Plasma Iron and Transferrin Iron-Binding Capacity Evaluated by Colorimetric and Immunoprecipitation Methods

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We evaluated plasma iron (PI) and total iron-binding capacity (TIBC) or transferrin in normal individuals and in patients with iron imbalance. The standard colorimetric measurements of PI and TIBC and the standard isotope-dilution measurement of TIBC were compared with an immunoprecipitation method and also with immunoelectrophoresis of transferrin. PI concentrations as measured by the standard and immunoprecipitation methods agreed closely for all individuals except those with saturating transferrin, where nontransferrin iron increased the results in the standard assay. This excess iron in saturated plasma may be derived from either free iron or iron-bearing ferritin. There were also differences in TIBC between the two methods. Iron-deficient sera gave higher values for transferrin when measured by immunoelectrophoresis. Unsaturated iron-binding capacity was increased in the isotope-dilution method in some iron-saturated plasma, compounding errors when added to erroneously high PI values to compute TIBC. Perhaps some exchange of iron occurred between added iron and transferrin iron in the isotope-dilution method. These measurements confirm the accuracy of the standard colorimetric method of measuring PI and TIBC except in iron-saturated plasma. However, the greater specificity of a polyclonal immunoprecipitation method of measuring PI and TIBC makes it particularly useful in differentiating transferrin-bound iron from nontransferrin iron.

Additional Keyphrases: iron-deficient anemia · hemochromatosis · thalassemia · isotope-dilution radioassay · differentiating transferrin-bound and non-transferrin-bound iron · reference values

Iron is transported to body tissues by a protein, transferrin, in plasma. This protein has two high-affinity binding sites for iron (1). Determination of total iron-binding capacity (TIBC) of the plasma therefore gives a measure of transferrin, although it can also be directly determined. Virtually all plasma iron (PI) normally is bound to transferrin, and measurement of PI is assumed to reflect the amount of transferrin iron. The expression "transferrin saturation," expressed as percent ([PI/TIBC] x 100), indicates the availability of iron to tissues. As transferrin saturation increases, there is an increase in the amount of diferric transferrin (2), which has a greater capacity to deliver iron than does monoferric transferrin (3, 4).

Measurements of PI, TIBC, and transferrin saturation have served several purposes in clinical medicine. The PI concentration and (particularly) transferrin saturation reflect the adequacy of iron supply. A saturation of <16% indicates a deficient iron supply (5), whereas a saturation of >60% as measured on more than one occasion represents excessive iron loading owing to increased iron absorption (6) or liver disease (7). An increased transferrin concentration as reflected in the TIBC indicates iron depletion if the effects of estrogen and pregnancy are excluded (1). Other characteristic changes in both PI and TIBC are useful in the differential diagnosis of various diseases, for example, the decrease in transferrin saturations associated with a decreased transferrin concentration in inflammatory states (8). Accurate measurement of PI and TIBC is particularly important in determining erythroid marrow function by ferrokinetic techniques.

In any of these clinical uses, it is essential that the PI accurately represents transferrin iron and that the TIBC represents the true iron-binding capacity of transferrin. In the present study we have assessed the validity of the standard PI and TIBC measurements in subjects with various states of iron balance by comparison with a more specific immunoprecipitation method. We also compared immune methods of measuring transferrin directly with other measurements of TIBC.

Materials and Methods

Blood was obtained from 15 healthy subjects of both sexes, and from 12 subjects with iron-deficiency anemia, 16 subjects with thalassemia, and eight with idiopathic hemochromatosis. Serum or heparinized plasma, obtained with care to prevent hemolysis, was stored frozen until the various procedures described were performed.

The colorimetric method for PI was that described by the International Committee for Standardization in Hematology (9), except that the trichloroacetic acid-treated plasma solution was not heated to 56 °C (this omission did not alter results). A 30-min centrifugation was adequate to obtain an optically clear supernate, and additional centrifugation did not change the results. We used 1-bathophenanthroline color indicator for determining ferrous iron. The coefficient of variation (CV) for the standard PI method was 2.4, as determined in 12 consecutive runs done on the same day.

TIBC was determined as recommended by the International Committee for Standardization in Hematology (10) with use of ar-grade magnesium carbonate (Mallinckrodt, Inc., St. Louis, MO 63147) to remove excess iron, leaving transferrin-bound iron in the supernate after centrifugation. We then measured the iron content of the supernate colorimetrically as in the PI method. We also determined TIBC by the isotope-dilution techniques, determining unsaturated iron-binding capacity (UIBC) from the amount of radiolabeled iron solution that remained bound to transferrin after excess iron was removed by adsorption onto magnesium carbonate. From the activity in the supernate and the specific activity of the radiolabeled iron solution, the UIBC could be determined and used to calculate TIBC: UIBC + PI = TIBC.

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2 Nonstandard abbreviations: TIBC, total iron-binding capacity; PI, plasma iron; UIBC, unsaturated iron-binding capacity.

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To determine transferrin-bound PI more specifically, we precipitate human transferrin by highly purified sheep antibody to human transferrin (raised by Kent Laboratories, Redmond, WA 98052; we supplied the antigen and purified the antibody). This antibody was freed of transferrin by column elution and of excess iron by treatment with desferrioxamine and dialysis (accepted for publication in J Lab Clin Med). We determined the precipitating capacity of the antibody by titration with plasma from apparently normal subjects after having labeled the transferrin with $^{59}$Fe. In the assay we used 1.5 times the amount of antibody required for complete (>95%) precipitation of radioactivity, i.e., roughly sufficient to precipitate the protein equivalent of an iron-binding capacity of 5 mg/L.

In determination of PI, we mixed 0.3 mL of plasma and 0.45 mL of antibody solution and kept this in crushed ice for 30 min. We then added 2 mL of bicarbonate–saline solution (5 mL of 0.95 mol/L sodium bicarbonate in isotonic 0.15 mol/L saline). The mixture was centrifuged (2000 $\times$ g, 30 min, 4 °C). After decanting the supernate, we added to the precipitate 0.3 mL of a “coctail” (0.26 mol of thiglycolic acid per liter of 0.25 mol/L acetate buffer, pH 3.3), vortex-mixed the mixture for a minute three different times, and kept it at room temperature for a total of 30 min. From this point on, the procedure was as described for the standard PI method: precipitating proteins with trichloroacetic acid and reacting a color reagent with the iron in the centrifuged supernate. The CV for the immunoprecipitation method was 2.5% (n = 12).

We also measured the TIBC by immunoprecipitation. In this procedure, 0.3 mL of normal plasma is mixed with 1.5 $\mu$L of $^{59}$FeS$_2$O$_4$, pH 2, in 0.2 mL of isotonic saline, incubated for 15 min at room temperature, then shaken with antibody solution and placed on ice. After 30 min 2.0 mL of the bicarbonate–saline solution is added, mixed, then centrifuged (2000 $\times$ g, 30 min, 4 °C). The supernate is then completely removed and discarded. We measured the radioactivity in the precipitate and calculated binding capacity as in other isotope-dilution procedures. After counting its radioactivity, we treated the precipitate as described above for the immunoprecipitation determination of PI.

Transferrin protein was determined by “rocket” immunoelectrophoresis (11, 12) and was standardized by use of purified human transferrin (no. T5791; Sigma Chemical Co., St. Louis, MO 63178, or our own preparation (13)). About 50 mg of either form of apotransferrin was dissolved in 5 mL of iron-free water, and the solution was centrifuged and passed over a sterile filter (0.45-μm pore size, Millipore; Millipore Corp., Bedford, MA 01730) to remove traces of Tyndall-active insoluble material. The solution was then diluted 10-fold and the absorbance measured at 280 nm. The measurement was converted to transferrin concentration by use of the equation $1.09 \cdot A_{280} = 1$ g/L. The theoretical iron-binding capacity of 1.4 mg per gram of protein was verified by spectrophotometric titration at 465 nm for iron (14, 15). We encountered considerable difficulty in preparing a standard solution having a known concentration of transferrin, owing to the presence of variable amounts of residual water and other contaminants in the lyophilized preparations. Although we observed no change in rocket electrophoresis with different degrees of iron-loading of transferrin, the presence of various plasma components somewhat increased the apparent amount of transferrin as compared with a purified transferrin solution.

To determine whether the iron measurements we used were affected by contaminating hemoglobin, we made labeled preparations of these substances, ferritin by in vitro tagging as described elsewhere (16) and hemoglobin by injecting $^{59}$Fe into a rat and from the harvested erythrocytes preparing a hemolysate to contain 7–12 μg of iron per deciliter (1.25–2.15 μmol/L) (17). Trichloroacetic acid, “iron-free,” was from GFS Chemicals, Columbus, OH 43223. In studies of the effect of copper on PI determination, we measured copper by atomic absorption (18).

Differences between results by the various methods were evaluated by the Spearman rank correlation test.

**Results**

PI values by standard and immunoprecipitation methods are shown in the first two columns of Table 1. In normal subjects, results by the standard colorimetric method exceeded by an average of 5% those obtained by immunoprecipitation. In the case of iron-deficient subjects, however, results by both methods were nearly identical. In partly iron-saturated patients with thalassemia, the mean PI value by the standard method was 3% less than that by the immunoprecipitation method. In patients with saturated transferrin due to thalassemia or hemochromatosis, mean values for PI by the standard method were respectively 9 and 11% higher than by the immunoprecipitation technique ($p < 0.01$). These latter results suggest that the plasma of patients with saturated transferrin contained additional chromogenic material, presumably iron, that was measured by the standard colorimetric method but not by the immunoprecipitation method.

Mean values for TIBC are shown in columns 3 through 6 in Table 1. Standard and immuno methods were each performed by colorimetric measurement and by the isotope-dilution technique. Results by all four determinations agreed well for each of the normal subjects. In iron-deficient subjects, the mean value by the standard colorimetric method was 5% more than by the standard isotope-dilution method, while that by the immunoprecipitation-colorimetric method was 2% more than by the corresponding isotope-dilution method. In thalassemic patients with unsaturated transferrin, values by the colorimetric methods for TIBC were about 8% lower than by immunoprecipitation methods. In thalassemia patients whose transferrin was saturated with iron, the mean by the standard colorimetric method was 14% less than by the standard isotope-dilution method ($p < 0.01$), while the immune precipitation colorimetric method was 7% less than the corresponding isotope dilution method ($p < 0.01$). In patients with idiopathic hemochromatosis, results by three of the four methods agreed closely, but by the standard isotope-dilution method TIBC was 6% higher ($p < 0.05$).

By the different methods, mean values for transferrin saturation in normal subjects ranged from 28 to 30%; those for 12 iron-deficient subjects ranged from 2–7%, with mean values of 4–5%. Of particular interest was the over-saturation of transferrin values by the standard colorimetric method in nine of 10 thalassemic subjects with iron overload and in six of eight patients with idiopathic hemochromatosis. Over-saturation was also observed in five of 13 thalassemic patients with iron overload when the immune precipitation result for plasma iron was compared with the colorimetric immune precipitation result for TIBC. Over-saturation was not observed in these thalassemic patients with isotope-dilution methods of measuring TIBC, and in only one patient with idiopathic hemochromatosis.
Direct measurements of transferrin concentration by rocket immunoelectrophoresis are also included in the final column of Table 1. Mean values were similar to other TIBC measurements and the only significant difference was in subjects with iron deficiency (p < 0.01).

One concern in measurements of plasma iron was whether color-producing substances other than transferrin iron may have been included in the results. One such substance was copper. Addition of various amounts of copper, either as copper sulfate or as ceruloplasmin, gave a color reaction that was read as iron in an amount equivalent to 4% of the copper present. However, atomic absorption analysis of the immunoprecipitate showed no detectable copper. Other substances that might contribute iron to the PI determination include ferritin, hemoglobin from hemolyzed blood, and free iron. When ferritin was added to plasma, about a third of its iron was detected by the standard colorimetric PI determination. But only 1 to 2% of the iron in ferritin was measured in the immunoprecipitation method of determining PI. Further, in the standard colorimetric method for TIBC, half the radioactivity of radioiron-tagged ferritin was removed by magnesium carbonate, presumably owing to adsorption of the ferritin itself. Results of neither the standard method nor the immune precipitation method were affected by hemolysis, even when hemolyzed plasma containing 500 to 5000 μg of hemoglobin iron per liter (9 to 90 μmol/L) was repeatedly frozen and thawed, then stored for a week in the frozen state before analysis. Free iron, added as ferrous sulfate to normal and transferrin-saturated plasma, was detected by the standard colorimetric method for iron in plasma (Table 2). Only iron bound to transferrin was detected by the immune precipitation method.

Discussion

The validity of the standard methods for measuring plasma iron and TIBC has been examined by comparing results with those by an immunoprecipitation method in which transferrin is selectively precipitated from plasma and its iron content determined. The greater specificity of the latter method was shown by its lack of reactivity with copper, ferritin, hemoglobin, and free plasma iron. For normal subjects, excellent agreement was found between results by the two methods, and these compare well with data from the literature. Mean values for PI and TIBC from individual reports vary considerably, but if one collates several reports of 10 or more subjects (18–32), the overall mean value for PI is about 100 μg/dL (18 μmol/L) and for TIBC about 3.34 mg/L (60 μmol/L) with a transferrin saturation of 28%. Our normal values for PI average 96 μg/dL (17 μmol/L) and for TIBC 337 μg/dL (61 μmol/L), with transferrin saturation of 28%.

The clinical utility of these methods lies in their use in the detection of abnormalities in either PI or TIBC. Therefore we examined individuals with iron deficiency and overload. Our results in cases of iron deficiency again are similar for the two methods, but in iron overload differences appeared. Values for plasma iron were significantly (p <0.01) higher with the colorimetric method than with the immunoprecipitation method in patients whose transferrin was nearly or completely saturated. That the presence of nontransferrin iron explains this difference seems further substantiated by the 9% lower than expected measurement for transferrin protein. Larger differences were reported by others in comparing a standard colorimetric TIBC with a measurement.

Table 2. Effect of Added Ferrous Sulfate on Results of Colorimetric and Immunoprecipitation Assays

<table>
<thead>
<tr>
<th>Added Fe** (μg/dL)</th>
<th>PI</th>
<th>Fe Increase</th>
<th>TIBC</th>
<th>PI</th>
<th>Fe Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>106</td>
<td>—</td>
<td>298</td>
<td>107</td>
<td>—</td>
</tr>
<tr>
<td>20</td>
<td>125</td>
<td>19</td>
<td>310</td>
<td>122</td>
<td>21</td>
</tr>
<tr>
<td>60</td>
<td>175</td>
<td>69</td>
<td>306</td>
<td>169</td>
<td>68</td>
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<tr>
<td>200</td>
<td>249</td>
<td>25</td>
<td>231</td>
<td>222</td>
<td>—7</td>
</tr>
<tr>
<td>600</td>
<td>303</td>
<td>54</td>
<td>231</td>
<td>226</td>
<td>—3</td>
</tr>
<tr>
<td>Thalassemia plasma with saturated transferrin</td>
<td>299</td>
<td>—</td>
<td>254</td>
<td>—</td>
<td>251</td>
</tr>
<tr>
<td>20</td>
<td>322</td>
<td>21</td>
<td>257</td>
<td>247</td>
<td>—4</td>
</tr>
<tr>
<td>60</td>
<td>374</td>
<td>75</td>
<td>259</td>
<td>259</td>
<td>8</td>
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</tbody>
</table>

Table 1. Plasma Iron and Binding-Capacity Measurements

<table>
<thead>
<tr>
<th>SC</th>
<th>IPC</th>
<th>TSC</th>
<th>TSI</th>
<th>TIPC</th>
<th>TIPi</th>
<th>TF</th>
<th>SSC</th>
<th>SSI</th>
<th>SIPC</th>
<th>SIPI</th>
</tr>
</thead>
<tbody>
<tr>
<td>μg per deciliter of plasma, mean ± SD</td>
<td>Iron saturation of transferrin, %</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

I. Normal (n = 15)

Mean | 99 | 94 | 334 | 341 | 335 | 336 | 325 | 30 | 29 | 28 | 28 |
SD  | ±26| ±23| ±34 | ±37 | ±19 | ±21 | ±46 | ±6 | ±8 | ±6 | ±8 |

II. Iron deficiency (n = 12)

Mean | 16 | 17 | 386 | 369 | 377 | 369 | 409 | 4  | 4  | 4  | 5  |
SD  | ±5 | ±5 | ±75 | ±73 | ±65 | ±61 | 90  | ±2 | ±2 | ±1 | ±1 |

III. Thalassemia

(a) unsaturated Tf (n = 6)

Mean | 100 | 103 | 157 | 160 | 168 | 174 | 171 | 65 | 63 | 62 | 60 |
SD  | ±22 | ±23 | ±34 | ±29 | ±26 | ±32 | ±24 | ±13| ±13| ±12| ±11 |

(b) saturated Tf (n = 10)

Mean | 235 | 210 | 210 | 222 | 240 | 211 | 114 | 96 | 96 | 98 | 91 |
SD  | ±58 | ±55 | ±46 | ±61 | ±49 | ±58 | ±43 | ±15| ±1 | ±12| ±2  |

IV. Idiopathic hemochromatosis (n = 8)

Mean | 232 | 212 | 226 | 241 | 226 | 229 | 241 | 101| 96 | 94 | 93 |
SD  | ±19 | ±23 | ±17 | ±16 | ±27 | ±24 | ±21 | ±3 | ±4 | ±6 | ±1 |

* Abbreviations: SC, standard colorimetric method; IPC, immunoprecipitation method; TSC, standard colorimetric method; TSI, standard isotope method; TIPC, immunoprecipitation-isotope dilution method; TF, transferrin by immunoelectrophoresis; SSC, SC/TSC; SSI, FC/TSl; SIPC, IPC/TIPC; SIPI, IPC/TIPi.
based on transferrin determination (33–36). Other investigators have added radioiron to the plasma of patients with iron overload and have demonstrated incomplete binding (37), although this method can only demonstrate the proportion of added iron that does not bind to transferrin. While these studies, particularly those involving the Hershko technique, indicate the presence of a fraction of easily chelatable and possibly free iron, the presence of iron-bearing ferritin in circulation is also to be considered (38).

The four TIBC determinations showed little difference between normal and iron-deficient subjects, but differences were observed in iron-saturated subjects. Of particular interest was the higher TIBC with isotope-dilution techniques; for example, compare columns 3 and 4 and columns 5 and 6 in Table 1 for patients with thalassemia and saturated transferrin (p <0.05). That the values in columns 4 and 6 are too high is suggested by the significantly lower values by transferrin analysis (p <0.01).

In our hands, direct measurement of TIBC by rocket immunoelectrophoresis was less accurate than by the other methods. The principal problem encountered, however, was in calibration of the method, which depends on a preparation of transferrin standard that demonstrates an iron-binding ratio of 1.4 or, alternatively, standardization against TIBC as determined by the standard colorimetric method. We could not confirm reports that the immunoelectrophoretic method is influenced by the degree of transferrin saturation with iron (39), but we saw a slight difference between results for pure transferrin and transferrin added to plasma. Direct measurements of transferrin by rocket immunoelectrophoresis in normal subjects were in general agreement with TIBC measurements except for significantly (p <0.01) higher transferrin values in iron-deficient subjects.

While this study has dealt with a comparison of methods for determining plasma iron and TIBC, other problems than those discussed must be kept in mind. A constant concern is the presence of external contamination of the plasma sample with iron. Fortunately, the use of disposable plastic syringes and tubes and of aluminum needles has minimized this problem, but special attention to the iron content of reagents is needed and it would seem important to keep the reagent blank to <12 µg/dL (2.2 µmol/L). Plasma copper is a consideration, but the 4% increase in apparent iron in plasma that may be caused by copper interference when bathophenanthroline is used, as in these studies, does not constitute a serious problem. It may have been responsible for the 5% difference in normal subjects between the standard and immunological measurements of plasma iron but, if so, it is peculiar that we did not see the same contribution from plasma copper in patients with iron deficiency. In instances where copper concentrations are markedly increased—as in certain patients with Wilson's disease, a significant error can exist.

Much greater effects on plasma iron have been observed after injection of iron dextran, which is partly measured by the standard method (40) and some of which was also found to adhere to the precipitate and be measured by the immuno method. The other cause of a very high iron in plasma is the presence of tissue ferritin released from the liver or some other damaged tissue of high iron content. This may be suspected when isoceptive-dilution measurements indicate the presence of unsaturated transferrin in the presence of a plasma iron concentration exceeding 4 mg per liter of plasma (72 µmol/L).

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References


