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Validated HPLC assay for iron determination in biological matrices based on ferrioxamine formation

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Abstract

A simple, robust and reproducible HPLC method has been developed and validated for iron determination in biological matrices. It is based on chelation with desferrioxamine (DFO) and the measurement of the chelate ferrioxamine (FO). The method was developed to permit monitoring of iron bio-kinetics and estimation of iron status in experimental animals. The chromatography was performed on a stainless steel XTerra MS C18 column (Waters; 250 mm × 4.6 mm i.d., 5 μm) using a gradient of Tris–HCl buffer (10 mM, pH 5) and acetonitrile. The method was validated in terms of selectivity, linearity (0.3–80 nmol on-column), limit of detection (0.2 nmol on-column), low limit of quantification (0.3 nmol on-column), recovery (91–102%), intra- and inter-day reproducibility, stability, and robustness. The method's universal applicability was illustrated by monitoring plasma and heart iron kinetic profiles in rats after a single intraperitoneal (i.p.) injection of 200 mg/kg iron dextran.

Keywords: Iron; RP–HPLC; Ferrioxamine; Iron bio-kinetics

1. Introduction

Iron is essential for a wide spectrum of biologic functions, including oxygen transport, mitochondrial electron transfer, and DNA synthesis. Iron determination is indispensable in the evaluation of iron metabolism disorders such as iron deficiency and iron excess (overload). Although biological iron exists in different forms, total serum (plasma) iron determination is a critical reference point for the estimation of transferrin saturation (serum iron/total iron binding capacity), as well as for the calculation of unsaturated iron binding capacity (total iron binding capacity—serum iron). Total iron is also pivotal in defining tissue, organ and organelle burden, particularly when monitoring iron load in transfusion-dependent patients during iron chelation therapy.

Iron's ability to form intensely colored, stable chelates with a variety of ligands, suitable for spectrophotometric determination, has been exploited extensively for analytical purposes. Ligands have included ferrozine [1–3], phenanthroline derivatives [4,5], thiocyanate [6,7]. Several ligands, including ethylenediaminetetraacetic acid (EDTA) and its analogs [8], ethylenediimine derivatives [9–11], and 5-diethylaminophenol derivative [12], have permitted reverse-phase liquid chromatographic (RP–HPLC) iron determination.

A naturally occurring trihydroxamic acid, desferrioxamine (DFO), is a potent iron chelator with little affinity for other metal ions [13]. An RP–HPLC separation of DFO and its iron chelate ferrioxamine (FO) at 1:1 stoichiometric ratio, was reported by Cramer at al. [14]. This method was applied by Gower et al. to identify DFO available iron in biological tissues, while the total iron content was determined by atomic absorption spectrophotometry [15]. The intent of our work was to establish a simple, selective, sensitive, and robust RP–HPLC method for determination of total iron in biological material and to permit quantification of both DFO-available iron and total iron by a single, widely available

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instrumental technique. The method was developed in order to monitor iron status in iron-overloaded experimental animals, to determine bio-kinetics of iron, and to estimate the efficacy of iron chelation treatment. A procedure comprising sample digestion with nitric acid, elemental iron chelation with DFO, and iron determination as FO, was developed and validated.

2. Experimental

2.1. Chemicals

The following chemicals were used: deferoxamine mesylate (Sigma Chemical Co., St. Louis, MO, USA), Tris–hydrochloride (tris(hydroxymethyl)-aminomethane hydrochloride, Merck KGaA, Darmstadt, Germany), ultra-pure nitric acid (Seastar Chemicals Inc., Sidney, BC, Canada), high purity spectroscopy standard of 1 mg/mL elemental iron in 2% nitric acid (Seastar Chemical Inc., Charleston, SC, USA), acetonitrile HPLC grade (Fisher Scientific, Canada), and deionized water (Millipore, the resistance >18 MΩ). Rat and mouse lyophilized and pulverized liver tissue samples used for method validation were obtained from the NIST (National Institute of Standards and Technology, Geithersburg, MD, USA) and from our previous studies (unpublished results). The iron concentration in these samples was pre-determined by inductively coupled plasma–mass spectrometry (ICP–MS).

2.2. Animal experiment

Sprague–Dawley male rats ($N = 33$), body weight about 250 g on arrival, were supplied by The Charles River Laboratories (Montreal, Canada). The animal experiments were approved by the University of Toronto Animal Care Committee, and the experimental procedures conformed to the requirements of the Canadian Council on Animal Care.

Thirty rats were administered a single intraperitoneal elemental iron dose of 200 mg/kg body weight in form of ferric hydroxide dextran complex (Iron Dextran, Sigma Chemical Co., St. Louis, MO, USA). Additionally, three rats received no treatment and were used as controls. The animals, divided in groups of three, were euthanized at 6, 24, 48, 72 h, and on the 6th, 8th, 10th, 15th, 22nd, and 29th day after the injection. Cardiac blood samples, collected into heparinized tubes to harvest plasma, were taken before the injection (control animals) and at the above-mentioned time points. The heart was collected, rinsed with deionized water and stored at 20 °C for iron analysis.

2.3. Sample preparation

2.3.1. Plasma

An aliquot of 0.3 mL plasma was mixed thoroughly with 0.5 mL ultra-pure nitric acid, and the sample was digested for 1 h at room temperature. A portion of the digested sample (0.6 mL) was evaporated to dryness and the residue was dissolved in 0.2 mL of 20 mM DFO solution in 10 mM Tris–HCl (pH 5). After 3 h, the sample was centrifuged for 5 min at 10,000 rpm. The clear supernatant was then chromatographed.

2.3.2. Heart tissue

The whole organ was weighed, cut into small pieces, lyophilized, pulverized and digested in 1 mL ultra-pure nitric acid. The acid digestion was completed after overnight mixing in a metabolic shaker at room temperature. Further treatment was as described for the plasma.

2.3.3. Reference liver samples

A 10–50 mg amount (depending on iron concentration) of the lyophilized and pulverized liver tissue was digested with 1 mL ultra-pure nitric acid. The acid digestion was completed after overnight mixing in a metabolic shaker at room temperature. Further treatment was as described for the plasma.

2.4. Chromatography

A Hewlett-Packard model series 1100 HPLC system with an autosampler and diode-array detector, and a stainless steel XTerra MS C18 column (Waters; 250 mm × 4.6 mm i.d., 5 μm) were used in the present study. Gradient-based chromatography was performed using a Tris–HCl buffer (10 mM, pH 5) and acetonitrile binary mobile phase as shown in Table 1. The total elution time was 38 min at a mobile phase flow rate of 0.8 mL/min. UV–vis detection wavelengths were 214 and 430 nm. All the measurements were performed at ambient temperature.

2.5. FO calibration curve

A 55.85 mcL aliquot of elemental iron solution (1 mg/mL in 2% nitric acid) was evaporated to dryness and the residue was dissolved in 1.0 mL of 20 mM DFO freshly prepared in 10 mM Tris–HCl, pH 5.0. After 3 h the volumes, from 0.025 to 80 mcL, of the FO solution, were chromatographed. The calibration curve range, defined as on-column FO, was from 0.025 to 80 nmol.

Table 1
Gradient chromatography conditions

Time (min)	Acetonitrile (%)	Tris–HCl 10 mM, pH 5 (%)
0	10	90
6	10	90
14	15	85
18	15	85
20	40	60
30	40	60
30.5	10	90

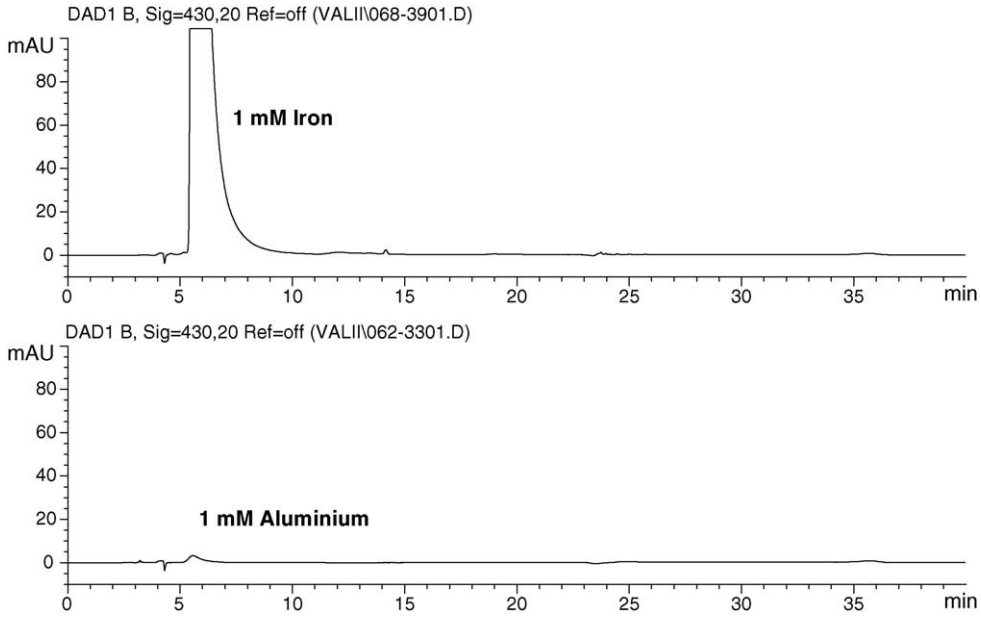

Fig. 1. Chromatograms of 1 mM iron as FO and 1 mM aluminium as Al-DFO detected at 430 nm.

3. Results and discussion

3.1. Selectivity

DFO ability to capture iron selectively in the presence of other metals has been described in the literature [13,16]. We tested the HPLC method selectivity in terms of its ability to measure iron without interference in the presence of other metals that potentially form chelates with DFO, such as zinc, magnesium, calcium, manganese, mercury, and aluminum. The 1 mM chelates of zinc, magnesium, calcium, manganese, mercury, and aluminum with DFO were prepared individually by applying the procedure analogous to the FO preparation as described under Section 2.5. An aliquot of each solution (20 μL) was chromatographed and the detector response was monitored at 430 nm. Among the above-mentioned elements, aluminum showed the greatest competition with iron for chelation with DFO. At equimolar Al-DFO and FO concentration of 1 mM, the area of Al-DFO peak was less than 0.5% of the FO peak area (Fig. 1). The peak areas of other

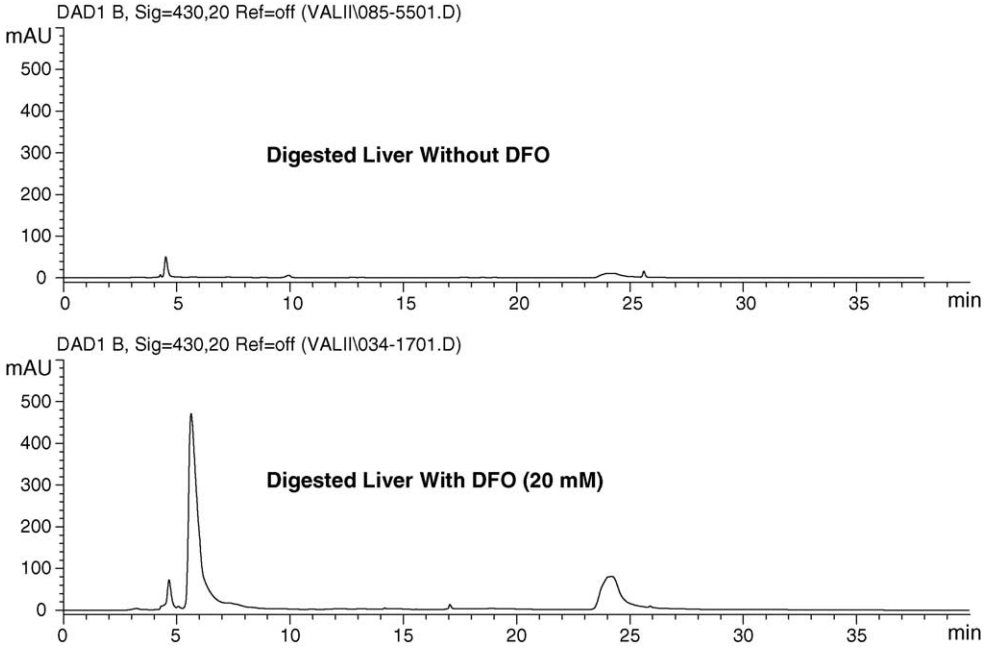

Fig. 2. Chromatograms of the digested liver sample with and without DFO, detected at 430 nm.

metals measured under the analogous conditions were even smaller. Potential FO chromatographic interference, caused by unknown endogenous material, was tested by dissolving sample residue, prepared as described under Section 2.3, in 10 mM Tris–HCl without DFO. The chromatograms of the liver sample at 430 nm prepared with and without DFO, as presented in Fig. 2, demonstrate selectivity of iron determination as FO in the presence of the aforementioned endogenous material.

3.2. Kinetics of FO formation

The complexation kinetics of aqueous iron(III) by DFO has been studied by Lentz et al. [17]. The overall second order rate constant was determined for three different equimolar iron(III) and DFO concentrations, at three different pHs and temperatures. The authors concluded that the complexation was sufficiently rapid to remove excess iron from blood. However, to optimize experimental conditions, we studied the complexation further in order to define the reaction time necessary to achieve the maximum formation of FO for iron concentrations varying from 0.1 to 10 mM, and a constant DFO concentration of 20 mM in Tris–HCl buffer, pH 5. The samples were prepared as described under Section 2.5. Aliquots of 5.585, 55.85, 279.25 and 558.5 mcL of iron atomic absorption solution were transferred into separate test tubes, evaporated to dryness and the residues were dissolved in 1.0 mL of freshly prepared 20 mM DFO in 10 mM Tris–HCl, pH 5.0. The iron concentrations in the prepared solutions were 0.1, 1, 5, and 10 mM. The solutions were chromatographed immediately after preparation and at different time points up to 72 h after mixing. To obtain comparable FO peak areas for iron concentrations of 0.1, 1, 5, and 10 mM, the volumes injected were 100, 10, 2, and 1 μL, respectively. The FO peak area at 430 nm was measured. Time-dependent FO formation at different Fe:DFO ratios, and the stability at room temperature, are illustrated in Fig. 3.

Table 2
FO unweighted and weighted ($1/x$ and $1/x^2$) linear regression parameters (±standard deviation)

Weight	Slope	Intercept	Correlation coefficient (r^2)
No	205.6 ± 0.6	13.7 ± 18.4	0.9998
$1/x$	206.1 ± 0.6	4.30 ± 1.7	0.9997
$1/x^2$	203.8 ± 2.3	4.9 ± 0.4	0.9961

Based on these results it was evident that a minimum equilibration time of 3 h was needed after mixing the samples with 20 mM DFO solution to ensure complete complexation. Once formed, FO was stable at room temperature for several days.

3.3. Linearity and sensitivity (LOD, LLOQ)

Non-linear FO calibration dependence has been reported by Kraemer and Breithaupt [18]. This occurred when FO was formed after post-column derivatization and then measured instantaneously. The quadratic calibration observed by the authors was related to the second-order reaction kinetics. Under our experimental conditions chromatographic response was measured after FO formation and thereby the phenomenon was avoided; our observed calibration dependence was linear. Calibration parameters, determined by plotting peak areas versus on-column iron amount in nanomoles, were evaluated from three individual calibration data sets. We fitted all the data simultaneously and compared unweighted and weighted ($1/x$ and $1/x^2$) regression parameters (Table 2).

The residual scatter for weighted ($1/x$ and $1/x^2$) linear regression at low FO concentrations (0–1 nmol on column), showed no advantages over the unweighted regression line. Therefore, the unweighted regression line parameters were employed for iron determination as FO.

The limit of detection (LOD) and lower limit of quantification (LLOQ), determined from the unweighted calibration data, based on the intercept and its standard deviation (LOD), and on the percentage deviation from the nominal concentra-

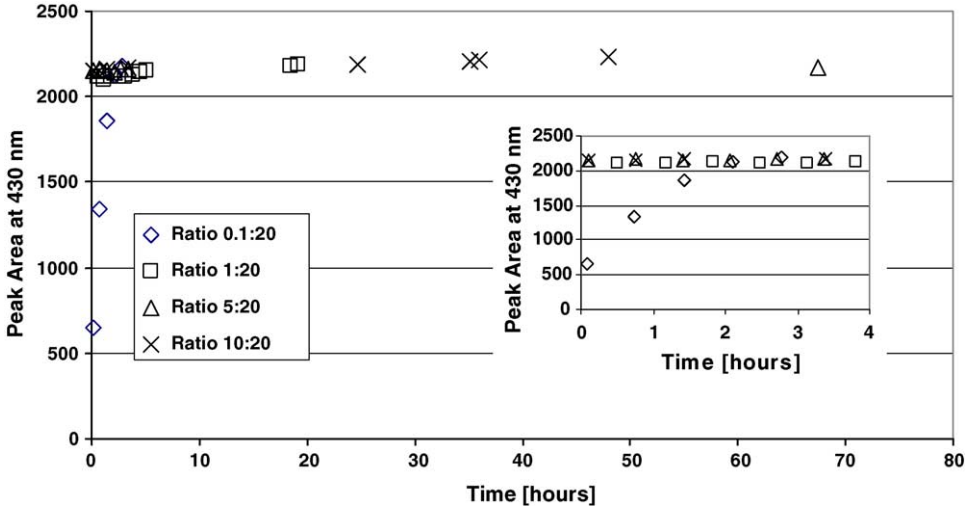

Fig. 3. Time-dependent FO formation and stability at different Fe:DFO ratios over a 4 h (inserted graph) and 72 h at room temperature.

Table 3
Recovery of iron determination from rat and mouse liver samples

Sample code	Iron concentration (mcg/g dry tissue) by ICP–MS	Mean iron concentration ± S.D. (mcg/g dry tissue) by RP–HPLC ($N = 4$)	Recovery (%)
NIST	180	183 ± 5.2	101.6
M336	595	598 ± 4.8	100.5
M328	2778	2519 ± 4.8	90.7
F385	16907	17027 ± 6.3	100.7

Table 4
Intra- and inter-day reproducibility (mean ± S.D., $N = 4$) of iron determination as FO by HPLC method

Sample	Day 1	Day 2	Day 3	Day 4	Inter-day
NIST	183 ± 5.2	190 ± 5.5	182 ± 11.8	164 ± 14.4	180 ± 10.2
M336	598 ± 4.8	662 ± 11.9	610 ± 11.9	621 ± 7.4	623 ± 9.5
M328	2519 ± 4.8	2644 ± 5.1	2614 ± 10.3	2386 ± 5.3	2541 ± 7.4
F385	17027 ± 6	17166 ± 5	16884 ± 5	15644 ± 3	16547 ± 7

tion (LLOQ), were 0.2 and 0.3 nmol on-column, respectively. The tested on-column FO linearity range was from 0.3 to 80 nmol.

3.4. Recovery, accuracy, reproducibility, and precision

Iron is present in all biological materials in variable concentrations. Therefore, instead of spiking a biological test material with iron for our recovery studies, we used rat and mouse liver samples as reference materials. The iron concentration in these samples was previously determined by ICP–MS. The samples were prepared in quadruplicate as described under Section 2.3. The results of sample analysis are presented in Table 3.

Deviations from the ICP–MS values by less than 10% are considered evidence of the method's good recovery and high accuracy. The coefficient of variation for repeated measurements was between 4.76 and 6.29% illustrating the method's precision.

Intra- and inter-day reproducibility was tested by preparing the samples in quadruplicate on four different days and analyzing them by HPLC. Intra-day stability was evaluated from the measurements performed on the same day, while for inter-day stability the measurements from four different days were obtained. The results of intra- and inter-day reproducibility, expressed as mean values with corresponding standard deviations, are summarized in Table 4.

3.5. Stability

As already described under Section 3.2, FO stability at room temperature was tested by injecting repeatedly the same solution over a period of several days. The samples were stable up to three days after preparation as shown in Fig. 3. The relative standard deviations of the peak areas measured over this time period were between 0.30 and 1.13%.

FO stability in solution after a single freeze-thaw cycle was tested for concentrations of 0.25, 0.5, and 1 mM. The solutions, prepared as described under Section 2.5, were divided into two portions after an equilibration period of 3 h. One portion was analyzed at room temperature (19–22 °C), another was stored in a freezer at −20 °C for a period of one week, and then analyzed after thawing at room temperature. Each sample was injected six times into the HPLC column. A paired one-tailed t-test was applied for the statistical evaluation. The results of stability testing are presented in Table 5.

3.6. Robustness

To test the method's robustness, the TRIS buffer was prepared and the analysis was performed by two analysts, using two XTerra MS C18 columns from different batches (Waters, W21191L 007 and W13201K 016, respectively) and two HPLC instruments (Agilent 1100 and Agilent 1050, Agilent Technologies, USA). A small change in FO retention time (from 5.9 to 6.4 min), and in the column dead time (from 4.1 to 4.3 min), resulting in a shift of the capacity factor from 0.44 to 0.49, were observed as a consequence of the above mentioned changes. Chromatographic repeatability was tested by injecting 20 μL of a solution as described under Section 2.5 on six occasions into each of two tested

Table 5
Stability of FO in solution

FO concentration in solution (mM)	FO peak area at 430 nm (±S.D., $N = 6$) at room temperature	FO peak area at 430 nm (±S.D., $N = 6$) after thawing	*t* Critical (alpha 0.05)	*t* Statistical
1	7638 ± 47	7800 ± 90	2.02	−7.05
0.5	3940 ± 36	3947 ± 62	2.02	0.72
0.25	1796 ± 24	1796 ± 17	2.02	−0.03

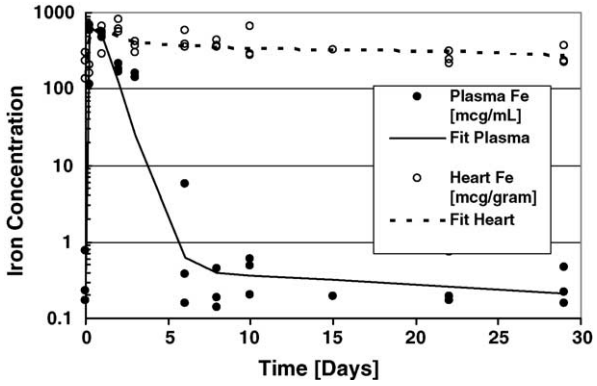

Fig. 4. The time-dependent plasma and heart iron data simultaneous fit to a two-compartment open model with elimination from the central compartment. The symbols represent the observed values and the lines represent the simultaneous computer fits.

systems comprising different HPLC equipment, different column batches, and analysts. The relative standard deviation of the FO peak areas was 0.94 and 0.84%, while the relative standard deviation of the retention times was 0.25 and 0.05%, respectively.

Other factors, such as chemical purity, the source, and preparation of FO for quantitative measurement can affect the method robustness. According to our knowledge the literature does not provide precise information about these factors. In this paper, we report the source of materials and the method for preparation of FO for the calibration curve. In additional experiments not presented herein, we observed the effect of the mobile phase composition on FO determination. It was observed that ethyl acetate and MOPS buffer (3-[*N*-morpholino]propanesulfonic acid) tend to interfere with FO measurement. Stainless-steel chromatographic equipment and columns generally might cause a contamination that interferes with the measurement, as well. The addition of EDTA (4 mM) to the mobile phase, as reported by others [14,15], could prevent the formation of FO during the chromatographic procedure. We, however, due to possible FO dissociation in the presence of another chelating agent, avoided addition of EDTA to the mobile phase. Under our experimental condition (10 mM Tris–HCl buffer, pH 5) the above-mentioned interferences were not encountered.

3.7. Method applicability

Iron determination in Sprague–Dawley rats' plasma and lyophilized heart tissue, after a single intraperitoneal (i.p.) injection of iron dextran solution (Sigma), is presented to illustrate the method's applicability in iron bio-kinetic studies. The plasma and heart data versus time, as presented in Fig. 4, were simultaneously fit (Scientist MicroMath Scientific Software, MicroMath Research, St. Louis, Missouri, USA) to a two-compartment open model with elimination from the central compartment. The plasma iron concentration versus time profile showed rapid iron absorption after i.p. injection leading to a C_{max} at about 6 h. The plasma elimination rate constant and a terminal half-life were 0.03 day^{-1}, and 23 days, respectively. The iron concentration in heart reached a maximum at 24 h after i.p. injection and showed a slow decline until and including the 29th day.

4. Conclusions

The main purpose of this work was to develop an HPLC assay for iron determination in biological samples. The presented method is based on FO formation, and is particularly applicable to iron bio-kinetic studies. The reaction between DFO and Fe(III) although generally rapid, requires some time to reach the equilibrium, especially for low Fe(III) concentrations, as described under Section 3. Therefore, a minimum equilibration time of 3 h was recommended. Once formed, FO is stable for several days at room temperature. A freeze-thaw cycle does not affect FO stability. The method permits total iron measurement in biological matrices and is useful for diagnostic purposes. Our validated method is simple, reproducible, robust and linear over the tested range and permits iron determination in biological material in a wider linearity range in comparison to atomic absorption spectrometry. Additionally, we experienced no necessity for background corrections, so common in atomic absorption spectrometry [19]. The rat plasma and heart tissue iron concentration versus time profile after a single intraperitoneal iron dextran dose illustrates the method's applicability for iron determination in biological matrices. We propose that the method has more universal applicability for a variety of matrices (e.g. food, pharmaceutics). Furthermore, the HPLC instrumentation is commonly used in analytical laboratories and the method therefore, requires no specialized equipment dedicated for metal analysis.

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