New Enzymatic Assay of Iron in Serum

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A new enzymatic method for assaying iron in serum samples, suitable for automated analyzers, is reported. Three reagent mixtures are used: dilution buffer (pH 3.0; ascorbate), reagent 1 (pH 6.7; apoaconitase), and reagent 2 (pH 7.7; citrate, magnesium, and isocitrate dehydrogenase). Sera are diluted with dilution buffer. Fe^{3+} is liberated from transferrin in sera under acidic conditions, and then reduced by ascorbate. Reagent 1 is added to diluted specimens, and apoaconitase is reactivated by Fe^{2+} at neutral pH. The resulting solutions are mixed with reagent 2, so that holoaconitase hydrolyzes citrate to isocitrate and the isocitrate and $NADP^+$ are converted to 2-oxoglutarate, NADPH, and CO_2. Serum iron is determined linearly up to 70 μmol/L, with within-run CVs ≤2.4% and day-to-day CVs ≤2.9%. This method (*y*) gives results correlating with those of a Reference Method (*x*) proposed by the International Committee for Standardization in Haematology: $y = 0.98x + 0.38$ μmol/L (n = 72, $r = 0.996$, $S_{y|x} = 0.63$ μmol/L). The mean (±SD) serum iron concentrations measured by our method were 18.5 ± 5.4 and 15.2 ± 6.0 μmol/L for 63 males and 166 females, respectively.

Indexing Terms: *aconitase/apoenzyme/holoenzyme/transferrin/intermethod comparison*

In human serum, most iron is bound to transferrin, a carrier protein for iron (*1*). The concentration of iron in serum is a useful index for diagnosis of various anemias and liver diseases (*2*).

Matsubara (*3*) reported a method for assaying serum iron, using bathophenanthroline as the iron-binding chromogen. The International Committee for Standardization in Haematology (ICSH) proposed a Reference Method with bathophenanthroline sulfonate (*4*).[2] Later, they proposed the deproteinization of serum specimens before assay to improve accuracy (*5*) and, in 1990, proposed replacing the chromogen with ferrozine or ferene, which were more sensitive and cheaper, and reducing the volume of the specimens from 2.0 to 0.5 mL (*6*). These methods all involved manual procedures, which were too complicated to be practiced routinely in clinical laboratories.

Yamada et al. (*7, 8*) reported a simpler method involving chromazurol B or 2-nitroso-5-(*N*-propyl-*N*-sulfopropylamino)phenol (nitroso-PSAP); 20–50 μL of specimen was required but deproteinization was not. However, bilirubin, always present at different concentrations in sera, introduced error into these methods involving iron chromogens.

We report here a new enzymatic rate assay of serum iron with automated analyzers. Our method is based on the fact that aconitase (EC 4.2.1.3), which is inactivated by removing the bound iron, is reactivated by the iron liberated from transferrin by acidification of serum specimens and reduced with ascorbate. Our method requires 5 μL of specimen, and is not influenced by substances such as bilirubin, hemoglobin, and lipid, even at the highest concentrations ever found for these in abnormal serum specimens.

Principle

The sequence of reactions in our method is shown below. Transferrins bound with and without iron are called ferritransferrin and apotransferrin, respectively.

$$\text{Ferritransferrin} \xrightarrow{\text{acidic pH}} Fe^{3+} + \text{apotransferrin} \quad (1)$$

$$Fe^{3+} + \text{ascorbate} \xrightarrow{\text{neutral pH}} Fe^{2+} + \text{dehydroascorbate} \quad (2)$$

$$Fe^{2+} + \text{apoaconitase} \rightarrow \text{holoaconitase} \quad (3)$$

$$\text{Citrate} + H_2O \xrightarrow{\text{holoaconitase}} \text{isocitrate} \quad (4)$$

$$\text{Isocitrate} + NADP^+ \xrightarrow{\text{ICD}} \text{2-oxoglutarate} + \text{NADPH} + CO_2 \quad (5)$$

The overall reaction for the assay is thus:

$$\text{Ferritransferrin} + \text{ascorbate} + \text{apoaconitase} + \text{citrate} + NADP^+ + H_2O \rightarrow \text{apotransferrin} + \text{dehydroascorbate} + \text{holoaconitase} + \text{2-oxoglutarate} + \text{NADPH} + CO_2$$

The serum iron in the specimen can be determined by measuring the production of NADPH.

Materials and Methods

Apparatus

We used the Model 7150 automated analyzer from Hitachi (Tokyo, Japan; also known as the Hitachi 717). In some cases, a conventional spectrophotometer (Model UV-2100S; Shimadzu Seisakusho, Kyoto, Japan) was also used.

Reagents

Chemicals. We used chemicals of reagent grade obtained from Wako Pure Chemical Industries (Osaka,

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[2] Nonstandard abbreviations: AAS, atomic absorption spectrophotometry; ICD, isocitrate dehydrogenase; ICSH, International Committee for Standardization in Haematology; nitroso-PSAP, 2-nitroso-5-(*N*-propyl-*N*-sulfopropylamino)-phenol; and PIPES, 1,4-piperazinediethanesulfonic acid.
Received June 1, 1993; accepted January 8, 1994.

Japan) except where mentioned. Cysteine was from Sigma Chemical Co., St. Louis, MO; disodium 4,7-diphenyl-1,10-phenanthroline disulfonate, 1,4-piperazinediethanesulfonic acid (PIPES), and EDTA were from Doujindo Lab., Kumamoto, Japan; and bilirubin, hemoglobin, and lipid (Interference Check A) were from International Reagent, Kobe, Japan.

Enzymes. Isocitrate dehydrogenase ($NADP^+$) (EC 1.1.1.42; ICD) from yeast (10 kU/g at 25°C) was a product of Oriental Yeast. Aconitase (EC 4.2.1.3) was purified from pig heart muscle (Nisshin Ham, Yonago, Japan), essentially by the method of Villafranca and Mildvan (*9*). Preparations of intact aconitase (holoaconitase), 2–3 g/L protein, were dissolved in 15 mmol/L Tris-HCl (pH 6.9 at 25°C) containing, per liter, 5 mmol of citrate, 0.1 mmol of EDTA, and 5 mmol of *o*-phenanthroline (iron-chelating reagent) and incubated at 4°C overnight. The resulting solutions were then desalted by passage through a Sephadex G-25 (Pharmacia, Uppsala, Sweden) column previously equilibrated with 15 mmol/L Tris-HCl (pH 6.9 at 25°C) containing 5 mmol/L tricarballylate. The desalted solutions were concentrated to ~10 g/L protein by filtration through a PM10 membrane (Amicon, Danvers, MA). The resulting enzyme solutions did not show any activity. The activity of the inactivated enzyme, called apoaconitase, can be restored by incubation with Fe^{2+}. The activities of intact aconitase and apoaconitase were measured by the method of Fansler and Lowenstein (*10*), modified as follows: We mixed 50 μL of the enzyme solution (~1 kU/L) with 50 μL of 50 mmol/L Tris-HCl (pH 8.5 at 25°C) containing, per liter, 10 mmol of L-cysteine and 2 mmol of ferrous ammonium sulfate and incubated the mixture at 37°C for 30 min. To the incubated mixture we then added 3.0 mL of 50 mmol/L Tris-HCl (pH 7.4 at 25°C) containing, per liter, 0.2 mmol of citrate, 0.5 mmol of $NADP^+$, 50 μmol of manganese sulfate, and 200 U of ICD and measured the absorbance change of the resulting mixture at 340 nm and 25°C with a spectrophotometer. The specific activity of the apoaconitase preparations was ~10 kU/g protein.

Standard reagent mixtures for assay of serum iron. Our standard procedure included three reagent mixtures as follows (the concentrations in parentheses are those in the reaction mixture for the overall reaction): Dilution buffer, 0.2 mol (14 mmol) of acetate buffer (pH 3.0 at 25°C) and 0.2 mol (14 mmol) of ascorbate per liter; reagent 1, 0.15 mol (71 mmol) of PIPES-NaOH (pH 6.7 at 25°C) and 2 kU (0.95 kU) of apoaconitase per liter; reagent 2, per liter, 0.1 mol (43 mmol) of PIPES-NaOH (pH 7.7 at 25°C), 1.0 mmol (0.43 mmol) of citrate, 1.0 mmol (0.43 mmol) of magnesium sulfate, 1.0 mmol (0.43 mmol) of $NADP^+$, and 4 kU (1.7 kU) of ICD. The dilution buffer was stable for 2 weeks or longer, and reagents 1 and 2 for at least 1 week at 4°C.

Iron solution. The iron calibrator solution (1.0 mmol/L) was prepared by dissolving 392.1 mg of ferrous ammonium sulfate hexahydrate in 1.0 L of 10 mmol/L HCl. We diluted this to various concentrations with 10 mmol/L HCl just before use to produce the iron calibrators.

Assay Procedure

Serum specimens for assay were diluted fourfold with dilution buffer in the sample cups of the Hitachi automated analyzer and then incubated at room temperature for 5 min. The analyzer then added 20 μL of the diluted specimens and 100 μL of reagent 1 to the reaction cells, and incubated these for 5 min before adding 90 μL of reagent 2 to each. For the reagent blank, isotonic saline (9 g/L NaCl) was substituted for the specimens. Absorbance at 340 nm (A_{340}) was measured for each reaction mixture and corrected for the reagent blank. The reaction rate (ΔA_{340}/min) was measured 3–4 min after addition of reagent 2 at analyzer settings of kinetic assay mode. The concentrations of iron in serum specimens were estimated on the calibration curve, produced with the iron calibrator solutions (0–70 μmol/L).

Comparison method. We also measured serum iron by the ICSH method reported in 1978 (*5*) and routinely practiced in clinical laboratories, as follows: We added 2 mL of aqueous solution containing 0.6 mol of trichloroacetic acid, 0.4 mol of thioglycollic acid, and 1 mol HCl per liter to 2 mL of the specimen (serum, calibrator, or pure water) in a Corning 15-mL centrifuge tube with screw cap (obtained from Iwaki Glass Co., Itabashi, Japan), and vortex-mixed thoroughly for 1 min before incubating at 56°C for 15 min. The incubated mixture was centrifuged at 1580*g* for 10 min (Model J-6B centrifuge; Beckman Instruments, Palo Alto, CA). To 2 mL of the supernate, we added 2 mL of 1.5 mol/L sodium acetate containing 0.5 mmol/L disodium 4,7-diphenyl-1,10-phenanthroline disulfonate, mixed thoroughly, and let the samples stand for 10 min at room temperature. The absorbances (A_{serum}, A_{cal}, and A_{blank}) at 535 nm were then measured against pure water with the spectrophotometer. The serum iron concentration of specimens was calculated from the formula: serum iron (μmol/L) = $[(A_{serum} - A_{blank})/(A_{cal} - A_{blank})] \times 40$. A_{blank} was generally ~0.014 in this study.

Results

Optimization Studies

Type and pH of buffer for reagent 2. We examined the effects on aconitase activity of various kinds and pH values of buffers in reagent 2: triethanolamine-HCl (50 mmol/L, pH 6.0–8.0 at 25°C), PIPES-NaOH (50 mmol/L, pH 7.0–9.0 at 25°C), and Tris-HCl (50 mmol/L, pH 6.5–9.5 at 25°C). Activities were maximum at pH 7.5–8.0 in the first two buffers, and at pH 7.5–8.5 with the last one. Although Tris-HCl showed the greatest activity at ~pH 8, we chose to use PIPES at pH 7.7, which showed the least effect of temperature change on pH. ΔpK_a/°C was −0.0085 for PIPES-NaOH, −0.020 for triethanolamine-HCl, and −0.028 for Tris-HCl (*11, 12*).

Effects of citrate concentrations on rate. We examined the effects of citrate concentrations on the rate of the overall reaction. Using a 20 μmol/L iron calibrator as the specimen, we followed the assay procedure as described, except for varying the concentration of citrate in reagent 2. Because the reaction rate reached a max-

imum at 1.0 mmol/L citrate in reagent 2, we used this concentration in our method.

Dissociation of iron from ferritransferrin. We examined the effects of various pHs in dilution buffer on the dissociation of iron from ferritransferrin. A pooled serum (serum 1) was diluted fourfold with various buffers, ranging in pH from 2 to 4 (0.2 mol/L acetic acid-HCl or acetic acid-NaOH containing 0.2 mol/L ascorbate). In some experiments, we substituted 0.15 mol/L PIPES-NaOH (pH 6.7 at 25°C; the buffer in reagent 1) containing 0.2 mol/L ascorbate for these buffers. The resulting mixtures were incubated for 10 min at 25°C and then assayed by our method. The measurable concentration of iron was 0 at pH 6.7, but increased with decreasing pH to a maximum (19.1 μmol/L) at pH 3.0 (Table 1). Assay by atomic absorption spectrophotometry (AAS) showed that serum 1 contained 20.5 μmol/L iron. Serum 1 also contained 25 mg/L hemoglobin, which bound 1.5 μmol/L iron. Doubtless, the difference between the values obtained by AAS and our method resulted from hemoglobin-bound iron, whereas our method measured only the ferritransferrin in the serum specimen.

We then supplemented serum 1 with 540 mg/L hemoglobin (serum 2) and diluted sera 1 and 2 fourfold with dilution buffer (pH 3.0). After incubation at 25°C for various lengths of time, we analyzed the resulting solutions for iron by our method (Table 2). For serum 1, the measurable concentration of iron increased slightly at the start of the incubation, reached a maximum (18.9–19.2 μmol/L) at 2–3 min, and hardly changed further, even at 360 min. For serum 2, however, the measurable iron increased appreciably with prolonged incubation time (1–24 h), as the iron dissociated from hemoglobin. However, the measurable concentrations of iron were almost the same for sera 1 and 2 at the incubation time of 5 min.

Reactivation of apoaconitase. We examined the reactivation of apoaconitase by various iron concentrations at various pH values for reagent 1. The iron calibrator solutions (0–200 μmol/L) were assayed by our proposed method, with the pH of 0.15 mol/L PIPES-NaOH in reagent 1 varied from 6.3 to 6.9. The rate of the overall reaction became faster with higher pH values (Fig. 1). It also increased with increasing concentrations of iron (Fe^{2+}). The increase was practically linear for Fe^{2+} up to 200 μmol/L at pH 6.3 and 6.5, up to 100 μmol/L at pH 6.7, and up to 60 μmol/L at pH 6.9. We used pH 6.7 for reagent 1.

Table 1. Effects of pH of dilution buffer on dissociation of iron from ferritransferrin.

pH	Iron conc (serum 1), μmol/L
2.0	18.2
3.0	19.1
3.5	18.5
4.0	13.8
6.7	0.0

Table 2. Effects of incubation time on dissociation of iron from ferritransferrin and hemoglobin in serum.

	Iron conc, μmol/L[a]	
Incubation, min	Serum 1	Serum 2
1	18.2	—
2	18.9	—
3	19.2	—
4	19.0	—
5	19.0	19.2
60	—	20.7
120	—	22.2
360	19.2	28.5
1440	—	41.2

[a] Serum 1 and 2 contained 25 and 565 mg/L of hemoglobin, respectively; thus, their iron contents were 1.52 and 34.4 μmol/L, respectively.

Assay Evaluation

Kinetics of reaction and linearity. We examined the kinetics of the overall reaction and the linearity of our method. Fig. 2 shows the results for assaying the iron calibrators (0–100 μmol/L) and the time courses for the rates of the overall reaction. There was a lag phase for 3 min after addition of reagent 2, whereas the reaction

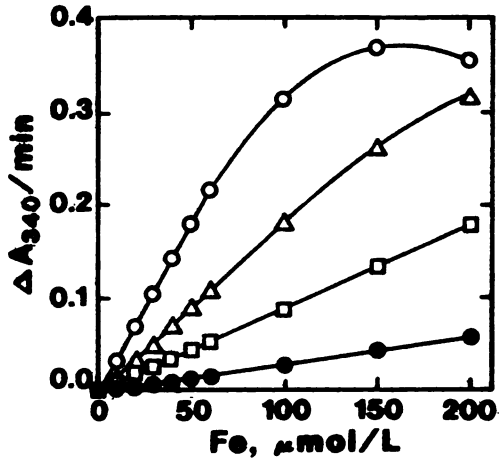

Fig. 1. Effects of PIPES pH in reagent 1 on overall reaction rate for assay of iron calibrators (0–200 μmol/L).
●, pH 6.3; □, pH 6.5; △, pH 6.7; ○, pH 6.9.

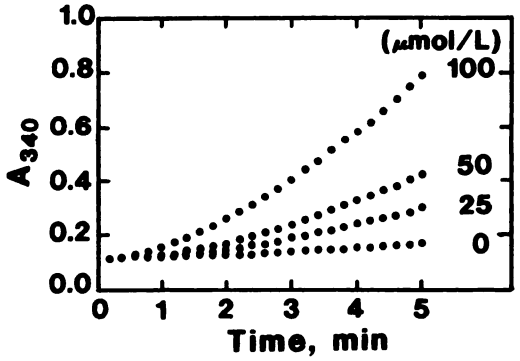

Fig. 2. Time courses for overall reaction rates with various concentrations of ferrous iron (0, 25, 50, and 100 μmol/L).
A_{340} was read at 12-s intervals after the addition of reagent 2 to start the overall reaction.

was practically linear at 3–4 min for all the concentrations tested. To study the linearity of the calibration curve, we assayed in duplicate the iron calibrator (eight concentrations, 0–70 μmol/L). The linearity between reaction rate (ΔA_{340}/min) (y) and iron concentration (μmol/L) (x) was $y = 0.0018x + 0.0016$ ($r = 0.9996$, $S_{y|x} = 0.0012$). The calibration curve was straight for iron concentrations up to 70 μmol/L.

Detection limit. We examined the detection limit of our method by assaying the 0 standard (9 g/L NaCl) 10 times. The result (mean ± SD) was 0.15 ± 0.31 μmol/L. The detection limit, defined as the mean iron concentration of the 0 standard + 2.6 SD, was therefore 0.96 μmol/L.

Precision. To examine the precision of our method we assayed two pooled human serum specimens containing different concentrations of iron. Within-run CVs for samples reassayed 20 times in the same assay were 2.4% (iron concentration, 17.0 ± 0.4 μmol/L) and 1.9% (31.0 ± 0.6 μmol/L) for the two specimens. Day-to-day CVs in assays repeated 20 times were 2.9% (17.2 ± 0.5 μmol/L) and 2.3% (30.8 ± 0.7 μmol/L).

Analytical recovery. We examined the recovery of serum iron by our method by adding various concentrations of iron to two pooled serum specimens and assaying. The analytical recoveries of added iron were 100–103% (Table 3).

Interference. We examined potential interferences in this assay of serum iron. A pooled serum specimen was supplemented with various cations and anions, ascorbate, bilirubin, lipids, and hemoglobin at concentrations significantly higher than the normal values (*13*): The resulting solutions were then assayed by our method and by the ICSH-proposed Reference Method (Table 4). Our results were hardly influenced by all the substances tested, except that NaCl and $CuSO_4$ caused negative errors of ~7% at concentrations as great as 0.45 mol/L and 60 μmol/L, respectively. Hemoglobin showed no effect at 16 μmol/L. The ICSH method (*5*), however, was slightly affected by 60 μmol/L $CuSO_4$ and 16 μmol/L hemoglobin.

Table 3. Analytical recovery of serum iron added to two sera.

	Iron conc, μmol/L			
Original	Added[a]	Measured	Difference[b]	Recovery, %[c]
15.4	5.0	20.7	15.7	102
	10.0	25.8	15.8	103
	20.0	35.6	15.6	101
34.9	10.0	44.9	34.9	100
	20.0	55.5	35.5	102
	30.0	65.6	35.6	102

[a] Calculated from the dilution factors for the added iron calibrator solution.
[b] Measured minus added.
[c] 100% × difference/original.

Method comparison. We examined the correlation between our method (y) and the ICSH method (x) for serum specimens containing <30 mg/L hemoglobin (*5*) obtained from 72 patients (Fig. 3). The regression equation was $y = 0.98x + 0.38$ μmol/L ($r = 0.996$, $S_{y|x} = 0.63$ μmol/L).

Reference interval. To determine the reference interval for serum iron by our method, we assayed pooled serum specimens from 219 apparently healthy individuals. Measurement of serum iron in these specimens individually gave a mean (±SD) concentration of 16.7 ± 5.7 μmol/L. We estimated the reference intervals for iron by a nonparametric statistical method because of its simplicity (*14*). The ranges for females and males were 5.1–27.6 μmol/L (mean ± SD, 15.2 ± 6.0 μmol/L, ages 9–78 years, n = 166) and 9.3–28.8 μmol/L (18.5 ± 5.4 μmol/L, ages 3–79 years, n = 63), respectively.

Table 4. Effects of various substances on proposed assay and ICSH Reference Method.

	Our method		ICSH method	
Substance added, mmol/L[a]	Measured iron, μmol/L	Recovery, %	Measured iron, μmol/L	Recovery, %
None	21.5	(100)	21.6	100
NaCl, 450 (136–145)	20.0	93	21.8	101
KCl, 20 (3.5–5.0)	21.7	101	22.1	102
NH_4Cl, 0.2 (0.013–0.075)	21.6	100	22.1	102
$CaCl_2$, 10 (1.1–1.3)	22.2	103	22.2	103
$MnCl_2$, 0.005 (—)	21.3	99	21.7	100
$CuSO_4$, 0.06 (0.011–0.024)	20.0	93	24.3	113
$ZnCl_2$, 0.1 (—)	21.6	100	21.6	100
$MgCl_2$, 3 (0.74–1.23)	21.5	100	22.0	102
Na_2HPO_4, 10 (0.31–0.73)	21.5	100	21.7	100
Na_2SO_4, 3 (—)	21.3	99	21.4	99
Ascorbate, 0.57 (0.023–0.085)	21.3	99	21.5	100
Bilirubin, 0.34 (0.0051–0.019)	21.7	101	22.5	104
Lipid[b] (—)	22.0	102	21.6	100
Hemoglobin, 0.016 (—)	22.2	103	23.0	106

[a] Range of normal values found in serum is listed in parentheses.
[b] Formazine turbidity, 2500°.

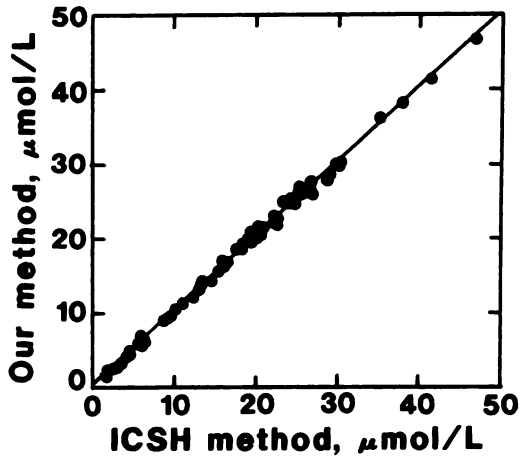

Fig. 3. Correlation between our method and the ICSH Reference Method for sera from 72 patients.

Discussion

Transferrin has a molecular mass of 77 kDa (*15*), and is capable of strongly binding 2 mol of iron per mole (*1*). In serum, ~30% of transferrin is bound with iron. In transferrinemia, the concentration of serum iron is extremely low (*16*), indicating that the iron is mostly bound to transferrin in either normal or abnormal sera. In clinical diagnosis, one must be able to measure iron concentrations of sera lower than the normal value.

Various methods for assaying serum iron with bathophenanthroline (*3*), bathophenanthroline sulfonate (*4, 5*), ferrozine, ferene (*6*), or nitroso-PSAP (*7, 8*) as the iron chromogen have been reported. The first two methods require large specimens (0.5–2.0 mL) and are difficult to automate, and the nitroso-PSAP method is affected by bilirubin.

After detailed optimization studies, we developed a new enzymatic method that determines concentration of serum iron. Intact aconitase is bound with 4 mol of iron per mole (*17*). The enzyme, if treated with appropriate iron-chelating reagent, is easily inactivated. The inactivated apoenzyme becomes reactivated when incubated with Fe^{2+}. Almost all the iron in the serum is bound to transferrin (*1*). Ferritransferrin releases Fe^{3+} at acidic pH, whereas apotransferrin binds it at neutral and alkaline pH (*18*). When we mixed serum specimens with a dilution buffer of acetate (pH 3) and ascorbate, the ferritransferrin present rapidly released all the Fe^{3+} (Eq. 1). Given that the reducing ability of ascorbate is high at neutral and alkaline pH but low at acidic pH, we supplemented mixtures of serum specimens and dilution buffer with reagent 1 (containing PIPES-NaOH, pH 6.7, and apoaconitase), so that the ascorbate could reduce all the Fe^{3+} to Fe^{2+} (Eq. 2). Simultaneously, the inactive apoenzyme was bound to Fe^{2+} and reactivated (Eq. 3). When the resulting solutions were further supplemented with reagent 2 (containing PIPES-NaOH, pH 7.7, citrate, Mg^{2+}, $NADP^+$ and ICD), the reactivated holoenzyme catalyzed the hydrolysis of citrate to isocitrate (Eq. 4), which was followed by the conversion of isocitrate and $NADP^+$ to 2-oxoglutarate and NADPH (Eq. 5). The rates for reduction of $NADP^+$ in our method were proportional to the concentrations of serum iron in the specimens.

In summary, our new enzymatic rate assay of serum iron is suitable for use with automated analyzers. Highly specific for serum iron, this method is safe and is not affected by other cations, bilirubin, or lipemia. The detection limit of <1 μmol/L demonstrates that this is a highly sensitive method. The mean values of iron concentration measured in serum specimens from healthy subjects by our method generally agree with earlier reports with the ICSH method (*19*). Because our method is simple, accurate, and sensitive, we conclude it may be useful in routine clinical diagnosis with automated analyzers.

We are grateful to T. Horio for discussions and encouragement and to Y. Hirayama for providing specimens.

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