Enzyme-Labeled Immunoosorbent Assay for Serum Ferritin: Method Evaluation and Comparison With Two Radioassays

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We report our evaluation of a commercially available procedure and reagents for determination of ferritin in serum by enzyme-labeled immunoosorbent assay (ELISA). Results by our procedure and the "Fer iron" (Ramco Labs) procedure show a degree of association (r) of 0.95 and a regression equation of y = 1.03x – 0.3. Similarly, our procedure compared to the "Gamma Dab" (Clinical Assays) ferritin procedure shows a degree of association of 0.98 and a regression equation of y = 0.93x – 11. Between-day standard deviations were 6 and 22 µg/L (n = 24 and 20) for ferritin concentrations of 20 and 300 µg/L, respectively. Ferritin values showed no correlation with total iron concentration, but show a broad inverse relationship with iron-binding capacities. The favorable correlation with existing procedures and the speed of the analysis commend the use of ELISA for measurement of ferritin in serum.

Additional Keyphrases: "kit" methods · total iron in serum · total iron-binding capacity

Until recently, body iron stores were estimated by examination of bone-marrow aspirates or assessment of gastrointestinal iron absorption (1). These procedures are lengthy, traumatic for the patient, and at best only semiquantitative. Subsequent development of sensitive and specific in vitro radioassay techniques has led to investigations of ferritin concentrations in the serum of normal individuals and of patients with various hematologic disorders. Most available evidence now supports the conclusion that ferritin concentrations increase and decrease proportionately with iron stores (2–4).

Hitherto, serum ferritin has been determined primarily by laboratories with equipment and license for radioassays; however, the recent introduction (5, 6) of enzyme-linked immunoabsorbent assay (ELISA) has brought the analysis within reach of any laboratory that can make spectrophotometric measurements. We present the results of an evaluation of a commercially available ELISA procedure and reagents for determining ferritin in serum.

Materials and Methods

Materials

We used a Statas III spectrophotometer (Gilford Instruments, Inc., Oberlin, OH 44074), a clinical laboratory rotator (Scientific Products Co., Bedford, MA 01730), Oxford samplers and tips (Fisher Scientific Co., Medford, MA 02155), IRMA procedure and reagents by "Fer-Iron" (Ramco Laboratories, Houston, TX 77098), radioimmunoassay procedure and reagents by "Gamma Dab" Ferritin (Clinical Assays, Cambridge, MA 02139), and ELISA procedure and reagents by "ELA-01-Ferritin" (New England Immunology Associates Inc., Cambridge, MA 02139).

Reagent Preparation

Buffer: The buffer used in the ELISA is phosphate-buffered isotonic saline containing, per liter, 30 g of bovine serum albumin (phosphate, 10 mmol/L, in NaCl, 0.1 mol/L) and 0.5 g of NaNO₂ as preservative, adjusted to pH 7.0 with a 1.0 mol/L solution of H₃PO₄.

Calibrators: A stock calibrator containing 50 µg of purified human liver ferritin per liter, furnished by New England Immunology Associates, is diluted in buffer to prepare a set of calibrators.

Other reagents: All other reagents are either used as received from the supplier or prepared as directed in the procedure. These reagents include antibody-coated polystyrene tubes; alkaline phosphatase (EC 3.1.3.1)-conjugated antibody; and 1.2 g of p-nitrophenyl phosphate per liter, buffered to pH 10.0 in 1.0 mol/L diethanolamine containing, per liter, 0.5 mmol of MgCl₂; and a 0.5 mol/L solution of NaOH.

Procedure

The ELISA is performed as follows: Add 0.3 mL of buffer and 0.1 mL of each calibrator to duplicate antibody-coated tubes. Add 0.3 mL of buffer and 20 µL of serum or control, undiluted and diluted 11-fold, to duplicate antibody-coated tubes. (The second of each of the duplicate serum tubes receives the prediluted serum.) Rotate all antibody-coated tubes for 2 h at ambient temperature on a clinical rotator with the head inclined at a 45° angle. Decant and drain the antibody-coated

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4 Incubating 5 ng of ferritin in a volume of 0.3 mL produces the same response as incubating 5 ng in volumes up to 0.6 mL. This implies that, within these limits, the immobilized antibodies on the tube surface respond to mass rather than to concentration; consequently, the 0.08-mL difference between calibrators and samples has no effect on the assay.

5 Assay of 20 µL of undiluted serum will accommodate ferritin concentrations ranging from 0 to 250 µg/L; inclusion of 11-fold pre-diluted serum extends the concentration range to 2750 µg/L.
tubes (it is unnecessary to wash the tubes at this point). Add 0.5 mL of alkaline phosphatase-conjugated antibody to each tube and rotate again for 2 h. Decant the supernate from all tubes and wash their interiors three times with distilled water. Add 1.0 mL of p-nitrophenyl phosphate and again rotate at ambient temperature for 30 min. Terminate the enzymatic hydrolysis of the colorless substrate to yellow p-nitrophenol by adding 0.3 mL of 0.5 mol/L NaOH to each tube and mixing thoroughly. Measure the absorbance of each tube at 405 nm and plot the absorbance of calibrators vs. the mass of ferritin. The relationship is linear. From it, the ferritin (in nanograms per tube) in the serum samples can be directly determined from their absorbance. Multiply these values by 50 (mass to concentration conversion) to obtain the result in micrograms per liter.

Results

Specificity: The antigenic ferritin extracts from human liver tissue and the antisera from rabbits were produced and characterized previously (5). Their homogeneity and specificity are reflected in the parallelism and comparison studies presented here.

Kinetics: The rate of ferritin binding to the antibody-coated tube is shown in Figure 1. Essentially all ferritin present (2.5 ng/tube) is bound within 2 h. Other ferritin doses ranging from 0.3 to 5.0 ng/tube were tested and found to approach equilibrium within 2 h. Similar investigations of the enzyme-conjugated antibody and the substrate hydrolysis reactions revealed that these reactions exhibit linear kinetics for 25 and 2 h, respectively. In practice, however, the initial reaction is allowed to reach equilibrium but the conjugated antibody and enzymatic hydrolysis reactions are terminated at 2 h and 30 min, respectively.

Calibration and parallelism: Calibration of the ELISA is shown in Figure 2. The mass of ferritin per tube in the original procedure as supplied by New England Immunology Associates is fivefold what we recommend. We find this unacceptable because the calibration plot curves to become nearly parallel to the mass axis at higher concentrations, thereby making almost indistinguishable the absorbances of samples containing more than 250 µg/L of ferritin. This nonlinearity

is attributed to either a high-dose "hook" effect (7) or a negative deviation from Beer's law (8). We did not experiment to determine whether one or both of these effects are responsible for the problem, because the response can be made linear by simply decreasing the mass of ferritin per tube.

Figure 2 also shows the parallelism observed when a serum sample with a high ferritin concentration is serially diluted. The parallel response reflects both the specificity of the antibody for ferritin and the corresponding lack of matrix effects.

Precision: Within-day standard deviations of the method

![Fig. 1. Binding of ferritin to antibody-coated tubes](image1)

![Fig. 2. Ferritin calibration (---) and parallelism (---)](image2)

![Fig. 3. Method comparison: (top) log ELISA values vs. log IRMA values; (bottom) log ELISA values vs. log RIA values](image3)
were 4 and 6 \( \mu g/L \) (n = 24 and 20) at ferritin concentrations of 20 and 300 \( \mu g/L \), respectively. Similarly, the between-day standard deviations are 6 and 22 \( \mu g/L \) for the same concentrations and number of samples.

**Method comparison:** ELISA results are compared with those obtained by the IRMA (9) procedure (Fer-Iron) performed at Bio-Science Laboratories on 43 casually selected serum samples in Figure 3 (top). Values range from 2 to 2300 \( \mu g/L \) with a degree of association (r) of 0.95 and a regression equation (10) of \( y = 1.03x - 33 \). Because the standard error of the intercept is 35, the apparent bias is not considered significant (11).

ELISA results are similarly compared with those obtained by the RIA procedure (Gamma Dab) performed at Clinical Assays on 34 samples (Figure 3, bottom). Values range from 8 to 1600 \( \mu g/L \) with a degree of association (r) of 0.98 and a regression equation of \( y = 0.93x - 11 \).

**Correlation with iron and iron-binding capacities:** The data presented in Figure 4 show the lack of relation between serum ferritin as determined by ELISA and iron concentrations and the broad inverse relation between ferritin and iron-binding capacity. This latter pattern appears to nearly duplicate the one previously reported when ferritin was determined by an IRMA procedure (3).

**Discussion**

Our data demonstrate that ferritin values as measured by ELISA are equivalent to those obtained by IRMA and RIA. Our modified procedure has a span of 0 to 2750 \( \mu g/L \) and shows no "hook" effect (7) within this range. Because specimens are routinely determined undiluted and diluted 11-fold, we also monitor parallel responsiveness in each individual sample. Furthermore, most samples fall within the calibration range, obviating repeat determinations at different dilutions.

ELISA technology also appears to have practical features that are worthy of mention. (a) The entire assay is done in a single tube without the need for time-consuming transfer or separation steps and the potential losses that may result. (b) Enzyme-labeled antibodies tend to have a longer shelf life than do their radioactive counterparts. (c) Enzyme wastes are readily disposed of, thereby eliminating the need for licensure and the precautions necessary in handling radioactive materials. (d) Any device that will measure absorbance at 405 nm replaces expensive radioactivity-counting equipment (12).

Because the three procedures considered herein compare well, it can be inferred that ferritin-antigen preparations from two different organs do also. Spleen is the organ source for the ferritin used in the IRMA, liver for that used in the ELISA and the RIA. Ferritin is a large, complex molecule (24 subunits, \( M_r \) about 450 000) with the potential to elicit the production of a heterogenous population of antibodies. In practice, however, extracts from selected tissues have produced relatively pure isoferritin fractions characteristic of the tissue involved (13). Production of antisera from these relatively pure extracts yields antibody populations that cross react to widely varying degrees with ferritin extracts from other tissue sources (14). Consequently, immunological reagents used in a sensitive immunoassay for serum ferritin require careful scrutiny.

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**References**

Evaluation of Homogeneous Enzyme Immunoassay of Serum Thyroxine with the Gilford 3500 Analyzer

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I evaluated homogeneous enzyme immunoassay (EMIT®) of serum thyroxine with the Gilford 3500 analyzer. The samples are pretreated before quantitation on the Gilford 3500; the procedure thereafter is entirely mechanized. Forty-four samples may be analyzed per run. Precision and accuracy are good, and results correlate well with those by radioimmunoassay.

Homogeneous enzyme immunoassay, first introduced by Rubenstein et al. (1), has become a very important analytical tool in the clinical laboratory. Recently, Ullman et al. (2) describe a method for so measuring serum thyroxine. Conjugation of a thyroxine derivative to pig-heart mitochondrial malate dehydrogenase (EC 1.1.1.37) results in powerful inhibition of enzyme activity, with an increase in "apparent" $K_m$ of the substrate. Addition of anti-thyroxine antibodies reactivates the enzyme. Thyroxine in a patient's sample competes with enzyme-labeled thyroxine for antibody binding sites and thereby decreases antibody-induced activation of the enzyme. NAD$^+$ and malate are used in the system; the reaction is monitored by observing the absorbance change at 340 nm (3).

An evaluation of the thyroxine enzyme immunoassay (EMIT, a registered trademark of the Syva Co., Palo Alto, CA) is presented with use of the Gilford 3500. After pretreatment of the samples, the procedure is completely mechanized.

Materials and Methods

Apparatus

Gilford 3500 (Gilford Instrument Laboratories, Oberlin, OH 44074).
Auxiliary Pipetter/Diluter (Gilford).

Reagents

All reagents were obtained from the Syva Co. The manual thyroxine configuration was used for this study.

Antibody reagent A. Reagent A contains antibodies to a thyroxine derivative. When reconstituted, the reagent contains a standardized preparation of $\gamma$-globulin from immunized sheep, NAD$^+$, a stabilizer, and a preservative (pH 5.2). The reagent is stable for 16 weeks when stored at 2-8 °C, after initial room temperature equilibration.

Reagent A diluent. The diluent contains 0.15 mol of glycine per liter, with stabilizer and preservative (pH 5.0). Reagent A diluent must be used cold and is stable for 16 weeks when stored at 2-8 °C.

Working reagent A. Prepare by combining one volume of antibody reagent A with nine volumes of cold reagent A diluent. Allow working reagent A to equilibrate at room temperature for 8 h (or overnight) before use. Working reagent A can be used for one week when stored at 25 °C. Each assay requires 0.5 mL of working reagent A.

Enzyme reagent B. Reagent B contains thyroxine chemically coupled to malate dehydrogenase in a 400/600 (by vol) mixture of glycerol and phosphate buffer (40 mmol/L, pH 9.2), with stabilizers and preservatives. Enzyme reagent B is stable for 16 weeks at 2-8 °C.

Enzyme working reagent B. To ensure stability, enzyme working reagent B is prepared during the sample-treatment period of the assay. Prepare it by combining one volume of enzyme reagent B with one volume of de-ionized water. Each assay requires 110 $\mu$L of enzyme working reagent B. At the end of each run, any excess must be discarded.

Serum pretreatment reagent. A solution containing NaOH (0.5 mol/L) with "Liplex" (a proprietary sequestering agent for free fatty acids) is provided.

Pretreatment working reagent. Prepare by combining one part serum pretreatment reagent with four parts of de-ionized water. The solution is stable for one week at 25 °C. Each assay requires 250 $\mu$L of pretreatment working reagent.

Thyroxine buffer. The buffer is a 0.3 mol/L solution of

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