

## Original Research Article

# Comparison of direct total iron binding capacity by light MgCO<sub>3</sub> and heavy MgCO<sub>3</sub>

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### ABSTRACT

**Background:** The aim of the study is focused to measure TIBC by calculated method (TIBC using the magnesium carbonate- MgCO<sub>3</sub> adsorption), measurement of TIBC by calculatory method from automated UIBC and automated Iron measurement. Latter these two values are to be added and would give an additive result of TIBC. The experiment would be performed by using the light MgCO<sub>3</sub> powder and the heavy MgCO<sub>3</sub> powder and check for any differences in their respective values. Regression analysis, Bland Altman analysis or histogram analysis of TIBC results obtained by calculated methods in patient samples to find correlation between the two methods is being performed.

**Methods:** Samples received for analysis of tests, regardless of patient identification with sample volume serum were used for the study. After complete analysis and reporting of the sample, the leftover serum was used. Serum was kept at room temperature. Then the calculated TIBC from UIBC and the IRON were compared after doing batch calibration for UIBC and lot calibration of Iron.

**Results:** It was found that, this study had positive bias by the usage of the heavy MgCO<sub>3</sub> powder. Positive bias in unsaturated iron binding capacity observed by both methods is studied and further investigated using data obtained during the experiment.

**Conclusions:** There is a higher yield of serum obtained with the heavy MgCO<sub>3</sub> powder, TIBC calculated from iron and unsaturated iron binding capacity as compared to TIBC measured directly using the light MgCO<sub>3</sub> powder.

**Keywords:** Iron, UIBC, TIBC, MgCO<sub>3</sub>, Calculated method, Automatic analyser

### INTRODUCTION

Laboratory measurement of ferrum status is very much common and utmost requirements by clinicians when a case of anemia is investigated. Of many tests required for investigations of a case of anemia, serum UIBC (unsaturated iron binding capacity), serum iron and serum TIBC (total iron binding capacity) are frequently ordered by clinicians, especially in women who are pregnant and children to know the status of iron nutrition in patients. At physiological pH, iron is rapidly oxidized to the insoluble ferric (Fe<sup>+3</sup>) absorption occurs by the enterocytes by divalent metal transporter-1 (DMT-1) present mainly in the duodenum and upper jejunum from

duodenal mucosa iron reaches to the blood from which, it reaches to the site of the erythropoiesis site- bone marrow by the transferrine.<sup>1,2</sup> Ferroportin interacts with hephaestin which has ferroxidase activity. Membrane-bound ferroxidase hephaestin which interacts with ferroportin catalyses the reoxidation into Fe<sup>+3</sup> and thus ferroportin-mediated efflux of Fe<sup>+2</sup> occurs.<sup>3</sup> So, this process is very dynamic and undergoes more than 10 times daily turnover to sustain erythropoiesis and reticulo endothelial cells export Fe<sup>+2</sup> from its plasma membrane through ferroportin. Ceruloplasmin re-oxidised the Fe<sup>+2</sup> to Fe<sup>+3</sup> and then Fe<sup>+3</sup> loaded to the transferrin.<sup>4</sup> GI HCL and Ascorbic acid also reduces Fe<sup>+3</sup> to Fe<sup>+2</sup> so enhances the absorption. Oxalates, Phosphates, and phytates from

vegetables, polyphenols, calcium, and peptides from partially digested proteins, decrease Fe<sup>2+</sup> absorption by making insoluble complexes.<sup>5</sup> Measurement of Iron, UIBC and TIBC should be taken as a challenge by the laboratories because iron is present in microgram quantity and prone to analytical and pre-analytical interferences and influences. Microgram quantity of Iron and related parameters makes it essential to have automation in their measurement to ensure clinically acceptable precision and accuracy. Moreover, manual steps increases turnaround time (TAT) and further decreases utility of method to clinicians.

Total iron binding capacity measurement can be done by two principle methods. The first method, excess and extra iron is added to saturated transferrin. Transferrin unbound iron is extracted by adsorption with MgCO<sub>3</sub>, followed by iron measurement at acidic pH. The method is huge and tedious and its modification is used as a reference method for measurement of TIBC. However, it needs manual steps. The second is more common method, Iron is added in a sample and unbound iron is measured at alkaline pH, at which transferrin do not release bound iron. This method does not require MgCO<sub>3</sub> adsorption step, it is fully automated. However, it's sensitive to pH of the reaction mixture. Iron status is monitored in a patient by serial measurements of various laboratory tests over time. Since, patient samples analysed serially over the course of time may go to different laboratories and may be analysed by different methodologies, interpretation of such test results can be done reliably only if different assay methodologies give comparable results. This study aims at comparing TIBC with heavy MgCO<sub>3</sub> and the other by light MgCO<sub>3</sub>. Various essential minerals like calcium, magnesium, phosphate, zinc, selenium, sodium, potassium cobalt, copper, iron, are widely distributed in food and drink products and most people eating a mixed diet are likely to receive adequate intake. So these minerals in our body are all required for structural function, involved in membrane function, functions as prosthetic groups in enzymes etc.

From all of these elements, iron is a biologically essential element of living organism. Iron from the diet occurs in two forms: heme and nonheme. Newborn children depend upon iron reserves received from mother during pregnancy. Milk and cereals are poor sources on which infants depend initially. Hence the iron supplements are usually given. Iron is recycled in our body so it is so conserved by the body.

#### **Aims and objectives**

Aim of the current study is the measurement of TIBC by direct method (TIBC using both heavy and light Magnesium Carbonate- MgCO<sub>3</sub> adsorption).The method involves manual steps for preparation of samples. Measurement of TIBC once by heavy MgCO<sub>3</sub> and again the other by light MgCO<sub>3</sub>.The method do require manual step. Comparing heavy and light MgCO<sub>3</sub> are required.

Objectives of current study were; to perform regression analysis of Heavy MgCO<sub>3</sub> and the other by light MgCO<sub>3</sub>, TIBC results obtained by other by heavy and light MgCO<sub>3</sub> methods in patient samples to find correlation between the two methods and to perform Bland Altman analysis of results obtained by heavy and light MgCO<sub>3</sub> methods in patient samples to find relative bias in TIBC results obtained by both methods in patient samples.

#### **METHODS**

This study is planned for patients in new civil hospital, Surat in between January-2020 to December-2020. It is an experimental study comparing two different types of analytical methods. So within a span of one year samples received from the patients in the laboratory were randomly collected. A total of 60 samples were found to be appropriate. After complete analysis the left over patient's samples from OPD or IPD patients are taken in account for the experiments if approximately 2 ml serum is left in the vacutainer.

#### **Inclusion criteria**

Samples received in plain vacutte to the hospital's clinical biochemistry laboratory, Surat with leftover 2 ml of serum after requested tests were performed, are included in this study.

#### **Exclusion criteria**

Exclusion criteria for current study were; hemolysed samples, samples collected in EDTA or fluoride vacutainer, serum less than 3 ml, lipemic samples, vacutainer devoid of patients needed information.

#### **Generalized sample preparation**

Leftover samples received for testing of tests by the hospital's clinical biochemistry laboratory, Surat, devoid of patient identification were used for the study. Laboratory technicians were told to give a list of such samples which consists of leftover 2 ml of serum after requested tests were performed on a daily basis. After samples being received, were given a unique lab ID number and registration was done in LIS (laboratory information system).

Each of the samples was centrifuged for 10 minutes at 3000 rotation per minute. For the first phase, samples were run for their requested test parameters. When complete analysis and reporting of the sample was done, the leftover samples were separated in another Eppendorf cup and sealed with parafilm for this study. Samples were kept at room temperature till 60 samples were collected. Then for TIBC heavy and light MgCO<sub>3</sub> was used for analysis. For both TIBC procedures, manual steps needed for sample preparation are described later.

### TIBC measurement

The serum in the sample is treated with excess Fe (+3) to saturate the iron binding sites on transferrin. Excess of Fe+3 is removed by adsorption with light magnesium carbonate powder and precipitated then the Iron content in the supernatant is measured to give the TIBC.

### Reagent preparation

Following chemicals are to be used: acetic acid, sodium acetate, Brij-35 (30%), thiosemicarbazide, ferrozine, hydroxylamine HCl, concentrated HCl, ferric ammonium sulphate, magnesium carbonate and DI water. To make R1, around 700 ml DI water was taken in 1000 ml beaker and add above all chemicals according to its concentration needed (except ferrozine). Thiosemicarbazide takes some time to dissolve; glass rod was used to dissolve it. Brij was added as it will prevent protein precipitation at acidic pH. Hydroxylamine HCL was used as a reducing agent. It is safer and easy to weigh acetic acid than that of measuring by its volume; it was made up to 1 litre solution using DI water. No need to adjust the pH, it will be acidic around 4.5. To make R2, 1 gram of ferrozine was added to 100 ml of R1. Both of these reagents were stable onboard for long. These reagents were stored at 2-4°C. Ratio for R1/R2 was 200:20, so 900 ml R1 needs 90 ml R2. For preparing R4, 4.32 gram ferric ammonium sulfate was added in 10 ml concentrated HCL and the volume was made upto 100 ml with water. Then iron standard solution was being prepared. For this, 1 ml of R4 is taken and made up to 1000 ml DI with water. Separate cups were made containing 100 mg of magnesium carbonate which were used in sample preparation.

**Table 1: Final concentration of chemicals in reaction mixture in TIBC.**

Chemical	mmol/l	Weight to be measured for 1 litre
Sodium acetate	250	20.5075 g
Acetic acid	250	15.028 g
Thiosemicarbazide	-	1 g
Brij-35% (30%)	-	10.5 g
Hydroxylamine HCl	-	20.84 g
Ferrozine	-	1 g
Ferric ammonium sulphate	-	4.32 g
Concentrated HCl	-	10 ml

### Sample preparation

The sample preparation was divided into two parts: for heavy MgCO<sub>3</sub>, and the other for light MgCO<sub>3</sub>. Both the procedures are almost same except that the powders are different.

Heavy MgCO<sub>3</sub>; sample preparation, it has many manual steps. 400 µl iron standard solution is first added to the 200 µl serum sample which was kept at room temperature for 10 minutes. Then 100 mg of heavy MgCO<sub>3</sub> is added to each sample. Again it was kept for 20 minute incubation at room temperature. After the incubation phase, centrifuge for 20 minutes at 3000 rpm. At about 150 µl of supernatant found. The supernatant was separated in another eppendorf cup and centrifuged for 10 minute at 3000 rpm. The supernatant was again separated in another eppendorf cup. Run iron from this sample. It will give TIBC results. system parameters set in an automatic chemistry analyser ERBA XL\_1000 to be used for TIBC measurements, like same way, light samples was also prepared.

Light MgCO<sub>3</sub>; sample preparation, it has many manual steps. 400 µl iron standard solution is first added to the 200 µl serum sample which was kept at room temperature for 10 minutes. Then 100 mg of light MgCO<sub>3</sub> is added to each sample. Again it was kept for 20 minute incubation at room temperature. After the incubation phase, centrifuge for 20 minutes at 3000 rpm. At about 150 µl of supernatant was found. The supernatant was separated in another eppendorf cup and centrifuged for 10 minute at 3000 rpm. Again the supernatant was separated in another eppendorf cup. Run iron from this sample. It will give TIBC results. System parameters set in an automatic chemistry analyser ERBA XL\_1000 to be used for TIBC measurements. The results were exported and imported into excel software for data analysis. Regression analysis and Bland-Altman analysis was performed.

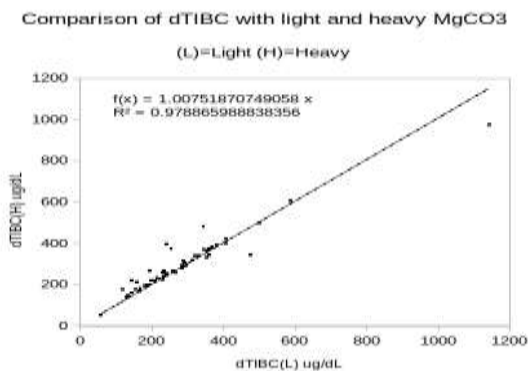
### RESULTS

Sample interference which had been affecting in TIBC measurement can be taken from observing reaction graphs which was generated in the automated analyzer machine. These interferences are associated with initial blank on mixing R1 (buffer) with sample and final absorbance during initial incubation of sample and buffer.

Although light MgCO<sub>3</sub> TIBC method is semi automated, in contrast to semi automated heavy MgCO<sub>3</sub> TIBC method, when sample interference with TIBC measurement is suspected, use of semi automated heavy MgCO<sub>3</sub> TIBC method measurement is advisable. This strategy is though laborious as compared to indirect method of calculated UIBC and iron, the analyst gets an upper hold by having more amount of samples (serum) in hand. If the analyst wants, can go for preparations of the sample, and can rule over any kind of interferences during the analytical procedure.

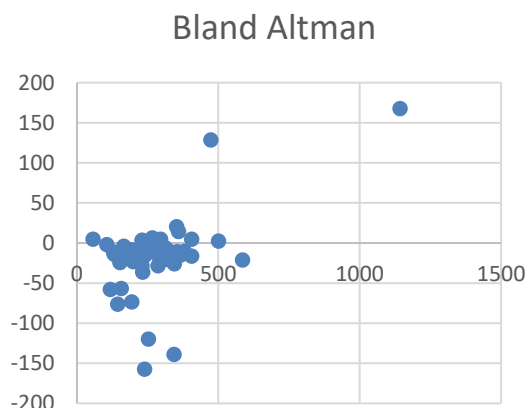
The demographic data and the characteristics of the populations were not noted though in the study. Any patients sample of any gender or age or race group was used for the study. There was a good correlation between light MgCO<sub>3</sub> powder and heavy MgCO<sub>3</sub> powder being

used as a precipitator being explained by regression analysis (Figure 1).



**Figure 1: Comparison of dTIBC with light and heavy MgCO3 by linear regression graph.**

The chart above shows slope of 1 and R2 of 0.97, indicating good correlation between direct TIBC values obtained using heavy and light MgCO3 for adsorption of excess iron in direct TIBC measurement. In order to find any sample interference, reaction kinetics of direct TIBC was visually observed in Erba XL-1000 fully automated biochemistry analyser. There was a good correlation between light MgCO3 powder and heavy MgCO3 powder being used as a precipitator being explained by Bland-Altman analysis (Figure 2).



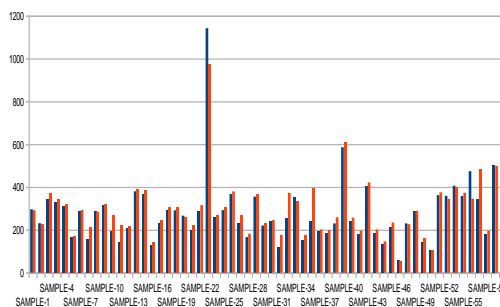
**Figure 2: Comparison of dTIBC with light and heavy MgCO3 by Bland-Altman analysis.**

The graph is plotted on the XY axis where X represents the difference of the two measurements, and the Y-axis shows the mean of the two measurement which can also be plotted using percentages or ratios. For other relevant measures, it was recommended here that 95% of the data points should lie within  $\pm 1.96$  SD of the mean difference-limits of agreement. Bland-Altman plot of differences for the light and heavy MgCO3. These differences are plotted against the average concentration. The mean difference (15 mg/dl) with standard deviation of differences is shown. A plot of residuals standardized to

unit standard deviation. The homogeneous scatter supports the assumed proportional error model and the assumption of linearity. Standardized residuals plot with indication of the outlier. The three lines represent mean of differences called bias and rest two lines are limits of agreement  $\text{mean} \pm 1.96$  SD.

The researcher should decide the level to which the error would be acceptable. It more than 50% of the values lie outside the limit, than it indicates that there is no agreement between the tests. As a general rule implied goals whether biological or clinical goals could define if the agreement interval is wide or narrow for any purpose. Here in the picture it depicts that there is a good correlation between light MgCO3 powder and heavy MgCO3 powder being used as a precipitator being explained by histogram analysis.

A graphical device for displaying large set of dTIBC results is the frequency distribution, also called a histogram. The differences (the delta values) of the two different methods are calculated and plotted in a frequency distribution histogram. The histograms values will be very different because the two method groups differ in severity of results.



**Figure 3: Comparison of dTIBC with light and heavy MgCO3 with histogram analysis.**

## DISCUSSION

So as estimated earlier from the method section, the sample was collected within a span of one year samples from the patients in the laboratory randomly. It's also mentioned that no any other special sampling technique is followed for measuring the size of the 60 sample which was being analysed. After complete analysis the left over samples are taken for the experiments which is approximately 2 ml serum is left in the vacuttainer.

There is approximately equal amount of values obtained with TIBC result calculated with light and heavy MgCO3 adsorption rates. Some samples had been having interference in TIBC measurement, probably due to high precipitation, high hemolysis and high turbidity. This interference which has been resulted is having an irregular correlation between the various results of the above two methods. The study could not find exact cause

for high initial absorbance observed in some of the samples which was taken into consideration as interferences from any pre-analytical condition. So the minerals present in the human body are required for structural function, are involved in membrane function, functions as prosthetic groups in enzymes etc. Amongst all of these elements, Iron plays a crucial role and is a biologically essential element of living beings. Iron absorbed from the diet occurs in two forms: heme and nonheme are as such. Newborn children depend needs this supplements being received from mother during the period of pregnancy. Milk and cereals are some of the sources, though poor, on which infants initially depend. So, the iron supplements replenishment is given usually. It is recycled in the body and is conserved by the body.

Interference in the sample which being affecting in TIBC measurement can be taken in to account from reaction graphs observed which we got in the automated analyzer machine. Associated of interference is related to the initial blank of the reagent solution on mixing R1 (buffer) with sample and final optical density will be found during initial incubation period being maintained by the procedure of sample and buffer. Light  $MgCO_3$  TIBC method is semi automated though, but in contrast to semi automated heavy  $MgCO_3$  TIBC method are also used, which, when sample interference with TIBC measurement is suspected, usage of this semi automated heavy  $MgCO_3$  TIBC method measurement is recommended as it yield more serum after centrifugation. This strategically a tedious and laborious process is though as compared to indirect method of calculated UIBC and iron, the analyst gets an upper hold by having more amounts of samples (serum) in hand. In the experiment if the analyst wants, can go for preparations of the sample, and can rule over any kind of interferences during the analytical procedure.

Dietary iron occurs in two forms: heme and nonheme. Iron is a biologically essential element of living organism.<sup>6,7</sup> Results of TIBC obtained by using heavy and light  $MgCO_3$  were entered in a spreadsheet. Heme iron's bioavailable (15%-35%) and dietary factors have little effect on its absorption, whereas nonheme iron's absorption is much lower (2%-20%).<sup>8</sup> The scatter plot of TIBC with heavy  $MgCO_3$  vs. TIBC light  $MgCO_3$  is depicted in (Figure 2). Primary sources of heme iron are hemoglobin and myoglobin from consumption of meat, poultry, and fish, whereas nonheme iron is obtained from cereals, pulses which is a growth limiting factor.<sup>9,10</sup> Iron contacts with oxygen and forms oxides, which are highly insoluble, and is not easily available by organisms for uptake and takes part in synthesis of oxygen transport proteins (myoglobin, hemoglobin), formation of heme enzymes and iron-containing enzymes of electron transfer and oxidation-reductions.<sup>11-13</sup> Approximately 75% of iron's present in RBCs as hemoglobin, 25% is present in iron store which is labile and left 15% is present in muscles, at physiological pH, ferrous iron ( $Fe^{+2}$ ) is oxidized to ferric ( $Fe^{+3}$ ) form and is absorbed by the

enterocytes's divalent metal transporter-1(DMT-1 ).<sup>14,15</sup> Mostly clinical chemistry analyzers measure UIBC because it is more easily automated than TIBC along with measuring iron. The precision of measurement of UIBC is good at high concentrations as found in iron depletion but worsens at low concentrations in the presence of iron overload.<sup>16</sup>

One of the crucial elements of the body is iron and is required for various functions; depletion of iron store is measured by estimation of serum iron, serum TIBC, serum transferrin level (by immune-chemistry method). Measurements of TIBC, serum iron, and the ratio of serum iron to TIBC (transferrin saturation) are widely used for the clinical diagnosis and monitoring of treatment for iron-deficiency anemia and chronic inflammatory diseases, as well as for screening tests for other clinical purposes like anemia.<sup>17-20</sup> Myoglobin resembles to a single Hb subunit because it does not form tetramers and lacks the allosteric oxygen-binding properties of Hb. In the circulation, iron is bound to free sites on the plasma Fe-transport protein transferrin. Excess iron is added to serum to saturate the iron-binding capacity.

The unbound iron is removed by using phenanthroline or ion-exchange resin or magnesium carbonate, and then the bound iron is extracted and determined colorimetrically.<sup>21</sup> The amount of serum-iron (SI) and the unsaturated iron-binding capacity of serum (UIBC) are determined, respectively, and then the two values are added.<sup>22-25</sup> The histogram represents the result distribution all tests methods, orange for light  $MgCO_3$  and blue for heavy  $MgCO_3$ . The histogram shows the distribution of 60 serum TIBC concentrations measured in consecutive clinical specimens.

Examination of the histogram serves to safeguard misapplication or misinterpretation of statistical methods, and it may reveal valuable information about the data and visual inspection of a histogram is a reliably better method for identification of possible outliers. Here, the histogram shows a non-Gaussian distribution of the above frequencies graphed according to length. The experiment was tabulated in three different analytical charts and it was found in all three formats that there is a nominal difference which is negligible and if ever there are outliers, than it could not be taken in account. Others have mentioned about comparing direct total iron binding capacities (dTIBC) with calculated total iron binding capacities (cTIBC) which has influenced us to go further with our new experiment for two different kinds of precipitating agent  $MgCO_3$ .

### **Limitations**

The study is limited to local demographics of Surat and nearby areas which caters diagnostic services to the patients. Another limitation is that the study did not take in account genders and any particular age groups. It

scopes for the usage of heavy MgCO<sub>3</sub> powder as it helps to extract higher amount of serum from the supernatants.

## CONCLUSION

Our team had been experimenting with cTIBC and dTIBC with MgCO<sub>3</sub> powder (light), but seemed the necessity to finalize a particular precipitating agent which could give us higher amount of supernatant, because of the scarcity of the supernatants proper analysis was disturbed and hardly any sample would be left for repetitions. Current study proceeded with experimenting with two different powder types of MgCO<sub>3</sub> (heavy and light). As per our assumptions both powders give almost the same results with a nominal difference being explained earlier. The purpose of using heavy MgCO<sub>3</sub> powder was successfully established as there no such big difference.

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