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Chelator-facilitated removal of iron from transferrin: Relevance to combined chelation therapy.

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Short title: Iron removal from transferrin

SYNOPSIS

Current iron chelation therapy consists primarily of the use of desferrioxamine (DFO), which has to be administered via intravenous infusion, together with deferiprone and deferasirox, which are orally active chelators. These chelators, although effective at decreasing the iron load, are associated with a number of side effects. Grady suggested that the combined administration of a smaller bidentate chelator and a larger hexadentate chelator, such as DFO would result in greater iron removal than either chelator alone (Grady, Bardoukas & Giardina (1998) *Blood* 92, 16b). This, in turn, could lead to a decrease in chelator dose. To test this hypothesis the rate of iron transfer from a range of bidentate hydroxypyridin-4-one (HPO) chelators to DFO was monitored. Spectroscopic methods were utilised to monitor the decrease in the concentration of the Fe-HPO complex. Having established that the shuttling of iron from the bidentate chelator to DFO does occur under clinically relevant concentrations of chelator, studies were undertaken to evaluate whether this mechanism of transfer would apply to iron removal from transferrin. Again, the simultaneous presence of both a bidentate chelator and DFO was found to enhance the rate of iron chelation from transferrin at clinically relevant chelator levels. Deferiprone was found to be particularly effective at 'shuttling' iron from transferrin to DFO, probably due to its small size and relative low affinity for iron compared with other analogous HPO chelators.

Keywords: Iron chelation; enhanced scavenging activity of iron chelators; transferrin; deferiprone; desferrioxamine; hydroxypyridin-4-ones.

INTRODUCTION.

Although current chelation therapy is effective at improving life expectancy of individuals with transfusion-dependent anaemias, its use causes numerous disadvantages. Desferrioxamine (DFO) is not orally active and must be administered by subcutaneous infusion over 8h periods up to 5 times per week. This property decreases compliance with the drug. Two orally active iron chelators have also been approved for clinical use; namely, deferasirox [1] and deferiprone (1,2-dimethyl-3-hydroxypyridin-4-one) [2]. A comparative study of the safety and efficacy of deferiprone and DFO in β -thalassaemia major patients has demonstrated a similar ability to remove iron from the liver, but a greater increase in cardiac function was observed with deferiprone treatment [3]. Interest in the application of iron chelators in the treatment of non iron overload diseases has also increased over the last 5 years with considerable effort being concentrated in two important fields, the treatment of cancer [4] and the treatment of neurodegeneration [5,6].

It has previously been suggested that the combined administration of a bidentate and a hexadentate chelator can lead to enhanced scavenging activity of iron from certain iron-containing proteins [7]. In principle, the smaller, kinetically labile bidentate chelator can rapidly access intracellular iron pools. The scavenged iron is subsequently transferred to the kinetically inert hexadentate chelator, forming a more stable complex, which is excreted. In this way, the bidentate chelator acts as a 'shuttle' molecule and the hexadentate chelator as a 'sink'. The concept of combined chelation therapy has previously been utilised to remove excess metals from the body in the cases of both plutonium and cadmium poisoning [8]. A combination of diethylenetriaminepentacetic acid (DTPA) and salicylic acid, for instance, was utilised in the removal of plutonium. Combined administration of these two chelators had a greater effect than either DTPA or salicylic acid alone.

Enhanced scavenging, if operational, will be dependent on the transfer of iron from the bidentate to the hexadentate chelator (Figure 1). It is therefore of interest to investigate whether appreciable rates of transfer can occur at clinically relevant chelator concentrations.

It has been previously established that DFO cannot remove iron from transferrin at physiological concentrations, probably due to its relatively large size [9]. However, it is hypothesised that in

the presence of a smaller bidentate chelator, iron could be 'shuttled' from transferrin to DFO. Experiments investigating possible synergistic effects of combined administration of chelators in the removal of iron from transferrin have been carried out previously [10,11]. However, these studies were undertaken using high chelator concentrations. The observation that deferiprone is able to chelate transferrin-bound iron [9], indicates that it may also be able to access other iron pools that are inaccessible to DFO. Thus, the combination of deferiprone with DFO may permit the chelation of iron, which would not normally be removed by DFO therapy alone. As deferiprone has been shown to be able to access and remove iron from cardiac tissue [3], the combination of deferiprone and DFO, for instance, might be expected to facilitate the removal of cardiac iron more efficiently.

This paper investigates the transfer of iron from bidentate chelators to DFO in an attempt to establish whether appreciable rates are achievable under clinical conditions and whether this phenomenon will lead to an increase in the rates of iron removal from transferrin, when compared to either chelator alone.

EXPERIMENTAL.

Materials.

1,2-dimethyl-3-hydroxypyridin-4-one (deferiprone) was purchased from Sigma Chemical Co. (Poole, U.K.). Nine hydroxypyridin-4-one (HPO) chelators were used in this study and each was prepared as previously described [12,13,14,15,16]. These chelators fall into three main categories, the 'low' affinity chelators (deferiprone, CP95 and CP40, $pFe^{3+} < 20$), chelators with a large ring substituent (CP357 and CP358) and the 'high' affinity chelators (CP363, CP375, CP502 and CP511, $pFe^{3+} > 20$). The pFe^{3+} value is defined as $-\log [Fe^{3+}]$ with $1\mu M$ total iron and $10\mu M$ total ligand at pH 7.4. The structures of these chelators are presented in table 1. All chemicals required for urea gel electrophoresis were purchased from National Diagnostics (Atlanta, U.S.A). All other chemicals were purchased from Sigma Chemical Co. (Poole, U.K.).

Purification of transferrin

Commercially available transferrin was not utilised for the iron removal studies since these preparations are frequently contaminated with other proteins and low molecular weight molecules, which could interfere with the iron removal process. The presence of such low molecular weight contaminants, for instance citrate, could facilitate the transfer of iron from transferrin to DFO thereby resulting in artefacts. For these reasons, transferrin was purified from human serum. Solid ammonium sulphate was added to the serum sample (approximate volume 500ml) to give a 45% saturated solution. The amount of ammonium sulphate required was calculated as previously described [17]. The solution was left stirring overnight at 4°C and subsequently centrifuged for 30 min at 8,000g at 4°C. A further amount of ammonium sulphate was added to the supernatant to increase the saturation to 70% and the solution was left to equilibrate for 3h at 4°C with stirring. The solution was then centrifuged for 1h at 16,000g at 4°C, after which the supernatant was discarded and the pellet was re-dissolved in 50mM sodium bicarbonate. The sample was then dialysed using 12kDa molecular weight cut-off tubing (Medicell International Ltd., London) overnight against de-ionised water at 4°C to remove the ammonium sulphate. 0.1M Fe-NTA solution (calculated as in equation 1) was added to completely saturate the transferrin present in the sample. In all cases Fe-NTA was prepared by the addition of the free acid to ferric chloride in a 1:1 ratio. This solution was freshly prepared at the time of use. Sodium bicarbonate was then added to the solution to give a final concentration of 5mM, together with a solution of Tris/HCl (pH 8), which was added to a final concentration of 10mM.

$$\text{Volume 0.1M FeNTA } (\mu\text{l}) = \frac{(2 \times \text{Molarity of Transferrin}) \times \text{Volume of Transferrin solution } (\mu\text{l})}{\text{Molarity of FeNTA (0.1M)}} \dots\dots\dots(1)$$

The partially purified transferrin was applied to a DEAE Sepharose column (30cm, radius 4cm) equilibrated with 500ml of 20mM Tris/HCl (pH 8). Both albumin and caeruloplasmin have a greater net negative charge than transferrin and therefore displace it from the DEAE Sepharose column. As the solution was passed through the column, albumin (brown) and caeruloplasmin (blue) were found to bind more tightly and transferrin (red) was collected in the eluent. Albumin and caeruloplasmin were displaced from the column by elution with 2M NaCl. The column was

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re-equilibrated with 20mM Tris/HCl (pH 8) and the process repeated twice until transferrin bound to the column without being displaced, indicating that the other proteins had been removed. Once bound to the column, transferrin was eluted using a salt gradient of 20mM Tris/HCl and 0.2M NaCl. Fractions were pooled and samples run on an SDS gel to confirm the purity of the purified transferrin. Selected fractions were then dialysed against de-ionised water overnight at 4°C and subsequently freeze-dried.

Preparation of diferric transferrin

Although an excess of Fe-NTA was added to the solution to completely saturate transferrin during the purification process, a mixture of all four forms of transferrin are usually present in the final product. In order to completely saturate the transferrin, the freeze-dried product was dissolved in 0.1M sodium bicarbonate and 0.1M Fe-NTA was added, the volume required being calculated using equation 1. A Sephadex G-25 column (30cm, radius 3cm) was equilibrated with 25mM ammonium bicarbonate and the transferrin solution was applied. This process removed excess Fe-NTA. The transferrin sample was again freeze-dried and stored at -20°C.

Methods.

Transfer of iron from hydroxypyridinones to desferrioxamine.

A 1mM stock solution of hydroxypyridin-4-one (HPO) was prepared in water and treated with atomic absorption standard iron. The solution was made up with 50mM MOPS (pH 7.4). The final total concentrations of iron and HPO in the solution were 10µM and 30µM, respectively. The HPOs are bidentate chelators, therefore a 3-fold molar excess of HPO over iron is required to fully complex the iron in solution. When an additional 300µM citrate or 100µM HPO was required, a calculated amount of either citric acid or HPO was subsequently added to the preformed iron(III) complex.

A solution of DFO was prepared in water and added to the Fe-HPO solution, to give a final concentration of 10µM. The transfer of iron was monitored by spectroscopy at room temperature. The Fe-DFO complex has an absorbance maximum at 430nm and the Fe-HPO complexes have maxima in the region 450nm-460nm, therefore the transfer of iron from one

chelator to the other can be monitored by UV-visible spectroscopy. In most cases the absorbance was monitored at a wavelength of 455nm. The rates of iron transfer were calculated using a method proposed by Rose in 1964 [18].

Iron removal from transferrin – urea gels.

Diferric transferrin (32.5 μ M) was prepared in 50mM MOPS buffer (pH 7.4). A combination of bidentate chelators followed by DFO were added and the sample was incubated for 2h at 37°C. The sample was then run on a urea gel. 6M urea gels were prepared as previously described [19]. Transferrin samples were dissolved in sample buffer prior to loading on the gel. The sample buffer contained 0.01% (w/v) bromophenol blue and 10% (w/v) glycerol in running buffer. The gel was run at 80V overnight. The bands were visualised by the addition of Coomassie Blue stain. 0.25% (w/v) Coomassie Blue was dissolved in 90ml acetic acid and 455ml methanol and made up to 1 litre with de-ionised water. After de-staining in a solution of 10% acetic acid and 10% methanol, the relative percentages of each of the forms of transferrin were calculated using densitometry scanning.

When iron removal from transferrin was investigated in the presence of serum, the sample buffer was replaced with Rivanol solution. Rivanol is a dye, which precipitates out the γ -globulins leaving transferrin in the supernatant [19]. A solution of Rivanol was prepared by the addition of 0.375% (w/v) Rivanol and 10% (w/v) glycerol to the running buffer and made up to volume with de-ionised water. A 25 μ l aliquot was removed from the serum mixture and 200 μ l of Rivanol solution added. This mixture was centrifuged at 2,000g for 3 minutes and 20 μ l of the supernatant was loaded onto the gel. The gel was then run at 80V overnight. Normal serum samples were iron loaded with FeNTA. The amount of FeNTA added was calculated so that the transferrin would not be completely saturated, thereby decreasing the likelihood of the presence of non-transferrin-bound iron (NTBI). The approximate saturation of transferrin in these experiments was 80% as determined by densitometry scanning of urea gels.

Percentage iron saturation of transferrin was calculated by determining the percentage of each of the four forms of transferrin, using densitometry scanning of urea gels and calculated using equation 2.

$$\text{Percentage Iron Saturation} = \% \text{ Diferric Tf} + \frac{1}{2} (\% \text{ N-monoferric Tf}) + \frac{1}{2} (\% \text{ C-monoferric Tf}) \dots\dots\dots(2)$$

Iron removal from transferrin – ferrioxamine (FO) assay.

Diferric transferrin (32.5 μ M) in 20mM MOPS (pH 7) was incubated with a combination of bidentate chelators and DFO at varying concentrations at 37°C. Aliquots were removed at regular time intervals and ultrafiltered through 30kD filters (Whatman, Brentford, U.K.) for 5 minutes at 15,000g to remove the protein; the filtrate (50 μ l) was then injected on to the HPLC column for analysis.

A metal-free HPLC system (Waters Bio-system 625 with 911 Photodiode Array detector) with PEEK (polyether-ethyl ketone) tubing was used for these measurements in order to prevent iron contamination of the samples. A Chrompak octadecylsilane (ODS) glass column was adopted and the flow rate was set at 0.4ml/min. The temperature of the injector was maintained at 4°C during the analysis to minimise any changes in the rate of reaction while the samples were in the autosampler. The detection wavelength was set at 430nm to detect FO. The mobile phase consisted of 20mM phosphate buffer pH 7, with 6% acetonitrile, yielding a retention time of 6 mins for FO. All samples were prepared in 20mM MOPS (pH 7). Concentrations of FO in the samples were determined by use of a standard curve of FO.

Speciation plots.

Speciation plots of the iron complexes of deferiprone and CP511 were generated using the computer programme Hyperquad Simulation and Speciation (HySS) [20]. The parameters incorporated into this model were $\log K(\text{FeOH}) = 2.563$, $\log K(\text{Fe}(\text{OH})_2) = 6.205$, $\log K(\text{Fe}(\text{OH})_4) = 21.883$, $\log K(\text{Fe}_2(\text{OH})_2) = 2.843$, $\log K(\text{Fe}_3(\text{OH})_4) = 6.054$ [21]. For deferiprone, $\log K_1 = 15.1$, $\log K_2 = 11.51$, $\log K_3 = 9.27$ [22], $\text{pK}_{a1} = 3.68$ and $\text{pK}_{a2} = 9.77$ [23]. For CP511, $\log K_1 = 14.5$, $\log K_2 = 10.49$, $\log K_3 = 7.47$, $\text{pK}_{a1} = 2.32$ and $\text{pK}_{a2} = 6.66$ [16].

RESULTS.

Iron transfer between hydroxypyridinones and desferrioxamine.

An absorption trace recording iron transfer from CP358 to DFO is shown in figure 2. It can be seen that the transfer of iron from the bidentate chelator to DFO follows a biphasic pattern, with a relatively fast initial transfer followed by a slower phase. A similar pattern was observed for the rate of iron transfer from all the HPO chelators tested in this study. This observation can best be explained by a two-step iron transfer mechanism; the initial fast phase corresponding to the formation of a ternary Fe.HPO.DFO mixed ligand complex followed by the slower formation of the Fe-DFO complex (Figure 1). The initial rates of transfer of iron from various Fe-HPO complexes (10 μ M:30 μ M) to 10 μ M DFO are presented in figure 3. It can be seen that there is an appreciable variation in the rate of iron transfer. CP95 was found to be the most effective at transferring iron under the experimental conditions adopted. The rate of iron transfer from deferiprone and CP40 to DFO was approximately 1.5-fold slower than that observed from CP95 to DFO. CP363 and CP375, which are higher affinity chelators, had rates of iron transfer similar to that of deferiprone. The slowest rates of iron transfer to DFO were observed with CP357, CP358, CP506 and CP511. These iron transfer experiments were repeated in the presence of 300 μ M citrate, a physiological iron chelator present in the plasma and the rate of transfer of iron from the majority of the HPO chelators to DFO was reduced by approximately 1.5-fold. However, the converse was noted in the case of iron transfer from CP357 and CP358 to DFO, the addition of citrate leading to a 2-fold increase in the rate of iron transfer to DFO (data not shown).

Under *in vivo* conditions, a major proportion of the administered dose of a chelator would be present in the unchelated form. It was therefore decided to examine the rate of iron transfer from HPO to DFO in the presence of 100 μ M excess HPO chelator (Figure 4). The data is presented as the ratio of the rate of iron transfer to DFO in the presence of excess HPO chelator to the rate of iron transfer to DFO in the presence of a 1:3 ratio of iron to HPO (that is, no excess chelator). All of the rates are markedly reduced in the presence of excess chelator. Significantly, the rates of iron transfer from the lower affinity chelators CP95 and deferiprone were the least affected by the presence of excess HPO. The presence of excess HPO has the greatest effect on the transfer of iron from the chelators CP357, CP358, CP506 and CP511.

Iron transfer from the Fe-DFO complex, ferrioxamine (FO) to deferiprone was investigated at both pH 5.5 and pH 7.4 in order to verify whether FO will act as an efficient 'sink' and prevent the re-distribution of iron under both extracellular conditions and conditions found in the lysosome. At deferiprone concentrations less than 300 μ M, there was no detectable transfer of iron to deferiprone at either pH 5.5 or 7.4. As the concentration of deferiprone was increased above 300 μ M, transfer of iron occurred, with the transfer at pH 5.5 being greater than at pH 7.4 (Figure 5). However, these latter deferiprone concentrations would not be achieved under clinical conditions.

Iron exchange between transferrin and chelating agents.

Having established that the transfer of iron from the bidentate chelators to DFO will occur under clinically relevant chelator concentrations, it was of interest to examine whether this 'shuttling' of iron would result in enhanced scavenging of iron from transferrin. An example of a urea gel is shown in figure 6. Lane 1 shows a single band corresponding to diferric transferrin. Lanes 2 and 3 show the presence of 4 bands, namely, apotransferrin, C-monoferric transferrin, N-monoferric transferrin and diferric transferrin, following a 2h incubation of diferric transferrin with 200 μ M and 600 μ M deferiprone, respectively. The iron saturation of transferrin decreases with increased chelator concentration. A combination of bidentate chelators and DFO were incubated with transferrin and iron removal was monitored using urea polyacrylamide gel electrophoresis. Three chelators were chosen for this study, namely, deferiprone, CP358 and CP502, both CP358 and CP502 are analogues of deferiprone (Table 1). CP358 is a larger chelator, which has a bulky side-chain and is therefore not expected to be able to efficiently chelate iron from the iron-binding sites of transferrin. CP502 is a small, higher affinity iron chelator, which should be more effective at iron removal from transferrin when compared to deferiprone. No iron removal was observed in the presence of 100 μ M DFO alone. Transferrin was incubated with a range of deferiprone concentrations in the presence and absence of 100 μ M DFO (data not shown). At deferiprone concentrations greater than 1mM, a large percentage of the iron was removed from transferrin (approximately 60%). At this deferiprone concentration, the further addition of 100 μ M DFO resulted in a synergistic increase in iron removal with approximately 75% iron removal from transferrin. As the concentration of deferiprone was increased up to a concentration of 5mM almost all the iron was removed from transferrin, but the further addition of DFO had virtually no effect. Following a 25mg/kg dose of deferiprone in man, the maximal

concentration of the drug in the plasma is approximately 100 μ M [24]. The steady state concentration of DFO during a 40mg/kg intravenous infusion is 10 μ M [25]. Iron removal from transferrin was therefore investigated in the presence of a range of HPO chelators (100 μ M) in the presence and absence of 10 μ M DFO (Table 2). In all cases an increase in iron removal from transferrin was observed when DFO was combined with HPO compared to HPO alone, however statistical significance was not reached. When the concentration of HPO was further reduced to 50 μ M, while maintaining the DFO concentration at 10 μ M (Table 2), weakly enhanced scavenging of iron was seen when DFO was combined with deferiprone and CP358, but not with CP502.

The rate of iron removal from 80% saturated transferrin in serum was also investigated. Combinations of HPO and DFO were incubated with the serum samples (Figure 7), the amount of iron removed by 100 μ M deferiprone alone in the presence of serum was found to be comparable to that removed from purified transferrin (Table 2). Addition of 10 μ M DFO did not result in an increase in the amount of iron removed from transferrin when compared to 100 μ M HPO alone. However, in the presence of equimolar concentrations of deferiprone and DFO, significant increases in iron removal from serum transferrin were observed when compared with deferiprone alone. The addition of 200 μ M deferiprone did not result in any further increase in iron removal compared to 100 μ M deferiprone. The combination of CP502/DFO did not result in enhanced scavenging activity even at high concentrations of chelator, however the combination of 100 μ M CP358 and 100 μ M DFO did result in marginal synergistic effects (Figure 7).

Iron removal from iron-saturated transferrin was also monitored using the ferrioxamine (FO) assay (Figure 8). The addition of 10 μ M DFO alone resulted in marginal (non significant) iron removal from transferrin. The addition of 30 μ M deferiprone resulted in a large increase in the rate of iron removal. Addition of 100 μ M deferiprone to 10 μ M DFO resulted in slightly greater FO formation compared to that observed in the presence of 30 μ M deferiprone and 10 μ M DFO (data not shown). It is clear that the FO assay is more sensitive than the urea gel method for determining iron removal from transferrin at these lower chelator concentrations.

DISCUSSION

Although current chelation therapy, namely the use of either desferrioxamine or deferiprone is effective at increasing life expectancy of patients with transfusion-dependent anaemias, there are some disadvantages associated with their use. It has been hypothesised that the combined administration of these two chelators may increase the total amount of iron removed from the body, without the need for increasing chelator dose [7]. Studies have been carried out which indicate that combined chelation therapy may not only prove to be effective at decreasing body iron burden, but may also decrease cost and increase compliance with these pharmaceuticals [26].

It has been suggested that enhanced scavenging activity of combined chelation therapy is based on the transfer of iron from the smaller bidentate chelator to the hexadentate chelator (Figure 1) and is therefore dependent on the rate of iron transfer between the two [7]. In this work, the rate of iron transfer between a range of bidentate HPO chelators and DFO was determined using UV-visible spectroscopy (Figure 2). The lower affinity chelators deferiprone and CP95 (pFe^{3+} values 19.4 and 19.9 respectively) were the most effective at iron transfer to DFO. The rates of iron transfer from CP357 and CP358 to DFO were approximately 2-fold lower than the rate of iron transfer from CP95. Both of these compounds possess long side-chains at the 2-position on the pyridinone ring. The presence of this side-chain could possibly interfere with the formation of the postulated DFO ternary complex (Figure 1). The high affinity chelators are the slowest iron donors to DFO, and this is probably due to a decrease in the rate of dissociation of the $Fe(HPO)_3$ complex to the intermediary $Fe(HPO)_2$ complex [27], which is suggested to be necessary for DFO chelation (Figure 1). From these data it appears that there is a limited relationship between the pFe^{3+} value and the rate of transfer of iron to DFO, with an increase in pFe^{3+} resulting in a decrease in the rate of transfer. However, other structural factors must also be involved in controlling the rate of transfer of iron from the bidentate chelator to DFO, since both CP95 and CP40, which have a similar pFe^{3+} values to deferiprone have much slower rates of transfer. In the presence of citrate, the rate of transfer of iron from HPO to DFO was decreased by approximately 1.5-fold in most cases (data not shown). The high concentration of citrate could possibly encourage transfer of iron to citrate. Once formed, dimeric and oligomeric iron-citrate species [28], which are not readily accessible to DFO, would be predicted to retard the rate of iron transfer to DFO.

The marked retardation in rate of exchange in the presence of excess chelator (Figure 4) is almost certainly associated with a shift in equilibrium of the dissociation process (Figure 1). In the presence of excess chelator, the equilibrium will adjust, favouring the formation of the $\text{Fe}(\text{HPO})_3$ complex, which will not donate iron to DFO, thereby reducing the rate of transfer. The lower affinity chelators such as deferiprone, CP40 and CP95 are not as greatly affected as the higher affinity chelators and the bulky chelators. Thus, even in the presence of excess chelator, the lower affinity chelators are more likely to dissociate than the higher affinity chelators and therefore the rate of transfer of iron from these chelators is likely to be greater. The relative proportions of the partially co-ordinated $\text{Fe}(\text{HPO})_2$ complex under any given condition can be calculated from speciation plots. Comparing the speciation plots obtained for deferiprone and CP511 at pH 7.4 provides an insight into the reasons for the variation in rates of transfer of iron from these two chelators to DFO (Figure 9). The presence of a higher proportion of the $\text{Fe}(\text{deferiprone})_2$ complex compared to the $\text{Fe}(\text{CP511})_2$ complex, demonstrates that the higher pFe of CP511 results in a decrease in the dissociation of the $\text{Fe}(\text{HPO})_3$ complex compared to deferiprone and thereby decreases the rate of iron transfer to DFO. Even in the presence of a 3-fold molar excess of HPO compared to iron, a proportion of partially coordinated $\text{Fe}(\text{HPO})_2$ will exist in solution, the exact concentration of which depending on the pFe of the chelator.

To determine the efficacy of DFO as a 'sink' molecule, the rate of transfer of iron from the Fe-DFO complex to deferiprone was investigated. The slow rates of transfer demonstrate that once chelated by DFO, it is highly improbable that the iron could be mobilised under clinically achievable HPO concentrations. Thus, although greater iron removal was observed at pH 5.5, the concentration of deferiprone required to mobilise iron was much higher than would be found under clinical conditions. Clearly DFO acts an effective 'sink' molecule and is a good candidate for combined chelation therapy with HPOs such as deferiprone.

Following on from the experiments demonstrating that transfer of iron from bidentate chelators to DFO occurs under clinically relevant concentrations of chelator, the interaction of chelators with transferrin was investigated. Transferrin iron is not readily accessible to DFO and so it was of interest to determine whether this iron could be chelated in the presence of both a bidentate HPO and DFO. At high deferiprone concentration the combination of deferiprone and DFO resulted in marginally greater iron removal from transferrin compared to deferiprone alone.

Having demonstrated that enhanced scavenging activity is possible for iron removal from transferrin at high deferiprone concentrations, chelator concentrations were reduced to simulate conditions achievable *in vivo* (Table 2). In the absence of DFO, CP502 was found to be the most effective at removing iron from transferrin. CP502 has the highest affinity for iron of these three chelators and is also relatively small, thereby enabling access to the iron-binding sites on transferrin. CP358 would be restricted in its access to the iron-binding sites on transferrin due to the larger size of the molecule. In the presence of DFO there is also a variation in the enhancement in iron removal observed with each HPO chelator, with a greater increase observed with the combination of deferiprone and DFO (approximately 2.4 fold increase in iron removal, compared to deferiprone alone) and lower increases observed with the combination of CP502/DFO and CP358/DFO (approximately 1.3 fold and 1.7 fold increases in iron removal, respectively, compared to either HPO chelator alone). The reason for this discrepancy in scavenging activity between the HPO chelators is probably due to their relative abilities to transfer iron to DFO. The rate of iron transfer from CP502 and CP358 to DFO is slower when compared to deferiprone (Figure 3).

Although purified transferrin has been shown to be an effective model for iron removal studies, experiments were also carried out using iron-loaded serum samples (Figure 7). The total iron removed from transferrin in serum by 100 μ M HPO alone was comparable to that achieved from purified transferrin. The addition of equimolar concentrations of deferiprone and DFO resulted in appreciable synergistic effects. In contrast, the combinations of CP358/DFO and CP502/DFO failed to demonstrate such an effect. The most probable reason for this marked difference in behaviour is that both CP358 and CP502 do not donate iron(III) to DFO as readily as deferiprone (Figures 3 and 4).

It was not possible to monitor iron removal from transferrin in the presence of low concentrations of bidentate chelator (for example, 30 μ M) using urea gels due to the insensitivity of the method, therefore, an alternative procedure based on the quantification of FO was adopted to monitor iron removal from transferrin at lower concentrations of bidentate chelator. The use of this method provided a time-course for the removal of iron. Any increase in FO formation in the presence of deferiprone must occur due to shuttling of iron from transferrin to DFO by the bidentate chelator, as DFO is unable to directly remove iron from transferrin under these conditions. Iron removal using a combination of 30 μ M deferiprone and 10 μ M DFO results in faster iron removal compared to DFO alone (Figure 8). The complete conversion of DFO to FO takes approximately

5h. A similar effect was observed with 100 μ M deferiprone. This observation confirms the findings previously reported, relating to the iron removal from transferrin by deferiprone [9].

The experiments described in this work demonstrate that the combination of the bidentate chelator, deferiprone, and hexadentate chelator, DFO, results in enhanced scavenging activity of iron from transferrin at clinically achievable levels of both chelators. In the presence of DFO, this mobilised iron will be donated to the hexadentate ligand (Figure 3 and 4). Thus, it is possible to predict that the simultaneous administration of the two ligands will lead to more efficient removal of iron from iron-overloaded patients. Furthermore, it is suggested that the combination of a chelator such as deferiprone with a 'sink' molecule, such as DFO, could further improve the efficacy of chelation of tissue iron by ligand exchange of iron. It should be noted, however, that enhanced scavenging activity of iron may also lead to increased toxicity due to enhanced iron removal from essential iron-containing enzymes. HPO chelators have been shown to inhibit lipoxigenase activity *in vitro* [29], an effect, which may be accentuated in the presence of a 'sink' molecule such as DFO. Inhibition of ribonucleotide reductase [30] and tyrosine and tryptophan hydroxylases [31] have also been shown to occur in the presence of HPO chelators. Limited information exists regarding the toxic effects of combined chelation therapy.

Combined chelation therapy with deferiprone and DFO has been demonstrated to enhance iron scavenging activity *in vivo* in a number of clinical cases and has been shown to be especially beneficial in decreasing cardiac iron burden [32]. This phenomenon has been attributed to the 'shuttling' of iron from the bidentate chelator to the hexadentate chelator [7, 33]. It has previously been established that deferiprone removes iron from transferrin, however the present study is the first to conclusively demonstrate that in the presence of both deferiprone and DFO *in vitro*, the chelated iron is 'shuttled' onto DFO to form the FO complex, which, in the absence of deferiprone does not form. This phenomenon demonstrates the possibility of 'shuttling' iron from a bidentate chelator to a hexadentate chelator and the subsequent removal of iron from a source unavailable to DFO alone.

In summary, the results presented here demonstrate that in the presence of a bidentate and hexadentate chelator enhanced iron scavenging activity occurs, at clinically relevant chelator concentrations and that amongst the HPO chelators, deferiprone would appear to be particularly well suited for such applications.

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Table 1. Structure of hydroxypyridin-4-ones**Table 2. Percentage iron removal from transferrin in presence of 50 μ M HPO \pm 10 μ M DFO and 100 μ M HPO \pm 10 μ M DFO.**

32.5 μ M diferric transferrin was incubated with 50/100 μ M HPO in the presence and absence of 10 μ M DFO for 2h at 37°C in 50mM MOPS (pH 7.4). Results are expressed as mean \pm s.d (n=3). No statistical significance was observed between the values for iron removal from HPO alone compared with HPO and DFO using the Students t test. Percentage iron removed was calculated using densitometry scanning.

Figure 1. The proposed mechanism of iron transfer from Fe-HPO to DFO.

The fully co-ordinated $\text{Fe}(\text{HPO})_3$ complex exists in equilibrium with the partially co-ordinated $\text{Fe}(\text{HPO})_2$ complex in solution. DFO is able to form a ternary complex with $\text{Fe}(\text{HPO})_2$ and subsequently displace the remaining HPO molecules from iron.

Figure 2. Rate profile of iron transfer from CP358 to DFO.

Iron transfer from CP358 to DFO measured as a decrease in absorbance at 455nm in 50mM MOPS (pH 7.4) at room temperature. $[\text{Fe}] = 10\mu\text{M}$, $[\text{CP358}] = 30\mu\text{M}$, $[\text{DFO}] = 10\mu\text{M}$.

Figure 3. Rate of iron transfer from Fe-HPO to DFO.

Initial rates of iron transfer from Fe-HPO ($10\mu\text{M}:30\mu\text{M}$) complexes to $10\mu\text{M}$ DFO in 50mM MOPS (pH 7.4) at room temperature. Data are presented as the mean \pm s.d (n=3). The black bars represent the higher affinity chelators, the gray bars represent the lower affinity chelators and the white bars represent the chelators possessing large ring substituents.

Figure 4. Ratio of the rate of iron transfer from Fe-HPO in the presence of excess HPO to DFO compared to the rate of iron transfer from a 1:3 molar concentration of Fe:HPO to DFO.

Rate of iron transfer from the Fe-HPO ($10\mu\text{M}:30\mu\text{M}$) complex in the presence of $100\mu\text{M}$ excess HPO to $10\mu\text{M}$ DFO in 50mM MOPS (pH 7.4) at room temperature. Data are presented as the ratio of the rate of iron transfer in the presence of excess HPO compared to the rate of transfer in the presence of a 1:3 molar ratio of Fe:HPO. The black bars represent the higher affinity chelators, the gray bars represent the lower affinity chelators and the white bars represent the chelators possessing large ring substituents.

Figure 5. Rate of iron transfer from DFO to deferiprone

Iron transfer from the FO (10 μ M) complex to various concentrations of deferiprone at pH 5.5 (20mM acetate buffer) (Δ) and pH 7.4 (20mM MOPS buffer) (\bullet). Incubations at room temperature. Data are presented as the mean \pm s.d (n=3).

Figure 6. A urea gel showing the four forms of transferrin.

A urea gel showing the effect of high deferiprone concentrations on the saturation of transferrin. Lane 1 depicts diferric transferrin. Lane 2 depicts apotransferrin, C-monoferric transferrin, N-monoferric transferrin and diferric transferrin, obtained following the incubation of diferric transferrin, with 200 μ M CP20 for 2h at room temperature. Lane 3 depicts apotransferrin, C-monoferric transferrin, N-monoferric transferrin and diferric transferrin, obtained following the incubation of diferric transferrin, with 600 μ M CP20 for 2h at room temperature.

Figure 7. Iron removal from serum transferrin.

Serum samples were incubated with equimolar concentrations of HPO and DFO (either 100 μ M each or 200 μ M each) at 37°C. (A) deferiprone and DFO (B) CP358 and DFO and (C) CP502 and DFO. Black bars denote HPO alone, grey bars denote HPO in the presence of equimolar DFO. Iron removal from transferrin was calculated using densitometry scanning. Data are presented as the mean \pm s.d (n=6). Asterisk denotes significant difference between HPO alone and the combination of HPO and DFO, using the Students t test at the 5% significance level.

Figure 8. Iron removal from transferrin measured by FO formation following incubation with deferiprone and DFO.

32.5 μ M transferrin was incubated with 30 μ M deferiprone and 10 μ M DFO (Δ) and 10 μ M DFO alone (\blacksquare). Samples prepared in 20mM MOPS (pH 7) and incubated at 37°C. Data expressed as mean \pm s.d (n=3). Asterisk denotes significant difference between DFO alone and the combination of deferiprone and DFO, using the Students t test at the 5% significance level.

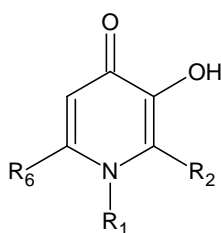
Figure 9. Speciation plots of iron-deferiprone and iron-CP511 complexes.

Speciation plots showing all complexes of iron and HPO and the predominant iron hydroxide species. (A) iron-deferiprone speciation plot, (B) iron-CP511 speciation plot. $[\text{Fe}]^{3+}_{\text{total}} = 1 \times 10^{-5}\text{M}$, $[\text{HPO}] = 3 \times 10^{-5}\text{M}$. Dashed lines show amounts of $\text{Fe}(\text{HPO})_2$ and $\text{Fe}(\text{HPO})_3$ present at pH 7.4. Plots generated using the computer programme Hyss [20].

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Table 1.



	R₁	R₂	R₆	Ref
Deferiprone	CH ₃	CH ₃	H	[12]
CP40	CH ₂ CH ₂ OH	CH ₃	H	[12]
CP95	CH ₂ CH ₂ CH ₃	C ₂ H ₅	H	[12]
CP357	CH ₃	CH ₂ NHCOCH ₂ CH ₂ NHCOCH ₃	CH ₃	[13]
CP358	CH ₃	CH ₂ NHCOCH ₂ CH ₂ CONHC ₃ H ₇	CH ₃	[13]
CP363	CH ₃	CH(CH ₃)OCH ₃	CH ₃	[14]
CP375	CH ₃	CH(C ₂ H ₅)OCH ₃	CH ₃	[15]
CP502	CH ₃	CONHCH ₃	CH ₃	[12]
CP511	H	CONHCH ₃	CH ₃	[16]

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Table 2.

	50µM HPO	50µM HPO + 10µM DFO	100µM HPO	100µM HPO + 10µM DFO
Deferiprone	4.7 ± 2.6	6.0 ± 2.1	5.8 ± 1.9	13.9 ± 5.2
CP502	5.5 ± 1.5	5.7 ± 1.1	13.5 ± 5.2	17.5 ± 8.0
CP358	2.8 ± 1.7	4.8 ± 1.4	8.3 ± 6.2	14.0 ± 2.2

Figure 2

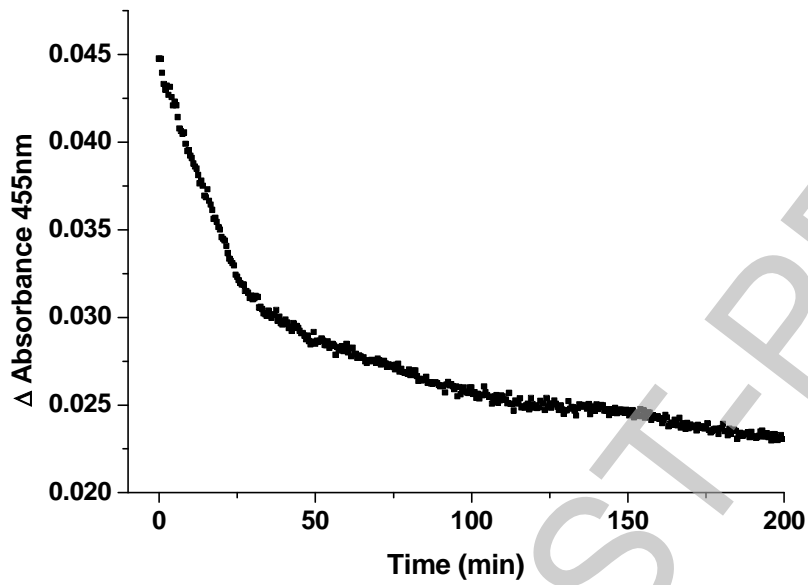
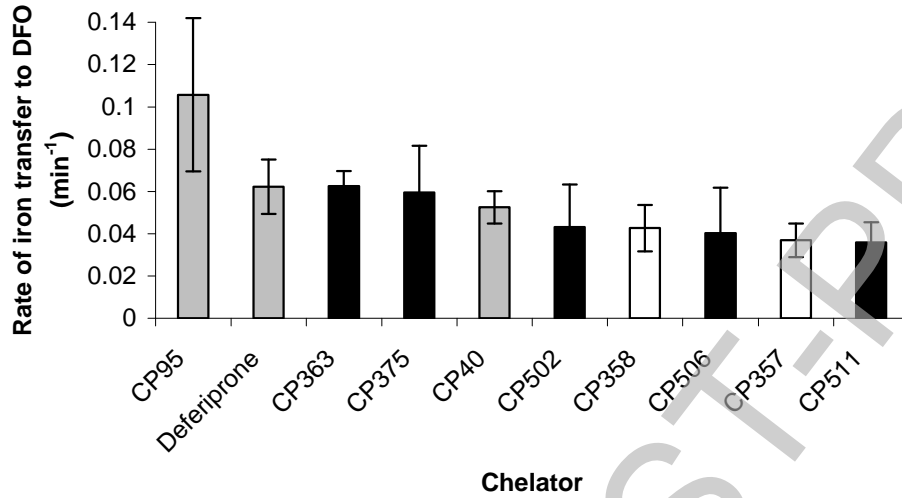


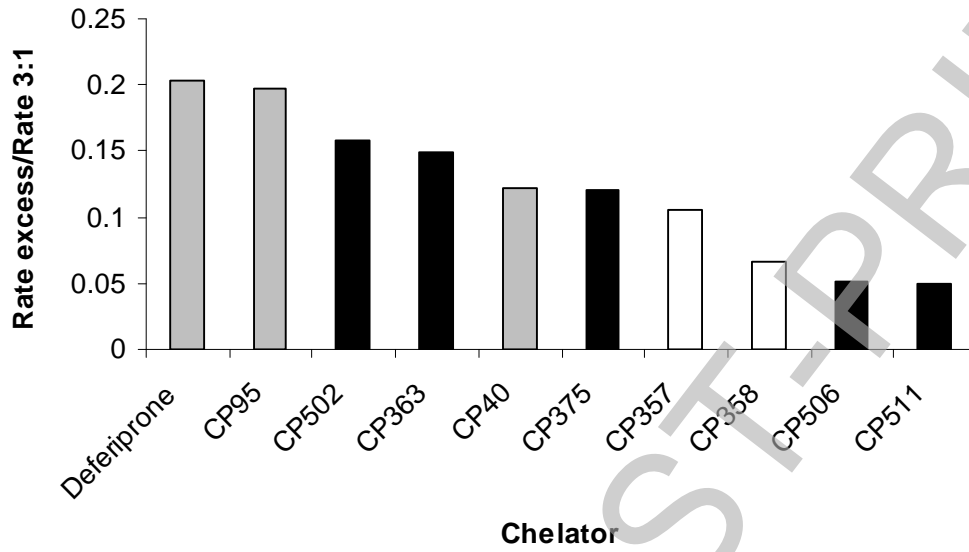
Figure 3



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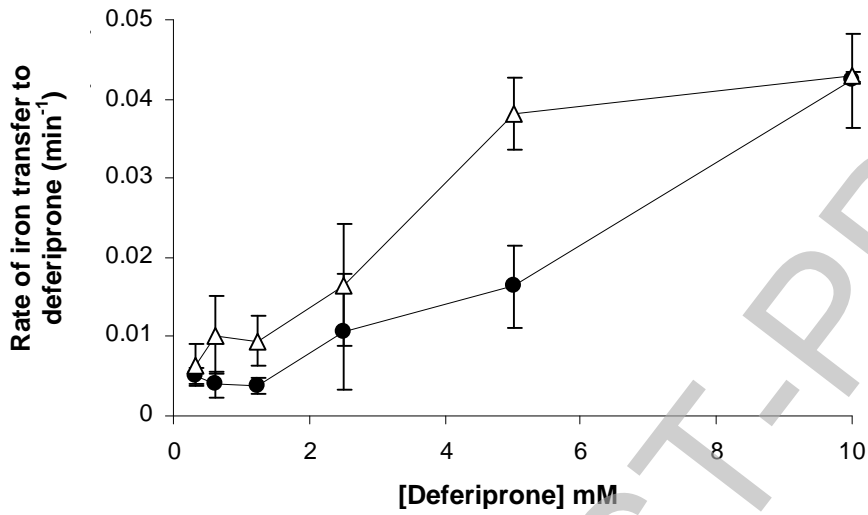
Figure 4



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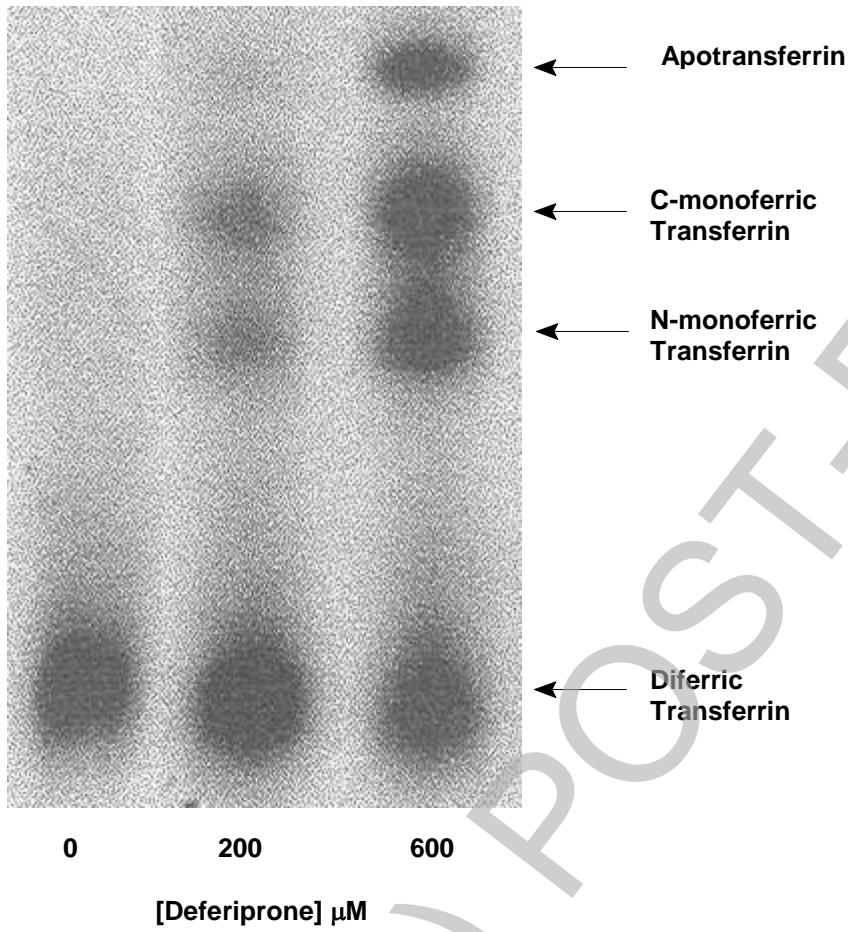
Figure 5



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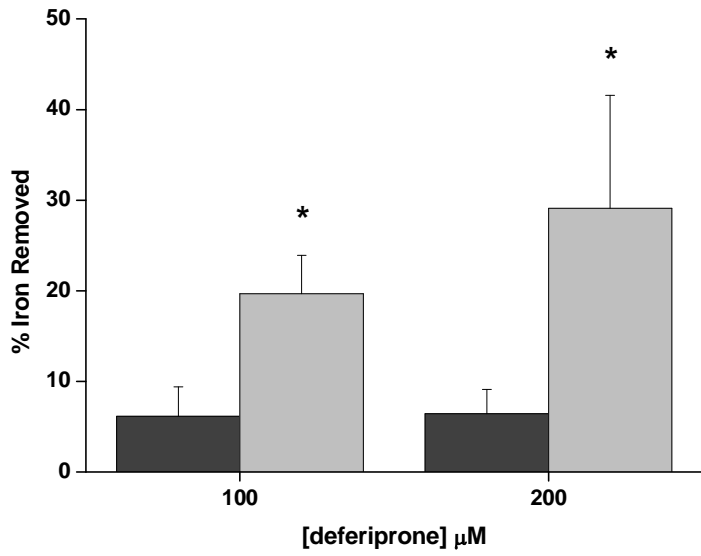
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Figure 6

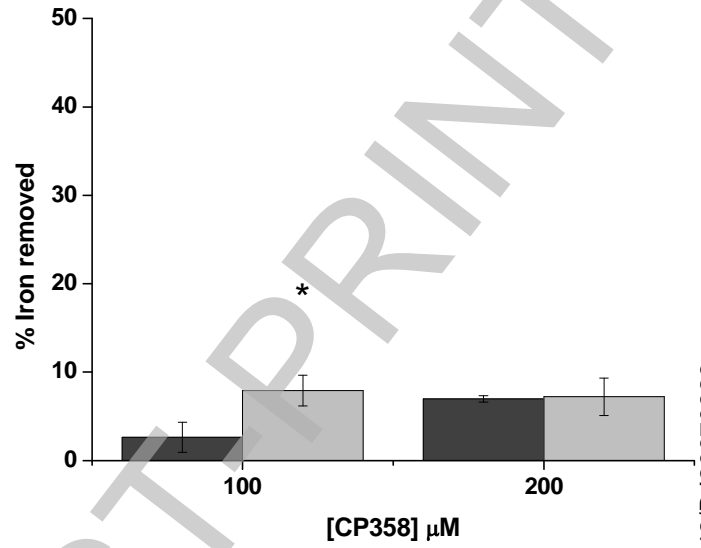


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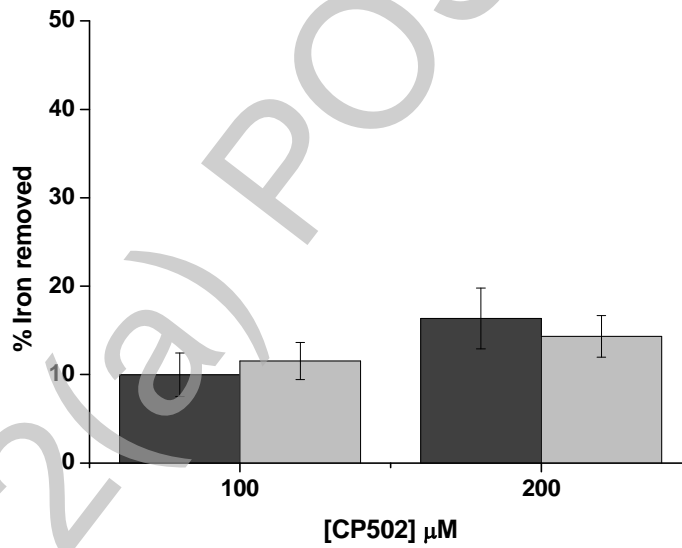
Figure 7



(A)



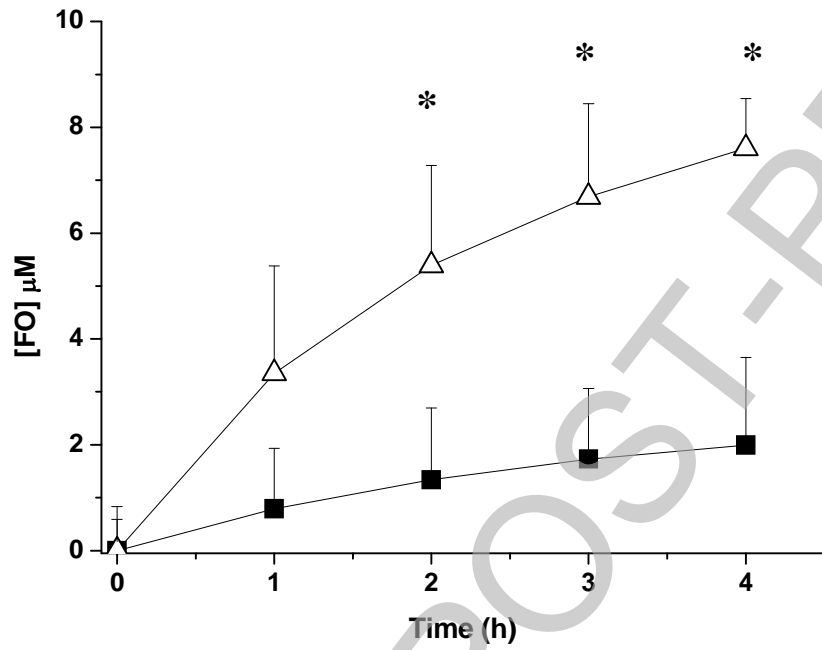
(B)



(C)

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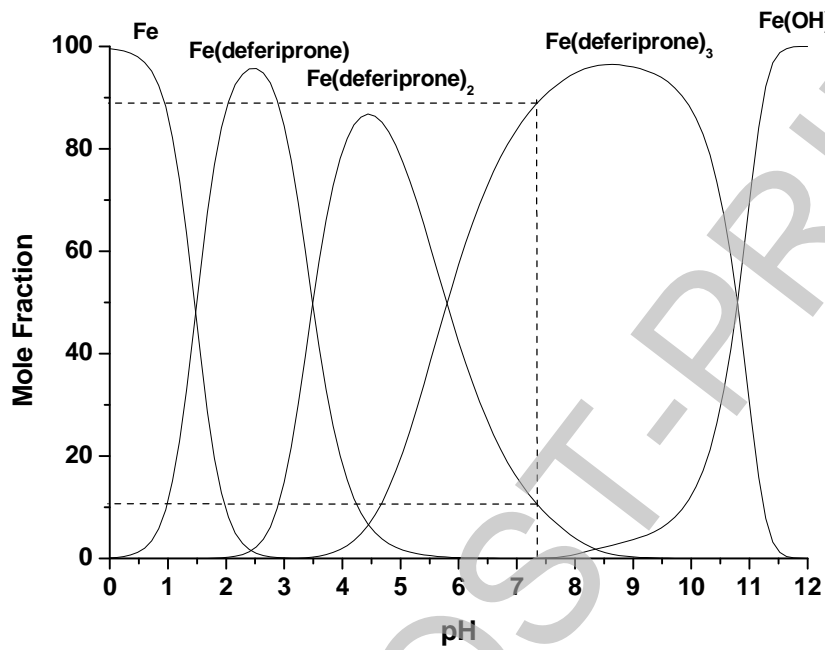
Figure 8



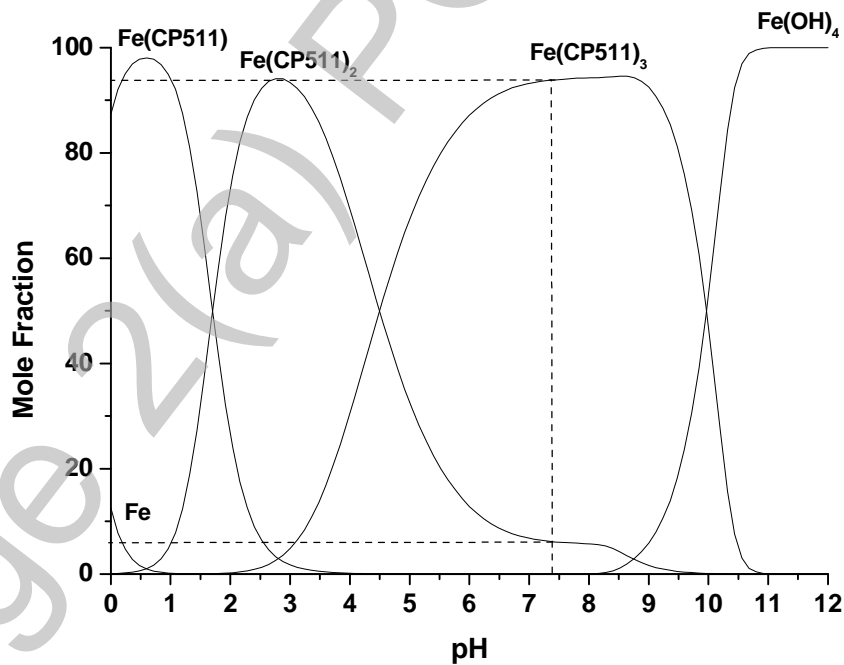
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Figure 9



(A)



(B)

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Abbreviations used: DFO, desferrioxamine; DTPA, diethylenetriaminepentacetic acid; FO, ferrioxamine; HPO, hydroxypyridin-4-one; NTBI, non-transferrin-bound iron; Tf, transferrin.

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