Quantitative determination of serum iron in human blood by high-performance capillary electrophoresis

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Abstract

A capillary electrophoretic (HPCE) method that can be used to quantitatively determine trace amounts of iron has been developed and applied to determine the iron level in human serum. After precipitation of serum proteins, Fe(III) in the serum is reduced to Fe(II) with hydroxylamine hydrochloride, and a stable Fe(II)–1,10-phenanthroline complex is formed by adding 1,10-phenanthroline to the supernatant containing 2.5 mM ammonium acetate–acetic acid at pH 5.0. The Fe(II)–1,10-phenanthroline complex, $[Fe(C_{12}H_8N_2)_3]^{2+}$, has a very strong absorbance at 270 nm (with a molar absorptivity of $\sim 9.2 \cdot 10^4$). By measuring the absorbance of $[Fe(C_{12}H_8N_2)_3]^{2+}$ at 270 nm, the iron level in human serum can be precisely quantified. The interference from copper, a major interference in serum, can be totally eliminated due to the complete separation of $[Fe(C_{12}H_8N_2)_3]^{2+}$ and the Cu(II)–1,10-phenanthroline complex. In addition, other problems that usually occurred with conventional spectrophotometric methods, such as co-precipitation and occlusion of iron during sample pretreatment, are significantly minimized due to the ability to wash the precipitate and the higher detection sensitivity. With this method, a single drop (10 μl) of serum would be sufficient to determine the serum iron concentration. The method is reliable, sensitive, rapid and reproducible. Thus it is highly suitable for use in the clinical laboratory.

1. Introduction

Iron (Fe) is the most important of the essential trace metals, and iron deficiency is one of the most prevalent disorders of humans. An appreciable number of human diseases are related to iron deficiency or disorders of iron metabolism. It is particularly common for children, young women and senior persons [1–5]. Measurement of serum iron concentration is widely used in the diagnosis of iron deficiency [6]. Although there are more than 50 different methods [7] for determination of serum iron concentration, none of these affords both sufficient accuracy and the use of only a small-volume serum sample. A wide variation in the iron concentrations measured resulted from the different methods and the bias could reach 15–58.5% for the same serum sample [8]. At present the recommended method for the clinical laboratory is the spectrophotometric method [9], which still requires 1–2 ml of serum for analysis. The method is difficult to use when a large number of samples has to be assayed, and/or when only a small volume of

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serum is available, e.g. in population surveys, pediatric work and experimental work on small laboratory animals [7]. The main sources of error of the recommended method are due to interferences and poor sensitivity. The method does not allow washing and dilution of serum during sample pretreatment, e.g. precipitation and extraction, and thus the method is inaccurate due to loss of iron during serum sample pretreatment caused by incomplete dissociation of iron from binding proteins, co-precipitation of iron with proteins [10,11] and spectrophotometric interferences from copper, and other compounds such as bilirubin and lipids present in the serum matrix [8,12]. Therefore, development of new methods for the determination of serum iron is still needed and important for iron deficiency diagnosis.

High-performance capillary electrophoresis (HPCE) has been proven to be a powerful technique for the separation of biological samples because of its high separation efficiency and small sample requirement [13–15]. Based on the strong absorbance of Fe(II)–1,10-phenanthroline at 270 nm, we have developed a new sensitive way to determine trace amounts of iron [16]. With this technique, the Fe(II)–1,10-phenanthroline can be separated from 1,10-phenanthroline and other interfering molecules. The technique is two orders of magnitude more sensitive than the conventional spectrophotometric method [16]. This is important with respect to accuracy and amount of serum sample required. It allows the serum sample to be diluted more than 10-fold during sample pretreatment, giving an iron concentration still high enough for HPCE analysis. Thus a 10-μl serum sample would be sufficient for analysis. In addition, the precipitate can be washed to prevent co-precipitation and occlusion of iron, which considerably improves the accuracy of the measurement.

In the present paper, an HPCE method was developed for the quantitative determination of iron in human serum. The factors affecting iron quantitation, such as the TCA concentration for protein precipitation, washing and shaking, will be discussed in detail.

2. Experimental

2.1. Reagents and samples

All reagents used were of analytical or ACS certified grade. Deionized water was prepared with a Milli-Q system (Millipore, Bedford, MA, USA). Ammonium acetate (NH_4Ac) with 0.5 ppm Fe, 1,10-phenanthroline ($C_{12}H_8N_2$), hydroxylamine hydrochloride ($NH_2OH \cdot HCl$) and ferrous ammonium sulfate [$Fe(NH_4)_2(SO_4) \cdot 6H_2O$] were purchased from Fisher Scientific (Fairlawn, NJ, USA). Acetic acid (HAc) and sodium hydroxide (NaOH) were obtained from Aldrich (Milwaukee, WI, USA). Trichloroacetic acid (TCA) was obtained from GFS Chemical (Columbus, OH, USA). Diethyl ether was purchased from Sigma (St. Louis, MO, USA). Serum samples were collected from student volunteers in Northeast Missouri State University.

2.2. Special treatment of glassware and plasticware

All glassware and plasticware used should be free from iron. Therefore, all glassware and plasticware were soaked in 6 M HCl for 5–6 h, and washed with deionized water.

2.3. Equipment

A Model 3850 HPCE system with a UV detector was purchased from ISCO (Lincoln, NE, USA). A positive high voltage was applied to the capillary by maintaining the injection end at a positive potential while the cathodic end was held at ground potential. A DataJet computing integrator (Spectra-Physics, Mountain View, CA, USA) was used to collect the data. A capillary column (60 cm × 75 μm I.D.) (Polymicro Technologies, Phoenix, AZ, USA) was used for separation. The polymer coating was burned off at 35 cm from the injection end to form a detection window. A UV absorbance detector was used at 270 nm.

2.4. Pretreatment of capillary column

All new capillary columns were cleaned with 0.1 M NaOH solution for 30 min. The column was then washed with deionized water and running buffer. The capillary was ready for use thereafter.

2.5. Preparation of background electrolyte

The background electrolyte (BGE) solutions for HPCE were prepared by dissolving 0.385 g NH_4Ac in 80 ml deionized water and the pH was adjusted to 5.0 with 5% HAc, and then diluting to 100 ml with deionized water to obtain 50 mM NH_4Ac concentration. The BGE was filtered through a 0.45-μm membrane filter (Fisher Scientific) and degassed before use.

2.6. Preparation of standard iron [Fe(II)] and Fe(II)–1,10-phenanthroline standard solutions

A 1.00 mM Fe^{2+} stock solution was prepared by dissolving 39.2 mg $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ in 70 ml deionized water and diluting to 100 ml. The 0.1 mM Fe^{2+} standard solution could be obtained by further dilution of the above prepared stock solution.

Different concentrations of $[Fe(C_{12}H_8N_2)_3]^{2+}$ standard solution were prepared by adding different volumes of 0.100 mM Fe^{2+} standard solution with an Eppendorf pipette, 0.500 ml 50 mM BGE with pH 5.0, and 0.800 ml 1.00 mM 1,10-phenanthroline to a 10-ml volumetric flask, and dilution to the mark with deionized water; then the solution was mixed well. These solutions were used to obtain the calibration curve for quantitation.

2.7. Serum sample treatment

The procedures for serum sample treatment have been significantly modified with respect to those described in the literature [17–19]. To a 10-μl serum sample 20 μl 10% trichloroacetic acid (TCA) was added. The mixture was agitated vigorously on a vortex-mixer for 30 s to assure

that all the lumps of precipitated protein were broken into small pieces. The precipitate was allowed to stand for 10 min, and then centrifuged at 5700 g for 10 min. The supernatant was transferred to a new 0.5-ml centrifuge tube. In order to prevent occlusion of iron within the precipitates, the precipitate was washed with 20 μl deionized water for two more times and vortex-mixed for 1 min. Then the proteins were again removed by centrifugation, and finally all the supernatants were combined. The combined supernatant was then extracted twice with 30 μl of diethyl ether to remove excess TCA. To the clear supernatant, 5 μl 50 mM NH_4AC–HAC (pH 5.0) was added, and 10 μl 2.0 mM NH_2OH were added to reduce the Fe(III) to Fe(II). The mixture was allowed to stand for 10 min and 10 μl 1.0 mM 1,10-phenanthroline was added. The sample solution was finally diluted to 100 μl with deionized water.

2.8. HPCE analysis

The sample solution was injected by hydrodynamic injection with an autoinjector for 15 s, and electrophoresis was carried out at 12.5 kV. Peaks were identified by injecting the standard and by the standard addition technique.

2.9. Relative recovery and Fe(II) quantitation

A standard calibration curve was used to quantify the concentration of iron (Fe^{2+}), and a standard addition method of Fe^{2+} and Fe^{3+} was used to verify the accuracy of the method and to calculate the relative recovery. The relative recovery of iron was calculated by:

$$\text{Recovery}\% = \frac{C_x}{C_o + C_s} \cdot 100$$

where C_x is the concentration of iron determined by HPCE after standard addition, C_o is the iron concentration measured by HPCE without standard addition, and C_s is the standard iron concentration.

3. Results and discussion

3.1. Optimization of separation and injection conditions

Since the presence of metal ions in serum will interfere with the determination of iron, one has to make sure that the separation conditions are optimized so that reproducible and accurate results can be obtained. Under ideal electrophoretic conditions [16], the $[Fe(C_{12}H_8N_2)_3]^{2+}$, $C_{12}H_8N_2$ and Cu–1,10-phenanthroline peaks can be well separated. A representative electropherogram of a serum sample is shown in Fig. 1,
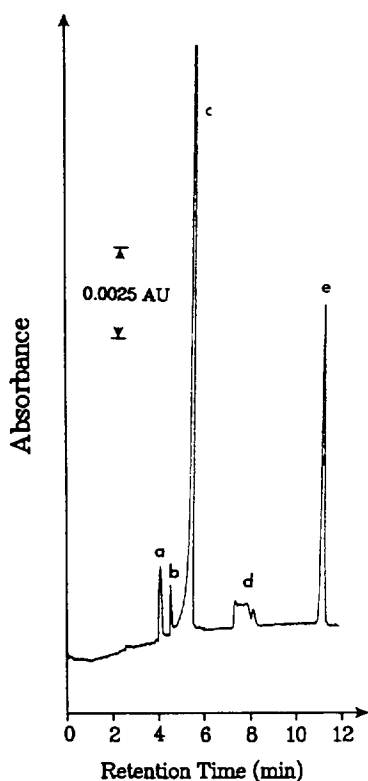

Fig. 1. Representative electropherogram of a typical serum sample diluted 10 times. BGE used: 50 mM NH$_4$Ac–HAc at pH 5.0. Injection: 15 s by hydrodynamic injection. Electrophoresis was carried out at +12.5 kV in a 60 cm × 75 μm I.D. capillary. Detection wavelength: 270 nm. Peak identification: a = Fe(II)–1,10-phenanthroline complex (the Fe concentration in serum = 3.14 μM); b = Cu(II)–1,10-phenanthroline complex; c = 1,10-phenanthroline; d = system peak; e = non-identified peak.

which shows that the interference from Cu (the major interference for iron determination in human serum) is completely eliminated. Identification of the peaks was carried out by co-injection of standards. Peak e in the electropherogram was from serum sample and does not interfere with the determination of iron. In a separate experiment it was shown that component e (peak e) does not form a complex with 1,10-phenanthroline, since the same peak (same migration time and peak size) was obtained when pure pretreated serum sample containing no $C_{12}H_8N_2$ was injected (Fig. 2). The peak has, however, not been identified yet. The electroosmotic flow-rate of the separation under the electrophoretic conditions used was $34.4 \cdot 10^{-5}$ cm^2 V^{-1} S^{-1}.

It was found that different injection techniques strongly influence the quantitation of the iron
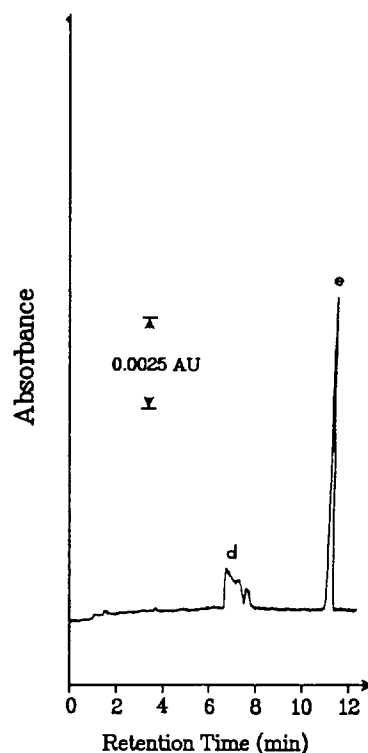

Fig. 2. Electropherogram of a pretreated serum sample containing no 1,10-phenanthroline. The electrophoretic conditions and peak identifications are the same as those in Fig. 1.

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level in serum. Since the ionic strength of the standard solutions which were used to prepare the calibration curve might be significantly different from that of the serum samples, and different serum samples might have different ionic strengths, the electrokinetic injection technique would cause discrimination during the injection, and would cause large errors in the quantitative analysis. Since hydrodynamic injection does not pose this problem, it would ideal for the present analysis.

It was also shown that the serum matrix does not affect the separation and detection of iron by HPCE. A serum sample was treated in the exactly the same way as described above except that no 1,10-phenanthroline was added to the sample solution. The results are shown in Fig. 2. Here we can see that there is no peak before 6 min, within which the Fe(II)–1,10-phenanthroline peak would co-migrate. Peak e is the same peak as that in Fig. 1. This means that serum components such as bilirubin and lipids do not interfere with the quantitation of serum iron.

The interference from cobalt and nickel with the determination of iron has been investigated. We found that under the current separation conditions the Co(II)–1,10-phenanthroline and Ni(II)–1,10-phenanthroline peaks can not be completely resolved from the Fe(II)–1,10-phenanthroline peak. However, it was found that 1% or less of Co or Ni (i.e. the ratio of C_{Ni} or C_{Co} over $C_{Fe} < 1\%$) does not influence the Fe signal. In human serum, fortunately, the concentrations of Co(II) and Ni(II) are in the range of about $8 \cdot 10^{-9}$ M [20], which is more than 1000 times lower than that of iron (the iron level in normal human serum is 11–35 μM) [20]. Therefore, interference from Co(II) and Ni(II) will be no problem for the iron level determination in human serum.

3.2. Effects of different serum treatment methods on the determination of the iron level

The method used for the pretreatment of serum samples significantly affects the accuracy of the determination of the iron level. In order to study the accuracy of our technique, we

Table 1
Effect of vortex-mixing and washing on accuracy of determination of serum iron

Sample treatment		Serum iron concentration[a] (μM)
Vortex-mixing	Washing	
No	No	24.19–24.72
Yes	No	25.35–26.31
No	Yes	30.05–31.11
Yes	Yes	31.11–31.88

[a] Data represent ranges of three measurements.
HPCE conditions: 50 mM NH_4AC–HAC (pH 5.0) served as a running buffer; samples are injected for 15 s by hydrodynamic injection and electrophoresis was carried out at 12.5 kV. All the data are from a single serum sample.

compared our method with the recommended method. The data listed in Table 1 show that vortex-mixing and precipitate washing are critically important for accuracy, especially the washing. With the conventional spectrophotometric method, the precipitate can not be washed due to the low sensitivity of detection. This means that the large amount of iron occluded within the precipitated proteins was totally ignored. The present study has proven that occlusion and co-precipitation is critical during the precipitation step. Fig. 3 shows that after protein precipitation without further washing, there is still a significant amount of iron co-precipitated with or occluded in the protein precipitate. Table 1 shows that the relative error caused by iron occlusion in proteins is > 22.2%. Due to the inconsistency found in the data for the same sample, a recent report [8] questioned the detection accuracy of the conventional spectrophotometric methods. To overcome this problem, vigorous agitation during precipitation and two washings of the precipitated proteins are required to guarantee the total release of iron molecules from the proteins. This procedure would increase the accuracy. Generally, after the second washing, the amount of iron occluded within the proteins was too small to be measured. In addition, the concentration of TCA which is used to precipitate the serum proteins also influences the accuracy. Experiments demonstrated that 10% TCA gives the
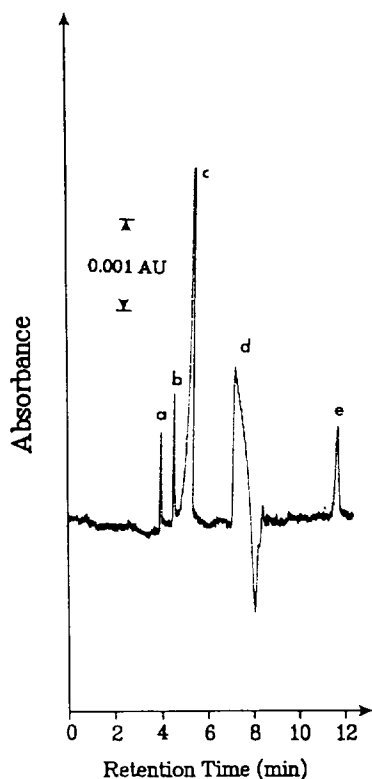
Fig. 3. Electropherogram obtained by injection of the washing solution of the precipitated proteins; Fe concentration = 0.40 μM. The conditions and peak identifications are the same as those in Fig. 1.

best results. When using a lower TCA concentration, the volume of TCA has to be increased, since otherwise the precipitation process is slow and the precipitation of proteins is incomplete. By using a higher TCA concentration, precipitation is very fast and the dissociation of iron from the binding proteins is incomplete; in addition, more severe occlusion and co-precipitation will occur, which will result in lower values even when double washing is used (see Table 2).

3.3. Sensitivity and linearity

The sensitivity and linearity of the response have also been studied. At 270 nm, the detection limit of the technique is as low as $5 \cdot 10^{-9}$ M iron with a signal-to-noise ratio of 5. This detection

Table 2
Effect of TCA concentration on the determination of serum iron

Serum sample	TCA concentration	Serum iron concentration[a] (μM)
1	10%	44.50 ± 0.10
	20%	42.40 ± 0.82
2	10%	42.97 ± 0.64
	20%	35.01 ± 0.45

[a] Data for four measurements ± standard deviation. Electrophoretic conditions as in Table 1.

limit has never been obtained by spectrophotometric methods, and is about 20 times more sensitive than that obtained with the same HPCE method at 508 nm, which is the normal wavelength adopted in spectrophotometric techniques. The assay is linear over more than two orders of magnitude (0.2–20 μM). However, since the iron concentration in human serum ranges form 10 to 35 μM, and the serum samples were diluted more than 10 times prior to analysis, linearity over the range 1.0–3.5 μM is very important for our studies. A good linear response was obtained over the range 0.5–6.0 μM iron and the corresponding regression coefficient is 0.997 ($n = 10$).

3.4. Recovery

The recovery has been verified by addition of standard iron [Fe(II) or Fe(III)] concentrations. The results are shown in Table 3. The recovery was found to be 90.64–110.57%, which means that the extraction is quantitative.

4. Conclusions

An HPCE technique for the quantitative determination of serum iron has been developed. The method is sensitive, rapid, accurate and requires only 10 μl of serum sample. The total separation takes only 5 min, and interferences from copper and other serum components are eliminated. A detection limit of $5 \cdot 10^{-9}$ M iron

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Table 3
The percent recovery of the HPCE method

C_{Fe} in serum (μM)	Standard $C_{Fe(II)}$ added (μM)	Standard $C_{Fe(III)}$ added (μM)	Measured total C_{Fe}^a (μM)	Percent recovery[a] (%)	S.D.[a] (%)
31.50	0.00	0	31.50	–	± 1.23
31.50	14.30	0	46.50	101.53	± 0.24
31.50	20.00	0	49.50	96.12	± 1.21
31.50	25.00	0	58.38	103.33	± 2.41
31.50	30.00	0	68.00	110.57	± 1.43
32.75	0.00	0	32.75	–	± 1.37
32.75	10.00	0	38.75	90.64	± 4.39
32.75	20.00	0	53.75	101.90	± 2.79
32.75	25.00	0	57.00	98.70	± 5.35
31.42	0.00	4.29	35.43	99.22	± 2.80
31.42	0.00	0	31.42	–	± 3.23

[a] $n = 5$; S.D. = Standard deviation.
Electrophoretic conditions as in Table 1.

can be obtained, which is 30 times lower than that obtained with the conventional spectrophotometric method, and about 20 times lower than that of a reported HPCE technique [21]. Therefore, this method is suitable for the quantitation of the serum iron level in population surveys, pediatric work and experimental work on small animals. Furthermore, the method is potentially useful for measuring the total iron-binding capacity in nutrition studies.

Acknowledgement

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