We established a method for fully automated measurement of total iron-binding capacity (TIBC) in serum without separation of the unbound excess iron after saturating serum transferrin. After saturation of serum transferrin with an excess amount of iron (first step), the unbound iron was eliminated by formation of a complex with ferrozine, which was used as a chromogenic reagent (second step). For the TIBC assay, iron dissociated from transferrin by shifting the pH to acidic was reacted with ferrozine, and the increase in the absorbance at 570 nm was measured (third step). Because the iron used as a calibrator, which was added to saturate transferrin, reacted completely with ferrozine in the second step (elimination of unbound iron), the change in the absorbance to generate a calibration factor could not be monitored in the third step. To solve this problem, we used N-(2-hydroxyethyl)ethylenediamine-N,N',N'-triacetic acid (HEDTA) to complex with the iron added to saturate transferrin in the second step. This made it possible to form an iron–ferrozine complex at acidic pH because iron was dissociated from HEDTA at acidic pH. The within-run CVs of this method were 0.66–2.43% at 17.7–77.0 μmol/L, and the day-to-day CVs were 1.06–1.57% at 29.9–60.4 μmol/L (n = 10). The correlation between the values obtained with this method (y) and those from the direct TIBC assay, which involved removal of unbound iron by ion-exchange resin (x), was: y = 0.963x + 0.29 μmol/L (r = 0.973, Sy,x = 2.83, n = 59), and with the TIBC values calculated from the serum iron concentrations and the unbound iron-binding capacities measured by a direct colorimetric method (x) was: y = 1.01x – 1.06 μmol/L (r = 0.994, Sy,x = 1.66, n = 51).

Total iron-binding capacity (TIBC) indicates the total amount of iron to saturate plasma or serum transferrin, which is a binding protein for iron. The measurement of serum iron, TIBC, and the percent saturation of transferrin with iron [(serum iron/TIBC)100] has been used to assess the state of iron deficiency and for other purposes in clinical medicine [1].

Among the several methods for measurement of serum iron, the direct colorimetric methods, which do not involve sample deproteinization, are widely used and have been modified as a fully automated assay for routine analysis [2,3]. On the other hand, the measurement of TIBC consists of three steps: saturation of transferrin by addition of an excess amount of iron, removal of unbound iron by absorption with magnesium carbonate [4–6] or ion-exchange resin [7], and finally determination of iron that is dissociated from transferrin at acidic pH.

However, the step for removal of unbound iron, requiring centrifugation, is an impediment to adaptation of the TIBC assay to a fully automated analysis. Moreover, it is difficult to generate a calibration factor necessary for the assay. When iron solution without binding protein is used as a calibrator, the unbound iron is removed in the elimination step, and therefore the reaction to obtain a calibration factor cannot be monitored in the next step. The ideal calibrator needs to bind a known amount of added iron at alkaline pH and dissociate completely at acidic pH, as serum transferrin does. To overcome this problem, we used as a calibrator a chelating agent that has a large stability constant with iron at an alkaline condition and succeeded in establishing a fully automated method for measurement of TIBC.

**Materials and Methods**

**REAGENT**
Analytical-grade sodium dihydrogen phosphate, manganese sulfate, copper sulfate, thiourea, ferric chloride, and

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1 Nonstandard abbreviations: TIBC, total iron-binding capacity; UIBC, unsaturated iron-binding capacity; HEDTA, N-(2-hydroxyethyl)ethylenediamine-N,N',N'-triacetic acid.
Preparation of Reagents
The first reagent (R1) was made up with 300 mmol/L Tris, 150 mmol/L sodium hydrogen carbonate, and 4.2 g/L Triton X-100 (pH 8.4). The second reagent (R2) was made up with iron calibrator solution (180 μmol/L Fe³⁺ in 10 mmol/L HCl), which was diluted 10-fold with R1. The third reagent (R3) was made up with 10 mmol/L ferrozine and 40 mmol/L L-ascorbic acid in Tris buffer (50 mmol/L, pH 5.0). The fourth reagent (R4) was made up with 600 mmol/L citric acid and 25.6 mmol/L thiourea, adjusted to pH 2.0 with 300 mmol/L sodium dihydrogen phosphate. Physiological saline (NaCl 150 mmol/L) and 20 mmol/L HEDTA in 0.03 mmol/L NaOH were used as zero and high calibrators, respectively.

Apparatus
The data obtained by the fully automated TIBC assay were compared with those obtained with conventional methods by use of the Model 7070 automated analyzer from Hitachi (also known as the Hitachi 911).

Assay Principle
Our fully automated assay consisted of three steps of reactions. Table 1 shows the scheme of each step in our method. We then used HEDTA as a calibrator to generate a calibration factor. This chelating agent has a large stability constant with Fe³⁺ metal ligand (log K⁵¹ = 19.8) at pH 7–12 [8].

In the first step, serum transferrin was saturated by the iron calibrator solution (180 μmol/L). Then the unbound iron was reduced to Fe²⁺ by ascorbic acid and eliminated by formation of a complex with ferrozine [9] used as a chromogenic reagent (second step).

Table 1. Scheme of the assay.

<table>
<thead>
<tr>
<th>Step</th>
<th>Sample</th>
<th>Reagent</th>
<th>What happens</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Serum, 50 μL or calibrator, 50 μL (HEDTA)</td>
<td>R1 (Tris buffer, pH 8.4), 200 μL</td>
<td>R2 is an iron solution used to saturate transferrin in serum. HEDTA (calibrator) chelates an iron calibrator. This takes 3.3 min.</td>
</tr>
<tr>
<td>2</td>
<td>From Step 1</td>
<td>R2 (iron calibrator solution), 50 μL</td>
<td>Ferrozine color reagent binds to all free iron but not to HEDTA iron. This takes 4.9 min.</td>
</tr>
<tr>
<td>3</td>
<td>From Step 2</td>
<td>R3 (ferrozine), 50 μL</td>
<td>Changes pH from 8.4 to 3.9. Iron released from transferrin and HEDTA reacts with ferrozine to produce additional color. Absorbance change between Step 2 and Step 3 provides direct measure of TIBC. This takes 5.9 min.</td>
</tr>
</tbody>
</table>

Triton X-100 were purchased from Wako Pure Chemical Industries; sodium hydrogen carbonate, citric acid, and L-ascorbic acid were purchased from Katayama Chemical. Ferrozine and N-(2-hydroxyethyl)ethylenediamine-N,N',N''-triacetic acid (HEDTA) were purchased from Dojindo Laboratory. Tris was purchased from Sigma Chemical Co., and lipemic turbidity and bilirubin conjugate were purchased from International Reagent. HemoLyse were purchased from Nippon Superior. Triton X-100 were purchased from Wako Pure Chemical Industries; sodium hydrogen carbonate, citric acid, and L-ascorbic acid were purchased from Katayama Chemical. Ferrozine and N-(2-hydroxyethyl)ethylenediamine-N,N',N''-triacetic acid (HEDTA) were purchased from Dijindo Laboratory. Tris was purchased from Sigma Chemical Co., and lipemic turbidity and bilirubin conjugate were purchased from International Reagent.
CUP, transferrin in the serum is saturated with the resin-bound iron, and all excess iron remains bound to the resin beads. We also calculated the TIBC value from the serum iron concentration and the unsaturated iron-binding capacity (UIBC) value obtained by Unimate and Unimate UIBC (Hoffmann-La Roche).

**Results**

**Optimization studies**

We examined the effect of ferrozine and ascorbic acid concentrations on the overall reaction. The maximal reaction rate was achieved when the ferrozine concentration was \(0.56 \text{ mmol/L}\) and the ascorbic acid concentration was \(2.22 \text{ mmol/L}\). We thus used 1.1 mmol/L ferrozine and 4.4 mmol/L ascorbic acid in the fully automated method.

We also examined the effect of the HEDTA concentration used as a calibrator, which behaved in the same manner as transferrin. When the HEDTA concentration was \(5 \text{ mmol/L}\), a slight increase in absorbance was observed after addition of R3 (elimination step). When the HEDTA concentration was \(20 \text{ mmol/L}\), there was no increase of absorbance, indicating that dissociated iron was not present. We thus used this concentration as the calibrator.

The pH of the mixture of the sample and calibrator after addition of R3 was 8.39 ± 0.03, and the pH of the mixture was 3.90 ± 0.05 at the final step. The typical time course of the calibrator and human serum is shown in Fig. 1. The reaction between unbound iron and ferrozine ended within 1 min, and dissociation of iron from transferrin and formation of the ferrozine–iron complex ended within 2 min after addition of R4.

To examine dissociation of iron from transferrin after addition of R4, we determined the serum iron concentration by our method but without saturation of transferrin and compared the results with those from the Unimate method. The correlation between values obtained with our method \((y)\) and the Unimate method \((x)\) was: \(y = 0.965x - 0.99 \text{ µmol/L}\) \((r = 0.995, S_{xy} = 0.93, n = 40)\).

**Assay evaluation**

To study the linearity of the calibration curve, we assayed the calibrator in duplicate with six concentrations of R2 (0–144 µmol/L). The calibration curve was straight for iron concentrations up to 180 µmol/L. We also assayed several human sera that were diluted with physiological saline. The dilution curve was linear for TIBC values up to 90 µmol/L (Fig. 2).

We also examined the detection limit of our method by assaying the zero calibrator (physiological saline) 10 times; the result (mean ± SD) was 0.63 ± 0.40 µmol/L.

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Fig. 1. Typical time course of overall reaction.

\(\Delta\), calibrator solution (20 mmol/L HEDTA); ○, zero calibrator (physiological saline); ●, human serum.

Fig. 2. Dilution curve of human serum.

Several human sera were serially diluted with physiological saline up to fivefold.
The detection limit, defined as the mean TIBC value of the zero calibrator + 3 SD, was 1.83 μmol/L.

To examine the precision of our method, we assayed pooled human sera. As shown in Table 2, the within-assay CVs ranged from 0.66% to 2.43% and the day-to-day CVs were from 1.06% to 1.57%. Our method was compared with the TIBC CUP method as well as the Unimate method.

As shown in Fig. 3, the correlation between values obtained with our method (y) and the TIBC CUP method (x) was: $y = 0.963x + 0.29 \text{ μmol/L (} r = 0.973, S_y/x = 2.83, n = 59)$ and with the Unimate method (x) was: $y = 1.01x - 1.06 \mu\text{mol/L (} r = 0.994, S_y/x = 1.66, n = 51)$.

We found no interference with TIBC ranging from 27.2 to 58.7 μmol/L by addition of 150 mg/L bilirubin, 100 μmol/L Mn$^{2+}$, 100 μmol/L Cu$^{2+}$, and 2460 formazine turbidity units of the lipemic material to pooled human sera. Table 3 shows the negative effects of hemolysis on this method. We then tested the absorption spectrum of the overall reaction in a hemolytic serum sample (100 mg/L hemoglobin). The reaction with unbound iron and ferrozine showed two peaks at 542 and 576 nm. After addition of R4, the increase of absorbance occurred in the range of 600 and 700 nm with a peak at 630 nm. The cause of this peak is unknown. When the absorbance at 660 nm (reference) was subtracted from the absorbance at 570 nm with the hemolytic sample, the absorbance change was about −25%, which was a negative error as compared with the nonhemolytic sample. This unknown peak was not observed when physiological saline was used as the sample and hemolysate was added.

We also examined the interference by ferritin iron. Serum samples containing ferritin concentrations of 2000–12 000 mg/L were measured by our method, and the results were compared with those obtained by the TIBC CUP method and the Unimate method. The values obtained by our method showed no positive error as compared with two other methods.

**Discussion**

Although several methods exist for measurement of TIBC [4–7, 10, 11], these methods need to absorb unbound iron after saturation of serum transferrin by magnesium carbonate or ion-exchange resin and removal by centrifugation. Therefore, it is difficult to adapt those methods to

<p>| Table 2. Precision for fully automated assay for TIBC. |</p>
<table>
<thead>
<tr>
<th>Mean ± SD, μmol/L</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within-run$^a$</td>
<td></td>
</tr>
<tr>
<td>17.7 ± 0.43</td>
<td>2.43</td>
</tr>
<tr>
<td>27.2 ± 0.51</td>
<td>1.87</td>
</tr>
<tr>
<td>51.8 ± 0.49</td>
<td>0.95</td>
</tr>
<tr>
<td>71.9 ± 0.70</td>
<td>0.97</td>
</tr>
<tr>
<td>77.0 ± 0.51</td>
<td>0.66</td>
</tr>
<tr>
<td>Between-run$^a$</td>
<td></td>
</tr>
<tr>
<td>29.9 ± 0.47</td>
<td>1.57</td>
</tr>
<tr>
<td>50.0 ± 0.53</td>
<td>1.06</td>
</tr>
<tr>
<td>60.4 ± 0.94</td>
<td>1.56</td>
</tr>
</tbody>
</table>

$^a$ n = 10 each.

<p>| Table 3. Effect of hemolysates. |</p>
<table>
<thead>
<tr>
<th>Hemoglobin added, g/L</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>25.6</td>
<td>57.1</td>
<td>60.8</td>
</tr>
<tr>
<td>0.2</td>
<td>23.9</td>
<td>54.0</td>
<td>60.5</td>
</tr>
<tr>
<td>0.4</td>
<td>22.3</td>
<td>52.6</td>
<td>58.5</td>
</tr>
<tr>
<td>0.6</td>
<td>19.8</td>
<td>51.1</td>
<td>56.3</td>
</tr>
<tr>
<td>0.8</td>
<td>14.6</td>
<td>47.7</td>
<td>53.1</td>
</tr>
<tr>
<td>1.0</td>
<td>11.9</td>
<td>44.8</td>
<td>50.9</td>
</tr>
</tbody>
</table>

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Fig. 3. Comparison of serum TIBC results.  
A, the present method vs the TIBC CUP method; B, the present method vs the Unimate method.
fully automated assay of TIBC. The TIBC value can also be calculated from the serum iron concentration and the UIBC value (TIBC = serum iron + UIBC), but we believe that the total amount of iron that is really bound to transferrin should be measured as TIBC.

A calibration factor could be generated by the use of HEDTA as a calibrator. The final absorbance of the calibrator after addition of R4 was the same as that of the zero calibrator (Fig. 1). This indicated that iron formed a complex with ferrozine completely in the presence of HEDTA.

The values obtained by our method without saturation of serum transferrin indicated that the serum iron concentrations correlated well with the values obtained with the Unimate method. There also was a good correlation between our method and the TIBC CUP method or other methods. These data suggested that the binding sites of serum transferrin were well saturated with added iron and that iron was dissociated from transferrin after addition of R4.

Recently, we reported in patients with hyperferritineemia that the serum iron concentration measured by the method proposed by the International Committee for Standardization in Haematology [12, 13] and a constant-potential coulometric method had positive errors caused by liberation of iron from circulating ferritin by deproteinization or the acidic solvent [14, 15]. However, no interference from ferritin was observed with the present method. This result means that transferrin-bound iron was released specifically from transferrin in the acidic condition, even at high concentrations of ferritin.

In summary, our fully automated method does not require any absorbents for removal of unbound iron or centrifugation. Moreover, the total amount of iron bound to serum transferrin is measured as TIBC without interference from other cations, bilirubin, lipemic material, or ferritin. We thus believe that our fully automated method for serum TIBC may be suitable for routine clinical use in the laboratory.

References