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A simple, rapid and improved colorimetric assay for nontransferrin thalassemia patients bound iron estimation inthalassemia patients

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Abstract

Non transferrin bound iron refers to the many kinds of plasma iron that are not carried by the plasma protein transferrin. Due to its redox activity, as shown by Haiber weiss and Fenton chemistry, it has been hypothesized that such iron compounds are poisonous. Non transferrin bound iron has been intensively investigated in patients suffering from iron overload disorders including hemochromatosis and thalassemia getting blood transfusion therapy owing to noticeable increased transferrin saturation. A number of scholars have proposed studying NTBI to evaluate the effectiveness of chelators in treating iron overload. Different researchers have proposed different ways for measuring the amount of iron that is not bound to transferrin, such as chromatography, fluorimetry, atomic absorption spectroscopy, spectrophotometry, etc. However, none of them have yet achieved widespread acceptance or been declared the gold standard because of their complicated methods, need for expensive specialist equipment, and questionable accuracy. To make the bathophenanthroline based colorimetric approach for non transferrin bound iron quantification more accessible in low-capacity labs without sacrificing accuracy, we have experimented with a number of variants. Patients with beta thalassemia and healthy controls were tested using the NTBI procedure that yielded the best stable findings. We discovered that the background noise was much reduced and the most consistent findings were produced by high-speed centrifugation with interstitial sample pre-incubation. In the beta thalassemia patient group, we found a considerably greater mean value for non-transferrin bound iron compared to the corresponding mean value in the control group.

Hemochromatosis, beta thalassemia, and bathophenanthroline are all diseases associated with an excess of iron that isn't bound to transferrin.

Key-Words: Non transferrin bound iron, transferrin, hemochromatosis, β thalassemia, bathophenanthroline

Introduction

Although iron is a necessary transitional metal that serves various important functions in the body, too much of it or iron in its free form may be harmful. Because it can go back and forth between its ferric (Fe3+) and ferrous (Fe2+) ionic states, it may produce reactive oxygen species via Haiber weiss and Fenton chemistry1, 2, 3. This is the primary cause of its toxicity. This quality mostly manifests when iron is allowed to exist in its free state, that is, without the classically protecting carrier molecule Transferrin (Tf). It is common to refer to this kind of iron as "non transferrin bound iron" (NTBI4). Nonetheless, it is fundamentally distinct from ferritin and haem and does not include apo-Tf. Normally, there wouldn't be any free iron floating about since the concentration of Tf would be high enough to occupy all the iron in the plasma. Due to this understanding, the NTBI was firstly checked for and detected in the patients with thalassemia followed by the patients suffering from hemochromatosis as a simple spill over mechanism[5].

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However later studies suggest the existence of such free iron in the conditions which are not linked with iron overload[6,7,8,9,10,11,12]. Even though being devoid of traditional carrier the iron can't really exist in absolutely free form, rather it is suggested to be bound with a numerous negatively charged molecules like albumin, citrate, DNA, acetate etc.[13,14,15]. As this fraction is not bound with Tf, it can escape the strict iron regulatory mechanism which is mainly focused on Tf bound iron and consequently get deposited intracellularly and cause the damage of the respective organ. NTBI is suggested to cause damage to heart, pancreas, liver, endocrine glands etc. The main way through which iron damage the organ free radical generation, which act detrimentally by depolymerising polysaccharide, inactivating enzymes, lipid peroxidation and damaging DNA[16,17]. Such deleterious effects are combated by Tf which binds to iron and cover its redox activity along with other anti oxidants like vitamin E, glutathione, bilirubin, urate etc.[18,19,20,21,22]. This free fraction of iron as a whole or its sub fraction is named differently by various researchers e.g. Labile plasma iron (LPI), Catalyticiron, free iron, redox active iron, NTBI, Non Plasma bound iron (NPBI), BDI (Bleomycin Detectable Iron), chelatable iron etc. depending on its biological characteristics or its accessibility to various chelators. Presently NTBI is suggested to be analyzed mainly for evaluation of iron overload and the efficacy of the chelator in such patients. However it is also found diagnostically significant in myocardial infarction, renal disease, diabetes, liver disease etc.[6,7,8,9,10,11,12].Presence of NTBI in non iron loaded condition have open the way for its wide scale indications in future, especially in diseases where oxidative stress plays an important role. Even after probable wide scale applications in diagnosis and prognosis of disease, no gold standard or even universally accepted method for NTBI estimation is available[23].

Several methods have been experimented by researchers to estimate NTBI, which are either based on indirect determination of NTBI with the help of Bleomycin or direct chelation of NTBI and its estimation with or without separating it from the biological fluid. Bleomycin based method is the oldest one but lengthy, tedious and extremely vulnerable to various factors like pH, source of bleomycin and application of chelax powder to minimize non specific iron interference increase the cost and complexity. The chelator based assays use different chelators to catch hold NTBI iron which is then either separated from the biological fluid usually by ultrafiltration and estimated with various analytical approaches like HPLC, atomic absorption spectroscopy, inductive conductiometric plasma spectrometry, and colorimetry or measured directly with fluorimeter without separation[24]. The fluorescent based methods are comparatively rapid and require least technical efforts but demand a specialized instrument fluorimeter, which may restrict its applicability. Chelation based methods exploring the ultrafiltration step to take off NTBI fraction from the biological fluid requires higher sample volume and the customized demand depending on the specialized detection system.

Amongst all we have look forward for the method which is least demanding and favorable to be opted in laboratories with restricted resources. One such method was suggested by Zang et al which is based on Bathophenanthroline (BPS) was first used for colorimetric determination of NTBI, but this method was modified by Nilsson et al., who proposed using BPS not only as a chromogen but also as a chelator13,25. BPS has been employed as a significant element in these approaches which traces the forms of NTBI interacting with it. Due to the heterogeneity of NTBI fractions reported various researchers26, it is unclear whether or whether this BPS detectable part represents the whole NTBI fraction.

Hemoglobin synthesis is impaired in beta thalassemia major, a hereditary autosomal condition of hemoglobin synthesis. It causes moderate to severe anemia, requiring regular blood transfusions to manage. These patients are at risk for conditions like iron overload, which might raise transferrin saturation and reveal the existence of NTBI because of the constant flow of blood.In order for the colorimetric technique to be used in everyday clinical practice13, we have experimented with a few different approaches to simplify it, speed it up, and make it user-friendly. We have performed analyses on thalassemia major patient samples to determine NTBI, serum total iron, % transferrin saturation, and Total Iron Binding capacity (TIBC), and have found the findings to be relatively trustworthy. The NTBI's relationship to the other iron-related factors has been investigated.The Stuff and How We Did ItLayout of the ResearchThe current investigation consisted of two parts.Phase 1 included experimental exploration of a variety of instruments accessible in medium-scale labs with the goal of improving the method's ease, simplicity, and speed without sacrificing dependability.Phase

2 of the research compared NTBI levels between the healthy control group and the sick case group. The connection of NTBI with the other analytes was examined.

Subjects

For this investigation, blood samples were taken from 365 participants. Three hundred and forty-five seemed to be healthy volunteers, but twenty were confirmed instances of -thalassemia major. Out of 345 people who seemed healthy, 290 were randomly selected to participate in the experiments, and 55 were used as controls.

Controls

The 55 participants in this study are all human adults from the south Gujarat area. They range in age from 18 to 50, and all seem to be in good health. Cases β Thalassemia major patients of south Gujarat region undergoing blood transfusion therapy have been included.

Informed consent was obtained from all the subjects included.

Sampling

In all the cases blood sample were collected by venipuncture.

Types of sample and anticoagulant

In phase 1 of the study, blood sample were collected in either heparinised or plain Vacutainer® with or without gel separator as per protocol design.

In the phase 2 of the study, the samples were collected in plain Vacutainer®.

Storage of the sample

All the samples collected with or without anticoagulant were centrifuged at 2000 rpm for 15 minutes at end of 30 minutes of collection. All the samples were either processed within next 4 hours or stored within 4 hour of the collection at -55 ^{0}C, till the time of processing, but not more than 3 months.

Biochemical parameters

NTBI, total serum iron, TIBC, % Transferrin saturation.

Materials required

Chemicals and consumables

All chemicals used were of analytical grade and of highest purity available. 4,7-diphenyl-1,10-phenantroline disulphonate (bathophenanthroline disulphonate) (BPS) (B 1375) and ferrous ammonium sulphate [(NH4)2Fe(SO4)2] (F 3754) were purchased from Sigma Aldrich chemical company. Ascorbic acid (103033E) was purchased from BDH Laboratory. De- ionized water was produced from Mili Q water purification system and used freshly. Four different types of BD Vacutainer® s were used as follows

a. Heparinised Vacutainer® without gel separator
b. Heparinised Vacutainer® with gel separator
c. Plain Vacutainer® with gel separator
d. Plain Vacutainer® without gel separator

All tubes and accessories used were disposable and made up of polystyrene to minimize iron interference. The syringe filter used was PVDF durapore membrane of 0.45 µ porosity (SLHVO33RS) from Millipore.

Instruments

High speed centrifugation was done with Revolutionary high speed table top refrigerated centrifuge of Remi, with R-248 rotor; the absorbance was measured with Spectroscan uv 2700 double beam uv-visible spectrophotometer of Chemitro.

Standard curve

Standard curve was prepared with 1 mM BPS and a graded concentration i.e. 0.1-25.6 µmol of iron prepared from ferrous ammonium sulfate, without areducing reagent ascorbate. After mixing the ferrous ions and BPS, the mixture was incubated for 15 minutes at room temperature to ensure completion of the reaction. After incubation the absorbance were read at 535 nm against water blank.

At the experimental level another standard curve was plotted in the same manner but with 1mM ascorbate.

Method

Methodology for phase I: Experimental protocol The experimentation includes 6 trials operated in asequential manner.

Trial 1: Blood samples were collected in heparinised Vacutainer® without gel separator; plasma was separated by centrifuging the Vacutainer® at 2000 rpmfor 15 minutes and processed in two different ways as follows:

- Plasma was filtered with filter mentioned in the materials and method before the reaction.
- Plasma was processed and after the reaction atthe end of incubation period

just before colorimetric reading the reaction mixture was filtered with the filter mentioned in the material and method.

Trial 2: Blood samples were collected in heparinised Vacutainer® without gel separator, plasma was separated as in trial 1 and processed in three different ways as follows:

- Plasma was re-centrifuged at 2500 rpm for 15 minutes and vortex mixed twice i.e. before the reaction and at the end of reaction before readingthe absorbance.
- Plasma was re-centrifuged at 2500 rpm for 15 minutes and absorbance was read at the end of reaction without application of vortex mixture.
- Plasma was processed and read without application of low speed re-centrifugation andvortex mixture.

Trial 3: Blood samples were collected in heparinised Vacutainer® with gel separator, plasma was separated as in trial 1 and processed in two different ways as follows:

- Plasma was processed and after the reaction at the end of the incubation period just before the colorimetric reading the reaction mixture was filtered with the syringe filter mentioned in the materials and method.
- Plasma was processed with the regular protocol without application of filter. In the fourth experiment, a Vacutainer® devoid of a gel separator was used to collect blood samples. After 15 minutes of centrifuging the Vacutainer® at 2000 rpm, the serum was extracted in one of two methods.
- The filter described in the "materials and methods" section was used to filter the serum before the reaction began, and the absorbance was measured thereafter.
- • After processing serum, the reaction mixture was filtered using the filter described in the materials and methods section right before colorimetric reading.
- Serum was separated as in Trial 4 and processed in three different methods, as follows: Trial 5: Blood samples were collected in simple Vacutainer® without gel separator.
- • The serum was centrifuged again for 30 minutes at 10,000 rpm. The serum's clear bottom was carefully pipetted into another tube using a micropipette. The top fatty layer generated at the conclusion of high speed centrifugation has been carefully protected from being disturbed. The serum's clear fraction was pre-incubated at 37 0 C for 15 minutes before being processed as usual.
- • The serum was centrifuged again for 30 minutes at 10,000 rpm. The serum's clear bottom was carefully pipetted into another tube using a micropipette. The top fatty layer generated at the conclusion of high speed centrifugation has been carefully protected from being disturbed. The clear serum fraction was subsequently processed according to standard operating procedure, without any pre-incubation of the serum samples.
- Regular centrifugation speeds and no pre-incubation were used to process the serum samples.
- In the sixth trial, blood samples were taken using a standard Vacutainer® including a gel separator.
- • The serum was centrifuged again for 30 minutes at 10,000 rpm. The serum's clear bottom was carefully pipetted into another tube using a micropipette. The top fatty layer generated at the conclusion of high speed centrifugation has been carefully protected from being disturbed. After separating the serum, the clear fraction was pre-incubated at 37 degrees Celsius for 15 minutes before being processed as usual.

  • The serum was centrifuged again for 30 minutes at 10,000 rpm. The serum's clear bottom was carefully transferred using a micropipette to another tube. An extra care has been taken to minimize the disturbance of top fatty layer formed at the end of high speed centrifugation. The separated clear portion of serum was then processed with regular protocol without in between sample pre incubation.
- The serum samples were processed without high speed centrifugation and pre incubation.

Figure 1: Experimental flow chart: shows all the experimental trials in brief.

Estimation of NTBI in serum/plasma

Phase 1:

Heparinized plasma / serum were processed with the specification noted in the experimental trials. To evaluate the consistency of results for each and every trial sample was processed in duplicate.

Phase 1 and 2:

Color was developed by mixing sample with

BPS and ascorbate 1mM each in final reaction mixture. 1mM Ascorbate was added along with BPS which can convert the ferric ions to ferrous ions which then react with BPS and generate the colored end product. The reaction mixture was then mixed and incubated for 10 min. at R.T. and read as test at 535 nm against reagent blank which is aqueous solution of BPS and Ascorbate 1mM each. For each and every sample, the sample blank was placed without BPS and ascorbate. The value of each sample blank and reagent blank were subtracted from value of test.

Methodology for phase 2

NTBI was measured from control and cases as perexperimental trial no 5A. In the samples collected fromthe subjects with β thalassemia, serum iron, TIBC and
% Tf saturation were analyzed.

Statistical analysis

SPSS 15 was used for box plotting the graphs and for descriptive analysis.

Results and Discussion

Colorimetric analysis of NTBI has been experimented by good number of research workers with the chromogen BPS. BPS is a bidentate ligand and best suitable for quantification of micro level of iron. Zang et al suggested to use the same chromogen for estimation of NTBI in biological sample along with an extra mobilizer, which chelates iron and then it can be estimated colorimetrically with the help of chromogen BPS[25]. Nilsson et al suggested of using BPS all aloneas it can play the dual role of chelator and chromogen[13]. This modification facilitates decrease in the assay time and reduction in the complexity of the procedure to a great extent. But the major limitation of this protocol was the high and fluctuating background color at 535 nm at which the absorbance has to be taken. They further suggested of using the multiscreen filtration system to minimize this background noise, which is not feasible to be used in routine pathological laboratories or hospital setups.

We have carried out experiments on the various routinely available techniques as shown in the experimental trial to minimize the non specific absorbance. One more significant modification we have done in the test protocol is the addition of 1mM ascorbate to the test mixture, which can convert ferric forms of iron to ferrous form and generate color with BPS. This modification has been done to estimate the level of total free iron (ferric + ferrous), and not only ferrous form. As in the body the conversion of ferric form to ferrous form takes place in the presence of reducing agents and ultimately leads to the generation of free radicals triggering the pathological events. No effect of the ascorbate on final color generation was ensured by making two standard curves with various aqueous dilutions of ferrous ammonium sulphate and BPS with and without ascorbate (data not given).

For aqueous solution, the linearity of the method was 0.1-50 µmol iron. The detection limit of the method is
0.1 µmol, which is comparable with the respective values reported by other workers using different methodology[27,28]. Below this level the corresponding absorbance became undetectable. Sensitivity of the spectrophotometer is extremely essential in this case; we had increased the path length which was of 10 mm, to enhance the sensitivity of the method. However while doing so the volume of sample demand had also been increased.

A summary of results for various trials experimented has been presented in the form of descriptive analysis table 1, which shows minimum, maximum, mean and standard deviation of NTBI. Vortex mixing and low speed re-centrifugation of the sample had been experimented to minimize the fluctuation in the absorbance but they failed to do so. Filtration of the sample is a routinely experimented technique which can increase the visible clearness and uniformity of the sample and thus can minimize the non specificfluctuation in absorbance. PVDF filter were used, which was already been successfully utilized by some other research workers in some other format for NTBI estimation[13]. In this study, we have used the PVDF filter in syringe format which is easily available, cost effective and doesn't need specialized accessories. As shown in the protocol, sample had been filtered boththe ways i.e. before and after reaction; unexpectedly the results exhibited a vast fluctuation in both the cases. This could be some non specific reaction taking place in reaction mixture when it comes in contact with We did find a weak but significant relationship between NTBI and % Tf saturation (r =0.55) and serum total iron (r =0.7), but no such relationship was detected between NTBI and TIBC (r =0.15). We found that the NTBI in patients with thalassemia major varied from 0 to 1.6 mol. The mean values calculated were 0.61 + 0.54 µmol which is substantially higher than the corresponding mean value of control (p= 0.001). While some have reported higher levels for NTBI, the mean value we found in individuals with thalassemia major is lower. Direct determination of

NTBI, without application of the additional mobilizor which may extract the iron even from the other iron bound ligands which can't release iron on binding with BPS, may explain the lower trend of the findings in the current work.

We conclude that high-speed centrifugation followed by sample pre-incubation may more effectively eliminate non-specific background noise and provide reliable findings. Colorimetric measurement of NTBI in pathology labs and hospitals might benefit from this straightforward and user-friendly approach. We obtained a value for NTBI in the control group that is consistent with previous reports from researchers who have used methods of similar sensitivity. Using the current approach, we observed that the average NTBI values in patients with thalassemia were greater than in the control group but lower than in the same patient group as reported by other researchers using alternative methods.

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