

Direct Serum Total Iron-binding Capacity Assay Suitable for Automated Analyzers

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Background: Present methods for measuring serum total iron-binding capacity (TIBC) involve manipulation of samples or performance of two assays on each sample. We developed a direct automated assay (DTIBC) for TIBC.

Methods: We added to serum a saturating amount of iron bound to an excess of chelating dye at a low pH, recorded a blank reading that represented the sum of the saturating amount of iron plus the serum iron, and then added a strong neutral pH buffer. The decrease in absorbance (as transferrin extracts iron from the iron-dye complex) is directly proportional to the TIBC. TIBC values for 125 patients were determined by DTIBC, alumina column TIBC (AC), magnetic particle TIBC (MTIBC), and the UIBC method (UIBC) on Roche COBAS FARA and Mira chemistry analyzers. In a separate study, TIBC values for 128 patients were determined on an Olympus AU400 by the DTIBC and the MTIBC methods.

Results: Methods comparisons on the COBAS analyzers yielded the following results: DTIBC = 1.05(MTIBC) – 1.0 $\mu\text{mol/L}$ ($r = 0.987$; $S_{y/x} = 2.6 \mu\text{mol/L}$); DTIBC = 1.07(AC) – 1.0 $\mu\text{mol/L}$ ($r = 0.982$; $S_{y/x} = 3.0 \mu\text{mol/L}$); and DTIBC = 1.14(UIBC) + 3.4 $\mu\text{mol/L}$ ($r = 0.982$; $S_{y/x} = 3.0 \mu\text{mol/L}$). A similar correlation study using the Olympus AU400 yielded DTIBC = 1.00(MTIBC) – 0.1 $\mu\text{mol/L}$ ($r = 0.983$; $S_{y/x} = 2.7 \mu\text{mol/L}$). The assay was linear from 12.5 to 125 $\mu\text{mol/L}$ (70–700 $\mu\text{g/dL}$) TIBC on the COBAS FARA. Within- and between-run imprecision (CV) was $\leq 4.8\%$ at two concentrations. Plasma samples were unsuitable for the method. No interference was seen with common interferants other than ascorbate, deferoxamine, and ferrous sulfate, and only at concentrations well above normal.

Conclusion: The new DTIBC assay is suitable for routine use in clinical laboratories and may improve the quality of iron metabolism studies.

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The serum total iron-binding capacity (TIBC) represents the maximum concentration of iron that can be bound by an individual's serum protein. Determination of TIBC is one of several commonly used assays in the assessment of iron status (1, 2), and TIBC is highly correlated with serum transferrin, the primary serum iron transport protein, because >95% of serum nonheme iron is bound by transferrin (1, 2). Usually, only ~30% of the available serum iron-binding sites are occupied, and changes in the ratio of serum iron to TIBC (calculated as the percentage of transferrin saturation) reflect changes in the body iron stores (1, 2). Clinically, TIBC determinations are useful in the diagnosis of iron-deficient anemia (3) and as a screening test in suspected cases of hereditary hemochromatosis, a common genetic disorder that causes chronic iron overload and severe health problems if untreated (4–6).

Several approaches are used at present to determine TIBC. One common method involves a pretreatment step. An excess of iron is added to fully saturate all the serum iron-binding sites; any remaining free iron is then removed by a solid-phase absorbent, and the TIBC is determined by a serum iron test. Various methods are used to remove the excess iron, including the following: magnesium carbonate adsorption and centrifugation, which is the proposed reference method (7, 8); alumina minicolumns (9); ion-exchange resins (10); and magnetically responsive alumina-containing particles (11). All of these methods require a separation step and involve manual manipulation of the samples. Alternative approaches include use of the unbound iron-binding capacity (UIBC), in which a fixed amount of iron is added to a sample at a neutral pH and the uptake of iron by the unoccupied serum iron-binding sites is measured by a serum iron method as a decrease in free iron. The TIBC is then calculated as the sum of the serum iron plus the UIBC. Although this approach can be automated, it re-

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quires two separate analyses: serum iron and serum UIBC. Existing UIBC methods generally exhibit a significant negative bias compared with TIBC methods [Ref. (12) and confirmed in this study]. This bias is also evident in proficiency surveys in which the calculated TIBC has a 7–10% negative bias relative to the proposed magnesium carbonate reference method. Although the reason for this negative bias has not been fully investigated, the use of an aqueous saturating/calibration solution with an assigned value rather than a serum-based calibrator is one possibility (12).

Another widely used method involves determination of the transferrin concentration by immunoassay, followed by a calculated conversion to TIBC (13). Recently, a method for the direct analysis of TIBC has been described in which a saturating amount of iron is added to the sample and any remaining excess iron is then reduced and reacted with ferrozine. This signal is removed by blanking the instrument. The pH is lowered, releasing iron from serum binding sites. This free iron is reduced and then measured colorimetrically as the TIBC. This approach requires three reagents (14, 15). We have developed an alternative direct method (patent pending) for the determination of serum TIBC that is suitable for automated analyzers and has several advantages over these existing methods.

Materials and Methods

DIRECT TIBC ASSAY PRINCIPLE

The Direct TIBC is a two-step assay. In the first step, the serum sample is added to reagent 1 (R1). R1 contains iron as ferric ion in sufficient quantity to saturate the highest anticipated TIBC in a complex with an excess of Chromazurol B in acetate buffer at pH 4.5. When the serum sample is added, the serum iron is released from transferrin because of the low pH. The iron from the sample then forms a complex with the remaining excess of Chromazurol B, increasing the absorbance. The resulting absorbance signal at the end of the first step is attributable to both the serum iron and the saturating amount of iron already present in R1. In the second step, reagent 2 (R2), which is strongly buffered and contains bicarbonate ions, is added. The affinity of transferrin for iron increases dramatically, and the transferrin extracts the iron from the iron–dye complex, decreasing the absorbance. The decrease in absorbance is directly proportional to the TIBC.

DIRECT TIBC REAGENT

The Direct TIBC reagent set consists of Reagent 1 (R1), which contains 166 $\mu\text{mol/L}$ Chromazurol B (Dojindo Molecular Technologies), 735 $\mu\text{mol/L}$ cetrimide (Sigma Chemical Co.), 4.1 mmol/L thiourea (Sigma), and 16 $\mu\text{mol/L}$ ferric chloride hexahydrate (Aldrich Chemical Co.) in acetate buffer, pH 4.55. Reagent 2 (R2) contains 338 mmol/L sodium bicarbonate (Sigma) in MOPS buffer, pH 7.3. The assay uses a serum-based calibrator with an

assigned TIBC value. Direct TIBC assays were run on a COBAS FARA II and the Olympus AU400.

DIRECT TIBC COBAS FARA METHOD

The following instrument settings were used for the Direct TIBC assay on the COBAS FARA: wavelength, 660 nm; temperature, 37 °C; instrument set for endpoint mode. The analyzer added 16 μL of sample to 200 μL of R1 and incubated the mixture for 5 min. The instrument then added 60 μL of R2 and immediately measured the absorbance for use as the blank. The mixture was then incubated for 7 min, and the final absorbance measurement was taken.

DIRECT TIBC OLYMPUS AU400 METHOD

The following instrument settings were used for Direct TIBC assay on the Olympus AU400: wavelength, 660 nm; temperature, 37 °C; instrument set for endpoint mode. The analyzer added 16 μL of sample to 200 μL of R1 and then incubated the mixture for 10 cycles. The instrument then added 60 μL of R2 and immediately measured the absorbance for the blank. The mixture was incubated for 17 cycles, and the final absorbance measurement was taken.

MAGNETIC TIBC METHOD

Magnetic TIBC reagent sets were from Equal Diagnostics, Inc. For studies using the COBAS instruments, supernates from the magnetic particle-treated samples were analyzed on a COBAS MIRA analyzer (Roche Diagnostics) with serum iron reagents from Thermo-DMA, Inc. For studies using the Olympus AU400, serum iron reagents from Olympus America, Inc. were used.

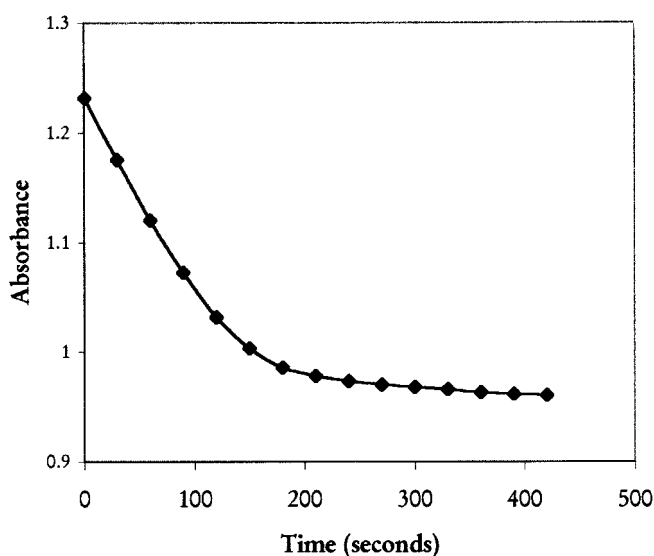


Fig. 1. Absorbance curve for a serum-based calibrator [assigned value, 64.1 $\mu\text{mol/L}$ (358 $\mu\text{g/dL}$)] in the Direct TIBC assay. Measured on the COBAS FARA II at 660 nm.

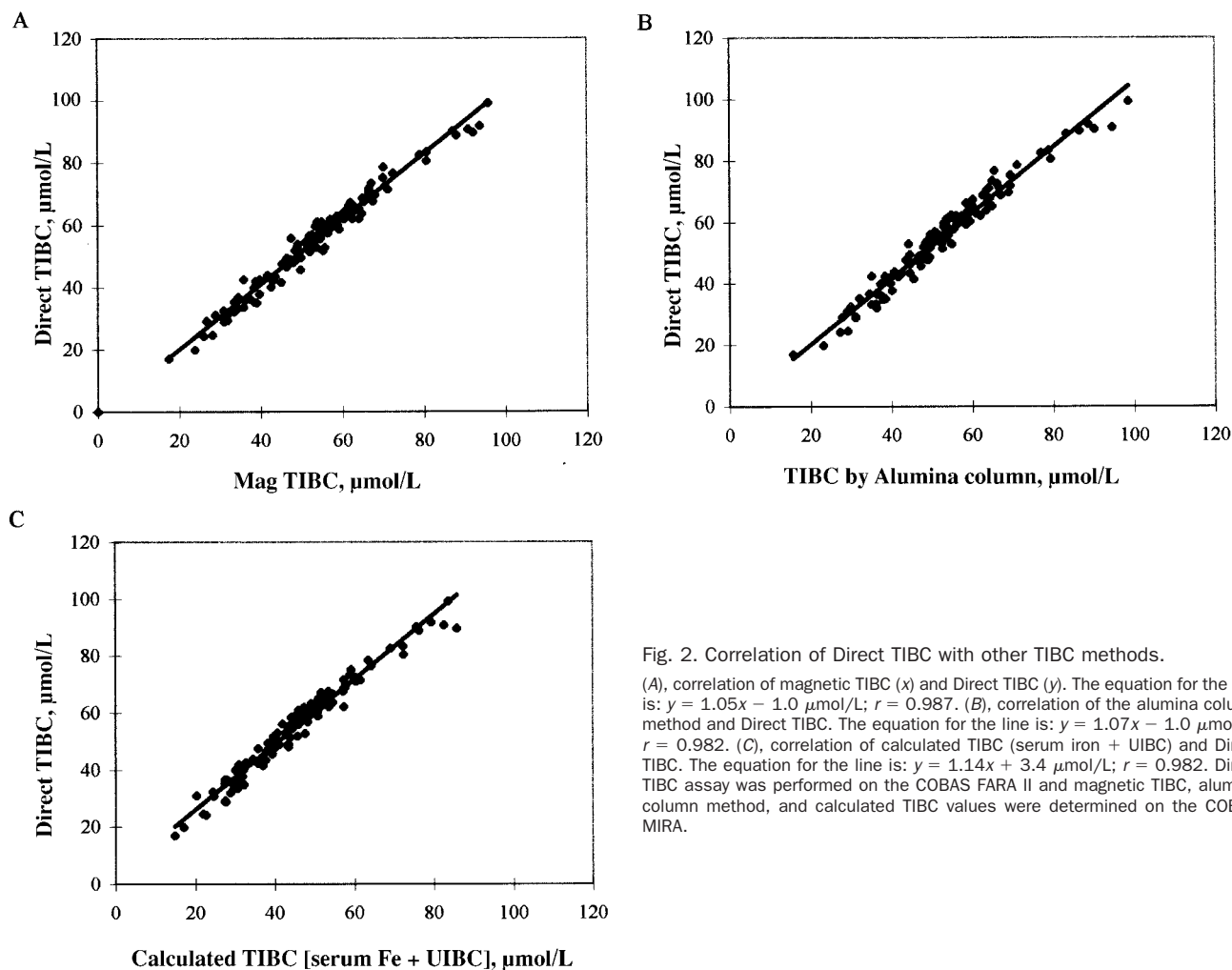


Fig. 2. Correlation of Direct TIBC with other TIBC methods.

(A), correlation of magnetic TIBC (x) and Direct TIBC (y). The equation for the line is: $y = 1.05x - 1.0 \mu\text{mol/L}$; $r = 0.987$. (B), correlation of the alumina column method and Direct TIBC. The equation for the line is: $y = 1.07x - 1.0 \mu\text{mol/L}$; $r = 0.982$. (C), correlation of calculated TIBC (serum iron + UIBC) and Direct TIBC. The equation for the line is: $y = 1.14x + 3.4 \mu\text{mol/L}$; $r = 0.982$. Direct TIBC assay was performed on the COBAS FARA II and magnetic TIBC, alumina column method, and calculated TIBC values were determined on the COBAS MIRA.

ALUMINA COLUMN METHOD

Alumina columns and saturating solution were obtained as a reagent set (J&S Medical Associates, Inc). Column-treated samples were analyzed on a COBAS MIRA analyzer with Thermo-DMA serum iron reagents.

CALCULATED TIBC BY UIBC AND SERUM IRON METHOD

Serum iron and UIBC were determined on a COBAS MIRA with serum iron/UIBC reagents from Thermo-DMA. The TIBC was then calculated as the sum of the UIBC and serum iron.

CORRELATION STUDIES

A total of 125 serum samples with TIBC values ranging from 17.0 to 99.2 $\mu\text{mol/L}$ (95–554 $\mu\text{g/dL}$) were analyzed on the COBAS MIRA analyzer by all three TIBC methods, and the results were compared by linear regression analysis. In a separate study, the results of another set of 128 serum samples with TIBC method values ranging from 23.6 to 98.8 $\mu\text{mol/L}$ (132–552 $\mu\text{g/dL}$) were analyzed by the Direct TIBC on an Olympus AU400 analyzer, and the results were compared with those obtained with the magnetic TIBC assay on the same analyzer.

PRECISION STUDIES

We assessed assay precision with two commercially available serum-based chemistry controls, Multiquel 1 and Multiquel 3 (Bio-Rad Laboratories), on both the COBAS FARA and Olympus AU400. Within-run precision was assessed by analysis of 25 replicates in one run. Between-run precision was assessed by analysis of 25 replicates in 25 separately calibrated runs during the course of these studies.

LINEARITY STUDY

We determined the linearity of the assay by adding additional human transferrin to a serum pool to make an increased-TIBC pool. This pool was then serially diluted with physiologic saline to create a set of linearity samples.

INTERFERENCE STUDIES

We assessed the effects of lipemia (expressed as triglycerides), hemolysis, bilirubin, ascorbate, copper, chromium, nickel, zinc, iron dextran, ferrous sulfate, and deferoxamine by adding each potential interfering compound at various concentrations to serum pools and

Table 1. Precision studies.

	Multiqual 1	Multiqual 3
COBAS FARA		
Within-run precision (n = 25)		
Mean	44.8 $\mu\text{mol/L}$ (250 $\mu\text{g/dL}$)	79.8 $\mu\text{mol/L}$ (446 $\mu\text{g/dL}$)
SD	1.6 $\mu\text{mol/L}$ (9.0 $\mu\text{g/dL}$)	1.5 $\mu\text{mol/L}$ (8.2 $\mu\text{g/dL}$)
CV, %	3.6	1.9
Between-run precision (n = 25)		
Mean	44.2 $\mu\text{mol/L}$ (247 $\mu\text{g/dL}$)	80.7 $\mu\text{mol/L}$ (451 $\mu\text{g/dL}$)
SD	1.7 $\mu\text{mol/L}$ (9.5 $\mu\text{g/dL}$)	1.9 $\mu\text{mol/L}$ (10.4 $\mu\text{g/dL}$)
CV, %	3.8	2.4
Olympus AU400		
Within-run precision (n = 25)		
Mean	43.5 $\mu\text{mol/L}$ (243 $\mu\text{g/dL}$)	83.4 $\mu\text{mol/L}$ (466 $\mu\text{g/dL}$)
SD	0.8 $\mu\text{mol/L}$ (4.7 $\mu\text{g/dL}$)	0.8 $\mu\text{mol/L}$ (4.2 $\mu\text{g/dL}$)
CV, %	1.9	0.9
Between-run precision (n = 25)		
Mean	43.8 $\mu\text{mol/L}$ (245 $\mu\text{g/dL}$)	85.2 $\mu\text{mol/L}$ (476 $\mu\text{g/dL}$)
SD	2.1 $\mu\text{mol/L}$ (11.7 $\mu\text{g/dL}$)	2.6 $\mu\text{mol/L}$ (14.3 $\mu\text{g/dL}$)
CV, %	4.8	3.0

testing for the recovery of TIBC. We assessed the effect of the anticoagulants heparin, EDTA, and oxalate/sodium fluoride by collecting blood from three volunteers simultaneously into a serum tube and heparin, EDTA, and oxalate/sodium fluoride plasma tubes and comparing the recovery in the Direct TIBC assay between serum and the various plasma samples. Informed consent was obtained from the volunteers.

STATISTICAL ANALYSES

Means, SDs, and CVs were calculated, and correlations were assessed by least-square regression analysis with Microsoft Excel software (Microsoft, Inc.) and a statistical macro-package, SPC KISS, Ver. 2.0 (Digital Computations, Inc.), which works as part of the Microsoft Excel program.

Results

STUDIES USING THE COBAS FARA II

A plot of the absorbance vs time for a serum-based calibrator with an assigned value of 64.1 $\mu\text{mol/L}$ (358 $\mu\text{g/dL}$) in the Direct TIBC assay on the COBAS FARA is shown in Fig. 1. The observed absorbance change was 3.9 mA per $\mu\text{mol/L}$ TIBC (0.76 mA per $\mu\text{g/dL}$ TIBC). To assess the linearity of the Direct TIBC assay, we prepared a set of samples by serial diluting an increased serum TIBC pool and assaying the dilutions in the Direct TIBC assay. The assay was linear between 12.5 and 125 $\mu\text{mol/L}$ (70–700 $\mu\text{g/dL}$). The Direct TIBC assay was highly correlated with both the magnetic TIBC and alumina column TIBC methods (see Fig. 2). Statistical analysis of the results yielded the following correlations: for magnetic TIBC vs Direct TIBC, $r = 0.987$ ($P < 0.001$), $S_{y|x} = 2.6 \mu\text{mol/L}$, slope = 1.05, y -intercept = $-1.0 \mu\text{mol/L}$ ($-5.4 \mu\text{g/dL}$); for TIBC by alumina column vs Direct TIBC, $r = 0.982$ ($P < 0.001$), $S_{y|x} = 3.0 \mu\text{mol/L}$, slope = 1.07, intercept = $-1.0 \mu\text{mol/L}$ ($-5.8 \mu\text{g/dL}$). The calculated TIBC and the

Direct TIBC were also highly correlated ($r = 0.982$; $P < 0.001$; $S_{y|x} = 3.0 \mu\text{mol/L}$), but with a proportional bias (slope = 1.14; $SE_{\text{slope}} = 0.2$) and a y -intercept of 3.4 $\mu\text{mol/L}$ (18.8 $\mu\text{g/dL}$; see Fig. 2). Analysis of a subset of these data by two other commercially available UIBC and serum iron methods yielded similar biases (data not shown). This bias is similar to that reported in other studies (12).

The precision of the Direct TIBC was assessed with two serum-based commercially available chemistry controls. The results are summarized in Table 1, and both the within- and between-run imprecision (CV) for the COBAS FARA was $\leq 3.8\%$. Comparison of plasma and serum samples demonstrated that plasma is not an acceptable sample for use in the Direct TIBC assay, most likely because of competitive chelation of iron by anticoagulants (see Table 2).

We assessed the specificity of the assay for iron vs other transition metals by supplementing serum pools with concentrations of copper (0–471 $\mu\text{mol/L}$), zinc (0–38.3 $\mu\text{mol/L}$), nickel (0–85 nmol/L), and chromium (0–962 nmol/L) that exceeded the highest reported reference values for those metals; no interferences were noted. This confirms other published studies on the use of Chromazuro B in iron determinations (16). We examined the effect of lipemia by mixing a specimen with increased

Table 2. Effects of anticoagulants on Direct TIBC assay.

Volunteer	Serum TIBC, $\mu\text{mol/L}$	Recovery, ^a %		
		Heparin-plasma	EDTA-plasma	Oxalate/NaF-plasma
A	64.9	105.1	25.8	29.8
B	60.3	112.4	38.0	48.1
C	71.6	129.3	37.5	48.6

^a Recovery = percentage of serum TIBC.

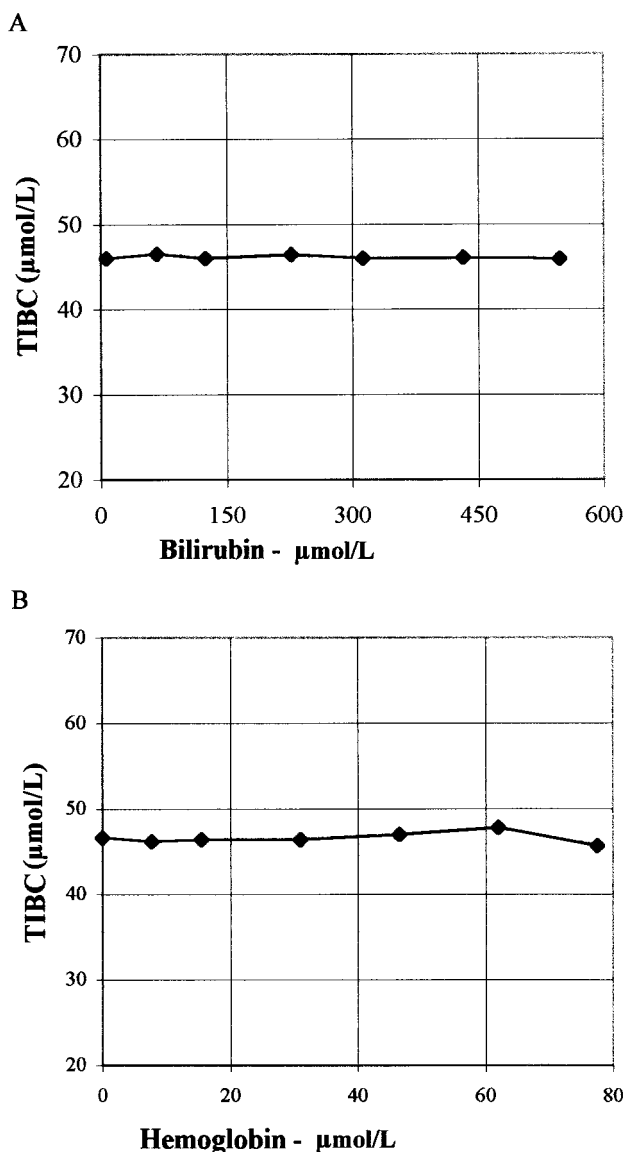


Fig. 3. Effect of increased bilirubin and hemoglobin concentrations on Direct TIBC assay.

Conventional units: bilirubin, 0–32 mg/dL; hemoglobin, 0–500 mg/dL.

triglyceride and TIBC concentrations proportionally with a specimen with low triglyceride and TIBC concentrations. Triglyceride concentrations up to 14.9 mmol/L (1318 mg/dL) had no effect on recovery. Bilirubin up to 547.2 µmol/L (32 mg/dL) and hemoglobin up to 77.5 µmol/L (500 mg/dL) demonstrated no interference (Fig. 3), and ascorbate decreased Direct TIBC values only at high concentrations well above those attainable even with megadose oral administration of ascorbic acid (17). We examined the effects of iron supplements by adding them to serum pools (Table 3). Iron dextran had no effect on assay performance. Ferrous sulfate >72 µmol/L (400 µg/dL) iron added decreased measured TIBC. This result is to be expected because the TIBC is exceeded and the free iron exceeds the capacity of the dye. However, serum

Table 3. Effect of iron supplements on results of the Direct TIBC assay.

Iron dextran added (as iron), µmol/L (µg/dL)	TIBC of % initial value
256 (1430)	100.9
192 (1072)	100.0
128 (715)	102.0
64 (358)	103.5
25.6 (143)	100.0
Ferrous sulfate added (as iron), µmol/L (µg/dL)	
358 (2000.0)	0.0
268 (1500.0)	13.4
179 (1000.0)	49.2
143 (800.0)	60.2
90 (500.0)	80.2
72 (400.0)	92.5
36 (200.0)	102.2

iron concentrations >72 µmol/L (400 µg/dL) would represent an acute overdose, not a therapeutic dose. The results suggest that the Direct TIBC assay, like other TIBC assays (18), would be of limited use in cases of acute overdose with iron. Added deferoxamine increased Direct TIBC values only at concentrations >44.6 µmol/L, well above therapeutic serum concentrations (19).

STUDIES USING THE OLYMPUS AU400

The Direct TIBC assay was highly correlated with the magnetic TIBC when both assays were performed on the Olympus AU400 (see Fig. 4). Statistical analysis of the results yielded the following correlations: magnetic TIBC vs Direct TIBC, $r = 0.983$ ($P < 0.001$; $S_{y/x} = 3.0$ µmol/L);

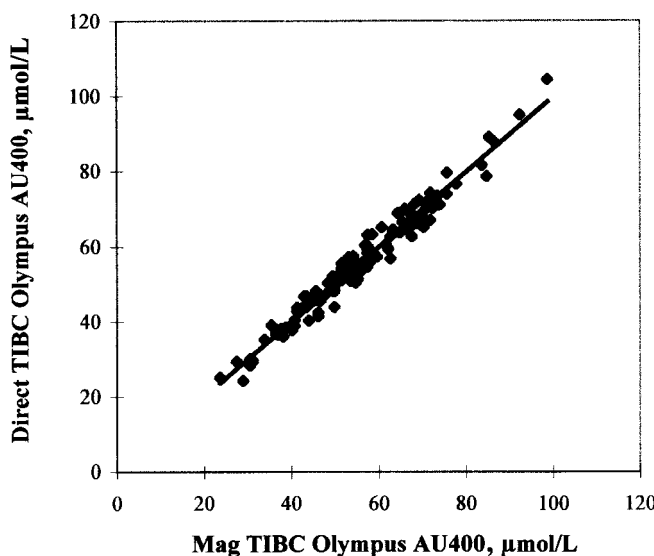


Fig. 4. Correlation of the Direct TIBC assay with magnetic TIBC on the Olympus AU400.

Equation for the line: $y = 0.997x + 0.1$; $r = 0.983$.

slope = 0.997; y -intercept = 0.1 $\mu\text{mol/L}$ (0.4 $\mu\text{g/dL}$). Within- and between-run imprecision (CV) were $\leq 4.8\%$.

Discussion

There is renewed interest in analyses measuring various aspects of iron metabolism. Recent studies have demonstrated that hereditary hemochromatosis is one of the most common genetic disorders (6), and the utility of screening the general population is being examined (5). Several approaches for screening the general population have been proposed. These include determining the percentage of transferrin saturation, which requires determination of serum TIBC or transferrin (4, 5), or alternatively, genetic testing for the C282Y and H63D mutations in the *HFE* gene (20, 21). Furthermore, TIBC is useful in the diagnosis of iron deficiency, which remains the most prevalent nutritional deficiency in the US (3). Present methods for determining serum TIBC have numerous drawbacks, including manual manipulation of the samples, separation steps, the requirement for more than one analysis, and the use of nonserum calibrators.

We have described a new, fully automated, Direct TIBC assay suitable for most chemistry analyzers. The method compares well with the existing alumina column and magnetic alumina particle TIBC methods, is specific for TIBC, and is unaffected by lipemia, bilirubinemia, hemolysis, and other potentially interfering compounds. Furthermore, the method is calibrated with a serum-based calibrator. In contrast to another recently reported direct TIBC method (14, 15), our method does not require a reduction step and requires only two reagents. We believe that this Direct TIBC method is suitable for routine use in clinical chemistry laboratories and will improve the quality of iron metabolism studies.

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