

# Bleomycin-detectable Iron Assay for Non-Transferrin-bound Iron in Hematologic Malignancies

LENI VON BONSDORFF,<sup>1\*</sup> ENNI LINDEBERG,<sup>1</sup> LEILA SAHLSTEDT,<sup>2</sup> JARI LEHTO,<sup>3</sup> and JAAKKO PARKKINEN<sup>1</sup>

**Background:** A microwell modification of the bleomycin assay for determining non-transferrin-bound iron (NTBI) was evaluated and compared with a chelation method.

**Methods:** The bleomycin assay reagent and sample volumes were halved, and measurements were done in microwell plates. Samples from patients treated for hematologic malignancies were studied. The chelation method was based on mobilization of NTBI with a chelator and measurement of the ultrafiltered iron-chelator complex. NTBI results were also compared with transferrin saturation and the distribution of transferrin iron forms by urea-polyacrylamide gel electrophoresis.

**Results:** The bleomycin assay intraassay imprecision (CV) was 7.7% and 8.2% and the interassay imprecision was 18% and 9.8% for a low (0.2  $\mu\text{mol/L}$ ) and a high (1.5  $\mu\text{mol/L}$ ) control, respectively. Hemolysis increased measured NTBI. A detection limit of 0.1  $\mu\text{mol/L}$  was established based on the interference of nonvisible hemolysis and on accuracy studies. In patient samples, NTBI exceeded the detection limits only when transferrin saturation was >80%. Compared with the chelation method, the bleomycin assay gave clearly lower NTBI concentrations. The chelation method also gave positive results at <80% transferrin saturation. The recovery of iron added as ferric nitrilotriacetate to serum was 33% by the bleomycin assay and 64% by the chelation assay.

**Conclusions:** The microwell version of the bleomycin assay is reproducible. When hemolyzed samples were excluded, bleomycin-detectable iron was found only when the transferrin saturation was >80%, suggesting high specificity. Bleomycin-detectable iron constitutes only a portion of the NTBI measured by the chelation method.

© 2002 American Association for Clinical Chemistry

Iron is bound with high affinity to transferrin, the iron carrier protein in serum. In some clinical conditions, transferrin becomes fully saturated, and non-transferrin-bound iron (NTBI)<sup>4</sup> appears in serum. NTBI is harmful because of its high redox activity and its potential for forming free radicals, thereby inducing cellular toxicity (1, 2). It is also known that iron has a critical role in the growth of bacteria and fungi, and NTBI may promote the growth of microorganisms and predispose patients to septic infections. NTBI has generally been detected in disorders with iron-overload conditions, such as thalassemia (3) and hemochromatosis (4, 5), and in certain other clinical conditions (6–8). One group of patients who typically have NTBI includes hematologic patients undergoing high-dose chemotherapy (9), particularly those treated with myeloablative therapy and stem cell transplantation (10, 11).

Beginning with the chelation method described by Hershko et al. (3), several assays have been developed to measure NTBI without interference of iron bound in a redox-inactive form to transferrin or to therapeutic chelating agents such as desferrioxamine. Two types of assays have been described: those in which loosely bound

<sup>1</sup> Finnish Red Cross Blood Transfusion Service, Kivihaantie 7, FIN-00310 Helsinki, Finland.

<sup>2</sup> Department of Medicine, PO Box 340, Helsinki University Central Hospital, FIN-00029 Helsinki, Finland.

<sup>3</sup> Department of Public Health, PO Box 41, University of Helsinki, FIN-00014 Helsinki, Finland.

\* Author for correspondence. Fax 358-9-5801429; e-mail leni.bonsdorff@bts.redcross.fi.

Received April 30, 2001; accepted November 26, 2001.

<sup>4</sup> Nonstandard abbreviations: NTBI, non-transferrin-bound iron; NTA, nitrilotriacetic acid; AAS, atomic absorption spectrophotometry; FeNTA, ferric nitrilotriacetic acid; PBS, phosphate-buffered saline; and PAGE, polyacrylamide gel electrophoresis.

iron is first mobilized with a chelating agent and the ultrafiltered complex is measured (3, 12–14); and assays in which a biologic reagent forms a complex with redox-active iron, causing a biochemical reaction that can be measured (15, 16). The most common chelating agent used in the chelation assays is nitrilotriacetate (NTA). The ultrafiltered chelated iron can be quantified by different methods, including HPLC, colorimetric measurement, and atomic absorption spectrophotometry (AAS). In the method first described by Breuer et al. (13), oxalate is used for chelation of NTBI, and quantification is based on fluorescence measurement in microwells coated with desferrioxamine in the presence of a fluorescent metallosensor, calcein. The bleomycin assay developed by Gutteridge et al. (15) is based on the direct complexation of redox-active iron with the antibiotic bleomycin. The iron–bleomycin complex causes degradation of DNA, and the degradation products can be measured. The detected iron is generally named bleomycin-detectable iron. The bleomycin assay has been compared with another biochemical assay that uses the iron-requiring enzyme aconitase (16), which gave results similar to the bleomycin method. Both the chelation methods and the bleomycin assay have been used for measuring NTBI in various clinical conditions.

One drawback of most chelation methods is the large amount (~1 mL) of serum needed for ultrafiltration, whereas the bleomycin assay requires only 100  $\mu$ L. In this work, we further reduced the scale of the bleomycin assay and introduced a spectrophotometric measurement in microwell plates. Because very limited information has been available on the performance of the bleomycin assay concerning assay precision, detection limit, and interference, we evaluated the assay. We also compared the bleomycin assay with a chelation assay for the determination of NTBI in the serum of patients with hematologic malignancies.

### Materials and Methods

#### PATIENT SAMPLES

Serum samples ( $n = 399$ ) were collected from patients with hematologic malignancies undergoing allogeneic stem cell transplantation and treated with cyclophosphamide (120 mg/kg) and fractionated total body irradiation (12 Gy). The patients were enrolled in clinical studies for chelation of NTBI by an investigational apotransferrin product. For the comparative analysis of NTBI measured by the chelation assay, additional samples ( $n = 21$ ) were analyzed from patients undergoing cytotoxic treatment with or without autologous stem cell transplantation. The samples were collected at the Helsinki University Central Hospital, Finland, and the studies were approved by the ethics committee of the hospital.

#### MICROWELL MODIFICATION OF BLEOMYCIN ASSAY

The bleomycin assay was carried out according to the method described by Evans and Halliwell (17) with the following modifications. All reactions were carried out in

one-half the volume, and the absorbance was measured at 535 nm rather than 532 nm, using a microwell plate reader. The reagent solutions, except the bleomycin, were treated overnight with Chelex (Bio-Rad), 300 mg in 10 mL or 800 mg in 20 mL of ascorbic acid solution, to remove any excess iron in the chemicals. To avoid iron contamination, only disposable plastic tubes were used. The assay components were added in the following order: 250  $\mu$ L of 1 g/L DNA (type I DNA from calf thymus; Sigma); 25  $\mu$ L of 1.5 kIU/L bleomycin sulfate (Sigma); 50  $\mu$ L of 50 mmol/L  $MgCl_2$ ; 25  $\mu$ L of sample, calibrator, or blank; and finally, 50  $\mu$ L of 8 mmol/L ascorbic acid (Merck). A predetermined amount of 25 mmol/L HCl (~7  $\mu$ L) was added to adjust the pH of reagent mixture to 7.4. The mixture was incubated at 37 °C for 1 h to allow the iron–bleomycin complexes to bind to DNA, releasing malondialdehyde from DNA degradation. The reaction was stopped by the addition of 50  $\mu$ L of 0.1 mol/L EDTA, and 250  $\mu$ L of 10 g/L thiobarbituric acid (Sigma) and 250  $\mu$ L of 250 mL/L HCl were added. The mixture was then incubated for 20 min at 80 °C to allow chromogen formation by the reaction of malondialdehyde with thiobarbituric acid. The samples were cooled to room temperature, 1.5 mL of butanol was added, and the chromogen was extracted into the organic phase by mixing. The samples were centrifuged for 20 min at 2500g to separate the phases, and 350  $\mu$ L of the clear top phase was pipetted into microwell plates (Nunc Maxisorp). The absorbance was measured at 535 nm in a microplate reader (Titertek Multiscan RC; Labsystems). The samples were measured in parallel with a corresponding blank without added bleomycin. The absorbance of the blank was subtracted from that of each sample. The absorbance of the reagent blank was subtracted from the absorbances of the calibrators, and a calibration curve with 0.1–3  $\mu$ mol/L iron (iron atomic absorption standard solution in 10 mL/L HCl; Sigma Aldrich) was calculated for each series. Data were transformed to logarithmic values before calculation of the calibration curve by linear regression to give weight to the calibrators with a low iron concentration (0.1–1  $\mu$ mol/L) before curve fitting.

We prepared positive controls by adding ferric nitrilotriacetic acid (FeNTA) to pooled, complement-inactivated, sterile-filtered normal serum. A 1 mmol/L FeNTA solution was prepared from  $FeCl_3$  and NTA (Sigma), in a 1:1 molar ratio. We added 30–40  $\mu$ mol/L FeNTA to obtain serum with fully saturated transferrin and NTBI. The normal serum without added FeNTA was used as a negative control.

#### NTBI ASSAY WITH IRON CHELATOR NTA

The NTBI assay with iron chelation and colorimetric quantification was carried out according to the method of Gosriwatana et al. (14). The serum samples were prepared by adding 100  $\mu$ L of 800 mmol/L NTA (pH 7.0), prepared from disodium and trisodium salts of NTA, to 900  $\mu$ L of serum and allowed to stand for 30 min at room

temperature. Serum filtrates were collected by ultrafiltration in centrifugal filter devices with  $M_r$  30 000 cutoff filters (Ultrafree-4; Millipore) that had been prerinsed with 10 mmol/L NTA and flushed with ultrapure water. For the colorimetric determination of iron, 400  $\mu$ L of serum ultrafiltrate was diluted with 400  $\mu$ L of 5 mmol/L MOPS buffer (pH 7.4), and 100  $\mu$ L of 120 mmol/L thioglycolic acid (Sigma) was added, followed by 100  $\mu$ L of 60 mmol/L bathophenanthroline disulfonic acid, disodium salt (Sigma). The mixture was then allowed to stand for 30 min at room temperature. The absorbance of 350- $\mu$ L samples in microwell plates was measured at 535 nm, using a microplate reader. Calibrators with 0–20  $\mu$ mol/L of the same iron calibrator as in the bleomycin assay were prepared in 80 mmol/L NTA, and a calibration curve was calculated by linear regression.

Chelated iron in the ultrafiltrates was also measured by graphite furnace AAS (Perkin-Elmer AAnalyst 600 equipped with Zeeman background correction). The samples and calibrators were diluted with ultrapure water, and  $Mg(NO_3)_2$  was used as a matrix modifier. The accuracy and precision of the method was monitored with standard reference material Seronorm<sup>TM</sup> Trace Elements (Sero AS).

#### OTHER METHODS

Serum iron was measured by colorimetric measurement without deproteinization using ferene-S as the chromogen (18). The interassay imprecision (CV) was 2.8% and 3.0% for a low (19.7  $\mu$ mol/L) and a high (51.4  $\mu$ mol/L) control, respectively. Serum transferrin was measured by an immunoturbidimetric method (Orion Diagnostica) with IMPRO/Nordic 96 calibrator (Labquality), which is standardized to the IFCC calibrator BRC-470. The imprecision of the controls was 3.2% for 0.97 g/L, 2.8% for 2.11 g/L, and 3.7% for 2.67 g/L. The percentage of transferrin saturation was calculated from the serum iron and transferrin concentrations, using the formula: serum iron ( $\mu$ mol/L)/transferrin (g/L)  $\times$  3.98. The formula is based on the maximal binding of 2 mol  $Fe^{3+}$ /mol of transferrin and a molecular weight of 79 570 for transferrin (19). Hemoglobin in serum was measured by a colorimetric method with *o*-toluidine (20). Serum ferritin was measured by a direct immunochemiluminometric method on an ACS:Centaur apparatus (Chiron Diagnostics).

The iron forms of transferrin were separated by urea polyacrylamide gel (6% acrylamide gels with 6 mol/L urea) electrophoresis according to Williams et al. (21) and visualized by immunoblotting. Serum samples with  $\sim$ 0.15  $\mu$ g of transferrin were separated in 10  $\times$  10-cm gels. Proteins were electroblotted from the gel onto polyvinylidene fluoride membrane (Immobilon-P; Millipore) in a transfer buffer containing 25 mmol/L Tris, 192 mmol/L glycine, and 200 mL/L methanol. The membrane was treated with 50 mL/L Tween in phosphate-buffered saline (PBS) overnight. Transferrin bands were visualized by immunostaining using rabbit anti-human transferrin IgG

(Dako A/S) as the primary antibody in PBS containing 10 g/L bovine serum albumin and 0.5 mL/L Tween 20 for 2 h at room temperature. The blots were washed three times with PBS containing 0.5 mL/L Tween 20 and incubated with anti-rabbit IgG conjugated with alkaline phosphatase (Jackson Immuno Research Laboratories) in PBS containing 10 g/L bovine serum albumin and 0.5 mL/L Tween 20 for 1 h at room temperature. After three additional washes with 0.5 mL/L Tween 20 in PBS, the blots were stained with 5-bromo-4-chloro-3-indolyl phosphate–nitroblue tetrazolium color development solution (Immuno-Blot Alkaline Phosphatase Assay reagent set; Bio-Rad). The reaction was stopped with 100 mmol/L sodium acetate (pH 5) containing 5 mmol/L sodium EDTA for 5 min. The blots were washed with distilled water and dried.

## Results

#### PRECISION AND ACCURACY OF THE BLEOMYCIN ASSAY

We prepared a calibration curve, using pure 0.1–10  $\mu$ mol/L iron calibrator in water; the calibration was linear up to 6  $\mu$ mol/L. In the standardized assay, we used iron calibrators in the range of 0.1–3  $\mu$ mol/L (Fig. 1) and determined the calibration curve by linear regression of the logarithmic values. The sensitivity of the assay for the iron calibrator was 0.316 absorbance units per  $\mu$ mol/L. The assay precision was measured using a high and a low serum control, which were prepared by adding FeNTA to normal serum to saturate transferrin and form different concentrations of NTBI (Table 1). The assay detection limit was determined by use of a normal serum control without added iron ( $n = 10$ ). The limit of detection, calculated as 3 SD above the mean of the negative control, was 0.05  $\mu$ mol/L.

To determine the accuracy of the bleomycin assay, iron in the form of FeNTA was added to normal serum in increments of 1  $\mu$ mol/L. The transition to full transferrin saturation was confirmed by urea-polyacrylamide gel

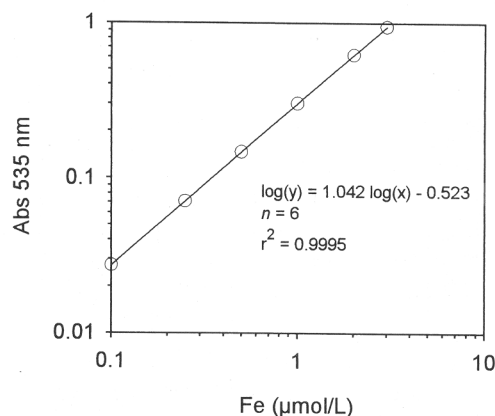


Fig. 1. Calibration curve for the bleomycin assay.

Pure iron calibrator in water was measured by the assay, and the iron concentration was plotted against the measured absorbances. The calibration curve was calculated by linear regression of the logarithmic values.

**Table 1. Bleomycin assay precision.<sup>a</sup>**

	n	CV, %	Mean, $\mu\text{mol/L}$	95% confidence interval
Intraassay				
Low control	10	7.7	0.21	0.20–0.22
High control	10	8.2	1.47	1.40–1.54
Interassay				
Low control	28	18	0.21	0.19–0.22
High control	28	9.8	1.54	1.48–1.59

<sup>a</sup> Controls were prepared by adding FeNTA to normal serum in amounts sufficient to saturate transferrin and produce different concentrations of NTBI.

electrophoresis (PAGE). When the transferrin was found only in the iron-saturated form, the NTBI was  $\geq 0.1 \mu\text{mol/L}$ , whereas the concentration was  $< 0.1 \mu\text{mol/L}$  when nonsaturated forms of transferrin could be detected by urea-PAGE. These results were from three experiments using different starting serum pools, with slight variations in the points for full transferrin saturation attributable to variations in iron and transferrin concentrations. A typical curve is presented in Fig. 2. The results suggested that the limit of detection of the assay was  $0.1 \mu\text{mol/L}$ . The NTBI concentration increased more steeply after the point of full saturation of transferrin. After the transferrin saturation, the slope of the curve indicated that  $\sim 33\%$  of the added iron was measured as NTBI.

#### INTERFERENCE STUDIES

To investigate the possible interference of hemolysis in the bleomycin assay, we mechanically caused different degrees of hemolysis in blood samples before separation of the serum and determined the percentage of transferrin saturation and concentrations of hemoglobin, NTBI, and iron forms by urea-PAGE (Fig. 3A). Hemolysis did not increase the amount of total iron, and the transferrin saturation remained the same. Serum hemoglobin in the samples ranged from 60 mg/L in samples without visible

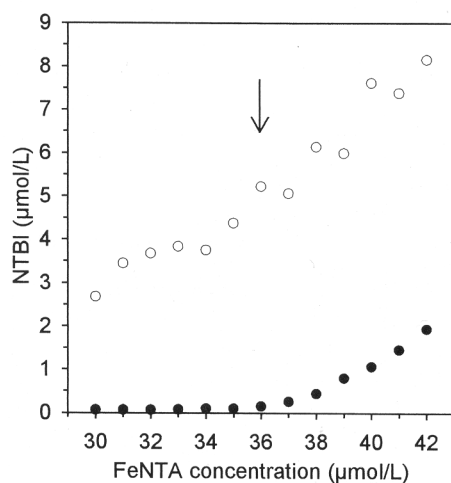


Fig. 2. Recovery of NTBI in normal serum with iron added as FeNTA. NTBI was measured by the bleomycin assay (●) or the chelation method (○). The arrow indicates the point of full transferrin saturation determined by urea-PAGE.

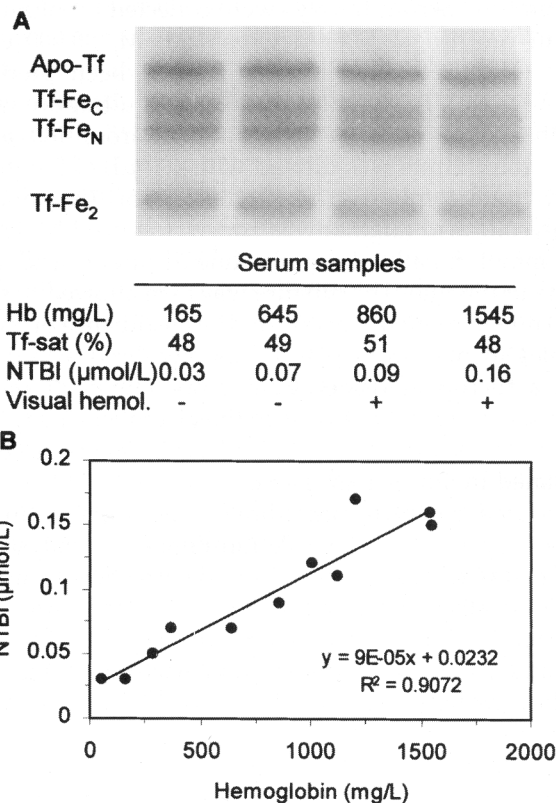


Fig. 3. Influence of hemolysis on NTBI measured by the bleomycin assay.

(A), influence of hemolysis on transferrin iron forms, as analyzed by urea-PAGE, and on hemoglobin (*Hb*), calculated transferrin saturation (*Tf-sat*), and NTBI in serum. *Apo-Tf*, apotransferrin; *Tf-Fe<sub>C</sub>*, monoferric transferrin with iron in C-lobe; *Tf-Fe<sub>N</sub>*, monoferric transferrin with iron in N-lobe; *Tf-Fe<sub>2</sub>*, diferric transferrin; *hemol*, hemolysis. (B), dependence of NTBI on the hemoglobin concentration in serum. Serum samples were prepared from blood hemolyzed to different extents *ex vivo*.

hemolysis to 1550 mg/L in samples with clearly visible hemolysis. A clear correlation between hemoglobin and the NTBI concentration could be seen (Fig. 3B). NTBI concentrations up to  $0.07 \mu\text{mol/L}$  could be measured when the hemolysis was not visually detectable and the hemoglobin was 290–645 mg/L. Any possible nonspecific effects attributable to hemolysis were corrected by use of the sample blank measured without added bleomycin, as described in *Materials and Methods*. It was concluded that, to exclude interference caused by nonvisible hemolysis, a limit of detection close to  $0.1 \mu\text{mol/L}$  should be used.

We found no interference of serum ferritin in the samples. Patient serum samples for ferritin analysis were taken 4 days before the samples for the bleomycin assay. At ferritin concentrations of 5–2470  $\mu\text{g/L}$  (mean  $\pm$  SD,  $568 \pm 733 \mu\text{g/L}$ ;  $n = 20$ ), all corresponding NTBI results were  $< 0.1 \mu\text{mol/L}$ . The reference intervals for ferritin were 7–100  $\mu\text{g/L}$  for women and 15–300  $\mu\text{g/L}$  for men. Even the high ferritin concentrations ( $> 500 \mu\text{g/L}$ ) found in one-third of the studied samples did not give detectable NTBI.

The interference of the cyclophosphamide used for

treatment of the patients was studied in normal serum and in patient sera with detectable NTBI. Cyclophosphamide was added in concentrations of 0.1–1.5 g/L, where the high concentration corresponded to a maximal theoretical concentration of the drug in serum. Cyclophosphamide did not interfere with NTBI results: in normal serum, NTBI was not detectable, and in the positive patient sera, the NTBI concentrations remained the same.

#### DETERMINATION OF NTBI IN PATIENT SERUM SAMPLES

NTBI concentrations were measured in 399 patient samples by the bleomycin assay, and the results were compared with transferrin saturation values (Fig. 4A). The NTBI varied between 0 and 0.97  $\mu\text{mol/L}$  in the samples without visible hemolysis, whereas the highest value

measured, 1.67  $\mu\text{mol/L}$ , was in a hemolyzed sample. On the basis of our results for assay accuracy and interference of hemolysis, we used a limit of detection of 0.1  $\mu\text{mol/L}$  for the patient samples. NTBI above the detection limit was found only in samples with transferrin saturation >80%. At transferrin saturation <80%, all samples had NTBI below the detection limit (Table 2). For comparison, we also calculated the number of samples that had <80% transferrin saturation and NTBI concentrations above the detection limit (0.05  $\mu\text{mol/L}$ ) determined from the negative control; we found 15 such samples (4% of total). The samples with <0.1  $\mu\text{mol/L}$  NTBI and transferrin saturation >80% were studied by urea-PAGE, and most (88%) had monoferric transferrin in addition to the diferric form, indicating that transferrin was not completely saturated. Of the positive samples with visual hemolysis, one sample with 0.12  $\mu\text{mol/L}$  NTBI and 80% transferrin saturation contained iron-free apotransferrin and was therefore considered a false positive. The results indicate a high apparent specificity of the assay, with no false positives at transferrin saturation <80% when hemolyzed samples were excluded.

#### COMPARISON WITH THE CHELATION METHOD

NTBI concentrations in patient samples were studied with another method that was based on the chelation of the NTBI in serum with NTA and colorimetric quantification of the chelated iron in the ultrafiltrate. The sensitivity of the colorimetric assay was 0.01 absorbance units per  $\mu\text{mol/L}$  and was clearly lower than that of the bleomycin assay. The limit of detection, calculated as 3 SD above the mean of a normal control serum ( $n = 10$ ), was 1.5  $\mu\text{mol/L}$ . The limit of detection was clearly higher than the corresponding value for the bleomycin assay (0.05  $\mu\text{mol/L}$ ) and reflected the larger variation of the results obtained by the chelation assay for the normal serum control ( $0.08 \pm 0.48 \mu\text{mol/L}$ , mean  $\pm$  SD).

We assayed the recovery of iron in serum samples in the same manner as in the bleomycin assay, using serum samples with added FeNTA (Fig. 2). The mean recovery for added FeNTA in three separate determinations was 64%, which was approximately twofold higher than the recovery of the bleomycin assay. However, the chelation method already showed gradually increasing positive NTBI values for the samples with added FeNTA before

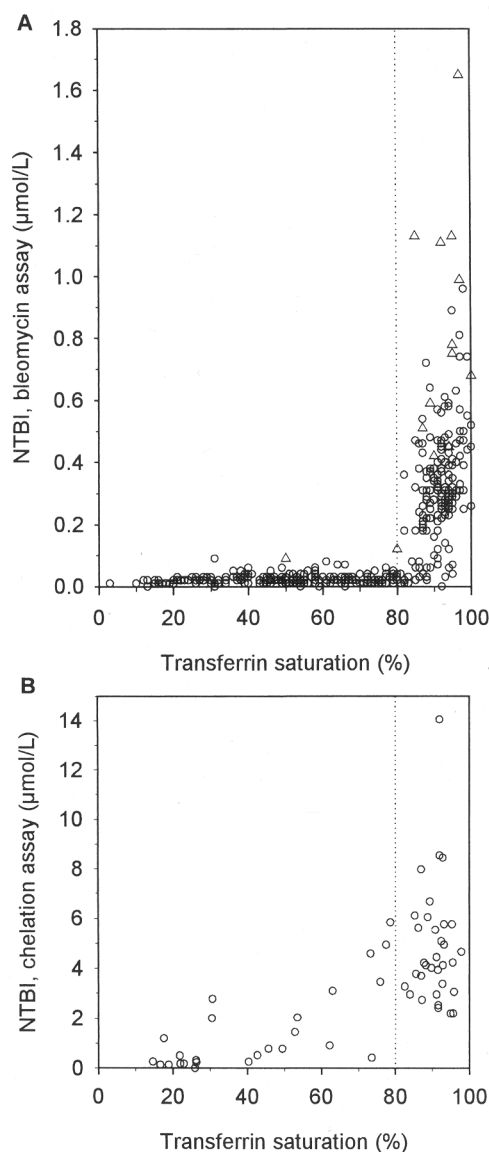


Fig. 4. Correlation between transferrin saturation and NTBI in serum from patients with hematologic malignancies.

NTBI was measured by the bleomycin assay (A) or by the chelation method (B).  $\circ$ , samples without visible hemolysis;  $\Delta$ , samples with visible hemolysis.

**Table 2. NTBI by the bleomycin assay compared with transferrin saturation in patients with hematologic malignancies.**

Bleomycin assay	n	%
Total number of samples studied	399	100
NTBI positive ( $\geq 0.1 \mu\text{mol/L}$ )	156	39
NTBI negative ( $< 0.1 \mu\text{mol/L}$ )	243	61
Positive samples with transferrin saturation <80%	0	0
Negative samples with transferrin saturation >80%	31	8

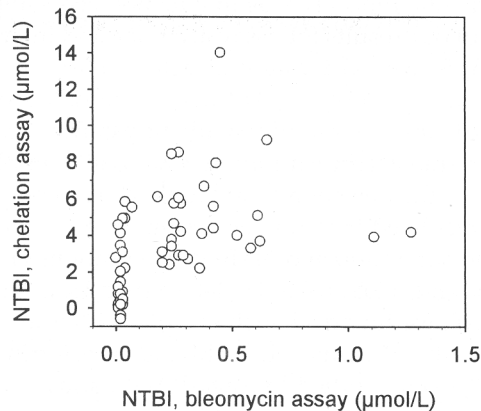


Fig. 5. Comparison of serum NTBI results obtained by the bleomycin assay and the chelation method in patients with hematologic malignancies.

the full transferrin saturation point, as determined by urea-PAGE (Fig. 2).

We studied 61 patient samples in parallel, using the bleomycin assay and the chelation-based NTBI assay, and found considerable variation between the two assays (Fig. 5). All samples that were positive in the bleomycin assay also were positive in the chelation assay, whereas one-third (11 of 30) of the samples with no bleomycin-detectable NTBI were positive in the chelation assay. A majority (8 of 11) of these samples had transferrin saturation <80% (Fig. 4B), and nonsaturated forms of transferrin could be detected in urea-PAGE. In samples with NTBI concentrations above the detection limit, no clear association between the concentrations of the two methods could be found, but clearly, higher absolute concentrations, up to 14  $\mu\text{mol/L}$ , could be measured by the chelation method (Fig. 5). Because the colorimetric quantification method had a low sensitivity, we measured the amount of chelated NTBI in parallel, using AAS to verify the results of the colorimetric method. The limit of detection of the AAS method, calculated as 3 SD above the mean of the standard without iron, was 0.02  $\mu\text{mol/L}$ , and the interassay imprecision (CV) was 2.2%. The results indicated that similar values for NTBI were obtained by both quantification methods [ $\text{NTBI}_{\text{AAS}} = 0.9373 (\text{NTBI}_{\text{colorimetric}}) + 0.7057; r^2 = 0.856; S_{y|x} = 0.693; n = 21$ ].

### Discussion

Our assay evaluation demonstrates that the microwell modification of the bleomycin assay can be carried out reproducibly using one-half the amount of reagents and serum compared with the original assay (17) and makes it possible to study large numbers of patient samples. In interference studies, we found that hemolysis increased the NTBI measured by the bleomycin assay; we also found a clear correlation between serum hemoglobin and NTBI. Slightly hemolyzed sera with no clearly visible hemolysis but with increased hemoglobin concentrations had measured NTBI concentrations of 0.05–0.1  $\mu\text{mol/L}$ .

In recovery studies, full transferrin saturation occurred when the measured NTBI was 0.07–0.1  $\mu\text{mol/L}$ . On the basis of these results, we used a detection limit of 0.1  $\mu\text{mol/L}$  as the positive cutoff for bleomycin-detectable iron in serum.

An earlier report (15) indicated that pure hemoglobin (1000 mg/L) does not interfere with the bleomycin assay. In our study, interference was observed in serum samples subjected to mechanical hemolysis during sample preparation; the interference may have been caused by heme released from hemoglobin or its degradation products. In line with our results, Pepper et al. (22) reported that some cardiopulmonary bypass patients who were subject to hemolysis during extracorporeal circulation had redox-active iron in their serum as measured by the bleomycin assay. Because the hemolysis took place *in vivo* in circulation, the presence of redox-active iron species in the serum of these patients probably was clinically important. Therefore, the cause of hemolysis should always be considered when evaluating the importance of redox-active iron in serum samples.

When we analyzed 399 serum samples from patients with hematologic malignancies by the bleomycin assay, we found NTBI-positive samples only when transferrin saturation was >80%, which suggested high specificity. The measured values were between 0 and 1  $\mu\text{mol/L}$ . Carmine et al. (23) found similar NTBI concentrations in patient samples, as measured by the bleomycin assay, whereas others have reported measured concentrations of 20–28  $\mu\text{mol/L}$  (9, 24). The higher total concentrations were found with an earlier version of the bleomycin assay (15) without the final butanol extraction step, which apparently reduces the risk of interference from precipitated proteins in the spectrophotometric measurement. Because these patients often have high serum ferritin concentrations, the ferritin interference was studied. No interference was found from ferritin, which was in accordance with results obtained using another type of NTBI assay based on iron chelation (10). The cyclophosphamide used for myeloablative treatment did not interfere with the bleomycin assay after *in vitro* addition. Furthermore, a sample blank value determined without the addition of bleomycin is always subtracted from each sample to assess possible interference from chemotherapy agents.

Transferrin saturation was calculated from the transferrin and iron determinations, which had assay CVs of 2.8–3.7%. An estimate of 4.8% for the imprecision of the transferrin saturation determination could be calculated from the respective assay CVs (25), indicating an acceptable precision for this method. The use of an immunochemical assay to measure transferrin in combination with a colorimetric iron assay is considered a reliable method to calculate transferrin saturation, provided that the correct molecular weight of transferrin is used (26). It has been suggested that this method shows less variability than the total iron-binding capacity method, especially at high ferritin concentrations (26, 27). In this study, the

point of full transferrin saturation was confirmed by urea-PAGE, in which only the diferric form of transferrin is detected at full saturation.

The bleomycin assay was compared with another method, which is based on the mobilization of NTBI with the iron chelator NTA and separation of the chelated iron by ultrafiltration. The iron in the ultrafiltrate was quantified by a chromogenic assay or AAS. The NTBI concentrations were in a range (0–10  $\mu\text{mol/L}$ ) similar to those found by others using the same type of assay (10, 14). The concentrations of chelatable NTBI showed variations in comparison with the bleomycin-detectable NTBI concentrations, with higher NTBI concentrations measured by the chelation assay. However, we found that unlike the bleomycin assay, in which positive samples were detected only when transferrin saturation was >80%, the chelation method also detected NTBI in some serum samples when the transferrin saturation was <80%. The presence of iron-free and monoferric transferrin in urea-PAGE confirmed the partial transferrin saturation in these samples.

The true nature in which NTBI exists in serum has been a matter of discussion. Ferric iron is virtually nonexistent as a free ion at neutral pH and readily forms colloidal, mostly insoluble hydroxide forms. On the other hand, serum contains small molecule-sized chelates such as citrate, which is considered an important complexing agent of NTBI (5). NTBI most likely also binds loosely to albumin (28) and to other plasma proteins, amino acids, peptides, and sugars. It has been suggested that the NTBI pool is heterogeneous with iron complexes with different affinities for chelators or iron scavengers at different proportions. The use of a chelator to mobilize NTBI in serum samples evidently is a reason for the higher NTBI concentrations measured by the chelation-based assay. The chelator probably mobilizes iron from complexes in which it is not available for the bleomycin reagent. Gosriwatana et al. (14) found that NTA was able to mobilize iron from albumin, but that it also mobilized small amounts of iron from other proteins, such as transferrin ( $\sim 0.5 \mu\text{mol/L}$ ) and ferritin ( $\sim 0.3 \mu\text{mol/L}$ ). The use of the chelating agent could also explain our finding of detectable NTBI in serum samples with <80% transferrin saturation if the chelating agent had mobilized a small amount of transferrin-bound iron. Further evidence of the risk of mobilizing a portion of transferrin-bound iron when using a chelating agent was provided by Breuer et al. (13), who used oxalate to chelate NTBI and shuttle it to immobilized desferrioxamine for detection. They found that in samples with transferrin saturation >60%, a substantial part (1–5%) of transferrin-bound iron was measured in the presence of oxalate, but not in its absence.

The recovery of added iron was higher (64%) in the chelation method than in the bleomycin method (33%), but neither assay could measure all of the FeNTA iron added to serum. FeNTA has been widely used to saturate transferrin in *in vitro* studies because of the better solubility of ferric iron in complex with NTA and its rapid

binding to transferrin (29). Addition of FeNTA to normal serum produced fully saturated transferrin, which was confirmed by urea-PAGE. However, it is not clear in which form the excess iron added to serum occurred and whether part of it formed insoluble ferric hydroxides. Such insoluble iron complexes may not be mobilized, even by NTA.

It is reasonable to conclude that the occurrence of NTBI is unlikely in serum with partial transferrin saturation, provided that there is no specific reason that iron binding by transferrin would be impaired, such as the severe ferrooxidase deficiency typically found in preterm babies (30, 31). The presence of NTBI at partial transferrin saturation has, however, been reported for patients with hemochromatosis measured both with the bleomycin assay and the chelation method (14, 32). It is therefore possible that some patient groups can have NTBI, or a portion of it, in a form that is not readily bound to transferrin. Our results with the bleomycin assay for patients with hematologic malignancies undergoing allogeneic stem cell transplantation did not indicate that NTBI might be present in any situations other than at >80% transferrin saturation unless the samples were clearly hemolyzed. The NTBI measured by the bleomycin assay is capable of binding to bleomycin and generating free radicals; it therefore is considered a measure of the redox activity of NTBI. The bleomycin assay thus specifically detects redox-active NTBI in serum samples, although probably cannot measure the total amount of NTBI.

## References

1. Halliwell B, Gutteridge JMC. Role of free radicals and catalytic metal ions in human disease: an overview. *Methods Enzymol* 1990;186:1–85.
2. Andersson GJ. Non-transferrin-bound iron and cellular toxicity. *J Gastroenterol Hepatol* 1999;14:105–8.
3. Hershko C, Graham G, Bates GW, Rachmilewitz EA. Non-specific serum iron in thalassemia: an abnormal serum iron fraction of potential toxicity. *Br J Haematol* 1978;40:255–63.
4. Porter A, Marshall L, Hider RC, Singh S. Kinetics of removal and reappearance of non-transferrin-bound plasma iron with deferoxamine therapy. *Blood* 1996;88:705–13.
5. Grootveld M, Bell JD, Halliwell B, Aruoma OI, Bomford A, Sadler PJ. Non-transferrin-bound iron in plasma or serum from patients with idiopathic hemochromatosis. Characterization by high performance liquid chromatography and nuclear resonance spectroscopy. *J Biol Chem* 1989;264:4417–22.
6. Hamill RL, Woods JC, Cook BA. Congenital atransferrinemia. A case report and review of literature. *Am J Clin Pathol* 1991;96:215–8.
7. Kime R, Gibson A, Yong W, Hider R, Powers H. Chromatographic method for the determination of non-transferrin-bound iron suitable for use on the plasma and bronchoalveolar lavage fluid of preterm babies. *Clin Sci* 1996;91:633–8.
8. Parkkinen J, von Bonsdorff L, Peltonen S, Grönhagen-Riska C, Rosenlöf K. Catalytically active iron and bacterial growth in serum of haemodialysis patients after *i.v.* iron-saccharate administration. *Nephrol Dial Transplant* 2000;15:1827–34.
9. Halliwell B, Aruoma OI, Mufti G, Bomford A. Bleomycin-detectable

- iron in serum from leukemic patients before and after chemotherapy. Therapeutic implications for treatment with oxidant-generating drugs. *FEBS Lett* 1988;241:202–4.
10. Bradley SJ, Gosriwatana I, Srichairatanakool S, Hider RC, Porter JB. Non-transferrin-bound iron induced by myeloablative chemotherapy. *Br J Haematol* 1997;99:337–43.
  11. Sahlstedt L, Ebeling F, von Bonsdorff L, Parkkinen J, Ruutu T. Non-transferrin-bound iron during allogeneic stem cell transplantation. *Br J Haematol* 2001;113:836–8.
  12. Singh S, Hider RC, Porter JB. A direct method for quantification of non-transferrin-bound iron. *Anal Biochem* 1990;186:320–3.
  13. Breuer W, Ronson A, Slotki IN, Abramov A, Hershko C, Cabantchik ZI. The assessment of serum nontransferrin-bound iron in chelation therapy and iron supplementation. *Blood* 2000;95:2975–82.
  14. Gosriwatana I, Loreal O, Lu S, Brissot P, Porter J, Hider R. Quantification of non-transferrin-bound iron in the presence of unsaturated transferrin. *Anal Biochem* 1999;273:212–20.
  15. Gutteridge JMC, Rowley DA, Halliwell B. Superoxide-dependent formation of hydroxyl radicals in the presence of iron salts. Detection of “free” iron in biological systems by using bleomycin-dependent degradation of DNA. *Biochem J* 1981;199:263–5.
  16. Mumby S, Masahiko K, Taniguchi N, Gutteridge JMC. Reactive iron species in biological fluids activate the iron-sulphur cluster of aconitase. *Biochim Biophys Acta* 1998;1380:102–8.
  17. Evans PJ, Halliwell B. Measurement of iron and copper in biological systems: bleomycin and copper-phenanthroline assays. *Methods Enzymol* 1994;223:83–92.
  18. Eskelinen S, Haikonen M, Räisänen S. Ferene-S as the chromogen for serum iron determinations. *Scand J Clin Lab Invest* 1983;43:453–5.
  19. Morgan EH. Transferrin. In: Haeberli A, ed. *Human protein data*. Weinheim: VCH, 1992.
  20. Beau AF. A method for hemoglobin in serum and urine. *Am J Clin Pathol* 1962;38:111–2.
  21. Williams J, Evans RW, Moreton K. The iron-binding properties of hen ovotransferrin. *Biochem J* 1978;173:535–42.
  22. Pepper JR, Mumby S, Gutteridge JMC. Blood cardioplegia increase plasma iron overload and thiol levels during cardiopulmonary bypass. *Ann Thorac Surg* 1995;60:1735–40.
  23. Carmine TC, Evans P, Bruchelt G, Evans R, Handgretinger R, Niethammer D, et al. Presence of iron catalytic for free radical reactions in patients undergoing chemotherapy: implications for therapeutic management. *Cancer Lett* 1995;94:219–26.
  24. Harrison P, Marwah SS, Hughes RT, Bareford D. Non-transferrin bound iron and neutropenia after cytotoxic chemotherapy. *J Clin Pathol* 1994;47:350–2.
  25. Kringler RO. Statistical procedures In: Burtis CA, Ashwood ER, eds. *Tietz textbook of clinical chemistry*, 2nd ed. Philadelphia: WB Saunders, 1994:384–453.
  26. Beilby J, Olynyk J, Ching S, Prins A, Swanson N, Reed W, et al. Transferrin index: an alternative method for calculating the iron saturation of transferrin. *Clin Chem* 1992;38:2078–81.
  27. Vernet M. Immunochemical assay of transferrin and iron saturation in serum. *Clin Chem* 1993;39:2352–3.
  28. Loban A, Kime R, Powers H. Iron-binding antioxidant potential of plasma albumin. *Clin Sci* 1997;93:445–51.
  29. Welch S. *Transferrin: the iron carrier*. Boca Raton, FL: CRC Press, 1992:1–291.
  30. Evans PJ, Evans R, Kovar IZ, Holton AF, Halliwell B. Bleomycin-detectable iron in the plasma of premature and full-term neonates. *FEBS Lett* 1992;303:210–2.
  31. Lindeman JHN, Lentjes EGWM, van Zoeren-Grobbe D, Berger HD. Postnatal changes in plasma ceruloplasmin and transferrin antioxidant activities in preterm babies. *Biol Neonate* 2000;78:73–6.
  32. Aruoma OI, Bomford A, Polson RJ, Halliwell B. Nontransferrin-bound iron in plasma from hemochromatosis patients: effect of phlebotomy therapy. *Blood* 1988;72:1416–9.