Determination of Serum Ferritin by a One-Step Immunoenzymoassay, and Comparison of Four Liver-Ferritin Standards

M. C. Revenant, J. Goudable, A. Beaudonnet, A. Maillavin, J. Pichot, and L. Monnet

We describe a one-step "sandwich"-type immunoenzymoassay for ferritin in human serum. The solid-phase consists of glutaraldehyde-treated polypropylene tubes coated with rabbit antibody to human ferritin. Liver ferritin is the standard. Peroxidase-conjugated antiseraum to ferritin and a sensitive chromogen, o-phenylenediamine, are used. The assay requires 90 min. The standard curve is linear up to 400 μg of ferritin per liter of serum. Within- and between-run CVs are <6% for low, high, and medium concentrations and are about 13.0% at the decision level for iron deficiency. Results by a two-step "sandwich" procedure (New England Immunology Associates kit) correlated well, r = 0.98. We assessed four liver ferritin standards from different manufacturers with the described method. The mean absorbance for the 40 μg/L ferritin standard was 1.5 for that from Diagnostics Biochem and National Institute for Biological Standards and Controls, 1.0 for that from Dako, and 0.4 for that from Sigma. Consequently, to standardize results, all liver ferritin standards should be calibrated vs. the National Institute for Biological Standards and Controls reference standard.

The concentration of ferritin in serum reflects mobilizable iron stores, and its determination requires radioimmunological or enzymoimmunoassays. The method we describe here is a one step "sandwich"-type immunoenzymoassay in which polypropylene test tubes are used as the solid phase, peroxidase as the label, and o-phenylenediamine (OPD) as the chromogen with H₂O₂. It is a modification of a previous method (1) in which immunological reactions are assayed successively. The standard is liver ferritin, and we report differences obtained with standards from four manufacturers, tested against an international reference ferritin.

Materials and Methods

Materials

Reaction tubes. Polypropylene test tubes (Sarstedt France, BP178 Strasbourg, France).

Rotator. All tubes, set at an angle of 45°, are rotated at 30 rpm.

Carbonate/HCl buffer, 0.1 mol/L, pH 9.0. Dissolve 10.58 g of sodium carbonate in 900 mL of distilled water, adjust the pH to 9.0 with hydrochloric acid, and dilute to 1 L with distilled water.

Glutaraldehyde (Baker Chemicals N.V., Deventer, Holland), 1 g per liter of carbonate/HCl buffer.

Carbonate (15 mmol/L)/bicarbonate (35 mmol/L) buffer, pH 9.6. Dissolve 1.59 g of sodium carbonate and 2.94 g of sodium bicarbonate in 900 mL of distilled water, adjust the pH to 9.6, and dilute to 1 L with distilled water.

Antiferritin rabbit antibodies (Dako, Copenhagen, Denmark). These are antibodies against human liver and spleen ferritin, and the working solution contains 10 mg of antibodies per liter, in carbonate/bicarbonate buffer.

Bovine serum albumin (BSA) buffer. Dissolve 2 g of bovine serum albumin in 100 mL of phosphate (0.01 mol/L)/NaCl (0.15 mol/L) buffer, pH 7.4.

Ferritin standards. We evaluated four different human liver ferritin standards:

Stock ferritin standard, 3.2 mg/L (Diagnostics Biochem Canada, Inc., London, Ontario).

Ferritin from human liver, type IV, 10 mg/L (Sigma Chemical Co., St. Louis, Mo 63178; cat. no. F 6754).

Ferritin from human liver, 10 g/L (Dako, Copenhagen, Denmark).

Ferritin from human liver, type IV, 10 mg/L (Sigma Chemical Co., St. Louis, Mo 63178; cat. no. F 6754).

Ferritin from human liver, 10 g/L (Dako).

Ferritin from human liver, 9.1 μg per bottle, i.e., 9.1 mg/L (μg/L) from the four stock ferritin standards, in BSA buffer.

Peroxidase-conjugated antiseraum to ferritin (Dako). The working solution of conjugate was obtained by diluting with BSA buffer, the dilution differing according to the batch (from 300-fold to 1500-fold).

Washing solution: sodium chloride, 0.15 mol/L, containing 0.5 mL of Tween 20 surfactant (ICI Americas, Inc.) per liter.

Acetocetate buffer, 0.1 mol/L, pH 5.0/hydrogen peroxide, 10 mmol/L. Dilute 1.7 mL of acetic acid and 9.57 g of sodium acetate 3H₂O to 1 L with distilled water, verify the pH, and add 12 mL of an 0.88 mol/L solution of hydrogen peroxide.

Chromogen: o-phenylenediamine (OPD), Merck. The working substrate solution contains OPD, 20 mmol/L. Dissolve 54 mg of OPD in 25 mL of acetocetate buffer.

Hydrochloric acid, 0.1 mol/L.

Citric acid, 0.4 mol/L.

Methods

Preparation of antibody-coated reaction tubes. Incubate 1 mL of a 1 g/L solution of glutaraldehyde in each tube for 3 h at 56 °C. Decant, and rinse the inside of the tubes three times with distilled water. Place 1 mL of 10 mg/L antiferritin antibody solution in each tube and incubate at 4 °C for at least three days. The tubes may be used for 10 weeks, but before use rinse them three times with washing solution.

Assay procedure. Stock ferritin from Diagnostics Biochem is used as standard. Each determination must be assayed in duplicate, including controls and standards. Dilute sera and controls 10-fold with BSA buffer. Add 0.1 mL of each sample (standards, sera, controls) and 0.25 mL of working conjugate solution to antibody-coated reaction tubes and incubate them on the rotator for 1 h. Discard the incubation mixture and rinse the interior of the tubes four times with distilled water. Place 1 mL of working substrate solution in each tube and incubate all tubes at room temperature, in darkness, for 30 min. Stop the color reaction by adding 1 mL of 0.1 mol/L hydrochloric acid or 1 mL of 0.4 mol/L citric acid and shake well. Measure the absorbance at 492 nm if the color reaction is stopped with hydrochloric acid, at 445 nm if the color reaction is stopped with citric acid.
Results

Analytical Evaluation of the Method

The influence of glutaraldehyde on coated tubes and the effect of rotation during immunological reactions are reported elsewhere. These two conditions as described give the best dose–response curve. The absorbance is increased about 2.5 times by use of glutaraldehyde and about fourfold as a result of the rotation. Absorbance values are increased threefold by acidification with hydrochloric acid, 1.5-fold if citric acid is used.

The standard curve is linear between 0 and 4 ng per tube, i.e., 0 and 400 µg/L in the serum. Figure 1 shows a typical standard curve. The mean absorbance for 4 ng of ferritin per tube is about 1.5 A.

Within-run precision was evaluated by assaying four different concentrations of serum ferritin 30 times each, in a single run. Between-run precision was evaluated by assaying four different serum ferritin concentrations in 15 consecutive assays. The results are shown in Table 1.

The "hook effect" is a paradoxical flattening of the response at high ferritin concentrations, resulting in a parabolic standard curve. We saw no hook effect, up to 330 ng of ferritin per tube, i.e., 33 mg/L in the serum.

We compared results with this procedure (y) with those obtained with a ferritin kit from New England Immunology Associates, Inc., Cambridge, MA 02138. This is a "sandwich" method, with alkaline phosphatase (a). For 73 serum samples covering the range 1 to 3510 µg/L, the averages were 3988 µg/L for x and 4140 µg/L for y, the coefficient of correlation (r) was 0.989, and the regression equation was y = 1.00x + 13.3.

![Figure 1. Example of linear calibration curve obtained with Diagnostics Biochem standard](image)

**Table 1. Precision of the Present Assay**

<table>
<thead>
<tr>
<th>Sample concn</th>
<th>Ferritin concn, µg/L</th>
<th>Mean</th>
<th>SD</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Within run (n = 30)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Very low</td>
<td>9.9</td>
<td>1.3</td>
<td>13.1</td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>30.9</td>
<td>1.4</td>
<td>4.7</td>
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<tr>
<td>Medium</td>
<td>150.2</td>
<td>6.2</td>
<td>4.1</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>280.2</td>
<td>10.1</td>
<td>3.6</td>
<td></td>
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<tr>
<td><strong>Run to run (n = 15)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Very low</td>
<td>12.8</td>
<td>1.7</td>
<td>13.7</td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>34.3</td>
<td>1.9</td>
<td>5.5</td>
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<tr>
<td>Medium</td>
<td>157.0</td>
<td>7.9</td>
<td>5.0</td>
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</tr>
<tr>
<td>High</td>
<td>288.5</td>
<td>9.4</td>
<td>3.2</td>
<td></td>
</tr>
</tbody>
</table>

Evaluation of Four Liver Ferritin Standards

The four liver ferritin standards (Diagnostics Biochem, Sigma, Dako, National Institute for Biological Standards and Controls) were tested with the present method. Figure 2 shows standard curves obtained. The standards from National Institute for Biological Standards and Controls and from Diagnostics Biochem showed the same reactivity, but those from Dako and Sigma showed a lower reactivity. The mean absorbance values obtained for the 40 µg/L ferritin standard (i.e., 400 µg/L in the serum before the 10-fold dilution) were 1.5 A with Diagnostics Biochem and National Institute for Biological Standards and Controls, 1.0 A with Dako, and 0.4 A with Sigma.

Discussion

Addition of 0.1 mol/L hydrochloric acid or 0.4 mol/L citric acid stabilizes the color for 60 min, but use of hydrochloric acid leads to spectral modification (maximum absorbance at 492 nm instead of 445 nm) and increases the color intensity by threefold. Citric acid increases the color intensity by 1.5 times, with no spectral modification (2). Thus either hydrochloric or citric acid is used, according to the sensitivity of the batch of conjugate.

The dose–response curve is linear up to 40 µg of ferritin per liter, i.e., 400 µg/L in the serum. Linear dose–response curves were established by Lee and Burgett (3) up to 250 µg/L or by Linpisarn et al. (4) up to 500 µg/L. On the other hand, many authors obtained no linear dose–response curves (5–7). Theriault and Page (8) realized a standard curve up to 1000 µg/L. The hook effect has been described by Miles et al. (9) in a two-step sandwich method. We found this phenomenon with the New England Immunology Associates ferritin kit for high ferritin concentrations in two sera, 12 690 and 8825 µg/L, where the results appear to be within the normal range if the sera are tested undiluted (10). Ng et al. (11) indicated that the hook effect can be eliminated if serum samples are tested at two different dilutions. We saw no hook effect up to 33 mg/L in the serum.

Within-run and between-run precisions are adequate and similar to those of other published enzyme immunoassays for ferritin (1, 3, 4, 12).

Ferritin is present in various organs, especially liver, spleen, heart, and bone marrow. The different ferritin-containing tissues studied also contained isoferritins. These isoferritins explain the different calibration curves obtained when the standards are derived from various tissues such as...
liver or spleen (13). In our study, we established four calibration curves with four ferritin standards from liver;
the reactivity of the standards is not the same, giving very different results for patients' serum samples. Consequently, it is necessary to establish the normal range for each method. Generally, there is a good correlation among the different methods ($r > 0.98$), but absolute values can vary greatly (14). To standardize the results, all liver ferritin standards should be calibrated vs the National Institute for Biological Standards and Controls reference standard (15).

In conclusion: the method described here is simple, fast, and presents the same precision and accuracy as the preceding (1) two-step method. The non-significant batch to batch variation (due to coating of tubes) does not affect the long-term reproducibility of the assay. We recommend use of the National Institute for Biological Standards and Controls reference standard to establish the standard curve. Furthermore, this assay procedure is less expensive than ferritin commercial kits.

References

Identification of Hyperthyroid Patients by Means of a Sensitive Assay for Thyrotropin

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We evaluated a new assay (TSH 1 MAIAClone) for thyrotropin (TSH) with improved sensitivity, testing a series of hospital inpatients with increased free thyroxin indices (thyroxin concentration $\times$ triiodothyronine uptake on resin). This assay involves use of three monoclonal antibodies and an antibody–magnetic particle conjugate that rapidly and completely separates bound and free tracer in a magnetic field. The assay turnaround time is 3 h. By the TSH 1 MAIAClone assay, 65% of these patients with an increased free thyroxin index were identified on the basis of a TSH value $<0.50$ mill-int. unit/L. In contrast, another commercially available assay for TSH detected suppressed TSH concentrations in less than 5% of these patients. We conclude that the TSH 1 MAIAClone assay markedly improves our ability to discriminate hyperthyroidism from euthyroidism.  

Additional Keyphrases: thyroid status, immunoassay by use of magnetizable solid phase, cutoff value

The regulation of pituitary secretion of thyrotropin (TSH)

1 Nonstandard abbreviations: TSH, thyrotropin (thyroid-stimulating hormone); T4, thyroxin; T3, triiodothyronine; FTI, free thyroxin index; TRH, thyroliberin (thyrotropin-releasing hormone); ECS, Environmental Chemical Specialties.