COMPARISON OF COLORIMETRY AND ELECTROTHERMAL ATOMIC ABSORPTION SPECTROSCOPY FOR THE QUANTIFICATION OF NON-TRANSFERRIN BOUND IRON IN HUMAN SERA

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Abstract. This paper describes a comparison of two analytical techniques, one employing bathophenanthrolinedisulfonate (BPT), a most commonly-used reagent for Fe (II) determination, as chromogen and an electrothermal atomic absorption spectroscopy (ETAAS) for the quantification of non-transferrin bound iron (NTBI) in sera from thalassemic patients. Nitrilotriacetic acid (NTA) was employed as the ligand for binding iron from low molecular weight iron complexes present in the serum but without removing iron from the transferrin protein. After ultrafiltration the Fe (III)-NTA complex was then quantified by both methods. Kinetic study of the rate of the Fe (II)-BPT complex formation for various excess amounts of NTA ligand was also carried out. The kinetic data show that a minimum time duration (> 60 minutes) is necessary for complete complex formation when large excess of NTA is used. Calibration curves given by colorimetric and ETAAS methods were linear over the range of 0.15-20 μM iron (III). The colorimetric and ETAAS methods exhibited detection limit (3σ) of 0.13 and 0.14 μM, respectively. The NTBI concentrations from 55 thalassemic serum samples measured employing BPT as chromogen were statistically compared with the results determined by ETAAS. No significant disagreement at 95% confidence level was observed. It is, therefore, possible to select any one of these two techniques for determination of NTBI in serum samples of thalassemic patients. However, the colorimetric procedure requires a longer analysis time because of a slow rate of exchange of NTA ligand with BPT, leading to the slow rate of formation of the colored complex.

INTRODUCTION

Iron is a vital element for all living organisms because it is essential in numerous metabolic pathways including oxygen transport, DNA synthesis and electron transport. Eighty percent of the human body's iron content is incorporated into hemoglobin in red blood cells and the rest is used in enzymatic processes or is stored within other cells. Under normal conditions, all of the iron present in the serum is bound to transferrin, the iron-transporting protein, which comprises only 0.1% of total body iron. Two iron atoms can bind to one transferrin molecule with serum transferrin normally 20-30% saturated with iron (Anderson, 1999; Emerit *et al*, 2001). However, in sera from patients with iron-overloaded conditions, such as thalassemia or hemochromatosis, transferrin may become more than 100 % saturated with iron, leading to the presence in the serum of various forms of iron not bound to transferrin, known as non-transferrin bound iron (NTBI) (Breuer *et al*, 2000). The chemical nature of NTBI in serum has not yet been identified. The iron may be associated with albumin, or may be bound to low molecular weight ligands, such as citrate, amino acids, peptides, and sugars (Anderson, 1999). NTBI is capable of generating harmful oxygen derivatives, leading to tissue and organ damage. It is, therefore, important to monitor and quantify the NTBI levels.

There are a number of articles that describe methods for the determination of NTBI level in serum including gel filtration (Hershko *et al*, 1978), aromatic hydroxylation assay (Singh *et al*, 1989), bleomycin assay (Bonsdorff *et al*, 2002; Evans and Halliwell, 1994), and fluorescence assay (Breuer

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and Cabantchik, 2001; Breuer *et al*, 2001). Nonetheless, many of these methods are either complicated or provide inaccurate NTBI level due to lack of specificity, reproducibility, and sufficient sensitivity. In 1990, Singh *et al* proposed a method to measure NTBI concentration based on the use of nitrilotriacetic acid (NTA) as the ligand to complex all of the NTBI in serum prior to ultrafiltration and analysis. This method is similar to the chelation ultrafiltration technique using EDTA as originally proposed by Hershko *et al* (1978). However, EDTA was used at concentrations which are capable of removing small amount of iron from transferrin, leading to an overestimation of NTBI values. NTA however removes and complexes only the low molecular weight iron compounds and iron nonspecifically bound to serum proteins, and does not mobilize iron from transferrin protein. The solution is then ultrafiltered to separate the resulting Fe (III)-NTA complex and determined for iron in the ultrafiltrate by high-performance liquid chromatography (HPLC) (Gosriwatana *et al*, 1999; Singh *et al*, 1990), colorimetry (Zhang *et al*, 1995) or electrothermal atomic absorption spectroscopy (ETAAS) (Jakeman *et al*, 2001).

In this article, the procedure, described by Zhang *et al* (1995), based on the use of bathophenanthroline as chromogen was investigated, particularly the kinetics of the rate of the tris (bathophenanthrolinedisulfonate) iron (II) complex formation in the presence of excess NTA ligand. The level of NTBI in serum samples from thalassemic patients was quantified by a modified assay and the results were compared with those obtained using an ETAAS method.

MATERIALS AND METHODS

Chemicals

All chemicals used were of analytical-reagent grade. Nitrilotriacetic acid (NTA), sodium thioglycollate (TGA), xylenol orange (XO), 8-hydroxy-7-iodoquinoline-5-sulfonic acid (ferron), hydroxylamine hydrochloride and 4-(2-hydroxyethyl) piperazine-1- ethanesulfonic acid (HEPES) were purchased from Fluka (Buchs, Switzerland). Bathophenanthrolinedisulfonic acid, disodium salt (BPT), 1,2-dihydrobenzene-3,5-disulfonate (tiron) and ascorbic acid were from Sigma (St Louis, MO, USA). Standard iron solution (1,000 mg/l) was obtained from Merck (Darmstadt, Germany). Glass volumetric flasks and plastic containers were soaked in 10% nitric acid overnight, thoroughly rinsed with de-ionized water produced by Milli-Q water purification system (Millipore, USA) to avoid iron contamination. Glass containers were avoided as much as possible, particularly for the storage of reagents, since iron leaches from the glass over time whereas plastic utensils were found to neither bind iron from nor leach iron into the stored solution.

Instrumentation

A Jasco Uvidec-650, double beam spectrophotometer (Japan), was used for colorimetric experiment. A Speedfuge HSC 10KAC (Savant Instrument Inc, Farmingdale, NY, USA) centrifuge was employed in the preparation of the serum ultrafiltrates. ETAAS measurements were carried out with a Perkin-Elmer Analyst 100 spectrometer (Norwalk, CT, USA) equipped with a deuterium-arc background corrector, an AS-72 autosampler and a cooling system for the HGA 800 heated graphite atomizer. Samples were injected using 25 μl sample volume with triplicate injections. The spectral bandwidth and lamp current were 0.2 nm and 20 mA, respectively.

Sample preparation

The ultrafiltration technique was carried out by a modification of the method employed by Singh *et al* (1990). Serum samples were stored frozen at -20°C until time of analysis and thawed before use. An aliquot of 450 μl of serum sample was mixed with 50 μl of 0.20 M NTA (pH 7.0) and allowed to stand at room temperature for 30 minutes. The solution was then ultrafiltered using an Amicon microcon YM-30 filter (MW 30,000 cut-off, Millipore Corporation, Bedford, MA, USA) with an applied centrifugal force of 5,000 rpm for 60 minutes to separate the resulting Fe (III)-NTA complex from transferrin. The Fe (III)-NTA complex present in serum ultrafiltrate was quantified using two different techniques, namely, colorimetric and ETAAS method. Standard iron solutions at various concentrations (0.15-20 μM) were prepared in 0.02 M NTA (pH 7.0) and analyzed by the same procedures as with the serum ultrafiltrate.

Determination of NTBI by colorimetric method

The method used in this study was modified from that described by Zhang *et al* (1995) as follows: an aliquot of 200 μl of serum ultrafiltrate was diluted 1:2 (v/v) with 0.50 M HEPES buffer (pH 7.0). Fifty μl of a reducing agent, 0.15 M

TGA, and 50 μl of 0.05 M BPT, a chromogen for iron (II), were then added to the solution for colorimetric measurement of the Fe (II)-BPT complex. The solution was then equilibrated for 90 minutes at room temperature in order for the formation of colored complex to reach equilibrium before measurement of absorbance at 537.0 nm. This solution was then used for the ETAAS method as validation of the quantitation.

Determination of NTBI by ETAAS

The same solution as used in the colorimetric study was analyzed for NTBI using ETAAS as a comparative method. Ten μl of solution followed by 15 μl of de-ionized water were sequentially injected into the graphite atomizer. The concentration of NTBI was determined using the furnace operating condition as shown in Table 1. The wavelength used for measurement of absorbance of gaseous atomic iron was 248.3 nm.

RESULTS

Optimization of colorimetric parameters

In order to obtain the optimal procedure for spectrophotometric determination of NTBI, a comparison of efficiency of various chromogens and reducing agents was studied. The objective of the experiment was to select for the chromogen giving the highest slope of calibration graph of a series of standard iron solutions (0.15-20 μM) in 0.02 M NTA (pH 7.0). Various chromogens for Fe (III) determination, such as 8-hydroxy-7-iodoquinoline-5-sulfonic acid (ferron), 1,2-dihydrobenzene-3, 5-disulfonate (tiron) and xylenol orange (XO) were chosen for comparison with BPT, a most commonly used reagent for Fe (II) determination (Fe (III) reduced to Fe (II) by TGA). Kinetic measurements given in Fig 1 demonstrate that the formation of Fe (II)-BPT complex required longer time periods than the ferric complexes to reach equilibrium. However, BPT clearly had a higher sensitivity for low levels of iron compared to the other chromogens, as shown in Fig 2. The effect of reducing agents, namely ascorbic acid, hydroxylamine and TGA on the sensitivity is summarized in Table 2. It was found that TGA gave higher sensitivity than the other reducing agents.

In addition, kinetic studies of the tris (bathophenanthrolinedisulfonate) iron (II) complex (Fe (II)-BPT complex) formation in the presence of excess NTA ligand were carried out. The Fe-NTA solution, containing 20 μM standard iron, was mixed with TGA and BPT as described in Materials and Methods for the two oxidation states of iron (Fig 3) and for various excess amounts of NTA (Fig 4), indicating that iron oxidation state had no effect on complex formation, whereas complex formation was slower in presence of excess NTA. The influence of temperature on the rate of formation of Fe (II)-BPT complex was also studied. Results showed that increasing temperature over the range

Table 1
Furnace operating condition for NTBI quantification in serum samples by ETAAS.

Step	Temperature (°C)	Ramp/hold time (sec)	Argon flow rate (ml/min)
Drying	100	10/20	250
Ashing	1,400	10/15	250
Pre-atomization	200	5/5	250
Atomization	2,500	0/5	50
Clean up	2,600	1/5	250

Table 2
The effect of reducing agents on the calibration line for standard iron solution[a].

Reducing agents	Slope ($\times 10^{-2}$)	Intercept ($\times 10^{-2}$)	r^2
Ascorbic acid	1.99	0.36	0.9945
Hydroxylamine	2.01	0.24	0.9986
Thioglycollate	2.28	0.12	0.9984

[a]n=3

Table 3
Validation parameters for determination of NTBI using the colorimetric method and the ETAAS method.

Validation parameters	Concentration of Fe (μM)	%RSD (n=10)	
		Colorimetric method	ETAAS method
Repeatability	2	1.19	5.31
	10	1.09	2.61
Reproducibility	2	9.32	10.47
	10	2.23	3.12

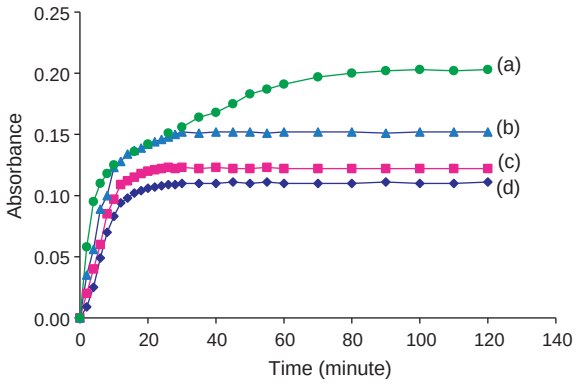

Fig 1–Effect of complexing agents on the kinetics of formation and absorbance of the iron complex. The concentrations of Fe (III) in 0.02 M NTA and complexing agents in all solutions were 20 μM and 5 mM, respectively. Reactions were initiated by mixing Fe (III)-NTA solution with 0.50 M HEPES buffer (pH 7.0) and adding one of the iron (III) complexing agents prior to monitoring changes in absorbance of iron complexes. For the measurement of formation of the Fe (II)-BPT complex, Fe (III)-NTA solution was mixed with 0.50 M HEPES buffer (pH 7.0) and a reducing agent (0.15 M TGA) was then added into the solution prior to adding BPT, an iron (II) complexing agent. All reactions were performed at room temperature. (a) Fe (II)-BPT (detection at 537 nm); (b) Fe (III)-XO (detection at 560 nm); (c) Fe (III)-tiron (detection at 492 nm); (d) Fe (III)-ferron (detection at 435 nm).

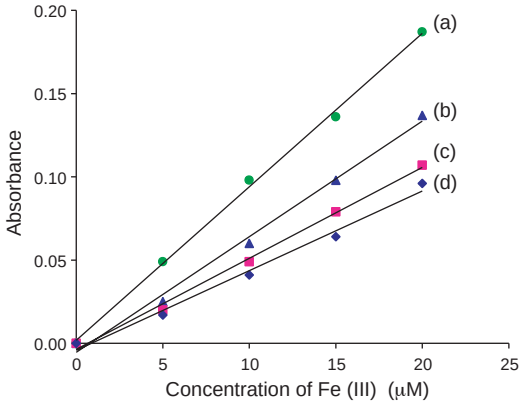

Fig 2–Relationship between the absorbance of the iron complexes and the concentration of Fe (III) in 0.02 M NTA solution for different chromogens. All solutions contained 5 mM chromogens and were carried out at room temperature. Other conditions are as described in Fig 1.

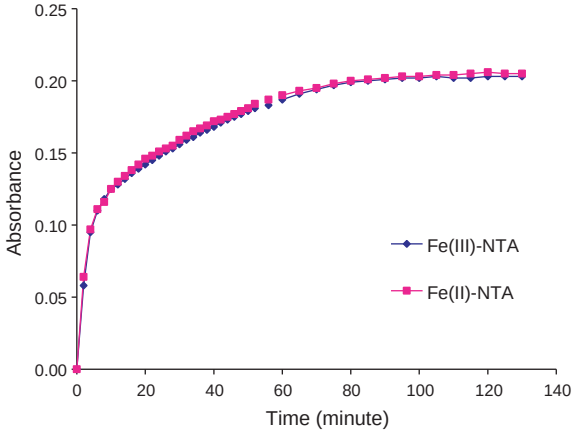

Fig 3–Kinetics of formation of colored Fe (II)-BPT complex for the two initial oxidation state of Fe-NTA complex. Reaction was initiated by mixing Fe-NTA solution (20 μM Fe, 0.02 M NTA) with 0.50 M HEPES buffer (pH 7.0) and a reducing agent (0.15 M TGA) and 0.05 M BPT was then added into solution prior to monitoring changes in absorbance at 537.0 nm. All reactions were carried out at room temperature. The data points are the mean of triplicate experiments.

30º-60ºC had only a small effect on the time for the complete formation of the Fe (II)-BPT complex (results not shown).

Analytical performances

The calibration curves for both methods were linear over the range of 0.15-20 μM iron (III) with correlation coefficient value > 0.99. Limits of detection were determined by ten replicate experiments of a reagent blank for both methods. The detection limit (3σ) was 0.13 and 0.14 μM for the colorimetric and ETAAS methods, respectively. The repeatability (intraday) and reproducibility (interday) were expressed as relative standard deviation (RSD) value of ten replicate analyses at two concentration levels covering the specified range, namely, 2 and 10 μM (Table 3). Recovery study in serum ultrafiltrate was carried out on normal human serum ultrafiltrate using both techniques. The ultrafiltrate from ten normal human sera were spiked with the Fe (III)-NTA standard solutions (2 and 10 μM). The results demonstrate that both colorimetric and ETAAS methods provide satisfactorily good recovery of the signal in the range 98-104% for 2 μM and 99-101% for 10 μM. For the sample separation step, the ultrafiltration technique appears to be the only suitable method currently available to separate the Fe (III)-NTA complex from proteins in serum

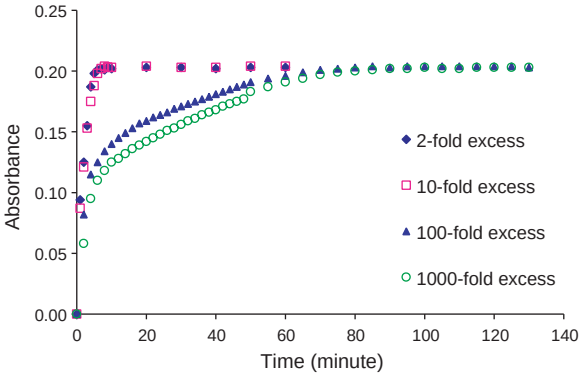

Fig 4–Effect of NTA ligand on the kinetics of formation of Fe (II)-BPT complex using TGA as reducing agent. The concentration of Fe (III) was kept at 20 μM in all solutions and the points are average readings from three experiments. Other conditions are as described in Fig 3.

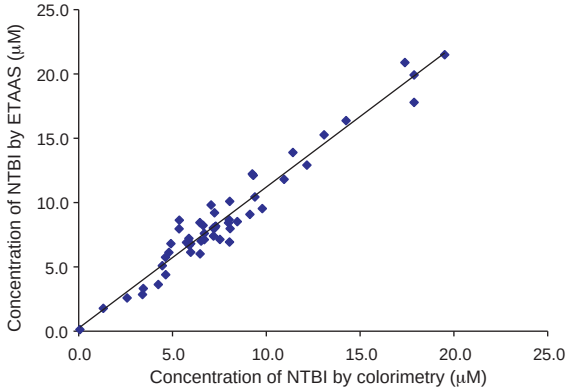

Fig 5–Plot of NTBI concentration from thalassemic serum samples as obtained using the colorimetric and ETAAS methods (n=55).

samples. The percentage recovery of Fe (III)-NTA complex using an ultrafilter is approximately 85% (Gosriwatana *et al*, 1999).

Validation and determination of NTBI in serum samples

Fifty-five serum samples from thalassemic patients were determined for NTBI levels using the ETAAS method and the method employing BPT as a chromogen. In the ETAAS method, NTBI concentration was calculated from a calibration equation using the peak area obtained. The two approaches, colorimetry and ETAAS, gave good agreement in the NTBI values, as shown in Fig 5. A linear regression analysis was performed at 95% confidence interval (Miller and Miller, 2000). The plot between the mean values of both techniques showed the absence of any analytical bias. The correlation coefficient (r) was 0.9693 and a good linear regression line was obtained (y = 1.03±0.07x + 0.63±0.64).

DISCUSSION

Optimization of spectrophotometric determination of NTBI in various chromogens and reducing agents were carried out. This study showed that BPT and TGA have a higher sensitivity for low levels of iron than the other chromogens and reducing agents, respectively. Therefore, BPT was chosen as the chromogen for the spectrophotometric quantification of NTBI in thalassemic serum samples, using TGA as reducing agent.

The kinetic of formation of Fe (II)-BPT complex for the Fe (II)-NTA and Fe (III)-NTA solutions showed that the reduction of Fe (III) to Fe (II) prior to forming the Fe (II)-BPT complex had no effect on the time period to reach equilibrium. The rate-determining step is thus the rate of Fe (II)-BPT complex formation. The effect of excess amount of NTA was studied, as shown in Fig 4. The inter-conversion of Fe (III)-NTA to the Fe (II)-BPT complex took place more slowly when large excess of NTA ligand was present. These results confirm that the exchange of the NTA ligand of Fe (II)-NTA complex with BPT is the rate-determining step. Zhang *et al* (1995) also noticed that formation of the iron complex with large excess ligand (NTA) requirs a period of time. Consequently, sufficient equilibration time for the colored Fe (II)-BPT complex formation is necessary whenever the use of large excess NTA ligand is required.

In this work, the large excess of NTA (0.02 M) was used for preparation of the samples to ensure complete complexation with NTA of all iron nonspecifically bound to serum proteins and iron bound to low molecular weight ligands. Hence, the solution was equilibrated for 90 minutes at room temperature in order for the formation of colored Fe (II)-BPT complex to reach equilibrium before measurement of the absorbance.

Both methods investigated, namely colorimetric and ETAAS, were validated using the optimum conditions. Calibration curves for both methods were linear over the range of 0.15-20 μM iron (III). The colorimetric and ETAAS methods exhibited detection limit (3σ) of 0.13 and 0.14 μM,

respectively. The intraday and interday precisions of the colorimetric method display a relatively low % RSD with a higher deviation for the ETAAS method. Determination recoveries were carried out to evaluate the accuracy of both analytical methods, giving good recovery in range 98-104%.

The NTBI concentrations from 55 thalassemic serum samples measured employing BPT as a chromogen were statistically compared with the results determined by ETAAS. The 95% confidence limit indicated that the slope and the intercept do not differ significantly from the ideal values. Consequently, there is no evidence for systematic differences between the NTBI concentrations obtained from the colorimetric technique and the ETAAS technique.

In summary, two analytical techniques, colorimetric and ETAAS method, were used for quantification of NTBI in thalassemic serum samples. Satisfactory validation data given by both methods were achieved for linearity, accuracy, and precision. Both methods gave desirably low value of detection limit (≥ 0.14 μM). This indicated that colorimetric and ETAAS techniques are sufficiently sensitive for measuring low levels of NTBI. Comparison of NTBI values in thalassemic sera obtained from two techniques shows that both methods are well correlated. This agreement of the results illustrates that it is possible to select any of these methods for determination of NTBI. The data from the kinetic study showed that the concentration of NTA ligand was an important factor in the rate of formation of the colored Fe (II)-BPT complex, due to the slow rate of exchange of the NTA ligand with BPT. The time required for complete formation of the colored Fe (II)-BPT complex should be considered when large excess of NTA ligand is used. The quantification of NTBI using a bathophenanthroline-based method is more convenient and utilizes simple instrumentation; nevertheless, this method requires longer analysis time due to the use of large excess of NTA, leading to the slow rate of formation of the colored Fe (II)-BPT complex.

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REFERENCES

Anderson GJ. Non-transferrin-bound iron and cellular toxicity. *J Gastroenterol Hepatol* 1999; 14: 105-8.

Bonsdorff L, Lindeberg E, Sahlstedt L, Lehto J, Parkkinen J. Bleomycin-detectable iron assay or non-transferrin bound iron in hematologic malignancies. *Clin Chem* 2002; 48: 307-14.

Breuer W, Cabantchik ZI. A fluorescence-based one-step assay for serum non-transferrin-bound iron. *Anal Biochem* 2001; 299: 194-202.

Breuer W, Ermers MJJ, Pootrakul P, Abramov A, Hershko C, Cabantchik ZI. Desferrioxamine-chelatable iron, a component of serum non-transferrin-bound iron used for assessing chelation therapy. *Blood* 2001; 97: 792-8.

Breuer W, Hershko C, Cabantchik ZI. The importance of non-transferrin bound iron in disorders of iron metabolism. *Transfus Sci* 2000; 23: 185-92.

Emerit J, Beaumont C, Trivin F. Iron metabolism, free radicals, and oxidative injury. *Biomed Pharmacother* 2001; 55: 333-9.

Evans PJ, Halliwell B. Measurement of iron and copper in biological systems: bleomycin and copper-phenanthroline assays. *Meth Enzymol* 1994; 233: 82-92.

Gosriwatana I, Loreal O, Lu S, Brissot P, Porter JB, Hider RC. Quantification of non-transferrin-bound iron in the presence of unsaturated transferrin. *Anal Biochem* 1999; 273: 212-20.

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.

Jakeman A, Thompson T, McHattie J, Lehotay DC. Sensitive method for nontransferrin-bound iron quantification by graphite furnace atomic absorption apectrometry. *Clin Biochem* 2001; 34: 43-7.

Miller JN, Miller JC. Statistics and chemometrics for analytical chemistry. 4th ed. Harlow: Pearson Education, 2000.

Singh S, Hider RC, Porter JB. Quantification of non-transferrin-bound iron in thalassemic plasma. *Biochem Soc T* 1989; 17: 697-8.

Singh S, Hider RC, Porter JB. A direct method for quantification of non-transferrin-bound iron. *Anal Biochem* 1990; 186: 320-3.

Zhang D, Okada S, Kawabata T, Yasuda T. An improved simple colorimetric method for quantitation of non-transferrin-bound iron in serum. *Biochem Mol Biol Int* 1995; 35: 635-41.