Micromethod for Determining Total Iron-Binding Capacity by Flameless Atomic Absorption Spectrophotometry

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Because of the large quantities of venous blood required, assays of total iron-binding capacity (TIBC) and serum iron are often omitted in diagnostic tests involving small children. As an alternative to the conventional method of assessing TIBC, we developed a micromethod based on flameless atomic absorption spectrophotometry (flameless AAS). Additionally, the standard microprocedure for determining serum iron was modified so that iron concentration could be estimated from the same iron standards used to calculate TIBC. With these procedures, only 20 µl of serum is required to prepare protein-free supernate for measurement of TIBC or serum iron with an atomic absorption spectrophotometer equipped with a graphite furnace and a recorder. The resulting TIBC, serum iron, and computed transferrin saturation values compared well with those obtained by conventional flame atomic absorption spectrophotometry. The correlation was statistically significant at the 99% confidence level. These flameless AAS micromethods provide an accurate and sensitive means of determining both TIBC and serum iron in a total of 40 µl of serum, a quantity easily collected from a fingertip puncture, and hence are appropriate for routine laboratory work in pediatric clinics and for large-scale nutrition surveys.

Additional Keyphrases: pediatric chemistry • nutrition survey method • iron in serum • transferrin saturation values • comparison with conventional AAS • diagnosis of Fe-deficiency anemia • trace elements

Transferrin saturation—defined as the ratio of serum iron to total iron-binding capacity, expressed as a percentage—is reportedly a specific and sensitive means of diagnosing iron deficiency and anemia secondary to iron deficiency (1-3). Conventional spectrophotometric (4, 5) and flame atomic absorption spectrophotometric (6) methods require 0.5-2.0 ml for each determination of TIBC or serum iron. Because these quantities of venous blood are difficult to obtain from small children, tests of TIBC and serum iron are often omitted from routine blood analyses and are virtually impossible to use in nutrition surveys of preschool children, a group in which iron deficiency is frequent. Clinically, it would be advantageous if the TIBC, serum iron, and other hematological values necessary to evaluate iron nutrition could be determined from one blood sample obtained by a fingertip puncture (“finger stick”).

The recent development of a carbon-rod atomizer and graphite furnace has lowered the detection limit and hence the sample size required for atomic absorption spectrophotometry. Consequently, flameless atomic absorption spectrophotometry is being used extensively to analyze for trace metallic elements (7-11). Despite the development of new techniques in which serum iron is measured by flameless spectrophotometry (9, 12, 13) in the supernatant fluid from 50 µl of serum (or with a smaller volume of untreated serum), there is currently no micromethod available for determining TIBC.

Reported here is a new flameless AAS-micromethod in which a graphite atomizer is used to determine TIBC with 5 µl of supernate prepared from 20 µl of serum. Also reported is our modification of an existing micromethod (12) for determining serum iron. This modified procedure permits calculations of serum iron from the same working standards used for TIBC determination.

Materials and Methods
Apparatus

For determining TIBC and serum iron by flameless AAS-micro- or macromethod,1 we used an atomic absorption spectrophotometer (Model 403;...
Perkin-Elmer Corp., Norwalk, Conn. 06856) equipped with a graphite furnace (HGH-2000, Perkin-Elmer) and a deuterium background corrector (Perkin-Elmer). A strip-chart recorder (Model 056, Perkin-Elmer) was used to trace the peak signal for estimation of absorbance values. The atomic absorption spectrophotometer was operated with an hollow-cathode lamp (for iron) at a wavelength of 248.4 nm, unless otherwise indicated, and a slit width of 0.2 nm. Power was supplied to the graphite tube at 100 °C for 20 s (drying), 1000 °C for 25 s (charring), and 2400 °C for 5 s (atomizing). A constant flow of nitrogen (1.5 liter/min) was directed to the graphite furnace to distribute the atomic cloud and to prevent oxidation of incandescent carbon. Tap water was circulated through the furnace at a flow rate of 3 liters/min, to dissipate heat quickly between analyses. An Eppendorf microliter pipet was used to transfer 5 μl of sample to the graphite tube, to prevent metallic contamination.

For analysis by conventional flame AAS-methods, an atomic absorption spectrophotometer (Perkin-Elmer, Model 403) mounted with a three-slot burner using an air-acetylene flame was operated at the same wavelength and slit width used for flameless AAS-methods.

Glassware

Disposable iron-free glassware was used throughout. Microplastic centrifuge tubes (5 mm × 49 mm) with integral stoppers were purchased from Evergreen Scientific, Los Angeles, Calif. 90058. Conventional polyethylene bottles were bought from Cole-Parmer Instrument Co., Chicago, Ill. 60648. Eppendorf microliter pipets and disposable polypropylene tips used for sample preparation were obtained from Brinkmann Instruments, Inc., Westbury, N. Y. 11590.

Reagents

Ferric chloride [FeCl₃·6H₂O], trichloroacetic acid (TCA), and magnesium carbonate [4MgCO₃·Mg(OH)₂·nH₂O], all reagent-grade, were purchased from J. T. Baker Chemical Co., Phillipsburg, N. J. 08865. De-ionized distilled water was used throughout the analysis.

Standard stock iron solutions were prepared with ferric chloride in de-ionized distilled water. The working standards represented 25, 50, 100, 150, 200, 250, and 300 μg/100 ml of serum diluted with TCA (50 g/liter) and water (1:1:2, by vol). They were prepared by transferring into 50-ml volumetric flasks containing 12.5 ml of the TCA, 0.625, 1.25, 2.50, 3.75, 5.0, 6.25, and 7.50 ml of 500 μg/100 ml stock standard, respectively, and diluting to 50 ml with de-ionized distilled water.

Sera were obtained from the clinical laboratory at this institution. Clinical chemistry control sera were purchased from Lederle Diagnostics, American Cyanamid Co., Pearl River, N. Y. 10965.

Procedures

Microprocedure for TIBC. Sera were processed according to the procedures of Olson and Hamlin (6) and Caraway (4), with the following modifications. Serum, 20 μl, was mixed with 40 μl of ferric chloride solution (500 μg of Fe per deciliter) in a micro-scale plastic centrifuge tube and allowed to stand for 10 min. After 10 mg of magnesium carbonate was added to precipitate unbound exogenous ferric iron, the contents of the tube were mixed vigorously every 6 min for 30 min and then centrifuged at 3000 rpm for 10 min. The resulting supernate (20 μl) was transferred to another micro-scale plastic centrifuge tube to which 20 μl of TCA (50 g/liter) had been added. After standing for 15 min at room temperature, the sample was diluted with 40 μl of de-ionized distilled water. The protein-free supernate obtained after centrifugation was used for TIBC determination by the flameless AAS-micromethod.

Microprocedure for serum iron. Protein-free supernate was prepared according to the method of Olsen et al. (12). Twenty microliters rather than 50 μl of serum was vigorously mixed with an equal volume of the TCA. After heating, the sample was cooled, diluted with 40 μl of de-ionized water and centrifuged.

 Macroprocedures for serum iron and TIBC. For analysis of serum Fe and TIBC by flame AAS-methods and flameless AAS-macromethods, 1.0 ml of serum was treated by the methods of Olson and Hamlin (6).

Calculation of iron concentration. According to the microporcedures described above, the final concentrations of TCA in standards and in supernate prepared for TIBC and serum iron determination were the same (12.5 g/liter). The absorbance values of standards and samples were corrected for the absorbance obtained with TCA, 12.5 g/liter, before calculating the iron concentration. Under the assay conditions, the absorbance of 5 μl of TCA, 12.5 g/liter, was about 0.020-0.025 absorbance unit, owing to iron contamination. Serum iron concentration was estimated from the absorbance-concentration curve or from the regression equation obtained from iron standards. Because serum was diluted 12-fold, and standards fourfold, TIBC was determined by multiplying the iron content by a factor of 3.

Results and Discussion

Calibration curves. As a means of selecting the most sensitive wavelength for iron determination, working standards prepared to represent the iron content of serum supernate diluted fourfold were measured for absorbance at different wavelengths: 248.4, 248.9, and 302.1 nm. The resulting values (Figure 1) were corrected for the absorbance obtained with TCA, 12.5 g/liter. Absorbance and iron concentration were linearly related between 25 and 300 μg/dl at all wavelengths tested. Because absorbance and sensitivity were greatest at 248.4 nm, we used this wavelength in subsequent analyses.
Evaluation of the flameless AAS-micromethod for determining TIBC. Accurate estimation of TIBC depends on complete saturation of transferrin by exogenous ferric iron and removal of excess iron (4, 6, 14). With this in mind, we established that with 20 μl of serum as a starting material, 40 μl of a solution containing, per deciliter, 500 μg of ferric iron and 10 mg of magnesium carbonate were optimal for preparing samples for TIBC determination. The correlated \( r = 0.926, P < 0.001 \) TIBC values obtained by the flameless AAS-micromethod vs. the flameless AAS-macromethod (Figure 2) confirmed this conclusion. We also found that incubating the supernate in hot water after adding TCA occasionally yielded extremely high values. Thus, the incubation step used in the conventional macromethod was omitted. Sera with a TIBC of 254 to 430 μg/dl as determined by the flame AAS-method were measured for TIBC by the flameless AAS-micromethod. The data are plotted in Figure 2. The linear regression equation, \( y = 0.816x + 69.528 \), where \( y = \text{TIBC} \) obtained by the flame AAS-method and \( x = \text{TIBC} \) obtained by the flameless AAS-micromethod, together with a high correlation coefficient \( r = 0.950, P < 0.001 \), indicated that results by the micromethod for determining TIBC agreed reasonably with the obtained by the conventional macromethod.

Reproducibility of the flameless AAS-micromethod for TIBC measurement was evaluated by using com-

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**Table 1. Reproducibility of TIBC Determination by the Flameless AAS-Micromethoda**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Within-run</th>
<th>1st TIBC, μg/dl</th>
<th>3rd</th>
<th>10th</th>
<th>24th</th>
</tr>
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<tbody>
<tr>
<td>LNb</td>
<td>285 ± 12 (9)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>4.2%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAc</td>
<td>542 ± 17 (9)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>3.1%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1d</td>
<td>259 ± 16 (20)</td>
<td>244 ± 23 (5)</td>
<td>264 ± 11 (5)</td>
<td>258 ± 18 (5)</td>
<td>267 ± 14 (5)</td>
</tr>
<tr>
<td></td>
<td>6.1%</td>
<td>9.4%</td>
<td>4.2%</td>
<td>6.7%</td>
<td>5.2%</td>
</tr>
<tr>
<td>2d</td>
<td>465 ± 17 (20)</td>
<td>469 ± 13 (5)</td>
<td>477 ± 22 (5)</td>
<td>457 ± 12 (5)</td>
<td>456 ± 11 (5)</td>
</tr>
<tr>
<td></td>
<td>3.6%</td>
<td>2.8%</td>
<td>4.6%</td>
<td>2.6%</td>
<td>2.4%</td>
</tr>
</tbody>
</table>

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* The data are expressed as mean ± SD, with the number of measurements on each sample given in parentheses. A coefficient of variation (%) is included below each value. ND = not done.

b Normal chemistry control serum (LN) had a TIBC of 276 ± 13 μg/dl as determined by flame atomic absorption spectrophotometer.

c Abnormal chemistry control serum (LA) had a TIBC of 532 ± 10 μg/dl as determined by flame atomic absorption spectrophotometer.

d Sera from the clinical laboratory, St. Jude Children’s Research Hospital.
mercially available normal control (LN) and abnormal control (LA) sera as well as sera obtained from our clinical laboratory. The coefficients of variation calculated from within-run analysis for LN, LA, and Samples 1 and 2 were 4.2%, 3.1%, 6.1%, and 3.6%, respectively, suggesting that an accurate TIBC within the normal and pathologic range can be determined by the flameless AAS-micromethod (Table 1). Similar reproducibility is indicated by the coefficients of variation (range: 2.4% to 9.4%) obtained from day-to-day determinations (Table 1).

Treatment of serum with TCA. Because hemoglobin interferes with spectrophotometric (5) and atomic absorption spectrophotometric (6, 7, 15) determinations of TIBC and serum iron, we investigated the effect of TCA on the TIBC test. Samples not treated with TCA before analysis always produced excessively high values of TIBC. This distortion, presumably caused by contamination by hemoglobin iron, was eliminated by precipitating sera with an equal volume of TCA (50 g/liter) (Table 2). Measurements of TIBC by the flameless AAS-micromethod and flame AAS-methods correlated closely when the samples were treated with TCA, suggesting that precipitation with this acid is required for accurate measurements. Sera with visible hemolysis may yield extraordinarily high values despite TCA treatment, and therefore should be discarded (5, 7).

Evaluation of the modified flameless-micromethod for serum iron measurement. For estimation of transferrin saturation, the serum samples used for TIBC assays were also used to determine serum iron. However, because the serum sample was not only small, but also was diluted during preparation, it was necessary to use the most sensitive wavelength, 248.4 nm, instead of 302.1 nm as suggested by Olsen et al. (12).

From comparative studies (Figure 3), we obtained a correlation coefficient of 0.938 (P < 0.001) for flameless AAS-micro- vs. macromethods and a value of 0.973 (P < 0.001) for the flameless AAS-micromethod vs. the flame AAS-macromethod, results that agree with those of Olsen et al. (12), who used 50 μl of serum; this suggests that with as little as 20 μl of serum the flameless AAS-micromethod gave results comparable to those obtained by the conventional flame AAS-macromethod for determining serum iron.

Aside from a decrease in sample size, the modified method allows one to determine serum iron from the same calibration curve obtained at 248.4 nm for use in TIBC analysis.

Evaluation of flameless AAS-micromethods for estimating transferrin saturation. Transferrin saturation was computed from the serum iron and TIBC data presented in Figures 2 and 3. The calculated data (Figure 4) indicated a close correlation (r = 0.965, P < 0.001) between the results obtained by flameless AAS-micromethods and flame AAS-methods. The regression equation, $y = 1.052x - 0.398$, where $y$ = % saturation obtained by flame AAS-methods and $x$ = % saturation obtained by flameless AAS-micromethods, implies that almost identical transferrin saturation values can be obtained by these methods. The correlation ($r = 0.937$, P < 0.001) between results of the flameless AAS-micro and flameless AAS-macromethods provides additional support for this conclusion.

The agreement of our flameless AAS-micrometh-

<table>
<thead>
<tr>
<th>Flameless AAS-Micro</th>
<th>Flame AAS</th>
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<tbody>
<tr>
<td>+TCA $^a$</td>
<td>-TCA $^a$</td>
</tr>
<tr>
<td>278</td>
<td>373</td>
</tr>
<tr>
<td>266</td>
<td>385</td>
</tr>
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<td>322</td>
<td>397</td>
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<td>352</td>
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<td>456</td>
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<tr>
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<td>432</td>
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<tr>
<td>292</td>
<td>362</td>
</tr>
<tr>
<td>407</td>
<td>396</td>
</tr>
</tbody>
</table>

For determination of TIBC samples were mixed with or without TCA (50 g/liter) as indicated in the preparation procedure (see "Methods and Materials"). Twenty microliters and 1.0 ml of serum were used to prepare protein-free supernate for TIBC determination by the flameless AAS-micromethod and flame of AAS-method, respectively. Sera were obtained from the clinical laboratory at St. Jude Children's Research Hospital.

$r = 0.937$ (P < 0.001) indicates that TCA is necessary for accurate quantitative determinations. Other comparisons produced poor correlations: $b$ vs. $a$, $r = 0.034$, and $d$ vs. $e$, $r = 0.032$.

**Fig. 3.** Correlation between flameless and flame AAS-methods for determination of serum iron.

Twenty microliters and 1.0 ml of serum were used to prepare protein-free supernate for the flameless AAS-macromethod and the flame AAS-method, respectively. The supernate prepared for flame AAS was also used in the flameless AAS-macromethod. Five microliters of the resulting supernate was injected into a graphite tube for both micro and macroflameless AAS. The correlation coefficients ($r$) for the flameless AAS-micro vs. flame AAS ($\bullet$), and flameless AAS-micro vs. flameless AAS-macromethod ($O----O$) were 0.973 and 0.936, respectively.
methods with conventional atomic absorption spectrophotometry suggests that they are sensitive and accurate procedures for determining TIBC and serum iron with a total of 40 μl of serum. These methods are suitable for routine laboratory work in pediatric clinics and for screening tests in large-scale nutrition surveys of small children.

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References