

# Removal of Fe<sup>3+</sup> and Zn<sup>2+</sup> from plasma metalloproteins by iron chelating therapeutics depicted with SEC-ICP-AES

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The iron chelation therapy drugs desferrioxamine B (DFO) and deferiprone (DFP) are used to treat iron overload patients, but not much is known about their adverse effects on other essential metals *in vivo*. After the addition of a clinically relevant dose of DFP or an equimolar dose of DFO to human plasma *in vitro*, the mixtures were analyzed by size exclusion chromatography (SEC) coupled to an inductively coupled plasma atomic emission spectrometer (ICP-AES). Simultaneous detection of the emission lines of copper, iron and zinc allowed the visualization of changes that these drugs exerted at the metalloprotein level. After the addition of DFP, a <10 kDa novel Fe-peak was detected and identified as (DFP)<sub>3</sub>Fe, whereas DFO resulted in the elution of a much smaller amount of Fe in this elution range. In fact, DFP was ~8-times more efficient than DFO regarding the removal of Fe from plasma proteins. The addition of both iron chelators also resulted in the elution of a <10 kDa novel Zn-peak. DFP abstracted twice as much Zn from plasma proteins compared to DFO. The identification of one of these peaks as (DFP)<sub>2</sub>Zn establishes a feasible biomolecular basis for the etiology of Zn-deficiency in patients that undergo long-term treatment with these drugs. Our results demonstrate that the analysis of plasma by SEC-ICP-AES can simultaneously provide insight into the efficacy of chelation therapy drugs and their adverse health effects at the metalloprotein level. Thus, SEC-ICP-AES emerges as a useful analytical tool to visualize health-relevant bioinorganic chemistry-related reactions of medicinal drugs in blood plasma *in vitro*.

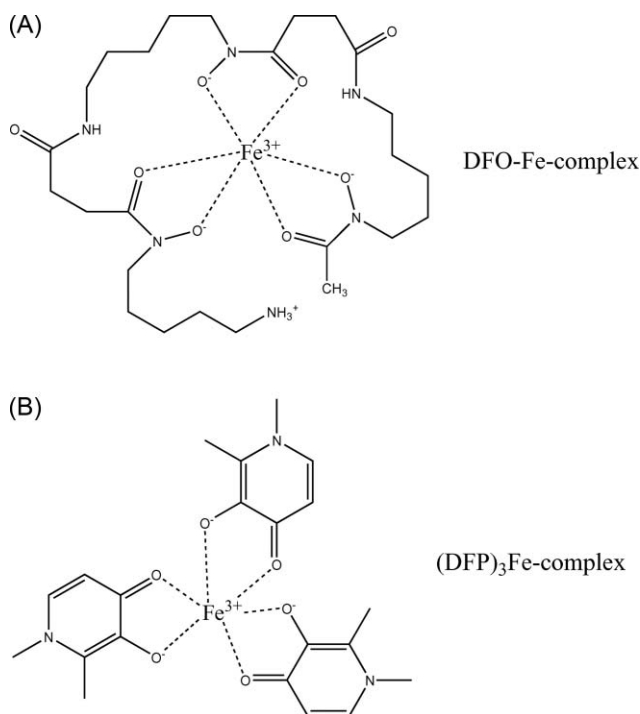
## Introduction

Metallomics represents a novel research area which may be broadly defined as the research efforts that aim to better understand the biomolecular mechanisms that govern metal-dependent life processes.<sup>1</sup> To visualize dynamic processes that involve metals inside organisms *in vitro*, however, bioanalytical techniques must be applied which can yield relevant information in spite of the presence of the considerable amount of matrix (*e.g.* proteins, salt *etc.*) that is inherently associated with the analysis of blood plasma<sup>2</sup> or bile.<sup>3</sup> To this end, the hyphenation of size exclusion chromatography (SEC) to an inductively coupled plasma atomic emission spectrometer (ICP-AES) has been recently employed to directly analyze rabbit plasma for the contained major Cu, Fe and Zn-containing metalloproteins/peptides.<sup>4</sup> Approximately 12 Cu, Fe and Zn metalloproteins/peptides were detected in rabbit plasma in <25 min and rather similar results were obtained after the analysis of human plasma.<sup>5</sup> Interestingly, the relative intensities and retention times of some metalloprotein peaks were different in human as compared to rabbit plasma, which indicates that biochemically relevant information can be obtained with this instrumental analytical technique. Despite some inherent limitations of this methodology, the analysis of plasma by SEC-ICP-AES nevertheless offers several unique advantages compared to other proteomic techniques, which makes it an interesting tool for metallomics studies.<sup>5</sup> SEC-ICP-AES, for instance, has

already been successfully applied to study the interaction of arsenobetaine with human plasma constituents *in vitro*.<sup>6</sup> This analytical approach, however, should also be ideally suited to visualize the transfer of a metal ion from one plasma protein to another entity (with a different hydrodynamic radius) *in vitro*. In order to investigate this inherent capability of SEC-ICP-AES, we studied the effect of iron chelation therapy drugs that were added to human blood plasma *in vitro* at the metalloprotein level.

Iron overload diseases, such as hereditary hemochromatosis (HH), are remarkably common in humans<sup>7</sup> and entail the accumulation of toxic levels of iron in various organs and tissues, including the liver, the heart and the pancreas.<sup>8,9</sup> Iron chelation therapy drugs, such as desferrioxamine B (DFO) and deferiprone (DFP), are frequently used to treat patients in order to ameliorate the adverse health effects that are associated with excess iron in tissues.<sup>10</sup> DFO (559 g mol<sup>-1</sup>) is a naturally occurring hydroxamate type siderophore, which is produced by *Streptomyces pilosus*.<sup>11</sup> When intravenously administered to humans, DFO forms a thermodynamically stable hexadentate 1:1 complex with Fe<sup>3+</sup> (complex formation constant log *K*<sub>f</sub> = 30.5,<sup>12</sup> Fig. 1A) which is subsequently excreted *via* urine and feces by mammals.<sup>11</sup> Conversely, DFP (139 g mol<sup>-1</sup>) is an orally active iron chelation therapy drug which is considerably cheaper and smaller than DFO and forms a 3:1 complex with Fe<sup>3+</sup> (Fig. 1B). Owing to its better lipid solubility, DFP readily enters mammalian cells and forms a (DFP)<sub>3</sub>Fe complex, which is subsequently mobilized from the cell and excreted predominantly *via* urine (70%).<sup>11</sup> In view of the fact that DFP is efficiently absorbed from the gastrointestinal tract, it offers a clear advantage over DFO (which must be intravenously administered), but it is also

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**Fig. 1** Molecular structure of the Fe<sup>3+</sup> complexes of (A) desferrioxamine B (DFO) and (B) deferiprone (DFP).

potentially more toxic since it can penetrate the blood–brain and the placental barrier. Nevertheless, DFP has been demonstrated to remove iron not only from liver cells, but also from heart cells.<sup>11</sup> In view of the fact that DFO and DFP both end up in the human bloodstream and taking into account that ferritin (Ft) and transferrin (Tf) are the major iron-containing plasma proteins in human plasma,<sup>5,13</sup> the addition of each of these iron chelators to plasma *in vitro* followed by the subsequent investigation of the Fe-distribution would represent a feasible way to directly observe the abstraction/removal of Fe<sup>3+</sup> from iron-containing plasma proteins. Furthermore, the simultaneous determination of the Cu- and Zn-distribution in plasma could reveal if DFO and/or DFP will also abstract these metals from their parent Zn- or Cu-containing metalloproteins.<sup>14,15</sup> The concomitant detection of small molecular weight complexes between Cu/Zn and DFO/DFP in plasma *in vitro* would therefore provide insight into biochemical mechanisms by which iron chelators disrupt the homeostasis of essential metals and thus explain some of the adverse side-effects that have been observed during the treatment of patients with these drugs.<sup>16</sup> In order to investigate if SEC-ICP-AES is amenable to visualize the abstraction of iron from plasma proteins by iron chelation therapy drugs, physiologically relevant doses of DFP or DFO were added to human plasma (from a healthy individual) and the obtained mixtures were analyzed. To eliminate the previously observed variation with regard to the concentration of metalloproteins between individual organisms,<sup>4</sup> a homogenous human plasma stock was used throughout this study. The obtained Fe-specific chromatograms were intended to reveal the comparative efficacy of these iron chelation therapy drugs with regard to their abstraction of Fe<sup>3+</sup> from plasma proteins, whereas the simultaneous monitoring of the emission lines of Cu and Zn was intended to visualize potential side-effects of these drugs.

## Experimental

### Chemicals

3-Hydroxy-1,2-dimethyl-4(1H)-pyridone (DFP, 98%), desferrioxamine mesylate (DFO) salt (≥95%, stored at –20 °C until use) and phosphate-buffered saline (PBS) buffer tablets were purchased from Sigma-Aldrich (St. Louis, MO, USA). ZnCl<sub>2</sub> (>98%) was obtained from Arcos Organics. PBS buffer (10 mM phosphate, 2.7 mM KCl, 137 mM NaCl, pH 7.4) was prepared by dissolving PBS tablets in the appropriate volume of water (followed by pH adjustment with dilute HCl) and the filtration of the obtained solution through 0.45 μm nylon-filter membranes (Mandel Scientific, Guelph, ON, Canada) before use. A mixture of protein standards which contained thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa) and vitamin B<sub>12</sub> (1.35 kDa) was obtained from Bio-Rad Laboratories (Hercules, CA, USA) to calibrate the employed Superdex 200 SEC column. All solutions were prepared with water from a Simplicity water purification system (Millipore, Billerica, MA, USA).

### Solutions

Doses of 25–60 mg kg<sup>–1</sup> body wt/day of DFO and 50–75 mg kg<sup>–1</sup> body wt/day of DFP are used in the clinic.<sup>16–18</sup> In view of the fact that DFP is administered ~3 times day, 25 mg kg<sup>–1</sup> body wt are administered orally per treatment. In our experiments the concentration of DFP in plasma after spiking was 0.58 mg DFP/mL and the corresponding concentration of DFO in plasma was 2.36 mg DFO/mL (this concentration is equimolar to that of DFP). We prepared stock solutions of DFO (61.308 mg mL<sup>–1</sup>) and DFP (15.158 mg mL<sup>–1</sup>) by dissolving the appropriate amount of each drug in the appropriate volume of PBS buffer (pH 7.4). The stock solution of DFP was prepared by incubating the solution at 37 °C and gentle shaking (100 rpm) until dissolution was complete. Both DFO and DFP solutions were used within 3 h of preparation.

### SEC-ICP-AES system

The SEC-ICP-AES system was comprised of a Smartline 1000 HPLC pump (Knauer, Berlin, Germany) and a Rheodyne 9010 PEEK injection valve (Rheodyne, Rhonert Park, CA, USA) which was equipped with a 0.5 mL PEEK injection loop (0.5 mL). A pre-packed Superdex™ 200 10/300 GL Tricorn™ high performance size-exclusion chromatography column (30 × 1.0 cm I.D., separates globular proteins between ~600 and ~10 kDa; GE Healthcare, Piscataway, NJ, USA) was used in conjunction with a PBS buffer mobile phase at a flow rate of 1.0 mL min<sup>–1</sup> (column temperature 22 °C).<sup>4,6</sup> Simultaneous multielement-specific detection of C (193.091 nm), S (180.731 nm), P (213.618 nm), Cu (324.754 nm), Fe (259.940 nm), and Zn (213.856 nm) in the column effluent was achieved with a Prodigy, high-dispersion, radial-view ICP-AES (Teledyne Leeman Labs, Hudson, NH, USA) at an Ar gas-flow rate of 19 L min<sup>–1</sup>, an RF power of 1.3 kW and a nebulizer gas pressure of 35 psi. A 7.0 min delay was implemented between injection and data acquisition based on the void volume (505 s/8.41 mL) that was determined by the injection of blue dextrane. A 1180 s data acquisition window was used. The obtained raw data were imported into Sigmaplot 11 software and smoothed using a

bisquare algorithm. Peak areas were determined using Sigmaplot 11 after baseline correction.

### Analysis of DFO/DFP spiked human plasma by SEC-ICP-AES

The collection of blood from humans was approved by the Calgary Conjoint Health Research Ethics Board (Approval No. E-21198). A human plasma stock was prepared from blood that was collected from a ~12 h fasted, male and healthy volunteer into heparinized trace metal testing blood collection tubes (Greiner-Bio-One Vacuette™, NC, USA). After centrifugation at 1100 *g* (4 °C) for 10 min, the buffy coat was removed and the supernatant plasma was removed and pooled. Aliquots of this homogenous plasma stock were transferred to cryovials and stored at –30 °C. Plasma (1.0 mL) was thawed at room temperature for 45 min and incubated at 37 °C for 4 min before the DFO or DFP solutions (40 μL) were added.<sup>4</sup> The pH of plasma (1.0 mL) after the addition of DFP (corresponding to a concentration of 0.60 mg DFP/mL plasma) was 7.73 and that of plasma after the addition of DFO (corresponding to a concentration of 2.44 mg DFO/mL plasma) was 7.71. The obtained mixtures were incubated at 37 °C for 10 min and then immediately injected onto the SEC-ICP-AES system. The rationale for the 10 min reaction time is based on the half-life of DFO in blood of humans, which is only about 10–15 min.<sup>7</sup> All experiments were carried out in triplicate and representative chromatograms are presented.

### Electrospray ionization-mass spectrometry (ESI-MS)

A Bruker Esquire 3000 instrument was used to detect positively charged ions in the <10 kDa SEC column fraction that was collected after the analysis of DFP-treated human plasma. The collected fraction was diluted two times with aqueous acetic acid (0.1%) and the obtained solution was introduced into the electrospray ionization (ESI) source at a flow rate of 600 μL h<sup>-1</sup>. The capillary voltage was 4000 kV and the experiment was run with a dry gas temperature of 350 °C.

### Carbon-13 nuclear magnetic resonance spectroscopy (<sup>13</sup>C NMR)

NMR experiments were performed on a Bruker Avance 400 MHz spectrometer equipped with a 5 mm broadband probe head at 37 °C. All NMR spectra were acquired and processed using Topspin 2.1 software (Bruker). Separate solutions of DFP (15 mg mL<sup>-1</sup> in D<sub>2</sub>O/PBS buffer, final pH 7.4) and the (DFP)<sub>2</sub>Zn complex [20 μL of a ZnCl<sub>2</sub> solution (371 mg mL<sup>-1</sup> in H<sub>2</sub>O)] were added to 1.0 mL of the DFP solution and the pH was adjusted to 6.6 with 4.0 M NaOH were prepared in D<sub>2</sub>O and <sup>13</sup>C NMR spectra were acquired using the waltz-16 <sup>1</sup>H decoupling scheme. A sweep width of 25 kHz, acquisition time of 0.65 ms, π/6 pulse length of 8.50 μs and pulse delay of 2 s were employed. A total of 848 scans were averaged and an apodization function of 2 Hz was applied prior to Fourier Transform. <sup>13</sup>C NMR spectra were referenced to an external sample of methanol in D<sub>2</sub>O at 49.15 ppm; *J*-modified and HMBC experiments were also performed for the solutions of DFP and the (DFP)<sub>2</sub>Zn complex to allow conclusive assignment of carbon signals (data not shown).

## Results and discussion

In the context of ameliorating genetic human illnesses that involve the dyshomeostasis of essential metals, a plethora of chelation therapy drugs have been developed over the last 60 years.<sup>10</sup> Even though the underlying biomolecular mechanisms of action have been extensively studied, many questions regarding an understanding of their *in vivo* effects at the molecular level remain elusive<sup>19</sup> and serious side-effects have often been described only on a phenomenological basis. Conceptually, side-effects of chelation therapy drugs must be ultimately attributed to the complexity of biological fluids (*e.g.* blood plasma contains up to as much as 10 000 different proteins)<sup>5</sup> and the comparatively high probability that an administered drug will not only interact with the intended target molecule (*e.g.* the desired metal), but also with unintended target sites (*e.g.* other essential metals *etc.*). Although the iron chelation therapy drugs DFO and DFP have been demonstrated to be effective in removing iron from various internal tissues in patients,<sup>10,11</sup> side-effects including Zn-deficiency have been frequently observed in patients that are treated for long periods of time.<sup>10,16,17</sup> The detailed mechanism(s) by which iron chelators remove iron from Tf, however, are not well understood at the molecular level.<sup>20,21</sup> Even though the DFP-induced Zn-deficiency which has been repeatedly reported in patients can be routinely managed by oral Zn-therapy,<sup>16</sup> the biomolecular basis of its etiology as well as that of other toxic side-effects is not understood.<sup>20</sup>

In principle, DFO or DFP can interact either with Fe<sup>3+</sup> that is bound to the major iron-containing plasma metalloproteins Ft and/or Tf,<sup>4</sup> and/or with the intracellular labile iron pool.<sup>8</sup> In view of the fact that both DFO and DFP eventually enter the human bloodstream regardless of how they are administered, studies into the interaction of each of these drugs with metalloproteins in plasma *in vitro* may contribute not only to a better understanding of their comparable efficacy (with regard to the removal of Fe<sup>3+</sup> from plasma proteins), but could also possibly provide important new insights into the biomolecular basis of some of their toxic side-effects.<sup>16,20</sup> Such a strategy, however, would require a bioanalytical technique which must allow one to visualize the DFO- or DFP-induced abstraction of Fe<sup>3+</sup> from iron-containing plasma proteins and to detect the small molecular weight DFO/(DFP)<sub>3</sub>Fe-complexes that are formed. We have employed SEC-ICP-AES to visualize this Fe-redistribution event by adding clinically relevant doses of DFP or DFO to human plasma and by analyzing the obtained mixture for the contained Cu-, Fe- and Zn-metalloproteins. In order to compare the efficacy of DFO to that of DFP, we chose a DFP dose that is commonly used in patients and compared the results that were obtained after the analysis of DFP-treated human plasma by SEC-ICP-AES to those that were obtained after the addition of an equimolar dose of DFO. In view of the fact that the results that were obtained with thawed human plasma were essentially identical to those obtained for fresh human plasma (data not shown), the findings that are described below cannot be attributed to the freezing of plasma. In addition, it is important to emphasize that based on our data it is impossible to delineate whether the changes in the observed plasma metalloproteome pattern that were caused by the addition of iron chelators to plasma were completed after the 10 min incubation period, or if chemical reactions between

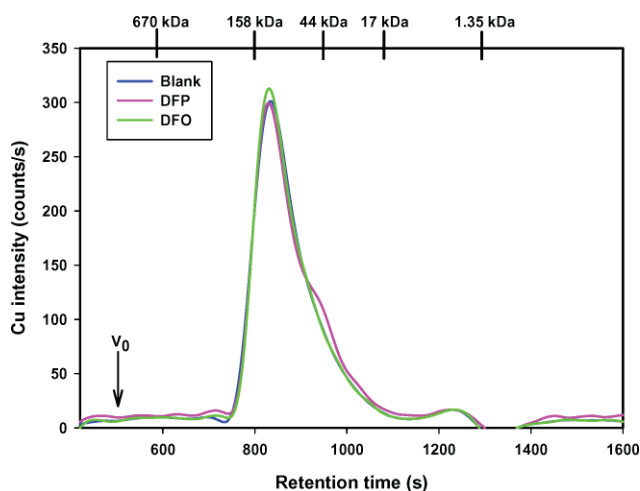
**Table 1** Total peak areas of all Cu, Fe and Zn peaks obtained after the analysis of human plasma and DFO or DFP spiked human plasma by SEC-ICP-AES ( $n = 3$ , mean  $\pm$  SD). Abbreviations: desferrioxamine (DFO), deferiprone (DFP)

	Plasma	Plasma + DFP	Plasma + DFO
Fe	22281 $\pm$ 779	22732 $\pm$ 675	22506 $\pm$ 840
Cu	44171 $\pm$ 2481	42339 $\pm$ 1474	43128 $\pm$ 2710
Zn	10012 $\pm$ 259	10486 $\pm$ 265	9689 $\pm$ 160

plasma proteins, the iron chelator and the salt from the mobile phase continued during the chromatographic separation process.

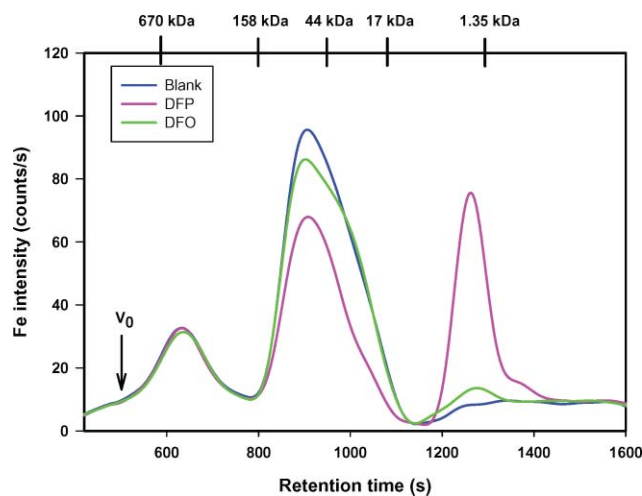
The combined area of all detected Cu, Fe and Zn peaks that were obtained after the analysis of plasma and DFO- or DFP-spiked plasma are displayed in Table 1. Overall, the addition of DFO or DFP to plasma did not affect the total peak area of all detected metal peaks, which is expected since a homogenous human plasma stock was used throughout this study.

Fig. 2 represents the Cu-specific chromatograms that were obtained after the analysis of plasma and DFO or DFP spiked plasma. Essentially a single Cu-peak (retention time 830 s) was detected in all instances. Based on the retention time of this peak and the previously reported instability of some Cu-metalloproteins contained in rabbit plasma over a 2 h period,<sup>4</sup> the detected Cu-peak (Fig. 2) is identified as ceruloplasmin, Cp. In accord with our earlier studies, labile plasma Cu-metalloproteins were not detected.<sup>4</sup> These results demonstrate that Cu-ions were not abstracted from Cp by DFO or DFP, which is in good agreement with previous studies.<sup>22</sup> This can be readily explained by the fact that all six Cu atoms in Cp are integrated into the structure and are therefore not exchangeable. Our results further suggest that the previously reported DFO-induced increased elimination of Cu from  $\beta$ -thalassemia patients<sup>14</sup> must therefore be attributed to the interaction of DFO with intracellular Cu-metalloproteins as is indicated by the fact that this iron chelator had a more pronounced effect on the faecal loss of Cu than on urinary loss.<sup>14</sup>



**Fig. 2** Representative Cu-specific chromatograms that were obtained for the analysis of human plasma on a Superdex 200 10/300 GL ( $30 \times 1.0$  cm I.D.,  $13 \mu\text{m}$  particle size) SEC column at  $22^\circ\text{C}$  using PBS buffer (0.15 M, pH 7.4) as the mobile phase. Flow rate =  $1.0 \text{ mL min}^{-1}$ , injection volume =  $500 \mu\text{L}$ , detector: ICP-AES at  $324.754 \text{ nm}$  (Cu). The retention times of the molecular weight markers are depicted on top of the figure.

The Fe-specific chromatograms that were obtained when plasma and DFO or DFP spiked plasma were analyzed by SEC-ICP-AES are depicted in Fig. 3. In agreement with earlier results only two Fe peaks were detected in unspiked plasma,<sup>5</sup> with the first one (retention time  $\sim 634$  s,  $\sim 23\%$  of total Fe) corresponding to Ft and the second one (retention time  $905$  s,  $\sim 77\%$  of total Fe) corresponding to Tf (Table 2; Ft and Tf were previously identified in column fractions following the analysis of human plasma immunoassays).<sup>4</sup> Even though it cannot be excluded

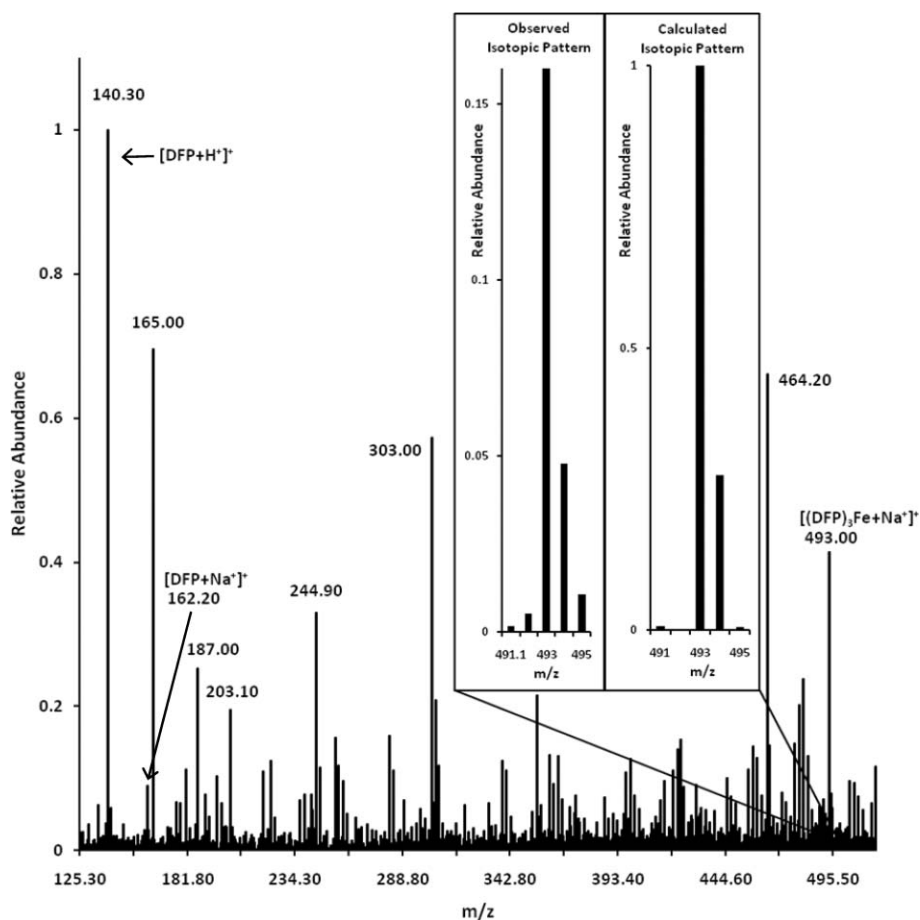


**Fig. 3** Representative Fe-specific chromatograms that were obtained for the analysis of human plasma on a Superdex 200 10/300 GL ( $30 \times 1.0$  cm I.D.,  $13 \mu\text{m}$  particle size) SEC column at  $22^\circ\text{C}$  using PBS buffer (0.15 M, pH 7.4) as the mobile phase. Flow rate =  $1.0 \text{ mL min}^{-1}$ , injection volume =  $500 \mu\text{L}$ , detector: ICP-AES at  $259.940 \text{ nm}$  (Fe). The retention times of the molecular weight markers are depicted on top of the figure.

that the second Fe-peak—based on the limited resolution of SEC chromatography—may also contain another Fe-containing protein of similar hydrodynamic radius,<sup>4</sup> it will be referred to as ‘Tf’ in the remainder of this manuscript. The Fe-specific chromatogram that was obtained after the analysis of DFO-spiked plasma was subtly, but significantly different from that of unspiked plasma (Fig. 3). In particular, an additional  $<10 \text{ kDa}$  Fe-peak was detected and the Fe-peak corresponding to Tf was slightly less intense. The determination of the peak area of all three Fe-peaks revealed that DFO had abstracted  $\sim 4\%$  of  $\text{Fe}^{3+}$  from Tf or  $\sim 3\%$  of total plasma Fe (Table 2). This result is in general agreement with previous observations that this iron chelator is rather ineffective at removing  $\text{Fe}^{3+}$  from this plasma protein within a 10 min time period.<sup>7,23,24</sup> The fact that at least some  $\text{Fe}^{3+}$  was removed from Tf by DFO is—albeit seemingly negligible—remarkable and deserves to be commented on. Considering that salt has been demonstrated to play an essential role in the redistribution of  $\text{Fe}^{3+}$  from Tf to DFO (salt essentially acts as a mediator),<sup>21,25,26</sup> it is possible that the interaction of the DFO-spiked plasma with the salt from the mobile phase (immediately after the injection of plasma onto the SEC-column) may have contributed to the results that were obtained. The fact that  $\sim 4\%$  of  $\text{Fe}^{3+}$  were removed from Tf by DFO is somewhat surprising since this iron chelator has been deemed too big to access the  $\text{Fe}^{3+}$  binding sites on Tf.<sup>27</sup> Nevertheless, our data indicate that DFO can abstract  $\text{Fe}^{3+}$  from Tf binding

**Table 2** Total peak areas and relative percentages of all Cu, Fe and Zn peaks obtained after the analysis of human plasma and DFO or DFP spiked human plasma by SEC-ICP-AES ( $n = 3$ , mean  $\pm$  SD). Abbreviations: desferrioxamine (DFO), deferiprone (DFP)

	Fe		Zn		
	Ft	Tf	<10 kDa	>17 kDa	<10 kDa
Plasma	5060 $\pm$ 223 23%	17222 $\pm$ 556 77%	—	10012 $\pm$ 259 100%	—
Plasma + DFP	5077 $\pm$ 138 22%	11627 $\pm$ 409 51%	6029 $\pm$ 139 27%	8731 $\pm$ 266 83%	1755 $\pm$ 12 17%
Plasma + DFO	5090 $\pm$ 271 23%	16753 $\pm$ 385 74%	663 $\pm$ 202 3%	8788 $\pm$ 148 91%	900 $\pm$ 14 9%



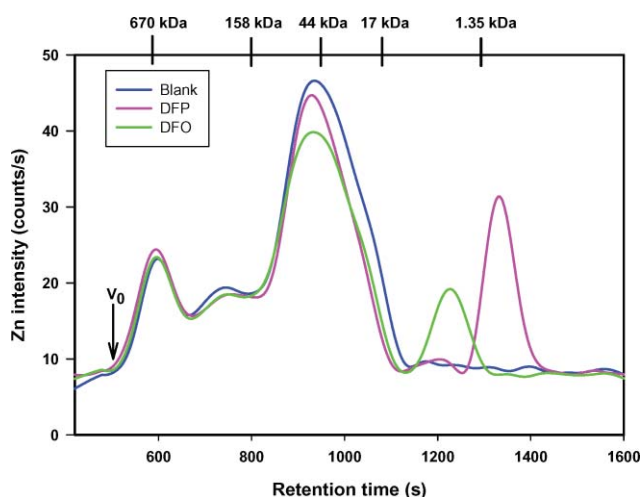
**Fig. 4** ESI-MS identification of  $(\text{DFP})_3\text{Fe}$  as the Fe-entity that eluted in the <10 kDa elution range (using SEC and 0.15 M PBS buffer at pH 7.4) after human plasma was spiked with DFP.

sites, even though the detailed underlying mechanism is unknown. In contrast to the results that were obtained for DFO, the addition of DFP to plasma abstracted  $\sim 34\%$  of  $\text{Fe}^{3+}$  (Fig. 3) from Tf (or  $\sim 27\%$  of total plasma Fe, Table 2), which is  $\sim 8$  times as much as was observed for DFO. Although the detailed biomolecular process by which  $\text{Fe}^{3+}$  is removed from Tf by these iron chelators is not entirely understood,<sup>21,26</sup> the considerably more efficient abstraction of  $\text{Fe}^{3+}$  from Tf by DFP can be rationalized in terms of its  $\sim 4$  times smaller molecular weight (Fig. 1) and its facilitated diffusion to the  $\text{Fe}^{3+}$  binding sites, which are buried in a crevice of each Tf-lobe.<sup>23,28–30</sup> With regard to the structural characterization of the Fe-species that eluted in the <10 kDa elution range, a fraction containing this species was identified as  $(\text{DFP})_3\text{Fe}$  (Fig. 1 and 4). Interestingly,

the ESI-MS data indicate that free DFP was also present in the collected fraction that contained the  $(\text{DFP})_3\text{Fe}$  complex. These findings indicate that neither free DFP nor  $(\text{DFP})_3\text{Fe}$  have a strong affinity for human plasma proteins, which contributes to make this iron chelation therapy drug quite effective in mobilizing iron. In contrast to the aforementioned removal of Tf-bound  $\text{Fe}^{3+}$  by DFO and DFP in human plasma, the intensity of the Fe-peak corresponding to Ft was virtually unchanged by both iron chelators (Fig. 3, Table 2). These results are in general agreement with previous studies, which demonstrated that the mobilization of  $\text{Fe}^{3+}$  from Ft by DFO or DFP is a very slow process (32–39% of  $\text{Fe}^{3+}$  were mobilized from Ft over 9.4 days)<sup>31</sup> that appears to be irrelevant on a biological time-scale.<sup>32–34</sup> Taken together,

the obtained Fe-specific chromatograms clearly demonstrate that DFP is an 8-fold better  $\text{Fe}^{3+}$ -chelator in human blood plasma than DFO, which is in general accord with previous observations.<sup>8,20,23,35</sup>

The Zn-specific chromatograms that were obtained after the analysis of plasma and DFO- or DFP-spiked plasma by SEC-ICP-AES are shown in Fig. 5. In general, the Zn-specific chromatogram that was observed for untreated plasma was in good accord with previous results<sup>5</sup> and three Zn-entities with retention times of 595, 742 and 930 s were detected. Zn-peak 1 was previously identified as  $\alpha_2$ -macroglobulin and Zn-peak 3 likely corresponds to albumin-bound Zn.<sup>4</sup> Interestingly, the addition of DFO to human plasma resulted in the elution of a <10 kDa Zn-peak (retention time 1225 s, ~9% of total Zn, Table 2), which was absent from the chromatogram obtained for unspiked plasma (Fig. 5). The decreased intensity of the Zn-peak corresponding to human serum albumin (HSA) indicates that DFO had abstracted  $\text{Zn}^{2+}$  from this plasma protein (Table 2); most likely from the weak  $\text{Zn}^{2+}$  binding



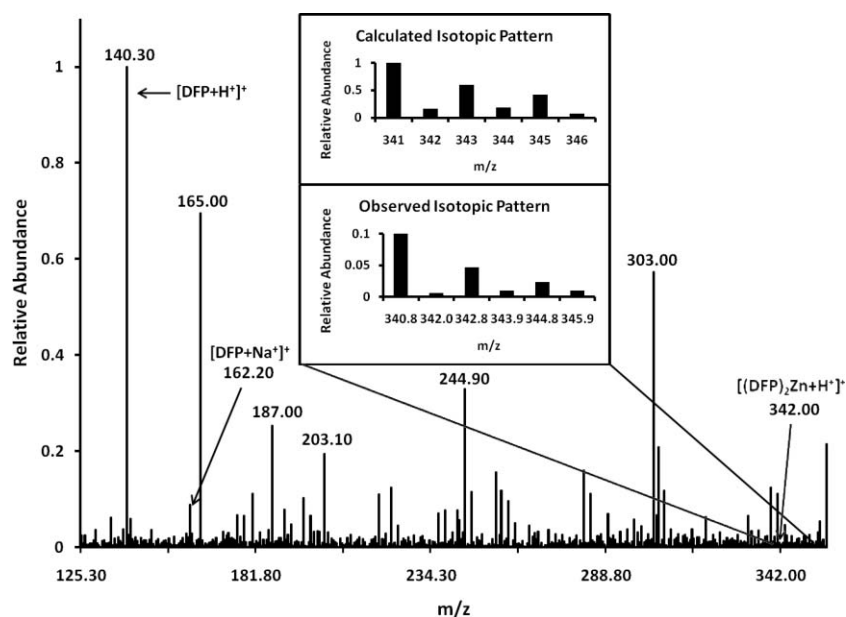
**Fig. 5** Representative Zn-specific chromatograms that were obtained for the analysis of human plasma on a Superdex 200 10/300 GL ( $30 \times 1.0$  cm I.D.,  $13 \mu\text{m}$  particle size) SEC column at  $22^\circ\text{C}$  using PBS buffer (0.15 M, pH 7.4) as the mobile phase. Flow rate =  $1.0 \text{ mL min}^{-1}$ , injection volume =  $500 \mu\text{L}$ , detector: ICP-AES at  $213.856 \text{ nm}$  (Zn). The retention times of the molecular weight markers are depicted on top of the figure.

site on HSA.<sup>36,37</sup> Despite several attempts to identify the putative DFO-Zn complex in a collected column fraction by ESI-MS and atmospheric pressure chemical ionization (APCI)-MS, we only observed peaks for free DFO (data not shown). The addition of DFP to human plasma also resulted in the elution of a Zn-peak in the <10 kDa elution range (Fig. 5), which—in contrast to DFO—contained ~17% of total Zn (Table 2). Compared to the <10 kDa Zn-peak that was detected after the addition of DFO to human plasma and had a retention time of 1225 s, however, the small molecular weight Zn-peak that was detected after the addition of DFP to plasma had a retention time of 1332 s, which implies a comparatively smaller Zn-species than the putative DFO-Zn complex. In agreement with this, the analysis of a column fraction containing this Zn-species revealed  $(\text{DFP})_2\text{Zn}$  complex (molecular weight  $343 \text{ g mol}^{-1}$ ) (Fig. 6). The *in vitro* formation of  $(\text{DFP})_2\text{Zn}$  in human plasma can be rationalized in terms of the putative abstraction of  $\text{Zn}^{2+}$  from the weak binding site of HSA and

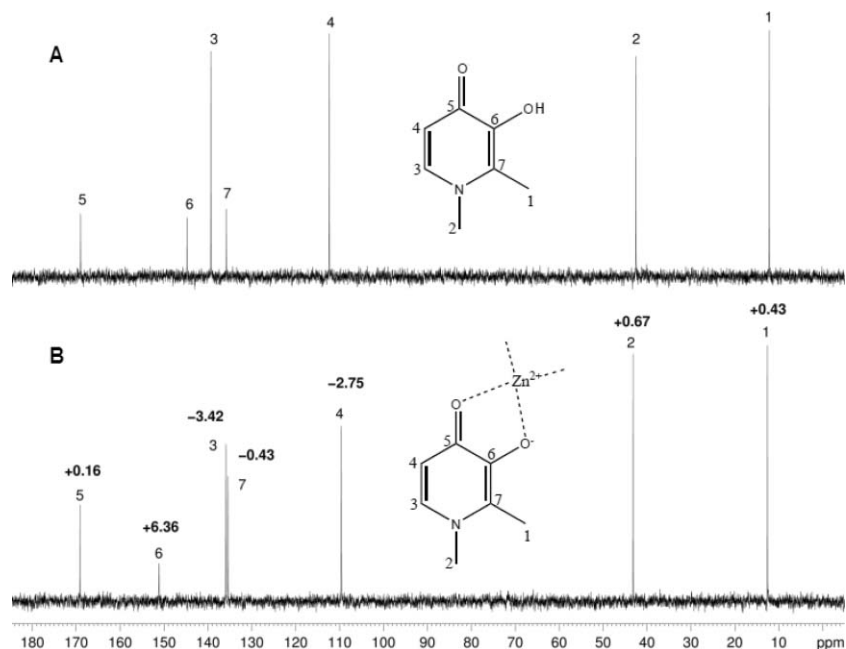
the moderate affinity of  $\text{Zn}^{2+}$  for 3-hydroxy-4-pyridinones.<sup>38</sup> The obtained ESI-MS data, however, only allow one to determine the overall stoichiometry of this complex, but not how  $\text{Zn}^{2+}$  binds to DFP. Based on the crystal structure of  $(\text{DFP})_2\text{Zn} \cdot 7\text{H}_2\text{O}$  (obtained by reacting DFP with  $\text{Zn}^{2+}$  in ethanolic solution) in which  $\text{Zn}^{2+}$  coordinated to two oxygen atoms of DFP,<sup>39</sup> it is likely that the  $(\text{DFP})_2\text{Zn}$  complex that was isolated from human plasma was structurally related. In order to verify that under physiological conditions  $\text{Zn}^{2+}$  can form a 1:2 complex with DFP in which the metal ion is coordinated to DFP in analogy to the synthetic  $(\text{DFP})_2\text{Zn} \cdot 7\text{H}_2\text{O}$  complex, we compared the  $^{13}\text{C}$  NMR spectrum of an aqueous solution of DFP in PBS buffer ( $\text{D}_2\text{O}$ , pH 7.4) with that obtained after the addition of a stoichiometric amount of  $\text{Zn}^{2+}$  to this solution (final pH 6.5). As is evident from Fig. 7, the addition of  $\text{Zn}^{2+}$  resulted in a considerable +6.3 ppm downfield chemical shift of the  $^{13}\text{C}$ -signal corresponding to carbon 6, whereas carbon 5 was only marginally affected (chemical shift +0.16 ppm). The  $^{13}\text{C}$ -signals corresponding to carbon 3 and 4, however, were shifted upfield, which indicates an increased electron density at these carbon atoms. The latter can be explained by the coordination of  $\text{Zn}^{2+}$  to the carbonyl group of DFP and the induced delocalization of the electron lone-pair from the nitrogen atom toward the double bond between carbon 3 and 4 (Fig. 7). Importantly, these results corroborate that under physiological conditions (PBS buffer,  $37^\circ\text{C}$ )  $\text{Zn}^{2+}$  ions indeed coordinate to both oxygen atoms of DFP, which suggests that the  $(\text{DFP})_2\text{Zn}$  complex that was isolated from human plasma was structurally indistinguishable from the synthetic  $(\text{DFP})_2\text{Zn} \cdot 7\text{H}_2\text{O}$  complex. In view of the fact that DFO-Zn and  $(\text{DFP})_2\text{Zn}$  complexes are known to be readily excreted in mammalian urine,<sup>40,41</sup> their *in vitro* formation in human plasma—which to the best of our knowledge is described here for the first time—provides a feasible biomolecular explanation for the DFO- and DFP-induced Zn-deficiency that is associated with the long-term therapy of iron overload patients.<sup>10,14,16,17,20,42</sup> Furthermore, it is possible that other side effects that have been previously described for these iron chelators in the literature, such as bone disease,<sup>7</sup> acute ocular toxicity,<sup>14,43</sup> renal toxicity/abnormal skeletal growth,<sup>43,44</sup> and auditory neurotoxicity<sup>43</sup> may also be ultimately traced back to the *in vivo* formation of DFO-Zn or  $(\text{DFP})_2\text{Zn}$ .

## Conclusion

The detailed biochemical reactions that unfold *in vivo* after patients are administered with the iron chelators DFO and/or DFP as well as their associated side-effects are incompletely understood.<sup>45</sup> The addition of physiologically relevant doses of DFO and DFP to human plasma from a healthy volunteer and the subsequent analysis of the obtained mixture (after incubation for 10 min at  $37^\circ\text{C}$ ) by SEC-ICP-AES revealed that DFP is ~8 times as effective as DFO with regard to the removal of  $\text{Fe}^{3+}$  from plasma proteins. Furthermore, our results revealed that DFP is twice as “effective” as DFO in abstracting  $\text{Zn}^{2+}$  from plasma proteins. Even though these findings were obtained using plasma from a healthy individual, they are relevant to the treatment of iron overload patients with these drugs because the underlying bioinorganic processes in plasma are in principle the same. Importantly, the detection of  $(\text{DFP})_2\text{Zn}$  and DFO-Zn in plasma likely represents the biomolecular basis for the Zn-deficiency that is induced in patients that undergo long-term treatment with DFO and/or



**Fig. 6** Identification of the Zn entities that eluted in the <10 kDa elution range after the analysis of human plasma that was spiked with DFP by SEC using 0.15 M PBS buffer (pH 7.4) by ESI-MS.



**Fig. 7**  $^{13}\text{C}$  NMR spectra of an aqueous solution of DFP (A) and a solution of DFP to which  $\text{Zn}^{2+}$  had been added in the stoichiometric ratio of 2:1 (B). Carbon peaks 1–7 were assigned based on the  $J$ -modified and heteronuclear multiple bond correlation (HMBC) spectra that were measured for both solutions.

DFP. The essentially systems-biology minded approach of analyzing whole plasma for the contained metalloproteins after the addition of DFO or DFP allowed us to observe the comparative efficacy of these chelation therapy drugs *and* to visualize their potential side-effects in a practical and expedited manner. SEC-ICP-AES therefore emerges as a useful tool that can be applied by bioinorganic chemists to study dynamic processes of other chelation therapy drugs in plasma *in vitro*. The experimental

verification of the capability of SEC-ICP-AES to simultaneously visualize intended biochemical reactions of chelation therapy drugs (*i.e.* the abstraction of the metal of interest from a plasma protein) as well as unintended ones (*i.e.* the abstraction of essential metals from other plasma metalloproteins) in their physiological context can now be applied to better understand bioinorganic chemical processes in plasma and therefore to advance human health in the 21st century.<sup>46</sup>

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## References

- 1 S. Mounicou, J. Szpunar and R. Lobinski, *Chem. Soc. Rev.*, 2009, **38**, 1119–1138.
- 2 K. Cottingham, *Anal. Chem.*, 2005, **77**, 197A–200A.
- 3 J. Gailer, S. Madden, G. A. Buttigieg, M. B. Denton and H. S. Younis, *Appl. Organomet. Chem.*, 2002, **16**, 72–75.
- 4 S. A. Manley, S. Byrns, A. W. Lyon, P. Brown and J. Gailer, *JBIC, J. Biol. Inorg. Chem.*, 2009, **14**, 61–74.
- 5 S. A. Manley and J. Gailer, *Expert Rev. Proteomics*, 2009, **6**, 251–265.
- 6 K. L. Pei and J. Gailer, *Metallomics*, 2009, **1**, 403–408.
- 7 K. R. Bridges, *Encyclop. Life Sci.*, 2001, 1–7.
- 8 R. S. Britton, K. L. Leicester and B. R. Bacon, *Int. J. Hematol.*, 2002, **76**, 219–228.
- 9 D. M. De Silva, C. C. Askwith and J. Kaplan, *Physiol. Rev.*, 1996, **76**, 31–47.
- 10 G. Crisponi and M. Remelli, *Coord. Chem. Rev.*, 2008, **252**, 1225–1240.
- 11 G. Faa and G. Crisponi, *Coord. Chem. Rev.*, 1999, **184**, 291–310.
- 12 W. Kaim and B. Schwederski, *Bioanorganische Chemie*, B. G. Teubner, Stuttgart, 1991.
- 13 W. Y. Craig, T. B. Ledue and R. F. Ritchie, *Plasma Proteins. Clinical Utility and Interpretation*, Dade Behring Inc., Newark, NJ, 2000.
- 14 S. De Virgili, M. Congia, M. P. Turco, F. Frau, C. Dessi, F. Argioli, R. Sorcinelli, A. Sitzia and A. Cao, *Arch. Dis. Child.*, 1988, **63**, 250–255.
- 15 I. Pashalidis and G. J. Kontoghiorghes, *Drug. Res.*, 2001, **51**, 998–1003.
- 16 A. V. Hoffbrand, *Best Pract. Res. Clin. Haematol.*, 2005, **18**, 299–317.
- 17 J. L. Kwiatkowski, *Pediatr. Clin. North Am.*, 2008, **55**, 461–482.
- 18 C. Vermynen, *Eur. J. Pediatr.*, 2008, **167**, 377–381.
- 19 G. M. Whitesides, P. W. Snyder, D. T. Moustakas and K. A. Mirica, in *Physical Biology. From Atoms to Medicine*, ed. A. H. Zewail, Imperial College Press, London, 2008, pp. 189–215.
- 20 G. J. Kontoghiorghes, K. Pattichi, M. Hadjigavriel and A. Kolnagou, *Transfus. Sci.*, 2000, **23**, 211–223.
- 21 P. F. Lindley, in *Handbook of Metalloproteins*, ed. A. Messerschmidt, R. Huber, T. Poulos and K. Wieghardt, John Wiley & Sons, Ontario, 2001, vol. 2, pp. 793–811.
- 22 P. F. Lindley, in *Handbook of Metalloproteins*, ed. A. Messerschmidt, R. Huber, T. Poulos and K. Wieghardt, John Wiley & Sons, Ontario, 2001, vol. 2, pp. 1369–1379.
- 23 S. Pollack, P. Aisen, F. D. Lasky and G. Vanderhoff, *Br. J. Haematol.*, 1976, **34**, 231–235.
- 24 I. Turcot, A. Stintzi, J. Xu and K. N. Raymond, *JBIC, J. Biol. Inorg. Chem.*, 2000, **5**, 634–641.
- 25 S. A. Kretchmar and K. N. Raymond, *Inorg. Chem.*, 1988, **27**, 1436–1441.
- 26 R. Kumar and A. G. Mauk, *J. Phys. Chem. B*, 2009, **113**, 12400–12409.
- 27 L. D. Devanur, R. W. Evans, P. J. Evans and R. C. Hider, *Biochem. J.*, 2008, **409**, 439–447.
- 28 F. N. Al-Refaie, C. E. De Silva, B. Wonke and A. V. Hoffbrand, *J. Clin. Pathol.*, 1995, **48**, 110–114.
- 29 G. J. Kontoghiorghes, A. Kolnagou, E. Eracleous and R. W. Evans, *Br. J. Haematol.*, 2005, **129**, 157–161.
- 30 W. R. Harris, Z. Wang, C. Brook, B. Yang and A. Islam, *Inorg. Chem.*, 2003, **42**, 5880–5889.
- 31 G. J. Kontoghiorghes, S. Chambers and A. V. Hoffbrand, *Biochem. J.*, 1987, **241**, 87–92.
- 32 R. R. Crichton, F. Roman and F. Roland, *J. Inorg. Biochem.*, 1980, **13**, 305–316.
- 33 M. C. Brady, K. S. Lilley, A. Treffry, P. M. Harrison, R. C. Hider and P. D. Taylor, *J. Inorg. Biochem.*, 1989, **35**, 9–22.
- 34 J. B. Porter, *Am. J. Hematol.*, 2007, **82**, 1136–1139.
- 35 C. E. Brook, W. R. Harris, C. D. Spilling, W. Peng, J. J. Harburn and S. Srisung, *Inorg. Chem.*, 2005, **44**, 5183–5191.
- 36 C. André and Y. C. Guillaume, *Talanta*, 2004, **63**, 503–508.
- 37 S. Ostojic, V. Dragutinovic, M. Kicanovic and B. R. Simonovic, *J. Serb. Chem. Soc.*, 2007, **72**, 331–337.
- 38 E. T. Clarke and A. E. Martell, *Inorg. Chim. Acta*, 1992, **191**, 57–63.
- 39 L. Saghale, G. Houshfar and M. Nishabor, *Iran. J. Pharm. Res.*, 2006, **3**, 179–189.
- 40 F. N. Al-Refaie, B. Wonke, A. V. Hoffbrand, D. G. Wickens, P. Nortey and G. J. Kontoghiorghes, *Blood*, 1992, **80**, 593–599.
- 41 G. J. Kontoghiorghes, M. A. Aldouri, L. Sheppard and A. V. Hoffbrand, *Lancet*, 1987, **329**, 1294–1295.
- 42 G. J. Kontoghiorghes, M. B. Agarwal, R. W. Grady, A. Kolnagou and J. J. Marx, *Lancet*, 2000, **356**, 428–429.
- 43 D. R. Crapper McLachlan, A. J. Dalton, T. P. A. Kruck, M. Y. Bell, W. L. Smith, W. Kalow and D. F. Andrews, *Lancet*, 1991, **337**, 1304–1308.
- 44 C. Hershko, A. M. Konijn and G. Link, *Br. J. Haematol.*, 1998, **101**, 399–406.
- 45 L. R. Perez and K. J. Franz, *Dalton Trans.*, 2010, **39**, 2177–2187.
- 46 E. Zeini Jahromi and J. Gailer, *Dalton Trans.*, 2010, **39**, 329–336.