Modified aca Method for Determination of Iron Chelated by Deferoxamine and Other Chelators

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Iron in serum and urine specimens containing deferoxamine (a chelator used in treating acute iron intoxication) can be measured by the DuPont aca discrete-analyzer method for iron if 0.5 mL of a 100 mL/L solution of thiglycolic acid (mercaptoacetic acid) is added to the IRN-1 and IRN-2 test packs before their use. This reagent releases the unreactive chelated iron, allowing reliable colorimetry. Because about 50% of hemoglobin iron is measured by the modified method, hemolysed samples should not be used (or results should be corrected for hemoglobin iron). The modification allows emergency determination of total serum iron and assessment of urinary iron during chelation therapy, as well as measurement of plasma iron in samples treated with sodium citrate or oxalate plus fluoride (the unmodified aca iron method gives falsely low iron values in the presence of these chelators). About 60% of iron present as the dextran complex (Imferon®) is measured by the modified aca method, as compared with <5% by the unmodified aca technique.

Additional Keyphrases: measurement of circulating chelated or complexed iron • toxicology • urine • urgent determinations • discrete analyzers • hemoglobin iron • monitoring therapy

Iron in serum is usually determined colorimetrically by reaction with chromogenic compounds that are selective for ferrous, rather than ferric, iron. These procedures involve use of a “reducing agent” such as hydroxylamine, hydradzine, sodium sulfite, sodium metabisulfite, ascorbic acid, thioglycolic acid (mercaptoacetic acid), or sodium hydrosulfite, to reduce any iron present and ensure reaction with the chromogen. Iron methods in which “mild” reducing agents such as hydroxylamine are used measure less hemoglobin iron than do methods with “strong” reducing agents such as thioglycolic acid (1). Because measurement of hemoglobin iron is regarded as an undesirable “positive interference” in routine serum iron measurements, the use of a mild reducing agent is advantageous under these circumstances. However, methods involving mild reducing agents will underestimate the iron concentration when deferoxamine, a chelator used in the treatment of acute intoxication with iron, is present in the sample. Because there are clinical situations in which estimation of total iron, including chelated iron, is important, several colorimetric methods have been developed for measuring iron in the presence of deferoxamine. These methods involve either wet ashing (3, 4) or strong reducing agents, such as sodium hydrosulfite (5), thioglycolic acid (6), and concentrated ascorbic acid (7).

Deferoxamine-bound iron can also be measured by atomic absorption spectroscopy, both in serum (8) and urine (9). The instrumentation for this is not available in many smaller laboratories, however, where it is also impractical to set up a separate manual or continuous-flow method to determine chelated iron for the occasional sample containing deferoxamine.

We have developed and evaluated a simple, inexpensive modification of the DuPont aca iron method, based on the addition of a strong reducing agent, thioglycolic acid, to the test packs, which allows rapid and accurate determination of deferoxamine-bound iron in serum and urine specimens. The modification was also evaluated for its effect on determination of iron bound to citrate, oxalate, hemoglobin, ethylenediaminetetraacetate (EDTA), or dextrans.

Materials and Methods

Apparatus

Iron determinations were performed in an aca (DuPont Automatic Clinical Analyzer; E.I. DuPont de Nemours & Co., Inc., Wilmington, DE 19898). The sample size was reduced to 125 μL per pack because most of the samples we analyze come from our pediatric patient population. Dilutions with iron-free distilled water were made necessary to bring results within the upper limit of linearity (10 g/L).

Atomic absorption spectroscopic iron determinations were done with an atomic absorption-emission spectrophotometer (Model 353; Instrumentation Laboratory, Inc., Lexington, MA 02173).

Spectrophotometric scans of aca test pack contents were performed with an Acta-III spectrophotometer (Beckman Instruments, Inc., Fullerton, CA 92834).

Random batches of tuberculin syringes (1-mL, 25-gauge, 5/8-in. needle; Becton Dickinson and Co., Rutherford, NJ 07070) were tested for iron contamination by washing them with the same 100 mL/L thioglycolic acid through successive syringes. The resulting solution had a negligible increase in “background iron” (less than 30 μg/L per syringe). Blood was collected in Vacutainer Tubes (Becton Dickinson and Co.) by direct venipuncture.

Glassware used in all experiments was soaked overnight in a 100 mL/L solution of nitric acid and doubly rinsed with iron-free distilled water after repeated rinses with tap water.

Serum and Urine Specimens

Serum used in the experiment with deferoxamine was pooled from clinical specimens received in our general chemistry laboratory. The pooled serum had a hemoglobin concentration of 20 mg/L. Urine and serum specimens were obtained from four patients; all of these specimens were assayed for iron by the unmodified and modified aca methods and by atomic absorption spectroscopy. The urine specimens were collected in iron-free plastic containers.

Case 1: Pre- and post-treatment urine samples were obtained from a 12-year-old black girl with sickle cell anemia, who was being treated electively with deferoxamine for transfusion siderosis.

Case 2: A serum specimen was obtained from a 22-month-

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old white girl during the course of deferoxamine therapy after accidental over-ingestion of multivitamin-plus-iron tablets.

Case 3: A 24-h urine collection after deferoxamine challenge was obtained from a 57-year-old black man suspected of having hemochromatosis.

Case 4: Consecutive 24-h urine specimens were obtained from a 12-year-old white girl being treated by continuous infusion of deferoxamine because of transfusion siderosis.

Reagents

Deferoxamine: Deferoxamine mesylate (desferrioxamine; Desferal™, Ciba Pharmaceuticals, Summit, NJ 07901) aqueous solutions containing 100 mg of deferoxamine per liter were found to be iron-free by atomic absorption spectroscopy.

Dextran iron: Imferon™ (Merrell-National, Cincinnati, OH 45215).

Thioglycolic acid (mercaptopoacetic acid), 980 mL/L. Two sources were used: Sigma Chemical Co., St. Louis, MO 63178, and Aldrich Chemical Co., Milwaukee, WI 53233. Aqueous 100 mL/L solutions from either source typically contained 300–350 µg of iron per liter as measured by atomic absorption spectroscopy. We were unable to remove this contaminant, either by distillation or other means. Because of this contamination, aca iron determinations with modified packs required a "background" correction, as described in Calculations.

Hemoglobin for use in the hemoglobin-iron recovery experiment was in the form of lysed (by freeze-thaw) erythrocytes.

Iron stock solutions: Concentrated iron stock, 100 mg/L, was prepared by dissolving 100 mg of pure iron wire in a minimum of hydrochloric acid and diluting to 1 L with water. This stock was diluted with iron-free distilled water to make aqueous standards for determination of "background" values and for use in calibrating the atomic absorption spectrophotometer. It was also added in appropriate dilution to pooled serum to approximate a toxic iron concentration of 5 mg/L.

Procedures

DuPont aca iron determinations: "Unmodified" aca results were obtained by following the manufacturer's recommendations. "Modified" aca results were obtained by injecting 0.5 mL of a 100 mL/L aqueous solution of thioglycolic acid through the lumen of the red-rubber inlet port of the IRN-1 and IRN-2 aca test packs, immediately before use, with a disposable 1-mL tuberculin syringe. These packs were used with the aca in the usual operating mode. We have performed hundreds of determinations using packs modified in this manner and have experienced no difficulty with mechanical malfunction or "pressure" error codes that could be attributed to the modified packs.

Atomic absorption spectroscopy was according to Olson and Hamlin (10), with trichloroacetic acid (200 g/L) as protein-precipitating reagent and thioglycolic acid (Sigma) as reducing agent. For the dextran iron determinations, samples were simply diluted as required with water to avoid precipitation of bound iron.

Hemoglobin measurements: Hemoglobin in serum samples was measured by the method (11) routinely used in our general chemistry laboratory.

Calculations: The "total iron" concentration of a specimen is calculated by the formula: Total iron, µg/L = (aca readout, using modified packs, µg/L X 1.1) - "background iron." The 1.1 is the ratio of the final volumes in the modified and unmodified aca packs (5.5 mL/5.0 mL).

The "background iron" value, obtained by using a 1000 µg/L aqueous iron standard as sample with modified and unmodified packs, is calculated as: (aca readout, using modified packs, µg/L X 1.1) - (aca readout, using unmodified packs). If the aca has been calibrated to read zero when distilled water is the sample, then distilled water may be used instead of an aqueous iron standard. The "background iron" value is a constant for any given thioglycolic acid reagent and reflects the iron contamination of the thioglycolic acid used for that run. In our experience, background iron values are typically 0.5 to 1.5 mg/L.

Results

Sera containing added deferoxamine in concentrations ranging from 10 to 2500 mg/L showed less apparent iron as measured by the unmodified aca (Figure 1), the decrease being proportional to the concentration of deferoxamine and varying logarithmically from 14% of baseline at 10 mg/L to 88% of baseline at 2500 mg of deferoxamine per liter. The modified aca method completely eliminated this interference. The results then obtained for "total iron" ranged from 5.19 mg/L to 5.40 mg/L.

Table 1 shows results of determinations made by the unmodified and modified aca methods and by atomic absorption spectroscopy for some clinical specimens. The unmodified aca method showed essentially complete suppression of color development in the case of urine specimens containing deferoxamine, and substantial suppression in the one serum

<table>
<thead>
<tr>
<th>Case</th>
<th>Unmodified aca</th>
<th>Modified aca</th>
<th>AAS</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>0.03</td>
<td>6.57</td>
<td>6.65</td>
</tr>
<tr>
<td></td>
<td>0.04</td>
<td>8.83</td>
<td>9.70</td>
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<tr>
<td>2a</td>
<td>0.95</td>
<td>1.88</td>
<td>2.00</td>
</tr>
<tr>
<td>3</td>
<td>2.80</td>
<td></td>
<td>3.00</td>
</tr>
<tr>
<td>4</td>
<td>0.20</td>
<td>30.40</td>
<td>34.00</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>72.80</td>
<td>64.00</td>
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<tr>
<td></td>
<td>0</td>
<td>48.45</td>
<td>53.50</td>
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<tr>
<td></td>
<td>0</td>
<td>27.60</td>
<td>29.00</td>
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</table>

* Serum specimen.
Fig. 2. Apparent iron concentrations of hemoglobin solutions by atomic absorption spectrophotometry as compared with aca results for iron before (unmodified) and after (modified) addition of 0.5 mL of 100 mL/L thioglycolic acid to reagent packs

specimen we examined. The modified aca method gave results very near those by atomic absorption spectroscopy.

Figure 2 shows results for iron as determined in aqueous solutions of hemoglobin by the modified and unmodified aca methods and by atomic absorption spectroscopy. The modified method measured 1.75 μg of iron per milligram of hemoglobin (half the actual total iron in hemoglobin as measured by atomic absorption spectroscopy). The unmodified aca measured negligible amounts of hemoglobin iron (5% or less of the total iron present).

Table 2 shows our results for determinations of iron in 20 sera by the modified and unmodified aca methods. If uncorrected for hemoglobin iron, the mean iron concentration was slightly higher by the modified method. By applying the correction factor of 1.75 μg of iron per milligram of hemoglobin to each sample, the mean serum iron obtained by the two methods are identical.

Addition of the thioglycolic acid reagent to the test packs lowers the pH of the reagent mixture at the end of the run from 4.2 (pH of unmodified test pack contents) to 2.8. We made a comparison of spectrophotometric scans of modified and unmodified aca test pack contents at the end of a run, using a 5 mg/L aqueous iron solution as sample. The curves could be superimposed. Evidently the different results obtained by the modified aca method should not be attributed to a non-specific shift in the absorbance curve of the chromogen, and the lower pH of the reagent mixture of the modified method does not qualitatively affect the absorbance characteristics of the chromogen. To further evaluate the effect of pH on color development, we injected packs with 0.5 mL of various concentrations of hydrochloric acid, and measured the pH of the reaction mixture at the end of the run, using a 5 mg/L aqueous iron standard as sample. Color development was not affected by lowering the pH to 2.8; at lower pH values, color development was less. Spectrophotometric scans of pack contents showed no difference whether the pH was lowered to 2.8 with thioglycolic acid or hydrochloric acid.

We also evaluated the effect of Imferon® and of the anticoagulants sodium citrate, oxalate-fluoride, and EDTA on aca iron results. Blood samples from healthy individuals were drawn simultaneously into the various 5-mL Vacutainer Tubes containing no anticoagulant (red stopper), citrate (blue stopper), oxalate-fluoride (gray stopper), or EDTA (lavender stopper). Average aca iron values for the plasma samples obtained by the unmodified aca method were 15, 20, and 90% lower, respectively, than the simultaneously drawn serum iron values (unpublished observations). By using the modified aca packs, the interference produced by citrate and oxalate was eliminated completely. However, the oxalate-fluoride anticoagulant caused moderate hemolysis, making a correction necessary to account for hemoglobin iron to avoid falsely high iron values by the modified aca method. The effect of EDTA could not be reversed by the modified method (the background iron value exceeded the aca iron readout by the modified method). Less than 5% of dextran-bound iron (in the form of Imferon® iron added in vitro) was detected by the unmodified aca method, but addition of thioglycolic acid resulted in measurement of about 60% of the added Imferon® iron.

Reagent stability and storage conditions: Aqueous 100 mL/L solutions of thioglycolic acid, stored in refrigerated glass bottles, are stable for a month or more; thereafter, they show increasing background and loss of "reducing power." Presumably the former is due to leaching of iron from the glass, the latter to oxidation of the reagent. The use of plastic bottles decreases the former effect but does not affect the latter. Aca packs stored for a period of weeks after addition of thioglycolic acid produce spuriously high iron readouts, apparently owing to opacification of the plastic forming the cuvette. We therefore prepare the modified aca packs immediately before use, using 100 mL/L thioglycolic acid that has been diluted within one month of use. Care should be taken to use clean pipettes and glassware, preferably acid-washed. If this is not available, disposable plastic items may be used. The "background iron" value serves as a check on the adequacy of preparation; values exceeding 2 mg/L probably indicate contamination during preparation.

Discussion

It is well established that many colorimetric methods for measurement of iron give falsely low results in the presence of iron chelators, notably deferoxamine (2, 8). To eliminate this interference, various methods have been developed involving commonly used iron chromogens, but with a pre-treatment step of wet ashing or the use of strong reducing agents (3–7). Atomic absorption spectroscopy does not exhibit interference in the presence of deferoxamine (8, 9), and has been advocated as an adequate method for such measurements. However, many clinical laboratories do not have an atomic absorption spectrophotometer and will not have available a special manual or wet-ashing procedure for an occasional determination of iron in a sample containing deferoxamine. In many instances, the only currently available iron methodology may be the widely used DuPont aca iron method, which severely underestimates iron in the presence of deferoxamine. Our modification of the aca method will allow measurement of deferoxamine-bound iron, a critical

Table 2. Iron Values, for 20 Sera, a as Measured by the Unmodified and Modified Methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Mean serum iron, μg/L (range) b</th>
</tr>
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<tbody>
<tr>
<td>Unmodified</td>
<td>630 (30–1520)</td>
</tr>
<tr>
<td>Modified, uncorrected for Hb iron</td>
<td>720 (110–1610)</td>
</tr>
<tr>
<td>Modified, corrected for Hb iron c</td>
<td>630 (80–1490)</td>
</tr>
</tbody>
</table>

a Selected without conscious bias.
b Distribution not gaussian.
c 1.75 μg of iron per milligram of hemoglobin (Hb) for the 100 mL/L solution of thioglycolic acid.
necessity in cases of acute iron poisoning when therapy with deferoxamine has been instituted, because the decision to terminate chelation therapy is partly based on sequential measurement of serum iron during therapy. The therapy is usually stopped when the serum iron falls below the pre-treatment total iron-binding capacity (12). Parenteral therapy with deferoxamine must be closely monitored because of the potential complication of hypotension (13). The decision to initiate deferoxamine therapy is largely based on measurement of serum iron and total iron-binding capacity on initial clinical presentation. Because of delays in obtaining these values, emergency semi-quantitative methods for detecting free iron in plasma have been developed (14).

Urinary iron is also assayed to follow the progress of cases of acute iron toxicity being treated with deferoxamine (12, 13). Partly because laboratories cannot provide emergency determinations of deferoxamine-bound iron, the presence or absence of significant urinary iron excretion is sometimes simply judged from the visual appearance of the urine, a reddish-brown color indicating the presence of the iron chelate, and its disappearance from urine as an indication to terminate chelation therapy (12).

In chronic iron-overload states, such as hemochromatosis or iron-loading anemias, urinary iron excretion after administration of deferoxamine is used both diagnostically and to follow the course of therapy (4), and accurate measurement of iron is necessary.

The therapeutic concentration of deferoxamine in serum has been estimated to be <130 mg/L (8). Concentrations in this range cause a significant depression of color formation with the unmodified "aca" iron method (Figure 1). The effect on clinical urine specimens is even more dramatic (Table 1). The higher values obtained by atomic absorption spectroscopy in Table 1 are explainable as an inherent bias between the two methods. A recent CAP survey showed that atomic absorption spectroscopy overestimated iron as compared with a reference method, whereas the "aca" iron method underestimated iron as compared with the same reference method (15). Other authors have also obtained higher results by atomic absorption spectroscopy than by colorimetric methods when measuring iron in samples containing deferoxamine (8).

We believe the modified "aca" method is suitable for serial measurement of serum iron in cases of acute iron toxicity being treated with deferoxamine. For this reason, our laboratories now offer emergency 24-h total iron-binding capacity determinations by "aca", in conjunction with the modified method for serum and urine iron. This eliminates the need for semiquantitative methods of detecting free iron in plasma.

The modified "aca" method is particularly well suited to urinary iron determination in the presence of deferoxamine. Hemoglobin is not normally present in urine, so there should be no need to correct for hemoglobin iron, as in some serum samples.

The mechanism by which the modified procedure measures chelated iron appears to be a combination of lowered pH and increased reducing ability. At low pH, deferoxamine has a much greater affinity for Fe₃⁺ than for Fe²⁺ (16). The stability constant for deferoxamine complexed with Fe³⁺ is 10⁶, as compared with 10¹⁰ for Fe²⁺ (17). Thus, the conditions created by addition of thioglycolic acid to the reagent pack favor release of iron from deferoxamine, allowing it to complex with the chromogen, bathophenanthroline. The approach of adding strong reducing agents in existing iron methods has been used previously. Gevirtz and Wasserman added sodium hydroxulfite to the reaction mixture to eliminate deferoxamine-induced interference (2).

The unmodified "aca" iron method is strikingly similar to the direct manual method developed by Jung (18) which does not react with hemoglobin iron. By adding thioglycolic acid to the reagent pack, we have converted the "aca" method from one using mild reducing conditions to one with strongly reducing properties at a more acidic pH. The combination of thioglycolic acid and bathophenanthroline has been used in several published methods for iron (19, 20). The reference iron method recommended by the International Committee for Standardization in Hematology uses this combination (21). Methods using this combination have been shown to be suitable for determination of iron in the presence of deferoxamine, both in serum (6) and in urine (9).

Our method for determining deferoxamine-bound iron has the advantages of speed, simplicity, and economy over atomic absorption spectroscopy or other colorimetric methods, for laboratories having "aca" iron methodology available.

A disadvantage of our method is the possibility of positive interference from hemoglobin iron. With ordinary care in specimen collection, there should not be a significant contribution from hemoglobin iron. The average hemoglobin iron contribution observed in random sera (90 mg/L, as shown in Table 2), would not be clinically significant in cases of acute iron toxicity. Visibly hemolysed specimens should not be used with the modified method unless the hemoglobin can be measured and the appropriate correction made.

One potential application of our method is that of monitoring deferoxamine therapy. At a given serum iron concentration, the per cent decrease in iron as measured on the "aca" given by (modified iron-unmodified iron/modified iron) × 100 would be proportional to the concentration of deferoxamine and thus an indirect measure of its concentration, as illustrated in Figure 1. This could prove useful in correlating toxicity with drug concentration. To our knowledge, there is no method of measuring deferoxamine in serum other than a research method involving the use of radioactively tagged deferoxamine (17). A method for measurement of ferrioxamine and deferoxamine in urine has been proposed (22).

The ability to overcome the interference caused by citrate and oxalate–fluoride mixtures is another application of the modified "aca" method, although it is of less clinical importance than the ability to overcome deferoxamine-induced interference.

Recovery of dextran-iron, although not quantitative, is sufficient to allow determination of the presence or absence of the drug, by noting the increase in apparent iron as measured by the modified method when the drug is present.

Iron reagent packs for this study were a gift from the DuPont Company, Instrument Products, Automatic Clinical Analysis Division, Wilmington, DE 19888.

References

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