductase**

Activity NADH-MR activity of erythrocyte haemolysate was assayed according to the method of Board et al. (1990). The reaction mixture contained 0.2ml tris-HCl/EDTA buffer pH=8.0, 0.2ml NADH and 0.35ml of distilled water, 0.2ml of K<sub>3</sub>Fe(CN)<sub>6</sub> and 0.05ml of erythrocyte haemolysate.

The 0.2ml tris-HCl/EDTA buffer pH=8.0, 0.2ml NADH and 0.35ml of distilled water were introduced into a test tube and incubated for 10minutes at 30°C. The content was transferred into a cuvette and the reaction was started by adding 0.2ml of K<sub>3</sub>Fe (CN)<sub>6</sub> followed by 0.05ml of erythrocyte haemolysate. The increase in absorbance of the medium at 30°C was followed spectrophotometrically at 340nm for 10 minutes at 60 seconds intervals against a blank solution.

#### **Calculation of NADH-Methaemoglobin Reductase Activity**

The equation below was used to evaluate erythrocyte NADH-MR activity in international unit per gram haemoglobin (IU/gHb).

$$E_A = \frac{100}{[Hb]} \times \frac{O.D/min}{\Sigma} \times \frac{V_C}{V_H}$$

Where, EA is the Enzyme activity in IU/gHb. [Hb] is the Haemolysate haemoglobin concentration (g/dl). O.D/min is the Change per minute in absorbance at 340nm. Σ is the Millimolar extinction coefficient = 6.22, in reaction in which 1mole of NADH + H<sup>+</sup> is oxidized.

VC is the Cuvette volume (total assay volume) = 1.0ml. VH is the Volume of haemolysate in the reaction system (0.05ml).

## **RESULTS AND DISCUSSION**

Erythrocyte methaemoglobin determination as a toxic endpoint in chemical poisoning and pathologic conditions has shown reliability in clinical diagnosis (Hopkins, 2000). The present study reveal that the two oral antiretroviral drugs investigated caused increase in methaemoglobin concentration and decrease in erythrocyte NADHMR activity in the rat

erythrocytes investigated.

Tables 1 and 2 present the mean  $\pm$  SD of methaemoglobin concentration and NADH-Methaemoglobin reductase activity in the presence of different concentrations of lamivudine and Abacavir sulfate. Significant ( $p < 0.05$ ) decrease was observed in NADHMR activity in the presence of abacavir sulfate, but the decrease due to lamivudine was not significant ( $p > 0.05$ ).

**Table 1: Rat erythrocyte meth and NADHMR in the presence of different concentrations of lamivudine**

DOSAGE (mg/100g body weight)	Lamivudine	
	%METH	NADHMR
0.00	1.52 $\pm$ 0.11 <sup>a</sup>	5.21 $\pm$ 0.09 <sup>a</sup>
0.20	1.55 $\pm$ 0.02 <sup>a</sup>	5.13 $\pm$ 0.12 <sup>a</sup>
0.40	1.57 $\pm$ 0.20 <sup>a</sup>	5.10 $\pm$ 0.11 <sup>a</sup>
0.60	1.60 $\pm$ 0.21 <sup>a</sup>	5.11 $\pm$ 0.22 <sup>a</sup>
0.80	1.61 $\pm$ 0.03 <sup>a</sup>	4.90 $\pm$ 0.02 <sup>a</sup>

Values are recorded as MEAN $\pm$ SD of triplicate determinations. Means with same superscript letters are not statistically different at 95% confidence limit ( $p < 0.05$ ).

**Table 2: Rat erythrocyte meth and NADHMR in the presence of different concentrations of abacavir sulfate.**

DOSAGE(mg/100g body weight)	Abacavir Sulfate	
	%METH	NADHMR
0.00	1.53 $\pm$ 0.11 <sup>a</sup>	5.50 $\pm$ 0.13 <sup>a</sup>
0.60	2.21 $\pm$ 0.01 <sup>b</sup>	4.52 $\pm$ 0.01 <sup>b</sup>
0.80	2.73 $\pm$ 0.20 <sup>c</sup>	4.11 $\pm$ 0.25 <sup>c</sup>
1.00	3.10 $\pm$ 0.20 <sup>c</sup>	3.90 $\pm$ 0.21 <sup>c</sup>
1.20	3.53 $\pm$ 0.03 <sup>c</sup>	3.74 $\pm$ 0.01 <sup>c</sup>

Values are recorded as MEAN $\pm$ SD of triplicate determinations. Means with same superscript letters are not statistically different at 95% confidence limit ( $p < 0.05$ ).

Methaemoglobin is a derivative of haemoglobin in which the iron of the deoxygenated heme complex has been oxidized to the ferric form. In this state, haemoglobin is incapable of being oxygenated, thus it is of no value for respiration. This is because methaemoglobin which is not accompanied by superoxide ( $O_2^-$ ) renders haemoglobin incapable of binding normal triplet  $O_2$  as it occurs in the air. Methaemoglobin levels are usually expressed as a percentage of total haemoglobin. A small amount of haemoglobin naturally becomes oxidized during oxygen transport, but endogenous mechanism exist to reduce methaemoglobin, so normally only about 1-2% of the bodies

haemoglobin exist as methaemoglobin (Wright *et al.*, 1999; Trapp and Will 2010). However, cyanosis becomes apparent when more than 1.5g % of methaemoglobin appears in the blood (Jaffe, 1981). A variety of naturally occurring substances including nitrates in vegetables, industrial chemicals and therapeutic agents are capable of causing methaemoglobin formation, either directly or indirectly through their metabolites (Bradberry, 2003; Trapp and Will 2010).

The decreases in NADH methaemoglobin reductase activity observed in this research were dose dependent. NADH methaemoglobin reductase is an enzyme that is required to convert methaemoglobin to haemoglobin. This enzyme therefore ensures that methaemoglobin is maintained in very low concentrations and that iron exist in a more useful ferrous form ( $Fe^{2+}$ ). By this act, NADH methaemoglobin reductase increases the oxygen binding capacity of haemoglobin making it useful for respiration. The decreases observed in the activity of NADH-methaemoglobin reductase in this work indicates that abacavir sulfate and lamivudine reduce the oxygen binding capacity of haemoglobin since they reduce the ability of NADH methaemoglobin reductase to reduce the  $Fe^{3+}$  in methaemoglobin to  $Fe^{2+}$ .

However, the significant ( $p < 0.05$ ) increase in the concentration of methaemoglobin in the presence of abacavir sulfate was expected because abacavir sulfate reduced the ability of the NADH dependent reductase to reduce methaemoglobin ( $Fe^{3+}$ ) to haemoglobin ( $Fe^{2+}$ ). Jaffe (1981) reported that deficiencies in the reductase systems and resulting clinical manifestations have been described. Thus the increases in methaemoglobin concentrations in the rats studied was expected in the presence of the both drugs since they decreased the activity of NADH methaemoglobin reductase, an enzyme responsible for the conversion of methaemoglobin back to haemoglobin.

Under normal conditions, this NADH-dependent enzyme also referred to as ferricyanide reductase, NADH-diaphorase or cytochrome b5 reductase is the only system within the erythrocyte that maintains hemoglobin in its oxygen-carrying reduced state (Rockwood *et al*, 2003), thus ensuring that methaemoglobin is maintained in very low concentration; and that the iron in haemoglobin exists in its useful form.

Increased methaemoglobin concentration as a consequence of decreased NADHMR activity as observed in this work was reported by some researcher. Lo and Agar (1986) and Whittington *et al.*, (1995) that showed that the capacity of non-human species to revert methaemoglobin ( $\text{Fe}^{3+}$ ) to haemoglobin ( $\text{Fe}^{2+}$ ) is inextricably connected with the NADH dependent reductase activity of the corresponding erythrocytes. Uwakwe *et al.*, (2015) reported this inverse relationship between methaemoglobin concentration and NADHMR activity in the presence of nevirapine and efavirenz in rat erythrocytes.

## CONCLUSION

The effect of increasing lamivudine and abacavir sulfate concentrations lead to increasing methaemoglobin concentration as a consequence of decreased NADHMR activity, which ultimately distort the  $\text{Fe}^{2+}$ ,  $\text{Fe}^{3+}$  ratio. The impact on the erythrocytes posed by abacavir sulfate was more than that posed by lamivudine. Both drugs adversely affected erythrocytes ability to maintain haemoglobin in its oxygen carrying state. Methaemoglobinemia is a potentially life-threatening condition that can be caused by a variety of drugs at therapeutic or supertherapeutic doses. Thus appropriate measures should be adhered to while prescribing these medicines to subjects considering these possible effects on the erythrocytes.

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