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The Quantification of Non-Transferrin-Bound Iron

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De hac ave dicitur, quod ferrum comedat et digerat, sed ego non sum hoc expertus: quia ferrum a me pluribus struthionibus obiectum comedere noluerunt.

ALBERTUS MAGNUS (*De animalibus*)

Acknowledgment

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Abstract

Nontransferrin-bound iron (NTBI) appears in the serum of individuals with iron overload and in a variety of other pathologic conditions. Currently established NTBI quantification methods require either expensive laboratory equipment or involve complicated, time consuming sample preparations.

The main focus of this investigation lay on development of an appropriate fluorescence-based quantification method for NTBI through simple method validation for required levels of analysis using CP691, a novel Pyridinone iron probe, on a multi-well plate reader. A standard curve was successfully established (R^2 >0.998) using 10% human blood serum and 1.5µM CP691 to measure 0-1µM Fe(III) nitrilotriacetic acid, the equivalent to the common range of 0-10µM NTBI in undiluted human blood serum from pathogenic conditions. The fluorescence-based assay was then compared to an ICP-MS-based method showing that the fluorescence-based assay can determine iron levels of unknown concentration with higher accuracy than the ICP-MS-based method. However, the application of the fluorescence-based method on human blood serum samples from patients suffering from various conditions leading to iron overload did show weak to no correlation between the fluorescence-based assay and established HPLC-based assays that were used to validate the method. The kinetic aspects of CP691 with established iron(III) chelators were compared in a time course experiment using a UV/Vis-Spectroscopy based method. CP691, was found to be able to exchange iron at the fastest rate ($T_{1/2}$ = 8.7 min) compared to desferrioxamine B, CP20 and CP692. Furthermore, investigations on CP691's potential for iron removal from transferrin in the presence of Fe-NTA/Fe-citrate did not only show that CP691 is the stronger iron chelator compared to apotransferrin (apo-Tf) but it is also able to remove iron from saturated transferrin, which could be leading to false positive NTBI results when applied on pathologic human blood serum samples.

These findings suggest that CP691 should not be used on the described direct measurement method of NTBI-levels in human pathologic serum samples.

Zusammenfassung

Nicht-Transferrin gebundenes Eisen (NTBI) tritt bei Menschen mit Eisenüberschuss und in einer Vielzahl anderer pathologischer Zustände auf. Bestehende NTBI-Messmethoden benötigen entweder teure Laborausrüstung oder bedürfen komplizierter, zeitaufwändiger Probenvorbereitung.

Das Hauptaugenmerk dieser Untersuchung lag auf der Entwicklung einer geeigneten fluoreszenzbasierten NTBI-Messmethode durch einfache Validierung des Verfahrens für die benötigten Analysenbereiche. Für die Messung von NTBI wurde CP691, ein neuer Pyridon-basierter Eisen-Indikator, an einem Multititerplatten-Reader verwendet. Durch die Verwendung von 10% humanen Blutserums mit 1.5µM CP691 zur Messung von 0-1µM Fe(III)-Nitrilotriessigsäure, entsprechend dem benötigten Bereich von 0-10µM NTBI in unverdünntem humanem Blutserum aus pathologischen Zuständen, konnte erfolgreich eine Eichgerade erstellt werden (R^2 >0.998). Die fluorsezenzbasierte Methode wurde anschließend mit einer an ICP-MS-basierten Methode verglichen. Dies zeigte, dass mit der fluoreszenz-basierten Methode Eisen-Werte unbekannter Konzentration mit höherer Genauigkeit bestimmt werden können als mit der ICP-MS-basierten Methode. Dennoch zeigte die Anwendung der fluoreszenzbasierten Methode auf Proben humanen Blutserums wenig bis gar keine Korrelation zwischen der fluoreszenzbasierten Methode und bekannten HPLC-basierten Methoden, welche für die Validierung verwendet wurden. Kinetische Aspekte von CP691 wurden mit denen bekannter Eisen(III)-Chelatoren in einem Time-Course-Experiment unter Verwendung von UV/Vis-Spektroskopie verglichen. Dabei konnte CP691 Eisen, verglichen mit Deferoxamin, CP20 und CP692 am schnellsten (T1/2 = 8.7 min) aufnehmen. Weitere Versuche betreffend CP691s Potential Eisen in der Gegenwart von Fe(II)-NTA/Fe(II)-Citrat von Transferrin zu entfernen zeigten nicht nur, dass CP691 verglichen mit Apotransferrin (apo-Tf) der stärkere Eisen-Chelator ist, sondern auch, dass es Eisen von gesättigtem Transferrin entfernen kann, was bei Anwendung auf pathologisches humanes Blutserum zu falsch-positiven NTBI-Ergebnissen führen könnte.

Diese Ergebnisse zeigen, dass CP691 nicht zur Bestimmung von NTBI in pathologischem humanem Blutserum in der beschriebenen Messmethode geeignet ist.

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Chapter 1

Introduction

Iron (Fe), element 26 in the periodic table, is the second most abundant metal and the most prevalent transition element in the Earth's crust, the sea and the human body. It is essential to all organisms, except for some few bacteria. Iron is an important trace metal serving various functions in the human body. Its distribution is heavily regulated in mammals. Iron is absorbed across cells in the duodenum and is transported via a specific protein, Transferrin (Tf), which tightly binds the iron. Iron found in the plasma not bound to Tf is referred to as non-transferrin bound iron (NTBI), a term introduced by Hershko et al 1975.[4]

NTBI has been detected in significant levels in sera of patients with iron-overloading conditions and in patients receiving long-term dialysis and chemotherapy.[5-10] The link between NTBI and iron overloading diseases raises concerns of possible toxicity risks associated with this freely circulating iron. Due to high reactivity of the iron there is a high probability of it contributing to free radical formation, which in turn affects the state of cellular homeostasis leading to destruction of various organs like heart and liver.[11, 12]

Thalassaemia and sickle cell anaemia are serious disease states, and increasingly chronic, conditions found in high frequency particularly in parts of the world with past or present falciparum, such as South Asia or parts of the African continent.[13-15] The increased medical need for therapy of these haemoglobinopathies raises the importance for research in these areas including the role of NTBI. The precise chemical nature of NTBI is still an unresolved question.[16, 17]

A task in NTBI research concerns the optimal quantification method for NTBI analysis. Different direct and indirect iron detection methods have been employed in NTBI analysis. Advances in NTBI-quantification will provide insight for the following areas:

- Early iron-overload diagnostics
- Efficiency of iron chelation therapy

A recently developed NTBI-quantification method forms the focus of this investigation.

1.1 Iron

1.1.1 Iron Physiology

Iron is one of the 13 essential trace elements in the human body needed for normal growth and development through participation in the many biological structures and biochemical functions[18]:

(i) Oxygen transport – iron-porphyrin in haemoglobin is responsible for O_2 binding

(ii) Iron proteins (nitrogenous complex) – molybdenum-iron protein, which catalyses the reduction of N_2 and nitrogenase reductase. Iron protein transfers electrons.

(iii) Co-enzymes – electron transport/substrate binding and activation through iron-sulfor centres in proteins.

(iv) Cell proliferation, DNA synthesis and cell recognition.[19, 20]

1.1.1.1 Iron distribution

In the balanced state, 1 to 2 mg of iron enters and leaves the human body every day. Dietary iron is absorbed by duodenal enterocytes. It circulates in plasma bound to Tf. The distribution of iron in tissue is shown in Figure 1. The concentration of iron normally is about 40mg Fe/kg body weight in women and 50mg Fe/kg in men.[21, 22] Most of this iron is incorporated into haemoglobin (28mg Fe/kg in women, 32mg Fe/kg in men). A further 5mg Fe/kg is found in muscle in the form of the oxygen storage protein myoglobin, and about 2mg Fe/kg as iron-containing and iron-dependent enzymes throughout the cells of the body. Most of the remaining iron (5-12mg Fe/kg) is stored in parenchymal cells of the liver, and reticuloendothelial macrophages, spleen, bone marrow, and to a lesser extent in muscle, as ferritin.

Macrophages provide most of the usable iron by degrading haemoglobin in senescent erythrocytes and reloading ferric iron onto Tf for delivery to cells. As mentioned previously iron homeostasis is crucial for life, and is maintained through highly regulated processes within the human body. However, serious problems can result if abnormal levels of iron are present.

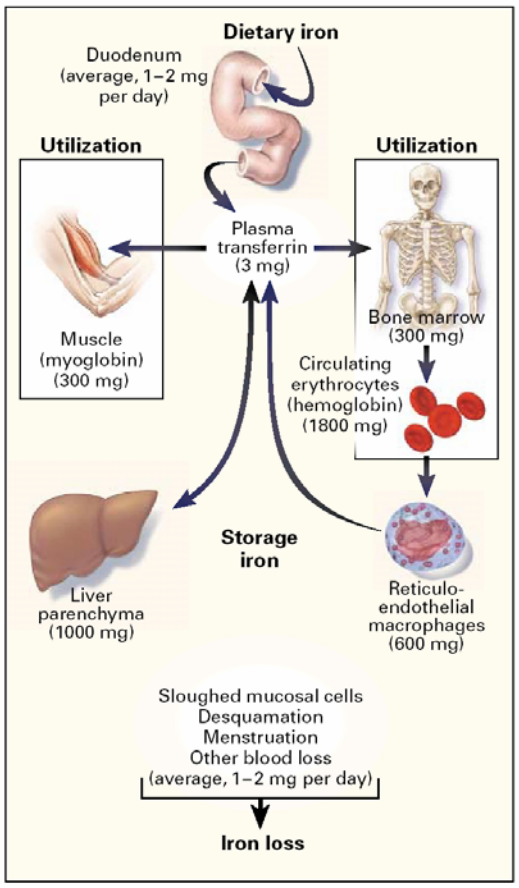

Figure 1. Distribution of Iron in adults[3].

1.1.2 Iron Toxicity

Iron exists in two valence states, Fe(II) (d^6) (ferrous) and Fe(III) (d^5) (ferric) of which the ferrous type is highly reactive in the formation of free radicals.[23] Thus, although iron is essential for maintaining normal body functions, it can also be toxic. Therefore, an extensive regulatory mechanism operates in the human body to monitor the iron balance, for example the decrease of iron absorption efficiency with increasing dosage.[24] The facile redox chemistry of iron renders the metal toxic when present in excess. In the presence of molecular oxygen, inappropriately coordinated iron can redox cycle between the two oxidation states thereby generating a range of oxygen derived radical species including superoxide $O_2^{-\cdot}$ and the hydroxyl radical $HO^{\cdot}$.[25]

$$Fe^{2+} + O_2 \leftrightarrow O_2^{-\cdot} + Fe^{3+} \quad \textbf{1.1}$$

$$2O_2^{-\cdot} + 2H^{+} \leftrightarrow H_2O_2 + O_2 \quad \textbf{1.2}$$

$$Fe^{2+} + H_2O_2 \leftrightarrow Fe^{3+} + HO^{-} + HO^{\cdot} \quad \textbf{1.3}$$

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2 \quad \textbf{1.4}$$

Scheme 1.1: Production of free radicals *via* Fenton reation.

The reduction of Fe(III) by superoxide (Equation 1.1) is followed by reoxidation of the metal by hydrogen peroxide (Equation 1.3) to generate ferric iron, hydroxide ion and a hydroxyl radical. This latter reaction is known as the *Fenton reaction*.[26] An important source of hydrogen peroxide is the dismutation of superoxide (Equation 1.2). If Fe(III) is reduced by Vitamin C of NADH then redoxcycling can occur (Equation 1.5).

$$Fe^{3+} + HO^{-} + HO^{\cdot} \xrightarrow{\text{Vitamin C/NADH}} Fe^{2+} + H_2O_2 \quad \textbf{1.5}$$

Scheme 1.2: Reduction of ferric iron catalyzed by Vitamin C/NADH.

The production of free radicals such as the hydroxyl radical can initiate a series of harmful reactions with lipids, proteins and DNA, leading to cellular damage, if the cellular levels of glutathione and other antioxidants[27] (e.g. ascorbate, tocopherol) fall abnormally low, since under normal conditions, the free radicals formed are controlled and removed by antioxidants (Equation 1.5), or if the cells are exposed to excessive quantities of a xenobiotic, initiating free radical production. Hence, iron must be acquired and stored in such a way that, its nutritional requirements are met, but oxidant-mediated damage is minimised.

1.2 Transferrin

Transferrins, first discovered in 1946, are a family of metal-binding proteins with *in vivo* preference for ferric iron. Transferrins are divided into four classes, namely: *serum transferrin (Tf)*, the iron transport protein, found in plasma and other extracellular fluids; *ovotransferrin*, from egg white[28]; *lactoferrin*, first discovered in milk, but also found in neutrophil granules and tears[29, 30] and *melanotransferrin*, a cell-surface glycoprotein present on most human melanomas.[31, 32]

Serum Transferrin is an iron carrying, single polypeptide glycoprotein consisting of 679 amino acid residues[33-37] and two N-linked glycan chains with molecular weight ~80,000.[38]

1.2.1 Structure

The molecular structure of serum transferrin is an alpha/beta protein of similar topology to human lactoferrin.[39] Two binding sites bind ferric iron at the inner end of a deep interdomain cleft within the two lobes of the molecule; the N and C lobes. Each lobe binds a single ferric ion and is further divided into two dissimilar domains. Each iron atom is octahedrally bound to four protein ligands: two tyrosines, a histidine, and an aspartic acid residue. Interestingly, optimal iron binding also involves the presence of a synergistic or binding anion such as carbonate that contains two oxygen functions.[1]

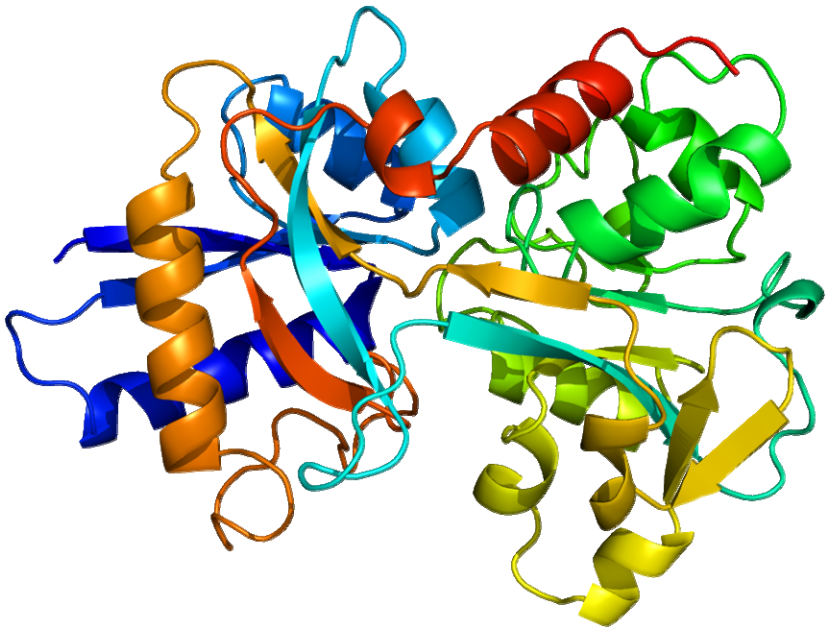

Figure 1.2: Human Serum Transferrin, recombinant N-terminal lobe[1]

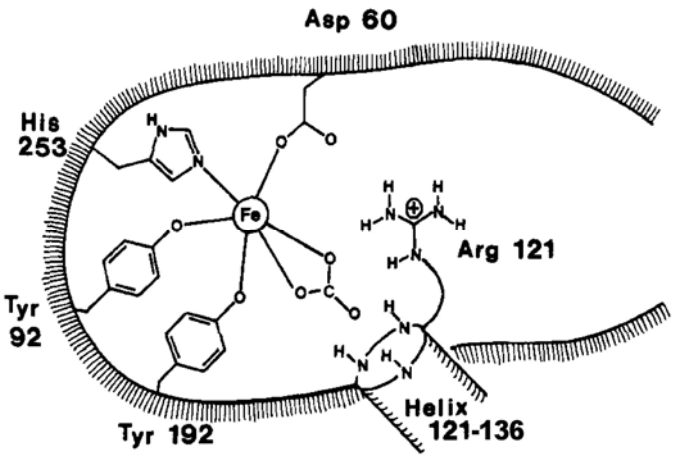

Figure 1.3: The metal binding site of transferrin.[2]

1.2.2 Function of Transferrin

Tf transports iron as a non-toxic, soluble form in plasma, and its major function is to transport iron from the intestine, liver and reticuloendothelial system to all tissues that require iron. At neutral pH, each Tf molecule combines with two iron atoms to form a stable complex that is pH dependent.[38] During the course of 1 day, approximately 45 mg of iron enters the blood plasma. A complete absence of plasma Tf is not compatible with life.

Tissues that have a continual requirement for iron utilise this element for normal growth and development. Tf-specific cell receptors have been detected on the cell membranes of most tissues. These Tf-receptors assist the cellular uptake of Tf-bound iron. The Tf-bound iron forms a Tf-receptor complex with the receptor, which becomes internalised. After iron release, the complex returns to the cell surface and apotransferrin (apo-Tf) is released back to the circulation.[40] Release of iron is normally associated with decrease of the endosomal pH. Each Tf molecule can repeat the process of Tf receptor mediated cellular uptake up to 300 times before degradation occurs.[41] Normal levels of total iron binding capacity (TIBC), which describes the blood's capacity to bind iron with transferrin, is 250–370 μg/dL (45-66 μmol/L) and Transferrin saturation levels in male are 20–50% and in female 15–50%[42]

1.3 Non-Transferrin-Bound Iron (NTBI)

1.3.1 The origins and forms of NTBI

As its name indicates, NTBI comprises all forms of iron in the plasma that are bound to ligands other than Tf. The iron binding capacity of Tf, the highest affinity ligand in the plasma with two iron binding constants of around 10^{20} [43] is normally in the range of 45-80μm. In a healthy individual, this suffices for efficient binding of even large boluses of iron entering the circulation. Upon binding to Tf, the incoming iron becomes effectively shielded from reactions leading to redox cycling and formation of reactive oxygen species via *Fenton reaction* (as shown in Chapter 1.1.2).

The main sources of plasma iron are absorption via the gastrointestinal tract and recycling of aged erythrocytes by macrophages. The precise mechanisms, by which ionic iron enters the plasma and is incorporated into Tf, have yet to be elucidated. Since plasma iron is

normally maintained at levels far below Tf-binding capacity (TIBC), its delivery into the plasma is believed to be tightly regulated. Thus, when non-Tf-bound iron (NTBI) appears in the plasma, it is assumed to result from an imbalance in iron metabolism. The simplest scenario for the emergence of NTBI is that of atransferrinemia, an autosomal recessive metabolic disorder characterized by the absence of transferrin causing all incoming iron to remain as NTBI. Another scenario occurs when plasma Tf becomes fully saturated, resulting in diminished plasma iron-binding capacity. Again, incoming iron is accumulated as NTBI. Such extreme iron-overload can be produced by massive iron ingestion or by repeated transfusions[44] such as those given to patients with haemolytic anaemia (e.g. thalassemia, aplasia, etc.). It is in these patients that the highest frequencies (Table 1.1) and levels of NTBI (up to 10 μM) are observed. This accumulation of iron, particularly in the heart, liver and pancreas, leads to fibrotic changes in these tissues resulting in organ failure and early death.[45]

There is sparse information about the biochemical nature of NTBI, but one may speculate that it is composed of a heterogeneous mixture of complexes whose composition might vary with the degree and type of iron-overload. The solubility of the ferric ion, Fe(III), in physiological salt solutions is extremely low, so unless ligands capable of forming multiple coordination points for stable binding are available, it forms insoluble polynuclear aggregates of chlorides and oxides.[46] The citrate anion is one such ligand, and analysis of NTBI in sera from haemochromatotic patients by high-performance liquid chromatography (HPLC) and high-resolution nuclear magnetic resonance (NMR) indicated the presence of citrate-iron and ternary citrate-acetate-iron complexes.[47] Discrete Fe-citrate compounds have been structurally characterized only since 1994. Lippard et al. have prepared the two dinuclear complexes $[Fe_2(cit)_2\ (H_2O)_2]^{2-}$ and $[Fe_2(citH)_3]^{3-}$depicted in Figure 4.[48] In the presence of excess citric acid, the two complexes are in equilibrium in aqueous solution.[49] Human serum albumin is another candidate ligand, and has been shown to form complexes with Fe(III) and with Fe(III)-citrate in vitro.[50, 51] Although the affinity of human serum albumin for trivalent metals is rather low, this might be compensated by its high concentration in serum (approx. 40mg/ml).

Patient group	Tf-sat range[a]	% NTBI positive[b]	References
Controls	25-35%	⩽1	[6, 8-10, 23, 52-57]
Hemolytic anemias	>85%	>90	[6, 52, 57, 58]
Hemochromatosis	12-104%	69-90	[23, 53-55, 59, 60]
Dialysis (ESRD)	15-55%	22	[55]
Chemotherapy		70-97	[8-10]
Cardiopulmonary bypass	>50%	13	[27, 61, 62]
Neonates		21	[63]
Premature neonates		50	[59]

[a]Approximate ranges

[b]Reported levels are generally in the range 1-10μM.

Table 1.1. Occurence of NTBI in various conditions

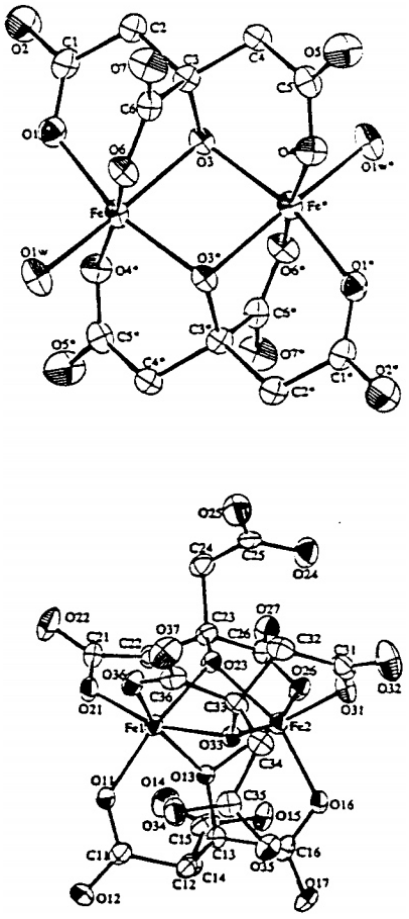

Figure 1.4 $[Fe_2(cit)_2\ (H_2O)_2]^{2-}$and $[Fe_2(citH)_3]^{3-}$[49]

1.3.2 Occurrence of NTBI

It is generally accepted that NTBI is a pathological manifestation and is never found in healthy individuals. In principle, any condition or treatment that produces a short- or long-term iron load can give rise to NTBI (Table 1.1). It can accumulate gradually over long-term as a result of repeated transfusions[44] (haemolytic anaemias such as hereditary spherocyotisis[64, 65]), dialysis (end stage renal disease, ESRD)[55] or hyperabsorption from the diet (hemochromatosis)[66], or it can be a short-term event, which is resolved over a period of hours or days. Examples of the latter are intravenous iron supplements, drug-induced imbalance in erythrocyte turnover, i.e., when the rate of erythrocyte degradation exceeds iron reutilization for erythropoiesis (chemotherapy[8-10, 67], myelodysplastic syndromes[68] or alcohol-induced[69]) massive hemolysis such as can occur during cardiopulmonary bypass operations and accelerated erythrocyte turnover which takes place in neonates. In most documented cases, the appearance of NTBI is preceded or accompanied by an increase in Tf-saturation, indicative of release of large amounts of iron into the circulation. Nonetheless, in some disease states of e.g. hemochromatosis which are associated with positive NTBI values, it has been reported that Tf is incompletely saturated.[23, 53]

1.3.3 Iron overload treatment

The goal of iron chelation therapy in most cases is to prevent and treat iron-mediated injury to cells. For the past 30 years, desferrioxamine B (DFO, DFB) (Figure 1.5) has been the only clinical useful drug available for relieving iron overload.[70] DFO is a natural siderophore, a growth-promoting agent produced by *Streptomyces pilosus* to scavenge iron form the environment.[71, 72] It is a powerful hexadentate ligand, which binds to Fe(III) in a 1:1 molar ratio and with an extremely high stability constant. Furthermore, the affinity of DFO for Fe(II) and other biological metal ions, such as copper, zinc, calcium and magnesium, is much lower.[73]

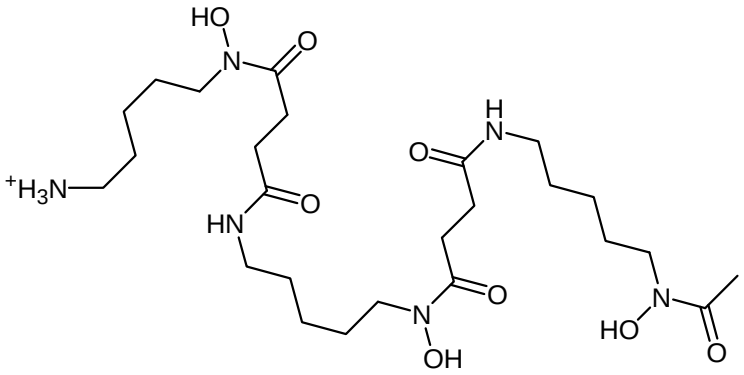

Figure 1.5: Desferrioxamine (DFO)

Follow ups of patients on life long transfusion programmes has shown that the regular use of the drug prevents the development of iron overload and its pathological consequences, like abnormal sexual maturation,[74] diabetes mellitus,[75] cardiac hypertrophy and dilatation, myocardial fiber degeneration or fibrosis,[75-78] hepatic fibrosis whilst transfusion-dependent patients not taking DFO succumb to iron toxicity in their late teens or early twenties. Adequate therapy with DFO has led to survival curves that approximate to those of the normal population (Figure 1.6). [75, 78-87] Unfortunately, DFO has the disadvantage of being orally inactive and must therefore be parenterally administered, in order to be effective.

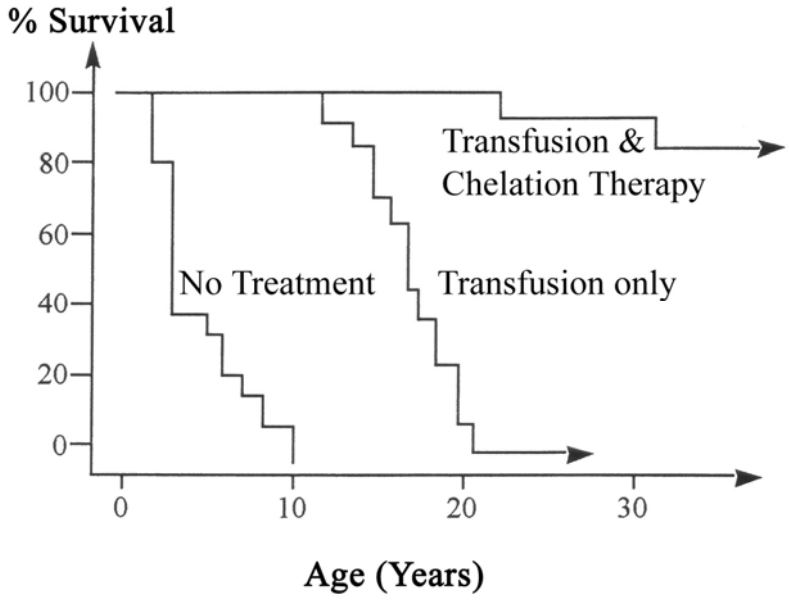

Figure 1.6: The effect of transfusion and chelation therapay on β-thalassaemic patient mortality.[86, 87]

Moreover, DFO is rapidly cleared from the circulation, due to its short plasma half-life of 5-10 minutes.[88] It only causes sufficient iron excretion to keep pace with transfusion regimes when administered as either a subcutaneos or intravenous infusion, 8-12 hour per day, five to seven days per week, at a dose between 40-60mg/kg. For this reason many patients find it difficult to comply with the treatment, some discontinuing chelation therapy altogether.[79, 85, 89] Finally, DFO is an expensive drug and, although it is available to most patients in the Western world, only a small proportion of such patients in poorer countries are so treated. Consequently, an orally active, selective, inexpensive iron chelator to replace desferrioxamine is highly sought after.

Such an orally active iron chelator is Deferiprone (CP20, L1) (1,2 dimethyl-3-hydroxyryridin-4-one) (Figure 4) which is a member of a family of hydroxypyridin-4-one (HPO) chelators[90] that require three molecules to fully bind Fe(III), each molecule providing two coordination sites (bidentate chelation). CP20 (Figure 1.7) is the world's first orally active chelator that haas been approved and is widely used as an alternative to DFO in Italy, Greece, UK and India.[91-93]

However, severe side effects such as agranulocytosis, neutropenia, arthropathym zinc deficiency, and gastrointestinal disorders have been reported.[94] It has also been reported that long-term use of CP20 is ineffective in maintaining hepatic iron concentrations below hepatotoxic levels.[94, 95]

Hider and co-workers have attempted to improve the efficacy of the hydroxypyridinone family, and to minimise their side effects by applying the prodrug concept to this molecular class. Such prodrugs would lead to efficient liver first-pass kinetics.[96]

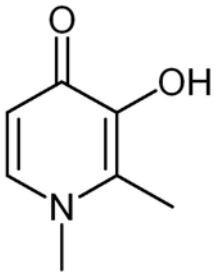

Figure 1.7: Deferiprone (CP20)

1.3.4 Detection of NTBI

At the present time there is no generally accepted method for the accurate quantification of NTBI. Both direct and indirect ways of measuring NTBI have been employed. Direct measurement involves the use of scavenging molecules like ethylenediaminetetraacetic acid (EDTA) [4, 52, 97, 98] (Figure 1.8), citrate, oxalate[7] and nitrilotriacetic acid (NTA) [54, 56, 97, 99] (Figure 1.9) which bind the non-specifically bound iron followed by separation and detection.

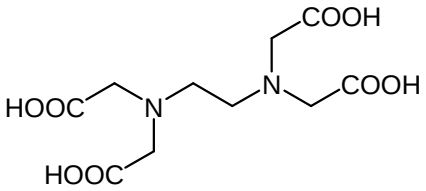

Figure 1.8: Ethylenediaminetetraacetic acid (EDTA)

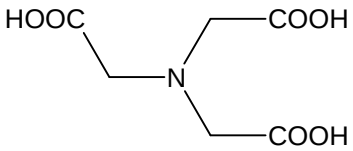

Figure 1.9: Nitrilotriacetic acid (NTA)

Various methods of detection have been used including high-performance liquid chromatography (HPLC), colorimetry, atomic absorption and inductively coupled plasma spectroscopy.[54] In each case, valid identification of an iron fraction as *bona fide* NTBI depends entirely on the demonstration that these agents do not extract iron from Tf. Since Tf-bound iron in normal serum is typically 14-32 μM and can be doubled in iron-overload conditions[6], even a fractional release of iron from Tf could give rise to NTBI values of several μM. Usually, assay selectivity is established by demonstrating the absence of detectable NTBI in control sera from healthy subjects. A more rigorous proof of selectivity is provided by adding iron-saturated Tf to the assay system and demonstrating that it does not contribute to the signal.[54, 55]

Methods currently used for NTBI measurement:

- DEAE Sephadex A50 catechol disulphate gel filtration[100]
- Aromatic hydroxylation assay[101]
- Chelation ultra filtration based on EDTA, NTA, determined, by colourimetry[6, 52, 102], HPLC[54, 56], fluorescence spectroscopy [103, 104]
- Inductively coupled plasma linked to an atomic emission spectrophotometry or a mass spectrometer, ICP-AES, [105] ICP-MS [54]
- Bleomycin assay [106, 107]

Overestimation of NTBI levels, lack of specificity and sensitivity are common features of NTBI measuring techniques. For correct determination of NTBI in serum samples it is important for the chelating agent not to displace iron from transferrin.[54] This problem has been partly overcome with the introduction of more direct methods based on the use of a large excess of a low affinity ligand such as Nitrilotriacetic acid (NTA).[56] This method is similar to the ultrafiltration method based on the use of EDTA originally developed by Hershko *et al.*[6] The addition of excess NTA to the serum results in the removal and complexation of iron that is non-specifically bound to serum proteins and low-molecular-weight ligands. In principle, all the NTBI so scavenged is quantitatively converted to the Fe-NTA complex. The solution is then subjected to ultrafiltration and the iron in the ultrafiltrate is determined by either HPLC[56] or colorimetry.[6, 52, 102] The main problem with these NTBI quantification methods is that they represent complicated techniques and provide difficulty to quantify accurately. The choices of measuring method for research is dependent on level of accuracy required. As NTBI levels are in the μM range, methods of high sensitivity are required. As mentioned previously indirect NTBI measurement methods can generate an artificially low NTBI value in the presence of apo-Tf. Therefore, most NTBI quantification methods require selectively blocking of the vacant iron-binding sites on transferrin with using Co(III). This cannot be achieved with simple Co(II) or Co(III) salts, but requires the use of *tris*-carbonatocobaltate(III) trihydrate.[108] The method described in this work avoids such difficulties.

1.4 Inductively coupled plasma linked to a mass spectrometer (ICP-MS)

From the various methods used for NTBI analysis, inductively coupled plasma linked to a mass spectrometer is considered to offer a very specific and sensitive iron analysis. The component of the ICP-MS system includes [109]:

- Sample introduction system – peristaltic pump, a nebulizer and spray chamber
- ICP-torch – the excitation source being the ICP plasma
- Interface – links the atmospheric pressure ICP ions source of ICP-MS
- Vacuum system – providing high vacuum for ion optics
- Quadrupole – the mass filter sorting the ions by their mass to charge ration (m/z)
- Detector – electron multiplier counting the ions pass through the quadruple
- PC with specified software – data processing

ICP-MS offers many benefits to trace metal analysis with detection limits equal to or better than that offered by Graphite Furnace Atomic Absorption spectroscopy (GFAAS). The principles of ICP-MS involve heating the sample to a very high temperature (6000°C), which is achieved with argon gas in the inductive plasma. The extremely high temperature completely breaks up the molecules present in the sample to elemental ionisation. These ions are then separated in the MS in order of mass and detected through ion counting allowing for individual isotope determination for each element.
The available isotopes for Fe are summarized in Table 1.2.[110] The Fe^{56} which is the most abundant natural iron isotope is not accessible to use for measurements as it interferes with ArO a component in the analytical gas.

Isotope	Relative abundance (%)	Preference for measurement
Fe^{54}	5.80	1
Fe^{57}	2.20	2
Fe^{56}	91.7	-
Fe^{58}	0.25	3

Table 1.2: Preferred Fe Isotopes for ICP-MS measurement

1.5 Fluorescence Methodology

1.5.1 Principles of Fluorescence

Fluorescence is the phenomenon in which, absorption of light of a given wavelength by a fluorescent molecule, is followed by the emission of light at longer wavelengths. Thus, a compound may absorb radiation in the ultraviolet region and emit visible light. This increase in wavelength is known as the *stokes' shift*.

At the atomic level, most compounds and molecules occupy the lowest energy state, which is referred to as the "ground" state (S_0). These species can in turn absorb electromagnetic radiation as long as the incident photon contains an energy level equal to the difference between two "allowed" energy states ($h\upsilon_{EX}$). When a quantum of radiation of sufficient energy is absorbed by a molecule, it becomes excited (S_1'), possessing a different electronic distribution. In the spectrometer this energy is in the form of a photon from an incandescent lamp or a laser. A molecule in an excited electronic state produces a molecule that is in an excited rotational state.[111, 112]

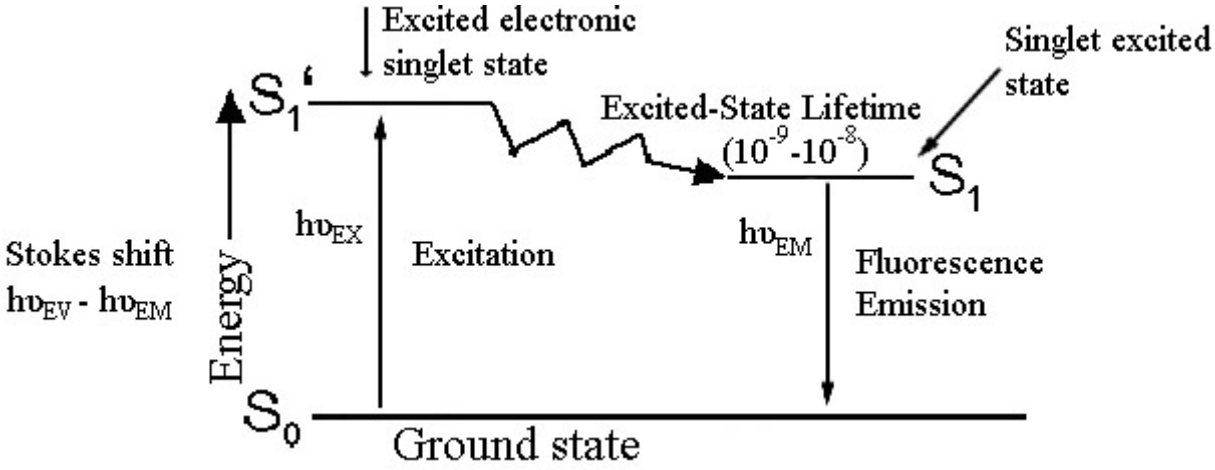

Figure 1.10: Jablonski diagram illustrating the processes involved in the creation of an excited electronic singlet state by optical absorption, and subsequent emission of fluorescence.[113]

Since the excited state is not the state of lowest energy, the molecule will not stay there indefinitely. The excited state exists typically for 1-10x10^{-9} seconds.[114] In the excited state, some of the energy is partially lost or dissipated to surrounding molecules, which gives the excited molecule a relaxed singlet excited state (S_1). If the excited molecule is stable, it will then return to the ground state (S_0), by emitting radiation of a different

wavelength from that administered. Transitions to lower energy states are divided into two categories: radiative transitions, which are responsible for fluorescence, and non-radiative transitions, which do not emit light because the energy is either moved around within the molecule, or is captured by another particle.
However, not all the molecules initially excited by absorption, return to the ground state (S_0) by fluorescence emission. The system can also lose the energy (i.e. depopultate S_1) by[114]:

- Internal conversion (heat)
- External conversion (collisional quenching)
- Intersystem crossing (phosphorescence)
- Fluorescence energy transfer

The dissipation of energy is crucial in fluorescence, as this is what makes the emission of a different wavelength. Fluorescence emissions are almost always larger in wavelength than the excitation radiation, meaning that they possess lower energy.
A spectrum of emitted fluorescence is recorded and can be used to monitor the abundance of certain fluorescent compounds within assays.

1.5.2 Fluorescence Instrumentation

Fluorescence-measuring instruments are primarily of three types, each providing distinctly different information:

(i) Spectrofluorometer and Microplate Reader
- measures the average properties of bulk (µl or ml) samples.

(ii) Fluorescence Microscope
- resolves fluorescence as a function of spatial co-ordinates in two dimensions for microscopic objects (less than 0.1mm diameter)

(iii) Cytometer
- measures fluorescence per cell in a flowing stream, allowing subpopulations within a large sample to be identified and quantified.

Each type of instrumentation produces different measurement artefacts and makes different demands on the fluorescent probe. For example, although photobleaching is often a significant problem in fluorescence microscopy, it is not a major impediment in flow cytometry because the time of stay of individual cells in the excitation beam is short.

A typical spectrofluorometer, such as the PERKIN ELMER LS 50B and the PERSEPTIVE BIOSYSTEMS Cytofluor© 4000, both used in this assay, contains the following[115] (Figure 1.11):

- An excitation source (e.g. xenon lamp in LS 50B and Cytofluor© 4000)
- Sample cell
- Wavelength filters – isolate emission photons from excitation photons
- Fluorescence detector – registers emission photons and produces recordable output, usually as an electrical signal or a photographic image.

Regardless of the application, compatibility of these elements is essential for optimising fluorescence detection.

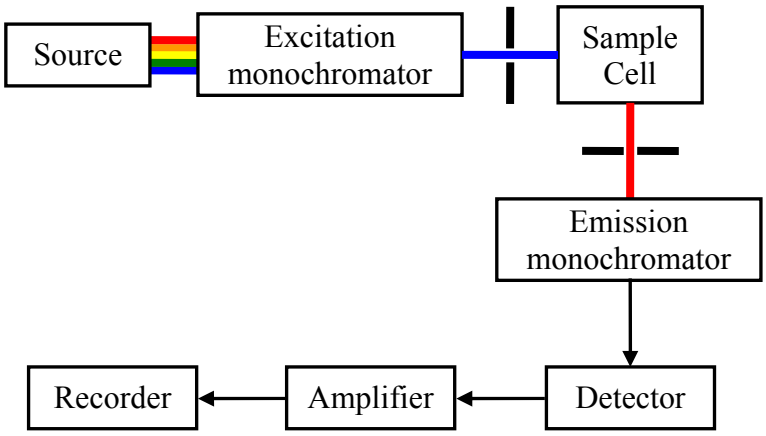

Figure 1.11: Schematic representation of a flourimeter

Molecules in solution are usually excited by UV-light and the excitation source is usually a deuterium or xenon lamp. Broad-band excitation light from a lamp passes through a monochromator, which passes only a selected wavelength (the activation wavelength). The fluorescence is dispersed by another monochromator (selecting the fluorescent wavelength) and detected by a photomultiplier tube.

Fluorescence intensity is quantitatively dependent on the same parameters as absorbance (defined by the Lambert-Beer law as the product of the molar extinction coefficient (ε), optical path length (l) and solute concentration (C); i.e. A = ε.C.l), as well as on the fluorescence quantum yield of the probe, the excitation source intensity and fluorescence collection efficiency of the instrument.

1.5.3 Fluorescent Probes

To trace the iron in biological fluids, the introduction of a probe or metal sensing agent is necessary. In recent years, the use of fluorescent dyes has become very important for visualising biological rooted ions, utilising the principle of chelation properties of metal chelators in biological systems. An important use for iron specific probes is the monitoring of NTBI levels in human sera. Fluorescent indicator molecules for the detection of chelatable iron consist of a fluorophore coupled with a chelating ligand, which should be as selective for iron as possible. The probe binds to the iron and can then be monitored for fluorescence intensity. This can be translated to calculate the actual concentration of the iron within the analyte.[112, 116]

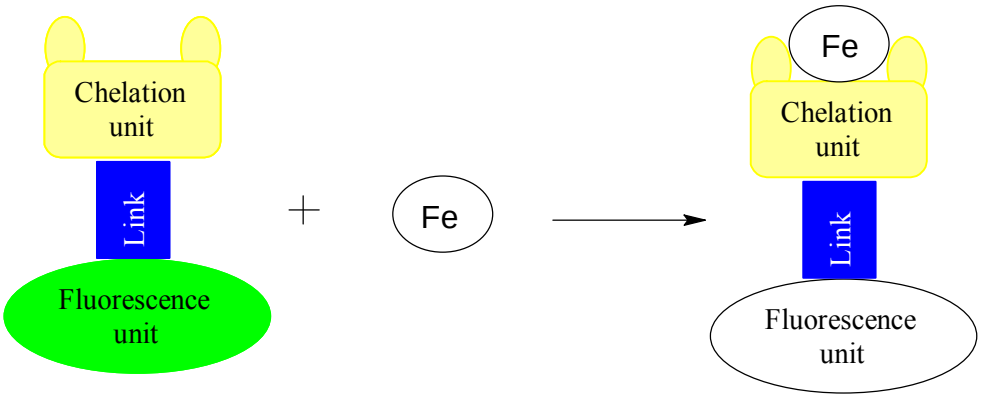

Figure 1.12: Principles of fluorescent probe based iron quantification.

1.5.3.1 Development of Fluorescent Iron Chelators

In the early 1990's, the development of fluorescent indicators for the detection of chelatable iron in biological systems started with the design of indicators based on naturally occurring (microbial) or synthetic siderophores coupled to small, neutral

fluorophores.[117] Fluorescent analogues of siderophores[118-120] were used for detecting iron ions in cell-free systems, as well as for recording the transport of iron into microorganisms and plants, for detecting pathogenic microorganisms[121] and for mobilization from cells. NBD-DFO, the 7-nitrobenz-2-oxa-1,3-diazole derivative of DFO and fluorescein-desferioxamine (FL-DFO), commonly used in the early 1990's, showed artificially increased levels of the chelatable iron pool and thus, with regard to several other methodical disadvantages, should be not suitable for detecting the physiological concentration of this pool.[117] In the mid 1990's Breuer et. al. introduced the so far most frequently used fluorescence spectroscopic method for determination of the chelatable iron pool of viable cells.[122] This method, using calcein as fluorescent agent, was later modified for the detection of NTBI.[55]

1.5.3.1.1 Calcein

Calcein (Figure) is a derivative of fuorescein which has two binding moieties and is a sensitive iron chemosensor, which can be used for quantification of low molecular weight iron in biological fluids.[123, 124]

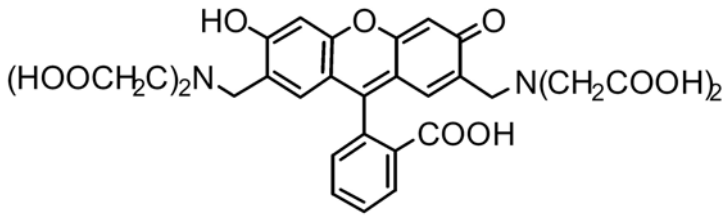

Figure 1.13: Calcein

Calcein binds iron in a 1:1 stoichiometric manner and its fluorescence is quenched in the presence of iron. This quenching effect of calcein is specific for low molecular weight iron (LMW). Thus a lower fluorescence intensity recorded indicates a higher concentration of LMW iron. Calcein is a very versatile probe as it binds to iron readily, and only releases it to chelators with a higher affinity for iron. A rise in fluorescence is observed when the calcein bound iron is removed. Calcein based assays showed a more than ten times higher sensitivity than the colorimetric assays which were carried out before the calcein introduction. A major drawback in the use of this probe is the fact that calcein is not iron specific. It is quenched by other non iron related compounds in the serum.[124] Although

the contribution of other metal ions is neglectable, circumstances can arise, sometimes under pathological conditions where for example substantial amounts of Cu(II), an ion which is usually less prevalent than iron, can be released from cells, and potentially bias the result of iron detection. This means the specificity of the assay is affected considerably, and the determination of iron levels is not as precise as would have been desired.

This method can also lead to an underestimation of NTBI values as DFO, the iron chelating agent used in this assay, was reported to be inaccessible to NTBI in the serum of hemochromatosis patients.[104] Furthermore, the experimental procedure is complicated.[7] Another limitation of calcein as a probe for iron detection is the fact that excessive citrate within the serum of the cell competes for binding with LMW iron. This can result in a wide variation between assays due to diminished quenching of fluorescence, which gives an imprecise estimation of NTBI. Thus, research for new iron-selective, sensitive probes is essential.

1.5.3.1.2 Novel Pyridinone Probes

An essential requirement for fluorescent probes is the stable chemical linking of iron to the fluorescent dye. Pyridinone fluorescent probes possess a high affinity for iron (III) and are therefore suitable for NTBI measurement. CP691[125], a recently designed probe is in the focus of the investigation.

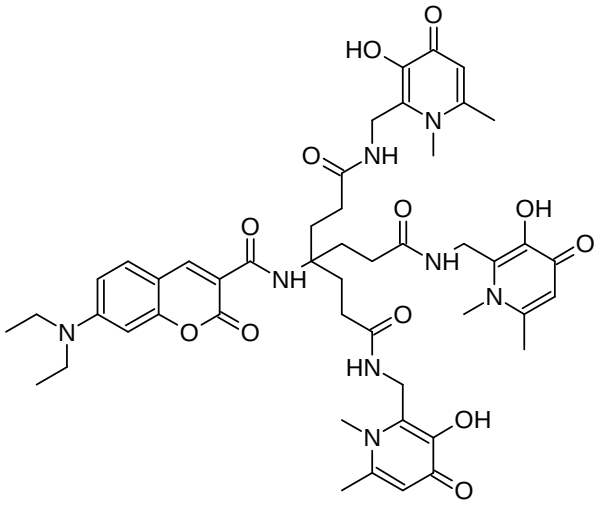

Figure 1.14: CP691: 3-hydroxypyridin-4-one with a coumarin substituent forming a bidentate ligand for chelation of iron (III) over the pH range of 6.0-9.0[125]

Chapter 2

Aims of Study

According to Singh *et al.* (1990)[56] an ideal fluorescent iron chelator should fulfill some minimum requirements such as:

(i) not detecting transferrin bound iron

(ii) not detecting ferritin bound iron

(iii) quantifying all iron bound to low molecular weight ligands in plasma

(iv) quantifying all iron non-specifically bound to plasma proteins

(v) being simple, rapid, reproducible and easily automated to analyse large numbers of clinical samples

Since currently established NTBI quantification methods which are described in Chapter 1.3.4, represent techniques either requiring expensive laboratory equipment such as HPLC [54, 56], ICP-MS[54] or ICP-AES [105] involving complicated, time consuming sample preparations or using iron chelators inaccessible to certain forms of NTBI which therefore tend to lack of specificity and sensitivity[104], it was found, that it is necessary to develop a novel fluorescence-based assay for NTBI measurements.

The main focus of this investigation lies on

(i) Development of an appropriate fluorescence-based quantification method for NTBI through simple method validation for required levels of analysis.

Furthermore, CP691, the novel pyridinone probe mainly used in this assay, is undertaken studies aiming on

(ii) Comparing the kinetic aspects of CP691 with established iron(III) chelators

and

(iii) CP691's potential for iron removal from transferrin in the presence of Fe-NTA/Fe-citrate

Finally the described novel fluorescence-based assay for NTBI measurements is undertaken evaluation by

(iv) Comparison of the fluorescence-based assay with an established NTBI-quantification method.

Chapter 3

Materials and Methods

3.1 Materials and Solutions

3.1.1 Equipment

- Fluorescence Plate reader: PerSeptive Biosystems Cytofluor® Series 4000
  Multi-well plate reader, computer linked;
  Software: Cytofluor Fluo Fluorescence Reader vers. 4.1, copyright 1997 by Perseptive Systems

- UV-Spectrophotometer: Perkin-Elmer UV/VIS Spectrometer Lamda 2, computer linked; Software: Perkin-Elmer PEC DD Software-Package for Lamda 2,15/17,9 Version 4.2 Nov/91

- ICP/MS: Perkin Elmer SCIEX ELAN DRC PLUS, linked to Poly Science® cooling pump; Software: ELAN instrument control

- Pipettors: Eppendorf Reference®, adjustable-volume 2-20µl and 50-200µl, yellow control button for 100µl and 300µl pipette tips
  Eppendorf Research®, adjustable-volume 20-200µl, yellow control button for 100µl and 300µl pipette tips
  Eppendorf Research®, adjustable-volume 100-1000µl, blue control button for 1000µl pipette tips

Eppendorf Research®, adjustable-volume 500-5000µl, violet control button for 5000µl pipette tips
Eppendorf Research® pro, electronic 8-channel pipettor, adjustable-volume 20-300µl, yellow control button for 300µl pipette tips

- Water purification: Millipore Simplicity 185 with UVPhoto-Oxidation (suitable for HPLC) cartridge: Millipore Simpak® prod. Date: 25-Nov-2004, feed with deionised water

- pH meter: MP 230 pH meter, METTLER-Toledo GmbH (Schwerzenbach, Switzerland)

- Blood tube mixer: DENLEY SPIRAMIX 5

- Shaking water bath: GRANT OLS200

- Stirer: IKAMAG ® RCT heat able stirrer

- Mixer: FISONS whirlimixer ™

3.1.2 Chemicals

All Chemicals were obtained from Aldrich (Dorset, UK) and Sigma (Dorset, UK) without further purification unless otherwise stated.

- CP691 MW 1050g/mol (designed and synthesised by Yongmin Ma)

- 3-[N-morpholino]propansulfonic acid (MOPS) MW 209.3g/mol, minimum 99.5%, CAS [1132-61-2]

- Human Serum Albumin, Sigma product A1653, Fraction V Powder, remainder mostly globulins, 96-99%[126], CAS [70024-90-7]

- Iron atomic absorption standard solution, Aldrich product 30595-2, Containing 989µg/ml of Fe in 1.1 wt. % HCl d1.010 [17.66mM]

- Fluka® Iron ICP Standard Solution

- Steril Human Mixed Pool Serum, batch HUT2285/NHS4107, First Link (UK), Birmingham

- Dimethyl sulfoxide (DMSO), Aldrich, 99+% ACS grade reagent, CAS [67-68-5]

- Chelex® 100 sodium form, Sigma, 50-100 dry mesh CAS [11139-85-8]

- Nitrilotriacetic acid (NTA), Aldrich, MW 191.14g/mol, mp 245°C, 99+% purity, CAS [139-13-9]

- Nitrilotriacetic acid (NTA), disodium salt, MW 235.11g/mol, mp >300°C, 99+% purity, CAS [15467-20-6]

- Sodium hydroxide (NaOH), AnalR® reagent, CAS [1310-73-2]

- Citric acid monohydrate, MW 210.11g/mol, CAS [5949-29-1]

- Citric acid trisodium salt, MW 258.11g/mol, CAS [68-04-2]

3.1.3 Consumables

- Microplates: Microplate Sterilin, flat bottom, polystyrene, not-treated, non-sterile, black, 96-well by Bibby Sterilin Ltd.

- Quartz-cuvettes obtained from SCIENSCO Ltd.

- Disposable cuvettes: 1.5mL and 4.5mL from KARTELL suitable for UV measure

- Fluorescence cuvettes: 4.5mL from VWR

- Pipettor tips: Eppendorf epTips, blue type, 50-1000µl; Eppendorf, epTips reloads 300µl in Eppendorf epTips box; Gilson Diamond D-200, 200µl, non-sterile; Greiner bio-one yellow type pipette tip to fit Eppendorf, 200µl; Biohit/Eppendorf blue type, 101-1000µl, non-sterile, multi-compatible; natural type 1-5ml, non-sterile

- Bottles: 500ml/1000ml fisherbrand, borosilicate glass, reagent and media, screw neck with polypropylene cap (for mops buffer stocking); 500ml polyethylene wide neck wash bottle Azolon/Fisherbrand

- Containers: 5ml polyethylene container, Elkay-brand, with screw neck (for biohazardous goods handling); 30ml polyproylene, plain labled, universal container with polyethylene cap, Greiner bio-one, aseptic production (for general purpose, Fe(III) stock solutions, biohazardous goods handling); 30ml glass, with polyethylene cap (for NaOH stocking), Polyethylene lunchbox (for soaking/washing procedure)

- Flasks: 50ml borosilicate glass, conical, narrow neck, Fisherbrand

- Tubes: Elkay, 1.5ml microcentrifuge tube, polyethylene (sample preparation, sera/sample/probe stocking)

- Pipettes: Disposable glass and plastic pipettes

3.1.4 Other experimental equipment

- Stirrer: IKAMAG® RCT, heated magnetic stirrer

- Mixer: FISONS whirlimixer®

- Balances: Mettler AJ150 (general purpose), Sartorius Masterpro® (analytical balance)

3.2 Precautions when handling potentially infectious materials

Some of the materials used in this assay were obtained from mammalian sources and therefore are potentially infectious. Even though all potentially infectious materials had been tested negative on HIV I and HIVII, HCV and HBsAg by their suppliers, special precautions when handling these materials must be taken.

For details on handling and waste disposal of bio hazardous material and current laboratory standards see the World Health Organization - Laboratory bio safety manual.[127]

Nitrile gloves, laboratory glasses and a laboratory coat suitable for handling bio hazardous material must be worn at all times!

3.3 Preparation of precursors

3.3.1 Washing procedure

Even when 96-well plates, tubes and pipettor tips are new, they may still be contaminated with iron or other chelatable ions. Thus it was necessary to wash them before use in order to minimize iron contamination: 96-well plates, ultracentrifuge tubes, 1ml, 5ml tips were all washed. Washing 200µl tips would have been too time consuming.

1. Preparation of washing solution (1% nitric acid): 50ml of 20% nitric acid are added to 1900ml purified water (Millipore water) and stirred afterwards.
2. Pipettor tips, microplates, containers and tubes are washed with 1% Triton detergent and rinsed thoroughly with purified water.

3. Pipettor tips, microplates, containers and tubes are soaked in the 1% HNO_3-Solution in a plastic container and are stirred from time to time (solution should reach all surfaces). Afterwards they are left to stand overnight.
4. Pipettor tips, microplates, containers and tubes are removed from the washing solution using a suitable net.
5. Pipettor tips, microplates, containers and tubes are rinsed thoroughly using plenty of high quality purified water. Afterwards the water is discarded using a net. This step is repeated two times.
6. Pipettor tips, microplates, containers and tubes are dried using an appropriate method (Air drying at room temperature is suitable)

3.3.2 Preparation of 50mM MOPS buffer pH 7.4 [Preparation A]

10.465g MOPS are diluted to 1l using Millipore purified water. After stirring the solution on the magnetic stirrer to dissolve the MOPS completely, the pH, which is around 3.5 at that time, is adjusted to 7.4 using 1N and 6N NaOH.

MOPS: M (FW) = 209.3, n = 0.05M
m = n x M = 0.05 x 209.3 = 10.465g

3.3.3 Preparation of 1/10mM CP691 stock solution [Preparation B/C]

10.50mg CP691 are diluted to 1ml using DMSO. For preparation of 1mM stock solution, solution from above was diluted ten-fold using MOPS buffer (50mM, pH = 7.4).

Due to light sensitivity of the fluorescent probe the solution must not be exposed to direct sunlight or other light sources. Reagents must be used fresh; however, they can be completely prepared, stored frozen at -20°C in portions, and thawed only once. Repeated freeze-thaw is not recommended because it causes loss of fluorescence!

CP691: M (FW) = 1050, n_1 = 0.01M
$m_1 = n_1$ x M = 0.01 x 1050 = 0.0105g

CP691: M (FW) = 1050, n_2 = 0.001M
$m_2 = n_2$ x M = 0.001 x 1050 = 0.00105g

Preparations of 1mM stock solutions of CP692, CP20 and DFO in DMSO and MOPS buffer [50mM] are being prepared analogously.

CP692: M (FW) = 1080, n_2 = 0.001M
$m_2 = n_2$ x M = 0.001 x 1080 = 0.00108g

CP20: M (FW) = 139.15, n_2 = 0.001M
$m_2 = n_2$ x M = 0.001 x 139.15 = 0.000139g

DFO: M (FW) = 560.68, n_2 = 0.001M
$m_2 = n_2$ x M = 0.001 x 560.68 = 0.00056g

3.3.4 Preparation of 1mM Fe(III)$[NTA]_2$ stock solution [Preparation D]

An equivalent of Fe(III)HCl is being dissolved in 3mM NTA solution giving 10ml of 1mM Fe(III)$[NTA]_2$ stock solution (m_2).

10ml of a 100mM NTA disodium solution in purified water is prepared.

NTA · 2Na: M (FW) = 235.11, n = 0.1M
m = n X M = 0.1 x 235.11 = 23.11g/l = 0.0231g/10ml

300µl of the above solution are added to 9134µl of purified water and mixed using a whirlimixer afterwards. To this solution 566µl of FeHCl solution [17.66mM] from the Atomic analysis standard (which normally is about 18mM iron concentration; checking of the individual packing label for precise concentration is required) is added.

n_1 = FeHCl [17.66mM] = 0.01766M , n_2 = 0.001M, m_2 = 10ml

$m_1 \times n_1 = m_2 \times n_2$

m_1 = 0.56625ml = 566µl

m_2 = 9134µl H_2O + 300µl NTA · 2Na [100mM] + 566µl FeHCl [17.66mM]

Obeying the described order is crucial to maintain an pH level below pH = 8 to prevent the production of non-measurable oligomeric iron within the solution!

A series of standard solutions of Fe(III)$[NTA]_2$ of different concentrations (100µM/10µM) in MOPS buffer (50mM, pH 7.4) is prepared.

n_1 = Fe(III)$[NTA]_2$ = 0.001M, n_2 = 100µM = 0.0001M, n_3 = 10µM = 0.00001M

$m_1 \times n_1 = m_2 \times n_2 = m_3 \times n_3$

$m_2 = m_1 / 10$

$m_3 = m_1 / 100$

Some experiments also require solutions containing of Fe(III)$[NTA]_2$ concentrations at 10µM/5µM/1µM/0.2µM in MOPS buffer (50mM, pH 7.4) which are being prepared analogously.

Because of iron-NTA solutions' light sensitivity it is recommended to not expose the solutions to direct sunlight!

3.3.5 Preparation of a 100µM Fe(III)$[NTA]_2$ stock solution for ICP-MS measurement [Preparation E]

55.83µL of Fluka® Iron ICP Standard Solution [17.906mM] are added to 30µL NTA in water [100mM] and afterwards mixed with 9914µL purified water.

n_1 = Fe Fluka® [17.906mM] = 0.017906M , n_2 = 0.0001M, m_2 = 10ml

$m_1 \times n_1 = m_2 \times n_2$

m_1 = 0.055847ml ~ 55.85µl

3.3.6 Preparation of 1µM Fe(III)citrate solution [Preparation F]

First a 100mM Citric acid monohydrate solution in purified water is being prepared.

Citric acid monohydrate: M (FW) = 210.1, n = 0.1M
m = n x M = 0.1 x 210.1 = 21.01g/l = 21mg/ml

1000µl of the above solution is added to 8430µl of purified water. To this solution 566µl of FeHCl solution [17.66mM] from the Atomic analysis standard (which normally is about 18mM iron concentration; checking of the individual packing label for precise concentration is needed) is added and then incubated at 37°C for 30 minutes. After incubation the pH is adjusted to 7.4 by addition of sodium hydroxide 25% (w/v) solution.

n_1 = FeHCl [17.66mM] = 0.01766M , n_2 = 0.001M, m_2 = 10ml
$m_1 \times n_1 = m_2 \times n_2$
m_1 = 0.56625ml = 566µl

m_2 = 8430µl H_2O + 1000µl Citric acid monohydrate [100mM] + 566µl FeHCl [17.66mM]

3.3.7 Preparation of 3%/6% human serum albumin solutions [Preparation G_1/G_2]

Human serum albumin (300/600mg) is dissolved in 10ml MOPS buffer (50mM, pH 7.4). The solutions are shaken gently to minimize the production of foam or even worse, cell disruption.

An ultrasonic bath must not be used for dissolving human serum albumin!

Albumin from human serum lyophilized powder and its solutions must be kept in a dry and cool place (2-8°C)!

It is essential that all standard solutions and samples are freshly prepared!

3.4 Construction of a standard curve

In order to measure chelatable non-transferrin bound iron in human patients' pathological blood serum samples a standard curve for the fluorescence-based NTBI measuring method described in chapter 3.5 had to be established.

3.4.1 Standard samples with human blood serum and albumin

In this assay a final volume of 200µl/well at a concentration of 0.12% human serum albumin, 0-2 µM Fe(III)$[NTA]_2$ and 2% Human Blood Serum was measured with the use of 2µM CP691.

0.24% Albumin + Fe(III)$[NTA]_2$ [0-4µM] in 100µl MOPS [*Solution* $A_{1\text{-}12}$]
4% Human Blood Serum + 4µM CP691 in 100µl MOPS [*Solution B*]

100µL of each Fe concentration with 0.24% Albumin [*Solution* $A_{1\text{-}12}$] were transferred in triplicate to 96-well plates using a single channel pipette (Eppendorf Research® single-channel pipette). Thereafter, *Solution B* was added into the wells. The samples' fluorescence was measured with a 96-well plate reader, PerSeptive Biosystems Cytofluor¬® Series 4000 (excitation 450 ± 50 nm, emission 530 ± 25 nm, gain: 90; 3 reads per well).

Procedure

1. Preparation of Fe(III)$[NTA]_2$ [10µM]:

10µl Fe(III)$[NTA]_2$ + 990µl MOPS are mixed and well stirred.

2. 40µl human serum albumin 3% + 0-200µl Fe(III)$[NTA]_2$ [10µM] are diluted to 500µl using MOPS-buffer [50mM]

Human serum albumin [3%] x m = Human serum albumin [0,24%] x 500µl
m = 40µl

3. put at rest for 30min in a dark place to form Fe-Albumin complexes

4. 16µl CP691 [1mM] + 160µl Human Blood Serum + 3824µl MOPS-buffer [50mM]

CP691 [1mM] x m = CP691 [4µM] x 4000µl
m = 16µl

Human Blood Serum [100%] x m = Human Blood Serum [4%] x 4000µl
m = 160µl

4000µl – 160µl Human Blood Serum [100%] – 16µl CP691 [1mM] = m x MOPS-buffer [50mM]
m = 3824µl

5. pipet 100µl of the solution prepared in step 4 in well A1-C12

6. ad 100µl of the solutions from step 2 (e.g. solution with Fe [0µM] in 1A, 1B, 1C, solution with Fe [0.1µM] in 2A, 2B, 2C…)

7. incubate for 30min in a dark place at room temperature

8. measure with the PerSeptive Biosystems Cytofluor¬® Series 4000 plate reader (excitation 450 ± 50 nm, emission 530 ± 25 nm, gain: 90; 3 reads per well).

The procedure is repeated 3 times.

c (Fe) [µM]	Albumin 3% µL	Fe [10µM] µL	MOPS µL
0	40	0	460
0.1	40	10	450
0.2	40	20	440
0.3	40	30	430
0.4	40	40	420
0.5	40	50	410
0.6	40	60	400
0.8	40	80	380
1.0	40	100	360
1.2	40	120	340
1.4	40	140	320
2.0	40	200	260

Table 3.1: Preparations for the standard curve using 2µM CP691, 2% human blood serum, 0.12% human serum albumin and Fe(III)$[NTA]_2$ 0-1µM

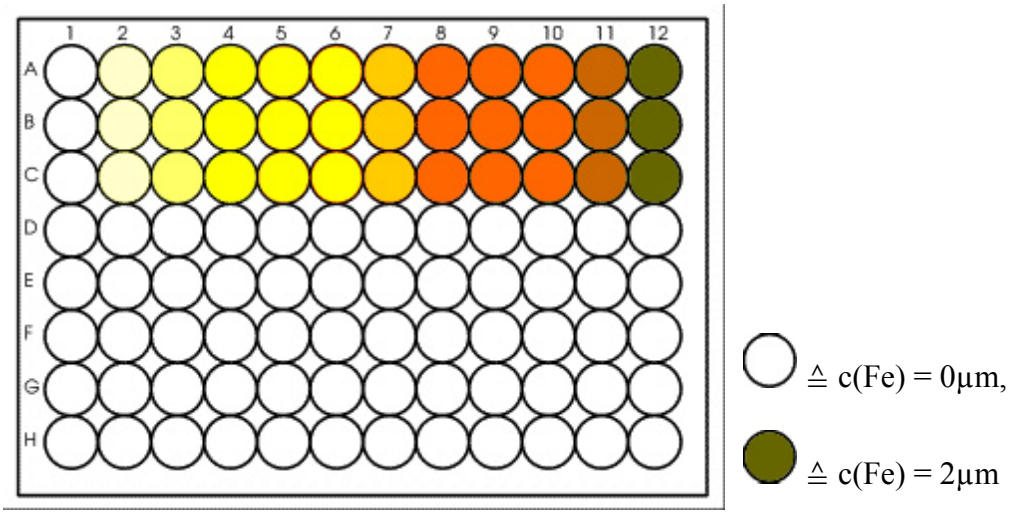

Figure 3.1: Scheme of NTBI quantification using Eppendorf Research ® single-channel pipette on a 96-well plate

3.4.2 Standard samples with human blood serum

To obtain more *in vivo* like results whilst minimizing the effect of human blood serum's autofluorescence it was found suitable to create a standard curve using 10% human blood serum and 1.5µM CP691 to measure $Fe(NTA)_2$ 0-1µM which is the equivalent to the common range of 0-10µm NTBI in undiluted human blood serum from pathogenic conditions.

$Fe(NTA)_2$ [0-1µM] in 10% human blood serum was measured with CP691 [1.5µM]. The final samples' concentrations for $Fe(NTA)_2$ were 0, 0.1, 0.2, 0.3, 0.4, 0.6, 0.8 and 1.0 µM..

Procedure

20µl of CP691 [150µM] are mixed with 0-200µl of $Fe(III)[NTA]_2$ [10µM] stock solution and the diluted with MOPS buffer, 50mM, pH 7.4 to 1ml. 100µl of each concentration is pipetted in 8 different wells (e.g. A1-H1, Figure 3.2). Afterwards 100µl of 20% human blood serum are added to each well and the mixture is incubated in a dark place at room temperature for 30min. Afterwards fluorescence levels are being measured using the 96-well plate fluorescence reader. (excitation 450 ± 50 nm, emission 530 ± 25 nm, gain: 90; 3 reads per well).

c (Fe) [µM]	Fe [10µM] µL	CP691 [150µM]	MOPS µL
0	0	20	980
0.1	20	20	960
0.2	40	20	940
0.3	60	20	920
0.4	80	20	900
0.6	120	20	860
0.8	160	20	820
1.0	200	20	780

Table 3.2: Preparations for the standard curve using 1.5µM CP691, 10% human blood serum and $Fe(III)[NTA]_2$ 0-1µM

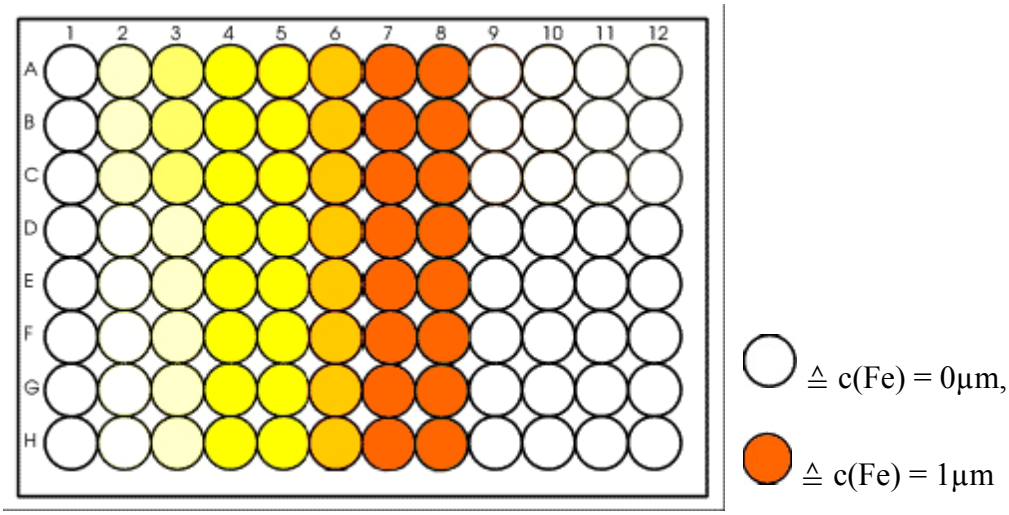

Figure 3.2: Scheme of NTBI quantification using Eppendorf Research ® pro multi-channel pipette on a 96-well plate

3.5 Pathological samples

A series of NTBI blind samples containing blanks, ferrous iron in unknown concentrations and pathological human blood serum from patients suffering from various conditions with iron overload such as β-Thalassaemia were measured for chelatable iron with CP691 as ligand using fluorescence spectroscopy. All samples were collected and provided by Patricia Evans of the University College Hospital London. Results for measured NTBI levels were validated by the use of two different HPLC-based NTBI-measuring methods (referred to as *HPLC 1* and *HPLC 2*) described in [54] and [104]. The HPLC-based assays were conducted by my colleges Patricia Evans and Dingyong Liu.

Procedure

Frozen unknown samples were defrosted by overnight storage at 4°C to avoid cellular disruption. Samples were then characterized by describing their colour and turbidity.
200µL of unknown sample are mixed with 50µL CP691 [60µM] and 1750µL MOPS buffer pH 7.4 giving a 10 fold dilution of unknown sample with CP691 [1.5µM] in MOPS buffer pH 7.4. A sample of 2000µL containing 10% Human Serum with no labile iron in CP691 [1.5µM] in MOPS buffer (50mM, pH 7.4) is prepared as a control sample.

After mixing the samples with the fluorescent probe they are incubated for 10 min at 37°C. Afterwards 200µL of each solution is transferred in octuplicate to 96-well plates. The fluorescence in the wells is determined in the PerSeptive Biosystems Cytofluor¬® Series 4000 (excitation 450 ± 50 nm, emission 530 ± 25 nm, gain: 90; 3 reads per well).

Values for the fluorescence of the control sample are being set as 100% and those for the unknown samples are being expressed as percentage of the control sample. Iron levels in unknown samples are then being calculated using the linear function describing the standard curve in Chapter 3.4.2. (Equation 4.1)

$$c(Fe)[\mu M] = \frac{\left(\frac{(\text{average fluorescence unknown sample})}{(\text{average fluorescence control sample})} x100\right) - 98.398}{-74.982} * 10$$

Equation 3.1: Calculation for iron concentration c(Fe) [µM] in unknown samples using the linear function from the standard curve obtained in Chapter 3.4.2.

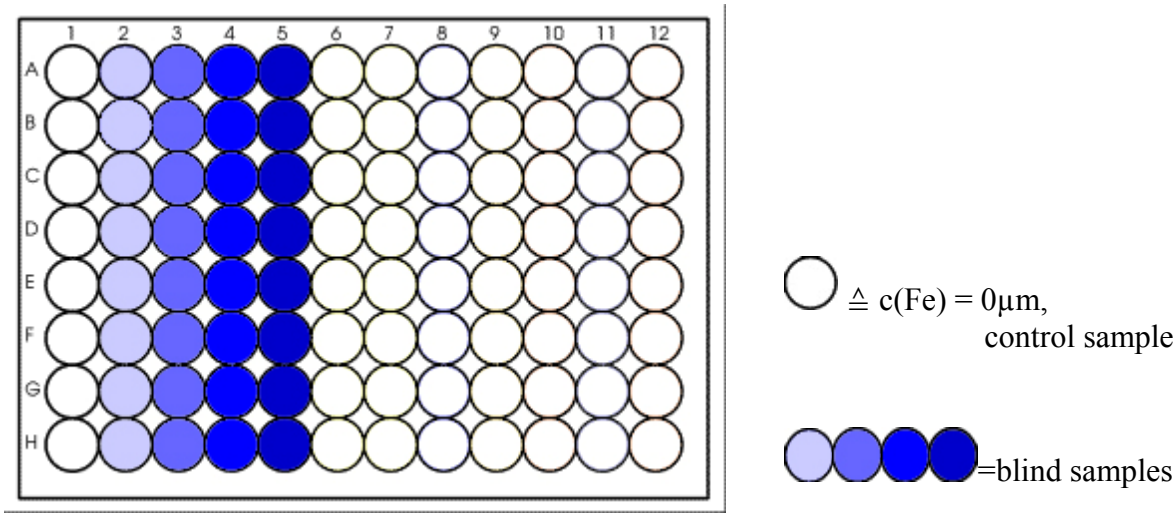

Figure 3.3: Scheme of NTBI quantification from pathological samples using an internal standard; Eppendorf Research ® pro multi-channel pipette on a 96-well plate

3.6 Reaction kinetics

3.6.1 CP691's potential for iron removal from transferrin in the presence of Fe-NTA/Fe-citrate

In this assay it was investigated whether CP691 or apo-Tf was the stronger iron chelator (Sample 1). Furthermore the assay should prove if aqueous iron citrate complexes were formed in the presence of citric acid without any non-transferrin bound iron being available which would result in false positive results (Sample 2).
Total iron binding capacity for transferrin (TIBC) (see Chapter 1.2.2), which describes the blood's capacity to bind iron with transferrin, is 250–370 μg/dL (45-66 μmol/L) and Transferrin saturation levels in male are 20–50% and in female 15–50%. To simulate *in vivo* situation a concentration of apo-Tf (32.5μM) and CP691 (4μM) both half loaded with Fe^{2+} was chosen.

Preparation of samples

Solution 1: 2mL of apo-Tf (65μM) in $NaHCO_3$ (50mM) solution is being prepared
Solution 2: *Solution 1* + 2mL of MOPS (50mM, pH 7.4)
Solution 3: Fe^{2+} (1mM) [$Fe(NH_4)_2(SO_4)_2$) in H_2O]
Solution 4: *Solution 2* + Fe^{2+} (32.5μM) are mixed and incubated for 2 hours at room temperature
Solution 5: CP691 (4μM) + Fe^{2+} (2μM) in MOPS buffer (4mL)

Sample 1: 2mL *Solution 4* + 2mL *Solution 5*
Sample 2: 2mL *Solution 4* + 2mL *Solution 5* + 30μL citric acid (10mM)
Internal Std: 3mL MOPS buffer + 6μL CP691 (1mM) + 3μL Fe^{2+} (1mM) + 1mL of citric acid (400μM)

Procedure

Fluorescence levels of Sample 1 and Sample 2 are being compared against an internal standard over a period of 21 hours. Measuring is done on a fluorometer (PERKIN ELMER LS 50B) set at excitation 450 ± 50 nm, emission 530 ± 25 nm.

3.6.2 Reaction kinetics of CP691 with Fe-NTA/Fe-citrate compared to those of other iron chelators (CP692, DFO, CP20)

Besides the question of accessibility, the rapidity and duration of response of non-transferrin-bound iron (NTBI) to chelation therapy are largely unknown. Therefore a test was designed to compare kinetic aspects of established iron chelators, namely DFO and CP20, with two novel fluorescent iron chelating probes, CP691 and CP692 using UV-Vis chromatography.

Desferrioxamine (DFO), a hexadentate chelator, a growth-promoting agent secreted by the microorganism *Streptomyces pilosus* is presently the therapeutic agent of choice for the clinical treatment of chronic iron overload.[128]

1,2-dimethylhydroxypyridinone (Deferiprone, CP20), a bidentate chelator, was the only orally active iron chelator available for clinical use at the time of design of this test.[128]

CP691 and CP692 are novel fluorescent iron chelating probes, designed to determine iron overload levels *in vitro*.

3.6.2.1 Determination of isosbestic point of DFO, CP691, CP692, CP20

Before being able to compare reaction kinetics of CP691, CP692, CP20 and DFO with Fe-citrate using UV-Vis spectroscopy their isosbestic point (the specific wavelength at which the chemical species have the same molar absorptivity) had to be determined.

Therefore, the absorption spectra of 10µm of the *hexadentate* iron(III) chelators DFO, CP691 and CP692 and 30µm of the *bidentate* iron(III) chelator, CP20, all in MOPS (50mM, pH 7.4) with 10µm $Fe^{3+}(NTA)_2$ each were compared. Absorption levels were measured in the range of 500-300nm wavelength using a Perkin-Elmer UV/VIS Spectrometer.

3.6.2.2 Reaction kinetics of CP691, CP692, CP20 and DFO with Fe-citrate

A time series was set to compare reaction kinetics of CP691, CP692, CP20 and DFO with Fe-citrate to give an insight on the chelation potential of investigated iron chelating probes.

10µm of the *hexadentate* iron(III) chelators, DFO, CP691 and CP692 and 30µm of the *bidentate* iron(III) chelator, CP20, each mixed with 10µm Fe(III)citrate were compared using UV/Vis spectroscopy. Specific wavelengths determined in the assay described in Chapter 3.6.2.1 were used.

To compare the different iron chelators investigated, the results gained from the UV/Vis Spectroscopy had to be subtracted by the values emitted by the free ligands.

$$A(absolute) = \text{ligand with iron citrate - free ligand}$$

Equation 3.2: Calculation of absorption levels of different iron chelators using UV/Vis spectroscopy.

$$A(relative) = \frac{(\text{ligand with iron citrate - free ligand})}{(\text{ligand with iron NTA - free ligand})} x100$$

Equation 3.3: Calculation of the relative absorption potential of different iron chelators with iron citrate compared to absorption levels of different iron chelators compared to iron NTA using UV/Vis spectroscopy.

3.6.3 Reaction kinetics of CP691 with Fe-NTA/Fe-citrate in the presence of physiological concentration of human serum albumin

For standardisation of the NTBI-measuring method it was found necessary to compare kinetic aspects of CP691 with Fe-NTA and Fe-citrate. In order to create more *in vivo*-like conditions kinetics were compared in the presence of physiological concentration of human serum albumin.

3.6.3.1 Choosing a suitable wavelength for measuring absorption of CP691 with Fe-NTA/Fe-citrate in the presence of physiological concentration of human serum albumin

Before being able to compare kinetic aspects of the reaction of CP691 with Fe-NTA/Fe-citrate a suitable wavelength had to be determined using UV/Vis Spectroscopy. Ideally, the spectrum should provide a specific wavelength where the difference between absorption values of "*Albumin + CP691+ Fe-citrate"* and "*Albumin + CP691+ Fe-NTA"* is as small as possible, whereas the difference of those to the absorption value of *"Albumin + CP691"* at this wavelength is as large as possible. Furthermore the interference of *Albumin* should be as small as possible at the determined wavelength.

Solution preparation

Albumin [40mg/mL]:
40mg of human serum albumin is dissolved in 1mL MOPS buffer [50mM]

Albumin [40mg/mL] + CP691 [10µM]:
990µL Albumin [40mg/mL] is added to 10µL CP691 [1mM]

Albumin [40mg/mL] + CP691 [10µM] + $Fe^{3+}(NTA)_2$ [10µM]:
980µL Albumin [40mg/mL] and 10µL $Fe^{3+}(NTA)_2$ [1mM] are mixed and then incubated for 30min at 37°C before being added to 10µL CP691 [1mM]

Albumin [40mg/mL] + CP691 [10µM] + Fe^{3+}citrate [10µM]:
980µL Albumin [40mg/mL] and 10µL Fe^{3+}citrate [1mM] are mixed and then incubated for 30min at 37°C before being added to 10µL CP691 [1mM]

Absorption levels of the prepared solutions were compared in the range of 500-300nm wavelength using a Perkin-Elmer UV/VIS Spectrometer.

3.6.3.2 Kinetics of Fe-citrate- compared to Fe-NTA-chelation in presence of physiological human serum albumin concentration using CP691

To find out to what extent Fe-citrate was accessible to CP691 compared to Fe-NTA over a certain period of time a time series experiment was set up.

Solution preparation

Albumin + CP691 [10µM] + Fe^{3+}citrate [10µM]:
980µL Albumin [40mg/mL] and 10µL Fe^{3+}citrate [1mM] are mixed and then incubated for 30min at 37°C before being added to 10µL CP691 [1mM]

After mixing the Albumin and Fe^{3+}citrate solution with CP691 the sample is measured immediately for a course of 6 minutes.

Results obtained from the time series experiment measuring the reaction of Fe-citrate with CP691 in the presence of a physiological concentration of human serum albumin were set into relation with the absorption levels given by Fe-NTA with CP691 in the presence of physiological human albumin concentration at λ=475nm in the UV/Vis spectrum described in Chapter 4.3.3.1. The results were calculated using the following equation (Equation 3.3):

$$A(relative) = \frac{(\text{CP691 with albumin and iron citrate} - \text{CP691 with albumin})}{(\text{CP691 with albumin and iron NTA} - \text{CP691 with albumin})} x100$$

Equation 3.4: Calculation for reaction kinetics of Fe-citrate compared to Fe-NTA-chelation in the presence of physiological human serum albumin concentration using CP691.

3.7 Comparison of the fluorescence-based assay with an ICP-MS-based method

The fluorescence-based assay established in Chapter 3.4.2. was validated with an ICP-MS-based method using a blind sample assay containing ferric iron over the range of 0-10µM. ICP-MS was chosen to compare the fluorescence-based assay because it is considered to be the most accurate of all existing iron quantification methods.[54]

Sample Preparation

Samples containing unknown concentrations of $Fe(NTA)_2$ in MOPS buffer (50mM, pH 7.4) in the range of 0-10µM were being prepared by my college Yongmin Ma using different concentrations of thawed **Preparation E** (see Chapter 3.3.5) in MOPS buffer (50mM, pH 7.4).

sample #	c (Fe) [µM]	$Fe(NTA)_2$ [100µM] µL	MOPS µL
0	0	0	1800
1	4	72	1728
2	10	180	1620
3	1	18	1782
4	2	36	1764
5	7	126	1674
6	3	54	1746
7	6	108	1692

Table 3.3: Preparations for unknown samples containing various concentrations of $Fe(NTA)_2$ in MOPS buffer (50mM, pH 7.4)

e.g. sample nr. 3:

$n_1 = Fe(NTA)_2$ [100µM], $n_2 = Fe(NTA)_2$ [1µM], $m_2 = 1800µl$

$m_1 \times n_1 = m_2 \times n_2$

$m_1 = 18µl$

Procedure for the fluorescence-based assay

200µl of sample (#0-7) are added to 20µl CP691 [150µM] and then added to 780µl of MOPS buffer (50mM, pH 7.4). Afterwards 100µl of the above are added to 100µl of a 20% Human Blood Serum in the 96-well plate giving a final 10 fold dilution of the sample in 10% Human Blood Serum. The samples' fluorescence is then measured using the PerSeptive Biosystems Cytofluor® Series 4000. The measured fluorescence of the blind samples is recalculated using the standard curve containing 10% Human Blood Serum and 1.5µM CP691 established in Chapter 3.4.2 to obtain the samples' iron concentration. Afterwards the results are being compared to those given by the ICP-MS measurement.

Sample c (Fe) 10%	Human Blood Serum 10%	CP691 [1.5µM]

Table 3.4: Scheme for final sample concentration for 96-well fluorescence plate reader measurement of unknown samples

Procedure for the ICP-MS-based method

First the Perkin Elmer SCIEX ELAN DRC PLUS is rinsed with 0.5% HNO_3 to get rid of any contaminations. Before measuring the blind samples the system is set under vacuum for the daily performance check and the calibration for Fe^{54} isotope is performed. Measurements on the ICP-MS were performed by my college Mark Sykes and Wei Luo.

Settings:
Vacuum: 7.4×10^{-6} Torr
Nebulizer Gas Flow: 0.98L/min
ICP RF Power: 1100 Watts
Lens Voltage: 5.25 Volts
Analog Stage Voltage: -2400 Volts
Pulse Stage Voltage: 2100 Volts

Chapter 4

Results

Iron is an important trace metal serving various functions in the human body. It is transported via the protein, transferrin, which tightly binds the iron. The iron found in the plasma not bound to transferrin is referred to as Non-Transferrin-bound Iron (NTBI). A task in NTBI research concerns the optimal quantification method for NTBI analysis.
An easy-to-handle one-step fluorescence-based assay was in the focus of this study.
Standard curves for various dilutions of human blood serum and typical levels of non-transferrin-bound iron with a fluorescent iron probe were prepared and applied on pathological human blood serum samples.
Reaction kinetic tests were conducted comparing the iron chelating probe to other commonly used iron chelators, illuminating its potential for iron removal from transferrin.
Finally the fluorescence-based assay was being compared with an ICP-MS-based method.

4.1 Construction of a standard curve

4.1.1 Standard samples with human blood serum and albumin

A standard curve for 0.12% human serum albumin, 0-2 μM Fe(III)$[NTA]_2$ and 2% Human Blood Serum with 2μM CP691 was prepared.

The results shown in Figure 4.1 show a direct, linear correlation (R^2=0,9983) between iron concentrations and fluorescence quenching of the fluorescent chelator probe (CP691). Fluorescence quenching was linear in the presence of 2% human blood serum of not only

solutions of $Fe(NTA)_2$ in MOPS buffer (50mM, pH 7.4) but also of Fe(III)Albumin complexes, which are presumably formed in pathologic iron overload states.[50]

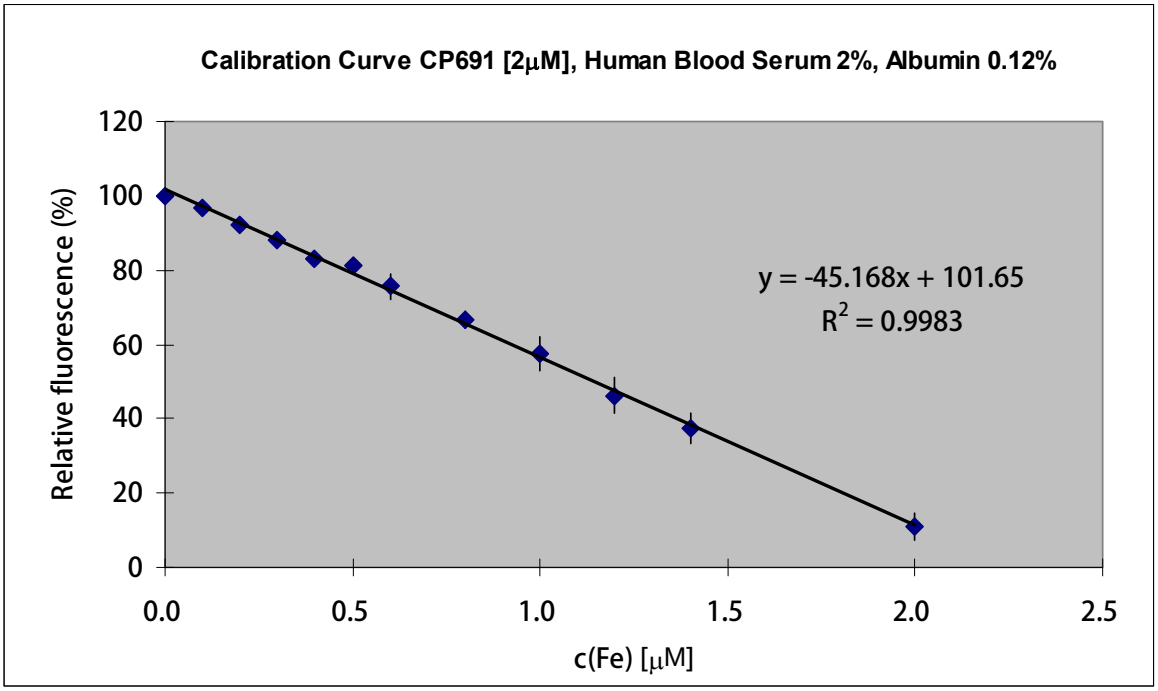

Figure 4.1: Standard curve of CP691 fluorescence quenching by ferrous iron in MOPS with 2% Human Blood Serum and 0.12% human serum albumin. Fluorescence of the probe, CP691 [2µM] in MOPS (50mM, pH 7.4), with 2% Human Blood Serum and 0.12% human serum albumin were set at 100%. The fluorescence of the probe in presence of human blood serum and various iron concentrations was expressed at the percentage of that of iron absence. Measurement was performed on a Spectrofluorometer at 450/530nm excitation/emission. Track shown is the average of 4 experiments performed on different days.

4.1.2 Standard samples with human blood serum

In order to obtain more *in vivo* like results whilst minimizing the effect of autofluorescence of human blood serum it was found suitable to create a standard curve using 10% human blood serum and 1.5µM CP691 to measure $Fe(NTA)_2$ 0-1µM which is equivalent to the common range of 0-10µm NTBI in undiluted human blood serum from pathogenic conditions.

$Fe(NTA)_2$ [0-1µM] in 10% human blood serum was measured with CP691 [1.5µM]. The final samples' concentrations for $Fe(NTA)_2$ were 0, 0.1, 0.2, 0.3, 0.4, 0.6, 0.8 and 1.0 µM.

The standard curve of probe in MOPS and serum is displayed in Figure 4.2. When iron concentration is between 0 to 1µM, it was found that the relative fluorescence intensity is strongly dependant on the iron concentration and a linear relationship is obtained (R^2 > 0.998).

$$y = -74.982x + 98.398$$

Equation 4.1: Linear function describing the standard curve using CP691 [1.5µM] with Human Blood Serum 10% in MOPS buffer (50mM, pH 7.4) with various iron concentrations $Fe(NTA)_2$ [0-10µM]. y = c(Fe)[µM], x=relative fluorescence

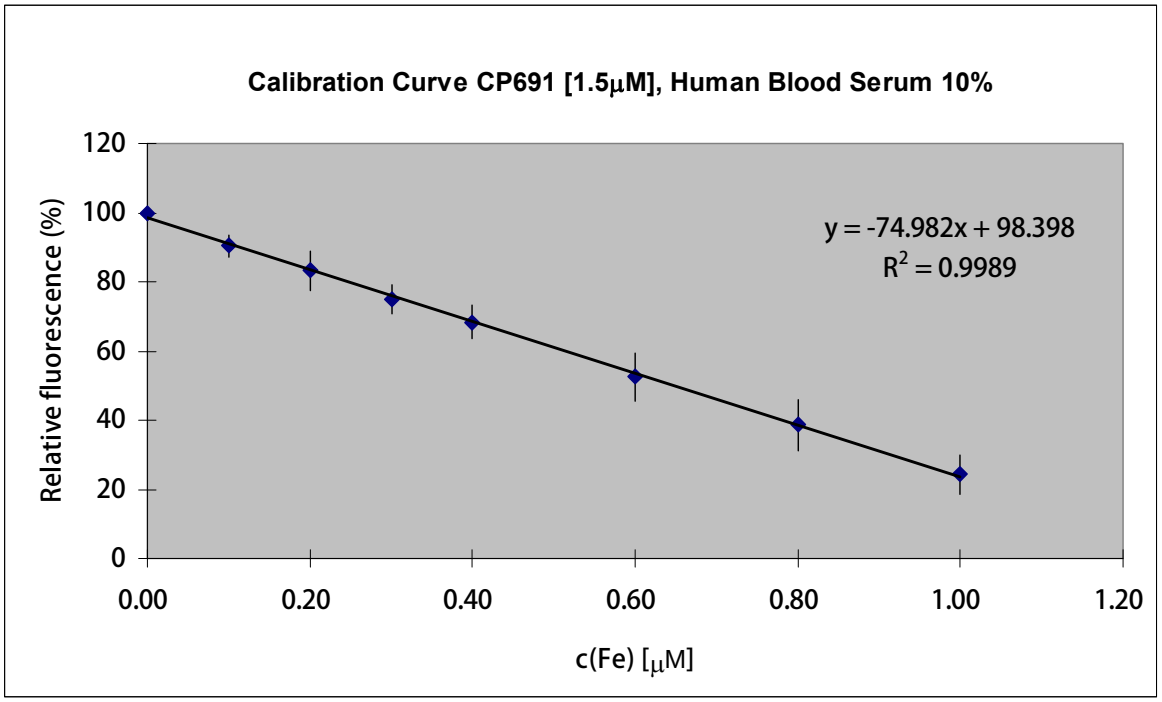

Figure 4.2: Standard curve of CP691 fluorescence quenching by ferrous iron in MOPS with 10% Human Blood Serum. Fluorescence of the probe, CP691 [1.5µM] in MOPS (50mM, pH 7.4), with 10% Human Blood Serum was measured at 450/530 nm excitation/emission by a Spectrofluorometer and set at 100%. The fluorescence of the probe in presence of human blood serum and various iron concentrations was expressed at the percentage of that of iron absence. Track shown is the average of 8 experiments.

4.2 Pathological samples

A series of NTBI blind samples containing blanks, ferrous iron in unknown concentrations and pathological human blood serum was measured for chelatable iron with CP691 as

ligand using fluorescence spectroscopy. Results for measured NTBI levels were validated by the use of two different HPLC-based NTBI-measuring methods (referred to as *HPLC 1* and *HPLC 2*) described in [54] and [104]. The HPLC-based assays were conducted by my colleges Patricia Evans (University College London) and Ding Yong Liu (King's College London).

Results displayed in Table 4.1 and Figure 4.3 show weak to no correlation between the NTBI-levels measured with the fluorescence-based assay and the HPLC-based assays. Results of two HPLC-based assays show weak to no correlation among themselves either. CP691 responds reliably to iron in the fluorescence-based assay when the iron is presented in a aqueous solution of $Fe(NTA)_2$ or Fe-citrate in the presence of 10% human blood serum from a standardized pool (see Chapter 3.4.2). Thus, the established standard curve could not be applied for calculating NTBI levels from other human blood serum pools.

sample #	NTBI c[µM] (CP691)	NTBI c[µM] (HPLC 1)	NTBI c[µM] (HPLC 2)	colour	turbidity
1	12.4	2.8	4	clear	-
2	12.3	1.3	8	clear	-
3	2.2	3.0	4.3	light brown	+
4	3.7	4.2	0	light brown	~
5	2.7	4.1	4	brown	++
6	12.4	2.6	10	clear	-
24	3.3	2.1	-	brown	++
25	3.6	3.4	-	light brown	++++
26	2.7	2.6	-	light brown	+
27	-	2.7	-	brown	+
28	-	1.9	-	brown	~
29	-2.0	3.9	-	red-brown	+++
30	2.0	3.9	-	brown	++~
31	-1.1	1.7	-	light brown	++
32	-	1.4	-	red-brown	+
33	-0.9	2.7	-	red-brown	++++
34	-0.1	3.4	-	dark brown	+~
35	0.3	2.5	-	brown	~
36	4.0	2.2	-	very light brown	~

Table 4.1: Calculated iron levels of human blood serum samples from patients suffering from various conditions with iron overload and other non-serum samples of unknown composition that were measured using 3 different methods (one fluorescence-based and two HPLC based methods). Furthermore samples'

were characterized by their colour and turbidity. Clear samples were identified as non-serum samples of unknown composition, "-" indicates that this sample has not been measured with the referring method.

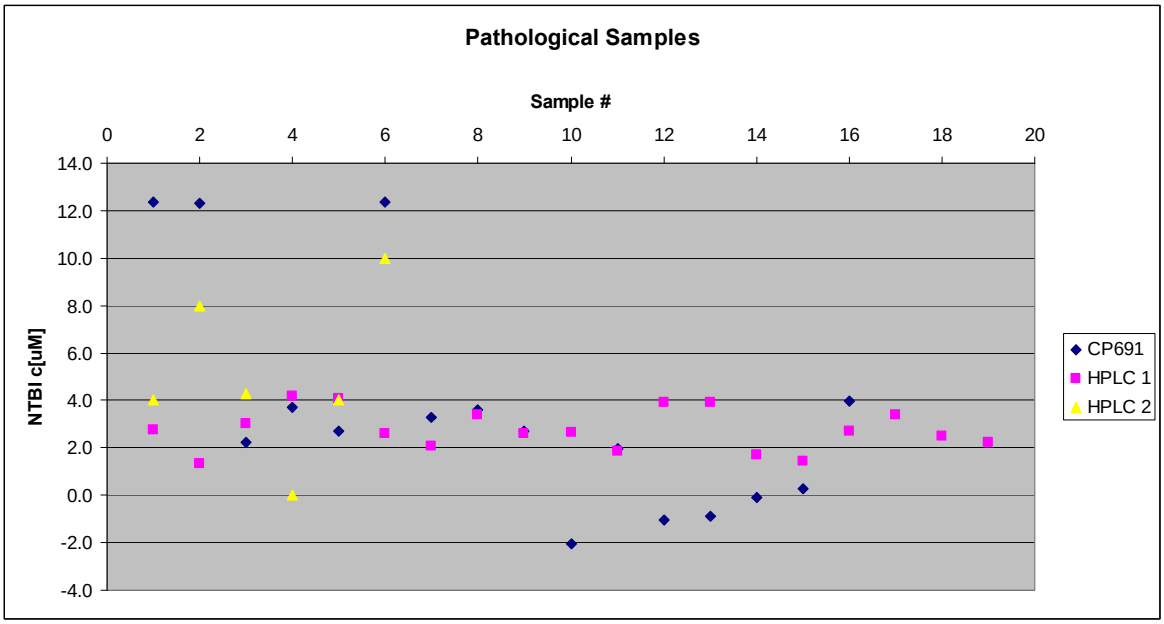

Figure 4.3: Plot shows the results for pathological human serum samples containing unknown levels of NTBI. No linear relationship between all 3 methods used (one fluorescence-based method and two HPLC based methods) could be established

4.3 Reaction kinetics

4.3.1 CP691's potential for iron removal from transferrin in the presence of Fe-NTA/Fe-citrate

Figure 4.4 shows the results for Sample 1 compared to the internal standard show that CP691 is a stronger iron chelator than apo-Tf. CP691 is slowly removing iron from Transferrin which leads to the expectation that CP691 used as a probe to determine human NTBI levels *in vivo* could give false positive results to a minor extent. This will only occur when measured after a period of more than 2 hours after sample preparation. See Chapter 3.6.1 for description of the sample preparation.

The results for Sample 2 (also Fig. 4.4) compared to the internal standard show that CP691 is not only the stronger iron chelator compared to apo-Tf but is also able to chelate iron in the presence of iron citrate complexes.

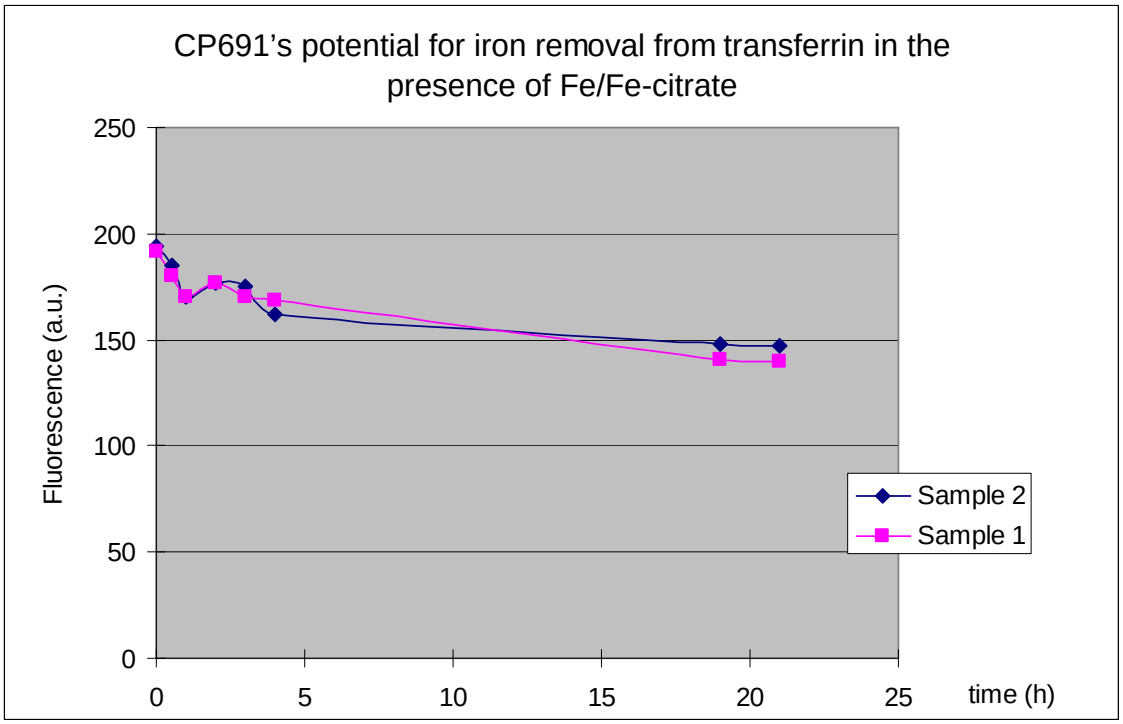

Figure 4.4: Fluorescence quenching of CP691 [4µm] by Fe(II)/Fe-citrate in the presence of apo-Tf. The Fluorescence intensity is measured by a Perkin-Elmer Spectrofluorometer and recorded in arbitrary units (a.u.). CP691 is slowly removing iron from Transferrin which leads to the expectation that the probe would lead to false positive results when used for detection of NTBI in human blood serum.

4.3.2 Reaction kinetics of CP691 with Fe-NTA/Fe-citrate compared to those of other iron chelators (CP692, DFO, CP20)

Besides the question of accessibility, the rapidity and duration of response of non-transferrin-bound iron (NTBI) to chelation therapy are largely unknown. Therefore a test was designed to compare kinetic aspects of established iron chelators, namely DFO and CP20, with two novel fluorescent iron chelating probes, CP691 and CP692 using UV-Vis chromatography.

4.3.2.1. Determination of isosbestic point of DFO, CP691, CP692, CP20

Results show that the isosbestic point can only be determined between CP20 and CP691 at 475nm. CP20's absorption maximum is at 460nm and DFO's absorption peak is found at 430nm. Therefore following wavelengths for further studies on kinetic aspects of the iron chelators in focus of this assay were set as indicated in the table below. (Table 4.2)

free ligand	wavelentgh (λ) [nm]	A
DFO	430nm	0.0229
CP691	475nm	0.0427
CP692	475nm	0.0564
CP20	460nm	0.0414

Table 4.2: measuring wavelengths determined for time drive experiments using UV/Vis spectroscopy

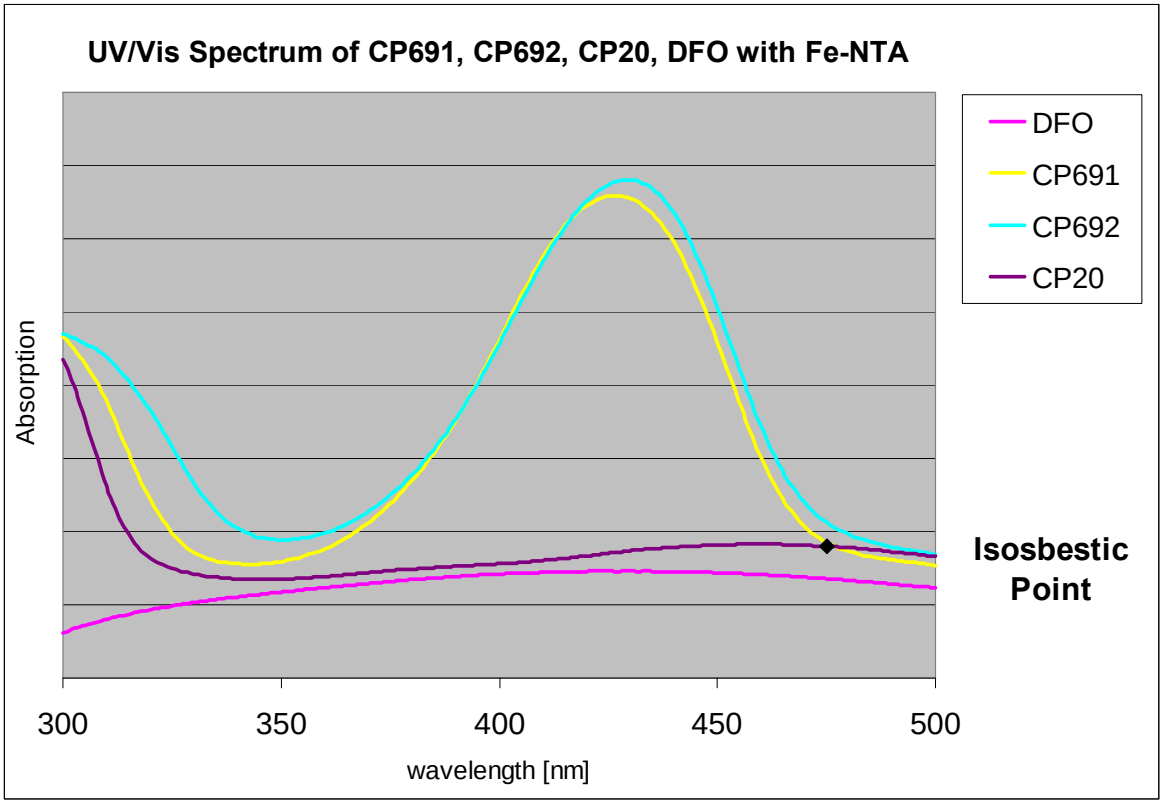

Figure 4.5: Determination of isosbestic point (cutting point of axis) of DFO [10µM], CP691 [10µM], CP692 [10µM], CP20 [30µM] in MOPS (50mM, pH 7.4)

4.3.2.2. Reaction kinetics of CP691, CP692, CP20 and DFO with Fe-citrate

In order to give an insight on the chelation potential of the investigated iron chelating probes a time series was set to compare reaction kinetics of CP691, CP692, CP20 and DFO with Fe-citrate.

Setting the values at the probes' specific wavelength obtained from the assay determining the isosbestic point (see Chapter 4.3.2.1 and Figure 4.5) in relation with the results obtained from the time series shows the probes' power to access iron from iron citrate complexes compared to freely accessible iron from $Fe^{3+}(NTA)_2$ solution. Results are depicted in Figure 4.7.

free ligand	A [max]
DFO [430nm]	0.0093
CP691 [475nm]	0.0147
CP692 [475nm]	0.0146
CP20 [460nm]	0.0047

Table 4.3: Measured absorption of free ligands (DFO, CP691, CP692 and CP20) at specific wavelengths

ligand with iron NTA	A [max]
DFO [430nm]	0.0229
CP691 [475nm]	0.0427
CP692 [475nm]	0.0564
CP20 [460nm]	0.0414

Table 4.4: Measured absorption of ligands (DFO, CP691, CP692 and CP20) with iron NTA at specific wavelengths

ligand with iron citrate	A [max]	t [max]	t [1/2]
DFO [430nm]	0.0259	21564sec	7194sec
CP691 [475nm]	0.0448	7136,5sec	520sec
CP692 [475nm]	0.3403	12495sec	2744sec
CP20 [460nm]	0.0458	12523sec	882sec

Table 4.5: Measured absorption of ligands (DFO, CP691, CP692 and CP20) with iron citrate at specific wavelengths

DFO was found to be the slowest chelator of those compared for iron from iron citrate ($T_{1/2}$ = 120 min) (Table 4.5 and Figure 4.6). Compared to DFO, CP20 was found to have a shorter $T_{1/2}$ (14.7 min). Not surprisingly, CP691, a hexadentate pyridinone ligand, was found to be able to exchange iron at the fastest rate ($T_{1/2}$ = 8.7 min). In contrast CP692, a pyranone counterpart obtained a markedly slow exchange rate ($T_{1/2}$ = 45.7 min) (Table 4.5 and Figure 4.6). Moreover CP692 failed to scavenge all iron from this iron citrate solution, even after 3 ½ hours.

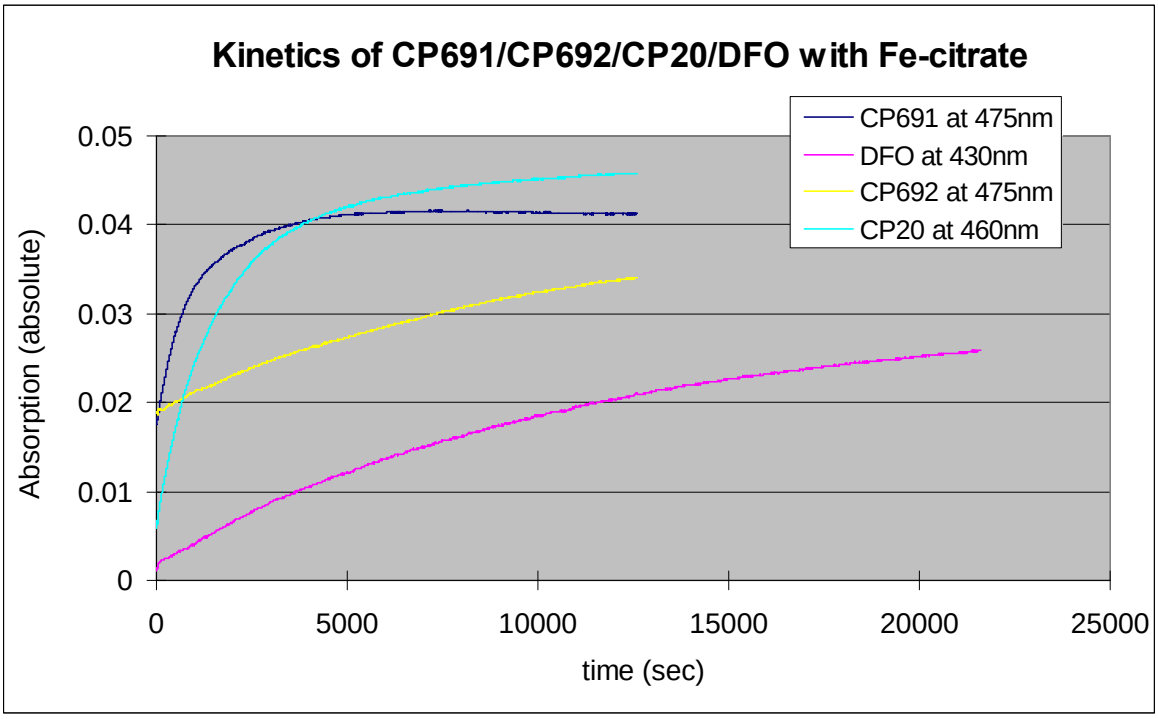

Figure 4.6: Comparison of reaction kinetics of CP691, CP692, CP20 and DFO with Fe-citrate. The measurements were performed in a time course under a UV/VIS spectrometer. The absorption of CP20 [30µM] and Fe-citrate [10µM] was measured at. The absorption of DFO [10µM], CP691 [10µM], and CP692 [10µM] at 430nm, 475nm and 475nm in the presence of Fe-citrate [10µM] was respectively measured in a time course.

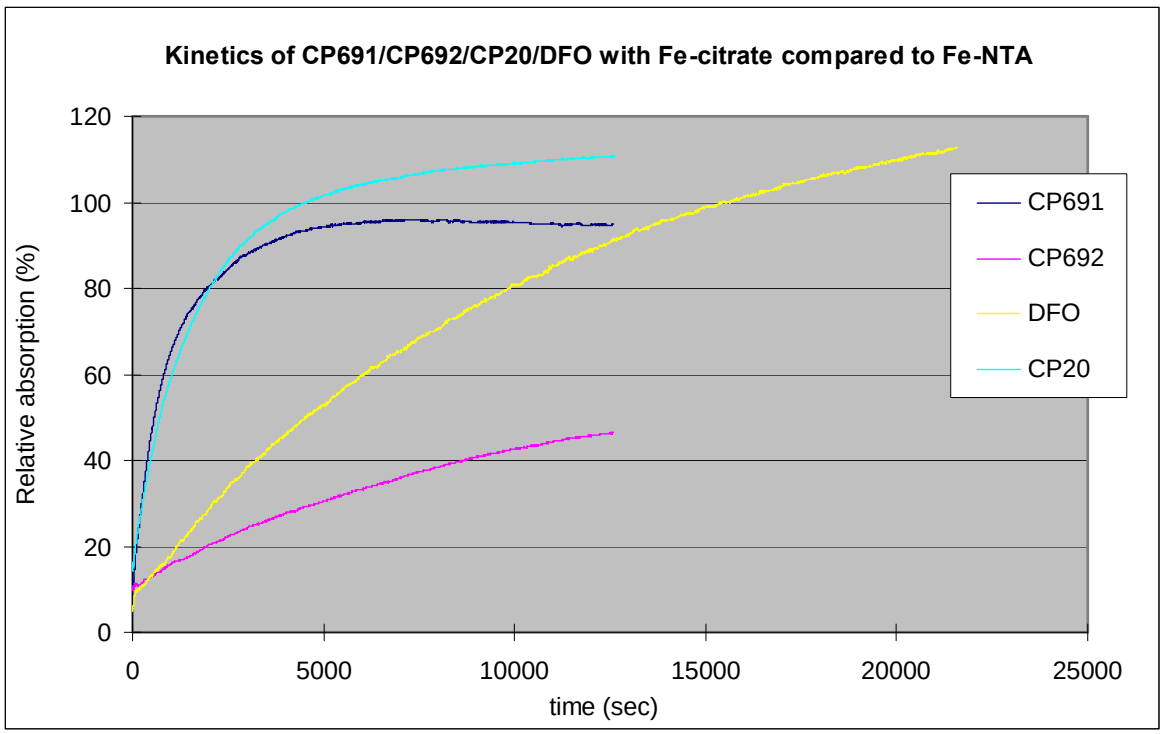

Figure 4.7: Comparison of reaction kinetics of CP691, CP692, CP20 and DFO with Fe-citrate in relation to ligand-iron NTA complexes. The measurements were performed in a time course under a UV/VIS spectrometer. The absorption of CP20 [30µM] and Fe-citrate [10µM] was measured at 460nm and set into relation with the absorption of CP20 [30µM] and $Fe(NTA)_2$ [10µM]. The absorption of DFO [10µM], CP691 [10µM], and CP692 [10µM] at 430nm, 475nm and 475nm in the presence of Fe-citrate [10µM] was measured in a time course and expressed as a percentage of the absorption of these ligands with $Fe(NTA)_2$ [10µM] at the same wavelength.

4.3.3 Reaction kinetics of CP691 with Fe-NTA/Fe-citrate in the presence of physiological concentration of human serum albumin

For standardisation of the NTBI-measuring method it was found necessary to compare kinetic aspects of CP691 with Fe-NTA and Fe-citrate. In order to create more *in vivo*-like conditions reaction kinetics were compared in the presence of physiological concentration of human serum albumin.

4.3.3.1 Choosing a suitable wavelength for measuring absorption of CP691 with Fe-NTA/Fe-citrate in the presence of physiological concentration of human serum albumin

The ideal wavelength obtained from the UV/Vis Spectrum is at **475nm** (Figure 4.8). The influence of human serum albumin present in physiological concentration seems to be neglectable at 475nm. Absorption levels of "*Albumin + CP691+ Fe-citrate"* (**0.1071 a.u.**) and "*Albumin + CP691+ Fe-NTA"* (**0.1 a.u.**) is very similar, they differ <8% after 30min of incubation each.

	λ [475nm]
Albumin [40mg/mL]	**0.0634**
Albumin [40mg/mL] + CP691 [10µM]	**0.0823**
Albumin [40mg/mL] + CP691 [10µM] + Fe3+(NTA)2 [10µM]	**0.1071**
Albumin [40mg/mL] + CP691 [10µM] + Fe3+citrate [10µM]	**0.1**

Table 4.6: Absorption values for Albumin [40mg/mL], Albumin [40mg/mL] + CP691 [10µM], Albumin [40mg/mL] + CP691 [10µM] + Fe^{3+}(NTA)2 [10µM] and Albumin [40mg/mL] + CP691 [10µM] + Fe^{3+}citrate [10µM] measured with UV/Vis Spectroscopy at λ=475nm.

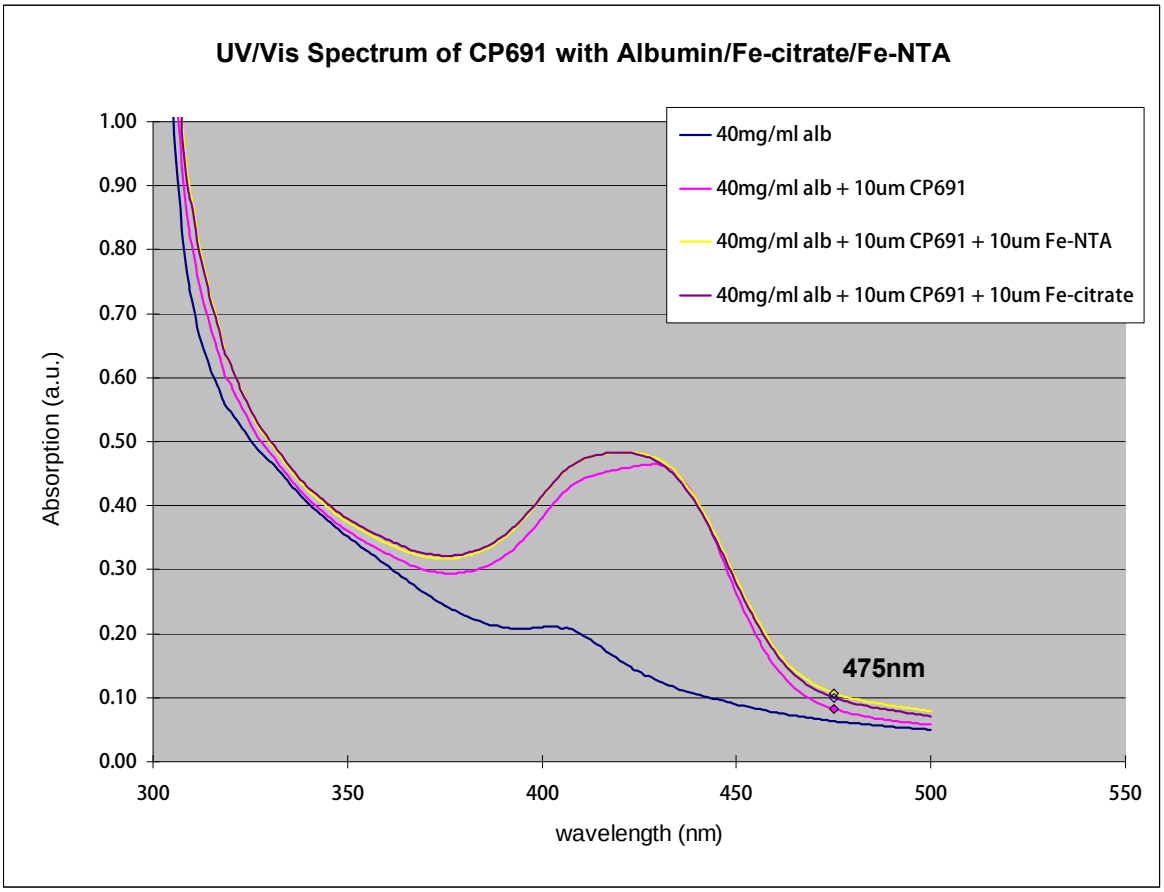

Figure 4.8: Peak height shows the absorption values for Albumin [40mg/mL], Albumin [40mg/mL] + CP691 [10µM], Albumin [40mg/mL] + CP691 [10µM] + Fe^{3+}(NTA)2 [10µM] and Albumin [40mg/mL] + CP691 [10µM] + Fe^{3+}citrate [10µM] measured with UV/Vis Spectroscopy at λ=475nm. Absorption levels are

displayed in arbitrary units. The ideal wavelength for measuring NTBI levels with CP691 as ligand using UV/VIS Spectroscopy was found to be at 475nm.

4.3.3.2 Kinetics of Fe-citrate- compared to Fe-NTA-chelation in presence of physiological human serum albumin concentration using CP691

Results displayed in Figure 4.9 show that Fe-citrate in presence of a physiological human serum albumin concentration is better chelatable compared to $Fe^{3+}(NTA)_2$ when using CP691 as an iron chelator.

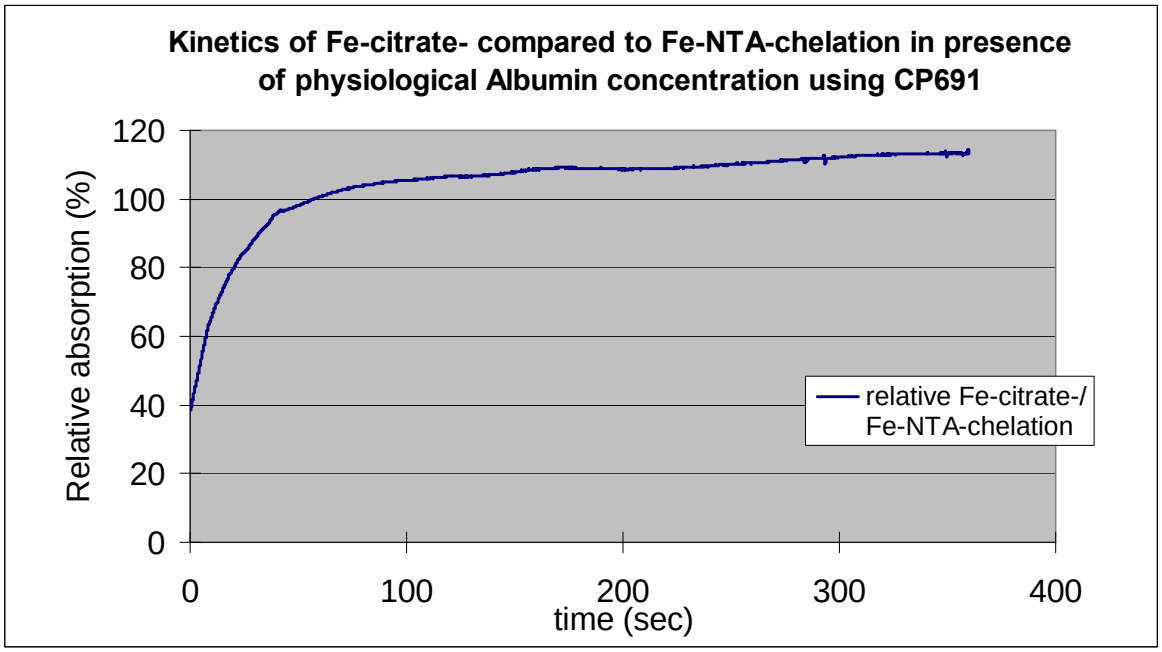

Figure 4.9: Comparison of reaction kinetics of CP691 [10µM], with Fe-citrate [10µM] in relation to ligand-iron complexes (CP691 [10µM] with $Fe(NTA)_2$ [10µM]) in the presence of physiological human serum albumin concentration (40mg/mL)

4.4 Comparison of the fluorescence-based assay with an ICP-MS-based method

The fluorescence-based assay established in Chapter 3.4.2 was validated with an ICP-MS-based method using a blind sample assay containing ferric iron over the range of 0-10µM.

In the presence of 10% Human Blood Serum iron levels of unknown concentration can be determined with higher accuracy with the fluorescence-based method compared to the ICP-MS-based method.

sample #	c (Fe) [µM]	c (Fe) [µM] Fluorescence-based method	c (Fe) [µM] ICP-MS
0	0	-0.214	-0.019
1	4	4.013	4.969
2	10	8.770	12.533
3	1	0.2899	1.2433
4	2	1.8488	2.2878
5	7	5.9529	8.214
6	3	2.5899	3.4482
7	6	5.1563	6.8302

Table 4.7: Results measuring unknown samples using the fluorescence-based and the ICP-MS-based method

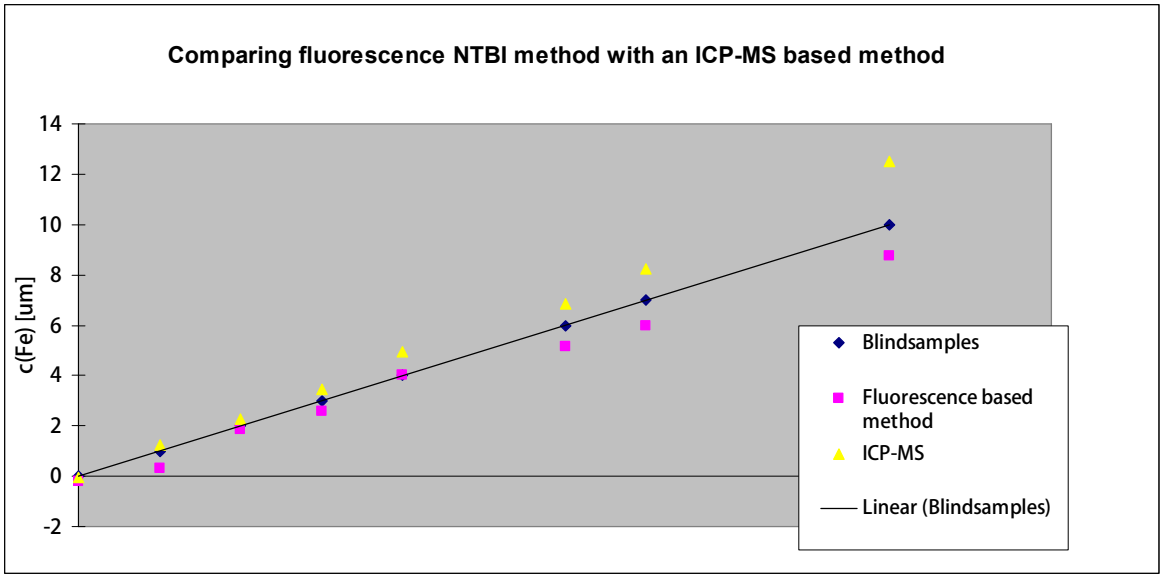

Figure 4.10: Calculated results from measuring blind samples using the fluorescence-based and the ICP-MS-based method. Results for unknown samples measured with the fluorescence-based assay show higher accuracy compared to those measured with the ICP-MS-based method.

Chapter 5

Conclusion and Discussion

Currently established NTBI quantification methods require either expensive laboratory equipment or involve complicated, time consuming sample preparations. Therefore, the development of an appropriate fluorescence-based quantification method for NTBI using CP691, a novel iron chelator, was in the focus of this investigation to create a time and cost-efficient alternative. Observations made during the experiments, conclusions and proposals for further studies will be discussed here.

5.1 Experimental part and Results

5.1.1 A general comment on fluorescence spectroscopy

Fluorescence spectroscopy is a highly sensitive method; therefore the emission spectrum can be distorted by numerous artefacts in the absorption of the sample. By tuning the gain and amplification of the fluorometer even a non-fluorescent sample can give measurable signals to the instrument. One may also observe interference to background fluorescence from solvents or the sample itself, light leaks or stray light within the instrumentation's optics and/or light scattered by turbid solutions. In order to obtain reliable, reproducible data, one needs to be aware and in control of these factors.

5.1.2 Construction of a standard curve

In order to measure chelatable non-transferrin bound iron in human patients' pathological blood serum samples a standard curve for developing the fluorescence-based NTBI measuring method, described in Chapter 3.4.2, had to be established.
Before, there had been no established fluorescence-based one step method for measuring non-transferring bound iron levels using the CP691 chelator on a multi-well plate reader such as the PerSeptive Biosystems Cytofluor¬® Series 4000 used in this assay the time these experiments were conducted. Therefore different levels of concentration of iron and chelator, different gain levels on the measuring instrument and measuring after elapsing at different amounts of time after sample preparation had to be used in an trial and error approach. Varying all these parameters in a logic consistent order did only lead to satisfactory results for a standard curve with the use of a 8-channel pipettor, especially designed for the use on a 96-well plate. Gained results showed an average low variation coefficient and acceptable coefficient of determination (R^2 >0.998) on the standard curve which was then found to be applicable for further experiments on human patients' pathologic blood serum samples.
Furthermore CP691's limit of detection for ferric iron in the presence of 10% Human Blood Serum [c(Fe) = 0.1µM] can be regarded as excellent.

5.1.3 Pathological samples

When using non standardized human patients' pathologic blood serum samples, such as in the method described in Chapter 3.5, samples' colour and turbidity (characterized in Table 4.1) seem to have a huge impact on the measured fluorescence. (Results are displayed in Table 4.3). This problem cannot be overcome using the described method.

However, the huge variety in samples' colour and turbidity cannot explain the non-corresponding results from the two HPLC methods applied by my colleges to validate the results obtained from the fluorescence-based method.

Because NTBI assays must detect very low concentrations of iron, possible iron contamination and different preparation methods in the different labs involved in the validation of the fluorescence-based method might have added to the erroneous results gained in this assay.

Another possible source of error for the observed differences in mean NTBI levels between the used methods could be the existence of different isoforms of NTBI. Different forms of NTBI might be differently selected by various measuring methods, meaning that different forms of NTBI might not be equally well detected by each NTBI assay.

5.1.4 Reaction kinetics

5.1.4.1 CP691's potential for iron removal from transferrin in the presence of Fe-NTA/Fe-citrate

The experiment of testing CP691's kinetics for iron removal from transferrin in the presence of Fe-NTA/Fe-citrate showed that CP691 is slowly removing iron from transferrin (Figure 4.4). This leads to the expectation that the application of the probe for measuring NTBI in human blood serum could give false positive results.

The results for Sample 1 and Sample 2 (Figure 4.4) were quite similar compared to the internal standard which leads to the expectation that either iron from iron citrate complexes was entirely accessible to the chelating agent or that citric acid is the weakest iron chelating agent of those investigated. Validation for this hypothesis could be subject of further investigation.

5.1.4.2 Reaction kinetics of CP691 with Fe-NTA/Fe-citrate compared to those of other iron chelators (CP692, DFO, CP20)

With the repeated run of a time course experiment using a UV/Vis-Spectroscopy based method the kinetics of different iron sensitive chelators with iron citrate could be analysed. CP691, a hexadentate pyridinone ligand, was found to be able to exchange iron at the

fastest rate ($T_{1/2}$ = 8.7 min clearly showing the probe's large iron chelation potential) compared to DFO, CP20 and CP692.

CP691's potential for chelating iron from iron citrate solutions compared to iron from solutions of iron NTA was average (relative absorption >95%) to those from other typical iron chelators (Fig. 4.6 and Fig. 4.7). These results show CP691's good potential for accessing the suspected biochemical forms of the labile iron pool (see Figure 1.4).

When comparing the kinetics of Fe-citrate- to Fe-NTA-chelation in presence of physiological human serum albumin concentration using CP691 the results were in contrast to those from the previous investigations (Fig. 4.9). One reason for that might be that iron citrate might have donated iron to human serum albumin during sample incubation and is therefore easier accessible to iron when measured later on. Further investigation for verification of this hypothesis should be considered.

5.1.5 Comparison of the fluorescence-based assay with an ICP-MS-based method

The validation of the fluorescence-based assay established in Chapter 3.4.2. with an ICP-MS method for ferric iron in the presence of 10% human serum albumin showed the clear advantage of the easy to use, low cost but fluorescence-based assay. The new established assay did not only show a similarly low limit of detection, but also a higher accuracy compared to the ICP-MS-based method.

5.2 Proposals for further studies

The fluorescence-based method used in this assay gave a good insight of the pros and cons of fluorescence spectroscopy when applied directly on non-standardized samples such as human patients' pathologic blood serum samples used in this assay.

The direct one step measurement of NTBI-levels in human pathologic serum samples using the described assay showed huge variation in colour and/or turbidity between samples

leading to possible interference in fluorescence. Therefore, the assay cannot be recommended as an eligible approach for further studies on developing an easy to handle, low cost NTBI-measuring method.

In future work CP691, the fluorescent iron chelator that was in the main focus of this investigation, should therefore not be used for direct measurement method of NTBI-levels in human pathologic serum samples described in this assay.

Variation in NTBI results between the methods described above show the need for better standardization of the used research methods before it is used in clinical practise. Further studies should include thorough characterization of the methodology and construction of standard curves and information on how iron contamination was avoided should be provided.

Nevertheless, CP691's excellent low limit of detection and its' high sensitivity for ferric iron makes it an ideal chelator for further investigations using fluorescence spectroscopy especially for detecting ferric iron levels in experimental settings that are less susceptible to fluorescence distortion (e.g. environmental analysis of non-fluorescent and artefact free (clear) substances such as water)

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