Elimination of Fibrinogen Interference in a Dye-Binding Method for Iron

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Serum iron concentrations >90 μmol/L were measured in samples from hemodialysis patients by a Ferrozine dye-binding method. Reanalysis by coulometry showed these results to be spuriously high. Turbidity resulting from precipitation of fibrinogen was identified as the source of the interference. High concentrations of heparin enhanced the effect. We conclude that the persistence of fibrinogen in serum samples from patients treated with anticoagulants is a potential interference in Ferrozine dye-binding methods performed without prior deproteinization. Adding guanidine·HCl to the acid buffer reagent is a simple way to eliminate this interference.

Additional Keyphrases: Ferrozine · hemodialysis · analytical error

We have observed above-normal concentrations of serum iron (>90 μmol/L) measured by a commercial direct dye-binding method in some patients undergoing hemodialysis. This artifact did not arise from known interferences such as hemolysis, lipemia (1), iron–dextran therapy (2), or the presence of monoclonal immunoglobulins (3). The use of plasma is known to affect certain iron assays (4), although the mechanism has not been elucidated. Because our patients are given large doses of heparin during dialysis, we investigated the possibility that the spurious iron results were due to incomplete coagulation of blood samples.

The increasing use of heparinized blood samples for clinical analyses has led to reports of interference from fibrinogen and heparin in routine assays (5–7). Here, we studied the effect of heparin and fibrinogen on a Ferrozine iron assay performed with a centrifugal analyzer.

Materials and Methods

Materials

Citrate/phosphate buffer, pH 4.5, containing sodium citrate (0.1 mol/L; BDH Chemicals, Toronto, Ontario) and dibasic sodium phosphate (0.1 mol/L; Fisher Scientific, Fair Lawn, NJ 07410), sodium acetate buffer (pH 4.5, 0.1 mol/L; Fisher), and isotonic saline (NaCl, 150 mmol/L; Fisher) were prepared with de-ionized water. Fibrinogen was quantitated by radial immunodiffusion with NOR-Partigen plates (Behringwerke, Somerville, NJ) according to manufacturer's instructions; the reference interval is 2.05–4.39 g/L. Vacutainer Tubes were from Becton Dickinson (Rutherford, NJ 07070). Guanidine·HCl (Grade 1) was obtained from Sigma Chemical Co. (St. Louis, MO 63178).

Samples

Routine samples from patients in the hemodialysis unit were collected (usually at the start of dialysis) into Vacutainer Tubes without anticoagulant, allowed to clot, and centrifuged at 1000 × g for 5 min. Serum samples were stored at 4 °C and analyzed for iron and fibrinogen within two days. Random plasma and serum samples obtained from hospitalized patients were used to study the effect of lithium heparin and of guanidine·HCl on the assay.

Saline, serum, or plasma containing various concentrations of lithium heparin were prepared by adding appropriate amounts of each to heparinized Vacutainer Tubes. The samples were analyzed for iron on the Cobas-Bio centrifugal analyzer (Roche Analytical Instruments, Nutley, NJ). We also used the analyzer to monitor at 20-s intervals the absorbance change after mixing the samples with the acid buffer used in the assay.

To investigate the effect of fibrinogen on the assay, we dissolved fibrinogen at concentrations between 0.2 and 6.0 g/L in denatured serum. We used this medium because fibrinogen is only marginally soluble in saline, and the addition of fibrinogen to fresh serum induces coagulation rapidly. The serum was stored at room temperature for 48 h (to deplete coagulation factors V and VIII), heparinized, and heated to 37 °C before use. Despite this pretreatment, measurement of fibrinogen concentration showed inconsistent and poor recovery of the added fibrinogen.

Because we could not quantify the interference by using lyophilized fibrinogen, we used serially diluted heparinized plasma of known fibrinogen concentration. Paired plasma and serum were obtained from six patients. Serum was heparinized (28 USP units/mL) and used to dilute the corresponding plasma sample to yield fibrinogen concentrations between 0 and 2.5 g/L. The samples were analyzed for iron immediately.

To study the effect of fibrinogen in isolation from heparin, we prepared nonheparinized fresh plasma from 20 mL of blood drawn from healthy volunteers (n = 10) into polypropylene syringes and centrifuged immediately in polypropylene tubes to minimize coagulation. The plasma obtained was analyzed immediately for...
Iron, and an aliquot was placed in a heparinized Vacutainer Tube (15 USP units/mL) to prevent coagulation. This aliquot was stored at −20 °C for subsequent fibrinogen quantification. A corresponding serum sample was obtained from blood collected in a glass tube.

**Methods**

**Iron assays:** Serum iron determination by a dye-binding method (Iron/UBC/TIBC, no. 565 colorimetric assay; Sigma Diagnostics) was performed with the Cobas-Bio centrifugal analyzer. Briefly, transferrin-bound iron is dissociated at acid pH, the ferric ion is reduced, and a complex is formed with Ferrozine [3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine, monosodium monohydrate; A_{max} 560 nm]. The instrument measures the initial absorbance of the buffer blank and the auxiliary absorbance of each sample 60 s after addition of the iron-dissociating buffer. Ferrozine dye is then added and, after 3 min, the final absorbance is measured. Iron concentration is calculated from the difference between the final and auxiliary absorbances after the buffer blank is subtracted. Our reference interval is 5–28 μmol/L. The assay was modified by adding guanidine · HCl to final concentrations of 1–4 mol/L in the buffer reagent.

Iron was also determined by constant-potential coulometry with the Ferrochem II (ESA, Bedford, MA 01730) and by dry-film technology with the Ektachem 700 (Kodak, Rochester, NY 14650). The dry-film technique is a colorimetric assay similar to the Sigma assay.

**Results**

**Effect of potential interferents:** Of the 16 samples studied, six had iron concentrations >90 μmol/L by the Cobas-Bio analyzer. The mean fibrinogen concentration for these samples was 5.7 g/L (range 3.8–6.6 g/L). The 10 samples with normal results for serum iron concentrations (6.7–28.3 μmol/L) had no detectable fibrinogen.

Lithium heparin in saline, even at extreme concentrations (80 USP units/mL), had no effect on the assay. In plasma, concentrations of heparin >35 USP units/mL resulted in a linear decrease in iron values. Serum samples showed minimal increases at high heparin concentrations (Figure 1).

In nonheparinized fresh plasma from all 10 volunteers, the final absorbance on the Cobas-Bio analyzer was increased markedly, with calculated iron results >90 μmol/L. The iron concentrations of the corresponding serum samples were within the physiological range. Fibrinogen concentrations of heparinized aliquots ranged from 3.00 to 5.43 g/L but were undetectable in the serum samples. Auxiliary absorbances were normal for both nonheparinized plasma and serum samples.

In studies to quantify the interference, increases in both lyophilized and endogenous fibrinogen produced linear increases in iron values over a small range but at different calculated concentrations. With native fibrinogen, apparent iron values increased linearly in plasma containing >0.3 g of fibrinogen per liter. At fibrinogen concentrations of ~1.5 g/L, iron results were >90 μmol/L for all patients (Figure 2). When lyophilized fibrinogen was used, linear increases in iron values occurred at calculated fibrinogen concentrations between 1.0 and 4.0 g/L and exceeded 90 μmol/L at fibrinogen concentrations >5.0 g/L. Possible interference by citrate (present in the lyophilized fibrinogen) was excluded by adding to one of the serum pools increasing amounts of sodium citrate to correspond to concentrations in the fibrinogen-supplemented samples. Coulometric determination of iron was not affected by fibrinogen.

**Elucidation of the interference:** Addition of the iron-dissociating buffer to plasma samples produced visible turbidity in the affected samples. The absorbance spectrum was shifted upwards one absorbance unit and no longer showed an A_{max} at 560 nm. Similar observations were made with an in-house acetate buffer and with citrate/phosphate buffer (pH 4.5).

Because of the high auxiliary absorbances in affected samples, we studied the change in absorbance after adding acid buffer to the sample. For plasma samples, serial absorbance readings at 560 nm showed increasing absorbance after a lag period of 20 s. Absorbance reached a plateau at ~120 s. In plasma samples with high heparin concentrations, absorbance increased rapidly in the first 60 s and thereafter dropped off slightly. Serum samples showed little change in absorbance over this period. Icterus (total bilirubin; as great as 550
(35) interferences the fibrinogen patients increased dialysis organic gravitated However, pmol/L DIscussIon clonal routine guanidine ized measurements fibrinogen 2.0 concentration (Figure 1.6 pmol/L) Fig. 2. Effect of fibrinogen on apparent iron values in heparinized plasma of six patients (■) and serum with added lyophilized fibrinogen (×)

µmol/L) had little effect on the absorbance. Hemolysis and lipemia caused marked increases in absorbances (Figure 3).

Effect of guanidine · HCl: As the guanidine · HCl concentration in the buffer increased, the interference from fibrinogen decreased. At a guanidine concentration of 2.0 mol/L (1.6 mol/L in the final reaction mixture), iron measurements in plasma samples were equal to those obtained by the dry-film method, even in overheparinized plasma (Figure 4). Using this concentration of guanidine · HCl did not affect iron measurement in routine serum samples, including samples with monoclonal immunoglobulins, high bilirubin content, or hemolysis: iron values obtained (n = 57) were within 4 µmol/L of the value obtained without guanidine · HCl. However, with lipemic samples, adding guanidine aggravated the interference. Removal of lipoproteins with organic solvents did not eliminate the problem.

Discussion

While investigating abnormally increased iron concentrations (>90 µmol/L) in serum samples from hemodialysis patients, we found that fibrinogen in the samples interfered with the Ferrozine dye-binding assay used on our centrifugal analyzer. The combination of increased fibrinogen synthesis, commonly seen in renal patients on hemodialysis (8), and retardation of clotting by heparin used during dialysis leads to persistence of fibrinogen in serum at concentrations sufficient to cause the interference. Fibrinogen in serum causing other interferences has been encountered previously (9) and may be more frequent than suspected.

Our studies suggest that turbidity due to protein precipitation is the cause of the interference. The pH of the iron-dissociating buffer is close enough to the pI of fibrinogen (4.5 vs 5.6, respectively) to decrease the solubility of the protein. Although heparin is not necessary for this to occur, at high concentrations it facilitates the precipitation. This property of heparin is used to precipitate fibrinogen extracorporeally in the management of familial hypercholesterolemia (10). In addition, low- and very-low-density lipoproteins are precipitated, which is the principle behind many methods for determining high-density-lipoprotein cholesterol (11). This may explain the increase in iron values seen with high heparin concentrations in serum samples (Figure 1). The main effect of heparin on serum is lipoprotein precipitation; with plasma, however, fibrinogen precipitation predominates. Therefore, in any given sample, the amount of turbidity is dependent on fibrinogen, lipoprotein, and heparin concentrations.

Similar observations have been made for other assays. Heparinized plasma causes interference in a γ-glutamyltransferase assay (5), in a dye-binding method for albumin (7), and in a centrifugal analyzer method for creatine kinase (6). In the report of interference in the albumin assay, fibrinogen or heparin alone did not interfere; only heparinized fibrinogen had an albumin-like reactivity. In the creatine kinase assay, low-density lipoprotein, but not fibrinogen, was recovered in the precipitate, possibly because of the higher pH of the
reaction mixture (pH 6.1). This resulted in creatine kinase values in plasma samples that were less than in serum. At high heparin concentrations, measurement of plasma creatine kinase concentrations increased above concentrations in serum. With our iron assay the increased iron values observed with plasma decreased when heparin concentrations were increased. The serial absorbance readings of sample–buffer mixture provide an explanation for this phenomenon. At low heparin concentrations, the absorbance is still increasing at 60 s ($A_{aux}$), hence fibrinogen precipitation is a significant contributor to the final absorbance. The result is a large change in absorbance ($\Delta A = A_{final} - A_{aux}$). Conversely, with overheparinized samples, precipitation is almost complete when $A_{aux}$ is measured, leading to a small $\Delta A$. This underscores the importance of heparin concentrations in studies of interference due to serum protein precipitation and the variable effect that may be seen if heparinized Vacutainer Tubes are not completely filled.

Guainidine has been used to solubilize proteins and to prevent interference of monoclonal immunoglobulins in direct dye-binding assays (3). We did not encounter this interference in 38 patients with monoclonal immuno-
globulin concentrations >10 g/L (range 10–62, mean 33 g/L). However, the use of guainidine · HCl in the acid buffer eliminated fibrinogen interference. In contrast to the high concentration of guainidine · HCl used previously (3, 12), we found 2 mol/L to be sufficient under the present assay conditions, even in overheparinized plasma samples. At this concentration, analysis of serum samples was not affected, except in markedly lipemic samples.

Laboratories using a Ferrozine dye-binding method for iron determination without prior deproteinization should be aware of interferences from various proteins. The National Committee for Clinical Laboratory Standards’ proposed method for iron determination is a Ferrozine method with protein denaturation and precipitation by trichloroacetic acid before analysis (13). Although this was adapted recently for the Cobas-Bio centrifugal analyzer (1), the pretreatment step is time consuming. Addition of guainidine · HCl to the buffer is a simple alternative. With the increasing use of heparin therapeutically and for sample preservation, incorporation of this step will reduce errors.

References