Modified Method for Analysis of Serum Iron

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ABSTRACT

An improved method for analysis of serum iron is described which is simple, rapid, precise and convenient for routine use in clinical laboratories. Serum proteins are precipitated with trichloroacetic acid-hydrochloric acid solution, with simultaneous release of Fe(III) from transferrin. Fe(III) is reduced to Fe(II) by sodium ascorbate, and Fe(II) is reacted with ferrozine to form a lavender complex, which is measured by spectrophotometry at 562 nm. Measurements of iron in 183 serum samples by this method were compared with measurements by a "direct" spectrophotometric method without deproteinization, as previously described. Close agreement was obtained in 171 of these 183 pairs of analyses (93.5 percent). Discrepancies (> 12 μg per dl) were noted in the remaining 12 serums, which were attributed to interference in direct spectrophotometric analyses of iron, owing to (1) hemolysis, (2) lipemia, (3) jaundice, (4) protracted storage or (5) repeated freezing and thawing of the serums.

Introduction

In 1974, Horak and Sunderman described a "direct" semi-micro method for serum iron and latent iron-binding capacity without deproteinization. This technique was shown to provide close correlations with (1) measurements of serum iron and iron-binding capacity by the atomic absorption method of Olson and Hamlin, and (2) measurements of serum iron by the reference procedure that has been proposed by the International Committee for Standardization in Hematology, with substitution of ferrozine for bathophenanthroline as the chromogen. During routine use of the direct semi-micro method for serum iron in our hospital laboratory, spurious increases were noticed in the concentrations of iron in a few serum samples, which were either (1) stored for more than one week at 4° to 10° C; (2) subjected to repeated freezing and thawing; or (3) grossly hemolyzed, lipemic or jaundiced. These sources of error have been avoided by the modification which is described in this paper, in which serum proteins are precipitated with trichloroacetic acid prior to spectrophotometric analysis of iron by the ferrozine color reaction. The present modified method for serum iron can be used conveniently in conjunction with measurements of serum latent iron-binding capacity by the original Horak-Sunderman method.

Method

PRINCIPLE

Serum is deproteinized with trichloroacetic acid-hydrochloric acid solution, with si-
multaneous release of Fe(III) from transferrin. Fe(III) is reduced to Fe(II) by sodium ascorbate, and Fe(II) is reacted with ferrozine to form a lavendar complex, which is measured by spectrophotometry at 562 nm.

REAGENTS

(Precautions. Distilled, demineralized water is used for preparation of all reagents. Glassware and plasticware are washed with dilute nitric acid and rinsed with copious distilled, demineralized water.)

Iron Stock Standard Solution, (50 mg per dl). Iron wire (99.999 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 6 ml of concentrated HC1*. 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 four 100 ml volumetric flasks are transferred 1, 2, 3 and 4 ml of Iron Intermediate Standard Solution. After the contents of these flasks have been diluted to 100 ml with water, they contain 50, 100, 150 and 200 μg Fe per dl, respectively. These standards are used to construct the calibration curve, which adheres to Beer’s law. For routine measurements, only the 100 μg per dl Fe standard is employed.

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.

Trichloroacetic Acid-Hydrochloric Acid Solution. Into a 500 ml volumetric flask are placed 50 g of trichloroacetic acid (“metal-free reagent,”† and approximately 300 ml of water. After the trichloroacetic acid crystals have dissolved, 60 ml of concentrated HC1 (12 mol/liter)* are added, and the contents are diluted to the mark with water.

Sodium Acetate Solution, (2 mol per liter). Into a 500 ml volumetric flask are weighed 136.1 g of sodium acetate (CH3COONa-3H2O),§ The contents are dissolved in water and diluted to the mark with water.

Sodium Acetate-Ascorbate Solution. Immediately before use, 250 mg of sodium ascorbate§ are dissolved in sodium acetate solution in a 50 ml volumetric flask, and diluted to the mark with the sodium acetate solution.

EQUIPMENT

Spectrophotometer.** This provides stable digital read-out 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 (1.000 cm light path, 3 ml volume) which contains water. All absorbance measurements are made versus this “water blank”.

PROCEDURE

Into respectively labelled plastic test-tubes are pipeted (1) 1 ml of H2O (“reagent blank”), (2) 1 ml of iron working standard solution (100 μg Fe per dl) and (3) 1 ml of serum. Trichloroacetic acid-hydrochloric acid solution (1 ml) is added to each test-tube, and the contents are mixed with a “Vortex” mixer. The tubes are allowed to stand at room temperature for 20 min, and then are centrifuged at 900 × g for 10 min. Into respectively labelled cuvets are

*Catalog No. X259, Eastman Kodak Co., Rochester, NY.
†Hach Chemical Co., Ames, IA.
§“Analyzed” reagent, Baker Chemical Co., Phillipsburg, NJ.
**Model 25, Beckman Instrument Co., Fullerton, CA.
transferred (1) 1 ml of each clear supernatant fluid, (2) 1 ml of sodium acetate-ascorbate solution and (3) 0.2 ml of ferrozine reagent. The cuvets are allowed to stand at room temperature for 10 min, and the absorbances of the cuvets are measured at 562 nm.

CALCULATION

Serum Fe (µg per dl) =

\[
\frac{[A \text{ (serum)} - A \text{ (reagent blank)}]}{[A \text{ (standard)} - A \text{ (reagent blank)}]} \times 100
\]

PROCEDURAL NOTES

After serum proteins have been precipitated with trichloroacetic acid-hydrochloric acid solution and removed by centrifugation, the supernatant fluid should be optically clear. If the fluid is hazy, it may be heated at 56°C for 15 min and recentrifuged, as suggested by Rice and Fenner.7

Especial attention should be directed to the hazard of iron contamination of plastic test-tubes and cuvets, as emphasized by Fielding and Ryall.8

Results

Simultaneous measurements of serum iron by (1) the original "direct" technique without deproteinization,4 and by (2) the present modified technique with protein precipitation were performed upon 183 samples of serum (figure 1). These serum specimens were submitted to the hospital laboratory for determinations of serum iron and iron-binding capacity, and they were analyzed during 20 successive runs. The paired analyses of serum iron by the two techniques agreed within 8 µg per dl in 171 of the 183 sera (93.5 percent). In the remaining 12 sera, the iron concentrations which were obtained by the original method were more than 12 µg per dl higher than by the modified procedure. Three of these 12 sera were markedly lipemic; one

Figure 1. Comparisons of measurements of serum iron in 183 serum specimens by the modified method with deproteinization (abscissa) and by the original "direct" method of Horak and Sunderman4 (ordinate). Data points for 12 serum samples with discrepant results (> 12 µg per dl) are encircled. L = lipemia, H = hemolysis; and I = icterus.
was icteric; and one was grossly hemolyzed. The remaining seven serums had been repeatedly frozen and thawed, and/or had been stored in a refrigerator at 4 to 10° C for more than one week. When these 12 serums were eliminated from the series of 183 serum samples, there was no significant difference between the iron concentrations which were obtained by the original and by the modified techniques, based upon a paired sample “t” test. The correlation coefficient of iron analyses in the 171 serum samples was 0.9965. The slope of the regression line was Y = 1.01X – 1.01, which was practically identical to that which was previously reported by Horak and Sunderman.4

Measurements of iron in 26 serums were performed by the present, modified method and by the reference procedure which has been proposed by the International Committee for Standardization in Hematology,1 with substitution of ferrozine for bathophenanthroline as recommended by Rice and Fenner.7 Based upon a paired sample “t” test, there was no significant difference between the results which were obtained by the two methods. The correlation coefficient was 0.9929. The slope of the regression line was Y = 1.03X – 1.83, which corresponded closely to the theoretical line.

Comparisons were made of hemoglobin interference in measurements of serum iron by the original and modified techniques. A specimen of heparinized blood was obtained from a healthy subject. The plasma was removed, and the erythrocytes were (1) washed three times with NaCl solution (8.5 g per liter); (2) resuspended in distilled water and (3) lysed by freezing and thawing three times. The erythrocyte membranes were removed by centrifugation (1 hr; 3000 × g; 4° C), and the hemoglobin concentration in the clear hemolysate was measured.5 Five dilutions of the hemolysate were prepared in distilled water, and 0.1 ml of each dilution was mixed with 2 ml aliquots of a serum sample in order to provide additions of 25, 50, 100, 200 and 300 mg of hemoglobin per dl, respectively. The concentrations of serum iron (with and without added hemoglobin) were measured by the original and modified methods. This procedure was performed upon serum specimens from three persons, and results are given in table I. Visible hemolysis (100 to 300 mg of hemoglobin per dl) caused interference in measurements of serum iron by the original method, but not by the modified method with deproteinization.

Iron analyses by the original and by the modified methods were performed upon five commercial quality control serums, which had been lyophilized and reconstituted. In these quality control serums, measurements of iron by the original method gave spuriously elevated results, whereas measurements of iron by the modified method were within 10 μg per dl of the “target” values.

The “within-the-run” precision of the modified method for serum iron analysis was determined on the basis of duplicate measurements of iron in 62 serums. These duplicate analyses yielded a relative standard deviation of 0.6 percent. In com-

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Comparison, the "within-the-run" precision of the original method was 1.3 percent, based upon duplicate analyses of 154 sera. The "day-to-day" precision of the modified method was determined by 26 consecutive measurements of a "normal" and an "abnormal" quality control serum. The relative standard deviations which were obtained by daily measurements of iron in these lyophilized and reconstituted sera were 4.3 and 5.6 percent, respectively.

Discussion

Based upon the results of the present investigation, the authors have concluded that serum specimens should be deproteinized prior to spectrophotometric measurements of iron by the Horak-Sunderman procedure. Our findings are consistent with the opinions which have recently been stated by Rice and Fenner and by Brunelle. Rice and Fenner have emphasized that "direct" spectrophotometric methods for serum iron may suffer interference from serum constituents such as proteins, bilirubin, hemoglobin and lipids. Brunelle has found good agreement between analyses of serum iron by two "direct" spectrophotometric methods and by a conventional spectrophotometric method with deproteinization, but he has concluded that the deproteinization technique is preferable for routine use in clinical laboratories. The modified method for serum iron which is described in this paper is simple, rapid, precise and it is convenient for use in conjunction with the Horak-Sunderman method for serum iron-binding capacity.

Acknowledgment

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References