Interference of Imferon in Colorimetric Assays for Iron

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Imferon (Merrell-National), an iron–dextran complex, is widely used in patients with iron deficiency. It is present in the circulation in appreciable amounts for two to three weeks after administration and interferes with all tested colorimetric iron assays, both with and without deproteinization. The amount of the plasma Imferon iron interference depends primarily on the choice of reductant. With dithionite it is essentially 100%. In the presence of ascorbic acid and hydroxylamine, the interference depends also on assay conditions, especially temperature, but also incubation time and pH. The minimum interference in a homogeneous assay was about 3%. The relative amount of interference from hemoglobin iron under the various assay conditions is different from that of Imferon iron. In the presence of a reducing agent, the dextran–iron complex decomposes—instantaneously with dithionite, and gradually with sulfite, ascorbic acid, and hydroxylamine. The freed iron becomes dialyzable, can react with bathophenanthrolene, and elutes on a Sephadex G-50 or G-15 column in the same fractions as an ammonium ferrous sulfate.

Imferon, a dextran–iron complex, is widely used in the treatment of iron deficiency. In contrast to saccharated iron, it disappears slowly from the circulation. Substantial amounts are present for two weeks after an intravenous (I) and three weeks after an intramuscular (2) injection.

We became aware, in my laboratory, of the interference of Imferon after we started to assay serum iron routinely with Teepol as solubilizer, dithionite as reductant, and bathophenanthroline as color reagent at room temperature (3) (essentially the same assay method is used in kits from Boehringer Mannheim and J. T. Baker). Within a month we had two cases of patients with apparent serum iron values above 9.0 mg/L. Both proved to have received intramuscular Imferon injections. Webster (4) already determined plasma Imferon iron with dithionite as reductant, but at a temperature of 90 °C. I am, however, not aware of literature that indicates its interference at room temperature in a serum iron assay. McIntosh et al. (5) noted a small but clinically significant contribution of Imferon iron in some colorimetric methods involving ascorbic acid or hydroxylamine as reductant; with atomic-absorption spectroscopy, as expected, all the iron in the complex was measured.

I studied systematically the interfering effect of Imferon iron in different serum iron assays with and without deproteinization, and compared it with that of hemoglobin iron. For both agents the interference was strongly dependent on choice of reductant, temperature, pH, and time of incubation.

Materials and Methods

Serum iron was determined according to Sanford (6) (Teepol, ascorbic acid, pH 4.7, room temperature), Von Lauber (3) (Teepol, dithionite, pH 4.7–5.4, room temperature), Goodwin et al. (7) (0.6 mol/L buffer, pH 4.5, ascorbic acid, 37 °C), and a method with deproteinization (8) (HCl, trichloroacetic acid, centrifugation, ascorbic acid, room temperature).

I investigated the effect of the different agents on the interference of Imferon iron as follows: to 1.5 mL of 0.2 mol/L acetic acid buffer, which contained Teepol 610 and reductant in concentrations given below, add 0.5 mL serum or Imferon solution; vortex-mix, then incubate the solution for 20 min, unless otherwise stated, at the indicated temperature. Measure absorbance at 540 nm (A1); then add 20 μL of bathophenanthroline (91 g/L), vortex-mix, and measure the absorbance again at 540 nm (A2). Correct the difference in absorbance (A2 − A1) with a similarly treated water blank, and calculate the iron content by comparison with a standard containing 1.0 mg/L ferric iron, handled the same way. The linearity in each assay was checked with a standard curve consisting of 0.5, 1.0, 3.0, and 9.0 mg of ferric iron per liter.

The Teepol 610, which contained 34% active material like Teepol HB6, was made iron-free according to Von Lauber (3): mix 200 mL of Teepol 610 with 200 mL of MgSO4 (10 g/L); quickly add 100 mL of 1 mol/L NaOH and after 15 min centrifuge the solution at 3000 rpm for 15 min; add glacial acetic acid and solid sodium acetate to the supernate until the buffer is 0.2 mol/L at the desired pH. This solution is stable for months. At the day of its use, add the reductant to this Teepol–acetate buffer to a concentration of 30 g/L for dithionite, 3 g/L for ascorbic acid and hydroxylamine, and 0.1 mol/L for sodium sulfite. If necessary, readjust the pH.

I measured absorbance with a Coleman Jr. II, Model 6/20, in round 10 × 75 mm cuvets.

Gel filtration was on a Sephadex G-50 or G-15 column, 1.8 × 14 cm; the eluent was the Teepol–acetate buffer containing, when used, 20 g of reductant per liter for dithionite or 2 g/L for ascorbic acid. Sample volume was 1 mL, and column fraction volume 1.5 mL. The iron concentration in the sample was 20 mg/L Imferon, ferric chloride, or a ferrous salt complex [ammonium ferrous sulfate, (NH4)2Fe(SO4)2·6H2O, also called Mohr's salt].

Hemoglobin was obtained from washed human erythrocytes, hemolyzed in distilled water plus toluidine. The final concentration in the serum pool was 6.2 mg of hemoglobin iron per liter.

Imferon was obtained from Fisons Ltd., Loughborough, Leicestershire, England; Teepol 610, (and, more recently, Teepol HB6) from Shell Co; dithionite from Matheson Coleman & Bell; ascorbic acid from Harleco or Merck; hydroxylamine from Merck; bathophenanthroline from Sigma; Sephadex G-15 and G-50 from Pharmacia.

Kits for serum iron determination with Teepol and dithionite can be obtained from Boehringer Mannheim GmbH, Postfach 31020, 6800 Mannheim 31, F.R.G., and from J. T. Baker Chemical Co., Phillipsburg, N.J. 08865.

Results

Effect of Imferon on Different Assay Methods

I checked the amount of interference from Imferon iron in various serum iron assays at three different concentrations.

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However, 18 mg/L added, the serum turbid, at pH 5.8, the interference is approximately 3–5%. However, at a pH of 6 or above, the iron from the serum pool itself gave too low values. For hemoglobin iron the interference was also somewhat lower at higher pH.

The interference of Imferon iron and hemoglobin iron is strongly time dependent, as shown in Table 2. With Imferon the iron reduction and dissociation is complete after overnight incubation at room temperature.

Ascorbic acid. With ascorbic acid as reductant the recovery of the Imferon iron was strongly dependent on assay conditions. At pH 5.0, the results of incubation at room temperature (25 °C) are identical to those with the method of Sanford shown in Table 1. The results at 37 °C compare well with those by the method of Goodwin et al. (7), who incubated also at 37 °C, but without Teepol, in concentrated buffer. This indicates a lack of effect of the Teepol itself. At a temperature of 90 °C the Imferon iron is nearly completely dissociated. For hemoglobin iron the effect of incubation temperature is also very strong and much more extensive than with dithionite; the interference ranges from 1% at 95 °C, 5% at 37 °C, to 95% at 90 °C.

The amount of interference from Imferon iron is lower at higher pH. At pH 5.8, the interference is approximately 3–5%. However, at a pH of 6 or above, the iron from the serum pool itself gave too low values. For hemoglobin iron the interference was also somewhat lower at higher pH.

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Hydroxylamine and sulfite. With hydroxylamine at pH 5.0 the interference from Imferon alone at 25 or even 37 °C without serum was only about 1% or less, but under these conditions the serum iron in the pool was only partly recovered. Under my assay conditions the minimum usable temperature was 60 °C at pH 5.0 and 90 °C at pH 5.0 with hydroxylamine. Only 90 °C and pH 5.8 gave the correct results with sodium sulfite. Maybe lower temperatures are sufficient with solubilizers other than Teepol.

The same general dependence on assay conditions was found with hydroxylamine and sulfite as for ascorbic acid: at higher temperature and lower pH the interference of Imferon iron is stronger. With hydroxylamine at pH 5 and 60 °C the interference was 3 to 5%; at pH 5.0 and 90 °C the interference after 5 min incubation was approximately 5%, after 20 min 25%, and after 1 h 60%; at pH 5.8 and 90 °C the interference was approximately 7%. For hemoglobin iron the interference was 3% at pH 5.0 and 60 °C, 8% at pH 5.0 and 90 °C, and 10% at pH 5.8 and 90 °C. With sodium sulfite as reductant, the hemoglobin iron interference was certainly lower (<0.5%) than with all other tested agents, but the interference of Imferon iron was quite strong.

Effect of Reducing Agent on the Size of the Dextran-Iron Complex

Imferon (60 mg/L) in Teepol–acetate buffer was dialyzed at room temperature against a 10-fold volume of this buffer under conditions identical to those of the iron assay as described under Materials and Methods. Samples of the dialysate were taken at the indicated times and the iron content was determined with dithionite as reductant. The results are given in Table 3. The iron is released from the dextran–iron complex in a dialyzable form only if a reductant is present. The dialysis rate of a ferric chloride solution is, however, even higher than that of the iron released from the Imferon complex. To find an explanation I assayed by gel filtration on Sephadex G-15 and G-50 columns, as described under Materials and Methods. On Sephadex G-50 Imferon was eluted at the void volume (fractions 7–9), ferric chloride without reductant at the total volume of the column (fractions 23–30); reduced ferric chloride reduced Imferon, and it and an inorganic ferrous salt complex were eluted at the same rate and appeared in fractions 17–22. Comparable results were obtained on Sephadex G-15, in which case the reduced Imferon appeared only two fractions after the Imferon itself. Results were identical for Imferon that had been incubated only 1 min with dithionite or overnight with ascorbic acid.

<table>
<thead>
<tr>
<th>Source</th>
<th>% recovery</th>
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<tr>
<td>Imferon</td>
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<tr>
<td>Imferon + dithionite</td>
<td>2</td>
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<tr>
<td>Imferon + ascorbic acid</td>
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Table 3. Recovery of Iron in a Dialysate
Discussion

The interference of Imferon iron in colorimetric homogeneous serum iron assays depends strongly on reaction conditions. The most important factors are the choice of reductant and incubation temperature, but pH and incubation time also play a role. With dithionite, even at room temperature and after only 1 min, the Imferon iron is already completely reduced and dislodged. With ascorbic acid and hydroxylamine, the interference fluctuates between 3 and 100%; it is lowest at low temperature, short incubation time, and not too low pH. With hydroxylamine and sodium sulfite, less favorable conditions were required to free the serum iron from the transferrin. The lowest interference was obtained with ascorbic acid at room temperature and pH 5.8 or with hydroxylamine at 60 °C and pH 5.0. The noted 3% agrees with the values obtained by McIntosh et al. (5) and is still clinically relevant. Even two weeks after an intramuscular Imferon injection, 10.0 mg of Imferon iron can be easily present (2) per liter of plasma, which adds at least 0.3 mg/L to the real serum iron value. Whenever the presence of Imferon in a serum sample is suspected, it can be confirmed most easily by comparing serum iron results in duplicate assays with dithionite in one and ascorbic acid in the other.

When the serum is deproteinized with trichloroacetic acid or with trichloroacetic acid and HCl before the assay, the Imferon remains mostly in solution. Because of some coprecipitation with serum proteins approximately 80% of the Imferon remains soluble; without serum about 100% remains in solution. Thus the amount of Imferon iron recovered depends mostly on the subsequent iron assay conditions, especially on the choice of reductant and temperature.

I have no experience with continuous-flow methods. My results show clearly, however, that Imferon iron is dialyzable after reduction with dithionite or ascorbic acid. Thus the interference of Imferon in such an assay, although probably low, can be present if a reductant is included before the dialysis step (i.e., 9).

The conditions most favorable for removing the iron from the iron-dextran complex differ from those for hemoglobin iron. Hemoglobin iron shows the lowest interference when sodium sulfite is present, in accordance with the results of Ramsay (10), whereas under these assay conditions the Imferon iron interferes about 75%. For hemoglobin iron the sequence of increasing interference is sulfite < dithionite < hydroxylamine < ascorbic acid. For Imferon iron this sequence is hydroxylamine < ascorbic acid < sulfite < dithionite.

Imferon is a high-molecular-mass complex of a ferric-oxyhydroxide core attached to dextran polysaccharide chains. It is in itself stable in serum and non-dialyzable. Under reducing conditions the complex decomposes and the reduced iron can react with bathophenanthroline, when present. With dithionite this process is probably instantaneous, and the governing factor for its appearance in the dialysate is its diffusion rate. With ascorbic acid the release takes place gradually, but after 24 h the reaction is complete. This is in accordance with the time dependency for Imferon iron dissociation under assay conditions.

The results of gel filtration indicate a somewhat higher molecular mass for the iron released from Imferon than that of ferric chloride. The same elution rate was found for reduced ferric chloride and for Mohr's salt, which points to the release of an inorganic ferrous complex.

I thank Mrs. Y. Elsa-Coster for her valuable technical assistance.

References