Assay of the Level of Calcium, Magnesium and Inorganic Phosphorus in HIV Infected Patients in Owerri, Southeast Nigeria

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Abstract

HIV is a retrovirus, but differs from other retroviruses such as Human T Lymphotropic Viruses (HTLV) 1 and 2, is transmitted sexually and pre-natally. A total of eighty HIV/AIDS patients attending heart to heart in a Federal Medical center were assayed for the presence of Calcium, Magnesium and inorganic Phosphates which are among trace elements found in the human body, in relation to their CD4 cell counts. The mean (mMol/l) value and Standard Deviation of Calcium, Magnesium and inorganic phosphates on the HIV infected male patients were investigated to be 1.96 ± 0.49(Ca), 1.01± 0.80(Mg), and 1.03 ± 0.09(P) respectively, while the mean (mMol/l) value and Standard Deviation (SD) were 1.96 ± 0.49 (Ca), 0.99 ± 1.70 (Mg), and 1.07 ± 0.25 respectively. The infection rate was high on the female between the age range of 21-30 and 31-40 years with a prevalence rate of 33.5% (27) and 15.0% (12) respectively. Spectrophotometric assay using flow cytometer counter reported that the CD4 cell count of each individual infected with HIV is not dependent or have positive correlation or significant effects at (p>0.05) on the level of concentrations of Calcium, magnesium and inorganic phosphates in the plasma, when compared to the controls and the normal estimate value for trace elements in human plasma. Based on the assay and statistical significance, the CD4 cell count of each individual patient infected with HIV or not, was assumed to be dependent on the anatomical composition and physiological state of their body, dietary and environmental factors as well as educational exposure of the individual.

Keywords: CD4 cell; Calcium; Magnesium; Inorganic phosphates; HIV

Introduction

It has been reported that HIV prevalence in Owerri is about 10% [1]. Owerri is the capital city of Imo State, Nigeria with the vegetation characterized of tropical rain forest with an average annual rainforest of about 1,600mm and an average atmospheric temperature of 30°C. There are two distinct seasons, the wet and the dry seasons; the former takes place between April and October, while the later occurs from November to March. The main occupations of the people include subsistence farming (mainly yam, maize and cassava) with some involved in animal husbandry. Other professions are involved in activities such as civil Service, trading, artisans, and stone quarrying are also practiced [1].

HIV is a retrovirus, but differs from other retroviruses such as human T lymphotropic viruses (HTLV) 1 and 2 [2], is transmitted sexually, in blood or blood products and pre-natally. Those most at risk of acquiring HIV infection are homosexuals, injecting drug misusers and those with bisexual orientation. Others include individuals receiving unscreened blood or blood products and infants born of infected women [2].

HIV in humans originated from cross-species infections by simian viruses in rural Africa, probably due to direct human contact with infected primate blood [3].

The duration between primary infection and progression to clinical disease averages about 10 years [2]. An immune response to HIV occurs 1 week to 3 months after infection, plasma viremia drops, and level of CD4 cells rebound [4]. However, the immune response is unable to clear the infection completely and HIV-infected cells persist in the lymph nodes [4]. Three main body systems usually affected by AIDS are the respiratory system, the gastrointestinal tract and the central nervous system. Most of these conditions are due to the reactivation of latent organisms in the patient or exposure to the numerous microbial flora in the environment. The overall management of AIDS involves the continuous monitoring and treatment of these conditions with drugs that may cause some serious side effect [5].

The CD4 T lymphocytes are major targets responsible for virus production, which appears to have similar high turnover rates, it is estimated that every nucleotide of the HIV genome probably mutates on a daily basis [3]. Symptoms of acute HIV infection are non specific and include; fatigue, rash, headache, nausea and night sweat. The more serious symptoms in adults are often fatigue, malaise, weight loss, fever, shortness of breath, chronic diarrhoea, whites patches on the tongue (hairy leukoplakia, oral candidiasis), and lymphadenopathy [3].

HIV infection has been associated with renal disease which is characterized by nephritic-stage proteinuria (>3.5 g/dl), azotaemia, hypoalbuminemia and occasionally hypocalcemia, electrolyte abnormalities and altered mineral metabolism which occur in patients

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with HIV/AIDS have been found to contribute to bone diseases, cardiovascular disease and other clinical problem [4-6].

HIV patients are at a higher risk for attraction in calcium concentration in the body. Some low serum calcium levels may be due to low protein level in the blood [7]. It was reported that these trace elements of interest, when they are reduced below the normal values found in healthy individual, could lead to malabsorption of the intestinal mucosa and bone abnormalities [7]. Osteoporosis is asymptomatic until the development of a bone fracture, whereas severe osteomalacia can lead to bone pain, muscle weakness, and softness, while laboratory abnormalities showed low calcium and phosphorus levels [7]. Calcium has critical roles in intracellular signaling at the plasma membrane of cells, and in control of function of extracellular proteins, such as those in the coagulation cascade [8].

Alternatively, Magnesium is a cofactor for more than 300enzymes, required for enzyme substrate formation (e.g. MgATP), and an allosteric activator of many enzymes. Reducing the serum magnesium concentration results in increased neuromuscular excitability because magnesium comparatively inhibits the entry of calcium into neurons [8].

The amount of HIV in the blood (viral load) is of significant prognostic value, as continual rounds of viral replication and cell killing in each patient are at a steady-state and the level of viruses in the blood varies from individual to individual [9]. However, more recent data suggest a gender difference in this parameter in women, that the viral load may be less predictive of progression to AIDS. Plasma viral load measurements are critical element in assessing the effectiveness of antiretroviral drug therapy [3,10]. Phosphate in the form of inorganic and organic phosphate is an important and widely distributed element in the human body. Inorganic phosphate is the fraction measured in serum and plasma by clinical laboratories [11].

Phosphate is also an essential element of cyclic nucleotides (such as cyclic Adenosine Monophosphate (AMP) and Nicotinamide Adenine Dinucleotide Phosphate (NADP). It is important for the activity of several enzymes [11-14]. The clinical manifestations of serum phosphate depletion depend on the length and degree of the deficiency. Plasma concentrations <1.5 mg/dl (<0.48 mmol/L) may produce clinical manifestations. Phosphate is necessary for the formation of ATP, both glycolysis and cellular function is impaired by low intracellular phosphate concentrations. Muscle weakness, acute respiratory failure, and decreased cardiac output may occur in phosphate (<1mg/dl or <0.3 mmol/L) [11,12,14]. Thus, the aim of this assay is to investigate the correlation of these trace elements such as Calcium, Magnesium and Phosphorus in relation to the level of viral replication and other metabolic process in human. Also to determine their clinical significance as relates to possible reduction of the circulating CD4T lymphocytes counts in HIV/AIDS patients, especially in Owerri.

Material and Methods

Site and study population

This study was conducted in the department of Chemical Pathology, Federal Medical Center Owerri, Imo State between May and September, 2012. A total of 80 HIV/AIDS patients between the age’s 4-75years drawn from patients attending heart to heart centre at Federal Medical Center Owerri, Imo State were recruited for the study. 70 of the patients were already on HAART and immune boosters whereas the remaining 10 patients were not on HAART but were on immune boosters. Patients included were those whose HIV/AIDS status have been confirmed using the western blot method, whether symptomatic or asymptomatic or on HAART or not.

Collection of blood samples from patients

The 3 ml of venous blood of 80 infected patients and the blood of 20 apparently healthy volunteers, matched for age and sex were collected respectively. The twenty healthy volunteers without history of HIV/AIDS infection served as control respectively.

Socio-demographic data like age, sex and CD4 status were obtained from the case notes of the infected patients, while the controls were interviewed orally and visually to obtain their age and sex respectively. The blood from each individual was quickly transferred into a sterile heparinized container separately and was sent to the laboratory, where each of the blood samples were centrifuged at 1,500 rpm for 5 minutes. After centrifugation, the heparinized plasma was separated from the red cell and was transferred into a sterile container each for further analysis. The samples (plasma) that were not estimated the same day were stored in a freezer till the next day.

Estimation of total serum calcium

The working reagent was prepared using an equal volume of each of the buffer (2-amino-2-methyl-propan-1-06) and Chromogen(o-cresolphthalein complexone 8-hydroxyquinoline hydrochloric acid) in a quantity that gave enough reagent for the number of test that were analyzed (Randox, UK). 1000 µl of the working reagent was transferred into 3 test tubes containing 25 µl of distilled water, 25 µl of plasma sample, 25 µl of the standard reagent respectively. The set up (the 3 test tubes) was mixed and incubated for 5 minutes at 37°C in water bath and was read using a spectrophotometer at 578 nm using the 1cm cuvette. After measuring the sample absorbance with o-cresolphthalein complexone without deproteinization, 1 drop of Ethylene Diamine Tetracetic Acid (EDTA) was added to the samples and observed for a colourless reaction. The absorbance reading was repeated using 1cm cuvette. The reading obtained was subtracted manually from the previous sample reading to obtain the actual calcium value [15]. A sample blank was used instead of a reagent blank for samples with lipaemia or bilirubin. The values were calculated thus;

Normal Values: Serum Calcium 2.02 – 2.6 mmol/l (8.10 -10.4 mg/dl)

Calculation: Absorbance of Test x Concentration of standard
Absorbance of standard

Estimation of magnesium

Working Reagent 4ml of Reagent A (Sodium carbonate 0.1 mol/l, potassium cyanide 7.7 mmol/l, sodium azide 0.95 g/d) was mixed with 1ml of Reagent B (Glycine 25 mmol/l, Xyldilid blue 0.5 mmol/l, Chloroacetamide 2.6 g/l) (Biosystems Reagents and instruments, USA). The unused mixed reagent was refrigerated at 4°C and stored for further use. The set up comprising of 10 µl of plasma sample, 10 µl of the standard reagent and the 3rd test tube which served as blank (sulphuric acid 0.36 mol/L, sodium chloride 154 mmol/L, detergent). 1000 µl of the working reagent was transferred to each of the 3 test tubes respectively. The set up was thoroughly mixed and allowed to stand for 2 minutes at room temperature. The absorbance of the standard and samples were read at 520 nm against the reagent blank. Magnesium in the sample reacts with xyldilid blue in alkaline medium forming a coloured complex that can be measured by spectrophotometer.
Calculations

The magnesium concentrations in the samples were calculated each using the formula:

\[ \text{Concentration} = \frac{\text{Absorbance of sample} \times \text{Concentration of the Standard}}{\text{Absorbance of Standard}} \]

The reference values used were;

**Serum and Plasma =** 1.7 mg/dl - 2.4 mg/dl (0.70 mmol / l - 0.98 mmol/l).

**Estimation of inorganic phosphorus**

The methods of [8] was adopted for this assay. 1000 µl of the working reagent (28 ml of the molybdate reagent was mixed with 12 ml of the blank reagent to get the working reagent) (Randox, UK), and was transferred to each of the 3 test tubes respectively as follows; 10 µl of distilled water, 10 µl of patient’s sample (heparinized plasma), 10 µl of the standard each was pipette into 3 different test tubes. The set up was mixed and incubated for 5 minutes at 37°C in a water bath. The absorbance of the sample was taken at 340 nm [15].

**Assay of CD4 count**

This was done using the flow cytometry method with cyFlow counter. The beam of light of a single wavelength is directed onto a hydrodynamically-focused stream of liquid. Each suspended particle from 0.2 to 150 micrometers passing through the beam scatters the ray, and fluorescent chemicals found in the particle or attached to the particle are excited into emitting light at a longer wavelength than the light source (Forward Scatter or FSC) and several perpendicular to it (Side Scatter or SSC) and one or more fluorescence detectors. This combination of scattered and fluorescence light was picked up by the detectors, by analysing fluctuations in brightness at each detector, and various types of information about the physical and chemical structure of each individual particle were derived. FSC correlates with the cell volume and SSC depends on the inner complexity of the particle.

**Statistical analysis**

The statistical analysis was carried out using mean, standard deviation and correlation coefficient as stated below;

\[ \text{Coefficient of Correlation} = r = \frac{n \sum (X - \bar{X})(Y - \bar{Y})}{\sqrt{n \sum (X - \bar{X})^2 \sum (Y - \bar{Y})^2}} \]

\[ T - test \ for \ correlation \ coefficient \ (\alpha = 0.05) = r \sqrt{n - 2} \]

**Results**

The result of the analysis of the level of calcium, Magnesium and Inorganic phosphorus in the serum of HIV and non HIV infected individual. Analysis revealed that infection rate was high in the age range between 21-30 years in the female patients screened for human immune virus. This is followed by the age range of the female between 31-40 years old. Surprisingly none of the female patients between 61 years and above were infected, out of the total 80 patients screened and were positive for HIV infection (Table 1). Though analysis showed that the male were infected with the HIV when compared to the number of female infected, (Table 2) the mean value of Calcium (Ca) level of the HIV infected male and female were seen to be 1.96 ± 0.49 respectively, while the non infected male and female patients were observed to be 2.25 and 2.24 mmol/l respectively, showing that there is no significant difference in the Ca level of the infected male / female patients when compared to the volunteers, but there was little decrease in the Ca level when compared to the normal values (Table 2). Though the non infected (controls) had normal values of Ca concentrations in their plasma, revealing that Ca concentration level is not gender base especially on HIV infected patients(may be nutritional habits or food intake) . Similar trend was observed in the level of Magnesium (Mg) found in the infected individuals, though the data shows that (p> 0.05) that the level of Mg concentration on HIV infected patients is dependent on gender (that is based on the anatomical differences in male / female). While the level of Phosphorus (P) concentrations also shows that it does not depend on the gender differences, rather it depends on dietary intake. Furthermore, reports in this study shows that the level of CD4 cells of the HIV infected patients is not dependent on the level or concentrations of the Ca, Mg and P level in the plasma or blood of this individuals infected with the HIV (that is, there is no correlation between the level of concentrations of this trace elements and CD4 cell counts observed (Table 3).

**Discussion**

HIV/AIDS remains one of the clinical conditions of public health important with high morbidity and mortality worldwide, especially in developing countries [16]. The result of this assay showed that there is no relationship or correlation in the level of calcium and inorganic phosphatase concentration in the plasma and serum respectively

<table>
<thead>
<tr>
<th>Age limits (years)</th>
<th>Number of HIV infected patients</th>
<th>Total number of infected patients (controls)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 - 20</td>
<td>1 (1.25 %)</td>
<td>6 (7.5 %)</td>
</tr>
<tr>
<td>21 - 30</td>
<td>2 (2.5 %)</td>
<td>27 (33.8 %)</td>
</tr>
<tr>
<td>31 - 40</td>
<td>8 (10.0 %)</td>
<td>12 (15.0 %)</td>
</tr>
<tr>
<td>41 - 50</td>
<td>2 (2.5 %)</td>
<td>9 (11.3 %)</td>
</tr>
<tr>
<td>51 - 60</td>
<td>1 (1.25 %)</td>
<td>6 (7.5 %)</td>
</tr>
<tr>
<td>61 – Above</td>
<td>6 (7.5 %)</td>
<td>0 (0 %)</td>
</tr>
</tbody>
</table>

**Table 1:** Prevalence Rate of Patients Infected With HIV Virus Based on the Age Limits in the Hospital.

<table>
<thead>
<tr>
<th>Sex of Patients</th>
<th>Mean mMol/l Ca (SD)</th>
<th>Mean mMol/l Mg (SD)</th>
<th>Mean mMol/l P (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>1.96 ± 0.49</td>
<td>0.99 ± 1.70</td>
<td>1.07 ± 0.25</td>
</tr>
<tr>
<td>Female</td>
<td>1.96 ± 0.49</td>
<td>0.99 ± 1.70</td>
<td>1.07 ± 0.25</td>
</tr>
</tbody>
</table>

**Table 2:** The Mean Values of the Trace Elements in HIV and Non HIV Patients.
There is a gender associated with the concentration of magnesium in HIV/AIDS patients. Though in this study it was observed a mean value of 1.01 ± 0.80 in the male and 0.99 ± 1.70 in the female patients screened when compared to the normal values of magnesium which is about 0.70-0.98, in the human plasma. This is in line with the works of [19], who reported that from their study, serum magnesium in HIV/AIDS patients is low with average serum magnesium level of 17.6 ± 10.3 mg/dl (0.70 mmol/l-0.98 mmol/l). They also stated that there is a quadratic relation between the serum magnesium level and the number of CD4 Lymphocytes in HIV/AIDS patients studied. Researchers have found that magnesium, which is critical for efficient energy production and protein synthesis, is not adequately consumed by up to half of the HIV-positive people tested [19]. This report supports the assay in this study, as the magnesium concentration in the plasma of HIV/AIDS patients did not correlate with their CD4 counts. This implies that the level of magnesium concentration in the plasma of HIV/AIDS patients has nothing to do with reduction or increase in the CD4 cell counts. Rather the magnesium concentration may be affected by the type of antiretroviral drug taken, the dietary factor and the health conditions of the patients. The result of the assay in the level of inorganic phosphates showed that the level of phosphates in the HIV infected patients 1.03 ± 0.09 in the male and 1.07 ± 0.25 in female were higher, when compared with the normal value of 0.87-1.45 mmol/l of inorganic phosphates in the plasma. Analysis also revealed that there was no correlation between the level of serum inorganic phosphates concentration in HIV/AIDS patients and their CD4 count. This agrees with the reports of [20], that hypophosphatemia is relatively frequent in HIV patients on antiretroviral therapy and not necessarily with low CD4 count. It also agrees with the report of [11-13] that hypophosphatemia may be as a result of; (1.) Shift of phosphate from extra cellular to intercellular spaces, (2.) Renal phosphate wasting (3.) decreased intestinal absorption and (4.) Loss from intracellular phosphate. Furthermore, reports have it that low serum phosphate at the beginning of antiretroviral therapy in HIV patients was an independent predictor of early mortality among HIV patients starting antiretroviral therapy with severe malnutrition or advanced immune suppression [11-13]. Though, a rapid increase in serum phosphate may be associated with hypocalemia. Therefore symptoms may include tetany, seizures, and hypotension [12,14]. The body’s handling of extra cellular calcium is closely intertwined with that of phosphate and to somewhat lesser extent of magnesium. It is also intricately connected with the active cellular processes in bone, a metabolically and functionally important system on its own [8,21].

### Table 3: Correlation Coefficient between the Trace Elements Concentration in the Plasma of HIV/AIDS Positive Patients and Their CD4 Count Randomly Selected.

<table>
<thead>
<tr>
<th>Calcium (x)</th>
<th>Magnesium (x)</th>
<th>Phosphorus (x)</th>
<th>CD4 cell count (Y)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>F</td>
<td>M</td>
<td>F</td>
</tr>
<tr>
<td>1.52</td>
<td>1.65</td>
<td>0.75</td>
<td>0.90</td>
</tr>
<tr>
<td>1.55</td>
<td>2.33</td>
<td>0.80</td>
<td>1.17</td>
</tr>
<tr>
<td>1.87</td>
<td>1.44</td>
<td>0.76</td>
<td>1.44</td>
</tr>
<tr>
<td>1.87</td>
<td>1.44</td>
<td>0.93</td>
<td>1.08</td>
</tr>
<tr>
<td>1.23</td>
<td>2.33</td>
<td>1.30</td>
<td>1.42</td>
</tr>
<tr>
<td>2.32</td>
<td>1.82</td>
<td>0.93</td>
<td>0.98</td>
</tr>
<tr>
<td>1.94</td>
<td>1.18</td>
<td>0.89</td>
<td>0.99</td>
</tr>
<tr>
<td>2.46</td>
<td>2.22</td>
<td>1.02</td>
<td>0.90</td>
</tr>
<tr>
<td>1.44</td>
<td>2.22</td>
<td>0.99</td>
<td>1.22</td>
</tr>
<tr>
<td>2.30</td>
<td>1.84</td>
<td>0.90</td>
<td>0.05</td>
</tr>
<tr>
<td>2.48</td>
<td>2.35</td>
<td>0.94</td>
<td>1.09</td>
</tr>
<tr>
<td>2.38</td>
<td>2.16</td>
<td>1.15</td>
<td>0.99</td>
</tr>
<tr>
<td>2.14</td>
<td>2.13</td>
<td>0.17</td>
<td>0.89</td>
</tr>
<tr>
<td>2.18</td>
<td>2.33</td>
<td>0.97</td>
<td>0.98</td>
</tr>
<tr>
<td>1.82</td>
<td>2.30</td>
<td>1.59</td>
<td>0.75</td>
</tr>
</tbody>
</table>

| Z=29.5   | Z=29.44     | Z=15.09      | Z=14.85      | Z=15.38      | Z=16.11      | Z=6257       |

Coefficient of correlation r =-0.47(Calcium) and r²=0.22%, where F=Female and M=male
Coefficient of correlation r =0.15(Magnesium) and r²=0.25%
Coefficient of correlation r =-0.14(inorganic phosphorus) and r²=2.0%
Where r²=Coefficient of determination and p>0.05 (level of probability)
In conclusion, since there was a strong positive correlation in the concentration of magnesium level in the plasma between the male and female HIV/AIDS patients. It could be as a result of occupational exposure of the patient, the viral load (since the plasma viral load in women may be less predictive of progression to AIDS than in men) and the anatomical composition of the body [22].

Also since there are no relationship between gender and plasma calcium and serum inorganic phosphatase in HIV/AIDS patients and no correlation between the gender and calcium, magnesium and inorganic phosphatase in the body of non HIV/AIDS positive individuals, the variation in the concentration of these parameter could be as a result of anatomical compositions of the body, environmental factors, physiological state of the body, dietary factors, the type of drug intake and personal maintenance of the body and not their HIV/AIDS status, CD4 count and gender issue. It is recommended that HIV infected patients should take 1000-1500 mg of calcium and 800-1000iu of vitamin D daily as stated by [2,23].

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References