Macro and Micro Methods for the Determination of Serum Iron and Iron-Binding Capacity

Wendell T. Caraway*

Blood serum is incubated with ascorbic acid and hydrochloric acid to liberate protein-bound iron. Proteins are precipitated with trichloroacetic acid in the presence of chloroform. To an aliquot of clear supernatant is added a solution of tripyridyltriazine and sufficient ammonium acetate to adjust the pH between 4 and 5. Absorbance of the blue-colored complex is measured at 590 m\(\mu\). Iron-binding capacity is measured by saturation of serum with ferric iron followed by removal of excess iron with magnesium carbonate. Measurement of total bound iron is completed as above. Macro and micro technics for either determination require 2 ml. and 0.1 ml. of serum, respectively. Reliability of the methods has been established by recovery experiments, replicate analyses, and variation of reagent concentrations and incubation times.

Analytical methods for the determination of serum iron and iron-binding capacity have been reviewed by Ramsay (25). The more sensitive reagents available for the colorimetric determination of iron are 4,7-diphenyl-1,10-phenanthroline (bathophenanthroline) and 2,4,6-tripyridyl-s-triazine (TPTZ). Molar extinction coefficients of the iron complexes for the two reagents are comparable (22,350 and 22,600 respectively) and are twice that of the complex with 1,10-phenanthroline (7). TPTZ was selected for further study of its applicability to the determination of serum iron and total iron-binding capacity (TIBC). The complex with ferrous iron is soluble in acidified aqueous solution. Maximum absorbance occurs at 593 m\(\mu\), where background absorbance

---

*Fellow of the Flint Medical Research Foundation.
Received for publication Feb. 20, 1962.

From the Laboratories of McLaren General Hospital, St. Joseph Hospital, and the Flint Medical Laboratory, Flint, Mich.
from serum filtrates is less significant. Finally, the reagent has more uniform composition and is less expensive than the commercially available sulfonated bathophenanthroline. The general use of TPTZ has been described (5) as well as its application to determination of iron in protein-free filtrates of serum (36). The present study is concerned with an evaluation of variables involved in the determination of serum iron and iron-binding capacity and adaptation of the methods to micro technics. The clinical importance of such determinations has been reviewed elsewhere (11, 20, 25).

In the proposed method iron is simultaneously reduced and detached from serum protein with the aid of ascorbic acid and hydrochloric acid. Proteins are precipitated by trichloroacetic acid in the presence of chloroform. To an aliquot of water clear supernatant is added a solution of TPTZ and sufficient ammonium acetate to produce an optimum pH for color development.

TIBC is determined by incubating serum with excess ferric iron to insure complete saturation of siderophyllin. Excess free iron is removed by treatment with magnesium carbonate powder as described by Ramsay (24). An aliquot of clear supernatant is then analyzed for serum iron as a measure of the total iron-binding capacity.

**Materials and Methods**

**Glassware**

Glassware should be cleaned by soaking in approximately 6N HCl, followed by thorough rinsing with distilled or deionized water. Random contamination with iron is indicated by variable absorbance readings on blanks and standards and by wide discrepancies on duplicate determinations.

**Reagents**

*Ascorbic acid, 1% in 0.2N HCl*  Prepare by dissolving 0.1 gm. of ascorbic acid in 10 ml. of 0.2N HCl. This solution is stable up to 2 weeks if refrigerated.

*Trichloroacetic acid, 20% (w/v)*  Dissolve 20 gm. of crystals in water and dilute to 100 ml. Caution: Do not use metal spatulas when weighing out the acid.

*Ammonium acetate, 40% (w/v)*  Dissolve 40 gm. of ammonium acetate in water and dilute to 100 ml.

*TPTZ, 0.1%*  Dissolve 0.10 gm. of 2,4,6-tripyridyl-s-triazine in 2 ml. of glacial acetic acid and dilute to 100 ml. with water. This solution
is stable at room temperature. Do not contaminate TPTZ solution with ammonium acetate as TPTZ will precipitate at higher pH. (The reagent used in this study was obtained from G. Frederick Smith Chemical Co., 867 McKinley Ave., Columbus 22, Ohio.)*

Stock standard iron, 20 mg./100 ml. Transfer 1.404 gm. of ferrous ammonium sulfate hexahydrate (MW, 392) to a 1000-ml. volumetric flask. Dissolve in about 800 ml. of water, add 0.5 ml. of concentrated sulfuric acid, and dilute to the mark. This solution is stable. Equal results were obtained with standards prepared from pure iron wire or from ferrous ethylene diammonium sulfate (Oesper's reagent; 3).

Working standard, 200 μg./100 ml. Dilute 1.0 ml. of stock standard to 100 ml. with water. It is preferable to prepare this solution fresh on day of use.

Ferric iron solution, 500 μg./100 ml. in 0.005 N HCl. This is used for total iron-binding capacity only and is prepared as follows: (a) Dissolve 10 gm. of FeCl₃·6H₂O in and dilute to 100 ml. with 0.1N HCl. (b) Pipet 0.5 ml. of this mixture into a flask and dilute to 100 ml. with 0.1N HCl. (c) Pipet 5.0 ml. of the resulting mixture into a flask and dilute to 100 ml. with water. This is the final working solution and is stable. The concentration need not be exact.

Magnesium carbonate, anhydrous, light powder Adsorptive properties should be checked by substituting water for serum in the method for TIBC. Analysis of the supernatant should show no traces of iron.

Procedure for Serum Iron

Pipet 2.0 ml. of serum into a small test tube, add 1.0 ml. of ascorbic acid solution, mix, and let stand 5 min. Add 1.0 ml. of 20% trichloroacetic acid and 1 ml. of chloroform. Stopper and shake vigorously for 10–15 sec. Centrifuge for 10 min. Carefully decant the supernatant into another small test tube; leave behind a supernatant protein button. The solution should be water clear; if not, it is recentrifuged.

Pipet 2.0 ml. of clear supernatant into a 12-mm. Coleman cuvet or test tube. Set up a blank and standard as follows:

Blank: 1.0 ml. of water + 0.5 ml. of ascorbic acid solution + 0.5 ml. of 20% trichloroacetic acid.

Standard: 1.0 ml. of working standard (200 μg./100 ml.) + 0.5 ml. of ascorbic acid solution + 0.5 ml. of 20% trichloroacetic acid.

To each tube add 0.2 ml. of TPTZ solution and mix. Finally, add 0.2 ml. of ammonium acetate solution and mix very thoroughly. Color develops immediately.

*Should a precipitate form in the solution, add 0.1 ml. concentrated HCl.
Set to zero absorbance with the blank at 590 m\(\mu\) and measure the absorbance of the test and standard, preferably within the next 5 min.

\[
\frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 200 = \text{serum iron (\(\mu g./100\) ml.)}
\]

*Note:* The final volume obtained is 2.4 ml. For instruments requiring 3 ml. of solution for measurement, 0.8 ml. of 10% ammonium acetate may be substituted for 0.2 ml. of 40% ammonium acetate in the procedure.

**Procedure for Total Iron-Binding Capacity**

This determination should be started some 30 min. before the serum iron so that resulting steps will be coordinated.

Pipet 2.0 ml. of serum to a small test tube, add 4.0 ml. of ferric iron solution (500 \(\mu g./100\) ml.), mix, and let stand at least 5 min. Add 0.5 gm. of magnesium carbonate powder, stopper, and shake vigorously for 10–15 sec. Let stand for 30 min. but remix thoroughly 4–5 times during this interval. Centrifuge at high speed for 10 min. Pipet 2 ml. of clear supernatant into a small test tube and analyze for serum iron as described in the preceding section.

\[
\frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 200 \times 3 = \text{TIBC, \(\mu g./100\) ml.}
\]

**Micro Procedures**

All volumes are reduced by a factor of 20, and general technics of ultramicrochemistry are applied (4). Absorbance is read on the Beckman DU spectrophotometer with ultramicro attachments.

For serum iron, pipet 100 \(\mu l\). of serum to the bottom of a 1-ml. centrifuge tube, add 50 \(\mu l\). of ascorbic acid solution, mix, and let stand 5 min. Add 50 \(\mu l\). of 20% trichloroacetic acid and 0.05 ml. of chloroform. Mix vigorously by drawing the tube back and forth across a test tube rack or with the aid of a Vortex, Jr. mixer (Scientific Industries). Centrifuge for 2 min. in a micro centrifuge at 10,000 rpm. Transfer 100 \(\mu l\). of clear supernatant to a 6 \(\times\) 50 mm. tube. At this point include a blank consisting of 50 \(\mu l\). of water + 25 \(\mu l\). of ascorbic acid solution + 25 \(\mu l\). of 20% trichloroacetic acid, and a standard, consisting of 50 \(\mu l\). of working standard (200 \(\mu g./100\) ml.) + 25 \(\mu l\). of ascorbic acid solution + 25 \(\mu l\). of 20% trichloroacetic acid. To each tube add 10 \(\mu l\). of TPTZ solution, mix, then add 10 \(\mu l\). of 40% ammonium acetate, and mix thor-
oughly. Transfer to Lowry-Bessey micro cells and measure the absorbance of the test and standard against the blank at 590 m\(\mu\) in a Beckman DU spectrophotometer.

For TIBC, pipet 100 \(\mu\)l. of serum to the bottom of a 1-ml. centrifuge tube, add 200 \(\mu\)l. of ferric iron solution (500 \(\mu\)g./100 ml.), mix and let stand at least 5 min. Add 25 mg. of magnesium carbonate powder, cover the tube with Parafilm, and mix vigorously for 10-15 sec. Let stand for 30 min. but remix thoroughly 4-5 times during this interval. Centrifuge for 5 min. at 10,000 rpm. Since evaporation is appreciable under these conditions, cover the tube with Parafilm while centrifuging. Pipet 100 \(\mu\)l. of clear supernatant into a 1-ml. centrifuge tube and analyze for serum iron as described in the preceding paragraph. Multiply results by 3 to obtain TIBC.

**Results and Discussion**

TPTZ combines with ferrous iron in the ratio 2:1 to form a tridentate chelate ring. The blue color of the complex is developed fully between \(pH\) 3.4 and 5.8 with maximum absorption at 593 m\(\mu\) and is stable for hours (5). The complex may be extracted into nitrobenzene with slight increase in the molar extinction coefficient. Substances interfering with the determination of iron include \(Cu^{++}\), \(Co^{++}\), \(Ni^{++}\), \(Cr^{+++}\), oxalate, nitrite, cyanide, and molybdate. TPTZ forms a precipitate with \(Ag^{+}\), \(Hg^{++}\), and \(Bi^{+++}\). None of these interfering substances would be found in serum in significant amounts, with the exception of \(Cu^{++}\). Experiments with standard solutions of cupric sulfate indicate that the absorbance produced by copper under the conditions of the procedure is less than 5% of that produced by an equal weight of iron.

Iron bound to serum siderophyllin is in ferric form (30). Binding with serum protein is maximal above \(pH\) 7 (33, 34) although dissociation curves vary depending on the type of buffer present (29). Reducing the iron and acidifying the mixture appear to effect rapid dissociation of the bond. The desirability of adding a reducing agent prior to precipitation of proteins has been emphasized by others (16, 32). Apparently serum alone, when acidified or heated, must reduce iron to a considerable extent (17). In the present method, however, omission of ascorbic acid resulted in slightly lower recoveries.

Independent variations of experimental conditions were investigated. Ascorbic-acid-reagent concentrations were varied between 1

---

*Marathon Division, American Can Co., Menasha, Wis.*
and 5%; hydrochloric-acid-reagent concentrations were varied between zero and 0.6N; incubation times were varied between 5 and 30 min. None of these variations had any effect on the final result. If the 5-min. incubation period was omitted, results were slightly lower. Hydrochloric acid could be omitted entirely but is useful to stabilize ascorbic acid by lowering the pH during incubation. Some workers use more concentrated acid and longer periods of incubation to effect complete extraction of iron and imply that low results or incomplete recoveries are obtained otherwise. Actually, use of strong acid at this stage tends to produce chromogens and higher serum blanks which, if not corrected, would cause falsely elevated results (12, 13, 28). Higher concentrations of HCl could be used in the present method, provided more ammonium acetate is added for final buffering.

Concentrations of trichloroacetic acid reagent were varied between 10 and 30% with equal results. Chloroform is added to achieve better dispersion and coagulation of protein and to obtain a clear supernatant after centrifuging (23). The chloroform also extracts some chromogens from the serum. Supernatant solutions consistently exhibited zero absorbance at 590 m\(\mu\). Some workers let the mixture stand for 1 hr. after adding trichloroacetic acid, which provides only for better coagulation of protein (9). Others heat the mixture at this stage to insure extraction of all iron. In the present method heating will increase the contribution of iron from any hemoglobin present. Moreover, quantitative recoveries of added iron were obtained without heating.

TPTZ can be substituted for dipyridyl in the method proposed by Ramsay (23), but the molar extinction coefficient of the complex is less in mixtures that have been heated. Some attempts were made to apply the TPTZ color reaction directly to serum buffered at pH 5 in the presence of ascorbic acid (10, 19, 29), but results were low and not reproducible.

The amount of TPTZ employed was shown to be sufficient to develop full color for serum-iron concentrations up to 800 \(\mu g./100\) ml. Linear calibration curves were obtained between zero and 500 \(\mu g./100\) ml. on either the Coleman or Beckman instruments. Absorbance of the 200-\(\mu g./100\) ml. standard at 590 m\(\mu\) was approximately 0.28 on the Coleman Jr. Model 6A (12-mm. cuvets) and approximately 0.35 on the Beckman DU (10-mm. light path) against corresponding blanks. Blank absorbance against distilled water usually ranged from 0.02 to 0.03. Although the final color obtained on standards was exceptionally
stable, a slight but significant decrease was encountered in absorbance values on serum filtrates. For this reason it is suggested that readings be taken without delay after development of color.

Optimum concentration of ammonium acetate was determined by discontinuous spectrophotometric and potentiometric titration of standards and protein-free serum extracts prepared as described. Iron was added to pooled serum to approximate final values of 200 μg./100 ml. Figure 1 illustrates the change in pH and absorbance with progressive increase in the concentration of ammonium acetate reagent. Above 30% concentration the absorbance is constant. With 40% ammonium acetate the pH is approximately 4.5, and the system is well buffered. The serum extract curve is displaced to the left because the buffer action of serum partially neutralizes the trichloroacetic acid in the system.

Recovery studies were done by analyzing pooled serum to which iron had been added in amounts ranging from zero to 250 μg./100 ml. Results, shown in Table 1, demonstrate recoveries ranging from 98 to 102% at all levels, even in the presence of appreciable concentrations of neutral fat, bilirubin, and hemoglobin. Serum from one patient with an iron content of 161 μg./100 ml. was diluted twofold and fourfold with water and analyzed with results of 82 and 41 μg./100 ml., respectively. This further confirms the completeness of extraction on undiluted serum.

![Figure 1](image_url)

**Fig. 1.** Effect of various concentrations of ammonium acetate reagent on absorbance and pH of final reaction mixtures.
Table 1. Recoveries of Iron Added to Serum

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fe added (µg./100 ml.)</th>
<th>Fe found (µg./100 ml.)</th>
<th>Fe expected (µg./100 ml.)</th>
<th>% recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pooled</td>
<td>0</td>
<td>80</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Pooled</td>
<td>50</td>
<td>128</td>
<td>130</td>
<td>98</td>
</tr>
<tr>
<td>Pooled</td>
<td>100</td>
<td>183</td>
<td>180</td>
<td>102</td>
</tr>
<tr>
<td>Pooled</td>
<td>150</td>
<td>231</td>
<td>230</td>
<td>100</td>
</tr>
<tr>
<td>Pooled</td>
<td>200</td>
<td>276</td>
<td>280</td>
<td>99</td>
</tr>
<tr>
<td>Pooled</td>
<td>250</td>
<td>324</td>
<td>330</td>
<td>98</td>
</tr>
<tr>
<td>Lipemic*</td>
<td>0</td>
<td>90</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Lipemic*</td>
<td>133</td>
<td>218</td>
<td>223</td>
<td>98</td>
</tr>
<tr>
<td>Jaundiced†</td>
<td>0</td>
<td>83</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Jaundiced†</td>
<td>100</td>
<td>185</td>
<td>183</td>
<td>101</td>
</tr>
<tr>
<td>Hemolytic‡</td>
<td>0</td>
<td>131</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Hemolytic‡</td>
<td>100</td>
<td>225</td>
<td>231</td>
<td>98</td>
</tr>
</tbody>
</table>

*Neutral fat, 930 mg./100 ml.
†Bilirubin, 40 mg./100 ml.
‡Hemoglobin, 1 gm./100 ml.

Quantitative recovery of iron added to serum does not insure complete extraction and recovery of all native serum iron (8, 13, 20, 23). Small but variable quantities of iron may be present in other forms such as ferritin or as hemoglobin iron. Wet-ashing technics will insure recovery of all iron; however, since the clinical and diagnostic significance of serum iron is related to the iron in transport, it is important not to include hemoglobin iron in the measurement. Adequate recoveries of added iron suggest that measurement of native iron attached to siderophyllin is complete.

The macro method for serum iron was compared with the dipyridyl method of Ramsay (23) on random sera and on pooled serum containing added iron. Results are shown in Fig. 2. The TPTZ method in this study gave results that averaged 8 µg./100 ml. higher than Ramsay's method. It should be noted, however, that absorbance readings with the dipyridyl method are only one-third of those obtained with the TPTZ method and are in a range subject to greater instrument error.

Hemolysis was found to have less effect on the present method than on those methods which specify heating to precipitate proteins or to liberate iron. Fresh washed red cells were hemolyzed by freezing and added to serum to provide final hemoglobin concentrations of 0.1–0.4 gm./100 ml. With 0.1% hemoglobin, serum iron was increased approximately 5 µg./100 ml. With 0.4% hemoglobin, the increase averaged 13 µg./100 ml. These increases represent the equivalent of about 1% of the available hemoglobin iron. These amounts of hemoglobin
had no effect on the TIBC. It would appear, therefore, that some non-hemoglobin iron was contributed from the hemolyzed red cells since it could be removed by treatment with magnesium carbonate. This effect was accentuated when aged red cells were used as the source of contaminating hemoglobin.

![Graph](image)

**Fig. 2.** Comparison of dipyridyl and TPTZ methods for serum iron. Equation shown is that of best straight line through points.

Total iron-binding capacity of serum is related to the concentration of a beta,-globulin (transferrin; siderophyllin) capable of binding iron. Immunochemical methods indicate that the average concentration of siderophyllin in serum is 0.27 gm./100 ml. (14), corresponding to a TIBC of approximately 340 μg./100 ml. (20). The difference between TIBC and serum iron represents the unsaturated iron-binding capacity (UIBC). Various methods have been proposed to measure either UIBC or TIBC. In general, UIBC may be determined by measuring the increase in absorbance produced as iron is added to diluted serum to form the red-colored iron-siderophyllin complex (15, 26, 33). Alternately, iron may be added in excess of that required to produce
saturation and the excess iron determined colorimetrically (10, 19, 27, 29). Measurement of TIBC involves addition of sufficient iron to insure complete saturation and removal of excess iron with magnesium carbonate (24) or ion exchange resin (18, 22, 31), or by selective precipitation (1, 6, 17). The iron-saturated serum remaining is then analyzed for iron by conventional technics to obtain TIBC directly. Despite its low sensitivity, the method of Rath and Finch (26) as modified by Ventura (23) or Kaldor (15) appears satisfactory for routine determination of UIBC, provided the serum is not hemolytic, lipemic, or jaundiced. For the present study, the method of Ramsay (24) was chosen for TIBC as it is not affected by these factors and is more convenient to adapt to micro technics.

The initial steps in the determination of TIBC involve addition of ferric iron solution to serum and removal of excess iron with magnesium carbonate powder. The amount of magnesium carbonate and the time of standing were varied both independently and simultaneously over the range of 0.2-0.8 gm. of magnesium carbonate per 2 ml. of serum and from 15 to 60 min. Samples were shaken intermittently by hand every 5 min. With 0.4 gm. of magnesium carbonate, equal results were obtained at 15-, 30-, or 60-min. incubations. Lesser amounts of magnesium carbonate failed to remove completely excess iron. Larger amounts were satisfactory with 30-min. incubation. Use of 0.5 gm. of magnesium carbonate with 30-min. incubation appears to be optimal, in substantial agreement with Ramsay and with Morgan and Carter (21). The magnesium carbonate used in this study was obtained from Matheson, Coleman and Bell. Other preparations are easily checked for adsorptive capacity as described under Reagents.

Micro methods for the determination of serum iron have been described that require 20 or 50 µl. of serum (2, 33), but the reagents employed are less sensitive than TPTZ. Macro and micro procedures for serum iron and TIBC as described in this paper were compared by performing 10 replicate analyses on pooled serum. Results, shown in Table 2, indicate comparable precision and show no significant differences between the two methods. Similar results were obtained on random sera in comparison studies. In initial experiments, erratic results obtained on the micro method for TIBC were traced to evaporation losses in the microcentrifuge. When 0.3 ml. of water in an uncovered 1-ml. tube was centrifuged for 5 min. in the Misco centrifuge at 10,000 rpm, a loss of 10% in weight occurred. This would lead to appreciable
Table 2. COMPARISON OF MACRO AND MICRO METHODS

<table>
<thead>
<tr>
<th></th>
<th>Macro (µg./100 ml.)</th>
<th>Micro (µg./100 ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Mean</td>
</tr>
<tr>
<td>Serum iron</td>
<td>10</td>
<td>96</td>
</tr>
<tr>
<td>TIBC</td>
<td>10</td>
<td>298</td>
</tr>
</tbody>
</table>

concentration of sample but may be prevented by covering the tubes with Parafilm.

To investigate the stability of serum iron and TIBC, one lot of pooled serum was refrigerated at 4° and analyzed in duplicate at intervals for a period of 8 weeks. There was no significant change in either serum iron or TIBC over this interval (Fig. 3). The standard deviations shown indicate probable reproducibility of determinations under routine working conditions.

Normal values for TIBC were reported by Ramsay to range from 250 to 400 µg./100 ml. of serum (25). Similar values were reported by Morgan and Carter with use of Ramsay’s method (21). In this laboratory, analysis of serum from 60 selected patients revealed a mean value of 350 µg./100 ml. with a 95% range of 255–419 µg./100 ml. Other reports in the literature are in substantial agreement with this range (13, 19, 20, 22).

Normal values for serum iron are discussed in the review by Ramsay (25). No definite range of normal was given in view of the many variables of methodology, diurnal variations, and random fluctuations of serum iron in normal individuals. It is generally accepted that the

![Fig. 3. Stability of serum iron and TIBC at 4° showing mean values and standard deviations.](image-url)
serum iron concentration of normal adult females is some 10–15% lower than in males. A recent collection of normal values from the literature (19) includes nine separate studies with mean values ranging from 98 to 173 μg./100 ml. and an over-all normal range from 44 to 238 μg./100 ml. Some of the higher values are subject to criticism on the basis of inadequate serum blank corrections (28). It is apparent that excessively wide ranges of normal detract from the value of a test. Our experience with the present method supplemented by a review of the literature has led to a working normal range for serum iron of 65 to 150 μg./100 ml.

References