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Research Article

Biodistribution of anti-diabetic Zn(II) complexes in human serum and *in vitro* protein-binding studies by means of CZE–ICP-MS

Application of modern analytical technology for studying the fate of metallodrugs after administration to the blood is of utmost importance for drug development. Zn(II) compounds are under development as insulin-enhancing drugs with potential use in the treatment of diabetes. In comparison to the well-established vanadium compounds, especially the lower risk of adverse effects due to the essentiality of the element in biological processes is advantageous. Herein, CZE–ICP-MS studies on the interaction of Zn(II)-maltolato, -2-picolinato and -2,6-dipicolinato complexes with human serum proteins are discussed and modeling calculations were confirmed by experimental results. Studies with human serum reveal preference for HSA over other less abundant proteins and serum components.

1 Introduction

Diabetes mellitus (DM) is a disease from which people suffer all over the world, and with a continuously increasing number of patients, especially in the industrialized world. It is considered as a syndrome of disordered metabolism resulting in abnormal high blood glucose level (hyperglycemia) due to defects in either insulin secretion or insulin action. There are three forms of DM: the insulin-dependent type 1, the non-insulin-dependent or adult-onset type 2 and the gestational DM. Type 1 is an autoimmune disease caused by a lack of the insulin hormone due to the destruction of insulin-producing β-cells, and it is therefore treated by insulin replacement administered as daily injection. Type 2 DM is characterized by insulin resistance, *i.e.* lack of proper answer of the cells to the presence of insulin. This kind of DM can be usually treated by a well-controlled use of drugs, which are able to increase the

quantity of secreted insulin or the sensitivity of target organs to insulin, or to decrease the rate of glucose absorption from the gastrointestinal (GI) tract. The available drugs for DM type 2 are mostly oral hypoglycemic agents such as biguanide, sulfonylurea, thiazolidinediones and α-glucosidase inhibitors. Since these anti-hyperglycemic chemicals do not yield satisfactory results for all patients, there are efforts on the development of new anti-diabetic drugs with high efficacy and with no or minor adverse effects [1, 2].

In recent years, metal complexes and organometallic compounds have gained considerable interest for medicinal applications [3–5]. Cu, Cr, Mo, V, Zn, Mn, *etc.* species were found to exhibit insulin-enhancing activity [2, 6–8] and vanadium compounds in the oxidation states IV and V are among the most effective representatives [6–8]. More than 100 vanadium complexes have been developed and tested both *in vitro* and *in vivo* [7–10], out of which bis(ethylmaltolato)oxovanadium(IV) (Fig. 1) has completed phase I clinical trials and has advanced to phase II studies [11].

In addition to the V compounds, anti-diabetic Zn(II) complexes have gained considerable interest for the treatment of DM in the last few years [7]. They were found less effective than the vanadium complexes [7, 10], but may be introduced more easily into the medical treatment due to the fact that Zn(II) is an essential metal ion, less toxic and better bioavailable. Zn is a cofactor for more than 200 biologically important enzymes (which are particularly involved in protein synthesis) and plays a key role in the

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synthesis and action of insulin. Moreover, Zn supplementation has also demonstrated a protective effect in DM type 2 animal models [12].

Fugono *et al*. have developed numerous Zn(II) complexes and some of the complexes bearing, *e.g.* maltol (mal) or 6-methyl-picolinic acid ligands were found to be active in KK-A^y mice when administered orally [13]. The effective Zn(II) complexes have a favorable effect not merely on the blood glucose level, but on the concentrations of adipocytokines such as free fatty acids, resistin, leptin, adiponectin, *etc*. and also on obesity and the systolic blood pressure [14]. The mode of action of anti-diabetic Zn(II) complexes is still not completely understood: Most probably they act at different sites of insulin signaling due to the activation of tyrosine kinase (of the insulin receptor), phosphatidyl inositol-3 kinase, phosphodiesterase enzymes and glucose transporters, and due to the inhibition of the protein tyrosine phosphatase [14]. Furthermore, these drug candidates are regarded as prodrugs. Hence the active forms are assumed to differ from the administered species as a consequence of biotransformation processes in different biological environments such as the GI tract, blood serum or the cytosol. According to recent studies, the carrier ligand determines mainly the absorption from the GI tract and it may be partly or completely displaced by suitable endogenous metal ion binders in the human serum [15, 16].

Improved knowledge about the Zn(II) speciation in serum might contribute to a better understanding of the transport processes *in vivo*. Previous results obtained *in vitro* using ultrafiltration–ICP-atomic emission spectroscopy and modeling calculations on artificial serum showed that Zn(II) is mostly bound to the high molecular mass (HMM) fraction of the serum, and the binding toward HSA is predominant [16, 17]. However, analysis of *real* blood serum should provide more detailed information about the Zn(II) distribution in the case of insulin-enhancing metal complexes. Among the different separation techniques, CZE exhibits high resolution, fast and efficient separation of metal-containing species of biomolecules [18]. CZE hyphenated to an ICP-MS provides a powerful tool to detect metal ions with high sensitivity and is perfectly suited for analyzing biological samples [19–21].

Herein, the biodistribution of Zn(II) complexes of mal, 2-picolinic acid (pic) and 2,6-dipicolinic acid (dipic), which exhibit anti-diabetic effects [14, 22], in human serum

samples is described. A CZE–ICP-MS method was developed for analyzing such samples under conditions maintaining metal-protein bonds.

2 Materials and methods

2.1 Instrumentation

CZE experiments were performed with an HP^{3D} CZE system (Agilent Technologies, Waldbronn, Germany) interfaced to an Agilent 7500ce ICP-MS with a CETAC CEI-100 microconcentric nebulizer. For all experiments capillaries of 60 cm total length (75-µm id) were used (Polymicro Technologies, Phoenix, AZ, USA). Injections were performed by applying a pressure of 25 mbar for 4 s, and constant voltages of 15 or 30 kV were used for Tris or carbonate buffer as BGE, respectively. Prior to the first use, the capillary was flushed at 1 bar with 0.1 M HCl, water, 0.1 M NaOH and again with water (10 min each). Before each injection, the capillary was purged for 2 min both with water and the BGE followed by applying a voltage of 30 kV for 30 s and flushed for 1 min with BGE again. The cleaning procedure included purging with 0.1 M HCl, water and 0.1 M NaOH (each for 2 min). The operational values for the CZE–ICP-MS interface are shown in Table 1. The nebulizer was employed in self-aspiration mode with the sheath liquid closing the electrical circuit and spraying a fine aerosol. Analyses were only started if a sufficiently stable signal (RSD ^{72}Ge < 5%) was attained.

2.2 Reagents and solutions

A 0.1M $ZnCl_2$ stock solution was prepared by the dissolution of $ZnCl_2$ (Reanal) in a defined amount of HCl (0.007 M) and its Zn concentration was determined by complexometry *via* the EDTA complex. NaOH, HCl and NH_4HCO_3 were obtained from Fluka; mal, pic and dipic (*puriss*) were from Sigma Aldrich, as were HSA (approximately 99%, fraction V), apotransferrin (apoTf; approximately ≥ 98%) and human serum. The concentrations of the protein solutions were estimated from their UV absorption: $\varepsilon_{280\,nm}(apoTf) = 92300\ M^{-1}\,cm^{-1}$ and $\varepsilon_{280\,nm}(HSA) = 36850\ M^{-1}cm^{-1}$ [23, 24]. The ICP-MS tuning solution
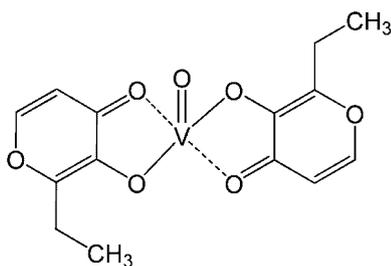

Figure 1. Structure of the vanadium compound BEOV (AKP-020), undergoing in clinical trials [11].

Table 1. ICP-MS conditions

Sampler	Ni (0.1 mm orifice)
Skimmer	Ni (0.4 mm orifice)
Plasma RF power, W	1500
Isotopes recorded	^{34}S, ^{72}Ge, ^{64}Zn, ^{66}Zn
Nebulizer gas flow rate, L/min	1.14
Sheath liquid	20 mM Tris with 20 ppb Ge or 10 mM ammonium bicarbonate with 20 ppb Ge
BGE	20 mM Tris or 10 mM ammonium carbonate

containing Li, Y, Ce, Tl and Co in 2% HNO_3 (each 10 mg/L) as well as the Ge standard were obtained from Agilent Technologies. High purity water used throughout this work was obtained from a Millipore Synergy 185 UV Ultrapure Water system (Molsheim, France).

2.3 Sample preparation

The Zn(II) complexes were synthesized *in situ*: A $ZnCl_2$ solution was added to the ligand in 1 mM Tris buffer or in 20 mM carbonate buffer (both pH 7.4). Depending on the denticity of the ligand (bidentate or tridentate) and the conditional stability constants of the formed complexes (at pH 7.4), the ratio $ZnCl_2$ ligand was varied (for dipic 1:2, for mal and pic from 1:2 to 1:6). For *in vitro* studies on the binding of the Zn compounds toward HSA and apoTf, samples containing 0.1 mM of $ZnCl_2$, 0.2 mM of the ligand and 0.1 mM of the serum proteins were prepared in 1 mM Tris buffer or 20 mM carbonate buffer. For the HSA–Zn-dipic titration, 100 µM HSA was mixed with 50–300 µM $[Zn(dipic)_2]^{2-}$ in carbonate buffer for 24 h. The samples for the competitive binding studies contained 100 µM of HSA, apoTf and Zn(II) and 200 µM dipic in carbonate buffer and were kept for 24 h at 25°C. The human serum was diluted 1:4 with the incubation buffer and the mixtures were incubated at 25°C for 24 h prior to the analysis. The experiments were repeated at least three times.

2.4 Data processing and calculations

Modeling calculations on the speciation of the Zn(II)-complexes in an artificial serum containing HSA, human serum transferrin (Tf), α_2-macroglobulin (α_2M) as HMM and L-cysteine (Cys), L-histidine (His), citric acid (cit) as low molecular mass (LMM) serum components were performed using the computer program PSEQUAD [25] based on the stability constants taken from Refs. [16, 17, 26–29] at physiological pH and at 25°C in the presence of HCO_3^-. The other LMM components of the serum were omitted from the modeling calculations because of their negligible Zn(II)-binding abilities [30]. The concentrations of the serum components used for the calculations were equivalent to those in the diluted blood serum [31].

The CZE–ICP-MS data was recorded using the Chem-Station software bundle (Agilent Technologies), data analysis was performed using Microsoft Excel 2003.

3 Results and discussion

Zn(II) is present at relatively low concentration in the human blood serum (the normal level is *appr.* 10–15 µM [31, 32]). When anti-diabetic Zn(II)-complexes are administered in animal DM type 2 models, the Zn(II) level was increased significantly to 100–200 µM [13]. HSA, α2M and Tf as HMM components, and Cys and His as LMM components are considered as the most efficient Zn(II) binders according to their blood concentrations and Zn(II)-binding abilities [16, 17, 26–29, 31, 32]. HSA is the most abundant serum protein (630 µM) with a molecular weight of *appr.* 67 kDa; it is negatively charged at physiological pH and has a key role in transport and/or storage processes of fatty acids, Cu(II), Ni(II), toxic metabolites (*e.g.* bilirubin) or drugs [18, 33, 34]. HSA is able to bind Zn(II) with conditional binding constants in the range of logK' = 7.1–7.9. The binding site is situated at the interface of domains I and II, which consists of two His-nitrogen atoms (His-67, His-247), two carboxylate-oxygen donor atoms (Asn-99, Asp-249) and a water molecule in the coordination sphere, and the Zn center adopts a distorted trigonal bipyramidal geometry [26, 34]. α2M is a fairly large protein with 720 kDa, but found at much lower concentration in the blood than albumin (2–6 µM), and with conditional formation constants of logK$_1$' = 7.49 and logK$_2$' = 5.12 it has also considerable Zn(II)-binding properties [29, 35–37]. Another possible Zn(II) binder is the 79 kDa iron transport protein Tf (37 µM in serum), but 30% of the binding sites are occupied by Fe(III) ions in the serum [27, 33]. The Zn(II)-binding constants are logK$_1$' = 7.8 and logK$_2$' = 6.4 (15 mM $NaHCO_3$) [27] and the presence of the synergistic HCO_3^- is also considered important.

Literature data show that most of the total serum Zn(II) is bound to the serum proteins (*appr.* 98%) and HSA is the primary target (80–90%) [32, 35, 37], followed by α2M (5-15%) [35–37]. However, the role of Tf is under discussion [36, 38] and also a minority of Zn(II) is circulating unbound or attached to LMM components (considered as the mobile portion of the metal ion).

In the case of anti-diabetic VO(IV) complexes the knowledge of the binding constants, characterizing their interactions with the HMM and LMM components of the blood serum, allows to calculate the distribution of VO(IV) among the various serum constituents. It was found that under physiological conditions in serum, Tf is the primary VO(IV) transporter and the binding ability of HSA is practically negligible, as confirmed by HPLC–ICP-MS analyses [39].

Zn(II) complex formation with mal, pic and dipic results predominantly in *bis* species in aqueous solution at physiological pH (see Fig. 2 for the structures) [22, 40], as demonstrated by the concentration distribution curves (for the Zn(II)-dipic system see Fig. 3). The bidentate mal and pic coordinate *via* (*O,O*) and (*N,O*) donor sets, respectively, to form $[ZnA(H_2O)_4]^+$, $[ZnA_2(H_2O)_2]$, $[ZnA_3]^-$ and $[ZnA_2(H_2O)(OH)]^-$ octahedral complexes ("A" stands for the fully deprotonated form of the ligands). Dipic is able to coordinate in a tridentate mode through the (*N,O,N*) donor atoms and forms the *bis* complex $[ZnA_2]^{2-}$, which is predominant in a wide pH range [22]. Conditional constants of the *bis* complexes of mal, pic and dipic are 8.13, 9.52 and 12.77, respectively, at physiological pH. The tridentate dipic forms a significantly more stable complex with Zn(II) as compared to the bidentate ligands mal and pic [22].
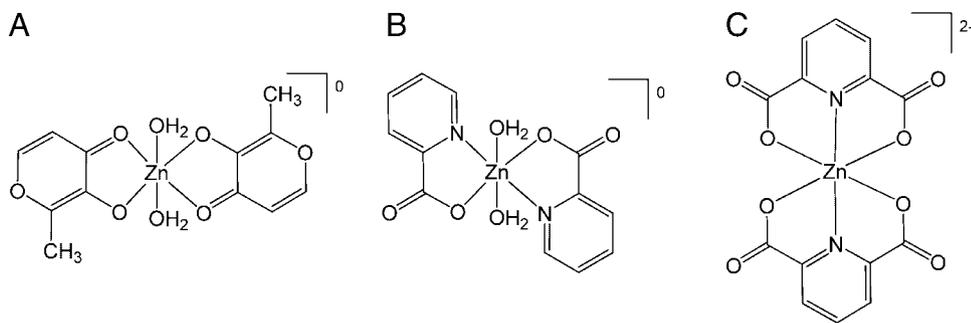
A   B   C

Figure 2. Structural formulae of the predominant species in aqueous solution at physiological pH: (A) $[Zn(mal)_2(H_2O)_2]$, (B) $[Zn(pic)_2(H_2O)_2]$, and (C) $[Zn(dipic)_2]^{2-}$.
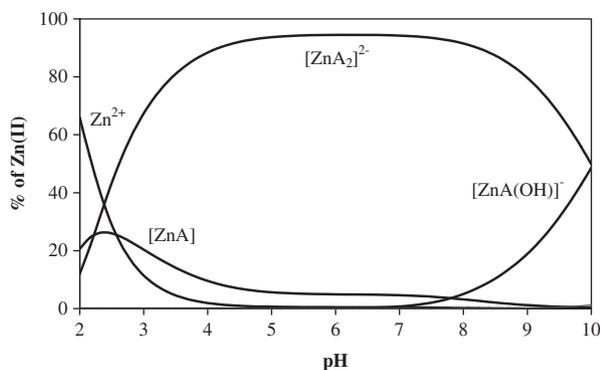

Figure 3. Concentration distribution curves for complexes formed in the Zn(II)–dipic system at 1:2 metal ion to ligand ratio; $c_{Zn(II)} = 0.1$ mM.

The migration behavior of the Zn(II) complexes was determined by CZE–ICP-MS with 1,2-dibromoethane as a neutral EOF marker. The species distribution of Zn(II)–ligand systems is highly dependent on the pH (see Fig. 3): At physiological pH the octahedral complex $[Zn(dipic)_2]^{2-}$ is the dominant species at a metal ion-to-ligand ratio of 1:2 and according to the expectations, a negatively charged species was observed in the electropherogram (Fig. 4). In the case of the bidentate ligands (mal and pic), the octahedral coordination sphere of the neutral *bis* complexes is not saturated, hence coordinated water molecules can be substituted by, *e.g.* separation buffer components. To shift the equilibrium to *tris* complexes, migrating then in the CZE mode slower than the EOF, the metal to ligand ratio was increased from 1:2 to 1:6 in the incubation solutions, since the extent of the formation of the negatively charged $[ZnA_3]^-$ species is higher at elevated ligand concentrations. In the case of the Zn(II)-mal system at a metal-to-ligand ratio of 1:2, no clear peaks were identified. When raising the ratio to 1:6, the neutral *bis* complex $[Zn(mal)_2(H_2O)_2]$ was detected in addition to a negatively charged *tris* species. Incubation of Zn(II) with an excess of pic resulted exclusively in a negatively charged complex, most probably $[Zn(pic)_3]^-$.

3.1 Interaction with HSA

To characterize the interaction between HSA and the Zn(II)-complexes with mal or dipic as ligands, samples containing
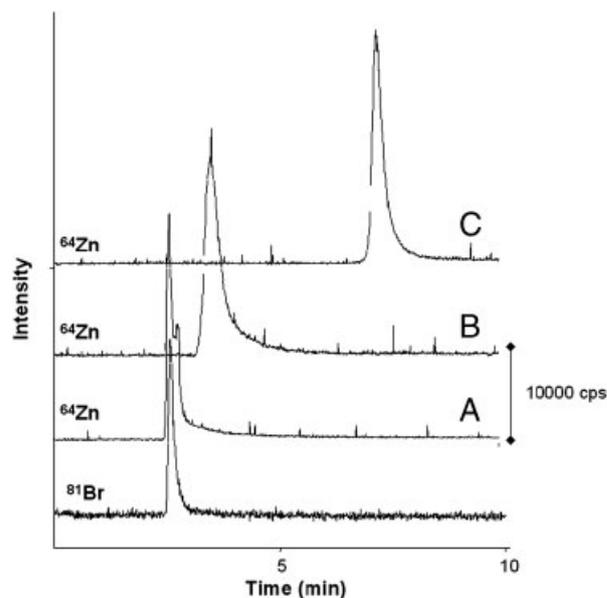

Figure 4. Comparison of the electropherograms of the Zn(II)-mal-system (1:6) (A), Zn(II)-pic system (1:6) (B) and Zn(II)-dipic-system (1:2) (C) after 24 h in carbonate buffer at pH 7.4. Shown are the traces of ^{64}Zn and ^{81}Br (EOF marker; 100 μM).

100 μM HSA, 100 μM $ZnCl_2$ and 200 μM of the ligand were prepared in 20 mM carbonate buffer (BGE: 10 mM carbonate solution at pH 7.4) or 1 mM Tris buffer (BGE: 20 mM Tris) and analyzed after an incubation time of 24 h at 25°C to ensure equilibrium conditions. Quantification of non-metal-containing proteins by ICP-MS is only accessible *via* analysis of the sulfur content. Polyatomic isotope interference from atmospheric gas and biological material can, however, cause serious errors in detection of sulfur. The most abundant ^{32}S isotope (94.93%) suffers from polyatomic interferences, such as $^{16}O^{16}O$, which cannot be resolved by conventional quadrupole instruments. The second most abundant isotope ^{34}S (4.29%) is interfered mainly by $^{16}O^{18}O$ and $^{16}O^{17}O^1H$, but quantification is still possible to a certain extent [41].

Figure 5 shows an electropherogram for the Zn(II)-dipic system in carbonate buffer, and approximately 60% of the total observed Zn content are bound to HSA. The second peak in the ^{64}Zn trace of the electropherogram was assigned to the parent complex $[Zn(dipic)_2]^{2-}$, based on the migration time and the absence of a sulfur peak. Switching
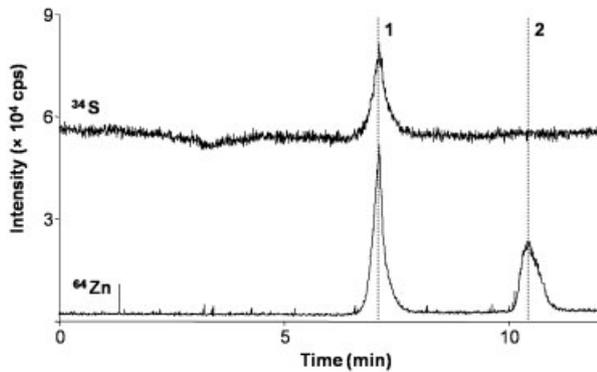
Figure 5. Electropherogram of $[Zn(dipic)_2]^{2-}$ incubated for 24 h with HSA in carbonate buffer. Shown are the traces of ^{64}Zn and ^{34}S (for HSA detection). Peak identification: 1 – HSA adduct, 2 – $[Zn(dipic)_2]^{2-}$; Conditions: see Section 2.

Figure 6. Titration of HSA with $[Zn(dipic)_2]^{2-}$ (incubated for 24 h). Legend: ■ $[Zn\text{-}dipic]_{exp}$, □ $[Zn\text{-}dipic]_{calc}$, ● $\Sigma[Zn\text{-}HSA]_{exp}$, ○ $\Sigma[Zn\text{-}HSA]_{calc}$; $\Sigma[Zn\text{-}HSA]$ stands for the summed concentrations of the binary and ternary species [Zn-HSA] and [Zn-HSA-dipic], respectively. Conditions: see Section 2.

the incubation/separation system to Tris buffer causes no significant changes. Under these conditions, *appr.* 55% of the total observed Zn content was found bound to HSA, whereas for Zn(II)-mal almost quantitative binding was observed. Modeling calculations on the Zn(II) distribution in the Zn(II)-ligand-HSA system were performed using equivalent conditions as for the CZE–ICP-MS measurements and based on the stability constants of the Zn(II)-ligand complexes, Zn(II)-binding ability of HSA and the HSA-ligand interactions [16, 17, 26] at equimolar ratio of the metal complex and HSA. Calculations show that in the case of dipic equimolar amounts of Zn are bound to the carrier ligand dipic and in a Zn(II)-HSA-ligand ternary complex. In contrast, the weak ligand mal is able to maintain merely *appr.* 6% of Zn(II) in a binary complex and the large majority of the Zn was found attached to HSA. These calculations are in good agreement to the experimental data.

Titration experiments with a constant amount of HSA (100 μM) with $[Zn(dipic)_2]^{2-}$ (from 50–300 μM; incubation time: 24 h) were performed. These studies show at low Zn(II)-dipic to HSA ratios that the majority of the Zn(II) is bound to albumin, whereas at increasing concentrations, $[Zn(dipic)_2]^{2-}$ becomes the predominant species in solution. The experimentally obtained results were compared with the calculated distribution and relatively good agreement was obtained (Fig. 6).

To characterize the binding kinetics for the reaction of $[Zn(dipic)_2]^{2-}$ with HSA, samples containing 100 μM HSA, 100 μM $ZnCl_2$ and 200 μM dipic were prepared in carbonate buffer and again analyzed by CZE–ICP-MS. Time resolved studies monitoring the peak area for the HSA adduct demonstrate that the binding of Zn(II) to HSA is very quick and already completed when the first data point was recorded (approximately 5 min after the start of the incubation).

3.2 Interaction with Tf

The same method as described for the analysis of the Zn-HSA system was transferred to the Zn-apoTf system
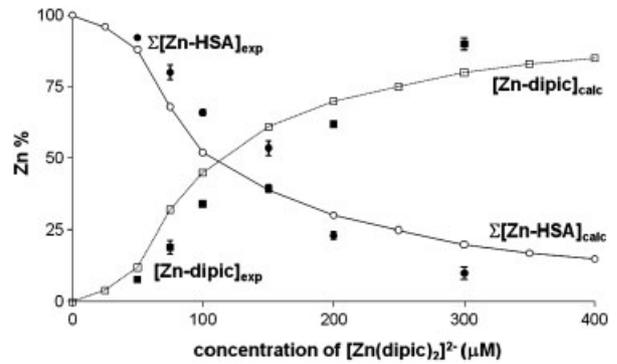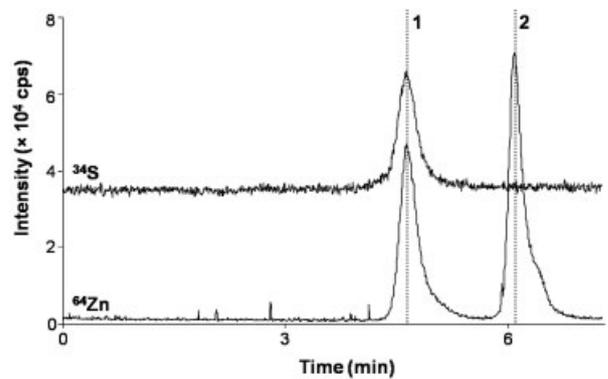

Figure 7. Electropherogram of $[Zn(dipic)_2]^{2-}$ incubated for 24 h with apoTf in carbonate buffer. Shown are the traces of ^{64}Zn and ^{34}S (for apoTf detection). Peak identification: 1 – apoTf adduct; 2 – $[Zn(dipic)_2]^{2-}$; Conditions: see Section 2.

(100 μM apoTf, 100 μM $ZnCl_2$, 200 μM dipic) and the Zn(II)-binding ability of apoTf was characterized. Incubation of apoTf with $[Zn(dipic)_2]^{2-}$ causes a partial carrier ligand displacement. In the presence of apoTf in carbonate buffer as incubation medium approximately 46% of the total observed Zn content is bound to apoTf (Fig. 7). The binding of Zn(II) to apoTf is strongly influenced by the incubation buffer: In Tris buffer nearly no interaction of Zn(II) with apoTf was observed and only 4% of the total Zn(II) content was found attached to apoTf after an incubation time of 24 h.

Hydrogencarbonate appears to act as a synergistic ion necessary for Zn(II) binding [42]. To study the role of hydrogen carbonate, the same set of samples was prepared in 1 mM Tris buffer containing 500 μM $NaHCO_3$ (incubation for 24 h) and the sample was analyzed using 20 mM Tris buffer or 10 mM carbonate buffer as BGE. When using Tris buffer as BGE, 20% of the total Zn(II) content was bound to apoTf, whereas with the carbonate buffer 50% of the Zn content was found attached to apoTf. These results demonstrate the importance of the choice of the BGE and

furthermore when using Tris as BGE, in addition to the adduct peak, broad peaks and an unstable baseline were visible in the electropherogram, most probably indicating the presence of mixed ligand species.

3.3 Competitive serum protein binding

Selective binding to a single protein after intravenous administration might be an option for exploiting a transport mechanism, *e.g.* known for Tf, causing enrichment of drugs in the desired tissue [43]. Therefore, competitive binding between HSA and apoTf was studied and it was found that, in contrast to VO(IV)-based insulin-enhancing complexes, HSA is the preferred binding partner at equimolar ratio of HSA to apoTf (42 *versus* 50 sulfur atoms (http://www.expasy.org/, ExPASy Proteomics Server, Swiss Institute for Bioinformatics): ∼64% of Zn(II) is bound to HSA, ∼25% to apoTf peak and the rest to the carrier ligand (Fig. 8); however, the peaks of the proteins are slightly overlapping and therefore no exact quantification is possible.

3.4 Interaction with proteins in human serum

To study the binding behavior of the Zn(II) complexes to serum proteins under more realistic conditions, serum samples were incubated with the Zn(II) compounds (0.1 mM; serum diluted with buffer; 1:4; 24 h; at 25°C; see caption of Fig. 10 for exact concentrations of the serum components). CZE–ICP-MS analysis revealed for the complexes with the dipic ligand that most of the Zn(II) was bound to HSA (approximately 70%), as identified *via* the ^{34}S trace. A minor amount of Zn(II) was still coordinated to dipic or other buffer components; however, no Zn(II) was detected at Tf (Fig. 9). This result might be explained by the low concentration of Tf in serum and also the iron-binding sites of transferrin are partly occupied by Fe(III), competing for the same binding site as Zn(II) [27].
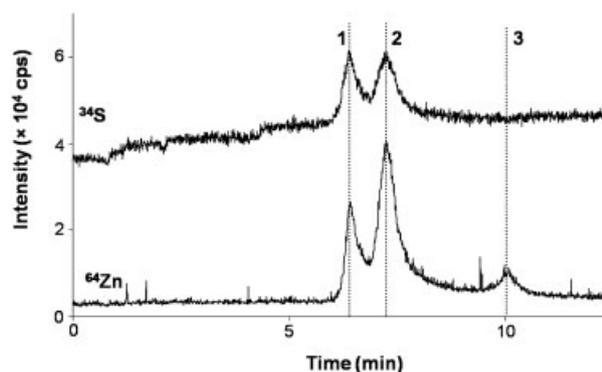
Serum incubation with $[Zn(mal)_2(H_2O)_2]$ and $[Zn(pic)_2(H_2O)_2]$ gives similar electropherograms, however nearly quantitative binding to HSA was observed.

Modeling calculations on the Zn(II) distribution in serum samples containing 100 µM of anti-diabetic *bis* complex were performed according to the conditions of the CZE–ICP-MS measurements. Formation of binary complexes [Zn(II)+carrier ligands; Zn(II)+LMM components], mixed-ligand species [Zn(II)+carrier ligands+LMM compounds] and the interactions between serum proteins and Zn(II) and between HSA and the carrier ligands were considered [16, 17, 26–29]. The modeling calculations for the Zn(II) distribution in artificial serum and with the most important HMM and LMM components (HSA, Tf, Cys, His, cit) showed that most of the Zn(II) is bound to the HMM fraction, mostly to HSA (Fig. 10) [16, 17]. At equimolar concentration of the *bis* complex and HSA, dipic is able to maintain Zn(II) at higher concentration in the LMM fraction compared to the binary ligands owing to its high potency to bind Zn(II). It was also revealed that HSA-ligand interaction affects the metal ion distribution. The results of the modeling calculation were also confirmed
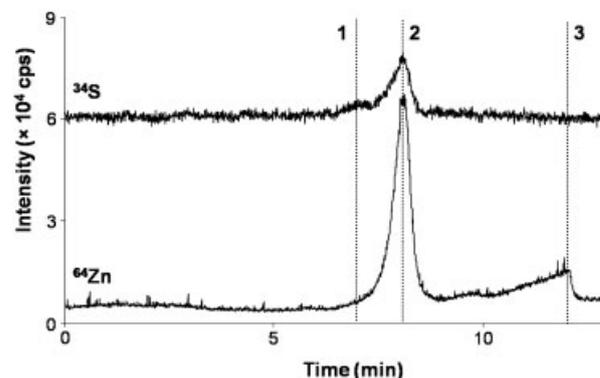

Figure 9. Electropherogram of $[Zn(dipic)_2]^{2-}$ incubated for 24 h with serum in carbonate buffer. Shown are the traces of ^{64}Zn and ^{34}S (for HSA and Tf detection). Peak identification: 1 – Tf; 2 – HSA adduct and 3 – $[Zn(dipic)_2]^{2-}$; Conditions: see Section 2.

Figure 8. Electropherogram of $[Zn(dipic)_2]^{2-}$ incubated for 24 h with apoTf and HSA at a ratio of 1:1:1 in carbonate buffer. Shown are the traces of ^{64}Zn and ^{34}S (for HSA and apoTf detection). Peak identification: 1 – apoTf adduct; 2 – HSA adduct and 3 – $[Zn(dipic)_2]^{2-}$; Conditions: see Section 2.

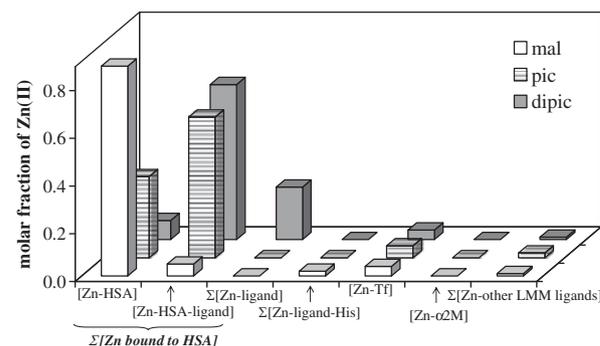**Figure 10.** Calculated Zn(II) speciation in the blood serum. Sample concentration of the anti-diabetic complex: c([Zn(ligand)$_2$]) 100 µM; of the relevant serum components: c(HSA) 158 µM; c(Tf) 6.3 µM; c(α2M) 1.5 µM; c(cit) 25 µM; c(His) 19 µM; c(Cys) 8 µM (pH = 7.40; *T* = 25°C).

Table 2. Conditional stability constants of Zn-protein, HSA-ligand and Zn-HSA-ligand complexes (pH = 7.40; $T = 25^{\circ}$C)

	dipic[a]	maltol[a]	pic[a]
Log β′ (HSA-L)[b]	6.0	3.47	4.33
Log β′ (HSA-(L)$_2$)[b]	8.9	–	–
Log β′ (Zn-HSA-L)[b]	13.3	10.77	11.63
Log β′ (Zn-HSA-(L)$_2$)[b]	16.2	–	–
Log β′ (Zn-HSA)[c]		7.30	
Log β′ (Zn-Tf)[d]		7.80	
Log β′ (Zn$_2$-Tf)[d]		14.20	
Log β′ (Zn-α2 M)[e]		7.49	
Log β′ (Zn$_2$-α2 M)[e]		12.61	

a) Constants for the (Zn-L); (binary Zn-LMM serum components) and (ternary Zn-L-LMM serum components) complexes are taken from Refs. [16, 17].
b) Constants taken from Ref. [16].
c) Constants taken from Ref. [26].
d) Constants taken from Refs. [27, 28].
e) Constants taken from Ref. [29].

experimentally by ultrafiltration–ICP-atomic emission spectroscopy studies [16]. HSA has a higher capacity to bind Zn(II) than α2M at elevated Zn(II) concentration, due to its much higher concentration and accordingly a higher number of available binding sites.

4 Concluding remarks

Zn(II) complexes are promising candidates as insulin-enhancing drugs for the treatment of diabetes. Zn(II) complexes with mal, pic and dipic ligands were prepared *in situ* and their reaction with human serum proteins was studied by means of CZE hyphenated to ICP-MS. As compared to the tridentate ligand dipic, the bidentate metal binders mal and pic form less stable complexes with Zn(II) and accordingly higher amounts of Zn were detected at both HSA and apoTf. Competitive experiments with HSA and apoTf revealed significant preference for HSA over apoTf at equimolar incubation ratios. However, under serum conditions, HSA is by far the preferred binding partner for all three Zn(II)-ligand systems. Modeling calculations based on solution speciation studies and stability constants of binary and ternary Zn-ligand (-biomolecule) systems can give a good estimation of the distribution of such bioelements in biological fluids, with kinetic factors and the time course of these metal transport processes potentially having a significant influence on this picture. Attempts to correlate the experimental data for the distribution of Zn(II) in the serum with calculated results demonstrated overall good agreement of data obtained by calculations and experiments. Such metal ion distribution results can be used for designing carrier compounds with the desired chemical characteristics for obtaining ideal transport properties and mobility features.

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5 References

[1] WHO, Reports of a WHO Study Group, WHO Technical Report Series 1985, pp. 727–876.

[2] Meyer, J. A., Spence, D. M., *Metallomics* 2009, *1*, 32–41.

[3] Guo, Z., Sadler, P. J., *Adv. Inorg. Chem.* 2000, *49*, 183–306.

[4] Hartinger, C. G., Dyson, P. J., *Chem. Soc. Rev.* 2009, *38*, 391–401.

[5] Jakupec, M. A., Galanski, M., Arion, V. B., Hartinger, C. G., Keppler, B. K., *Dalton Trans.* 2008, 183–194.

[6] Thompson, K. H., Chiles, J., Yuen, V. G., Tse, J., McNeill, J. H., Orvig, C., *J. Inorg. Biochem.* 2004, *98*, 683–690.

[7] Sakurai, H., Katoh, A., Yoshikawa, Y., *Bull. Chem. Soc. Jpn.* 2006, *79*, 1645–1664.

[8] Thompson, K. H., Orvig, C., *J. Inorg. Biochem.* 2006, *100*, 1925–1935.

[9] Rehder, D., Pessoa, J. C., Geraldes, C. F. G. C., Castro, M. M. C. A., Kabanos, T., Kiss, T., Meier, B. *et al.*, *J. Biol. Inorg. Chem.* 2002, *7*, 384–396.

[10] Sakurai, H., Kojima, Y., Yoshikawa, Y., Kawabe, K., Yasui, H., *Coord. Chem. Rev.* 2002, *226*, 187–198.

[11] Thompson, K. H., Lichter, J., LeBel, C., Scaife, M. C., McNeill, J. H., Orvig, C., *J. Inorg. Biochem.* 2009, *103*, 554–558.

[12] Taylor, C. G., *BioMetals* 2005, *18*, 305–312.

[13] Fugono, J., Fujimoto, K., Yasui, H., Kawabe, K., Yoshikawa, Y., Kojima, Y., Sakurai, H., *Drug Metab. Pharmacokinet.* 2002, *17*, 340–347.

[14] Yoshikawa, Y., Ueda, E., Kojima, Y., Sakurai, H., *Life Sci.* 2004, *75*, 741–751.

[15] Kiss, T., Jakusch, T., Hollender, D., Doernyei, A., Enyedy, E. A., Pessoa, J. C., Sakurai, H., Sanz-Medel, A., *Coord. Chem. Rev.* 2008, *252*, 1153–1162.

[16] Kiss, T., Jakusch, T., Hollender, D., Enyedy, É. A., Horváth, L., *J. Inorg. Biochem.* 2009, *103*, 527–535.

[17] Enyedy, E. A., Horvath, L., Gajda-Schrantz, K., Galbacs, G., Kiss, T., *J. Inorg. Biochem.* 2006, *100*, 1936–1945.

[18] Timerbaev, A. R., Hartinger, C. G., Keppler, B. K., *Trends Anal. Chem.* 2006, *25*, 868–875.

[19] Hartinger, C. G., Keppler, B. K., *Electrophoresis* 2007, *28*, 3436–3446.

[20] Hartinger, C. G., Timerbaev, A. R., Keppler, B. K., *Electrophoresis* 2003, *24*, 2023–2037.

[21] Yin, X.-B., Li, Y., Yan, X.-P., *Trends Anal. Chem.* 2008, *27*, 554–565.

[22] Enyedy, E. A., Lakatos, A., Horvath, L., Kiss, T., *J. Inorg. Biochem.* 2008, *102*, 1473–1485.

[23] Beaven, G. H., Chen, S.-H., D'Albis, A., Gratzer, W. B., *Eur. J. Biochem.* 1974, *41*, 539–546.

[24] Chasteen, N. D., Grady, J. K., Holloway, C. E., *Inorg. Chem.* 1986, *25*, 2754–2760.

[25] Zékány, L., Nagypál, I., in: Leggett, D. L. (Ed.), *Computational Methods for the Determination of Stability Constants*, Plenum Press, New York 1985, pp. 291–353.

[26] Masuoka, J., Saltman, P., *J. Biol. Chem.* 1994, *269*, 25557–25561.

[27] Harris, W. R., *Biochemistry* 1983, *22*, 3920–3926.

[28] Sun, H., Cox, M. C., Li, H., Sadler, P. J., *Struct. Bonding* 1997, *88*, 71–102.

[29] Adham, N. F., Song, M. K., Rinderknecht, H., *Biochim. Biophys. Acta Protein Struct.* 1977, *495*, 212–219.

[30] Pettit, L. D., Powell, H. K. J., *IUPAC Stability Constants Database*, ver. 5.5, Otley, Academic Software, 2000.

[31] Harris, W. R., *Clin. Chem.* 1992, *38*, 1809–1818.

[32] Lu, J., Stewart, A. J., Sadler, P. J., Pinheiro, T. J. T., Blindauer, C. A., *Biochem. Soc. Trans.* 2008, *36*, 1317–1321.

[33] Timerbaev, A. R., Hartinger, C. G., Aleksenko, S. S., Keppler, B. K., *Chem. Rev.* 2006, *106*, 2224–2248.

[34] Stewart, A. J., Blindauer, C. A., Berezenko, S., Sleep, D., Sadler, P. J., *Proc. Natl. Acad. Sci. USA* 2003, *100*, 3701–3706.

[35] Kiilerich, S., Christiansen, C., *Clin. Chim. Acta* 1986, *154*, 1–6.

[36] Boyett, J. D., Sullivan, J. F., *Metab. Clin. Exp.* 1970, *19*, 148–157.

[37] Foote, J. W., Delves, H. T., *J. Clin. Pathol.* 1984, *37*, 1050–1054.

[38] Charlwood, P. A., *Biochim. Biophys. Acta Protein Struct.* 1979, *581*, 260–265.

[39] Jakusch, T., Hollender, D., Enyedy, E. A., Gonzalez, C. S., Montes-Bayon, M., Sanz-Medel, A., Costa Pessoa, J. *et al.*, *Dalton Trans.* 2009, 2428–2437.

[40] Jakusch, T., Gajda-Schrantz, K., Adachi, Y., Sakurai, H., Kiss, T., Horvath, L., *J. Inorg. Biochem.* 2006, *100*, 1521–1526.

[41] Groessl, M., Hartinger, C. G., Polec-Pawlak, K., Jarosz, M., Keppler, B. K., *Electrophoresis* 2008, *29*, 2224–2232.

[42] Harris, W. R., Stenback, J. Z., *J. Inorg. Biochem.* 1988, *33*, 211–223.

[43] Hartinger, C. G., Jakupec, M. A., Zorbas-Seifried, S., Groessl, M., Egger, A., Berger, W., Zorbas, H. *et al.*, *Chem. Biodiversity* 2008, *5*, 2140–2155.