Speciation of metal complexes of medicinal interest: relationship between solution equilibria and pharmaceutical properties

Tamás Kiss [1,2,*], Éva A. Enyedy[1], Tamás Jakusch[1], Orsolya Dömötör[1,2]

[1] *Department of Inorganic and Analytical Chemistry, University of Szeged, Dóm tér 7, H-6720 Szeged, Hungary;* [2] *MTA-SZTE Bioinorganic Chemistry Research Group, University of Szeged, Dóm tér 7, H-6720 Szeged, Hungary*

* Corresponding author. Tel.: +36/36-62-544-337
E-mail address: tkiss@chem.u-szeged.hu

Abstract: Biospeciation of essential and toxic metal ions, metal complexes with biological or medicinal activity are discussed in the paper in order to emphasize the importance of the distribution of metal ions in biological milieu. The exact knowledge of the chemical species present in the different organs/compartments/fluids/cells may provide essential information about the pharmacokinetic properties and the biological effect of the metal ion or the drug candidate metal complex. The transport of essential and toxic metal ions in the blood serum is discussed first, which is followed by the description of biodistribution of several important metal complexes with medicinal interest such as (i) anticancer, (ii) insulin-enhancing and (iii) MRI contrast agents in biological fluids.

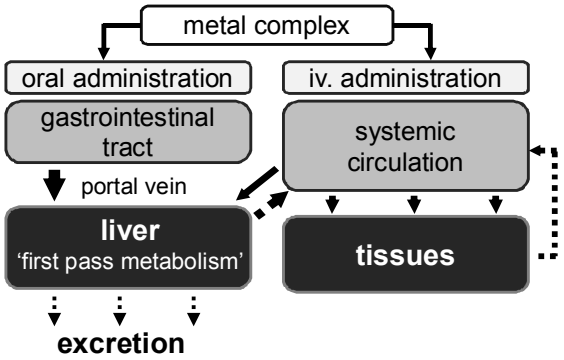
Keywords: chemical equilibrium, solution stability, serum proteins, modeling calculation, therapeutic metallodrugs, species distribution

1. INTRODUCTION

The experimental conditions of preparation of metal complexes with potential biological activity usually differ considerably from the milieu in the living systems, where they exert their biological effects. The medium, the pH of the biological fluids, cells and tissues might be significantly different. Moreover, various endogenous molecules may also be present, having high affinity to the metal ion and accordingly, these compounds may partly or fully displace the original metal binding ligand(s) and thus the original complex may undergo transformations during (i) the absorption processes in the gastrointestinal (GI) tract in the case of complexes administered orally, (ii) their transport processes in the blood stream interacting with the serum/plasma components, (iii) metabolism, and (iv) in the cell via targeting its final

target molecule(s). Accordingly, the original carrier ligand(s) might be fully lost in these processes and the real biological/physiological activity is connected to an entirely different chemical entity. [1]

The metal complexes often behave as pro-drugs, which means, that the active forms are considered to be different from the administered compounds due to the various biotransformation processes in biological fluids such as the GI tract and blood serum. Transformation processes of metallodrugs show a wider diversity compared to the case of the conventional organic (non-metallic) pharmaceutical drugs. In order to establish structure-activity relationships and to understand the mechanism of action the knowledge of the aqueous solution behavior of the metal complexes is highly important. One crucial point is the mode of administration (Scheme **1**). These metallodrugs are frequently administered intravenously: the solid state complex is dissolved in water (*e.g.* in physiological NaCl or glucose solution often with citric acid and/or lactate), then added to the infusion solution which reaches the systemic circulation. This route avoids the so called 'first pass' through the portal vein, and consequently the pre-systemic metabolism of the drug. By contrast orally administered complexes meet the fairly acidic gastric and/or the slightly basic intestinal juice containing numerous GI secretions before getting into the blood stream and then pass through the liver before they arrive to the final target [2]. In aqueous phase and in the presence of the blood serum components various transformation processes of a metallodrug also can take place.



Scheme 1. Schematic way of a metal complex in human body after oral or intravenous (iv.) administration.

Dissolving in aqueous solution the metal complex may decompose depending on its thermodynamic stability, its concentration and the pH, additionally Cl^-/H_2O ligand exchange, de(protonation) and formation of mixed hydroxido species are also possible. Interaction with low molecular mass (LMM) molecules or serum proteins (high molecular mass (HMM)

components) can lead to partial or complete displacement of the original ligand(s). These reactions change the original composition of the metal complex and have a strong impact on the pharmacokinetic properties as well. This is the reason why the deep knowledge of the kinetics and thermodynamics of these processes is needed. It is highly important to determine the rate of these reactions, the equilibrium constants for the various chemical equilibria, the stoichiometry of the formed species, and binding strength and sites on a protein. Since these transformation processes take place parallel in the real and multicomponent systems, the determination and the mathematical modeling of the speciation of a metallodrug in serum is very complicated. In the case of kinetically inert complexes the traditional equilibrium studies usually cannot be applied due to the slow ligand-exchange processes. This can also be the reason why available speciation data *e.g.* for anticancer metal complexes are generally very limited.

Accordingly, studying distribution of metal ions and biologically active metal ion containing compounds in biological systems (*e.g.* fluids and tissues) is essentially important in order to monitor their fate in living organisms.

2. THE ROLE OF THE HUMAN BLOOD SERUM IN TRANSPORT AND DISTRIBUTION PROCESSES

Human blood plasma is the formed element (red and white blood cells and platelets) free portion of blood, accordingly it contains all plasma proteins such as albumin (HSA), transferrin (Tf), globulins, fibrinogen and other specific proteins and small molecules. The fibrinogen and clotting factor free blood plasma (*i.e.* the fluid part of blood after coagulation) is called blood serum. After practical considerations this latter is used for *in vitro* studies instead of whole plasma.

From the point of view of speciation the serum constituents can be classified into two distinct groups: LMM and HMM serum components, the latter are mostly peptides and proteins. Interaction of metal complexes with serum proteins such as HSA and Tf has a profound effect on the ADME (absorption, distribution, metabolism, and excretion) properties and the toxicity. Notably fairly diversified scenarios are possible regarding to the serum protein binding modes and reaction rates in case of metallodrugs. Binding through non-covalent bonds (such as hydrogen and salt-bridge bonding, π-π stacking interactions, Van der Waals force) does not change generally the structure of the metal complexes; consequently the complex retains its original entity. Non-covalent and weakly-to-moderately strong

reversible binding to HSA might be advantageous for the targeted delivery due to the enhanced permeation and retention effect of solid tumors [3]. Furthermore binding to Tf can be profitable for the cellular uptake of antitumor drugs via the overexpressed Tf-receptors due to the higher iron demand of cancer cells. On the contrary, irreversible and strong binding generally takes place via coordinative bonds being accompanied by partial or complete displacement of the original ligand(s) by the donor atoms of the protein and ultimately may lead to the inactivation of the metal complexes, especially in the case of slow ligand-exchange processes. In this way the original complex cannot reach the desired site of action and even the metal ion cannot be released there effectively. The formation of covalent metallodrug-protein adducts might be responsible for *e.g.* side effects of the chemotherapy.

HSA is the most abundant protein in human blood plasma; it constitutes about half of the serum protein content, its concentration is typically 530 - 750 μM [4]. It maintains oncotic pressure, buffers pH, and transports among others thyroid- and other mostly fat-soluble hormones, fatty acids (FAs), unconjugated bilirubin and many drugs [4,6]. Serum albumin levels can affect the half-life of drugs [5]. On the other hand HSA is mainly an extravascular protein, 67% of its total amount is found interstitially. Its exchange rate across the capillary wall is relatively slow: 5% leaves the intravascular space per hour [6]. At the same time the microenvironment of solid tumors often shows more pronounced accumulation of albumin due to the enhanced permeability and retention effect and the recently discovered active transport mechanisms across blood vessels including gp60 (albondin) transport protein [3]. HSA greatly augments the transport capacity of serum due to its high concentration and extraordinary binding capacity [6]. There are seven distinct binding sites on HSA for long-chain FAs (FA1–FA7) [5]. On the other hand HSA has nonspecific binding pockets where chemically diverse endogenous and exogenous compounds can bind and the principal regions of these sites are located in subdomains IIA, IIIA, IB often called as Sudlow's site I and II and site III, respectively [7]. Each site forms a hydrophobic pocket: site I binds preferentially bulky heterocyclic anions, the prototypical ligand being warfarin; site II is preferred by aromatic carboxylates with an extended conformation; while site III seems to bind diverse assortment of acidic, neutral and basic molecules (Fig. **1**) [5,6,8]. These sites are allosterically coupled with each other and with FA binding sites as well, thus binding of an agent at one site may enhance or hinder the binding of another molecule(s) on the other site(s) [6,8].

Four types of metal ion binding sites of HSA have been described [7]: the N-terminal binding site (NTS) is specific primarily for Cu(II) and Ni(II) (often called as ATCUN motif as well) [4,9], the multi-metal binding site (MBS) which primarily binds Zn(II) and other

bivalent metal ions [10]; site B at which Cd(II) can be bound but Zn(II) as well [9,11], and the thiol group of Cys34 can bind gold and platinum containing compounds [9]. Furthermore HSA contains 16 His imidazoles (Fig. **1**) and 6 Met thioether groups from which the surface exposed donor atoms can serve as monodentate coordinating sites for various type of complexes: *e.g.* organoruthenium or platinum compounds [12,13].

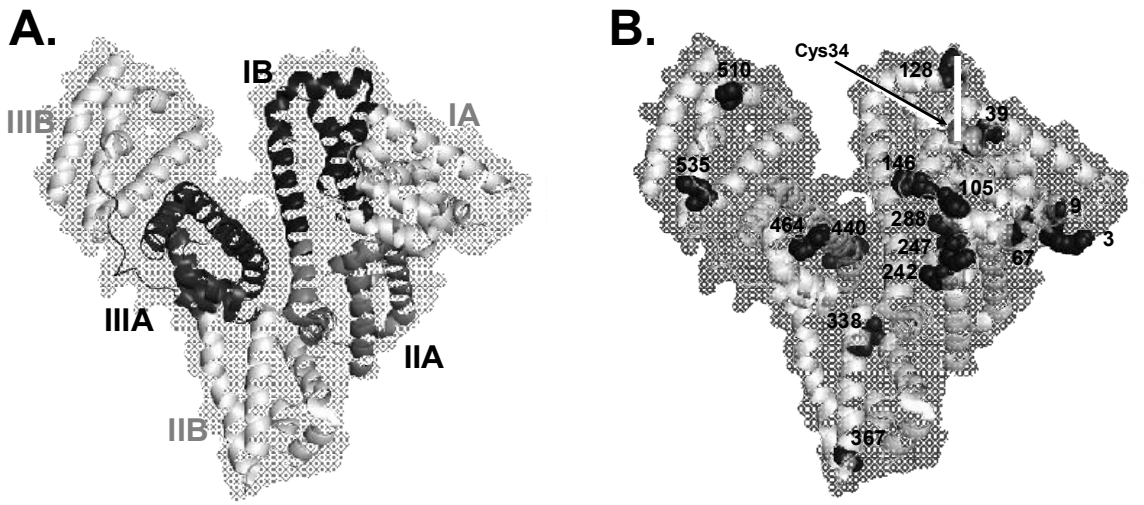


Fig. (1). (**A**) Organization of the subdomains in HSA. Subdomains IIA, IIIA, IB, containing the hydrophobic binding pockets site I, II and III respectively are marked with dark colors. (**B**) The positions of the histidine residues (numbered black fragments) and the single free cysteine (Cys34) within the protein.

Transferrins are iron-binding blood plasma glycoproteins that control the level of free Fe(III) in biological fluids [14]. Tf consists of a polypeptide chain containing 679 amino acids and two carbohydrate chains, and this protein is composed of α-helices and β-sheets that form two domains. The N- and C-terminal sequences are represented by globular lobes [15]. Tf contains two specific remarkably similar high-affinity Fe(III) binding sites. Each lobe of Tf contains a distorted octahedral Fe(III) binding site consisting of two Tyr, one His, one Asp, and one bidentate carbonate anion (the so-called 'synergistic anion') [16]. Tf saturated by metal ions undergoes a conformational change, the C- and N-terminal parts move closer to each other and takes up a closed conformation [17]. The concentration of Tf in the serum is *ca.* 37 μM and its Fe(III) saturation falls between 15–50% [18], therefore it binds efficiently other metal ions such as Ga(III), Zn(II), V(IV)O, Al(III), Ru(III) and Bi(III) [19].

Immunoglobulins are glycoproteins can react in an immune response to neutralize pathogens introduced or inoculated into the body. Immunoglobulins are divided into the

classes IgA, IgD, IgE, IgG, and IgM, these classes differ in their biological properties, ability to deal with different antigens and functional locations. The most important class is IgG since it represents 75% of the total immunoglobulin content in the blood. The IgG concentration in human blood serum is approximately 73 μM [20].

α2-macroglobulin is one of the largest plasma proteins. It mostly occurs in a tetrameric form and its main function is the inhibition of proteases. A CysGlu fragment of the protein seems to play an active role in the enzyme inhibition. Its concentration in human serum, however, is rather low and thus its ability in metal binding remains subordinate. With the exception of Zn(II) it has no reported role in metal ion transport in blood serum [21].

The role of LMM serum compounds is far not negligible as they can be responsible for the (partial) displacement of the original ligand(s), or for formation of ternary complexes. The LMM fraction of the serum consists of carboxylic acids such as oxalic acid (9.2 μM), hydroxycarboxylic acids, such as lactic acid (1.5 μM), citric acid (99 μM) and malic acid and numerous amino acids, His (77 μM), Cys (33 μM), cystine (Cis; 10.9 μM), Gln (80 μM), Asn being the most important as potential metal ion binders [22]. Besides the above mentioned organic acids, LMM fraction contains anions of inorganic acids as well, such as phosphate (1.10 mM), hydrogencarbonate (24.9 μM), sulphate (330 μM), and chloride (102 mM) ions (listed in decreasing strengths of their metal ion binding ability) [22,23]. It is noteworthy that chloride ions are often considered as coordinating ligands (in the case of *e.g.* Pt(II), Ru(III), Rh(III)) thus can influence the aquation and hydrolysis of the metal complexes. Besides, serum reductants such as ascorbate (55 μM) may reduce the metal center, consequently may initiate ligand exchange processes around the metal ion.

Concerning the metal ions, serum transports numerous endogenous essential metal ions: iron, copper, zinc, cobalt, calcium, magnesium, sodium, potassium. Besides these, depending on the circumstances various other essential (*e.g* as components of medicines, such as V(IV,V)) or toxic (Pu(IV) or Al(III) *etc.*) metal ions can occur and are transported in the serum.

3. SERUM SPECIATION OF SELECTED ESSENTIAL AND TOXIC METAL IONS

The serum speciation of Fe(III), as one of the most important serum transported essential transition metal ions, is simple since it binds exclusively to Tf [21]. Concerning the LMM components under healthy conditions they have negligible importance. Citrate might be the only binder of Fe(III) which may have a role [24]. The importance of Fe(III)-citrate

interaction in the serum may increase in case of iron overload. As it is very toxic it must be removed by suitable chelator molecules [25].

The serum speciation of the important transition metal ion the copper is much more complex as it is distributed in different forms in serum both in the HMM and the LMM fractions. The largest part of the copper is bound in the oxidation state of two in ceruloplasmin. Its serum level is 1.5-2.7 μM and 40-70% of the copper is bound to this protein [26,27]. 6-7 Cu/protein are tightly bound and buried in the ceruloplasmin in different environments mostly in non-exchangeable copper pool [27-28]. 20% of serum Cu(II) is bound to HSA almost exclusively at the specific ATCUN binding site [26,27,29]. The albumin bound copper means the exchangeable pool, which can deliver the metal ion to most of the cells. α2-macroglobulin and transcuprein also bind some copper in serum. They have high affinity binding sites for copper, higher than in HSA [27]. They guarantee fast exchangeable copper pool for the tissues [27,29].

The remaining part of copper is bound to the LMM fraction mostly in His containing binary and ternary complexes formed with Thr, Asn and Gln. Different modeling calculations were carried out for the Cu(II) bound in this fraction, one of them (probably the most accepted) is listed in Table **1**.

Table 1. Calculated distribution of Cu(II) bound to LMM constituents of human blood serum (based on data in Ref. [30]).

Cu(II)	%	Cu(II)	%
Cu(His)(Gln)	19	Cu(His)(LysH)$^+$	4
Cu(His)$_2$	16	Cu(His)(Gly)	4
Cu(His)(Thr)	15	Cu(His)(Asn)	4
Cu(His)(Ser)	8	Cu(His)(Val)	4
Cu(His)(Ala)	5	Cu(His)(Leu)	4

Zn(II) is bound almost exclusively to HMM components [29]. Tf can bind zinc at the iron binding sites [31], however, due to its relatively low concentration biologically it is not a relevant binder [19,32]. Albumin is its primary binder at the MBS site, or site A, 60-70% of Zn(II) is transported by HSA [7,33]. Despite its low serum concentration α2-macroglobulin binds *ca.* 30% of Zn(II); this Zn(II) is more tightly bound than in HSA. Elevated serum levels of α2-macroglobulin and concomitant low levels of serum Zn(II) seem to correlate with some medical conditions (*e.g.* cancer, inflammation) and aging [33-36]. The remaining 2-3% of

Zn(II) binds to the LMM fraction mostly to His and Cys containing binary and ternary complexes [29,32,37].

Extracellular Ca(II) concentration is relatively high (2.1-2.6 mM), and about half of it is bound in the HMM fraction, namely it binds to HSA and to seven other proteins [3637]. Among the LMM ligands hydrogencarbonate, citrate and lactate are the important Ca(II) binders. However, substantial fraction of the metal ion remains free. Practically a similar distribution is valid for Mg(II) too, although its concentration in the serum is significantly lower (0.6-0.95 mM).

Alkaline metal ions occur almost exclusively in free form in the serum; accordingly, they are very mobile.

According to present knowledge Ni(II) is a non-essential element for humans and binds preferably to HSA at the ATCUN binding site. Besides, small amount of Ni(II) binds to some other proteins of the serum, however due to their low concentration their biological relevance in Ni(II) transport is negligible. LMM fraction bound nickel is also significant (Table **2**).

Table 2. Calculated distribution of Cd(II), Ni(II), Pb(II) and Pu(IV) in human blood serum (based on data in Ref. [30]).

Cd(II)	**%**	**Ni(II)**	**%**	**Pb(II)**	**%**	**Pu(IV)**	**%**
Cd(Cys)	46	$Ni(His)_2$	51	Pb(Cys)	80	$Pu(citrate)(OH)_2^-$	71
Cd(Cis)	28	$Ni(Cys)(His)^-$	18	$Pb(Cys)(citrate)^{3-}$	7	Pu(citrate)(OH)	29
$Cd(Cys)(OH)^-$	14	$Ni(Cys)_2^{2-}$	11	$Pb(Cis)^+$	5		
$Cd(Cys)_2^{2-}$	8	$Ni(His)^+$	4				

Among the toxic metal ions Al(III) is fairly well studied, due to its potential involvement in neurological disorders [38] Absorbed Al(III) is transported by the blood stream but its speciation in blood serum is still not exactly known. It is sure that most of the Al(III) is bound by the HMM iron transport protein Tf. HSA and other serum proteins bind weakly the metal ion and are not able to compete for a significant amount of Al(III) [39]. From among the LMM binders of serum citrate and phosphate are the only efficient binders. However, the literature data are rather contradictory, which of them is the more potent binder. The reason for the contradictory speciation models is the lack of reliable solution stability data for the Al(III) complexes involved in the models and the differences in the experimental conditions of the speciation and modeling.

W.R. Harris *et al.* [40] using difference UV spectrophotometry, pH-potentiometry and electrospray ionization mass spectrometry (ESI-MS) measurements reported a model at ~10 μM Al(III) concentration, which is closer to the normal biologically relevant serum level (~0.1-0.3 μM) [41]. Their speciation model given in Table **3** (based on data in Refs. [40,42]) suggests that 93% of the total Al(III) is bound to Tf. Of the pool of LMM aluminium, 88% of Al(III) would be bound to citrate, 8% to hydroxide and ~2% to phosphate.

Table 3. Speciation of Al(III) at pH ~7.4 and 298 K (based on data in Refs. [40,42]).

	% of Al(III) bound	
	$c_{Al(III)}$ = 10 μM	$c_{Al(III)}$ = 1 mM
HMM components		
Albumin	–	–
Transferrin	93	77
LMM components		
Phosphate	0.1	14
Citrate	6	5
Citrate-Phosphate	0.8	4

At the same time at 1 mM total Al(III) concentration, that corresponds better to conditions with aluminium overload and when the thermodynamic equilibrium was surely attained (monitored by multinuclear NMR) the amount of phosphate bound Al(III) increased significantly and ternary Al(III)-citrate-phosphate complexes could also be detected (Table **3**) [43].

Cd(II) binds preferably to proteins: HSA and α2-macroglobulin, but besides them immunoglobulins and transthyretin (also referred to as prealbumin) also have some, but significantly smaller cadmium binding capacity. In the LMM fraction Cys and Cis are the main Cd(II) binders (Table **2**).

Concerning the exogenous metal ions Tf binds and transfers Ti(III), V(IV,V), Cr(III), Ga(III), which may have biological application (see later the cases of antidiabetic vanadium complexes and anticancer Ga(III) complexes). Although Ru(III) also can be bound to Tf at the iron binding sites with considerable strength, HSA is the primary transporter of Ru(III) anticancer compounds in serum as will be discussed in the subsequent section. Trivalent actinide ions such as Am(III) and Cm(III) are reported to bind relatively weakly to Tf, and HSA may play a more decisive role in their transport *in vivo*. On the contrary tetravalent actinide ions (*e.g.* Pu(IV)) and the UO_2^{2+} cation are bound to Tf much tighter than to HSA ; consequently, complexes of these ions formed with Tf may be the major actinide-containing

species in the human blood. However, the Tf complexes of these actinide ions bind weakly to the Tf receptors indicating that this serum protein does not play a major role in incorporation of these metal ions into cells. The potential use of Tf in technologies to selectively remove metal ions from waste water streams is considered to be limited [19].

In the subsequent sections the distribution of several biologically active compounds with (potential) medicinal *i.e.* therapeutic and diagnostic applications in the presence of the serum components will be given in order to shed light on their transport processes.

4. BIOSPECIATION OF ANTICANCER METAL COMPLEXES

In the field of metals in chemotherapy three Pt(II) complexes, namely cisplatin, carboplatin and oxaliplatin (Chart **1**) dominate and are used in almost 50% of the treatment regimes for patients suffering from cancer in mono or combination therapy [44]. The resistance and deleterious, highly debilitating side effects of anticancer drugs used in treatment have motivated the design and development of novel metal-based compounds that combine good efficacy, selectivity and low systemic toxicity. Among the potential candidates a diversity of metallodrugs is seen in the clinical trials or at advanced stage of preclinical developments such as various Pt(II/IV) and non-Pt *e.g.* Ru(III/II), Ga(III), Cu(II), Au(I/III) anticancer compounds [45-49]. Ru-based compounds have been recognized as promising alternatives to anticancer Pt complexes as they are less toxic in general and show less and more tolerable side effects. Among the antitumor non-Pt compounds sodium [*trans*-tetrachlorido*bis*(1H-indazole)ruthenate(III)] (NKP-1339, IT-139) [50] and *tris*(8-quinolinolato)gallium(III) (KP46) [51] are being in clinical trials with promising results (Chart **1**). According to the early hypothesis of the action of NKP-1339 its uptake is mediated via the overexpressed Tf receptors and Ru(III) is reduced to the active +2 oxidation state in the reducing environment of the cancer cells. Recently adduct formation with HSA is reported to be preferred to Tf, however the 'activation-by-reduction' hypothesis is still widely accepted [50,52]. Based on this theory, organometallic Ru(II) compounds attracted interest and numerous arene/arenyl half-sandwich compounds with remarkable antitumor properties were developed in addition to analogous isoelectronic Os(II), Rh(III), Ir(III) complexes (Chart **1**).

The biological activity of the metal-based chemotherapeutics is often governed by the metal ion, while the coordinated ligand has a decisive role in the protection of the metal ion against hydrolysis; and the type of the ligand markedly affects various fundamental physico-chemical properties of the complex such as size, charge, stability, lipophilicity, water-solubility, rate of (co)ligand-exchange and hydrolytic processes and ultimately the biological

activity. On the contrary in particular cases such as the hypoxia-activated Co(III) complexes the metal ion is considered as a specific carrier of the ligand with antitumor activity. The ligand is released selectively from the kinetically inert Co(III) complex by reduction in the hypoxic tumor microenvironment resulting in the much more labile and usually less stable Co(II) complex [53,54]. On the other hand cytotoxic/cytostatic complexes are accessible by incorporating a bioactive ligand into the structure that can operate in a synergistic way with the antitumor metal ion.

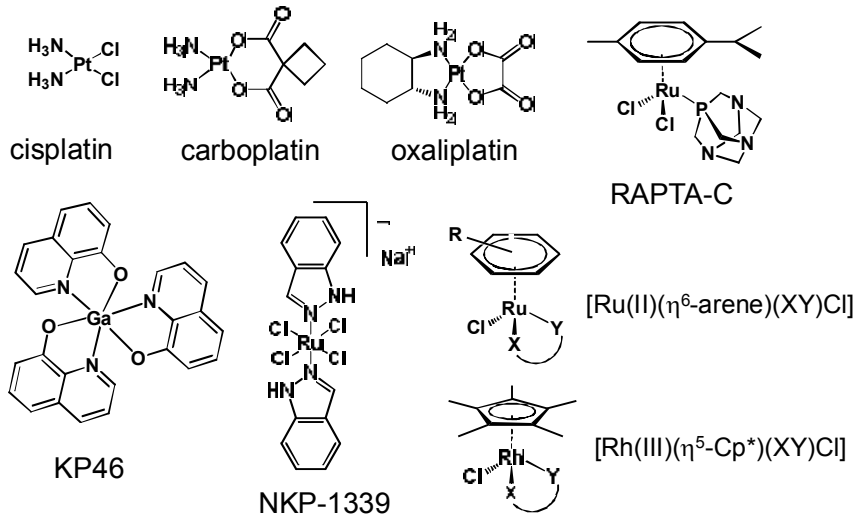


Chart 1. Chemical structures of some anticancer metal complexes, and general structures of the organoruthenium and organorhodium complexes bearing (XY) chelating bidentate ligand.

This chapter gives a brief overview about the solution equilibrium and blood serum speciation studies of some representative groups of anticancer metal complexes such as Pt(II), Ru(III), organometallic half-sandwich and Ga(III) complexes.

4.1. Antitumor platinum(II) complexes

Solution stability constants are rarely published for Pt(II) complexes due to their slow formation kinetics, which makes the description of such systems from the equilibrium point of view very difficult [55]. For this reason, complexation processes of the analogous Pd(II) complexes possessing several orders of magnitude higher ligand exchange rates are also studied as model systems [56-58]. In aqueous solution the Pt(II) complexes may suffer from various transformation processes such as aquation (cisplatin: Cl^-/H_2O exchange), deprotonation of the coordinated aqua ligand (formation of mixed hydroxido species) and loosing *e.g.* oxalate ligand (oxaliplatin). In the presence of the LMM components reactions with small peptides (*e.g.* glutathione (GSH)) and amino acids (Cys, Met) are probable [59].

All these processes are relatively slow and the extent of the reactions also depends on the concentration of the reaction partners such as chloride, hydroxide ions (thus pH) and LMM compounds. Aquation of the Pt–Cl bond is frequently studied as it is an important step of activation and has a key role in the DNA interaction. The major products of the Cl^-/H_2O exchange of cisplatin are the *mono*- and *bis*-aqua species *cis*-$[Pt(NH_3)_2(H_2O)Cl]^+$ and *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$, respectively [60,61]. Kinetics of the aquation and hydrolysis processes of cisplatin were studied in details under various conditions by Davis *et al.* and equilibrium constants (pK_1 = 2.07 and pK_2 = 3.49) obtained after 40 h at 298 K were also reported [61]. These transformation processes are unambiguously important, however, the analysis of samples from *in vivo* treated animals or patients showed the decisive role of the serum proteins in the distribution in the case of numerous antitumor Pt(II) drugs.

The protein binding profile is different for the three FDA approved Pt(II) drugs, namely the level of protein binding of carboplatin is the lowest, while oxaliplatin preserves its original form most weakly in the unbound/LMM fraction in real-world samples [59,62]. Cisplatin was found to bind predominantly to HSA, although the concentration of the free drug strongly depends on the time (up to 90% of Pt(II) bound to proteins after 3 h infusion) [59]. However, it is important to note that the increasing protein bound fraction of the platinum compound in time is more likely due to the fast renal excretion and short half life of the free drug and not to its extensive serum protein binding [62,63]. Most of Pt(II) ions were found to be covalently bound, and the coordination of the protein donor atoms, thus the exchange of the original ligand(s), is slow due to the kinetically more inert feature of this metal ion. Unfortunately comparative binding data are not available for the HSA adducts of the three Pt(II) complexes, even in the case of the 'gold standard' drug cisplatin large variations are reported in the literature regarding the number of binding sites (up to 5-12), and binding constants [13,59,64,65]. The major binding site of cisplatin on HSA involves a (S(Met298),N) macrochelate most probably [13], and conditional stability constants log*K*'HSA = 3.88 and 4.17 for cisplatin and oxaliplatin were reported, respectively by A.V. Rudnev *et al.* [65].

4.2. Anticancer ruthenium(III) complexes

Among the non-platinum tumor-inhibiting agents only one Ru(III) complex is presently being clinically tested, namely NKP-1339 (Chart **1**) [4950]. NKP-1339 and its indazolium salt analogue (= KP1019) have already completed phase-I studies [66,67]. However, KP1019 suffered from low water-solubility which limited the maximum dosage

[66], which is the reason why NKP-1339 was introduced and then furher used in the clinical trials. Imidazolium [*trans*-tetrachlorido(DMSO)(imidazole)ruthenate(III)] (NAMI-A) (DMSO = dimethyl sulfoxide) was the other promising Ru-based agent which was directed against the process of metastasis with a focus on lung cancer and reached phase I in monotherapy, and phase I/II in combination therapy with Gemcitabine [68], but the clinical trials were terminated.

NKP-1339 is administered intravenously and applied against solid tumors showing minor side effects [50]. After entering the bloodstream NKP-1339 may suffer from various chemical changes. Notably Ru(III) complexes are generally characterized by relatively high kinetic inertness and that is the reason for the lack of the traditional speciation studies in aqueous phase. The rate of aquation (Cl^-/H_2O exchange) of NKP-1339 and KP1019 is strongly affected by the pH, temperature and the concentration of chloride and HCO_3^- ions [6869,70]; on the other hand their redox potentials are in the rage of physiological reductants [50]. Thus aquation and the reduction by *e.g.* ascorbate would be possible under the serum conditions. However, the binding to serum proteins can hinder the possible aquation and reduction processes. In addition, interaction with serum proteins is of great relevance due their influence on the biodistribution, the half-life in the circulation and clearance, ultimately on the pharmacokinetic profile. HSA and Tf can act as drug carriers justifying the intensive studies on the interaction of NKP-1339 with these proteins. Fast binding of NKP-1339 and KP1019 to both Tf and HSA was reported by various authors [70-73]. Reaction rates of KP1019 with HSA and apotransferrin (apoTf) were determined by the capillary zone electrophoresis – inductively coupled plasma mass spectrometry (CZE)–ICP(MS) method (k(HSA) = 10.6×10^{-4} s^{-1}, k(apoTf) = 28.7×10^{-4} s^{-1}) showing the kinetically more favorable binding toward apoTf [74]. On the contrary the much slower binding of KP1019 to apoTf via covalent bonds was found by Walsby *et al.* using electron paramagnetic resonance (EPR) spectroscopy [75]. The majority of KP1019 and NKP-1339 is uniformly described by the different authors as being bound to HSA both in *in vitro* human serum and *in vivo* samples obtained from treated patients [66,74,75]. Firstly, the complex NKP-1339 (and KP1019) binds to HSA in a non-covalent manner due to the axial indazole ligand's interaction into the hydrophobic pockets proved by EPR measurements [7475]. Binding at sites IIA and IIIA was found by some of us with moderate binding strength (log$K$'(HSA)= 5.3–5.8), regardless of the counter ions (Na^+ or indazolium) using ultrafiltration-UV, CZE and fluorescence spectroscopy [71]. However, at longer incubation times NKP-1339 and KP1019 are converted to a covalently bound species. The slow binding of these complexes to side chain donor atoms of HSA via ligand exchange

(such as Cl^-/nitrogen donors of His) is suggested [75]. Therefore, the delivery of NKP-1339 by HSA to the cancer cells is assumed followed by the enzymatic degradation of HSA and the reduction of the Ru(III) complex in the hypoxic environment [50]. The reduction leads to the formation of the kinetically more labile Ru(II) complex (Section 4.3.), which is more prompt to react with the diverse targets (proteins, DNA)

4.3. Organometallic anticancer complexes

The activation-by-reduction mechanism of action suggested for the Ru(III) compounds such as NKP-1339 attracted the current interest in organometallic Ru(II) complexes with anticancer activity. The coordination of the π-bonded arene or arenyl ring can stabilize the +2 oxidation state of the metal ion and has a dramatic influence on the chemical and biological properties of these pseudo-octahedral half-sandwich ('piano-stool') complexes [76]. Numerous organometallic half-sandwich complexes (Ru, Rh) are effective catalysts for various organic reactions, *e.g.* $[Rh(\eta^5\text{-}Cp^*)(bpy)H_2O]^{2+}$ complex (Cp*: pentamethylcyclopentadienyl, bpy: 2,2′-bipyridine) is a widely used homogenous transfer hydrogenation catalyst for regeneration of the enzymatic cofactor nicotinamide adenine dinucleotide (NADH) [77,78]. This catalytic activity may have importance in the mechanism of cancer cell death [79]. The Ru(II)(η^6-arene) complexes are the best known among the anticancer half-sandwich organometallic compounds such as the [Ru(II)(η^6-arene)(L)Cl] (L: bidentate ligand) complexes developed by Sadler and coworkers [12,80,81], or by the group of Keppler [82,83]. RAPTA compounds developed by Dyson and coworkers are Ru(II)-arene complexes bearing the 1,3,5-triaza-7-phosphatricyclo-[3.3.1.1]decane (PTA) ligand [84]. Reduction of the growth of primary tumors by [Ru(η^6-*p*-cymene)Cl_2(PTA)] in preclinical models for ovarian and colorectal carcinomas was reported and this complex was considered as a promising drug for translation to clinical evaluation [85].

The available information about the blood serum speciation of this type of organometallic half-sandwich complexes using 'real-world' samples is fairly limited in the literature, although numerous data were reported about their aqueous solution behavior, kinetic aspects of their (co)ligand-exchange processes and interaction with endogenous LMM and HMM biomolecules. This chapter describes mainly the behavior of [Ru(II)(η^6-arene)(L)Z] (Z: leaving group) complexes regarding their solution equilibrium (stability, stoichiometry) and kinetic properties, their interaction with LMM components of serum,

proteins and protein models. In addition the analogous Os(II), Rh(III) and Ir(III) complexes are also considered briefly.

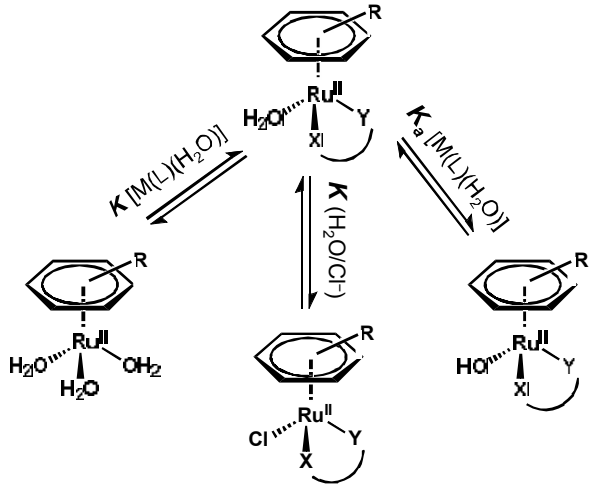


Chart 2. Possible ligand exchange processes of the half-sandwich η^6/η^5-organometallic Ru(II), Rh(III), Os(II) or Ir(III) complexes via the example of a Ru(II) complex with the general formula of [Ru(II)(η^6-arene)(XY)H_2O].

One of the best known antitumor organoruthenium compound is [Ru(η^6-biphenyl)(en)Cl][PF_6] (en: ethylenediamine) possessing an IC_{50} value of 7.6 μM measured in human ovarian cancer cells [81]. In the case of this type of complexes various equilibrium processes can take place in aqueous solution as Chart (**2**) shows. It is assumed that the aqua complex [Ru(II)(η^6-arene)(L)H_2O] embodies the bioactive species, accordingly the exchange of the chlorido ligand to water should proceed with adequate rate and extent [12]. Deprotonation of the coordinated aqua ligand leads to the formation of the less active mixed hydroxido [Ru(II)(η^6-arene)(L)OH] complex, thus a pK_a value higher than the physiological pH is advantageous [11]. The Cl^-/H_2O exchange rate in the compound [Ru(η^6-biphenyl)(en)Cl][PF_6] ($k(H_2O) = 1.23 \times 10^{-3}$ s^{-1}) is one order of magnitude higher than that of the cisplatin, and the displacement of the neutral en ligand by *e.g.* the negatively charged acetylacetonate (acac) increases the rate constant. On the other hand the type of the co-ligand (Z) has also a strong impact on the exchange process, namely the substitution of the chloride by bromide, iodide ions or by pyridine decreases the rate constant in this order and most probably the too slow pyridine/H_2O exchange process results in the inactivity of the pyridine complex [12]. The ratio of the aqua, chlorido and hydroxido species was calculated for [Ru(η^6-biphenyl)(en)Cl][PF_6] at equilibrium state at various chloride ion concentrations corresponding to the blood serum, cytosol and cell nucleus, respectively (Fig. **2**) [80]. The

predominant presence of the chlorido complex was found in the serum which may be beneficial for the protection of the original compound against undesirable reactions already in the serum.

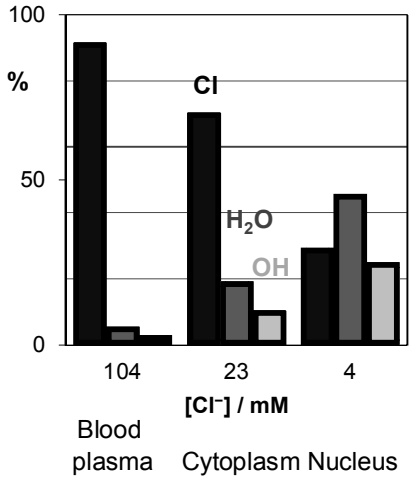


Fig. (2). Speciation of $[Ru(\eta^6\text{-biphenyl})(en)Cl]^+$ (5 μM) in blood plasma, cytosol and cell nucleus {based on the chloride ion concentrations and pH in these environments and the pK_a and Cl^-/H_2O exchange constant of the complex}. Adapted from Ref. [80].

The pK_a value of the complex determines the ratio of the aqua and the mixed hydroxido species at a given pH, and pK_a of 8.2 – 7.7 values were reported for the $[Ru(II)(\eta^6\text{-arene})(en)H_2O]$ complexes depending on the type of the arene ring. The coordination of (O,O) donor ligands such as maltol or acac increases the pK_a values [81]. However, these compounds were not so cytotoxic most probably due to the decomposition of the complexes in solution under physiologically relevant conditions. Detailed solution equilibrium studies were performed on the $[Ru(\eta^6\text{-}p\text{-cymene})(L)Cl]$ (L: maltol, acac) complexes by P. Buglyó *et al.* and their results revealed that these complexes are predominant at pH 7.4 in the mM concentration range (acac: 59%, maltol: 84%), however they suffer from significant decomposition at *e.g.* 1 μM concentration (acac: 6%, maltol: 28%) which leads to the appearance of the free ligand and the trihydroxido-bridged dimeric species $[Ru_2(\eta^6\text{-}p\text{-cymene})_2(\mu\text{-OH})_3]^+$. They also studied the solution stability of the $Ru(\eta^6\text{-}p\text{-cymene})$ complexes formed with other (O,O) donor ligands: deferiprone (dhp, 1,2-dimethyl-3-hydroxy-pyrid-4(1H)-one), and with the serum components lactate and oxalate [86]. As the basicity of these ligands differs, pM values can be calculated for comparison of the complex stabilities under identical conditions. pM is the negative logarithm of the summed equilibrium concentrations of the non-bound metal ion in its aqua and μ-hydroxido bridged dinuclear

forms. A higher pM value means the stronger metal ion binding ability of the ligand. On the basis of literature data pM values were computed for various Ru(η^6-*p*-cymene) complexes (Fig. **3**) [86-90]. Among the chosen bidentate compounds (such as the (O,O) donor containing ligands: dhp, maltol; the (O,N) donor ligand: 8-quinolinol. 8-quinolinol forms complex with the highest stability among these ligands. The metal binding ability of oxalate, acac, lactate and Ala is significantly lower. It can be concluded that even a large excess of lactate can not replace considerably the ligand dhp in the [Ru(η^6-*p*-cymene)(L)Cl] complex on the basis of the stability constants at pH 7.4 [866,87].

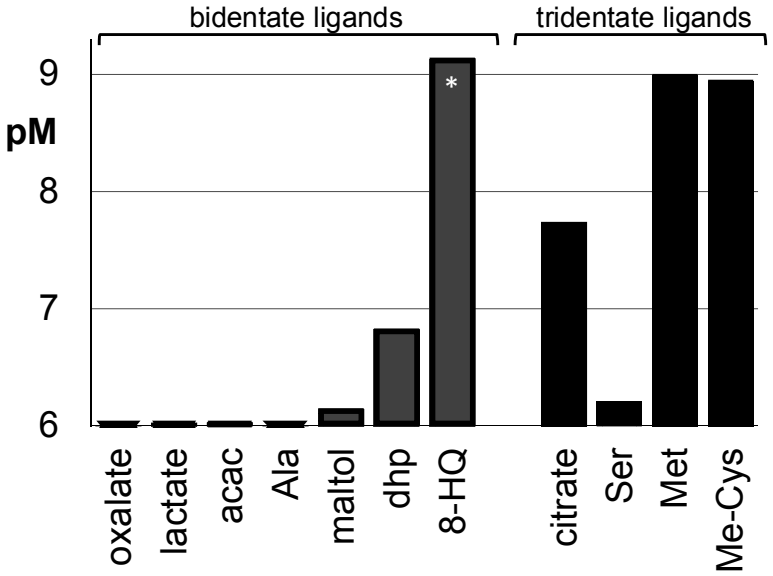


Fig. (3). pM values calculated for the Ru(η^6-*p*-cymene) complexes formed with various bi- or tridentate ligands at pH = 7.40; pM = – log[M], where [M] is the equilibrium concentration of the ligand-free, unbound organometallic fragment {$c_{Ru} = c_L$ = 1 μM, I = 0.20 M KCl or KNO_3 in case of 8-quinolinol (*)}. Calculations are based on data from Refs. [86-90].

Sadler *et al.* investigated the interaction of complex [Ru(η^6-biphenyl)(en)Cl][PF_6] with numerous bioligands such as His, Met and Cys as the side chain donor atoms of these amino acids are involved most probably in the interaction with proteins [12]. Interaction with His and Met resulted in the formation of 22% and 23% mixed ligand complexes, after 24 h and 48 h incubation period, respectively. While Cys was found to replace the ligand en and a sulfur-bridged dimeric complex was formed with 50% abundance [12].

The interaction of [Ru(η^6-*p*-cymene)(H_2O)$_3$]$^{2+}$ with Met and S-methyl cysteine (Me-Cys) was reported to be fairly fast by Buglyó *et al.* and the coordination via (N,COO^-,S) donor set was proved [90]. The stability of these complexes is much higher compared to that

of the analogous species formed with Ser that binds via (N,COO^-,O^-) donor set, due to the stronger preference for the sulfur donor atoms (see Fig. **3**) [87]. While the thiolato donor containing thiolactate and thiomalate ligands form exclusively dimeric sulfur-bridged complexes with this organometallic cation in a wide pH range [91]. Based on these findings the relatively low affinity of the $[Ru(\eta^6\text{-biphenyl})(en)Cl]^+$ complex towards Cys and Met (*vide supra*) is originated from its kinetic inertness [12]. Studies on complexation of $[Ru(\eta^6\text{-}p\text{-cymene})(H_2O)_3]^{2+}$ with the protein His side chain model N-methylimidazole revealed slow interaction, although the stability of the mono, *bis* and *tris* complexes formed is significantly high [92]. The coordination of the rather accessible surface imidazole nitrogen donors (His) of lysozyme and HSA to the Ru(II) centre was reported by Sadler in the case of $[Ru(\eta^6\text{-biphenyl})(en)Cl]^+$ complex [80,81].

It is noteworthy that varying the central Ru(II) ion to other isoelectronic metal ions (*e.g.* Os(II), Rh(III), Ir(III)) all kinds of thermodynamic and kinetic parameters of the organometallic complex are changed. *E.g.* the rate of the Cl^-/H_2O exchange is 40-fold lower in the $[Os(\eta^6\text{-biphenyl})(en)Cl]^+$ complex compared to the analogous Ru(II) species and the pK_a of the analogous Os(II) aqua complex is also lower by 1.4 order of magnitude [12,77]. The hydrolytic processes of the various organometallic cations such as $[Ru(II)(\eta^6\text{-}p\text{-cymene})(H_2O)_3]^{2+}$, $[Os(II)(\eta^6\text{-}p\text{-cymene})(H_2O)_3]^{2+}$, $[Rh(III)(\eta^5\text{-Cp*})(H_2O)_3]^{2+}$ and $[Ir(III)(\eta^5\text{-Cp*})(H_2O)_3]^{2+}$ also differ in aqueous solution as Fig. (**4**) shows in the absence and presence of chloride ions [93,94]. (In terms of metal ions or metal complexes hydrolysis means that coordinated water molecules are subject to deprotonation.) The tendency of these *tris*-aqua cations to hydrolyze is the following: Os(II) > Ir(III) > Ru(II) > Rh(III) in the absence of chloride ions, on the other hand the presence of chloride ions results in a shift of the appearance of the hydrolyzed species to the higher pH values.

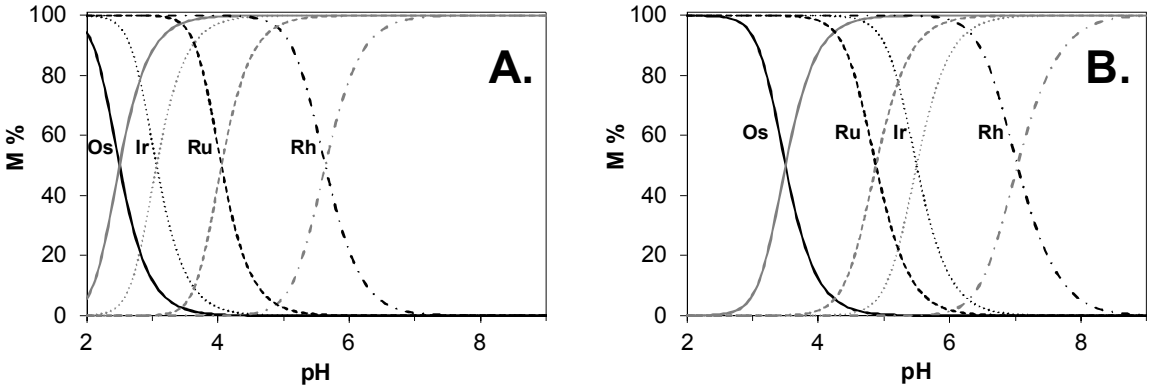


Fig. (4). Hydrolytic processes of the organometallic cations $[Ru(II)(\eta^6\text{-}p\text{-cymene})(H_2O)_3]^{2+}$, $[Os(II)(\eta^6\text{-}p\text{-cymene})(H_2O)_3]^{2+}$, $[Rh(III)(\eta^5\text{-Cp*})(H_2O)_3]^{2+}$ and $[Ir(III)(\eta^5\text{-Cp*})(H_2O)_3]^{2+}$ (black

curves) as a function of pH at I = 0.20 M KNO_3 (**A**) or KCl (**B**). Grey curves show the sum of μ-hydroxido dimeric species $[M_2(\mu\text{-}OH)_i]^{(4-i)+}$, i= 2, 3, M = organometallic fragment {c_M = 1.0 mM}. Calculations are based on data from Refs. [93,94]

Besides the characterization of the hydrolysis of the cation $[Rh(III)(\eta^5\text{-}Cp^*)(H_2O)_3]^{2+}$, its complex formation processes with various bidentate ligands were also studied in details via combined approaches using pH-potentiometry, ^1H NMR spectroscopy and UV-Vis spectrophotometry in the absence and presence of chloride ions [94-96]. On the basis of the determined stability constants concentration distribution curves were calculated for the $[Rh(III)(\eta^5\text{-}Cp^*)(H_2O)_3]^{2+}$ – maltol equimolar system and similar calculations were performed for the analogous Ru(II)(η^6-*p*-cymene) complexes (Fig. **5**). It can be concluded that the decomposition of the complexes leading to the formation of the dimeric $[Rh_2(\eta^5\text{-}Cp^*)_2(\mu\text{-}OH)_3]^+$ and $[Ru_2(\eta^6\text{-}p\text{-cymene})_2(\mu\text{-}OH)_3]^+$ species occurs at higher pH values in the case of the organorhodium complexes despite the higher stability constants of the organoruthenium complexes. This phenomenon is originated from the stronger affinity of the Ru(II)(η^6-*p*-cymene) fragment to the hydroxide ions [93,94].

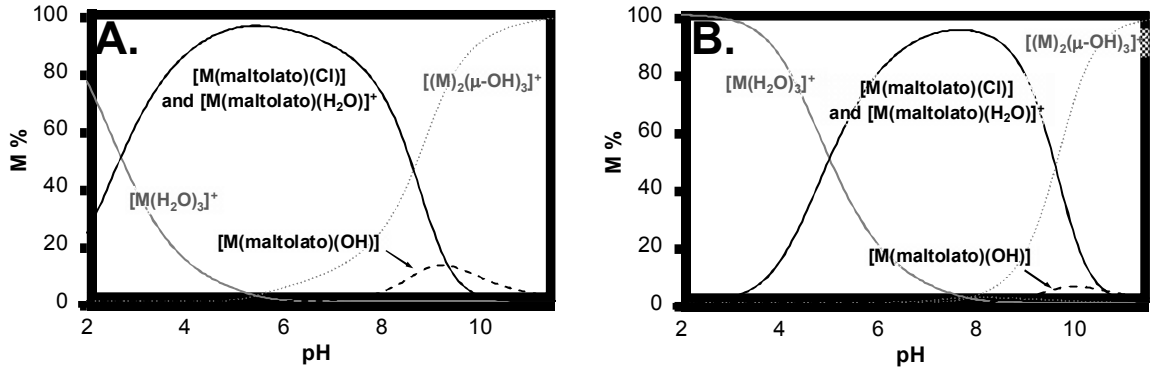


Fig. (5). (**A**) Concentration distribution curves for the $[Ru(II)(\eta^6\text{-}p\text{-cymene})(H_2O)_3]^{2+}$ or (**B**) $[Rh(III)(\eta^5\text{-}Cp^*)(H_2O)_3]^{2+}$ – maltol systems; M = organometallic fragment {c_M = c_{maltol} = 1.0 mM, I = 0.20 M KCl }. Calculations are based on data from Refs. [86,94]

Interaction of $[Rh(III)(\eta^5\text{-}Cp^*)(H_2O)_3]^{2+}$ and Rh(III)(η^5-Cp*) complexes of bpy, en, 2-picolinic acid (pic) and dhp with HSA was studied by ^1H NMR spectroscopy, ultrafiltration/UV-Vis and spectrofluorometry at 102 mM chloride ion concentrations [95]. It was pointed out that the Rh(III)(η^5-Cp*) fragment is able to bind at least 8 sites involving binding events at sites I and II. The complexes can be bound to HSA in various modes and

extent depending on the nature of the coordinating ligand, thus the stability of the complexes. Namely, the binding is mainly dissociative in the case of the lower stability dhp complex and the ligand is cleaved off, while the bpy and en complexes possessing much higher stability represent complex-protein adduct formation (Fig. **6**). The binding is most probable at the His nitrogen donor atoms of HSA [95].

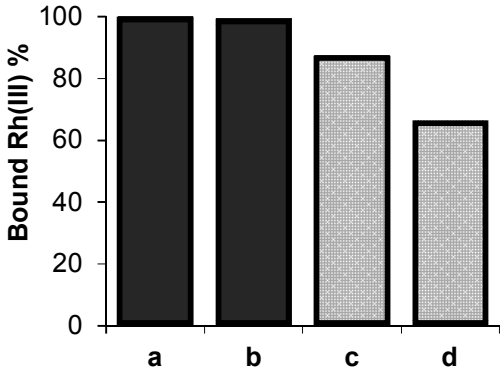


Fig. (6). HSA binding ability of the organometallic cation $[Rh(III)(\eta^5\text{-}Cp^*)(H_2O)_3]^{2+}$ (a) and its complexes $[Rh(III)(\eta^5\text{-}Cp^*)(L)Z]$ formed with L = dhp (b), bpy (c) or en (d); Z = Cl^- or H_2O {c_{HSA} = $c_{complex}$ = 50 μM, pH = 7.4 c_{Cl^-} = 0.10 M}. Black and grey bars show when the complex binds as Rh(III)(η^5-Cp*) fragment and as the whole complex retaining the ligand coordinated, respectively. Data are taken from Ref. [95].

4.4. Anticancer gallium(III) complexes

Numerous Ga(III) complexes are able to inhibit the proliferation of tumor cells and Ga(III) was the second metal ion after Pt(II), to be administered to cancer patients [97]. KP46 and *tris*(maltolato)gallium(III) (GaM) are the most promising orally active agents currently undergoing clinical trials [51,98]. In these octahedral Ga(III) complexes the coordination of the negatively charged bidentate ligands provides higher hydrolytic stability, lipophilicity and thus bioavailability compared to those of the simple inorganic salts such as $Ga(NO_3)_3$. The relatively good water-solubility of GaM allowed the determination of the stability constants of the $[GaL]^{2+}$, $[GaL_2]^+$ and $[GaL_3]$ complexes (where L is the completely deprotonated form of the ligand) formed in aqueous solution in the Ga(III) – maltol system by the traditional approach (combined use of pH-potentiometry and ^1H NMR spectroscopy in the mM concentration range). At the same time stability constants of the 8-quinolinolato Ga(III) complexes owing poor water-solubility were determined in highly diluted aqueous solutions

by UV-Vis spectrophotometry and spectrofluorometry, and were also determined from extrapolation of values obtained in 30% and 60% (w/w) DMSO/H_2O mixtures by pH-potentiometry [99]. The actual chemical forms of KP46 and GaM in aqueous phase can be estimated using these stability constants and can be compared to each other at various pH values. For this reason concentration distribution curves of the *tris*-ligand Ga complexes of maltol and 8-quinolinol were computed at a biologically relevant low concentration (Fig. **7**). In both cases, the $[GaL_3]$ species are the predominant complexes at pH 7.4, although partial decomposition resulting in free ligand and $[Ga(OH)_4]^-$ is more pronounced in the case of maltol due to the considerably lower stability of its complexes.

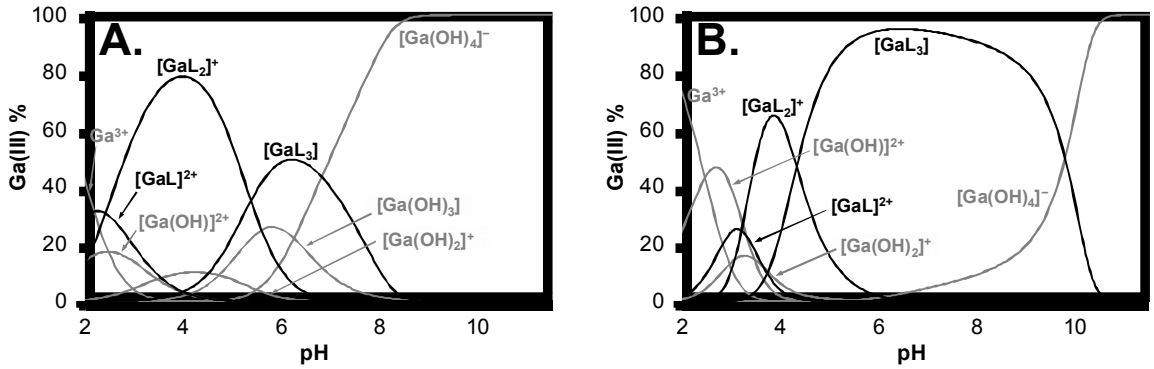


Fig. (7). (**A**) Concentration distribution curves of Ga(III) − maltol system and (**B**) Ga(III) − 8-quinolinol system at 1:3 metal-to-ligand ratio. {$c_{Ga(III)}$ = 20 μM, c_{ligand} = 60 μM, I = 0.20 M (KCl)}. Data are taken from Ref. [99].

As it is described here, despite the structural similarities of these Ga(III) complexes, KP46 possesses much higher solution stability and lipophilicity than GaM. These features have a profound effect on their binding processes with serum proteins [100]. GaM was reported not to be bound to HSA, while a moderate binding of KP46 to HSA via non-covalent bond retaining the coordination environment around the metal ion was proved ($\log K'$ = 4.04, K_D = 91 μM) by the combined use of fluorometric and saturation transfer difference NMR measurements. The binding of KP46 on HSA takes place at IB binding site based on modeling calculations. Tf is ready to compete for Ga(III) with maltol or 8-quinolinol, though the substitution of maltol occurs to a much higher extent in comparison with that of 8-quinolinol. Based on these findings equilibrium modeling calculations on the speciation of GaM and KP46 in the presence of HSA and Tf were performed; and an unequivocally different speciation was obtained (Fig. **8**). Tf plays significant role in the case of GaM, namely this protein is able to replace maltol completely at concentrations lower than 30 μM.

In contrast, KP46 was estimated to bind primarily to HSA under physiologically relevant conditions. The interaction of KP46 with HSA is reversible and does not change the original coordination mode of the complex, which might be beneficial with respect to increased half life and solubility. This finding also suggests a Tf-independent gallium uptake mechanism of KP46 [100].

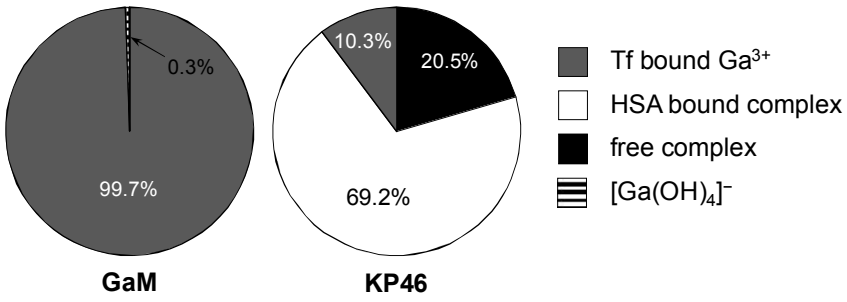


Fig. (8). Concentration distribution diagrams for the GaM − HSA – Tf and KP46 − HSA – Tf systems. {$c_{complex}$ = 20 μM, c_{HSA} = 630 μM, c_{Tf} = 37 μM considering that 30% of the binding sites on Tf are saturated with Fe(III), pH = 7.4}. Data are taken from Ref. [100].

5. BIOSPECIATION OF INSULIN-ENHANCING METAL COMPLEXES IN BIOLOGICAL FLUIDS

In the past years we have published several reviews in this field [1, 43, 101-105], and thus there may be significant similarity of this section with some of them. When a larger part of the text is very similar to one of these former reviews a reference number will be given. However, in most cases the original paper is cited.

Diabetes mellitus (‘diabetes’ or DM) is a group of metabolic diseases in which there is high blood glucose level over a prolonged, sometimes nearly a lifetime period. In the case of DM type 1 the insulin-producing cells in the body have been destroyed, while in the case of DM type 2 they are not producing enough insulin or the cells of the body are not responding properly (partial resistance) to the insulin produced. As of 2015, an estimated 415 million patients had type 2 DM worldwide [106], which usually appears in people over the age of 40, making up about 90% of the cases [107]. Type 1 diabetes can be only treated with insulin, a natural peptide (protein) hormone composed of 51 amino acids, also over 40% of patients with type 2 diabetes require, as part of their treatment, insulin injections. Since peptides currently cannot be taken orally because of their degradation in the GI tract, insulin is given as an injection [108]. This painful and expensive treatment should be replaced by an insulin ’mimicker’ which can be taken orally. Many metal ions such as Cr(III), Mn(II), Mo(VI), Se(V), V(IV/V), W(VI) and Zn(II) have insulin-like effect.

5.1. Insulin-enhancing vanadium compounds

Insulin-like effects of vanadium were first demonstrated *in vivo* by McNeill and co-workers [109] by simple adding of sodium orthovanadate (Na_3VO_4) to the drinking water of streptozotocin (STZ)-diabetic rats for several weeks. Later the only partly soluble vanadates were replaced by $VOSO_4$ resulting in decreased side effects [110-112]. However, the applied necessary dose of vanadium still was close to the levels at which adverse effects are observed [113]. The question became then, ‘‘is there some way to chemically improve potency of these drug candidate anti-diabetic agents?’’. Several vanadium complexes with different coordination modes were synthesized and characterized in order to answer this question. The most frequent basic structures (‘backbones’) are depicted in Chart (**3**). Most of them are neutral *bis* complexes formed with bidentate organic ligands, where the oxidation state of the vanadium is +4 [101]. The only exception is the $[VO_2(dipic)]^-$ with negative charge and +5 oxidation state of the central metal ion [114]. All these complexes are labile, and ready to take part in fast ligand exchange reactions.

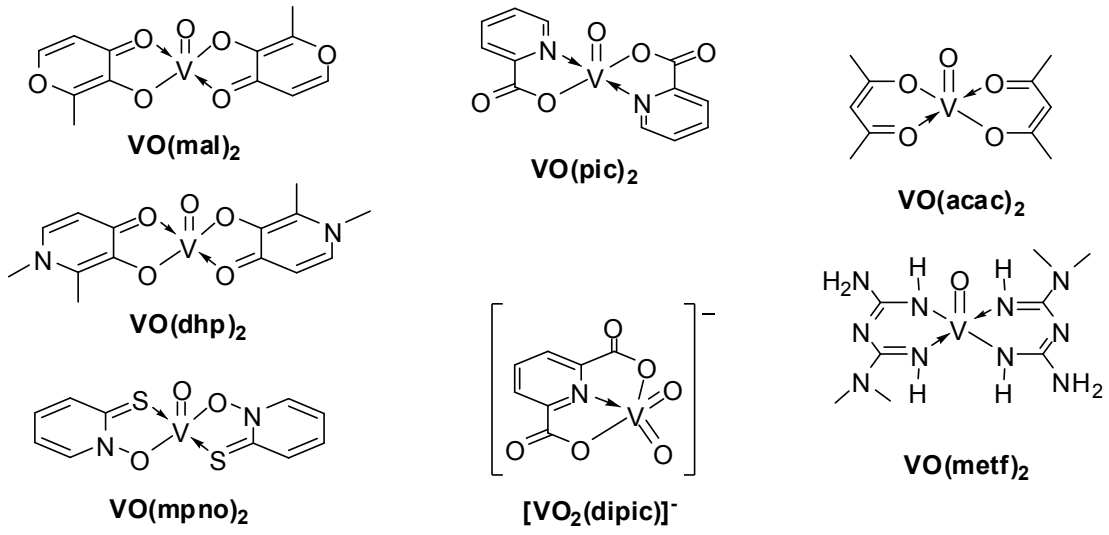


Chart 3. Basic structure of insulin mimetic vanadium complexes studied in detail. (The water molecules in the coordination sphere are omitted.)

Only one comparative study of some of these drug candidate vanadium complexes has been made, and it was pointed out that these complexes have 30–70% of the activity of insulin in insulin-depleted mice fibroblast cell culture tests [115]. Based on that publication it would be impossible to differentiate between the effectiveness of the vanadium compounds studied.

Confessing the truth, only one of these compounds were tested in humans: the Phase I clinical trial [113] of *bis*(ethylmaltolato)oxovanadium(IV) (BEOV, the ethyl- derivative of

$VO(maltolato)_2$) was completed in 2000, and the results of the Phase IIa clinical trial were first published in 2009 [116]. The clinical Phase I trials proved that an orally administered 10-90 mg BEOV single dose is safe and tolerable by healthy people, overall bioavailability of vanadium from BEOV was three times higher than that of vanadium from vanadyl sulfate and fasted subjects absorbed vanadium thirteen times better than fed subjects [113].

In Phase IIa tests over the course of a 28-day treatment period (daily dose 20 mg complex: 5.8 μmol or 3 mg vanadium), BEOV was consistently well-tolerated. A positive effect was observed in most of the treated subjects, such that reductions in fasting blood glucose were observed when compared to the two placebo subjects [116]. The tests have, however, been abandoned due to renal problems with some of the probands [117].

The orally administered vanadium complex should go first throught the GI tract and after the blood serum before arriving to the targeted cell. Biotransformation is possible in the entire route, and the absorption efficacy from the GI tract can be improved by formulation of the drug including encapsulation technique [118]. However, the original carrier ligand can be replaced by serum/plasma components or endogenous binding molecules, before the cells would be able to take up the complex.

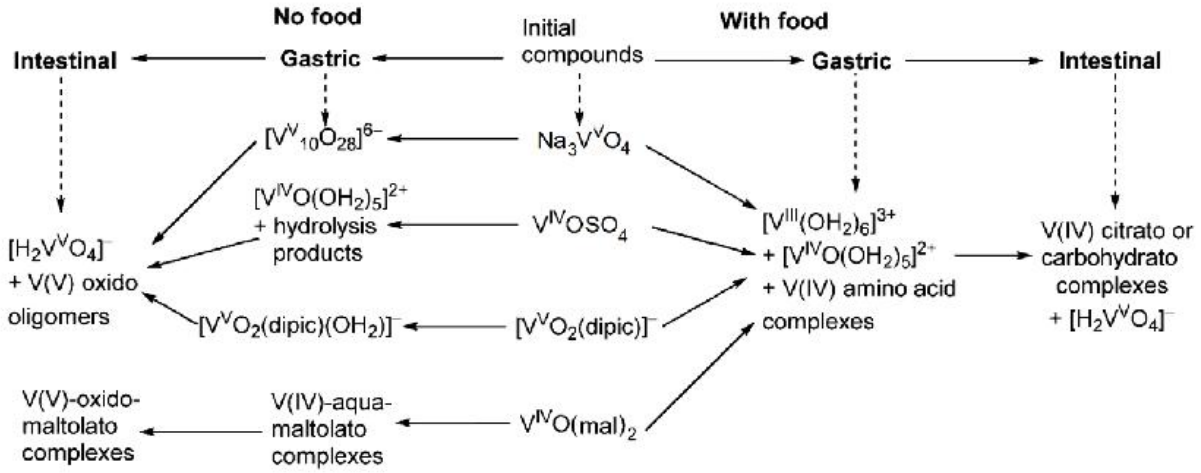
The active species is vanadate ($H_2VO_4^-$) most likely, one possible final product of the biotransformation of any kind of vanadium compound. The possible mode of action is the inhibition of a protein tyrosine phosphatase at the cytosolic site of the cellular insulin receptor and/or the activation of a tyrosine kinase in the signaling pathway [119].

5.1.1. Speciation of vanadium complexes in the GI tract

Based on speciation studies [101,102] it is clear that in consequence of the parallel processes of protonation of the metal-binding sites of the coordinating ligands, the neutral *bis* complexes ($VO(maltolato)_2$ [120]. $VO(dhp)_2$ [121], $VO(pic)_2$ [122], $VO(mpno)_2$ (where mpno = 2-mercaptopyridine-N-oxide) [123], $VO(acac)_2$ [124] and their derivatives) will certainly partly decompose in the acidic pH range, *e.g.* at the pH (*ca.* 2) of the gastric juice. The species formed in this way will be charged, and will possess entirely different membrane transport properties. The $[VO_2(dipic)]^-$ complex is stable at pH 2 but it has already a –1 charge.

Recently a useful speciation method, assessment of the chemical states of V, in biological environments was published [125]. Classification can be done on the basis of a three-dimensional diagrams of pre-edge and edge parameters in X-ray absorption near edge structure (XANES) spectra, developed on the basis of a library of model V(V/IV/III) complexes. Based on this method XANES results reported the speciation of four vanadium

compounds mimicking oral administration by artificial digestion [126], although the applied vanadium concentration was fairly high, namely 1.0 mM. (Artificial gastric/intestinal juice has been prepared,; incubations and commercial liquid semi-synthetic meals were used.)



Scheme 2. Proposed biotransformation of the initial vanadium compounds in GI environment based on XANES data taken after artificial digestion. (c_V = 1.0 mM) Taken from Ref. [126].

Typical anti-diabetic V(V) and V(IV) complexes undergo profound chemical changes in GI media. The main observation is that in the absence of food V(IV) is oxidized to V(V) only in the intestine and only the dipic dissociates (intestinal), while maltol does not. While in the presence of food reduction of V(V) to V(IV) and even to V(III) takes place already in gastric environment together with the total dissociation of the original carrier ligands (Scheme **2**). The observed significant difference between the presence and absence of food is in a complete coincide with the absorption difference of BEOV between the fasted/fed subjects reported in Phase I trial [113] (Section 5.1).

As the overall bioavailability of BEOV was three times higher than that of vanadium from vanadyl sulfate (Phase I, Section 5.1 [113]) it seems that passive diffusion of neutral species is the most effective absorption process, while the other possibilities *e.g.* vanadate like phosphate or $V(IV)O^{2+}$ species via M^{2+} uptake mechanisms or V(III)/Fe(III) pathways are less important.

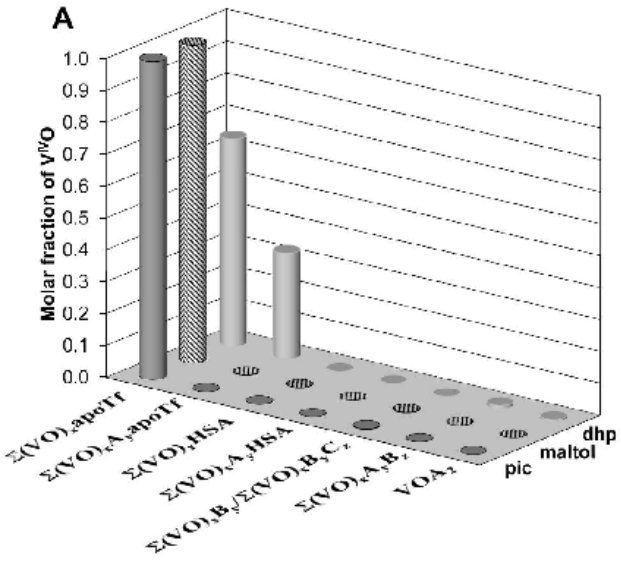
All exogenous and endogenous biomolecules being present in the stomach or intestines, where the complexes are absorbed, may play a role in V(IV)O binding. Interactions with these molecules could change the net charge of the complex unfavorably, which will decrease their absorption efficacy. This certainly has to be taken into account during the formulation of the drug (*e.g.* by encapsulation techniques, whereby these problems may well be overcome). Results of Sakurai *et al.* [118] support this prediction. In their study $VOSO_4$

was administered orally in various formulations: in solution, in gelatin capsules and in enteric-coated capsules. It was found that administration of the V(IV)O salt in encapsulated forms improved the metal ion absorption as compared with that associated with the simple solution form. As far as we know, up to now, no such experiment has been done with vanadium complexes.

5.1.2. Speciation of vanadium(IV) and vanadium(V) in blood serum

The maximum daily oral dose in the Phase I clinical trial was 95 mg BMOV, equivalent to 15 mg or 0.22 mg/kg vanadium for a 70 kg person) [113]. For an absorption efficacy of 30% [127] and an overall blood content of 5 L, if all of this vanadium enters the blood at the same time, the maximum concentration attainable would be ~20 μM. However, this is only a rough estimation, but it clearly shows a well-defined limit. In animal studies involving much higher doses up to 12 mg/kg vanadium, two independent research groups determined the maximum vanadium concentration in the blood to be 2–3 μg/mL, *i.e. ca.* 40–60 μM. The maximum value of the vanadium concentration in the human blood during treatment (Phase I-IIa) was not published. Modeling calculations were performed in order to explore the potential biotransformation processes in serum [101,102].

The speciation [1] of the metal ion among the LMM and HMM components of blood serum, and the original carrier ligands, including mixed ligand species was calculated based on stability constants and concentration data at three concentration levels of antidiabetic compounds (1, 10 and 100 μM), the results are summarized in Fig. (**9**).



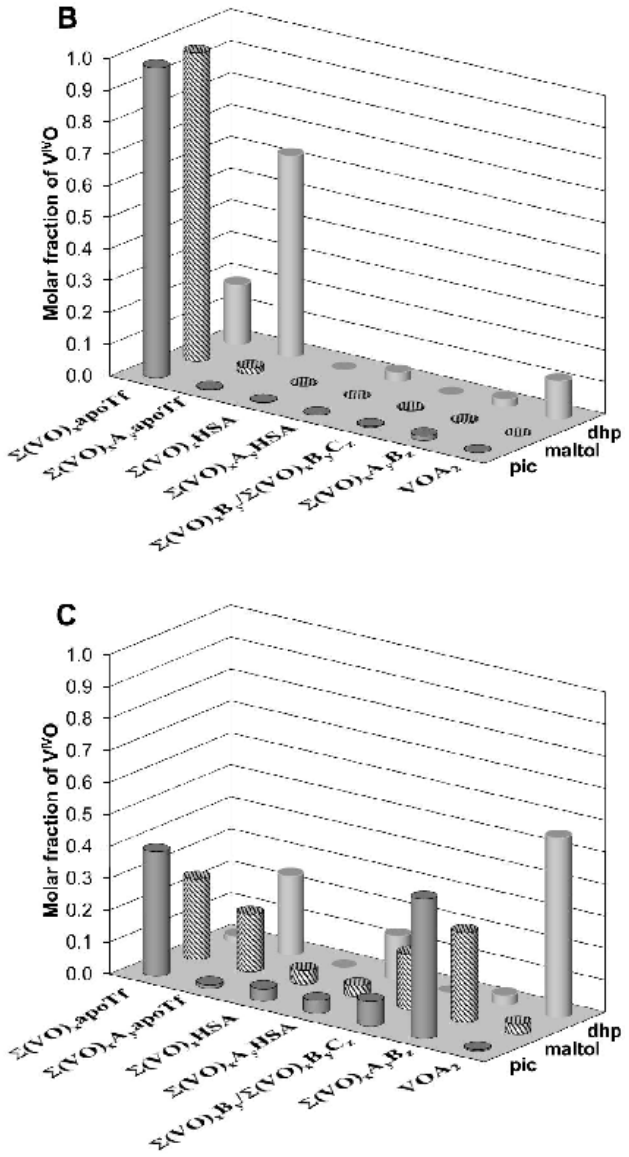


Fig. (9). Speciation of various potentially antidiabetic V(IV)O compounds (**A**: 1 μM, **B**: 10 μM, **C**: 100 μM) in serum at pH 7.4. In this presentation the sum of the concentration of the similar type of species are depicted: VOA_2: V(IV)O bound in the binary *bis* complex, $(VO)_xB_y$: binary species formed with the LMM components of the serum, $(VO)_xB_yC_z$: ternary species of the LMM components of the serum, $(VO)_xA_yB_z$: ternary species of an antidiabetic complex with LMM components of the serum, $(VO)_x$apoTf: binary species of V(IV)O with apoTf, $(VO)_xA_y$apoTf: ternary species of an antidiabetic complex with apoTf, $(VO)_x$HSA: binary species of V(IV)O formed with HSA, $(VO)_xA_y$HSA: ternary species of an antidiabetic complex with HSA; A: carrier ligands, pic, maltol, dhp; B and C: LMM components of the serum: citric acid, lactic acid, phosphate. "B" taken from Ref. [1] "A" and "C" calculated similarly based on the published data in Ref. [1].

The following conclusions could be drawn: (i) It is clear that apoTf, one of the two important HMM binders, is much more efficient than HSA and will displace 90-95% (1 μM and 10 μM V(IV)O compound concentration levels) of the original carrier from the complex or will form ternary complexes with them. (ii) Only the hydroxypyridinone derivative dhp is a strong enough carrier ligand to preserve a significant proportion of the V(IV)O in the original complex or still bound to V(IV)O in a ternary complex with apoTf. In the other two cases (pic, maltol), the carrier ligands are completely displaced by serum proteins. Similar behavior can be expected from the $VO(acac)_2$, as the acac ligand is much weaker metal ion binder than dhp, maltol and pic. (iii) Among the LMM binders, citrate is the main 'active' component, able to influence the solution state of these antidiabetics but only at 100 μM vanadium compound ($VO(pic)_2$,$VO(maltolato)_2$) level, when there is no Tf enough to bind all the metal ions. Among the LMM components the ternary complex(es) with the original carrier ligands dominate. (iv) The HSA containing fraction is negligible at 1 μM and 10 μM V(IV)O compound concentration level, similarly to citrate, it is able to bind the metal ion (or complex) only when the apoTf is already saturated with V(IV)O. The total vanadium containing HSA fraction is lower than 20% in all three cases even at 100 μM vanadium compound level. (v) The speciation is strongly concentration dependent in the 10-100 μM V(IV)O concentration range.

The dominance of apoTf in V(IV)O binding was confirmed with the use of native blood serum measurements by ultrafiltration, separation through a 10 kDa membrane, the LMM and the HMM fraction bound V(IV)O was measured by atomic absorption spectroscopy method [103,104]. Similarly, the protein bound V(IV)O was separated by anion exchange chromatography and determined by ICP-MS. Only Tf was able to bind V(IV)O, the binding ability of the other important serum protein HSA was negligible [103].

Accordingly, the most important role of the carrier ligand seems to facilitate the absorption of V(IV)O from the GI tract, but the complexes fall apart at last in the serum. Pharmacokinetic investigations proved this prediction by using labelled $V(IV)O(maltolato)_2$ complexes [128].

It should be mentioned, that even at higher concentrations of the V(IV)O complexes, such as mM level, which exceed the serum level of the strongest V(IV)O binder protein Tf, HSA also becomes an important binder of the V(IV)O species in serum, due to its significantly higher concentration [1]. However, there is no real clinical importance of this

observation, as for example in the whole blood the LC_{50} value of vanadate is in the 2-5 mM range (estimated based on [129]).

Among several drug candidate ligands (hpno/mpno/pic/dhp), the hpno (2-hydroxypyridine-N-oxide, the O derivate of mpno) forms the highest stability complex with V(V) at pH 7.4 [130]. The $[VO_2(dipic)]^-$ decomposes at this pH, as it is stable only in weakly acidic solution [1144]. Based on modeling calculation it is clear that at biologically relevant concentrations, c(V(V)) < 10 μM, the Tf is the only V(V) binder in the blood serum. Under such conditions, neither the carrier ligands, nor HSA nor the LMM biomolecules present in the serum (lactate, citrate, phosphate, Gly or His) form sufficiently strong complexes to compete with apoTf, even though *ca.* 5% of the V(V) exists as free $H_2VO_4^-$ ion in solution [130].

XANES speciation studies of antidiabetic vanadium complexes in the whole blood [131] were carried out using the same classification method on 3D diagrams of pre-edge and edge parameters similarly to GI speciation (Section 5.1.1). The outcome suggests an important role of the red blood cells in the biospeciation of vanadium, however the applied concentration of vanadium was 1 mM, which is therapeutically irrelevant, and makes the results questionable.

5.1.3. Speciation of vanadium in the cells

Vanadium, either in oxidation state IV or V, mainly binds to Tf in human serum (see Section 5.1.2). Accordingly, vanadium may be assumed to enter the cell through the Tf receptor following the iron pathway.

In the intracellular environment, reducing agents can redox-interact with vanadate(V). A frequently discussed candidate for the reduction is GSH [132]. A high intracellular excess of GSH increases the possibility of formation of V(IV)O and its complexation with either GSH or GSSG. Both have been shown to be reasonably potent binders for V(IV)O [132-134]. Other effective reducing agents, such as NADH or ascorbate, may cause the formation of even V(III) species [135,136]. Hydrolytic degradation of V(IV)O may be responsible for the reoxidation to vanadate(V).

Among the LMM binders, the widely distributed ATP may also be of importance [137], as it efficiently binds V(IV)O and is also present in millimolar concentration in cells. Comparing the V(IV)O complex-forming properties of ATP and GSH, it can be concluded that in the whole pH range ATP is a more efficient V(IV)O binder. When ATP and GSH are simultaneously considered as potential V(IV)O binders, GSH is not expected to be able to

compete with ATP for binding to V(IV)O. Since ATP is a strong V(IV)O binder, ATP will chelate the metal ion, forming binary and/or ternary complexes, and thus might somehow be involved in the antidiabetic action of the V(IV)O compounds.

XANES spectroscopic studies [138] on vanadium uptake and speciation in mammalian cells and cell culture media were carried out also by P. Lay and coworkers, similarly to the other two XANES studies (GI tract/blood). In this work they experimentally proved the earlier criticism of some of the authors of this paper: (Section 5.1.2) namely the 1.0 mM vanadium concentration is therapeutically irrelevant. Such conditions were toxic for the cells at >8 h treatments, but the authors can not use lower V concentration because of the X-ray fluorescence detection limit. [139] However, the conclusions of this study are that the easy interconversions of V(IV) and V(V) species in the cells, *i.e.* the antidiabetic V(V) and V(IV) complexes undergo profound transformations in cell culture media, and the resultant products are further metabolized by cultured mammalian cells.

After vanadium enters the body and undergoes several biotransformations finally it may be excreted or accumulated by different tissues. Direct comparison studies between ^{48}V-BMOV and $^{48}VOSO_4$ demonstrated a similar pattern of biodistribution to that of inorganic vanadium salts observed earlier: the order of relative accumulation is bone > kidney > liver. The absorption level is low, the bones retaining only the *ca.* 0.1% of an oral dose/g tissue 24 h after an oral dose of $VOSO_4$, however half-life of elimination is quite long (>10 d for ^{48}V in bone after a single dose 10 μM by oral gavage in rats). [105,113]

5.2. Speciation of insulin-enhancing zinc(II)-complexes in blood serum

However, Zn(II) is not so effictive in insulin mimesis as vanadium, as it is an essential metal ion it may be introduced more easily as a medical drug. Studies were also carried out with some Zn(II) complexes (*e.g.* $Zn(pic)_2$; $Zn(maltolato)_2$ $Zn(dhp)_2$ and $Zn(dipic)_2$) having insulin-enhancing activity in the concentration range of 100 μM level [1]. The possible interactions of the drug candidate complexes in blood with the LMM components were studied by pH-potentiometry in details [140,141], based on modeling calculation among these binders (without the HMM components) His and Cys and their ternary complexes are the primary Zn(II) binders [142].

HSA binds Zn(II) with conditional binding constants in the range of log$K$' = 7.1–7.9 [11,143]. α2-macroglobulin has also significant Zn(II) binding properties though found at much lower concentration in the serum than albumin (2–6 μM), with the reported conditional formation constants logK'_1 = 7.49 and logK'_2 = 5.12 [144,145]. The third possible Zn(II)

binder is apoTf, the Zn(II) binding constants are logK'_1 = 7.8 and logK'_2 = 6.4 (at 15 mM $NaHCO_3$) [146].

When both the HMM and the LMM serum binders are considered without the presence of any carrier ligand, the modeling calculations showed that most of the total serum Zn(II) is bound to the serum proteins (~98%) and HSA is the primary binder (80–90%) [37,140,147, 148] followed by α2-macroglobulin (5–15%) [144,147,148]. However, the role of apoTf is still under discussion [147,149], a minority of Zn(II) is mobile and circulating attached to LMM components or as free metal ion.

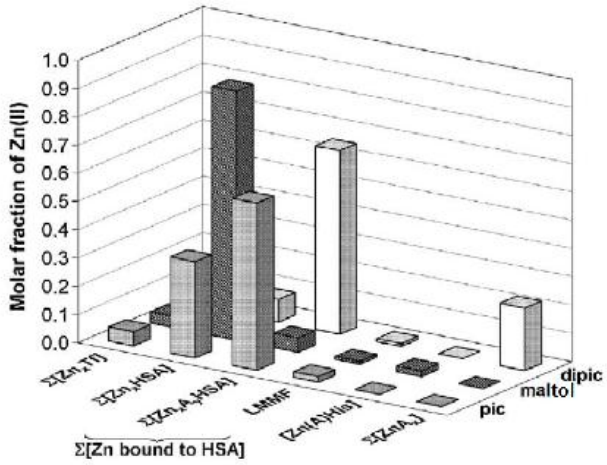


Fig. (10). Speciation of various potentially antidiabetic Zn(II) compounds in serum pH 7.4. Taken from Ref. [1]

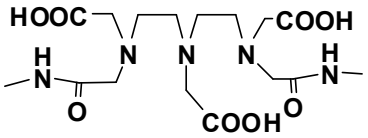
In order to investigate the binding properties of the Zn(II) compounds to blood serum components under more realistic conditions, human serum samples were incubated with the Zn(II) complexes. CZE experiments with ICP-MS detection was used to quantify the metal ion binding towards the different serum components. Most of the Zn(II) was bound to HSA (*ca*. 80–95%) as identified via the ^{34}S trace. A minor amount (<5%) of Zn(II) was still coordinated to the original carrier (or other buffer components); however no Zn(II) was detected bound by Tf [140].

The latest modeling calculation on the Zn(II) distribution in serum samples containing 100 μM of insulin-enhancing *bis*-ligand complex are depicted in Fig. (**10**). Formation of binary complexes [Zn(II)-carrier ligands]; [Zn(II)-LMM compounds], mixed ligand complexes [Zn(II)-carrier ligands-LMM compounds] and the interactions between serum proteins and Zn(II), and also between HSA and the carrier ligands were considered [140]. The

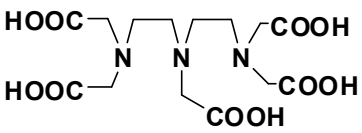
results of the modeling calculations (see above) were confirmed experimentally not only by CZE-ICP-MS but also by ultrafiltration-ICP-atomic emission spectroscopy [141] studies.

6. SPECIATION OF MRI CONTRAST AGENTS

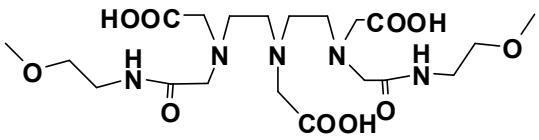
Magnetic resonance imaging (MRI), a powerful and indispensable medical diagnostics technique is used to image the anatomy and the physiological processes of the body. MRI has a wide range of applications in medical diagnostic and over 25,000 scanners are estimated to be used worldwide [150]. MRI is based upon NMR spectroscopy, the most frequently studied nuclei is ^{1}H. By varying the parameters of the pulse sequence, different contrasts can be generated between tissues, based on the relaxation properties of the hydrogen atoms therein. Contrast agents (CAs) usually shorten, but in some instances increase the value of spin-lattice relaxation time (T_1) of nearby water protons thereby altering the contrast in the image [151]. Approximately 35% of the clinical MRI applications apply paramagnetic complexes (or superparamagnetic, ferromagnetic substances) as CAs [152]. The trivalent Gd(III) is the most efficient relaxation agent among all paramagnetic cations due to its high electron spin (S = 7/2) and slow electron spin relaxation and therefore it is the most commonly used ion in MRI as CAs. Other paramagnetic metal ions, in particular the non-toxic biocompatible Mn(II) (S = 5/2) have also been considered as MRI contrast agent and their role becomes more and more important [153].



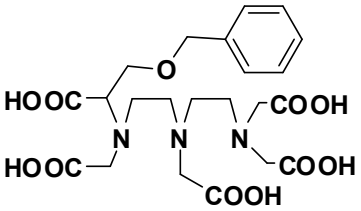
H_3DTPA-BMA
Gd(DTPA-BMA): Omniscan



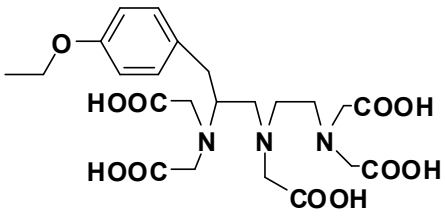
H_5DTPA
$(NMG)_2$Gd(DOTA): Magnevist



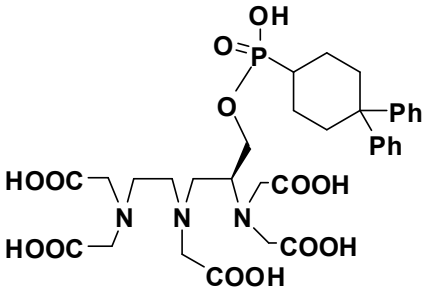
H_3DTPA-BMEA
Gd(DTPA-BMEA): OptiMARK



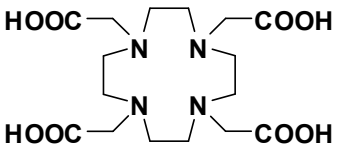
H_5BOPTA
$(NMG)_2$Gd(BOPTA): Multihance



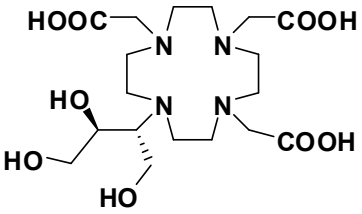
H_5EOB-DTPA
$(Na)_2$Gd(EOB-DOTA): Primovist/Eovist



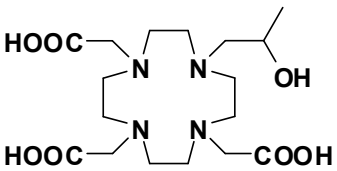
diphenyl-cyclohexyl phosphodiester-H_5DTPA
$(Na)_3$Gd(diphenyl-cyclohexylphosphodiester - DOTA): Vasovist/Ablavar



H_4DOTA
(NMG)Gd(DOTA): Dotarem



H_3DO3-butrol
Gd(DO3-butrol): Gadovist/Gadavist



H_3(HP-DO_3A)
Gd(HP-DO3A): ProHance

Chart 4. Chemical structures of ligands, formulae, and trade names of Gd(III) complexes used as contrast agents (NMG: N-methylglucosamine)

As the free Gd(III) ion is toxic [154], it is necessary to be chelated with appropriate multidentate ligands (Chart **4**), which ensure a high thermodynamic stability and/or kinetic inertness.

The first model calculation on Gd(III) speciation in serum conditions has been made by Jackson and his coworkers in 1990 [155]. They used mainly literature data with ionic strength and temperature corrections, or even estimated values when it was necessary. They already concluded that at the normally applied Gd(III) CAs level (>0.1 mM) there is no apoTf enough to bind significant amount of Gd(III), in the absence of chelator the Gd(III) supposed to be coordinated by LMM serum components mainly amino acids, lactate and citrate. The possible importance of the phosphate was proposed, as $Gd(III)PO_3$ is a precipitate. Theoretically it should form, however the authors were not able to include it into the speciation model. $Gd(III)PO_3$ was not possible to detect experimentally either, most probable due to the kinetically slow decomposition of the Gd(III)-containing CAs. A weak relationship between the thermodynamic stability of a Gd-CAs – calculated free Gd(III) concentration and the toxicity (LD_{50}) was observed.

The major part of the clinical Gd(III)-based agents, which mean approximately the 90% of all clinical contrast agent injections, are non-specific, have low molecular weight, and are localized in the extracellular space [152]. At present, nine different Gd(III)-aminopolycarboxylate complexes are used in the clinics, they are derivatives of the open chain H_5DTPA (diethylenetriaminepentaacetic acid) or the macrocyclic H_4DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) [156,157] (Chart **4**).

Table 4. The detected number of NSF and the conditional stability constant of different number of CAs. (Data are taken from Ref. [158])

Chemical name	Commercial name	Detected number of NSF[a]	log*K*' (conditional stability constant at pH 7.4)
Gd-DTPA-BMA	Omniscan	163	14.9
Gd-DTPA	Magnevist	85	17.7
Gd-DTPA-BMEA	OptiMARK	9	15.0
Gd-BOPTA	MultiHance	2	18.4
Gd-HP-DO3A	ProHance	0	17.1

[a] Total number of clinical applications of the individual drugs are not known, During the mid-2000s Omniscan (market share: ~50%) and Magnevist (market share: ~40%) dominated the application in the USA [159].

The expanding clinical use of Gd(III)-based contrast agents was halted in 2006, when the development of a newly observed disease, namely nephrogenic systemic fibrosis (NSF),

was assumed to be related to the use of these Gd(III) complexes [160,161]. NSF is a very rare, highly debilitating, life-threatening disease, which was observed only in patients with severe or end-stage renal disease [160-167]. The number of NSF cases reported up to the end of 2007 was around 400, but due to the recommendations of health authorities, the number of cases has dramatically reduced and no new cases have been reported [163,168,169]. About 75–80% of the known NSF cases could be associated with the use of Omniscan (Gd(DTPA-BMA)), where BMA = *bis*-(methylamide)) and far fewer diseases have been reported in patients treated with Magnevist (Gd(DTPA)), and only a few cases have been observed after the use of macrocyclic agents [160,164,170-174].

In Table **4**. the detected number of published NSF (FDA's Med-Watch database for NSF as of May 2, 2007) and the conditional stability constants log*K*' were collected together for five Gd(III)-CAs. Although some tendencies are clear, it is seen that there is no direct connection between the thermodynamic stability and the observed number of caused NSF patients. For example in spite of the lower stability constant, the chemical properties of Gd(DTPA-BMA) were considered to be favorable for *in vivo* use, because in parallel with the decreased stability of Gd(DTPA-BMA), the stabilities of the DTPA-BMA^{3-} complexes of Zn(II), Cu(II) and Ca(II), which may compete with Gd(III) in body fluids, decreased more markedly [175]. Moreover, other kinetic processes may play a role in the dissociation.

In body fluids, a lot of various metal ions and ligands may compete with the Gd(III) ion and the DTPA-BMA^{3-} ligand, respectively. Equilibrium calculations performed with a simplified plasma model including the Cu(II) / Zn(II) / Ca(II) cations, amino acids (Ala, Cys, Glu, Gly, His and Lys), HSA and Tf, and other small biomolecules (hydrogencarbonate, phosphate, citrate, lactate, malate and succinate) [176]. The model predicts, that under physiological conditions (pH = 7.4, T = 310 K, I = 0.15 NaCl) phosphate ion is the only endogenous ligand that can compete with DTPA-BMA^{3-} for binding of Gd(III). Approximately 17% of the Gd(DTPA-BMA) dissociate to form $Gd(PO_4)$ as a precipitate. As the ligand DTPA-BMA is also released it is able to form complexes with the endogenous metal ions such as Zn(II), Cu(II), and Ca(II). Due to the relatively high (0.35 mM) concentration of the contrast agent, the free DTPA-BMA could collect approximately ~80% of the serum Zn(II) and ~90% of the Cu(II) content [176].

However, decomposition of the Gd(DTPA-BMA) in human serum is controlled kinetically, not thermodynamically. The decomposition of the complex occurs parallel with its elimination from the body, the amount of Gd(III) released from Gd(DTPA-BMA) in body fluids depends on the rates of decomposition and elimination of the complex. Based on rate

data from pharmacokinetic studies on the elimination of Gd(DTPA-BMA) and the rate data characterizing the dissociation of the Gd(III) complex, a two-compartment open model has been developed (Fig. **11**) to assess the amounts of Gd(III) released in body fluids [176].

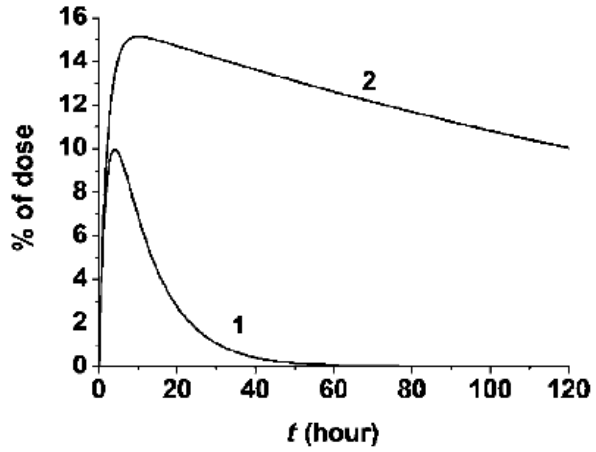


Fig. (11). Simulation of the amounts of 'free' Gd(III) released during the elimination of Omniscan from subjects with normal (1) and severely impaired (2) renal function (the dose of CA was 0.1 mmol/kg body weight). Taken from Ref. [176].

In patients with normal renal function the rate data predict the elimination of about 95% of the administered Gd(DTPA-BMA) within *ca.* 48 h. In patients with severe renal impairment, the elimination of the Gd(DTPA-BMA) is slow ($t_{1/2}$ ~30–40 h) and during the long residence time larger amounts of complex may dissociate and significant amounts of Gd(III) (approximately 12.5% in 5 d after the administration) may be deposited in their body [176].

7. CONCLUSIONS

In this review different examples were given how the behavior of the metal complexes (primarily the character of the metal ion) in the various biological milieu (fluids and tissues) can influence the reactions of these compounds with endogenous biomolecules, their decomposition, *etc*., which will basically affect their ADME properties.

The serum speciation of the metal ion will basically determined by its kinetic inertness or lability, the strength of its Lewis acidity and its binding ability to the different serum constituents. Low molecular mass binders provide the easily exchangeable, mobile forms of the metal ions, while high molecular mass components namely serum proteins, serve as the less mobile metal ion pool. Alkaline and alkaline earth metal ions will be hardly or relatively

weakly bound to serum components. On the other hand heavy metal ions can bind to biomolecules much stronger, which can be either advantageous (*e.g.* in metalloproteins), but disadvantageous as well, interfering normal function of essential biomolecules. For this reason, in any case when a metal ion with strong affinity to biomolecules is going to added to a biological system it has to be surrounded (wrapped) by a carrier molecule to hinder its interaction with the endogenous biomolecules.

Anticancer, antidiabetic compounds and MRI diagnostics were used to illustrate how the thermodynamic parameters and the kinetic behaviour of the compounds will affect their solution state in the biological milieu. What is the distribution of these compounds when they are transported in the blood serum between the LMM and HMM fractions (protein and non-protein bound mobile and less mobile fractions of these drugs which *e.g.* strongly affects their transport properties and their absorption In the case of the MRI contrast agents their thermodynamic and kinetic properties gave answer to the appearance of a side effect of the Gd(III) containg agents, which reflected attention to other metal ion (Mn, Fe) containg MRI agents.

FUTURE PERSPECTIVES

Several examples have been given in this paper representing how stability data and speciation modeling calculations can be used to provide detailed description of the distribution of metal ions or metal compounds with physiological activity in biological systems. Basic condition of making modeling speciation calculations is the need of correct speciation models and stability data. For this reason we need reliable databases. There are enormous amount of stability data in the literature. These, however, should be critically evaluated and classified into groups according to the needs of the potential users to help them to find more easily the data they need. This work has already been started and critically evaluated databases are also available in the literature (*e.g.* IUPAC Stability Constants Database), even some for special users, but there is still enough work to do in this field.

The parallel use of thermodynamic and structural investigating methods is needed with a complete identification of each species formed in the equilibrium system. In many cases the *in vitro* determination and quantification of the metal containing species confirm the *in vivo* results in biological fluids or tissues. Due to the not high enough sensitivity of the experimental methods, it is quite frequent that not a complete confirmation of the results of the modeling calculations can be achieved but only the sum of the concentration of a group of the species can be determined, and so only a kind of fractionation can be experimentally

verified. The other way of improving the reliability of modeling calculations is to increase the sensitivity of experimental techniques to provide *in vivo* confirmation of the modeling calculations for real systems. We can prove for the potential users with these efforts that modeling calculations can provide reliable results for conditions when there is no way for direct experimental measurements.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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ABBREVIATIONS

acac	acetylacetonate
apoTf	apotransferrin
ATCUN motif	amino terminal Cu(II)- and Ni(II)-binding motif
BEOV	*bis*(ethylmaltolato)oxovanadium(IV)
BMA	*bis*-(methylamide)
bpy	2,2′-bipyridine
CA	contrast agent
Cis	cystine
Cp*	pentamethylcyclopentadienyl
CZE	capillary zone electrophoresis
dhp	1,2-dimethyl-3-hydroxy-pyrid-4(1H)-one, deferiprone
DM	diabetes mellitus
DMSO	dimethyl sulfoxide
en	ethylenediamine
EPR	electron paramagnetic resonacne
ESI-MS	electrospray ionisation mass spectrometry
FA	fatty acid
GaM	*tris*(maltolato)gallium(III)
GI	gastrointestinal tract
GSH	glutathione
H_4DOTA	1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid
H_5DTPA	diethylenetriaminepentaacetic acid
HMM	high molecular mass

hpno	2-hydroxypyridine-N-oxide
HSA	human serum albumin
ICP-MS	inductively coupled plasma mass spectrometry
KP46	*tris*(8-quinolinolato)gallium
LMM	low molecular mass
maltol	3-hydroxy-2-methyl-4H-pyran-4-one
MBS	multi-metal binding site
mpno	2-mercaptopyridine-N-oxide
MRI	magnetic resonance imaging
NADH	nicotinamide adenine dinucleotide
NAMI-A	imidazolium [*trans*-tetrachlorido(dmso)(imidazole)ruthenate(III)]
NKP-1339	sodium [*trans*-tetrachlorido*bis*(1*H*-indazole)ruthenate(III)]
NSF	nephrogenic systemic fibrosis
NTS	N-terminal site
phen	1,10-phenanthroline
pic	picolinic acid
PTA	1,3,5-triaza-7-phosphatricyclo-[3.3.1.1]decane
Tf	human serum transferrin
UV-Vis	UV-visible
XANES	X-ray absorption near edge structure

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