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A simple, rapid and improved colorimetric assay for non transferrin thalassemia patients bound iron estimation in thalassemia patients

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Abstract

Non transferrin bound iron comprises the forms of plasma iron which are not bound to its traditional transporter plasma protein transferrin. Such forms of iron have been suggested to be toxic due to its redox activity through Haiber weiss and Fenton chemistry. Non transferrin bound iron has been widely studied in patients suffering from iron overload conditions like hemochromatosis and thalassemia receiving blood transfusion treatment due to obvious higher transferrin saturation. NTBI has been suggested to be studied by various researchers to determine the efficacy of chelators used to chelate iron in iron overload patients. Several researchers suggested a variety of methods based on diverse approaches like chromatography, fluorimetry, atomic absorption spectroscopy, spectrophotometry etc. to determine the level of Non transferrin bound iron. However none of them is still widely accepted or considered gold standard due to high complexity of methodology, requirement of sophisticated specialized instruments and accessories as well as debatable reliability. We have experimented different variation in bathophenanthroline based colorimetric method for non transferrin bound iron estimation to make it simple, rapid, cost effective and suitable to be opted in laboratories with limited resources without compromising the reliability of the results. From all the trials, protocol giving most consistent results was used to determine the NTBI value in β thalassemia patients as well as control group. We found that the high speed centrifugation with in between sample pre incubation had significantly decreased the background noise and generated the most consistent results. The mean Non transferrin bound iron value we got in β thalassemia patient group was significantly higher than the respective mean of control group.

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

Introduction

Iron, being an essential transitional metal performs several vital activities in the body but can be proven dangerous if present in excess or left in the free form. Its toxicity is mainly due to its ability to get interconverted between the two ionic states i.e., Ferric (Fe^{3+}) and Ferrous (Fe^{2+}), which makes it capable of generating reactive oxygen species by Haiber weiss and Fenton chemistry[1,2,3]. This property is revealed mainly when iron gets a chance to be present in free form i.e. devoid of its classical safeguarding carrier molecule Transferrin (Tf). Such portion of iron is often called as non transferrin bound iron (NTBI)[4]. However essentially it is not only free of apo-Tf but also not a part of ferritin and haem. In normal conditions the level of Tf is sufficient enough to completely occupy the iron present in plasma and prevent existence of free iron in the circulation.

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]. 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

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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), Catalytic iron, 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 colorimetric estimation of NTBI with the use of Bathophenanthroline (BPS) as a chromogen, it was modulated by Nilsson et al who suggested the use BPS as a chelator as well as chromogen[13,25]. BPS has been used as a key ingredient in these methods which traces the forms of NTBI reacting with it. This BPS detectable portion may or may not be complete NTBI portion due to the heterogenicity of NTBI fractions reported by researchers[26].

β thalassemia major is an inherited autosomal disorder of hemoglobin synthesis, wherein the impairment of β chain synthesis takes place. It results in moderate to severe anemia and such patients are treated with frequent blood transfusions. Due to the frequent blood inflow these patients are susceptible to the development of situation like iron overload which may increase the transferrin saturation and may exhibit the presence of NTBI.

We have experimented certain variations in colorimetric method to make it simple, rapid and user friendly so that it can be applied in routine clinical practice[13]. After achieving the reasonably reliable results, we have analyzed the β thalassemia major patient samples for NTBI, serum total iron, % transferrin saturation and Total Iron Binding capacity (TIBC). An attempt has been made to derive the correlation of the NTBI with the other iron related parameters studied.

Material and Methods

Study design

The present study was divided into two phases.

In the phase1, various tools available in the medium scale laboratories were experimentally explored to increase the ease, simplicity and speed of the method without compromising the reliability of the results.

In the phase 2 of the study, healthy controls and the clinical case groups were analyzed for the presence and change in the levels of NTBI. The correlation of NTBI with the other analytes was evaluated.

Subjects

Blood samples were collected from 365 subjects for this study. Out of these 345 were apparently healthy subjects and 20 were known cases of β thalassemia major. From 345 apparently healthy subjects 290 subjects sample were studied for various experimental trials, 55 were taken as control group.

Controls

This group consist of 55 apparently healthy human adults of the age group 18-50 years, who were either regular blood donors or attendants of patients, staff members and others from south Gujarat region.

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 a reducing 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 a sequential manner.

Trial 1: Blood samples were collected in heparinised Vacutainer® without gel separator; plasma was separated by centrifuging the Vacutainer® at 2000 rpm for 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 at the 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 reading the 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 and vortex 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.

Trial 4: Blood samples were collected in plain Vacutainer® without gel separator. Serum was separated by centrifuging the Vacutainer® at 2000 rpm for 15 minutes and processed in two different ways as follows:

- Serum was filtered with the filter mentioned in materials and method before the reaction and absorbance was taken at the end of reaction.
- Serum was processed and after the reaction at the end of incubation period just before colorimetric reading the reaction mixture was filtered with the filter mentioned in materials and method.

Trial 5: Blood samples were collected in plain Vacutainer® without gel separator; serum was separated as in trial 4 and processed in three different ways as follows:

- Serum was re-centrifuged at 10,000 rpm for 30 minutes. Using a micropipette, the clear bottom portion of serum was transferred cautiously 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 then pre incubated at 37 ^{0}C for 15 minutes and processed with regular protocol.
- Serum was re-centrifuged at 10,000 rpm for 30 minutes. Using a micropipette, the clear bottom portion of serum was transferred cautiously 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 then processed with regular protocol without in between sample pre incubation.
- The serum samples were processed regularly without high speed centrifugation and pre incubation.

Trial 6: Blood samples were collected in plain Vacutainer® with gel separator. Serum was separated as in trial 4 and processed in three different ways as follows:

- Serum was re-centrifuged at 10,000 rpm for 30 minutes. Using a micropipette, the clear bottom portion of serum was transferred cautiously 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 pre incubated at 37 ^{0} C for 15 minutes and processed with regular protocol.
- Serum was re-centrifuged at 10,000 rpm for 30 minutes. Using a micropipette, the clear bottom portion of serum was transferred cautiously 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 per experimental trial no 5A. In the samples collected from the 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 alone as 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 specific fluctuation 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 both the 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 either the filter or the accessories used. As compared to the protocol including filtration, we found lesser fluctuation in the absorbance when samples were processed without filtration, so the remaining protocols were designed without filtration step. In another trial, gel Vacutainer®s were used to minimize the fluctuation, which could not decrease the fluctuation in the absorbance, but certainly increased the serum/plasma volume output from the sample, which is also significant in this methodology. High speed filtration has been routinely practiced to minimize the non specific turbidity of the sample which has been tried in our experiment to minimize the non specific absorbance. Freshly collected samples after separation of serum or freshly frozen and stored serum samples were thaw and then re-centrifuged at high speed at 10,000 rpm for 30 minutes. An extra care has been taken to minimize the disturbance of top fatty layer formed at the end of high speed centrifugation. This approach had significantly reduced the non specific fluctuation and generated more consistent results. Another modification in the same approach has been successfully incorporated i.e. in between sample pre incubation at 37^0 C. Experimentally it has been shown that when sample is processed for electrophoresis, the mobility of the lipoproteins can enhanced when sample is pre-incubated at 37^0 C[29]. This was suggested to be due to solubilization of the lipoproteins present in the sample by activating LCAT enzyme. The same approach was tried in the present methodology as solubilization of lipoprotein may also decrease the non specific fluctuation in the absorbance. In the final protocol the samples were pre incubated after high speed centrifugation, where the major lipidic portion had been removed by high speed centrifugation and a little part present in the sample were made more soluble which gave the most consistent and reliable results. Results of the experimental trials operated in duplicates showed maximum consistency with the trial no 5A and 6A (results had not been shown). The serum has been suggested over plasma as the test samples due to obvious greater and long lasting apparent clearness and ease of collection, when the value of the analyte was suggested to be same in both.

The mean NTBI value for control group was 0.02 ± 0.06 µmol, which is comparable with the other methodology[11,27,30]. The significant cut off value we set was ≤ 0.3 µmol, which was the highest value found in control group, no control subject had the respective value > 0.3 µmol. The mean NTBI values for β thalassemia major patients were higher than the respective mean derived in the control group. When the mean ± SD was checked with the other clinical parameters, we found some correlation of NTBI with the serum total iron (r =0.7) and % Tf saturation (r= 0.55) but no significant correlation was observed with TIBC (r= 0.15). The range of NTBI we observed for β thalassemia major patients was 0 - 1.6 µmol. The mean values derived were 0.61 ± 0.54 µmol which is significantly higher than the respective mean value of control (p= 0.001). However the mean NTBI value we got in β thalassemia major patients is lower than the values reported by others[31]. This lower tendency of the results in the present study could be due to direct determination of NTBI, without application of the extra mobilizor which may extract the iron even from the other iron bound ligands which can't release iron on binding with BPS.

In conclusion we have found that the high speed centrifugation with subsequent sample pre incubation could more efficiently reduce the non specific background noise and generate more consistent results. This simple and easily operated technique can be proven cost effective modification for colorimetric estimation of NTBI in the pathological laboratories and hospital setups. The value of NTBI we found in the control group is comparable with the values reported by other workers with comparable sensitivity. The mean NTBI values we found in the β thalassemia patients using the present method were significantly higher than the control group but lower than the levels found by other workers using different methodology in same patient group.

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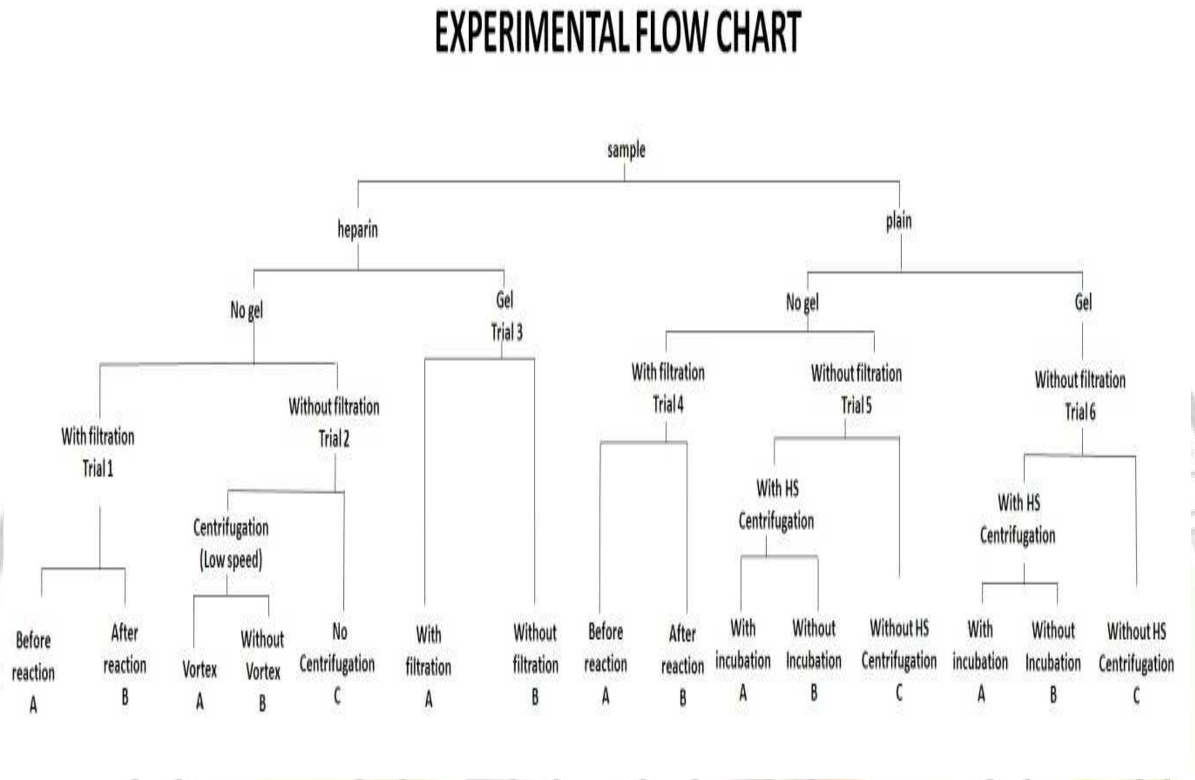

Fig. 1: Experimental flow chart of various trials

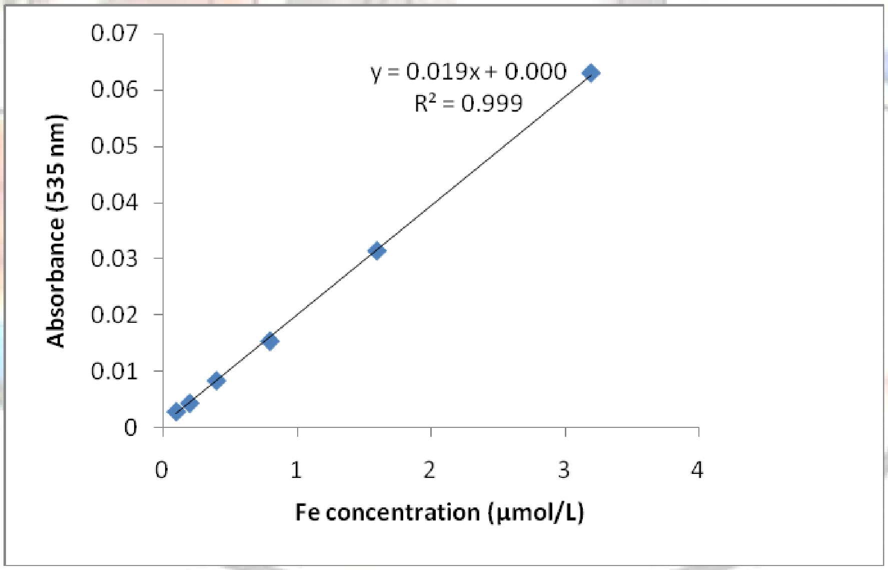

Fig. 2: Standard curve prepared from various concentration of Ferrous (Fe^{2+}) with BPS (1mM)

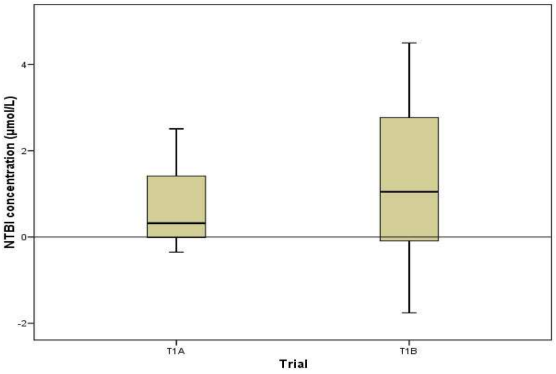

Fig. 3: Concentration of NTBI when heparinised plasma collected in a Vacutainer® without gel separator was: filtered before (1A) and after (1B) the reaction

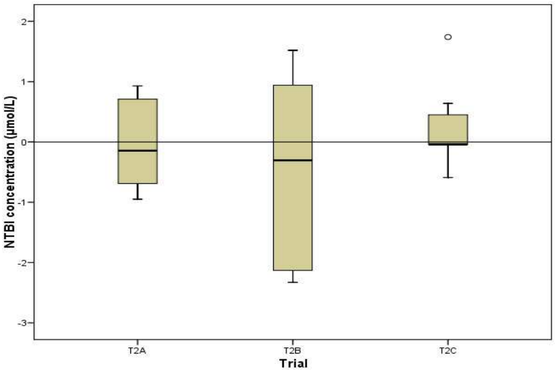

Fig. 4: Concentration of NTBI when heparinised plasma collected in a Vacutainer® without gel separator was: recentrifuged with low speed and vortex mixed before as well as after reaction (2A), recentrifuged without vortex mixing (2B) and as such without recentrifugation as well as vortex mixing (2C)

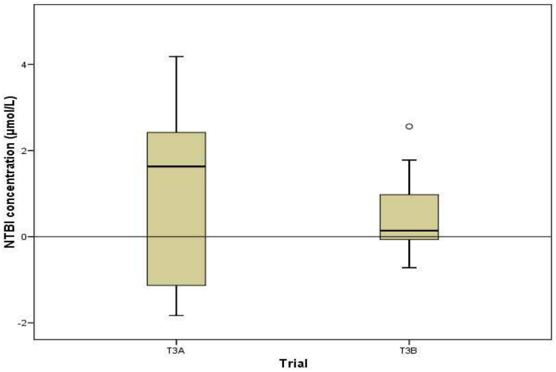

Fig. 5: Concentration of NTBI when heparinised plasma collected in a Vacutainer® with gel separator was: filtered at the end of reaction (3A) and processed without filtration (3B)

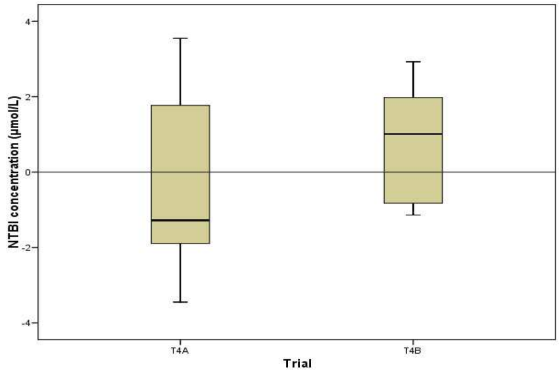

Fig. 6: Concentration of NTBI when serum collected in a Vacutainer® without gel separator was: filtered before reaction (4A) and after the reaction (4B)

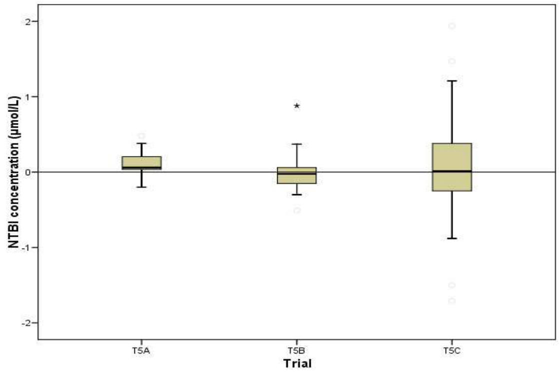

Fig. 7: Concentration of NTBI when serum collected in a Vacutainer® without gel separator was: treated with high speed centrifugation followed by pre incubation of the sample (5A), with high speed centrifugation without pre incubation (5B) and without high speed centrifugation as well as pre incubation (5C)

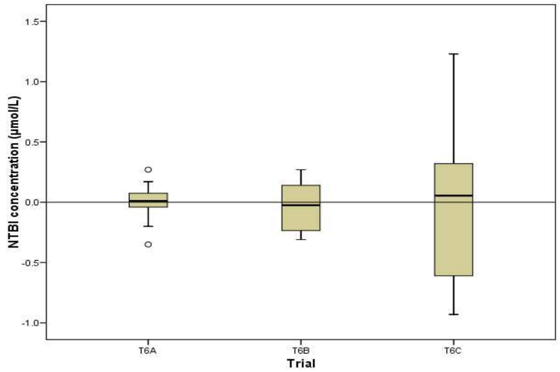

Fig. 8: Concentration of NTBI when serum collected in a Vacutainer® with gel separator was treated with: high speed centrifugation followed by pre incubation of the sample (6A), with high speed centrifugation without pre incubation (6B) and without high speed centrifugation as well as pre incubation (6C)

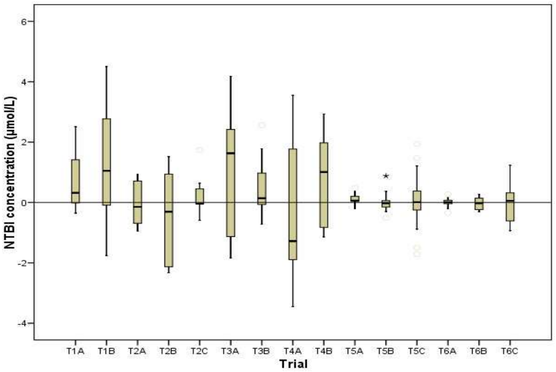

Fig. 9: A comparative account of concentration of NTBI in different experimental trials

Table 1: NTBI values in various experimental trials

Trial No.	Minimum	Maximum	Mean ± SD (µmol/L)
T1A	-0.35	2.51	0.82 ±1.49
T1B	-1.76	4.50	1.17 ± 1.60
T2A	-0.95	0.93	0.03 ± 0.75
T2B	-2.33	1.52	0.20 ± 1.60
T2C	-0.59	1.74	0.17 ± 0.61
T3A	-1.83	4.18	1.02 ± 2.16
T3B	-0.72	2.56	0.42 ± 0.79
T4A	-3.45	3.55	-0.20 ± 2.60
T4B	-1.14	2.93	0.73 ± 1.70
T5A	-0.20	0.48	0.10 ± 0.16
T5B	-0.51	0.88	-0.01 ± 0.25
T5C	-1.71	1.94	0.07 ± 0.79
T6A	-0.35	0.27	0.00 ± 0.16
T6B	-0.31	0.27	-0.03 ± 0.21
T6C	-0.93	1.23	-0.03 ± 0.65

Results of Phase II

Table 2: Mean and SD of NTBI values in different groups (n=55 for control, n=20 for β thalassemia)

Group	Mean NTBI (μmol)
Control	0.02 ± 0.06
β thalassemia	0.61 ± 0.54

Table 3: Other Biochemical parameters studied for β thalassemia major patients

Other biochemical parameters	Mean ± SD
S. Iron (µg/dl)	114.0 ± 22.72
S. TIBC (µg/dl)	279.80 ± 46.34
% Transferrin saturation	41.28 ± 7.74

Table 4: Pearson correlation coefficients (r) between NTBI and other clinical parameters

Name of the parameter	Pearson correlation coefficients (r)
S. Iron (µg/dl)	0.70
S. TIBC (µg/dl)	0.15
% Transferrin saturation	0.55