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December 1998

C61-A

Determination of Serum Iron, Total Iron-Binding Capacity and Percent Transferrin Saturation; Approved Standard

This document provides methods for determining serum iron and total iron-binding capacity; and describes the measurement of serum iron concentration as well as the determination of the percent saturation of transferrin with iron.

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Determination of Serum Iron, Total Iron-Binding Capacity and Percent Transferrin Saturation; Approved Standard

Abstract

This document describes the measurement of serum iron concentration, provides guidelines for the determination of serum total iron-binding capacity, and describes the determination of the percent saturation of transferrin with iron. The methods are linear over a wide range of iron concentrations, interference is negligible, and the precision is adequate. Although the methods, as described, are tedious and demand a large sample size and meticulous processing, they can be (semi)automated and the required amount of sample can be decreased. An example of automation is included. The document also provides reference ranges by age groups, race, and gender for serum iron concentration, total iron-binding capacity, and % transferrin saturation as determined during the U.S. National Health and Nutrition Examination Surveys, 1971-74, 1976-80, and 1988-94.

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Foreword

Human metabolic requirements for iron are met by a positive balance during the years of growth and by a rate of absorption that is physiologically matched with a relatively fixed rate of loss in the adult. Iron balance is unusual in that there is no physiological route for the excretion of excessive amounts and the avoidance of iron loading depends on the regulation of iron absorption. Many factors may influence iron absorption. Under physiologic conditions, however, only three factors appear to be important: the amount of iron ingested, its bioavailability, and the iron status of the patient. Absorption of iron is primarily controlled by the mucosal cells of the proximal small intestine, with a fraction of available iron absorbed inversely proportional to the body iron stores.^{1,2} Iron deficiency occurs when the dietary intake of bioavailable iron is inadequate, when absorption is impaired, or when bleeding occurs causing iron losses that exceed the capacity of the gastrointestinal tract to extract iron from the diet. Iron (over)loading may result from an abnormal increase in the amount of iron absorbed, from the parenteral administration of iron, or from blood transfusion.

Storage iron occurs predominantly in two forms: as a soluble component of ferritin which is composed of a soluble (FeOOH)_x core within an apoferritin protein shell, or as part of insoluble hemosiderin. There is evidence that the most recently formed hemosiderin has the most rapidly available iron for mobilization from storage to transport pools.³ Under conditions of iron overloading, hemosiderin iron increases relative to ferritin iron with massive deposits in parenchymal cells leading to tissue and organ damage.

Mammals have specific iron-binding proteins that move iron from sites of absorption and storage to sites of use. The best characterized of these proteins are the transferrins: two-sided single-chain iron-binding proteins widely distributed in physiologic fluids and cells. Two major types have been identified: serum transferrin and lactoferrin. Serum transferrin is the major carrier protein of iron in blood and tissues⁴; lactoferrins are found predominantly in tissue fluids and cells and seem to function in the body's defense against infection as well as in iron transport.⁵ Transferrin has a relative molecular mass of approximately 79 600⁶ and is a major serum protein, usually one-third saturated with iron, corresponding to a plasma iron concentration of 1 mg/L (17.9 μ mol/L; 100 μ g/dL)

Iron deficiency is the most common nutritional deficiency in both developing and developed countries. Iron deficiency results from a persisting negative iron balance due to inability to meet physiologic needs through diet. Iron deficiency not only causes anemia, but may have an effect on immunocompetence,^{7,8} and has been associated with behavioral abnormalities and reduced intellectual performance in children.^{9,10} In older children and adults, iron deficiency usually implies chronic blood loss.

The first, mildest stage of iron deficiency is iron depletion in which iron stores (*e.g.*, reticuloendothelial cells of liver, spleen, and bone marrow) are substantially reduced. In the second stage, iron stores are depleted but anemia is not yet demonstrable. The third and most severe degree of iron deficiency involves frank anemia, frequently with hypochromic and microcytic erythrocytes.

Plasma or serum ferritin concentration has proven a useful index of storage iron.¹¹ Direct evidence relating serum ferritin concentration to the size of the body iron stores has been obtained by comparison with chemical measurements of non-heme iron concentration in bone marrow,¹ and by studies in which the body iron store was estimated by phlebotomy-induced blood loss.¹²⁻¹⁴ Although the serum ferritin assay provides a useful and convenient way of detecting an absence of storage iron in uncomplicated iron deficiency, its usefulness is limited in many clinical situations in which iron deficiency coexists with infection and other inflammatory disorders, or with neoplasms, liver disease, or chronic renal disease. However, in these conditions, the usefulness of serum iron assays may also be quite limited.

Foreword (Continued)

	Normal	Fe depletion	Fe-deficient erythropoiesis	Fe-deficiency anemia
Storage Fe				
Hemoglobin Fe				
Stainable marrow Fe (0-6+)	2-3+	0-1	0	0
TIBC (mg/L)	3.4 ± 0.45	> 3.6	> 4.0	> 4.2
Serum ferritin ($\mu\text{g/L}$) (age/method dependent)	20-115	< 20	10-20	< 10
Serum Fe (mg/L)	1.0 ± 0.35	< 1.0	+	< 0.5
Transferrin saturation (%)	27 ± 10	< 25	< 15	< 10
Red cell morphology	normal	normal	normal	+ hypochromia, microcytosis

Laboratory test results during gradual development of iron deficiency (Modified from Bothwell et al. *Iron Metabolism in Man*. Oxford: Blackwell Scientific; 1979).

Once iron stores are depleted, the rate of delivery of iron to the bone marrow is limited and the serum iron concentration falls, as does the percent saturation of transferrin. On the other hand, the total iron binding capacity, TIBC, which is a measure of both unsaturated and saturated transferrin, is already affected before the iron supply fails: TIBC rises as storage iron decreases.^{15,16} The reliability of transferrin saturation, calculated as serum iron concentration/TIBC, as an index of iron deficiency is, however, limited by variability of the serum iron concentration, particularly because of marked diurnal variation. Even when specimens are collected at the same time of day, variability in the same person on different days may be as much as 40%.¹⁷

The most prevalent form of iron overload occurs as an inherited, nonhematological disorder of iron overabsorption: (idiopathic) hereditary hemochromatosis. In the early stages of the disease a number of screening tests may be helpful. Serum iron concentration is usually high and the circulating transferrin is usually completely or almost completely saturated; the TIBC of serum is usually reduced. Serum ferritin may be increased, although this is not invariably the case early in the iron-loading process. In clinical practice the best screening procedure for iron overload thus involves combined measurements of serum iron concentration, TIBC, and calculation of the percent transferrin saturation. In 1996 an Iron Overload Expert Advisory Panel to the Centers for Disease Control and Prevention recommended that determination of % transferrin saturation be used as a screening test for (idiopathic) hereditary hemochromatosis.¹⁸

Key Words

Hemochromatosis, iron deficiency, iron overload, percent transferrin saturation, (serum) iron, (serum) iron-binding capacity, TIBC, transferrin

Determination of Serum Iron, Total Iron-Binding Capacity and Percent Transferrin Saturation; Approved Standard

1 Introduction

The measurement of serum iron, total iron-binding capacity (TIBC), and calculation of the percent transferrin saturation have long been used as diagnostic procedures in the evaluation of iron deficiency. However, these tests are relatively insensitive for this purpose. A relatively sensitive test for the absence of storage iron in uncomplicated iron deficiency is the determination of serum ferritin, although the usefulness of this test is also limited, especially in situations where iron deficiency coexists with infections or inflammatory disorders. Calculation of the percent transferrin iron saturation, however, is a sensitive screening procedure for iron overload.

Laboratory errors in serum iron assays caused by iron contamination were common in the past, but have been largely eliminated by the use of disposable plastic supplies. The International Council for Standardization in Haematology, ICSH, has published recommendations for the determination of serum iron and TIBC.¹⁹⁻²³ The methods, however, remained subject to interference by copper and influenced slightly by heme iron,²⁴ and sources of variability in the determination of TIBC included the concentration of the saturating iron solution and the type and amount of magnesium used to remove the unbound iron.²⁵

Modifications developed at the Centers for Disease Control and Prevention, CDC, to maximize the sensitivity of the method and minimize interference included replacing thioglycolic acid by ascorbic acid as reducing agent, adding thiourea to the chromogen reagent,²⁶⁻²⁸ use of basic instead of "light" magnesium carbonate,²⁶ and a dilution factor of 3 instead of 2 in the determination of TIBC to bring the absorbance values in the same range as those of the serum iron determination.

The CDC-NCCLS-modified method has been compared to the original ICSH reference method and the results have shown to be comparable, if not identical;²⁹ and the method has been used in the evaluation of other, (semi)automated

methods.^{30,31} The methods have also been automated by CDC and used extensively during the National Health and Nutrition Examination Surveys (NHANES) I (1971-74), II (1976-80) and Hispanic (1982-84), without thiourea added to the chromogen reagent,²⁶ and NHANES III (1988-94), with thiourea added to the chromogen reagent.³² (See Appendix A.)

2 Scope

This standard describes the recommended method for the determination of serum iron, guidelines for the determination of TIBC, and the calculation of % transferrin iron saturation. Accurate determinations are required:

- for the evaluation of patient iron status and the differential diagnosis of anemia;
- for screening and diagnosis of iron overload syndromes; and
- in the evaluation of other manual or automated methods for the determination of these analytes.

This standard is thus intended primarily for clinical laboratory personnel.

3 Units of Measurement

Serum iron and TIBC values can be expressed as mass concentration, unit $\mu\text{g/L}$, mg/L , or as substance concentration, unit $\mu\text{mol/L}$.

The International Federation of Clinical Chemistry, IFCC, and the International Union of Pure and Applied Chemistry, IUPAC, recommend the exclusive use of the liter as unit of volume when reporting laboratory results using the Système International d'Unités (SI).³³

Although many published values are given per deciliter (dL; 10^{-1}L) as the unit of volume, this document, in conformance with ICSH, IFCC, IUPAC, and World Association of Societies of Pathology, (WASP), will use the liter and, where applicable, give both mass and substance concentration.

Conversion factors:

$\mu\text{mol/L}$	x	55.85	= $\mu\text{g/L}$; x 0.05586 = mg/L
$\mu\text{mol/L}$	x	5.585	= $\mu\text{g/dL}$
$\mu\text{g/L}$	x	0.1	= $\mu\text{g/dL}$
$\mu\text{g/L}$	x	0.017905	= $\mu\text{mol/L}$
mg/L	x	17.905	= $\mu\text{mol/L}$
$\mu\text{g/dL}$	x	10	= $\mu\text{g/L}$
$\mu\text{g/dL}$	x	0.17905	= $\mu\text{mol/L}$

4 Specimens

The recommended method for the determination of serum iron requires a 2 mL sample; the determination of TIBC requires 1 mL. The methods have been successfully "miniaturized" with respect to sample requirements.^{26,29,32}

Serum or **heparinized** plasma is suitable,³⁴ and may be either fresh or frozen. Serum iron and TIBC are stable and specimens may be stored (-20 °C or lower); serum iron levels are not affected by freeze-thaw cycles, TIBC values tend to decrease after two freeze-thaw cycles.³⁵

Specimens anticoagulated with ethylenediaminetetraacetic acid (EDTA) salts, with oxalate or with citrate are not suitable because these anticoagulants also chelate iron, thus preventing its reaction with the chromogen used in the iron determination. Grossly hemolyzed specimens are not suitable because of the potential contribution of iron from the hemoglobin.

For best results, fasting samples should be obtained. However, there is a marked diurnal variation of serum iron concentration (see Section 6.7.6). Even when specimens are collected at the same time of the day, the within-person day-to-day variability may be as high as 40%.¹⁷

5 Standard Precautions

Because it is often impossible to know which might be infectious, all patient blood specimens are to be treated with standard precautions. Specimens from any patient could be infected with human immunodeficiency virus, HIV, or hepatitis B or C virus, HBV, HCV. Proper blood collection techniques should be followed to minimize risk to the laboratory staff, and gloves should be worn when appropriate. For specific precautions for preventing the laboratory

transmission of bloodborne infection from laboratory instruments and materials; and recommendations for the management of bloodborne exposure, refer to NCCLS document M29—*Protection of Laboratory Workers from Instrument Biohazards and Infectious Disease Transmitted by Blood, Body Fluids, and Tissue*.

6 Recommended Method for the Determination of Serum Iron**6.1 Principle of the Method**

Addition of acid to serum releases iron from transferrin (by lowering the pH) and precipitates of serum proteins. The iron, Fe(III), in the supernatant is reduced to Fe(II) and determined quantitatively by photometric measurement of the absorbance of the colored complex formed between Fe(II) and FerroZine™ (or Ferene®) as chromogen. Thiourea is added to the chromogen reagent to complex copper [Cu(II)] which can also bind to FerroZine™ and yield falsely elevated iron values.

6.2 Materials**6.2.1 Instrumentation**

- Spectrophotometer, total bandwidth around 560 nm less than 8 nm; absorbance repeatability over the range $A = 0.000$ to 1.000 not to exceed 0.001 ; zero drift not to exceed 0.001 absorbance unit per hour
- Centrifuge
- Vortex mixer
- Cellulose acetate, or similar, membrane filter, $0.45 \mu\text{m}$ mean pore diameter

6.2.2 Chemicals

- Deionized water
- FerroZine™ iron reagent; 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine, monosodium, monohydrate ($\text{C}_{20}\text{H}_{14}\text{N}_4\text{O}_6\text{S}_2\text{Na}\cdot\text{H}_2\text{O}$). See Figure 1 for spectral characteristics. Alternatively, Ferene® may be used. See Section 6.8.
- Hydrochloric acid (HCl)
- Iron wire (Fe), 99.9% purity
- L-ascorbic acid ($\text{C}_6\text{H}_8\text{O}_6$)
- Sodium acetate, trihydrate ($\text{C}_2\text{H}_3\text{O}_2\text{Na}_3\cdot 3\text{H}_2\text{O}$)
- Thiourea ($\text{CH}_4\text{N}_2\text{S}$)
- Trichloroacetic acid (CCl_3COOH).

Related NCCLS Publications**

- C42-A Erythrocyte Protoporphyrin Testing; Approved Guideline (1996).** This document contains recommended guidelines for the measurement, reporting, and interpretation of erythrocyte protoporphyrin using hematofluorometric and extraction measurement methods.
- EP5-T2 Precision Performance of Clinical Chemistry Devices Second Edition; Tentative Guideline (1992).** Offers guidelines for designing an experiment to evaluate the precision performance of clinical chemistry devices; recommendations on comparing the resulting precision estimates with manufacturer's precision performance claims and determining when such comparisons are valid; and manufacturer's guidelines for establishing claims.
- EP9-A Method Comparison and Bias Estimation Using Patient Samples; Approved Guideline (1995).** This document addresses procedures for determining the bias between two clinical methods or devices and design of a method comparison experiment using split patient samples and data analysis.
- H3-A4 Procedures for the Collection of Diagnostic Blood Specimens by Venipuncture; Approved Standard Fourth Edition(1998).** This document provides procedures for the collection of blood specimens by venipuncture, including line draws, blood culture collection, and venipuncture in children. Includes recommendations on order of draw.
- H5-A3 Procedures for the Handling and Transport of Diagnostic Specimens and Etiologic Agents Third Edition; Approved Standard (1994).** American National Standard. Gives proper packaging, handling, and transport requirements for medical specimens and governing regulations.
- M29-A Protection of Laboratory Workers from Instrument Biohazards and Infectious Disease Transmitted by Blood, Body Fluids, and Tissue; Approved Guideline (1997).** Provides guidance on the risk of transmission of hepatitis viruses and human immunodeficiency viruses in the laboratory setting; specific precautions for preventing the laboratory transmission of bloodborne infection from laboratory instruments and materials; and recommendations for the management of bloodborne exposure.

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