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EVALUATION OF Fe AND Zn/Cu RATIO IN SERUM OF PATIENTS WITH SICKLE CELL ANEMIA BY TOTAL REFLECTION X-RAY FLUORESCENCE UNING SYNCHROTRON RADIATION

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ABSTRACT

Sickle Cell Anemia (SCA) is a blood disorder that affects hemoglobin, the protein found in red blood cells that help carry oxygen throughout the body. In this work we have analyzed serum samples from patients with SCA by using Total Reflection X-Ray Fluorescence using Synchrotron Radiation (SRTXRF). The SRTXRF measurements were performed at the X-Ray Fluorescence Beamline at Brazilian National Synchrotron Light Laboratory (LNLS), in Campinas, São Paulo using a polychromatic beam. We have studied forty-three patients aged 18-50 years old, suffering from SCA and Sixty healthy volunteers aged 18-60 years old. It was possible to determine the concentrations of the following elements: P, S, Cl, K, Ca, Fe, Cu, Zn, Br and Rb. Student's t-test was applied in order to check whether the two populations (CG x SCA) had the same mean values. It was observed that elemental concentration of P, Cl, K, Fe, Cu, Zn and Br differed significantly ($\alpha = 0.05$) between groups of healthy subjects and SCA. The concentrations of K, Fe and Cu in the serum samples of patients with SCA were larger 15%, 120 % and 20 %, respectively, when compared with the CG. On the other hand, the concentrations of P (-20 %), Cl (-6 %), Zn (-25 %) and Br (-22 %) were smaller than the values determined for the control group. The serum level Cu/Zn ratio was significantly higher (60%) in the serum samples of patients with SCA group than the CG. So, the Cu/Zn ratio can be used as an adjuvant index in enhancement for diagnosis of SCA. There are evidences of an association among Fe, Cu, Zn and Cu/Zn in the SCA pathogenesis process.

Keywords: Cu/Zn ratio**,** Sickle Cell Anemia, X-Ray Fluorescence and Synchrotron Radiation

1. INTRODUCTION

Sickle Cell Anemia (SCA) is a blood disorder that affects hemoglobin, the protein found in red blood cells that help carry oxygen throughout the body. It is a disease that originated in Africa and now is the most prevalent hereditary disease in the whole world because of the vast racial mixing that affects millions of people. SCA occurs when a person inherits two abnormal SS or HbSS [1]. Instead of being flexible and disc-shaped, these cells are stiffer and curved in the shape of the old farm tool known as a sickle. These irregular-shaped blood cells die prematurely, resulting in a chronic shortage of red blood cells. In addition, they can get stuck when traveling through small blood vessels, which can slow or block blood flow and oxygen to certain parts of the body. Moreover, this causes pain and can lead to serious complications: anemia, delayed growth, infections and acute chest syndrome. Therefore, the determination of trace element levels in human serum is of interest for biomedical area because several elements take part in all metabolic processes. So, it has been established that trace element levels in human serum can be utilized as indicators for several pathological conditions. In this way, the simultaneous detection of certain elements in the serum offers a very interesting approach in the diagnosis and treatment of various diseases including SCA [2]. The total reflection X-ray fluorescence (TXRF) is a multielemental technique widely used in the analysis of low concentration ($\mu g.g^{-1}$) in environmental, medical and biological samples. TXRF analysis is a well established analytical technique for the detection of major, minor and trace elements, especially suited for samples, whenever only small specimen mass is available. On the other hand, synchrotron radiation is an excellent source for exciting total reflection X-ray fluorescence [3].

In this study, the quantitative analysis of Fe, Cu. Zn and Cu/Zn in serum from patients with Sickle Cell Anemia (SCA) and healthy subjects was performed by Total Reflection X-Ray Fluorescence using Synchrotron Radiation. Moreover, the results can be used to analyze the role serum trace elements as an adjuvant index in enhancement for diagnosis of SCA.

2. EXPERIMENTAL

2.1. Sampling serum

Forty-three patients (15 males and 28 females) aged 18-50 years old were studied, who suffered from Sickle Cell Anemia and sixty healthy volunteers (41 males and 19 females) aged 18-60 years old. The diagnosis of sickling condition was established by medical history, physical examination and hematological studies. In addition, the medical history was evaluated in all patients to exclude other pathologies. All the serum samples provided by "*Instituto Estadual de Hematologia Arthur de Siqueira Cavalcanti*" (*HEMORIO*) were collected from people who lived in the urban area of Rio de Janeiro City, Brazil. This study was performed with the approval of the local ethics committee and all subjects volunteered for the study with informed consent.

2.2. Sample preparation

Blood was collected into vacutainers tubes without any additives (Greiner Bio-One International AG, VACUETTE). Immediately after collection, each blood sample was centrifuged at 3000g for 10 min in order to separate blood cells and suspended particles from serum. The sera were transferred into polyethylene tubes and stored in a freezer at 253 K until time for analysis. In environmental temperature, a volume of 500 μL of serum was taken and diluted with 1500 μL of ultrapure water, 18.2 MΩ from the Milli-Q water purification system (Millipore Systems Inc., Bedford, MA). An internal standard consisting of 30 μL of Gallium solution (Gallium ICP standard traceable, Merck) was added (100 mgL^{-1}). After that, the solution was homogenized by shaking and a small aliquot of 5 μL was pipetted on a precleaned Lucite carrier (Perspex® - Molecular formula: $(C_5O_2H_8)_n$ – rectangular-plate with dimensions: 30 x 15 x 2 mm). After the deposition, the samples were left to dry very slowly under an IR lamp. To avoid contamination from the recipients and volumetric flasks used, they were washed with detergent, soaked in a 1 molL^{-1} HNO_3 solution (*SUPRAPUR® Nitric Acid* 65% - Merck), rinsed with ultrapure water (Milli-Q water), filled with 10 molL^{-1} HNO_3, kept for at least one night and rinsed again. In addition, the Lucite carriers were cleaned with neutral detergent, rinsed with Milli-Q water and thereafter dried sufficiently. All samples were analyzed in triplicate. The validation of methodology was verified using a reference material (Multi element standard VII - ICP/MS calibration standards -CertiPUR®. - Merck)**.**

2.3. Experimental setup

The Total Reflection X-Ray Fluorescence technique using Synchrotron Radiation (SR-TXRF) analyses were performed at the X-Ray Fluorescence Beamline at Brazilian National Synchrotron Light Laboratory (LNLS), in Campinas, São Paulo, using a polychromatic beam with maximum energy of 20 keV for the excitation [4]. The detector was of HPGe with energy resolution of 140 eV at 5.9 keV. The Lucite carrier-detector distance was of 12 mm. The incidence angle (for carrier) was of approximately 1.0 mrad. All samples were excited for 100 s and X-ray fluorescence spectra obtained were evaluated by the software QXAS software package, distributed by the International Atomic Energy Agency, in order to obtain the X-ray fluorescence intensities [5]. All analyses were performed in natural atmosphere.

3. RESULTS AND DISCUSSION

Figure 1 shows a typical X-ray fluorescence spectrum of a serum sample using SR-TXRF. It was possible to detect the presence of ten elements: Phosphorus, Sulfur, Chlorine, Potassium, Calcium, Iron, Copper, Zinc, Bromine and Rubidium. The presence of Ga is explained because it was used as internal standard. In order to confirm the accuracy of multi-elemental analysis of serum samples by means SRTXRF, we have carried out elemental analysis of the CertiPUR® by Merck reference material. Student’s t-test was applied in order to check whether the two populations (control group and SCA group) had the same mean values. All statistic analyses were performed by SPSS 10.0 for Windows software. The t-test ($\alpha = 0.05$) was used to verify the variations in the elemental concentrations between patient group

(SCA) and control group (CG). The t-test has shown that concentrations of P, Cl, K, Fe, Cu, Zn and Br presented significant differences between group of patients with SCA and CG. On the other hand, we found no significant differences in the concentrations of S, Ca and Rb.

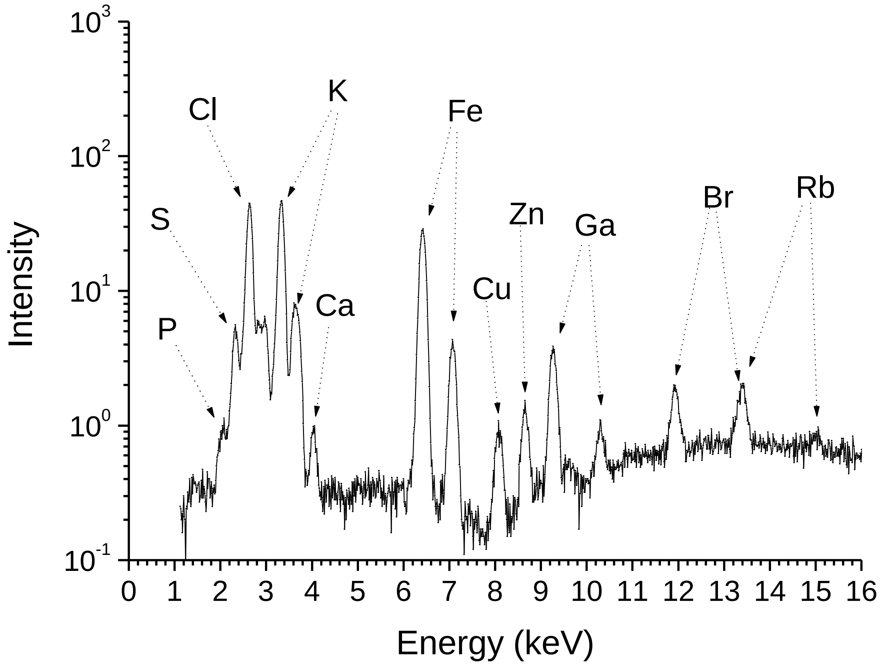

Figure 1 - X-ray fluorescence spectrum of a serum sample using SRTXRF

The results are shown in Table 1. The results obtained agree well with their respective nominal reference values. The accuracy of this method was approximately 3 % for Ca and 8 % for Sr and relative standard deviations were lower than 8.7 %. The detection limit (DL) varied from 250 μgL^{-1} for Sulfur to 1.5 μgL^{-1} for Zinc. DL values are in agreement with the values reported in the literature [6].

Table 1 - SRTXRF results for CertiPUR® by Merck reference material (mgL^{-1})

Element	K	Ca	Mn	Sr	Ba
Reference value	100 ± 5	100 ± 5	100 ± 5	100 ± 5	100 ± 5
SRTXTF [a)]	93 ± 8	97 ± 7	103 ± 9	92 ± 6	106 ± 8
Relative error	0.07	0.03	0.03	0.10	0.06
RSD[b)] (%)	8.6	7.2	8.7	6.5	7.5

a) Mean and SD for ten measurements.
b) Relative Standard Deviation.

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Table 2 shows the quantitative results (elemental concentrations) found between groups of patients with SCA and CG for Fe, Cu, Zn and Cu/Zn. Statistical significance was considered at $p < 0.05$.

Table 2 - Mean concentrations and confidence interval for human serum (mgL^{-1}).

Element	**Groups (Mean ± SD)**						
	Sickle Cell Anemia		**Control**				
	This work (n=43)	**[7] (n=84)**	**This work (n=60)**	**[8] (n=2233)**	**[9] (n=50)**	**[10] (n=560)**	**[11] (n=22)**
Fe	2.2 ± 0.2	-	1.0 ± 0.2	-	1.4 ± 0.4	-	1.5 ± 0.7
Cu	1.2 ± 0.1	1.3 ± 0.3	1.0 ± 0.1	1.5 ± 0.3	0.9 ± 0.2	1.5 ± 0.4	0.9 ± 0.2
Zn	0.6 ± 0.1	1.0 ± 0.1	0.8 ± 0.2	1.2 ± 0.2	1.0 ± 0.2	1.1 ± 0.3	1.3 ± 0.2
Cu/Zn	2.0 ± 0.3	1.3 ± 0.3	1.3 ± 0.3	1.3 ± 0.3	0.9 ± 0.3	1.4 ± 0.5	0.7 ± 0.1

Statistical significance was considered at $p < 0.05$.

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The concentration of K in the serum samples of patients with SCA was larger 15% when compared with the CG. On the other hand, the concentrations of P (-20 %), Cl (-6 %), Zn (-25 %) and Br (-22 %) were smaller than the values determined for the CG.
The concentrations of Fe and Cu in the serum samples of patients with SCA were larger 120 % and 20 %, respectively, when compared with the CG. In the case of SCA the elements Fe and Cu play important roles in several physiological and biochemical functions. The increase of Fe may be connected to folic acid ingestion (90% of patients with SCA ingest it) that helps in the maintenance of red blood cell production and the constant regular transfusions of red blood cells in which patients with SCA are submitted.
Previous studies have shown that the Fe overload that results from such therapy in other patient populations is associated with significant morbidity and mortality. The pathophysiology of iron overload in sickle cell disease is not well known. Whereas there are many studies of the cardiac, hepatic, and endocrine morbidity and mortality of iron overload in the setting of thalassaemia, there are little data on the clinical consequences of iron overload in Sickle Cell Anemia [12]
So the increase on Fe concentration may affect directly Cu concentration in human serum, due to its dependency in relation to important Cu-dependent enzymes as cerulosplasmin, Cu-Zn superoxide dismutase and hephaestion. In addition, Copper is used in several important biochemical processes such as origin of red blood cells and the autoimmune response of the patient, protecting different cellular structures against the action of reactive oxygen species.
In fact, there are evidences suggesting an association among Fe, Cu and Zn in the SCA pathogenesis process. On the other hand, in the case of iron concentration in SCA, it would be important to study this element in serum of patients with SCA that have not yet undergone treatment with acid folic intake. Fe supplementation can impair Zn retention in human pregnancy [13] and appear to agree with Fe and Zn bioavailability studies that suggested that the increased ingestion of elemental Fe depressed the Zn bioavailability by competition in the bowel wall.
The concentration of Zn found in the serum samples of patients with SCA were approximately 25 % lower values determined in control group. The decrease in Zinc concentration in samples of patients with SCA in this study could be related to decrease immunity and may also be indicative of problems related to growth. Both reduction immunity and growth problems are symptoms of SCA [14]. Zn is important for immune function, wound healing and is needed for DNA synthesis. One third of the Zinc found in plasma is attached loosely to albumin and about two thirds is firmly bound to globulins. Zinc also supports normal growth and development during pregnancy, childhood, and adolescence. Zinc deficiency is a common nutritional problem in adult sickle-cell disease patients [15] Certain complications of SCA, such as growth retardation, hypogonadism in men, and poor wound healing, have recently been related to zinc deficiency. Additional factors such as predominant dietary use of cereal protein and other nutritional factors that affect zinc availability adversely cannot be ruled out, and further investigations are required for proper evaluation of the pathogenesis of zinc deficiency in SCA.
The serum level Cu/Zn ratio was significantly higher (60%) in the serum samples of patients with SCA group than the CG. It is generally recognized that the increased level of Cu and the decreased level of Zn lead to a Cu/Zn ratio elevation [16]. The increase of Cu/Zn in the serum samples with SCA was probably induced by the constant inflammatory process that these patients suffer. Zinc maintains a balance with copper in blood, and the blood levels of the two tend to be inversely related, with low plasma zinc nearly always being associated with high serum copper. There are several published studies that state that the normal zinc to copper

ratio, in children and adults, is close to 1:1 [17]. Our results on serum samples from CG were close to this ratio. Dietary intake has been documented in several studies to be inadequate in SCA patients and to decline further as patient's age [18]. Careful nutritional studies will need to be performed to determine contributions of diet and of these elements malabsorption in SCA development. Regardless of the underlying etiology, these results suggest that all patients with SCA who are chronically transfused should have periodic nutritional evaluation and supplementation as necessary.

4. CONCLUSIONS

SRTXRF is a powerful tool for the determination of multielemental concentrations in human serum samples. The elements P, S, Cl, K, Ca, Fe, Cu, Zn, Br and Rb were identified and their concentrations were determined in serum of healthy subjects (CG) and patients with SCA. Only the concentrations of P, Cl, K, Fe, Cu, Zn and Br showed significant differences between the group of patients with SCA and the CG.

The values found in this study to the average concentrations of Cu and Zn are close to the values found in literature. The increase of Fe may be connected to folic acid ingestion that helps in the maintenance of red blood cell production and the constant regular transfusions of red blood cells in which patients with SCA are submitted. The increase of Cu/Zn in serum samples from patients with SCA was probably induced by inflammatory processes contained in these patients. Therefore, to determine Cu/Zn ratio may be more important for studies of these processes that the determination of the mean concentrations of Cu and Zn.

The investigation of concentrations of the elements Fe, Cu, Zn and Cu/Zn may help the studies on the development of SCA. The increase or decrease the concentrations of these elements may be associated with typical symptoms of SCA as liver disease, heart disease, endocrine disorders, autoimmune problems, reduced growth and decreased blood oxygenation. These elements can be used as an adjuvant index in enhancement for diagnosis of SCA.

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REFERENCES

1. S. Yuditskaya, A. Tumblin, G. T. Hoehn, G. Wang, S. K. Drake, Xiuli Xu, S. Ying A. H, Chi, A. T. Remaley, R. F. Shen, P. J. Munson, A. F. Suffredini, G. J. Kato, "Proteomic identification of altered apolipoprotein patterns in pulmonary hypertension and vasculopathy of sickle cell disease". *Blood*. **Vol. 113**, pp. 1122-1128 (2009).

2. M. JongHwa, K. SangHoon, C. YongSam, L. OkHee, 2007. "Elemental analysis of Korean women's blood serums using instrumental neutron activation analysis". J. *Radioanal. Nucl. Chem*. **Vol. 271**, pp. 155-158 (2007).

3. P. Wobrauschek, "Total reflection x-ray fluorescence analysis—a review". *X-Ray Spectrom*. **Vol. 36**, pp. 289-300 (2007).

4. C. A. Pérez, M. Radtke, H. J. Sánchez, H. Tolentino, R. T. Neuenshwander, W. Barg, M. Rubio, M. I. S. Bueno, I. M. Raimundo, J. J. R. Rohwedder "Synchrotron radiation X-Ray fluorescence at the LNLS: beamline instrumentation and experiments" *X-Ray Spectrom.* **Vol. 28**, pp. 320-326 (1999).

5. G. Bernasconi, A. Tajani, "Quantitative X-ray Analysis System (QXAS) Software", *Package: Documentation Version 1.2, International Atomic Energy Agency (IAEA)*, Vienna (1996).

6. R. Klockenkämper, 1997. Total-Reflection X-ray Fluorescence Analysis. *Wiley-Interscience Publication*, New York (1997)

7. A. S. Prasad, J. Ortega, G. J. Brewer, D. Oberleas, B. Eric, E. B. Schoomaker, "Trace Elements in Sickle Cell Disease", *JAMA*. **Vol. 235**, pp. 2396-2398 (1976).

8. S. M. R. Parizadeh, S. M. R. Kazemi-bajestani, A. Shapouri-Moghaddam, M. Ghayour-Mobarhan, H. Esmaeli, M. R. Majdi, A. Emadzadeh, M. Safarian, M. Azimi-Nezhad, G. H. Khodaei, S. J. Hosseini, S. M. J. Parizadeh,M. R. Oladi, G. Ferns, "Serun zinc and copper consentrations and socio-economic status in a larg Persian cohort". *Asian Biom.* **Vol. 5**, pp. 329-335 (2011).

9. C. C. Lin, J. F. Hunag, L. Y. Tsai, Y. L. Huang, "Selenium, iron, copper, and zinc levels and copper-to-zinc ratios in serum of patients at different stages of viral hepatic diseases". *Biol. Trace Elem. Res*. **Vol. 109**, pp. 15-23 (2006).

10. C. Abiaka, S. Olusi, A. Al-awadhi, "Reference Ranges of Copper and Zin and Prevalence on their Deficiencies in an Arab Population Aged 15-80 Years". *Biol. Trace Elem. Res*. **Vol. 91**, pp. 33-43 (2003).

11. F. Deveci, N. Ilhan, "Plasma Malondialdehyde and Serum Trace Element Concentrations in patients with Active Pulmonary Tuberculosis". *Biol. Trace. Elem. Res*. **Vol. 95**, pp. 29-38 (2003).

12. R. J. Adams, B. Brambilla, “Discontinuing Prophylactic Transfusions Used to Prevent Stroke in Sickle Cell Disease”. *N. Engl. J. Med*. **Vol. 353**, pp. 2769-2778 (2005).

13. E. B. Dawson, J. Albers, W. J. McGanity, 1989. “Serum zinc changes due to iron supplementation in teen-age pregnancy”. *Am. J. Clin. Nutr*. **Vol. 50**, pp. 848–852 (1989).

14. A. S. Prasad, 2002. “Zinc deficiency in patients with sickle cell disease”. *Am. J. Clin. Nutr*. **Vol. 75**, pp. 181-182 (2002).

15. A. S. Prasad, F. W. J. Beck, J. Kaplan, P. H. Chandrasekar, J. Ortega, J. T. Fitzgerald, P. Swerdlow, “Effect of Zinc Supplementation on Incidence of Infections and Hospital Admissions in Sickle Cell Disease (SCD)”. *Am. J. Hematol.* **Vol. 61**, pp. 194–202 (1999).

16. A. Ece, B.S. Yanik, A. Iscan, P. Ertan, M. R. Yigitoglu, “Increased Serum Copper and Decreased Serum Zinc Levels in Children with Iron Deficiency Anemia”. *Biol. Trace Elem. Res.* **Vol. 59**, pp 31-39 (1997).

17. S. Faber, G. M. Zinn, J. C. Kern, H. M. S. Kingston, “The plasma zinc/serum coper ratio as a biomarker in children with autism spectrum disorders”. *Biomarkers*. **Vol. 14**, pp. 171-180 (2009).

18. D. A. Kawchak, J. I. Schall, B. S. Zemel, K. Ohene-Frempong, V. A. Stallings, “Adequacy of dietary intake declines with age in children with sickle cell disease”. *J. Am. Diet. Assoc*. **Vol. 107**, pp. 843–848 (2007).