An Accurate Spectrophotometric Method for Serum Iron and Iron-Binding Capacity without Deproteinization or Centrifugation*†

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ABSTRACT

A simple, direct semi-micro method has been developed for serum iron and latent iron-binding capacity. For total serum iron, into a one cm cuvet are pipetted (a) 1.5 ml of Na acetate buffer (pH 4.5) containing Na ascorbate and (b) 0.3 ml of serum. The serum blank absorbance is measured at 562 nm, and 0.2 ml of ferrozine color reagent is added. After incubation (42°, 20 min), the absorbance is again measured. The concentration of serum iron is calculated by reference to a standard Fe solution (100 µg Fe per dl), with correction for the absorbance of serum and reagent blanks. For serum latent iron-binding capacity, into a one cm cuvet are pipetted (a) 1.5 ml of tris-HCl buffer (pH 7.8) containing Na ascorbate, (b) 0.1 ml of serum and (c) 0.2 ml of iron standard solution (400 µg Fe per dl). The mixture is incubated (42°, 10 min). The serum blank absorbance is measured at 562 nm, and 0.2 ml of ferrozine color reagent is added. The sample is incubated (42°, 20 min), and the absorbance is again measured. Correction for the serum blank absorbance is made, and the concentration of unbound iron in the sample is calculated. The amount of added iron minus the amount of unbound iron in the sample is equal to the latent iron-binding capacity. Measurements of serum iron and latent iron-binding capacity by this procedure are comparable in accuracy and superior in precision to measurements by atomic absorption spectrometry.

Introduction

The semi-micro technique for measurement of serum iron and iron-binding capacity which is described in this paper is extraordinarily simple and convenient for routine use, yet it does not sacrifice analytical accuracy or precision. The technique is a modification of the method of Persijn et al. and it incorporates certain features

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of the direct spectrophotometric methods which have been previously described by Levy and Vitacca,9 Goodwin et al,5 Askevold and Vellar,4 Piccardi et al,14 White and Flashka,16 Haeckel,6 Martinek,11 and Gemba et al.4 Ferrozine has been selected as the iron chromogen, following the recommendations of Stookey,15 Yee and Zin,17 Carter3 and Manasterski et al.10

Method

PRINCIPLE

Measurement of Serum Iron. Serum is acidified with acetate buffer (pH 4.5) in order to release Fe(III) from transferrin without precipitation of serum proteins. Fe(III) is reduced to Fe(II) by sodium ascorbate. Fe(II) reacts with ferrozine to form a lavender Fe-ferrozine complex, which is measured by spectrophotometry at 562 nm. The absorbance of the Fe-ferrozine complex is proportional to the serum iron concentration, provided appropriate correction is made for the "background" absorbance of the non-deproteinized serum.

Measurement of Serum Latent Iron-Binding Capacity (LIBC). Serum is mixed with tris buffer (pH 7.8), and an accurately measured volume of iron standard solution is added. The quantity of added iron greatly exceeds the latent iron binding capacity of the serum transferrin. The unsaturated iron-binding sites of serum transferrin become completely saturated during 10 minutes of incubation at 42°, despite the fact that the added iron is in the Fe(II) state, owing to reduction by sodium ascorbate. Van Kreel et al7 have recently shown by electron spin resonance spectroscopy that the iron which becomes bound to transferrin under these reaction conditions exists in the Fe(III) state within the metalloprotein complex. The unbound, excess Fe(II) is reacted with ferrozine, and the lavender Fe-ferrozine complex is measured by spectrophotometry at 562 nm. Correction is made for the "background" absorbance of the non-deproteinized serum. The iron which has become bound to serum transferrin (i.e. the LIBC) is calculated by subtracting the amount of unbound, excess iron from the amount of iron which was originally added. Serum total iron-binding capacity (TIBC) is calculated by addition of serum iron and serum LIBC.

REAGENTS

(Precautions: Distilled, demineralized water is used for the preparation of all reagents. The glassware is washed with dilute nitric acid and is rinsed with copious distilled, demineralized water.)

Iron Stock Standard Solution, (50 mg per dl). Iron wire (99.99 percent Fe) is cleansed with "Kimwipes," leaving no lint on the wire. Exactly 500 mg of the wire are placed in a one-liter volumetric flask and dissolved with gentle heating in six ml of concentrated HCl.§ When the iron is completely in solution, the contents of the flask are diluted to the mark with water.

Iron Intermediate Standard Solution, (5 mg per dl). Ten ml of iron stock standard solution are diluted with water to 100 ml in a volumetric flask.

Iron Working Standard Solutions. Into two 50 ml volumetric flasks are transferred one and four ml of iron intermediate standard solution. After the contents of these flasks have been diluted to 50 ml with water, they contain 100 and 400 μg Fe per dl, respectively.

Ferrozine Reagent. Into a 50 ml volumetric flask are transferred 250 mg of ferrozine (3-(2-pyridyl)-5,6-bis-(4-phenylsulfonic acid)-1,2,4-triazine).‖ The contents of the flask are dissolved in water and are diluted to the mark. This reagent is stored in a brown bottle.

§ "Ultrapure Reagent," E. Merck Co., Darmstadt, Germany, purchased from EM Laboratories, Inc., Elmsford, NY 10523.

‖ Hach Chemical Co., Ames, IA 50010.
Acetate Buffer, (pH 4.5). Into a beaker which contains approximately 600 ml of water are placed 42.9 g of sodium acetate (NaC₂H₃O₂·3H₂O) ("Analytical" reagent) and 28.6 ml of glacial acetic acid. The contents are dissolved and adjusted to pH 4.5 by addition of small amounts of sodium acetate or acetic acid. The contents are transferred to a one-liter volumetric flask and diluted to the mark with water.

Tris Buffer, (pH 7.8). Into a beaker which contains approximately 600 ml of water are placed 24.23 g of tris-hydroxymethylaminomethane (2-amino-2-hydroxy-methyl-1,3-propanediol). The contents are dissolved and adjusted to pH 7.8 by addition of 2.5 N HCl. The buffer solution is transferred to a one-liter volumetric flask and diluted to the mark with water.

Acetate Buffer-Ascorbate Solution. Immediately before use, 250 mg of sodium ascorbate ("Analyzed" reagent) are dissolved in acetate buffer in a 50 ml volumetric flask, and diluted to the mark with the buffer.

Tris Buffer-Ascorbate Solution. Immediately before use, 250 mg of sodium ascorbate are dissolved in tris buffer in a 50 ml volumetric flask and diluted to the mark with the buffer.

**Equipment**

Spectrophotometer Cuvets, square, 1.000 cm light path, 3 ml volume.

Spectrophotometer.†† This equipment provides stable digital readout of absorbance within ±0.001 A in the range from 0 to 1.0 A. The spectrophotometer is adjusted to 0.000 A at 562 nm with a cuvet containing distilled water, and all absorbance measurements are made versus this "distilled water blank."

Water Bath, 42°.

†† Baker Chemical Co., Philipsburg, NJ 08865.

<table>
<thead>
<tr>
<th>Table I: Effect of Incubation Temperature on Serum Iron</th>
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<tbody>
<tr>
<td>Serum Temperature (°C)</td>
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<tr>
<td>A (37°)</td>
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<tr>
<td>1</td>
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<td>3</td>
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<td>9</td>
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<tr>
<td>10</td>
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<tr>
<td>Mean:</td>
</tr>
</tbody>
</table>

A = 37°; B = 42°; C = 45°

AA = Analyses by atomic absorption

Procedure

Serum Iron. Acetate buffer-ascorbate solution (1.5 ml) is pipetted into the requisite number of one-cm cuvets. Into respectively labelled cuvets are pipetted (a) 0.3 ml of H₂O ("reagent blank"), (b) 0.3 ml of iron working standard solution (100 μg Fe per dl) and (c) 0.3 ml of serum. The absorbance of the cuvet containing serum is measured at 562 nm ("serum blank"). To each of the cuvets is added 0.2 ml of ferrozine reagent. The contents are mixed and all of the cuvets are placed in a water bath at 42° for 20 min. The cuvets are then allowed to stand at room temperature for 10 min and the adsorbances of all of the cuvets are measured at 562 nm.

Serum Fe (μg per dl)

\[
\text{Serum Fe (μg per dl)} = \left( \frac{[A(\text{serum}) - 0.9 \cdot A(\text{serum blank})] - A(\text{reagent blank})}{[A(\text{standard}) - A(\text{reagent blank})]} \right) \times 100
\]

Serum Latent Iron-Binding Capacity (LIBC). Tris buffer-ascorbate solution (1.5
ml) is pipetted into the requisite number of one-cm cuvets. Into respectively labelled cuvets are pipetted: (a) 0.3 ml of H₂O ("reagent blank"), (b) 0.1 ml of H₂O and 0.2 ml of iron working standard solution (400 μg Fe per dl) and (c) 0.1 ml of serum and 0.2 ml of iron working standard solution (400 μg Fe per dl). The contents of the cuvets are mixed and the cuvets are placed in a water bath at 42° for 10 min. The absorbance of the cuvet containing serum is measured at 562 nm ("serum blank"). To each of the cuvets are added 0.2 ml of ferrozine reagent and all of the cuvets are placed in the water bath at 42° for 20 min. The cuvets are allowed to stand at room temperature for 10 min and the absorbances of all of the cuvets are measured at 562 nm.

Calculations

Serum LIBC (μg per dl) = 800

\[
\frac{[A(\text{serum}) - 0.9 \cdot A(\text{serum blank}) - A(\text{standard}) - A(\text{reagent blank})]}{A(\text{standard}) - A(\text{reagent blank})} \times 800
\]

Serum Total Iron Binding Capacity (TIBC) = Serum Fe + Serum LIBC

Results

The incubation temperature of 42° proved to be critical for optimal color development by the ferrozine reaction in the presence of serum. In table I are given measurements of iron in 10 serums when the incubation was performed at 37°, 42° and 45° for 20 min. For comparison, in the righthand column of table I are measurements of iron in the same 10 serums by means of the atomic absorption method of Olson and Hamlin. When incubation was preformed at 37°, at least 40 min were required to achieve full color development. On the other hand, when incubation was performed at 45° for 20 min, faint turbidity frequently developed which artifactitiously increased the apparent concentration of serum iron. Excellent agreement with the atomic absorption method was accomplished with incubation at 42° for 20 min, with serums containing iron concentrations ranging from 35 to 190 μg per dl.

The effect of incubation temperature upon the latent iron-binding capacity of the same 10 serums is shown in table II. Twenty min incubation at 37° was insufficient for complete reaction of ferrozine with the unbound excess iron, and hence resulted in an apparent increase in the iron which became bound to transferrin. The fine turbidity which frequently developed during 20 min incubation at 45° caused an apparent decrease in LIBC. Excellent agreement with the atomic absorption method was accomplished with incubation at 42° for 20 min.

As shown in table III, use of acetate buffers ranging from pH 4.5 to 5.2 had very little effect upon the observed concentration of serum iron. Acetate buffer at pH 4.5 was selected, since on the basis of several experiments it appeared to provide closest correlation with iron measurements by atomic absorption. As shown in table IV, tris buffer at pH 7.8 gave a higher mean
value for serum latent iron-binding capacity than did tris buffers at pH 7.0 or pH 8.5. For this reason, pH 7.8 was selected for use.

Data for the precision of measurements of serum iron and TIBC by the present method and by the atomic absorption method of Olson and Hamlin\(^{12}\) are given in table VI. Based upon duplicate analyses, the relative standard deviation “within-the-run” by the present method was 0.5 percent for serum Fe and 0.9 percent for serum TIBC. These values were significantly less than the values obtained by atomic adsorption (1.9 percent and 1.9 percent, respectively). Similarly, the data for day-to-day precision, based upon daily means of duplicate analyses of a single frozen serum, were significantly less by the present method than by the atomic absorption method.

Measurements of recovery of iron added to serums in concentrations of 20 and 40 \(\mu g\) per dl are summarized in table VI. Iron was quantitatively recovered both by the present method and by the atomic absorption procedure.

TABLE III

<table>
<thead>
<tr>
<th>Serum</th>
<th>Serum Fe ((\mu g/dl))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>1</td>
<td>93</td>
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<td>8</td>
<td>166</td>
</tr>
<tr>
<td>Mean:</td>
<td>112</td>
</tr>
</tbody>
</table>

\(A = pH 4.5; B = pH 4.7; C = pH 4.9; D = pH 5.2\)

In figure 1 are compared measurements of serum iron in 122 serums by the present spectrophotometric method and by the atomic absorption procedure of Olson and Hamlin.\(^{12}\) The serums were analyzed in duplicate by both methods, and the means of the duplicates are compared on the slide. Based upon a paired sample t-test, there was no significant difference between results by the two methods. The correlation coefficient was 0.997. The slope of the regression line was \(Y = 1.010X - 1.34\), which corresponded very closely to the theoretical line.

In figure 2 are compared means of duplicate measurements of serum total iron-binding capacity in the same 122 serums by the present spectrophotometric method and by the atomic absorption procedure, which involves adsorption of unbound iron on magnesium carbonate, and subsequent removal of the unbound iron by centrifuga-
Figure 1. Comparisons of measurements of serum iron by the proposed spectrophotometric method and by the atomic absorption method of Olson and Hamlin\textsuperscript{12} in 122 serums from hospital patients.

Measurements of serum iron in 21 serums were performed by the present method and by the reference procedure which has been recommended by the International Committee for Standardization in Hematology (Bothwell et al\textsuperscript{18}), with substitution of ferrozine for bathophenanthroline as the chromogen. Based upon a paired-sample t test, there was no significant difference between the results by the two methods. The correlation coefficient was 0.999. The slope of the regression line was $Y = 0.973X + 2.47$, which corresponded very closely to the theoretical line.

Discussion

Measurements of serum iron and iron-binding capacity by the method which has been described are not influenced by icterus, hyperlipemia or hemolysis, such as
commonly occur in serums of hospital patients. Occasionally, a serum may be encountered which is turbid or deeply pigmented so that it is impractical to correct for the background absorbance of the serum "blank." In such cases, or in patients with paraproteinemia, measurements of serum iron should be performed after deproteinization with trichloroacetic acid as described by Bothwell et al., with substitution of ferrozine for bathophenanthroline as the chromogen. Artefactitious interference by drugs has not been detected during routine use of the method for six months in our hospital laboratory.

It is essential to employ a spectrophotometer such as the Beckman Model 25 which provides stable digital readout within ±0.001 absorbance unit. Under the reaction conditions which have been described, measurements of an iron standard solution which contains 100 µg Fe per dl yield a corrected absorbance at 562 nm of approximately 0.085. Calibration curves are linear with iron concentrations up to one mg Fe per dl.

Discrepancies may be encountered in measurements of serum iron and iron-binding capacity of commercial "quality control serums." Leggate and Crooks and Butler have emphasized that analyses of freeze-dried quality control materials of animal origin do not provide an accurate reflection of the routine analytical performance of clinical laboratories for determinations of serum iron-binding ca-

![Figure 2. Comparisons of measurements of serum total iron-binding capacity (TIBC) by the proposed spectrophotometric method and by the atomic absorption method of Olson and Hamlin in 122 serums from hospital patients.](image)

<p>| TABLE VI |
|-------------------------|-------------------|-----------------|------------------|
| <strong>Recovery of Iron Added to Serums</strong> |</p>
<table>
<thead>
<tr>
<th>Method</th>
<th>Fe Added (µg/dl)</th>
<th>No. of Serums</th>
<th>Fe Recovered (µg/dl)</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Present Method</td>
<td>20</td>
<td>15</td>
<td>19.9 ± 1.0</td>
<td>99.5</td>
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<tr>
<td></td>
<td>40</td>
<td>15</td>
<td>40.0 ± 1.3</td>
<td>100.0</td>
</tr>
<tr>
<td>Atomic Absorption</td>
<td>20</td>
<td>7</td>
<td>19.6 ± 0.8</td>
<td>98.0</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>7</td>
<td>39.8 ± 1.5</td>
<td>99.4</td>
</tr>
</tbody>
</table>
capacity. In the authors' laboratory, 50 ml of serum is obtained from a fasting, healthy person. Aliquots of the serum are frozen in one ml ampoules, and are stored at $-15^\circ$. One ampoule is thawed on each working day for use in quality control of measurements of serum iron and iron-binding capacity.

In summary, a simple direct method has been described for measurements of serum iron and iron-binding capacity in non-deproteinized serum. The bothersome use of magnesium carbonate or ion-exchange resins for adsorption of unbound iron in the determination of iron-binding capacity is avoided. Measurements of iron and iron-binding capacity can be made on 0.4 ml samples of serum, and complete analyses of 20 serums can easily be completed by this manual procedure within 90 minutes. The technique should be readily adaptable to automation by use of either continuous-flow or discrete-sample instrumentation.

References


