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Determination of Iron in Human serum samples of Thalassaemia patients by Flame Atomic Absorption

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Abstract

A simple and sensitive flame atomic absorption spectrophotometricmethod has been used forthe determination of iron concentration in blood serum for both(male and female) ofThalassemia patients group and normalgroup.The method is compared with a standard spectrophotometric method. The two methods are based on the reaction of iron with ferene reagent at acidic condition.The results of 84 samples showed that the distribution of iron concentration between serum samples isranged between (401.3) and (440.3) µg /dL for female and male respectively of thalassemia patients group, (66.3),(90.6) µg/dLfor normal female and male group, so theanalysis of iron levels in serum showed that iron levels in serum of thalassemia patients group were significantly higher than the normal group and the value of iron in the serum of the male of thalassemia patients group and normal group is higher than female. Values of iron content measured in the serum samples by flame atomic absorption method are in good agreement with the results obtained with the standard spectrophotometric method, the calculated *t* value was less than t tabulated value, therefore no significant difference between the two methods at 95% confidence level was found.

Key Words: Iron, serum, ferene, determination, thalassemia, atomic absorption, spectrophotometric.

1. Introduction

Thalassemia syndromes are a heterogeneous group of inherited hematologicdisorders characterised by deficiencies in the rate ofproduction of specific globin chains [1]. The patients ofthalassemia suffer from anemia because their bodiesare insufficient producer of red blood cells. As a resultof anemia caused in thalassemia major, patients arepale, fatigue, have slower rate of growth and mostsignificant is the expansion of bone marrow. Thisexpansion of the bone marrow forces the bones toexpand, and develop "Cooley's facies". The bodyattempts to compensate for the severe anemia byabsorbing more iron from food passing through thegastrointestinal tract. By absorbing more iron, the body exposes itself to newdanger- iron overload and this increased iron depositsin the various tissues and organs. Many of thecomplications of thalassemia seen are the result ofincreased iron deposition from repeated bloodtransfusions.

These complications of the chronicanemia is prevented or ameliorated by a program ofroutine red cell transfusions. There are about 75 mg ofiron in 100 mg of packed red cells. The transfusion of200ml packed cells into a child every 4 weeks addsabout 2 gm of iron per year.Adolescents receive more than twice that amount ofiron annually[1].Iron chelation therapy is just asupportive treatment for this disease which isassociated with serious complications. The prime goalof iron chelation therapy is to control body iron .Growth disturbances are a major clinical feature ofuntreated patients with thalassemia [2]. With current transfusion therapy normal prepubertal linear growthis usual, but there is a retardation of growth in thesecond decade and many patients fail to attain normalstature [3].In countries where patients do not receive adequatetreatment, chronic anemia and inadequate nutritionare the main cause of growth failure whereas, incountries where patients are well transfused but showpoor compliance to chelation treatment, iron overloadis the major cause of poor growth. However, in welltransfused and well chelated patients, high doses ofdeferoxamine may cause toxicity at the bone level,which ultimately delays growth. Impairment in growthand sexual maturation is directly related to ironoverload [4-6].

The control of thisnecessary but potentially toxic substance is animportant part of many aspects of human healthand disease [7]. Iron ions circulate bound to plasmatransferrin and accumulate within cells in the formof ferritin. The subsequent development ofpractical clinical measurements of serum iron,transferrin saturation, plasma ferritin, and red cell protoporphyrin permitted the definition and detection of the body's iron store status and iron-deficient erythropoiesis [8]. The difference in ironabsorption from various foods, meals or drugsdepends, in part, on the chemical properties ofiron [7]. The clinical effects of iron deficiency havebeen described in the medical literature datingback to the middle ages, in fascinating accounts ofa disorder called chlorosis. Large amounts offerrous salts are toxic, but fatalities arerare inadults [8]. Iron overload usually causes tissuedamages, so studying concentration of serum ironcontent has its diagnostic and pathologic value .

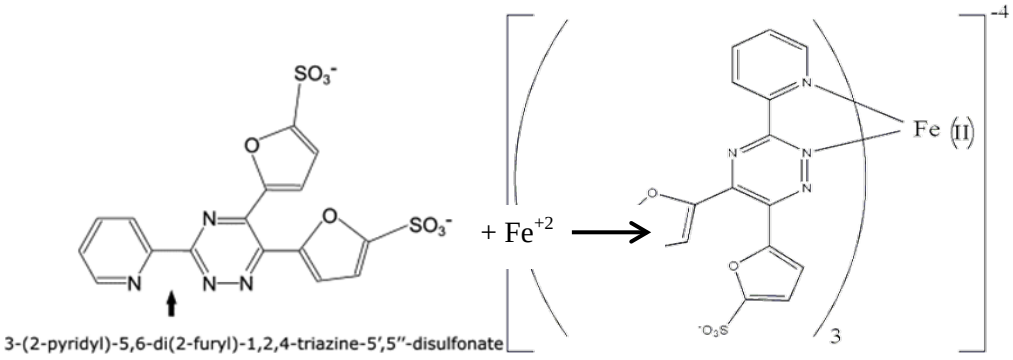
Serum iron determinations have madeimportant contributions to the diagnostic processfor several decades. Despite the importance of ironmeasurements, the accuracy of present routinemethods is suspect, speed and convenience ofmethods have taken the place of accuracy [9]. Mainsources of error in iron methods are incompletedissociation of iron from binding proteins, loss ofiron during protein precipitation, incompletereduction of iron (III) to iron(II), copper andhemoglobininterferences , spectrophotometricinterferences by compounds present in the serummatrix (e.g., bilirubin and lipids)[10] and proteins

mayproduce turbidity because of the high proportion ofserum that must be used and because thechromogenic reaction is usually performed at pH'sthat are near the isoelectric point of many serumproteins[11]. Usually, surfactants have been used toeliminate turbidity, but not always with completesuccess, owing to the variable protein composition of sera [11, 12]. In the field of clinical diagnostics, theamount of iron bound to transferrin is commonlyreferred to as the serum iron, there is essentiallyno difference between plasma and serum iron .Serum is usually used for the iron assay forreasons of technical capability. Normally theamount of iron in serum/plasma (not includinghemoglobin) is about 100 µg per l00ml of blood [13]. In the clinical laboratory, theamount of iron measured in serum can be done byknown methods. In one method, serum iron isassayed by adding a serum sample to a reagentbuffered at an acid pH. At this acid pH, ferric iondissociates from transferrin. The reagent includesa reducing agent, which aids in the dissociationprocess and reduces ferric ion to ferrous ion. Achromogenic reagent is then added and the chromogen complexes with ferrous iron to form acoloredcomplex . The colored complex is measured spectrophotometrically[13]. Results were obtained by the use of o-phenanthroline[14],protocatechuic acid [15]diphenyl-1:phenanthroline[16],ethanolicsolutionofbathophenanthroline [17]2 ,4,6 -Tripyridyl-s-triazine (TPTZ) [18], Catalytic photometric method[19], N-ethyl-2-methyl-3-hydroxypyridin-4-on(EMHP)[20]andmany other chelating reagents. Ferene wassynthesized in 1980 and reacts with iron(II) to forma stable, deep blue complex which is also verysoluble in water[21]. Iron content was determined byatomic absorption spectrophotometry "AAS" inundiluted plasma or a 1:1 dilution of plasma anddeionized water [22].

Flame Atomic Absorption spectrophotometry AAS (was used for the determination of Fe-II and Fe-III in water after their separation with Aspergillus niger immobilized on sepiolite[23] .Iron is traditionally measured in unhaemolysed serum by colorimetric assay. Thetwo major approaches are colored complexformation followed by spectrophotometry or AAS . Colorimetric procedures to quantify serum iron areusually available in hospital pathology departments and are suitable for rapid emergency analyses,although they suffer from at least one of threeundesirable features:[7] low sensitivity of the colorreaction employed[8] turbidity in the final colorsolution[9] andnonspecific background absorbancein the color solution. More sensitive and specificAAS"atomicabsorptionspectrometry "procedures can be applied to the analysis of iron inserum, plasma, whole blood and urine .

Therefore this study was carried out to estimatethe appropriate method for iron analysis in bloodserum. So the determination of iron concentration inblood serum with two different analytical methodswas done.In an acidic medium transferrin bound iron dissociates into ferric ions which are reduced to ferrous ions in the presence of ascorbic acid. The ferrous iron reacts with the chromogen ferene to form a blue complex which absorbs at 585 nm by spectrophotometric method and at 248.3 nm for iron by FAAS.The absorbance is directly proportional to the serum iron concentration, the equations below describe the reactions.

$$\text{Transferrin} -(Fe^{+3})_2 \xrightarrow{\text{Ascorbic acid}} 2Fe^{+2} + \text{Transferrin}$$



Ferene Blue complex

2. Experimental

2.1 Reagents and solutions

R1: Reductant

Citric acid	150 mmol/L
Ascorbic Acid	30 mmol/L
Thiourea	27 mmol/L

R2: Chromogen

Ferene	600µmol/L

R3: Standard

Iron	200 µg/dLor 35.8µmol/L

The working reagent prepared as follows:
R1 (50 volumes) + R2 (1 volume)

Two sets of solutions were prepared as follows:

Blank	1ml R1 + 200 µL Distilled water
Standard	1ml R1 + 200 µL Standard
Assay	1ml R1 + 200 µL Specimen

The standard solution and assay solution mixed gently,the absorbanceA_1 read for each one after 3 min. against the blank solution at 585 nm.

Blank	1ml working reagent + 200 µL Distilled water
Standard	1ml working reagent + 200 µL R3
Assay	1ml working reagent + 200 µL Specimen

The standard solution and assay solution mixed gently,the absorbance A_2read for each one after 3 min. against the blank solution at 585 nm.

All the reagents and solutions above were prepared according the instructions of

Biolabo reagents –Iron recipe kit (France).

For FAA method stock solution of Iron-II, concentration 100 mg/L was prepared in the following way:0.4974 g of $Fe(SO_4).7H_2O$ iron-II sulphateheptahydrate was dissolved indistilled water in 1000 ml volumetric flask.

Standard solutions of Iron-IIat the range (1-8) mg/L areequal to (100 – 800) µg/dLwas prepared daily from stock solution by appropriate dilution with distilled water as below:

200 µL of each one of the following standard solutions (100, 200, 300, 400, 500, 600, 700, and 800) µg/dL were prepared, the following volumes of working reagent (0.5, 1, 1.5, 2, 2.5, 3, 3.5,and 4 µL) were added respectively.

2.2 Instrumentation

All measurements were carried out with both AI 1200 Flame Atomic Absorption Spectrometer (Aurora Instruments Ltd., Canada), the instrumental parameters were adjusted according to the manufacturer's recommendations, Iron hallow cathode lamp was used as a radiation source, operated at 5 mA with a slit width 0.2 nm, the wavelength was set at 248.3 nm resonance line, the air-acetylene flame was used, fuel flow rate was 1.5 Lmin.$^{-1}$and UV-1100 Spectrophotometer -single beam(E-chrom tech Co.), λ_{max} 585 nm with quartz cells1cm path length.

3. Results and discussion

Calculation

The equation below is used to determine the Iron concentration in the assays. The dilution of the standard and specimen solutions were taken into account during the calculation.

$$\text{Iron [µg / dl]} = \frac{(A_2\text{-}A_1)\ \text{Assay}}{(A_2\text{-}A_1)\ \text{Standard}} \times \text{Conc. St.}$$

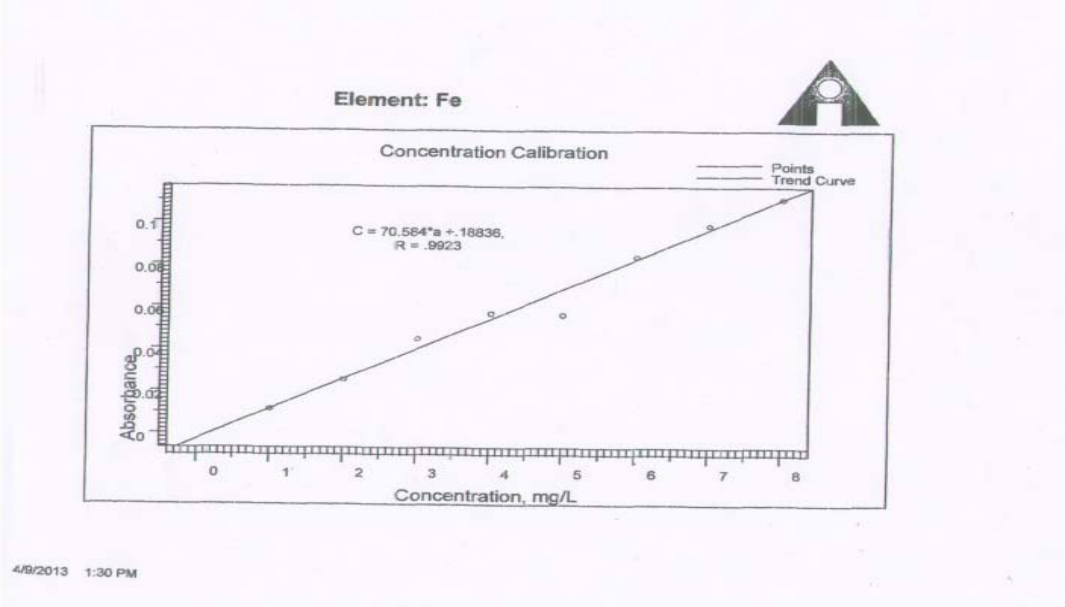


Fig1. The calibration curve of Iron obtained by FAAS

Table 1: Iron concentration in serum samples of Thalassemia patients determinate by bothspectrophotometry and flame atomic absorption spectrometery.

Sample	Iron µg / dL * Spectrophotometry	Iron µg / dL FAAS	Sample	Iron µg / dL ** Spectrophotometry	Iron µg / dL FAAS
1.	429.123	429.212	22.	514.948	514.888
2.	269.973	269.866	23.	843.249	843.231
3.	383.295	385.212	24.	757.424	757.430
4.	302.469	302.451	25.	340.799	340.811
5.	368.296	367.501	26.	469.119	469.124
6.	374.962	375.101	27.	249.975	249.875
7.	678.265	678.341	28.	142.485	142.555
8.	383.295	383.311	29.	453.288	453.341
9.	288.304	288.310	30.	471.619	472.111
10.	549.945	500.341	31.	325.800	326.212
11.	472.452	472.533	32.	408.292	408.331
12.	285.804	285.810	33.	434.956	434.889
13.	168.316	168.322	34.	452.454	452.462
14.	538.279	537.888	35.	349.965	349.863
15.	162.483	162.512	36.	189.147	189.185
16.	294.970	294.889	37.	522.447	522.453
17.	392.460	392.452	38.	315.801	315.821
18.	559.944	559.954	39.	351.631	351.643
19.	444.122	444.131	40.	421.624	421.671
20.	449.955	500.102	41.	292.470	292.478
21.	629.103	629.212	42.	938.239	938.235

* (Male)
** (Female)

Table2. Iron concentration in serum samples of normal groupdetermined by both spectrophotometry and flame atomic absorption spectrometery

Sample	Iron µg / dL * Spectrophotometry	Iron µg / dL FAAS	Sample	Iron µg / dL ** Spectrophotometry	Iron µg / dL FAAS
1.	56.150	56.142	22.	58.454	58.233
2.	64.612	64.608	23.	65.380	69.824
3.	69.222	69.225	24.	67.684	67.652
4.	153.839	153.941	25.	63.069	63.121
5.	66.150	66.145	26.	58.454	58.511
6.	76.914	76.867	27.	60.765	61.101
7.	61.531	61.553	28.	73.071	73.122
8.	121.524	121.533	29.	140.746	139.875
9.	119.985	119.963	30.	51.224	51.369
10.	129.215	130.121	31.	59.992	60.101
11.	89.995	89.977	32.	66.146	66.155
12.	126.139	125.982	33.	86.148	86.161
13.	81.533	80.899	34.	55.378	55.444
14.	67.689	67.642	35.	63.069	64.107
15.	84.605	84.589	36.	58.454	58.544
16.	104.603	104.591	37.	66.146	66.159
17.	115.371	115.354	38.	56.916	57.111
18.	78.452	78.576	39.	64.607	64.715
19.	67.684	67.597	40.	56.916	56.842
20.	96.911	96.821	41.	50.995	51.381
21.	71.533	71.751	42.	64.607	64.643

* (Male)
** (Female)

The calculated *t* value was (1.3240, 0.0226) for male and female of thalassemia patients group respectively and (1.8542, 1.2880) for normal male and female group respectively, these values were less than *t* tabulated value (2.086) at the 95% confidence level for 20 degree of freedom, so therefore no significant difference between the two methods at this confidence level was found.

Conclusion

The flame atomic absorption method with ferene reagent can be successfully used to the determination of iron in serum and can be used instead of spectrophotometric methods. No significant difference between the two methods was found. This study shows that the routine monitoring at regular interval is necessary to detect any disturbance in order to establish appropriate protocol for treatment

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تقدير الحديد في مصل دم مرضى الثلاسيميا
بمطيافية الآمتصاص الذري اللهبي

الخلاصة:

استخدمت طريقة بسيطة وحساسة وهي مطيافية الأمتصاص الذري اللهبي في تقدير تركيز الحديد في مصل الدم ولكلا الجنسين (ذكروانثى) لمجموعة مرضى الثلاسيميا ومجموعة اعتيادية من الأصحاءوقورنت النتائج مع الطريقة الطيفية القياسية ، اعتمدت الطريقتان على تفاعل الحديد مع كاشف الفرين في الوسط الحامضي . اظهرت نتائج قياس 84 نموذج اختلاف تركيز الحديد في نماذج المصل، ووجد ان الحديد يتواجد بمعدل (401.3) و (440.3) ميكروغرام / ديسي لترللأناث والذكور على التوالي لمجموعة مرضى الثلاسيميا ، وبمعدل (66.3) و (90.6) ميكروغرام / ديسي لترللأناث والذكور على التوالي في مجموعة الآصحاء.

اوضح نتائج التحليل ارتفاعا" في مستويات الحديد في مصل مجموعة مرضى الثلاسيميا عما هو عليه في مجموعة الآصحاء وكذلك ارتفاع قيمة الحديد في ذكور مرضى الثلاسيميا والحالة الاعتيادية عنه في الأناث .وجد إحصائيا" ان هناك اتفاق جيد في قيم محتوى الحديد في نماذج مصل الدم التي قيست بطريقة الأمتصاص الذري اللهبي مع النتائج التي حصل عليها بالطريقة القياسية ، وان قيمة t المقاسةكانت اقل من قيمة t في الجدول عند مستوى 95%في هذا المستوى من الثقة ولهذا لا توجد فروقات معنوية بين الطريقتين.