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Thermodynamics-based rules of thumb to evaluate the interaction of chelators and kinetically-labile metal ions in blood serum and plasma†

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Metal ions play a very important role in nature and their homeostasis is crucial. A lot of metal-related chemical research activities are ongoing that concern metal-based drugs or tools, such as chelation therapy, metal- and metabolite sensors, metallo-drugs and prodrugs, PET and MRI imaging agents, *etc*. In most of these cases, the applied chelator/ligand (L) or metal–ligand complex (M–L) has at least to pass the blood plasma to reach the target. Hence it is exposed to several metal-binding proteins (mainly serum albumin and transferrin) and to all essential metal ions (zinc, copper, iron, *etc*.). This holds also for studies in cultured cells when fetal calf serum is used in the medium. There is a risk that the applied compound (L or M–L) in the serum is transformed into a different entity, due to trans-metallation and/or ligand exchange reactions. This depends on the thermodynamics and kinetics. For kinetically-labile complexes, the complex stability with all the ligands and all metal ions present in serum is decisive in evaluating the thermodynamic driving force towards a certain fate of the chelator or metal–ligand complex. To consider that, an integrative view is needed on the stability constants, by taking into account all the metal ions present and all the main proteins to which they are bound, as well as the non-occupied metal binding site in proteins. Only then, a realistic estimation of the complex stability, and hence its potential fate, can be done. This perspective aims to provide a simple approach to estimate the thermodynamic stability of labile metal–ligand complexes in a blood plasma/serum environment. It gives a guideline to obtain an estimation of the plasma and serum complex stability and metal selectivity starting from the chemical stability constants of metal–ligand complexes. Although of high importance, it does not focus on the more complex kinetic aspects of metal-transfer reactions. The perspective should help for a better design of such compounds, to perform test tube assays which are relevant to the conditions in the plasma/serum and to be aware of the importance of ternary complexes, kinetics and competition experiments.

1 Introduction

1.1 Metals in biology

Metal ions play an important role in biological systems including humans. They can be distinguished into essential (required to survive) and non-essential metal ions. In humans, the well-established essential ions are Na, Mg, K, Ca, Mn, Fe, Co, Cu, Zn, and Mo.[1] Curiously, it is still controversial whether Cr is essential for humans.[2,3] Non-essential metal ions, which can be found in the body upon environmental exposure, are also relevant for humans, either because they can be toxic above a certain threshold or because they are constituents of drugs. Indeed, several therapeutic and diagnostic compounds contain non-essential metal ions, such as Pt in the anticancer drug cisplatin or Gd in contrast agents for magnetic resonance imaging (MRI).[4]

1.2 Therapeutic approaches using metal-based drugs

Metal-based drugs can be either genuine *metallodrugs*, *i.e.* drugs that contain metal(s) (metal–ligand complexes; M–L) or also mere chelators (L) that will form a metal complex in the body.[4,5]

In particular, several approaches can be considered for the application of metal-based drugs:

- Metal complexes (M–L) containing either essential or non-essential metals can be used as drugs for the treatment of diseases not directly caused by metal ion deregulation;

- Metal complexes containing a particular, essential metal can be administered to cope with its deficiency (systemic or local);

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†This article is dedicated to the memory of Prof. Maria C. Linder, a biochemist devoted to the study of Cu speciation in the blood.

- Chelators (L) can be used to bind and evacuate a toxic metal ion in the case of high exposure of a non-essential metal ion or of overload of an essential metal ion (chelation therapy);

- Chelators can also act as pro-drugs that acquire their therapeutic activity upon (essential) metal-binding in the body.

1.3 Endogenous factors challenging the design and application of metal-based drugs

The biological applications of metal-based drugs can be very challenging because, depending on the target and the administration mode, the metallodrug or chelator is exposed to several biological compartments that may undermine their stability or selective metal-binding capacity.

For instance, upon oral administration, drugs have to face the low pH of the stomach, which challenges the stability of metal complexes. Besides, most drugs pass *via* the blood and hence have to resist this extracellular medium, where important metal-binding proteins exist. Finally, drugs with an intracellular target (*e.g.* in the cytosol or nucleus) have to cope with a thiol-rich reducing environment. Indeed, mM concentrations of thiols can reduce efficiently redox-active metal ions such as Cu^{II} and Fe^{III} into Cu^I and Fe^{II}, respectively and bind them quite strongly. In this respect, it was recently shown that abundant thiol-containing glutathione and metallothioneins can efficiently deactivate Cu-complexes, particularly those that are highly redox active.[6] Overall, metal-based drugs have to resist quite different redox environments, which is particularly challenging for redox-active ions such as Cu and Fe.

1.4 Fate of metal-based drugs in the body

From the perspective of metal-based drug administration, different scenarios could be envisioned (see Fig. 1). Notably, chelators can be (i) sufficiently strong to compete with endogenous ligands and bind one (if selective) or different

metal ions or rather (ii) too weak and bind no metal ion. Metallodrugs could (i) be stable, (ii) exchange one or more ligands with an endogenous one (ligand-exchange), (iii) lose the metal (dissociation) and remain as free ligands or (iv) take up other metal ions (trans-metallation).
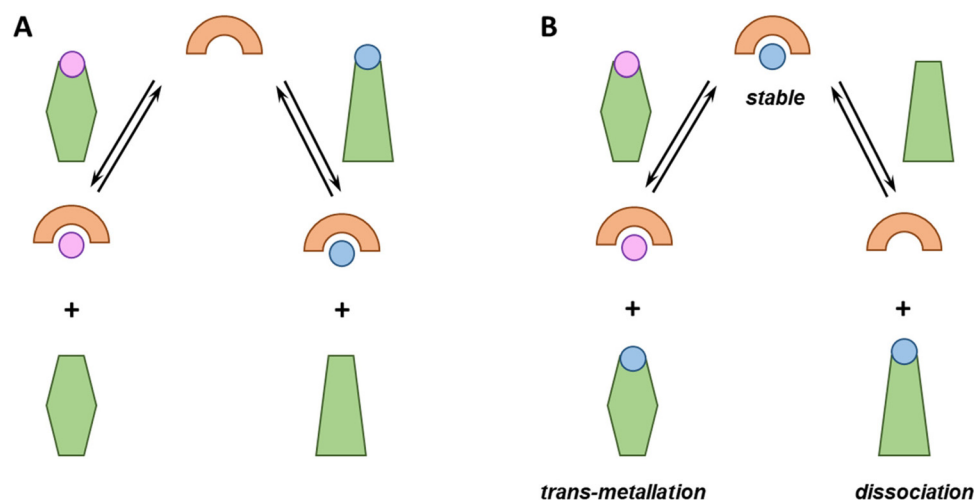
Each of these eventualities may fit or not with a particular therapeutic application. For instance, in the case of cytotoxic metallodrugs, the complex should be stable until it reaches the target. Conversely, in the case of a metal ion deficiency, the applied metal–ligand complex might be designed to release the metal to a suited recipient. Of note, in all these cases, metal-binding selectivity plays a major role, *e.g.* to prevent unsought trans-metallation or to ensure that a chelator binds, in the best case, the targeted metal ion only and does not interfere with the homeostasis of the other metal ions.[7]

1.5 Thermodynamic *vs.* kinetic control

In general, the fate of chelators and metal-based drugs is dictated by both thermodynamic and kinetic aspects. A thermodynamic driving force is needed for a reaction to occur. If the kinetics is much faster than the time during which the metal–ligand complex (M–L) resides in the biological entity or exerts the biological action, thermodynamics becomes determinant. In contrast, if the kinetics are much slower, the impact of thermodynamics is minor and kinetics becomes determinant.

In general, metal-exchange kinetics are difficult to predict. Although the rates of M–L complexes can be measured (k_{on} and k_{off}), this can only predict the metal transfer between two ligands in the case of a dissociative mechanism. However, at least for the essential d-block metal ions, associative mechanisms are very important and experimental measurements are needed to assess the kinetic aspects.

Nevertheless, some more general considerations can be made in relation to the type of metal ions and ligands. Essential metal ions, except for Co^{III}, are generally kinetically

Fig. 1 Possible non-exhaustive fates of chelators (A) or metal complexes (B) in a biological environment such as blood plasma. (A) A chelator can be able (or not) to compete with endogenous metal-binding proteins (green), picking up one or more metal ions (pink and blue circles); (B) A metal-complex can (i) be stable, (ii) dissociate or (iii) trans-metallate.

labile, and they are the focus of the present article. Labile M–L complexes undergo relatively fast ligand-exchange reactions and the thermodynamic equilibrium is often reached faster than the biological activity is exerted. Non-essential metal ions can be both labile (*e.g.* Cd^{II} and Hg^{II}) or inert (*e.g.* $Pt^{II/IV}$, Ir^{III}, and $Ru^{II/III}$).

The ligand architecture, mainly denticity, bulkiness and cyclisation, also plays a role in kinetic stability. The ligand exchange reaction of a metal ion in an M–L complex with another ligand L′, yielding M–L′, often occurs *via* a transient ternary complex (L–M–L′ in an associative mechanism or with H_2O in a dissociative mechanism). Hence the access of the L′ or H_2O to the metal ion in M–L and the formation of a ternary complex are key features for kinetics and can explain why cyclic and bulky ligands with high denticity (coordination sphere saturation) increase inertness.[8–11]

In conclusion, the lability of the M–L complex depends on both the type of metal ion and the ligand architecture. Hence, labile metal ions can become quite inert with suited ligands. For instance, the macrocyclic ligand DOTA forms rather inert complexes with lanthanides, which are otherwise labile.[12] On the other hand, inert metal ions can form inert complexes even with low denticity and low steric hindrance ligands.

Thanks to their inertness, these complexes can be stable under chromatographic or electrophoretic separations, and hence their stability can be monitored over time, for instance, *via* hyphenated ICP-MS methods.[13–15] Remarkably, these measurements can be also performed on biological samples (such as blood plasma) after *ex vivo* incubation or *in vivo* administration of the metal-based drugs.[16–18]

1.6 Aim of the perspective

Since blood serum represents the main distributor of molecules throughout the body, and hence an environment that most of the drugs that are not administered on the outer or inner surfaces of the body (*e.g.*, skin or gastrointestinal tract) will be exposed to, we aim here to easily estimate, based purely on thermodynamic considerations, if a kinetically labile metal–ligand complex is stable in a plasma-like environment or dissociates or transmetallates and if a ligand is able or not to chelate selectively a certain essential metal ion. This con-

sideration does not include the kinetics and cannot tell if the reaction occurs in a biologically relevant time frame. The weight is put on simplicity and the evaluation of metal complex fate in the context of blood plasma or serum and not on the accuracy of the binding affinity, for which several reviews and books or other approaches are available.[19–22] Here the idea is to get a simpler and faster estimate of a metal complex stability, but with an integrative view considering the essential metals and the different plasma proteins. We concentrate here on the chelators and complexes of essential metal ions due to the sake of simplicity, but the same considerations are also valid for non-essential metals.

2 Metal ions and their carriers in human blood serum

2.1 Overview of metal ions in the blood

Besides drugs, the blood is also a main distributor of essential metal ions. There are quite important differences in the form (often called speciation) in which the metal ions exist in the blood, *i.e.* the oxidation state, the ligand(s) and the coordination geometry. Indeed, the alkaline metal ions Na^{I} (~135 mM) and K^{I} (~4 mM) are mainly found as aqua complexes and are not considered to form defined and strong complexes with biomolecules. Hence, they are not further considered for the rest of the article, as we focus on the formation of complexes.

The earth-alkaline Mg^{II} and Ca^{II} are present in low mM concentrations partially bound to biomolecules and partially as aqua-complexes (see Table 1). The d-block metal ions (Mn, Co, Fe, Cu, Zn, and Mo) are much less abundant (≤μM) and are generally bound in defined complexes (aqua complexes are present at neglectable levels apart for Mn, see section 3.2). In particular, several proteins are known for essential d-block metal ion transport.[23–25] These pick up the metal ions at the enterocytes (cells in the intestine) and carry them through the blood plasma. Classically, in contrast to Ca^{II} and Mg^{II}, whose concentration exceeds that of endogenous or exogenous ligands, d-block metal ions are found in lower concentrations than their carriers (see Table 1).[26–29] In other words, there are more binding sites than

Table 1 Concentration of metal ions and their carriers in blood plasma

M	$[M]_{tot}$	Main protein	[Protein]	$\log K_d$ (pH 7.4)	M : BS[a]	$\log[M]_{exc}$
Mg^{II}	1 mM (ref. 26)	Several			<1	−3
Ca^{II}	2 mM (ref. 26)	Several			<1	−2.7
Mn^{II}	10 nM (ref. 27)	Albumin	~600 μM	−4.3[30]	>1	−8
Fe^{III}	~20 μM (ref. 31)	Transferrin (2 BS)	35 μM (70 μM BS)	−22.1/−22.7[32] or −20.7/−19.4[33]	>1	−4.7
Co^{III}	2 nM (ref. 31)	Albumin[b]				
Cu^{II}	~15 μM (ref. 28)	Ceruloplasmin	~3 μM		~1	
	~1 μM (ref. 28)	Albumin	~600 μM	−13[34]	>1	−6
Zn^{II}	15 μM (ref. 29)	Albumin	~600 μM	−7	>1	−4.75
$Mo^{VI c}$	6 nM (ref. 31)					

[a] BS = binding sites. [b] Endogenous Co is inertly bound to Vitamin B12; exogenous Co can bind to albumin. [c] Mainly MoO_4^{2-}, not considered further.

metal ions. This reasonably reflects their carrier function, as a transporter carrying cargo from A to B must be loaded when going from A to B, but at least partially empty upon return from B to A. Hence, these are proteins with a partially empty site and relatively high affinity for metal ions, and hence prime potential competitors for any exogenous ligand.

2.2 The essential d-block metal ions and their carriers

2.2.1 Iron. Iron is present at around 20 μM in blood plasma and is bound exclusively to Tf in its trivalent Fe^{III} state.[35] Normal average Tf concentrations are about 35 μM and Tf has two Fe^{III}-binding sites with an average $\log K_d$ of about −22 at pH 7.4 (−22.1 and −22.7 were reported under blood plasma conditions).[32,33] Hence, classically about one-third of Tf is loaded with Fe^{III} (holo-Tf).

2.2.2 Copper. There are two main Cu proteins in the blood. About 70–95% Cu is bound to Ceruloplasmin (Cp), where it is a catalytic cofactor for the oxidation of iron.[23] Cu is strongly and inertly bound and cannot be retrieved by chelators such as EDTA (ethylenediaminetetraacetic acid) unless very high concentrations (>10 mM) are used.[36] Nevertheless, Cp was proposed to be implicated in Cu-transport, but this would imply, at least partially, unfolding or degradation to retrieve Cu. Serum albumin (SA) binds about 5% Cu as Cu^{II} with relatively high affinity ($\log K_d \sim -13$ at pH 7.4) but is exchangeable and chelators such as EDTA can retrieve it at stoichiometric levels on a minute time scale. This is clearly in line with a transport function and the Cu-loading of SA is very low (~0.5%), whereas Cp seems to be fully loaded with Cu.[23,37,38]

2.2.3 Zinc. Zn^{II} is present at around 15 μM concentration and most of it (75–90%) is bound to SA with a $\log K_d$ of about 7. Hence about 2% of SA contains Zn.[29,39,40]

2.2.4 Cobalt. Cobalt is exclusively bound to vitamin B12, very inertly, so it is unlikely to be involved in any metal exchange reaction and does not have to be considered further unless exogenous Co salts or complexes are added to the blood, where it can bind to SA (Table 2).[41]

2.2.5 Manganese. Mn^{II} is found at around 30 nM concentration,[42] mainly bound to SA (>85%) and only very little to Tf (<1%), while its aqua complex has been estimated to be ~5%.[30]

2.2.6 Molybdenum. It does not exist in the cationic form, but as molybdate (MO_4^{2-}) or bound to organic cofactors. Molybdate seems to be transported mainly by serum albumin and α_2-macroglobulin.[43] Due to its anionic character, molybdate does not compete with metal cations.

Hence the main metal ions to consider, *i.e.* which can make strong coordination compounds with ligands or transmetallate, are Cu, Fe, Zn and Mn and the main metal-binding proteins are Tf and SA (see Table 2).

2.3 Fetal calf serum

Fetal calf serum is often added for studies of cells in culture at 2 to 10%.[45] This serum contains bovine SA that has very similar binding properties for Cu^{II} and Zn^{II} as human SA. In 10% FCS, about 40 μM bovine SA would be present.[45] Likewise, FCS also contains Tf concentrations similar to human serum, although its Fe-loading is higher (55–92%).[46] Hence, the considerations for human serum are also of relevance in this case but would have to be adapted due to the often-used dilution of the cell culture medium.

3 Thermodynamic considerations

Here we aim at estimating which M–L complexes are formed and to what extent when a ligand L is added in a biological sample where several metals and ligands including proteins (P) are present. This can be most accurately calculated for the desired concentrations and pH when all the stability constants are known.[47] However, simpler considerations can also help assess whether an M–L complex is likely stable or not. In the simplest system with two different ligands and one metal ion (equimolar concentrations), the question would be to which ligand the metal binds. This is particularly relevant for the d-block metals as the ligand : metal ratio is >1 in the plasma. The other simplest system includes two different metal ions and one ligand and the question is which metal will bind to the ligand. This situation is more relevant for abundant earth-alkaline metals. In the following sections, we discuss some concepts to compare metal–ligand affinities and thus evaluate whether a chelator is able to retrieve a metal ion from a protein or if a metal-complex releases the ion to the proteins. Of note, although the formation of ternary L–M–P complexes can be very important (see section 5.3), for the sake of simplicity, we limit the discussion to binary systems.

3.1 Affinity constants and their comparison

Chemists often characterize metal–ligand affinities by measuring the acid dissociation constants, K_a, of the metal-free and metal-bound ligands by potentiometry, from which dimensionless thermodynamic complexation constants K (or overall constants $\beta_n = K_1 \cdot K_2 \cdot ... \cdot K_n$, for the stepwise formation of complexes with 1 : 1; 1 : 2, ..., 1 : n metal : ligand stoichiometry) are obtained.

Simplistically, the complexation constant K or β_n is the affinity of the deprotonated ligand, *i.e.* the affinity at very high

Table 2 Affinities (−log K_d at pH 7.4) of transferrin and serum albumin for essential and some non-essential metal ions

		Transferrin[44]	Serum albumin[40]
Essential	Mn^{II}	4	4.3
	Fe^{III}	22	n/a
	Co^{II}	n/a	4
	Cu^{II}	10	13
	Zn^{II}	6	7
	Cr^{III}	17	n/a
Non-essential	VO^{II}	n/a	10.3
	Ni^{II}	4	6.8
	Ga^{III}	20	n/a
	Cd^{II}	7	5.5
	La^{III}	5	n/a
	Bi^{III}	19	n/a

pH, which generally is not relevant under biological conditions (in biology, pH varies mostly between *ca.* 2 to about 9). Even though conditional stability constants can be derived for any pH, their determination needs calculations involving all coordination and protonation species. Indeed, the conditional dissociation constant K_d at a given pH, here 7.4, can be estimated from absolute β by taking into account the K_a of the ligand (see Appendix for a simplified estimation). Besides, proteins are often not stable over the wide pH range required by potentiometric measurements and normally include too many protonation sites to yield reliable values. Hence, protein chemists mostly determine a conditional dissociation constant, K_d, at a given pH (normally 7.4). Such K_d values are often determined *via* isothermal titration calorimetry (ITC), spectrophotometric or fluorometric metal titrations or through competition experiments using metal-responsive chromophores or fluorophores, in the presence of a buffer to keep pH constant. Buffers can be weak chelators and this should be considered and corrected for; otherwise, the determined K_d is only valid at a certain buffer concentration.[48,49] Of note, the K_d value reflects the concentration of unbound/free metal ([M]) when half of the ligand is bound to the metal ([L] = [M–L]). It is also worth pointing out that it is essentially inappropriate and misleading to compare the K of 1 : 1 complexes with the β_n of 1 : n ($n > 1$) metal : ligand complexes.

3.1.1   Comparison of K_d. The K_d can be directly used to compare the affinity of ligands (L) and proteins (P) as long as they have the same total concentration and they both form a 1 : 1 complex (M–L and M–P).

For instance, at pH 7.4, Cu^{II} forms 1 : 1 complexes with EDTA and SA (considering the physiologically-relevant binding site only) with log K_d values of −15.9 and −13, respectively.[34,50] Thus, since the difference in K_d is almost 3 orders of magnitude, EDTA is able to withdraw Cu^{II} almost completely from SA at the same concentration (*e.g.* when one adds about 0.6 mM EDTA to plasma with the same concentration of SA). Remarkably, even at (10- or 100-fold) lower concentrations, EDTA withdraws most Cu^{II} from SA. As a rule of thumb, it can be estimated that when the concentration of SA is 2 orders of magnitude higher than EDTA (0.6 mM SA, 6 μM EDTA), the latter would retrieve ~90% of the Cu^{II} (see Appendix).

3.1.2   Concept of pM. When L and P concentrations are different or when 1 : 2 (M–L$_2$) or 1 : 3 (M–L$_3$) complexes can be also formed, the K_d is not sufficient to directly compare the metal-binding abilities of different ligands. In this case, L and P concentrations have to be considered, as well as m, the number of L bound to the metal ion. In such a case, it is more convenient to calculate and compare the concentration of "free" metal ion (aqua-complex) at certain concentrations of metal ion and ligand (note that the concentrations of the ligands have to be high enough so that all metal ions could bind, *i.e.* for an M–L complex [L] ≥ [M] and for M–L$_2$ [L] ≥ 2[M] *etc.*). Indeed, a lower concentration of "free" M corresponds to a stronger affinity. Thus, the aqua-complex serves as a kind of standard to compare with. The "free" M is often given as the

negative logarithm, p[M] or pM,[51] which can be calculated as follows:

$$K_d = \frac{[M][L]^m}{[M-L]} \tag{1}$$

$$[M] = K_d \frac{[M-L]}{[L]^m} \tag{2}$$

$$pM = -\log\left(K_d \frac{[M-L]}{[L]^m}\right) = -\log\left(K_d \frac{[M]_{tot}}{([L]_{tot} - m[M]_{tot})^m}\right) \tag{3}$$

The concentration of M–L at equilibrium can be approximated to the total M concentration (*i.e.* all metal is bound to L) as long as [M]$_{free}$ ≪ [M]$_{tot}$, which holds for the small K_d values considered here. Importantly, as shown in Fig. 2, the pM depends strongly on the ligand concentration ([L]) and the stoichiometry of the complex (m = 1, 2 or 3). Of note, as the pM is derived from a pH-dependent K_d, the pM is only valid at a certain pH and at the chosen concentrations of ligand and metal ion, which in the literature typically are [M]$_{tot}$ = 1 μM and [L]$_{tot}$ at 10 μM. For the following, we consider the pM at pH 7.4 only, the average pH of the blood. The concept of the pM value gives an accurate description in the case where two ligands compete for one metal ion at a given pH. In this case, the pM value has to be calculated at the same pH and for the same [M]$_{tot}$. In more complex systems like blood, pM is useful for the first estimation of how competitive a certain ligand is.

3.2   Estimation of pM in human plasma

Similarly, we can estimate the pM under classical blood plasma conditions, hereafter denoted as pM$_{plasma}$, considering the total concentrations of metal and protein in the blood (see Table 1). Such calculations for Mn^{II}, Fe^{III}, Cu^{II} and Zn^{II}, whose exchangeable pool is basically made of only one protein (SA or Tf), are reported in Table 3 and represented in Fig. 3. Interestingly, comparing the pM$_{1/10}$ values calculated according to the classical definition ([M]$_{tot}$ = 1 μM, [L]$_{tot}$ = 10 μM) and pM$_{plasma}$, which takes into account the actual concen-
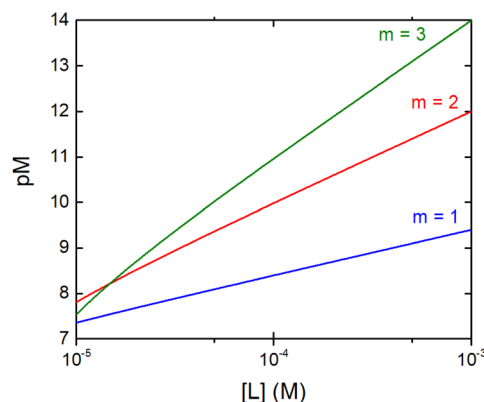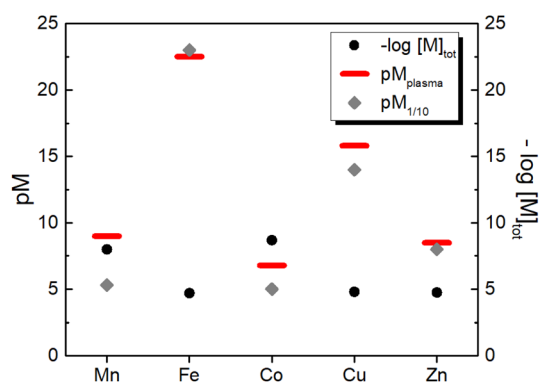

Fig. 2 Dependence of the pM on the ligand concentration [L] for different complex stoichiometries (m = 1—blue, 2—red, and 3—green). Sample curves were calculated for the Zn–Phen$_m$ complex (see below) considering [Zn]$_{tot}$ = 1 μM, log β_1 = 6.4, log β_2 = 12 and log β_3 = 17.[50]

Table 3 Thermodynamic parameters of the chelators discussed

		Mn^{II}	Fe^{III}	Cu^{II}	Zn^{II}	Prediction
Plasma proteins	$pM_{1/10}$	5.3	23.1	14	8	
	pM_{plasma}	9	22.5	15.8	8.5	
EDTA	$\log \beta^{50}$	16.7	25	18.8	16.4	Can chelate Cu, Zn, and
	$-\log K_d{}^a$	13.8	22.1	15.9	13.5	Mn and little Fe^{III}
	$pM_{1/10}$	12.8	23.1	16.9	14.5	
	$pM_{pl/100}$	16.8	22.7	17.9	14.2	
DOTA	$pM_{1/10}$		24.3[52]	19.3[53]	17.9[54]	Can chelate Fe, Cu, and Zn
	$pM_{pl/100}$		23.9	20.3	17.6	
Phen	$-\log K_d{}^{50}$	10.3 (1:3)	Not stable (reduced to Fe^{II})	16 (1:2)	17 (1:3)	Selective for Zn^{II}
	$pM_{1/10}$	0.8		11.8	7.5	
	$pM_{pl/100}$	6.3		14	8.8	
DFO	$\log \beta^{50}$		30.6	14	11	Selective for Fe^{III}
	$-\log K_d{}^a$		26	9.4	6.4	
	$pM_{1/10}$		27	10.4	7.4	
	$pM_{pl/100}$		26.6	11.4	7.1	
TRIEN	$pM_{1/10}{}^{55}$			17.1	8.4	Selective for Cu^{II}
	$-\log K_d$			16.1	7.4	
	$pM_{pl/100}$			18.1	8.1	
3AP	$pM_{1/10}$		15.7 (1:2)[52]	11.4 (1:1)[56]		Complexes not stable
	$-\log K_d$		19.9	10.4		
	$pM_{pl/100}$		16.1	12.4		
LH8	$pM_{1/10}{}^{57}$			15.5	12.5	Cu–L partially stable; it can
	$-\log K_d$			14.5	11.5	transmetallate to Zn–L
	$pM_{pl/100}$			16.5	12.2	

^{a} See Appendix.

Fig. 3 Comparison between the total concentration of the essential d-block metal ions in human blood plasma and the concentration of their aqua complexes calculated either as $pM_{1/10}$ (*i.e.* at 1 µM metal and 10 µM ligand) or as pM_{plasma} (*i.e.* at the actual concentrations of metals and their binding site in plasma (see Table 1) except for Co for which 1 µM was arbitrarily chosen).

trations of metals and proteins, a significant shift is observed for Mn and Cu, as their [M–L]/[L] ratio diverges most from 1 : 10 (see Table 3 and Fig. 3).

4 Evaluation of some biologically-relevant chelators

Since a higher pM, *i.e.* a lower concentration of the "free" metal ion, corresponds to a higher affinity, we can now compare, at first, the $pM_{1/10}$ of some classical chelators to the $pM_{1/10}$ of the corresponding metal-binding plasma protein, and hence esti-
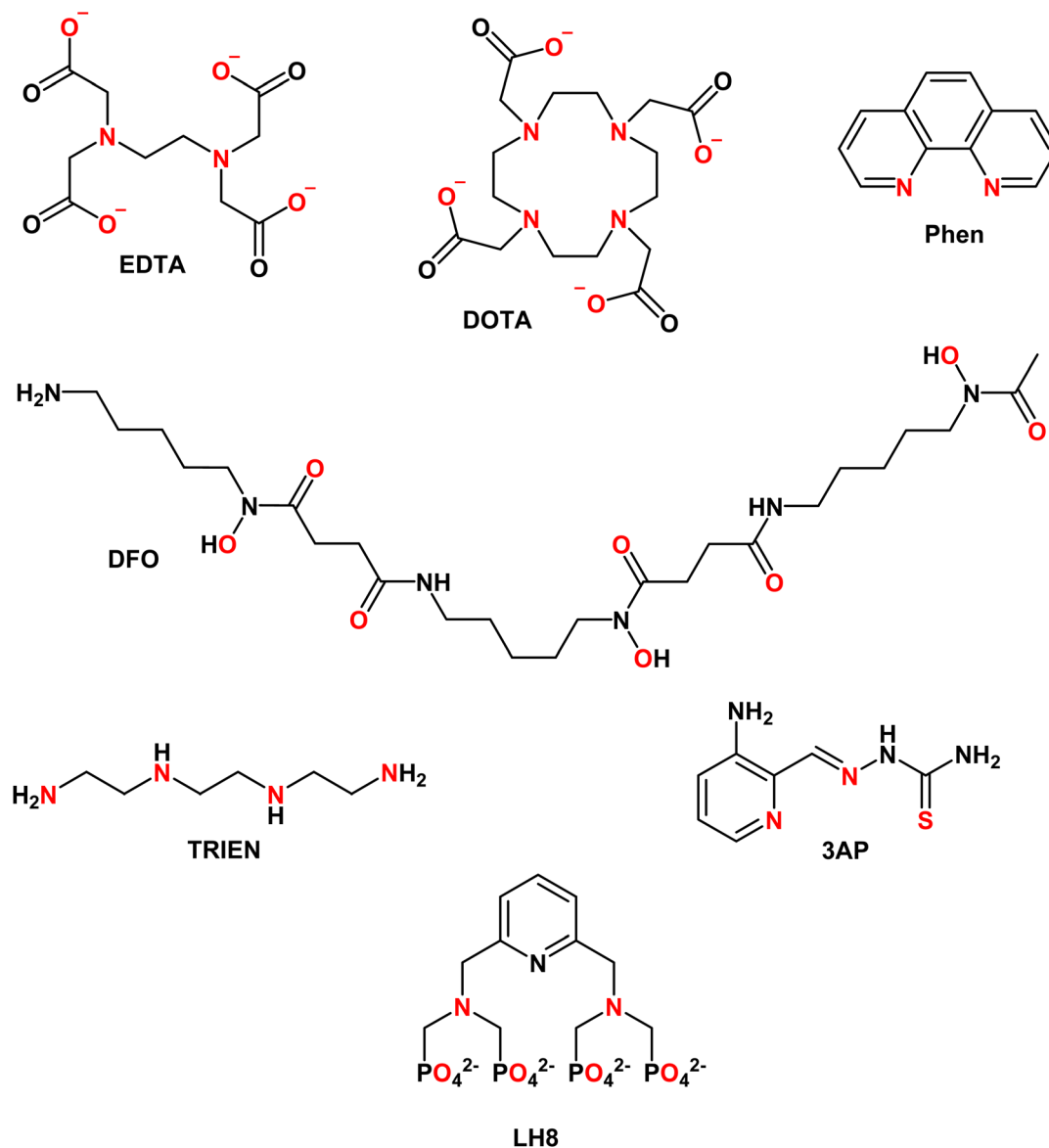
mate whether such ligands can thermodynamically pick up some and which metals from blood proteins. In other words, a ligand can bind roughly those metals for which $pM_{1/10}$(Ligand) > $pM_{1/10}$(Protein).‡ In order to have a more realistic estimation, the pM of the ligand can be also calculated by taking into account the real metal concentration in plasma and a higher concentration of the ligand, *e.g.* 100 µM. Such a $pM_{pl/100}$ can be then compared to pM_{plasma}. In the following, the metal binding ability and selectivity of certain common chelators, as well as the fate of some medicinally relevant metal complexes, will be evaluated based on the thermodynamic criteria discussed. In particular, we chose a few cases (see Table 3) that exemplify the different potential behaviours of chelators (selective for a certain metal or not) and metal complexes (*e.g.* stability *vs.* trans-metallation).
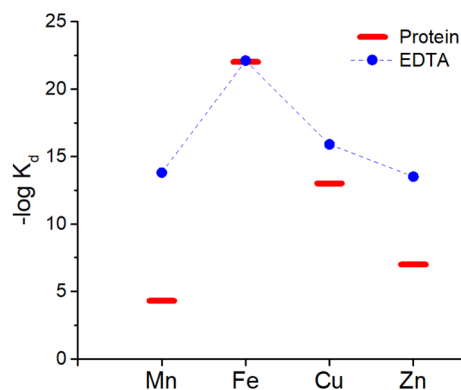
4.1 EDTA

EDTA (see Chart 1) is well studied and $\log K$ values for all essential divalent and trivalent metal ions can be found (see Table 3).[50] As metal ions form a 1 : 1 complex with proteins and EDTA, the competition between the latter can be at first evaluated using the conditional dissociation constants K_d at pH 7.4 (see Tables 2 and 3). Upon comparing the K_d values of M–P and M–EDTA (see Fig. 4), EDTA is found to be able to bind all the metal ions but to a lesser extent to Fe^{III} as Tf and EDTA show very similar K_d values for this ion. Notably, the

‡ In the case pM(Ligand) > pM(Protein) for more than one metal ion, it is not straightforward to estimate which metal would be bound most, but two parameters should be taken into account: the difference between the pMs and the metal concentration. Notably, the metal with the highest concentration and/or higher pM difference would be bound preferentially.

Chart 1 Structures of the chelators discussed. Metal-binding atoms are coloured in red.

Fig. 4 Comparison of the K_d between EDTA (blue dots) and plasma proteins (red bars). Mn, Zn and Cu are considered to be bound to SA while Fe to Tf.

comparison between the $pM_{1/10}$ (see Fig. 5A) or between the $pM_{pl/100}$ and the pM_{plasma} (see Fig. 5B) show a similar trend (see Fig. 4B and C).§ This consideration is in line with the use of EDTA as a general chelator for metal ions (even Fe^{III} can be totally removed when EDTA is used at higher concentrations).

4.2 DOTA

1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA, see Chart 1) and its derivatives are the most common chelators for theranostic applications (MRI, PET, …).[58]

§ Considering that the difference between $pM_{pl/100}$(EDTA) and pM_{plasma} is largest for Mn^{II} and Zn^{II}, these are likely the metal ions mostly bound to EDTA. Moreover, it should be considered that EDTA would very likely bind Mg^{II} and Ca^{II} also, which are much more abundant than the d-block metal ions.
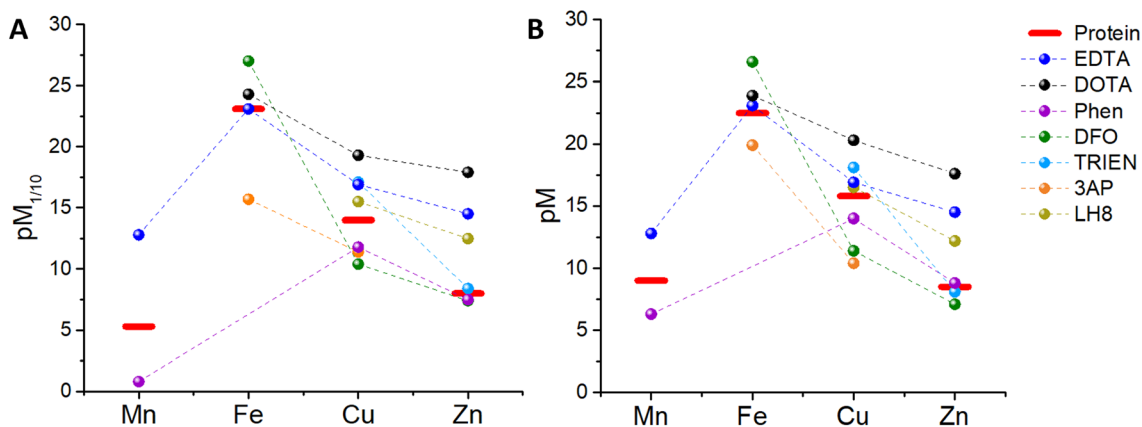
Fig. 5 Comparison between (A) the $pM_{1/10}$ of several chelators and plasma proteins, (B) the $pM_{pl/100}$ of the chelators and the pM_{plasma} (values takes from Table 3).

Similar to EDTA, DOTA is able to bind Cu^{II}, Zn^{II} and, to a smaller extent, Fe^{III} in plasma (see Table 3). However, DOTA complexes, such as Gd^{III} contrast agents or Ga^{III} radiopharmaceuticals, do not normally transmetallate with endogenous metal ions in the time between injection and clearance thanks to their high kinetic inertness. Hence, in this case, the mere thermodynamic considerations are not relevant to this biological application.

4.3 Phenanthroline

1,10-Phenanthroline (Phen, see Chart 1) has been used as a chelator to bind metal ions, mainly *in vitro*, and metal complexes of Phen and its derivatives have also been investigated as anticancer and antimicrobial drugs.[59,60] Phen makes complexes with up to 1 : 3 metal : ligand stoichiometry with Zn^{II}, Cu^{II}, Fe^{II} and Mn^{II}, although for Cu^{II} the third ligand binds with low affinity and hence mostly $Cu–Phen_2$ is considered. Interestingly, $Fe^{III}–Phen_3$ is unstable as reduction occurs due to its high redox potential (see Table 3). Hence, Fe^{III}-binding of Phen seems not to be relevant in blood plasma. As shown in Fig. 5A, the $pM_{1/10}$(Phen) is clearly below the $pM_{1/10}$ of plasma proteins for Mn and Cu, but only slightly smaller for Zn. Hence $M–Phen_m$ complexes are estimated to be not stable in plasma at 10 μM Phen concentration. Instead, if we consider the $pM_{pl/100}$ (see Fig. 5B), the pZn but not pMn nor pCu, overcomes slightly the pM_{plasma}, indicating that Zn^{II} will be selectively bound. Accordingly, the predicted instability of the Cu–$Phen_2$ complex, which has been recently shown in cell culture medium with 10% FCS,[45] jeopardises the application of Phen-type ligands as Cu-based drugs. Complexes of Cu–$Phen_2$ and Mn–$Phen_3$ are not stable in the μM regime, which considerably limits their application as anticancer agents. Zn–$Phen_3$ is stable in the higher μM range, roughly above 10 μM. Of note, these considerations neglect ternary complexes, although they are of utmost importance (see section 5.3).

4.4 Desferal (DFO)

This chelator (see Chart 1) is used to treat iron overload.[61] It shows a high affinity for Fe^{III}, but only a moderate affinity for Fe^{II} (see Table 3). The thermodynamic binding constants ($β$) have been determined for Fe^{III}, Zn^{II} and Cu^{II} (Mn could not be found) and hence the selectivity between these three ions can be evaluated in the plasma environment. First, the conditional K_d at pH 7.4 can be calculated by taking into account the pK_a of the ligand (see Table 3 and Appendix) and then the $pM_{1/10}$ and the $pM_{pl,100}$. Thus, we can see that considering either the $pM_{1/10}$ or the $pM_{pl/100}$, DFO competes with Tf for Fe^{III} but not with SA for Cu and Zn, indicating that DFO is quite selective for Fe^{III} (see Fig. 5). Although this also depends on the real concentration of DFO in the plasma, the selectivity will stay very good, especially considering that this chelator is used in the case of Fe overload. A more detailed consideration of DFO and other Fe^{III} chelators is provided elsewhere.[7]

4.5 Trientine (TRIEN)

TRIEN (see Chart 1) is a chelator used to treat Cu^{II} overload in Wilson's disease.[62] Based on $pM_{1/10}$, a selectivity for Cu^{II} over Zn^{II} is expected, although Zn^{II}-binding may still partially occur (see Fig. 5A). Actually, as shown in Fig. 5B, a higher selectivity for Cu^{II} over Zn^{II} is predicted from comparing the $pM_{pl,100}$ with the pM_{plasma}. Moreover, as TRIEN is used in the case of Cu overload, the selectivity will be further increased. The K_d of TRIEN for Fe^{III} could not be found, but this pure tetradentate N ligand is expected to have an affinity lower than Tf.

4.6 Triapine (3AP)

The $pM_{1/10}$ of triapine (see Chart 1 and Table 3), a thiosemicarbazone that went through several clinical trials as an anticancer agent, is below the $pM_{1/10}$(Protein) for both Fe and Cu and hence 3AP complexes are not expected to be stable or to be formed in the plasma.[52,56] Interestingly, recent experimental studies suggest that Cu^{II}-3AP may be stabilized *via* the interaction with SA.[63]

4.6 LH8

This compound (see Chart 1) was conceived as a ^{64}Cu-ligand for PET.[57] For such an application, its Cu complex should be

stable in the blood plasma. The reported $pM_{1/10}$, 15.5, is slightly below the $pM_{1/10}$ of SA for Cu^{II}, while it is above for Zn^{II}. Accordingly, a potential transmetallation from Cu to Zn could be envisioned for this ligand. However, the calculated $pM_{pl/100}$ for Cu^{II} appears to be higher than pCu_{plasma}, suggesting that Cu^{II}–LH8 can be partially stable in blood plasma. Nevertheless, since the difference between $pM_{pl/100}$ and pM_{plasma} is considerably higher for Zn^{II} compared to Cu^{II}, partial trans-metallation is a serious concern.

These examples show that several d-block metal complexes with some biomedically-relevant ligands have a $pM_{pl/100}$ lower than pM_{plasma}, and hence have a high risk to dissociate during their circulation in the blood. It does not mean that they cannot exist in the blood (see below) but the possible dissociation of ligands from metal ions should be taken into account.

Besides, it is also worth noting that, although in most cases the comparison of $pM_{1/10}$ (which does not take into consideration the real metal and ligand concentration) yields results qualitatively similar to the comparison of $pM_{pl/100}$ and pM_{plasma}, the latter enables more reliable predictions, particularly useful if the differences in $pM_{1/10}$ are small.
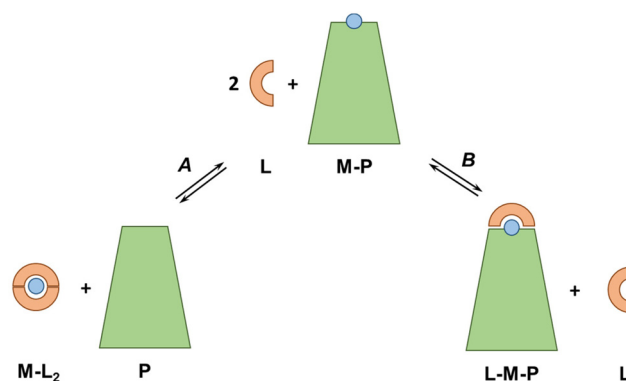
5 Limitations

5.1 Simplifications and modulators

The concepts and estimations made above are based on a simplified system including only the main (*i.e.* most abundant and with the highest affinity) metal-binding protein for each metal ion and neglecting other metal-binding biomolecules with likely lower affinity. It is also worth noting that the affinity of plasma proteins for metal ions, and hence the pM, can be modulated by co-ligand or allosteric effectors, like carbonate in Tf or fatty acids in SA.[64,65] Moreover, the discussion was mostly limited to the d-block metal ions, although, as seen with EDTA, the other metal ions could also be relevant.

5.2 Ternary complexes

In our discussion, we knowingly neglected the potential formation of ternary complexes between exogenous ligands and plasma proteins (see Fig. 6). Indeed, such ternary complexes, which can occur *via* a bridging metal ion (L–M–P) but also *via* metal-independent interactions of the ligand with the protein, can considerably change the thermodynamics and kinetics of metal-exchange reactions. A native example is Tf, which binds Fe^{III} coordinated to a carbonate ion, such that the Fe^{III} affinity depends on carbonate concentrations. Bidentate or tridentate ligands, such as Phen and 3AP, are particularly prone to form ternary complexes. For instance, tridentate thiosemicarbazones, such as 3AP, bind Cu mainly in a 1 : 1 complex, so any molecule in the blood serum (*e.g.* amino acid, His residues on SA) may bind to the fourth equatorial site (replacing water or weakly-bound anions) and increase the stability of the complex. For instance, some Cu-thiosemicarbazones have been shown to form a ternary complex with a His of SA in

Fig. 6 Schematic representation of the equilibria potentially occurring between a metal–protein complex (M–P) and a ligand (L) able to form 1 : 2 complexes (M–L₂): (A) L retrieves M from P forming M–L₂; (B) L form a ternary L–M–P complex with M and P. This is a simplified system and more states are possible, *e.g.* with other stoichiometries, weak interactions between L and P, allosteric modulators, *etc.*

crystals.[66–70] As this ternary complex is expected to have a higher pCu than the binary complex, it is possible that the ternary species is formed in the blood plasma.

Besides, Nunes *et al.* recently showed that Cu–Phen₂ decomposes almost completely in a cell culture medium with 10% FCS and ternary complexes of Cu, Phen and SA were formed.[45]

5.3 Redox state

Some of the metal ions can change their redox state upon binding to a ligand/protein. For instance, it has been proposed that Mn can bind to Tf as Mn^{III} with higher affinity. Therefore, although very little Mn^{II} is expected to bind to Tf,[30] the latter might bind more Mn (as Mn^{III}) than what is expected considering the affinity for Mn^{II}.[71]

6 Conclusions

Estimations of pM allow us to get a first glimpse of the selectivity and stability of M–L_m complexes. If they are much higher than the pM of plasma metal carriers (pM_{plasma}), for the metal in question, but lower than other metals, then the complex could be formed with high selectivity and stability. If it is far below, then dissociation would occur. If they are close, more accurate calculations (*i.e.* overcoming the simplifications and approximations used here), together with the use of software such as HYSS,[72] give more trustworthy predictions. In general, one can also point out that for metal–ligand complexes with kinetically-labile metal ions and low denticity ligands (mono- and bidentate), the partial dissociation of at least one ligand is very likely to occur, especially at typical drug concentrations, *i.e.* in the μM range or below. In any case, experimental testing by direct competition with either SA or Tf is always useful because:

- determination of overall stability constants (β), conditional dissociation constant (K_d) or pM is not easy, and the fit sometimes does not allow the unambiguous determination of the complex formation model; thus, they can vary between published works or not be very accurate; often the values are only available in the presence of organic co-solvents (*e.g.* DMSO);

- all the considerations mentioned do not include ternary complexes, although they are very important in particular for M–L$_m$ with $m > 1$; careful experiments can give hints to the existence and importance of ternary complexes;

- information on metal-bindings/transfer kinetics, which is another very important parameter, can also be gained.

In addition, more biologically relevant stability measurements could be performed directly in blood plasma or even *in vivo*.

The general conclusion is that the comparison between the pM values or as a less precise estimate of K_d of chelators or metal–ligand complexes and the pM$_{plasma}$ gives the first information on their selectivity and potential fate in the blood. If the differences are quite large (3 logs or more), then this can be taken as an argument for stability or instability and selectivity. For smaller differences, several factors might influence the stability and selectivity (such as ternary complexes) and further experiments seem to be of interest.

Conflicts of interest

The authors declare no conflict of interest.

Appendix

Simplified estimation of conditional dissociation constant, K_d, at pH 7.4 from the stability constant β

The overall stability constant β, commonly obtained by potentiometric pH titrations, can be considered the binding constant of the *fully deprotonated* ligand. However, since protons can compete with metal ions in coordination bonds, they weaken the metal affinity. Therefore, to estimate the conditional dissociation constant K_d at a certain pH, we have to consider all the pK_a of bases that coordinate the metal ion (the protonation state of the non-coordinating bases is neglected here). As a first simplistic approximation here we consider only the pK_a that is higher than the pH for which we want to determine the K_d (for an accurate determination of the conditional K_d from the whole set of ligand and metal–ligand complex pK_as, software such as HYSS[72] are particularly helpful).

For instance, the pK_a of EDTA are 10.3 (amine), 6.2 (amine), 2.7 (carboxylates), and 2.9 (carboxylates). As only the pK_a of 10.3 is above or close to pH 7.4 and the amine binds to the metal ions, we have to correct for that as follows (see Table 2):

$$-\log K_d = \log \beta - (\mathrm{p}K_a - \mathrm{pH}) = \log \beta - (10.3 - 7.4)$$
$$= \log \beta - 2.9$$

The pK_a of DFO are 10.8 (non-coordinating amine), 9.5, 9.0 and 8.3 (hydroxylamines). Hence,

$$-\log K_d = \log \beta - \sum(\mathrm{p}K_a - \mathrm{pH}) = \log \beta - 4.6$$

Finally, Phen has no pK_a higher than 6, so $\log K_d$ can be approximated to $\log \beta$.

Competition between EDTA and SA for Cu^{II}

In order to evaluate the competition between EDTA (L) and SA (P) for Cu^{II}, the conditional dissociation constant, K_d, can be compared. Indeed:

$$K_d^L = \frac{[\mathrm{Cu}][\mathrm{L}]}{[\mathrm{Cu-L}]}$$

and

$$K_d^P = \frac{[\mathrm{Cu}][\mathrm{P}]}{[\mathrm{Cu-P}]}$$

Hence,

$$\frac{[\mathrm{Cu-P}]}{[\mathrm{Cu-L}]} = \frac{K_d^L}{K_d^P} \frac{[\mathrm{P}]}{[\mathrm{L}]}$$

Since $\frac{K_d^L}{K_d^P} \approx 10^{-3}$, when $[\mathrm{P}] = [\mathrm{L}]$, $[\mathrm{Cu-P}] \approx 10^{-3}[\mathrm{Cu-L}]$, *i.e.* $\approx 99,9\%$ Cu is bound to EDTA.

If $[\mathrm{L}] \approx 0.01\,[\mathrm{P}]$, then $[\mathrm{Cu-P}] \approx 10^{-1}[\mathrm{Cu-L}]$, *i.e.* $\approx 90\%$ Cu is bound to EDTA.

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