METHODS IN HEMATOLOGY

IRON

Edited by

JAMES D. COOK

CHURCHILL LIVINGSTONE

Iron

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Iron Methodology: An Overview

James D. Cook

The generation of new knowledge in biomedical science is intimately linked to the development of new methods or the refinement of old ones. Iron metabolism is no exception. The advent of iron radioisotopes in the 1940's and their intensive application in the decade that followed accounts for much of our present knowledge of iron metabolism and red cell production. Similarly, the introduction during the past decade of radioimmunometric assays for measuring serum ferritin has produced a new wave of investigative activity that is producing important advances in our ability to detect and treat clinical disorders in iron balance.

In compiling this volume on iron methodology, I have made no attempt to catalog all the procedures that have been used to advance our knowledge. Methods such as x-ray crystallography for the study of ferritin molecular structure and Mössbauer spectroscopic analysis for the study of polynuclear iron complexes are too specialized to be included here. Other, more conventional procedures, such as gel chromatography or isoelectric focusing, are not unique to iron metabolism and are dealt with more appropriately in the biochemical literature. Noninvasive methods for assessing iron overload, such as nuclear resonance scanning or electron magnetic susceptibility measurements, are not sufficiently developed at the present time to warrant their inclusion in this volume.

The methods that I have chosen for detailed review encompass chemical, isotopic, histologic, and immunologic methodologies. Emphasis has been placed on clinical methods of iron status because these techniques have the widest application at the present time. Many of these, such as serum iron, iron binding capacity, free erythrocyte protoporphyrin (FEP), and serum ferritin, have now earned a permanent place in the hospital laboratory. The other methods covered here are tissue iron stores, food iron absorption, ferrokinetics, and electron microscopy, all of which are largely investigative in nature. Because each of these techniques occupies a unique position in the broad framework of iron metabolism, their current status will be reviewed separately.

About half of this volume is devoted to the specific details of each method, while the balance consists of a discussion of alternate techniques, interpretation of the results, and indications, either clinical or research, for per-

forming the measurement. The format for these chapters has not been rigidly standardized. Certain methods, such as serum ferritin, have been described in meticulous detail, largely because of the complexity of the method and the relatively brief descriptions in the published literature. With other methods, such as iron absorption, the protocol is much less detailed because the procedure has few, if any, clinical indications at the present time; its optimal application in a research setting requires a degree of methodologic flexibility in order to accommodate to the objective of a particular study. In all chapters, an effort has been made to provide a more detailed outline of the various procedures than is currently available in published accounts. There should be little need to seek other references in order to establish these methods in the laboratory.

SERUM IRON AND IRON BINDING CAPACITY

Although the measurement of circulating nonheme iron was first introduced more than half a century ago,[1] it remains an important measurement in the clinical and research laboratory. Plasma iron constitutes only a small fraction of total body iron but it is important clinically because plasma is the major pathway for internal iron transport. Measurement of transferrin, the specific iron transport protein in plasma, is equally important because levels of serum iron and transferrin are independently regulated and do not change in parallel in various disease states. As Dr. Fielding has emphasized in Chapter 2, maximal information is gained by performing the two measurements in tandem. The ratio of serum iron:iron binding capacity, i.e., transferrin saturation, is generally considered the most relevant index of iron supply to the erythroid marrow and the best measure of interchange between plasma iron and nonerythroid body tissues.[2]

There is a wide variety of techniques available for the determination of serum iron. Atomic absorption spectroscopy improves the sensitivity of these measurements, but colorimetric methods are still preferred for clinical purposes. The main differences between published methods relate to whether serum is initially deproteinized, what type of iron chromagen is used, and whether the method is manual or automated. Under optimal conditions (as with fresh normal sera), the choice of a particular method is not usually critical because systematic errors are small relative to the wide diurnal variation in serum iron that is seen in normal subjects.[3] Deproteinized methods are preferred in a clinical setting because of errors that can occur with measurements on hemolyzed or jaundiced sera. It is especially important to use a method that is little affected by hemolysis. Certain methods release significant amounts of hemoglobin iron which is then measured colorimetrically and gives a false elevation in serum iron. All automated methods should be validated by careful comparison with the International Committee for Standardization in Hematology (ICSH) technique described in Chapter 2.

An even greater spectrum of methods is available for determination of plasma transferrin. The traditional approach in the clinical laboratory is based on the high avidity of transferrin for inorganic iron. The most suitable

are the adsorbent techniques for colorimetric measurement of total iron binding capacity (TIBC) or radioactive measurement of unsaturated iron binding capacity (UIBC). With this approach, an excess of saturating iron is added to serum initially and the unbound excess of iron is subsequently removed by adding an insoluble iron adsorbent such as magnesium carbonate. In most hospital laboratories, the TIBC is employed in preference to radioactive UIBC because once the excess of saturating iron is removed, TIBC can be determined colorimetrically by the same method used for serum iron.

Highly satisfactory interlaboratory agreement has been achieved for serum iron using the ICSH method described in Chapter 2. However, extensive collaborative efforts by the ICSH Iron Panel over several years failed to identify an entirely satisfactory clinical method for assaying serum transferrin.[4] The magnesium carbonate method described in Chapter 2 is suitable with fresh or frozen sera but not with lyophilized specimens. This is not a serious drawback when standardizing laboratories are within the same country, but it imposes a major constraint in providing reference samples for international standardization programs. The difficulty in identifying a suitable reference method for TIBC may relate to the fact that most transferrin assays are based on protein function rather than chemical analysis. Lyophilization apparently alters the iron binding property of transferrin, or perhaps the serum matrix is altered in some way. In theory, the problem with lyophilized samples can be circumvented with a direct protein assay. Such immunologic measurements of transferrin have been described,[5] but they are not necessarily more accurate and often lack the precision and sensitivity of iron binding capacity methods.

Apart from these methodologic considerations, the most important question regarding serum iron and TIBC measurements is that of their future role in the clinical laboratory. The value of these traditional parameters of iron status has been questioned since the advent of more simplified methods such as FEP and more specific techniques such as serum ferritin. From a cost-effective standpoint, serum iron can be performed in clinical laboratories equipped with automated chemistry procedures at little or no additional cost. However, with the adsorbent methods for TIBC, manual processing of the sample is required prior to performing the colorimetric measurements of iron. As a result, TIBC or transferrin saturation measurements are still costly to perform. Nevertheless, it is doubtful that transferrin saturation will be eliminated from the hospital laboratory in the near future, mainly because it has played such an extensive role in the development of our knowledge of clinical disorders in iron and red cell metabolism. More experience with FEP and serum ferritin is required before serum iron and TIBC will be replaced.

FREE ERYTHROCYTE PROTOPORPHYRIN

FEP is an important new addition to the clinical laboratory for evaluating iron status. It is not actually a new measurement, but earlier assays were too cumbersome to be practical outside the research laboratory. Simplified pro-

cedures have been developed during the past decade that have made FEP measurements feasible and cost effective both for the hospital laboratory and for the prevalence studies of nutritional iron deficiency. A convenient and reliable method is described by Drs. Labbe and Finch in Chapter 3. As they point out, further simplification of the method can be achieved by using a hematofluorometer to measure fluorescence of zinc protoporphyrin on a single drop of blood.[6] This approach has enormous appeal for field studies of nutritional anemia because it requires no quantitative sample manipulation but only a source of electricity. Several instruments of this type are now available commercially, and their value for detecting lead poisoning is well established. However, there are few if any published studies indicating that sufficient accuracy can be achieved with this approach to measure the relatively modest elevations of FEP that accompany iron deficiency or iron deficient erythropoiesis.

For the detection of iron deficient erythropoiesis, FEP and transferrin saturation provide similar clinical information and have about the same degree of specificity. However, there are certain considerations that favor the use of FEP. Transferrin saturation is highly labile and can fall to abnormally low levels within a few hours of the onset of inflammation. Thus, a low value gives no indication of the duration of iron deficient erythropoiesis. On the other hand, FEP does not increase until iron deficient erythropoiesis has existed for several weeks and does not return to normal levels for many weeks following treatment of iron deficiency. This greater stability of FEP is an important advantage over transferrin saturation as a measure of iron status. However, FEP is affected by lead intoxication in the absence of iron deficiency anemia while transferrin saturation is not. Moreover, transferrin saturation can be used to screen for iron overload in population studies whereas FEP is not affected in iron overload states.

SERUM FERRITIN

The most important advance in iron methodology during the past decade is the measurement of serum ferritin. Except for presence of ferritin in the plasma of patients with hepatic malignancy,[7] it was long believed that ferritin is confined to intracellular compartments. The Cardiff laboratory was the first to report that using a sensitive radioimmunoassay, ferritin can be invariably detected in human serum at levels in normal subjects ranging between 10 and 300 ng/ml.[8] The acknowledged importance of serum ferritin as a measure of iron status is reflected by the huge volume of literature published since the original report, including several recent reviews on the subject.[9–12]

At first glance there would appear to be innumerable pitfalls in assaying serum ferritin by radioimmunoassay. One important variable is whether one uses an immunoradiometric assay (IRMA) with labeled antibody or a radioimmunoassay (RIA) with labeled antigen. Both procedures are described in detail in Chapter 4 by Dr. Worwood and some practical differences

between these two approaches are discussed. IRMA is inherently more sensitive than RIA, but the precision of the latter is adequate for clinical measurements. In our experience, iodination of antibody is more difficult than iodination of ferritin. However, IRMA is easier to perform in the laboratory because centrifugation is not required. Unfortunately, there are few if any studies in which an adequate side-by-side comparison of RIA and IRMA has been performed with respect to ease of performance, sensitivity, variability, and accuracy. Most iron laboratories are now using IRMA despite the fact that interlaboratory comparisons in both the United States and the United Kingdom have not identified major differences between the two procedures with measurements on normal sera.

There are two types of IRMA. In the original assay, described in 1972,[8] ferritin in the serum unknown is first reacted with an excess of purified ^{125}I antihuman ferritin; the excess labeled antibody is then removed by adding ferritin insolubilized on an immunoadsorbent. Radioactivity that remains in the supernatant after centrifugation is proportional to the concentration of ferritin in the original serum. The alternative method, described by Miles et al.,[13] is the 2-site IRMA in which serum ferritin is first reacted with unlabeled antibody bound to a solid phase, such as a polystyrene tube. The tube is rinsed to remove unreacted ferritin, and then radiolabeled antibody, which binds to the insoluble antigen, is added.

The radioactivity remaining after a final washing is proportional to the ferritin content of the original serum sample. A 2-site IRMA slightly modified from the original procedure is described in Chapter 4.

It now appears that ferritin actually consists of a spectrum of proteins or isoferritins that differ slightly with respect to molecular size, molecular charge, and immunologic reactivity. There are many aspects of RIA and IRMA measurement that could be affected by isoferritin composition and thereby lead to difficulties in standardizing measurements in different laboratories. For example, differences might occur depending on whether ferritin is isolated from liver or spleen and whether the protein is purified by heat and crystallization or by ultracentrifugation and gel filtration. Other important variables include the animal species used to prepare antisera, the method used to quantify ferritin protein, and the particular iodination technique. Nevertheless, when normal sera has been assayed in various laboratories with both RIA and IRMA, a surprisingly close agreement in serum ferritin values has been observed.

Two important problems relating to the 2-site IRMA, neither of which have been completely resolved, are outlined by Dr. Worwood. The first is the 'high-dose hook effect' in which serum containing very high concentrations of ferritin may give assay values within the normal range. Methods for minimizing the risk of this potential error are presented in Chapter 4. In our experience, we have never obtained a ferritin concentration below 200 ng/ml in sera shown by subsequent dilution to contain very high concentrations of ferritin. We therefore use this level as a cut-off point for repeating measurements at a greater dilution.

A more important problem with the 2-site IRMA is the fact that serum severely inhibits the assay. Thus, much higher ferritin values are obtained when purified ferritin is diluted with albumin rather than serum. This error is avoided to a large extent by maintaining a constant concentration of rabbit serum when diluting purified ferritin for the standard curve. However, this is still not completely satisfactory because the inhibitory effect of nonimmune rabbit serum is usually less than human serum, and even human serum may differ in its inhibiting effect. Further complexity arises because different batches of labeled antibody may differ in the extent to which they are inhibited by serum. We have performed various types of sample pretreatment, such as heating the sera, but have not been successful in eliminating this inhibitory effect.

The importance of serum ferritin in the detection and management of patients with iron deficiency or iron overload is well recognized. Because iron deficiency occurs more commonly than iron excess, the greatest demand is for evaluating anemic patients. Serum ferritin provides essentially the same information as bone marrow examination for stainable iron. For example, in 32 patients with iron deficiency anemia as proven by absence of stainable iron on marrow examination, only 2 had a serum ferritin above 12 ng/ml and none had a value greater than 14 ng/ml[14]; serum ferritin below 12 ng/ml is therefore diagnostic of iron deficiency. Serum ferritin measurements will be particularly useful for detecting iron deficiency in the physician's office and hospital out-patient clinic because if the value is low, the inconvenience, expense, and discomfort of a bone marrow examination can be avoided.

Serum ferritin is also useful for evaluating hospitalized patients with laboratory evidence of iron deficient erythropoiesis, i.e., low transferrin saturation, elevated FEP, or microcytic hypochromic anemia. Iron deficient erythropoiesis occurs not only in true iron deficiency but also in the anemia of chronic infection or malignancy where there is a block in iron release from the reticuloendothelial cell. In contrast with true iron deficiency anemia, anemia of chronic disease is characterized by an increase in marrow iron stores and a parallel increase in serum ferritin. However, there is a problem in using the serum ferritin to distinguish between the anemia of infection and true iron deficiency: the serum ferritin is disproportionately elevated by infection at any given level of marrow iron stores and this holds true even in patients with absent iron stores. In a patient with laboratory evidence of iron deficient erythropoiesis, a normal serum ferritin does not exclude iron deficiency. Thus, in hospitalized patients it is often more economical and more practical to perform bone marrow examinations at the outset.

One of the most important applications of serum ferritin measurements is the assessment of iron status in population studies.[15] Iron deficiency is commonly identified in nutritional surveys by the anemia that accompanies more severe deficiency. However, hemoglobin is relatively insensitive because of the wide range of values in normal individuals and because it lacks specificity. Transferrin saturation and FEP are more sensitive parameters of iron deficiency but, as noted previously, they do not distinguish between true iron

deficiency and the anemia of chronic infection. Serum ferritin is not only the most sensitive parameter of iron deficiency but it also provides quantitative information about iron status in the nonanemic segment of a population. In a recent study, based on measurements of hemoglobin, transferrin saturation, FEP, and serum ferritin, it was possible to obtain a frequency distribution of iron stores in a population of adult women.[16] If these results are confirmed in other laboratories, this battery of iron parameters will be valuable for determining baseline iron status in a population, for assessing efficacy of iron intervention strategies designed to combat iron deficiency, and for the long-term monitoring of iron balance. If resources do not allow all three measurements to be performed, serum ferritin is more useful than either transferrin saturation or FEP.

Iron overload is less common than iron deficiency but its clinical recognition is more imperative because it may be possible to arrest or reverse tissue damage with chelation therapy or therapeutic phlebotomy. Most methods for quantitating body iron stores, including desferrioxamine-induced iron excretion and histological examination of bone or liver biopsy, are not optimal screening methods. Serum ferritin, on the other hand, is a simple and convenient screening method for iron overload and for selecting patients for more detailed clinical measurements of iron status. Similarly, serum ferritin is valuable in patients on chronic renal dialysis who are given regular injections of parenteral iron to replace iron lost to the dialyzer or to the laboratory.[17, 18] Hemosiderosis due to excess parenteral iron has recently been reported in such patients[19]; regular monitoring of iron status with the serum ferritin reduces the risk of this iatrogenic iron overload.

Idiopathic hemochromatosis is a potentially fatal disease that can be effectively treated if recognized early. It is critically important to screen family members of patients with proven disease. It is also desirable to screen patients with clinical abnormalities that could be due to iron overload such as cardiomyopathy, cardiac arrhythmias, diabetes mellitus, cirrhosis, or poorly characterized arthropathy. Transferrin saturation has been used for screening in the past but it is a crude measure of iron status at best and it fails to distinguish between patients with minimal and far-advanced iron overload. Serum ferritin is more reliable because in patients with established idiopathic hemochromatosis, the level is invariably greater than 1,000 ng/ml. However, the ability to detect preclinical iron overload with serum ferritin measurements has been debated. Wands et al.[20] reported that the serum ferritin level may be normal despite significant iron-loading of the hepatocyte. This was not confirmed in a more recent study of 41 patients with idiopathic hemochromatosis and 199 of the first or second degree relatives. Of the 32 relatives (16 per cent) who had increased iron stores determined by desferrioxamine iron excretion or nonheme liver concentration on liver biopsy, serum ferritin was increased in all but one. False-positive results with serum ferritin occurred in less than 2 per cent of normal relatives as compared with 33 per cent with transferrin saturation. Some of these conflicting reports of family studies in idiopathic hemochromatosis might be due to methodologic differ-

ences in that some assays were not detecting all of the isoferritins circulating in patients with preclinical disease. Nevertheless, although the value of serum ferritin in detecting overload is not fully established at present, the weight of published evidence indicates that it is adequate for this purpose.

TISSUE IRON STORES

The iron measurements discussed in Chapters 2 to 4 are performed on plasma or whole blood and therefore provide only an indirect measure of body iron reserves. Since the adverse effects of iron deficiency or iron overload are expressed at the tissue level, it is often desirable to have a direct measurement of tissue iron or body iron stores. These tissue iron methods have been reviewed by Drs. Torrance and Bothwell in Chapter 5.

Quantitative phlebotomy is commonly regarded as the most accurate method for measuring body iron stores in normal and nonanemic patients. It is a highly specialized and time-consuming procedure which is applicable only in carefully selected situations as in the validation and calibration of serum ferritin as a measure of body iron stores. The accuracy of quantitative phlebotomy is probably less than is generally assumed because of errors in estimating the contribution of absorbed dietary iron during the phlebotomy period. The accuracy of the method can be improved by using ^{51}Cr-tagged red cells to measure the exact difference in circulating hemoglobin at the start and conclusion of the phlebotomy program, but the small gain in precision seldom justifies the additional effort. Body iron stores can also be measured by isotopic dilution as discussed in Chapter 5. However, this is an equally complicated procedure and has the added disadvantage of measuring only miscible rather than total storage iron. The phlebotomy and isotopic methods are suitable for use only in the research laboratory.

Available chemical methods for measuring tissue iron are reviewed in detail in Chapter 5. The importance of excluding heme iron in these assays is stressed, especially when performing measurements on tissue with normal or reduced levels of storage iron. The nonheme iron method selected by Drs. Torrance and Bothwell has the advantages that it can be scaled down for small samples (e.g., liver biopsy tissue), is much simpler to perform than most of the available techniques, and is satisfactory for measurements on either fixed or frozen tissue.

The choice between histologic evaluation of iron stores and the more quantitative chemical measurement of nonheme iron depends on the purpose of the study. In highly specialized laboratories, good agreement has been observed between histologic and chemical methods using either liver or bone marrow, although chemical measurements are more desirable for research purposes. Neither approach completely eliminates sampling errors that can occur, especially with percutaneous biopsies of cirrhotic liver tissue. With bone marrow, sampling errors can be minimized by using a biopsy specimen

rather than aspirated marrow, although the greater precision afforded by bone biopsy is not usually required for clinical purposes. It is important to remember that a false-negative result can occur in estimating marrow iron if the aspirated sample does not contain sufficient marrow stroma. This can be avoided by examining both a stained and unstained marrow slide, and by grading iron stores only when ample stroma has been obtained.

The choice between liver and bone marrow for estimating tissue iron is more important than the choice between histologic and chemical measurements because liver and spleen are functionally different iron storage compartments. The distinction is fully discussed by Drs. Torrance and Bothwell. Bone marrow contains reticuloendothelial iron stores and is therefore useful in diagnosing iron deficiency and studying various causes of anemia. A marked increase in reticuloendothelial iron stores can occur with little or no change in total body iron. However, bone marrow is of little value in detecting idiopathic hemochromatosis where there is an increase in liver parenchymal iron rather than reticuloendothelial iron. When a screening measurement such as transferrin saturation or serum ferritin suggests iron overload, liver biopsy should be used in tandem with desferrioxamine iron excretion to obtain the best baseline estimate of parenchymal iron excess before undertaking a program of therapeutic phlebotomy.

IRON ABSORPTION

Clinical disorders in iron metabolism occur when there is a significant increase or decrease in body content of the metal. It is an accepted tenet of iron metabolism that body iron stores are regulated almost entirely by gastrointestinal absorption and that there is no efficient mechanism for excreting an excess of the metal. Appropriately, iron absorption in man has been studied extensively over the past four decades in an attempt to identify primary disturbances in iron assimilation which may lead to a significant deficiency or excess of body iron. Although some useful information was obtained by chemical iron balance and by measuring serum iron following an oral dose of therapeutic iron, most of our present knowledge has been generated with radioisotopic methods.

There are several ways in which retention of orally administered radioiron can be determined.[12] Fecal radioiron balance was used in earlier studies and although reliable data can be obtained with this approach, it is time-consuming for the patient, unpleasant for the technologist, and suffers from errors due to unsuspected sample loss. The double isotope method in which one form of radioiron is given intravenously to measure red cell incorporation and a second form is given orally to measure absorption is also a valid approach, but it does not permit multiple absorption measurements in the same individual. As indicated by Dr. Hallberg in Chapter 6, the preferred method of measuring radioiron absorption depends on the objective of the study. When the form of administered iron is being examined, as in studies of

food iron assimilation, the best approach is to use incorporated red cell activity because the use of dual radioisotopes makes it possible to perform as many as four separate absorption measurements in the same individual. In patients with possible abnormalities in red cell iron incorporation, iron absorption studies should be performed with ^{59}Fe and whole-body counting. The whole-body counter must be adequately sensitive and the counting efficiency should be little affected by the size of the patient or the body location of absorbed radioiron.

Much of the published information on iron absorption in man, particularly clinical studies, was obtained by administering small doses of inorganic radioiron. As discussed by Dr. Hallberg in relation to reference doses, inorganic iron absorption is a reliable measure of the mucosal 'setting' for iron absorption but usually has little clinical relevance because the luminal phase of iron absorption is not tested. For example, defects in iron absorption occur in patients with achlorhydria or partial gastrectomy but these defects are usually detected only with measurements of dietary iron absorption. Moreover, absorption of inorganic iron cannot be extrapolated to food iron absorption, and because of this, it is not possible to accurately predict the long-term effects on body iron stores of disturbances in iron assimilation.

The method of dual isotope tagging for heme and nonheme iron absorption outlined in Chapter 6 has been validated in several laboratories. The method can be used to assess the contribution of the absorptive process to the development of iron deficiency or iron overload. In this method, small quantities of inorganic ^{59}Fe- and ^{55}Fe-labeled hemoglobin are thoroughly mixed with one or more standardized meals believed to be typical for a given population. By performing chemical measurements of heme and nonheme iron on this meal and measuring absorption of heme and nonheme iron with ^{55}Fe and ^{59}Fe, it is possible to calculate absolute absorption of dietary iron. Application of this method in a clinical setting will define more clearly the role of the absorptive process in pathogenesis of clinical disorders in iron balance.

In nutritional studies, it is usually unnecessary to measure the absorption of heme iron because it constitutes only a small portion of dietary iron in underdeveloped areas and because it is little affected by the composition of the diet; nonheme iron absorption measurements are therefore usually sufficient. The effect of subject-to-subject differences in iron status can be eliminated by performing multiple tests in the same individual. Studies of this type will be important in defining biochemical factors affecting iron absorption, in determining the importance of diet per se in the pathogenesis of iron deficiency anemia, in selecting intervention strategies for combating iron deficiency, and in measuring potential benefits and/or risks in food iron fortification programs.[22]

FERROKINETIC MEASUREMENTS

The majority of methods reviewed in this volume will have an expanding role in a clinical setting in the years to come. One exception may be ferro-