Complex formation equilibria of Cu^{2+} and Zn^{2+} with Irbesartan and Losartan

DOI:

[10.1016/j.ejps.2016.11.010](https://doi.org/10.1016/j.ejps.2016.11.010)

Document Version

Accepted author manuscript

[Link to publication record in Manchester Research Explorer](#)

Citation for published version (APA):

Lachowicz, J. I., Nurchi, V. M., Crisponi, G., Jaraquemada-Pelaez, M. D. G., Caltagirone, C., Peana, M., Zoroddu, MA., Szewczuk, Z., & Cooper, G. (2017). Complex formation equilibria of Cu^{2+} and Zn^{2+} with Irbesartan and Losartan. *European Journal of Pharmaceutical Sciences*, 97, 158-169. <https://doi.org/10.1016/j.ejps.2016.11.010>

Published in:

European Journal of Pharmaceutical Sciences

Citing this paper

Please note that where the full-text provided on Manchester Research Explorer is the Author Accepted Manuscript or Proof version this may differ from the final Published version. If citing, it is advised that you check and use the publisher's definitive version.

General rights

Copyright and moral rights for the publications made accessible in the Research Explorer are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

Takedown policy

If you believe that this document breaches copyright please refer to the University of Manchester's Takedown Procedures [<http://man.ac.uk/04Y6Bo>] or contact uml.scholarlycommunications@manchester.ac.uk providing relevant details, so we can investigate your claim.

Accepted Manuscript

Complex formation equilibria of Cu^{2+} and Zn^{2+} with Irbesartan and Losartan

Joanna Izabela Lachowicz, Valeria Marina Nurchi, Guido Crisponi, Maria de Guadalupe Jaraquemada-Pelaez, Claudia Caltagirone, Massimiliano Peana, Maria Antonietta Zoroddu, Zbigniew Szewczuk, Garth J.S. Cooper

PII: S0928-0987(16)30489-4
DOI: doi:[10.1016/j.ejps.2016.11.010](https://doi.org/10.1016/j.ejps.2016.11.010)
Reference: PHASCI 3796

To appear in:

Received date: 9 September 2016
Revised date: 28 October 2016
Accepted date: 7 November 2016

Please cite this article as: Lachowicz, Joanna Izabela, Nurchi, Valeria Marina, Crisponi, Guido, de Guadalupe Jaraquemada-Pelaez, Maria, Caltagirone, Claudia, Peana, Massimiliano, Zoroddu, Maria Antonietta, Szewczuk, Zbigniew, Cooper, Garth J.S., Complex formation equilibria of Cu^{2+} and Zn^{2+} with Irbesartan and Losartan, (2016), doi:[10.1016/j.ejps.2016.11.010](https://doi.org/10.1016/j.ejps.2016.11.010)

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Complex formation equilibria of Cu²⁺ and Zn²⁺ with Irbesartan and Losartan.

Joanna Izabela Lachowicz^{*a}, Valeria Marina Nurchi^a, Guido Crisponi^a, Maria de Guadalupe Jaraquemada-Pelaez^a, Claudia Caltagirone^a, Massimiliano Peana^b, Maria Antonietta Zoroddu^b, Zbigniew Szewczuk^c and Garth J. S. Cooper^{d,e}

^a *Department of Chemical and Geological Science, University of Cagliari, Cittadella Universitaria, I-09042 Monserrato-Cagliari;*

^b *Department of Chemistry and Pharmacy, University of Sassari, Via Vienna 2, I-07100 Sassari, Italy;*

^c *Faculty of Chemistry, University of Wrocław, F. Joliot Curie 14, 50-383 Wrocław, Poland;*

^d *Central Manchester NHS Hospitals Foundation Trust, and School of Biomedicine, The University of Manchester, and Manchester Academic Health Sciences Centre, Manchester, UK;*

^e *School of Biological Sciences and Maurice Wilkins Centre for Molecular Biodiscovery, Faculty of Science, University of Auckland, Auckland 1010, New Zealand*

** Corresponding author: Joanna Izabela Lachowicz, Department of Chemical and Geological Science, University of Cagliari, Cittadella Universitaria, I-09042 Monserrato-Cagliari, tel. +39 0706754471, lachowicz@unica.it*

Abstract

We conducted a thorough study of Cu^{2+} complex formation equilibria with Irbesartan and Losartan, the two primary drugs for the cure of cardiovascular diseases, with the aim of recognising if these drugs could exert a chelating action toward Cu^{2+} . We used different complementary techniques to gain a clear picture of the involved protonation and complexation equilibria. The low solubility in water of the ligands and of the formed metal complexes prevented the use of water as solvent, so we had to perform the measurements in mixed methanol-water solvents. Further, we studied the related equilibria with Zn^{2+} for evaluating a potential interference of this essential metal ion, largely present in biological fluids. Our study provided a strong evaluation of the formed complexes and of the relative stability constants. The binding of both metal ions takes place through the tetrazole moiety except for the Zn^{2+} -Irbesartan system. In this last case, NMR measurements gave evidence of a tautomeric equilibrium involving the imidazole ring and the aliphatic chain. The estimated complexation model, and the related stability constants, allowed a speciation study in human plasma, based on a number of simplifying assumptions, which remarked that both drugs, Losartan and Irbesartan, could exert a chelating action, scavenging non-negligible amounts of Cu^{2+} from the organism.

Cardiovascular disease is the leading cause of death and disability in patients with diabetes.[1] Defective copper regulation is widespread in diabetes, where it is likely one of the causes of cardiovascular disease, [2] and is now serving as a target for experimental therapeutic intervention by treatment with a Cu^{2+} -selective chelator.[3] In diabetes, impaired copper regulation manifests as enhanced urinary Cu^{2+} excretion, elevated systemic copper balance and defective myo-cellular copper regulation,[2, 4, 5] and it has been involved in increased pro-oxidant stress, defective antioxidant defenses,[6] and progressive damage to the blood vessels, heart, kidneys and nerves (the ‘diabetic complications’).[7] Cardiovascular disease in diabetic patients tends to be more aggressive and disabling than in matched non-diabetic patients.[1, 8]

Scheme 1. Molecular structures of Irbesartan (A) and Losartan (B). The structural differences between molecules are marked in red.

A general opinion is that sartans exert their therapeutic effects through actions as angiotensin receptor blockers (ARBs). However, it is becoming increasingly apparent that, under certain circumstances, they may elicit some of their therapeutic effects through actions other than as ARBs. For example, Valsartan has been shown to improve arterial stiffness in type-2 diabetes independently of blood-pressure-lowering,[9] raising the possibility that it is acting through a different mechanism in this case. Notably, treatment with a Cu^{2+} selective chelator improves diastolic stiffness and arterial fibrosis in animal models of diabetic cardiac and arterial disease.[6, 10-12] In the extra-cellular space, copper is present mainly in the form of Cu^{2+} ions.[7]

The present work takes origin from joint research efforts of two groups with different aims and expertise, a clinical group working on different aspect of diabetes, and a chemical group interested

in metal complex formation of chelating agents for the treatment of metal ion toxicity. The working hypothesis is that some drugs in use in diabetic patients could also act as copper scavenging agents. Sartans share molecular properties with other clinically used Cu^{2+} -binding molecules, such as triethylenetetramine, in use as a second line treatment for Wilson's disease and in the experimental therapeutics of the diabetic complications;[13, 14] both the two classes of molecules contain several nitrogen atoms spaced at distances consistent with potential Cu^{2+} -binding sites.[15],[16],[17]

The copper-binding properties of the sartans are unknown. Based on a preliminary structural analysis, we hypothesized that sartan molecules could demonstrate Cu^{2+} binding properties. Here we report the determination of the Cu^{2+} binding properties of two representative sartan molecules, Irbesartan and Losartan, registered in the USA and in Europe for the treatment of cardiovascular disease in diabetic and non-diabetic patients. Due to the low water solubility of Irbesartan and Losartan, and especially of their metal complexes, for potentiometric equilibrium studies, we performed our measurements in mixed methanol/water solvents[§]. The aim of this work was to give evidence of the interaction of copper with the two drugs, and to highlight the complexation scheme, though in an environment outside that of physiological conditions. The obtained results, even in the limits of the inherent experimental restraints, gave a clear indication of a possible copper scavenging action of Irbesartan and Losartan.

Regarding Zn^{2+} ions, it has been found that therapy with Losartan in patients with hypertension causes an increase in urinary zinc excretion, resulting in zinc depletion, further intensified by the addition of hydrochlorothiazide.[18] For this reason we extended the work to the interaction with this essential metal ion, disregarding that with essential metal ions as Fe^{3+} , Mg^{2+} and Ca^{2+} since the pronounced hard character limits their interaction with nitrogen based ligands.

2. Experimental

2.1. Reagents

Irbesartan and Potassium Losartan were purchased from TCI Europe. CuCl_2 , ZnCl_2 , NaOH, NaOD, NaCl, HCl, DCl, DMSO- d_6 , D_2O , MeOD and methanol from Sigma Aldrich. All the reagents, analytical grade, were used without further purification.

2.2. Potentiometry

Protonation and complex formation equilibrium studies were carried out in a thermostatted glass cell equipped with a magnetic stirrer, under Argon flow. The potentiometric apparatus consisted in Metrohm 691 pH-meter, Dosimat 665 Metrohm titrator and Metrohm LL UNITRODE glass

electrode. All the titrations were performed at 25.0 °C using 0.1 M NaOH at ionic strength 0.1 M NaCl, in 30:70 and 80:20 MeOH/H₂O mixtures. The electrode was daily calibrated for hydrogen ion concentration by HCl/NaOH titration in the same experimental conditions as above, and the results were analysed with Gran procedure.[19] Working ligand concentration was 0.5 mM both for protonation and for complex formation titrations. In the last case, 1:2 and 1:4 metal/ligand molar ratios were used. The obtained titration data were processed by Hyperquad 2013 program.[20]

2.3. Spectrophotometry

A Varian Cary 50 UV/Vis Spectrophotometer was used for UV-Visible studies. Ligand protonation equilibria were studied by titrating 20 ml of 0.5 mM ligand solutions with 0.1 M NaOH at 0.1 M NaCl ionic strength, in 30:70 and 80:20 MeOH/H₂O mixtures, using a joined potentiometric-spectrophotometric procedure.[21] Spectra were recorded in the 200–400 nm spectral range with a 0.2 cm path length fiber-optic probe. The obtained data were processed by HypSpec program[22]. Solubility studies of ligand in water were made at pH 3 with the use of calibration curve previously prepared at the same conditions.

2.4. Spectrofluorimetry

A Varian Cary Fluorimeter was used for Fluorescence spectra. Ligand working concentrations for the study of protonation equilibria were 0.032 mM in 30:70 and 80:20 MeOD/H₂O solutions. Batch measurements at variable pH were performed by adding proper amounts of HCl and NaOH. Since UV-Vis spectra at different pH values revealed an isosbestic point at 280 nm, this wavelength was chosen for excitation. Emission spectra were registered in the 290-490 nm spectral range.

2.5. NMR

NMR measurements were performed on a Bruker Ascend™ 400 MHz spectrometer equipped with a 5 mm automated tuning and matching broadband probe with z-gradients, as previously described.[23-25] 5-7 mM concentrations of the ligands in different solvents, D₂O, MeOD or DMSO at 25°C in 5 mm NMR tubes were used. The pH of the solutions was adjusted with diluted DCl and NaOD on a pH meter Crison micro TT 2050 with an electrode Mettler Toledo InLab 422. The pD values measured in D₂O solutions were converted to the pH values by using the deuterium isotopic relation $\text{pH} = \text{pD} - 0.4$. [26, 27] A stock acidic deuterated aqueous solution of ZnCl₂ salt was used to achieve the concentration of Zn²⁺ ions. The metal ion solution was added to Irbesartan and Losartan ligand solutions (80:20 MeOD/D₂O solution) and the pH was then set to the right value just before the acquisition of the spectra. 2-D ¹H-¹³C heteronuclear correlation spectra

(HSQC) were acquired by using a phase-sensitive sequence employing Echo-Antiecho-TPPI gradient selection with a heteronuclear coupling constant $J_{XH} = 145$ Hz, and shaped pulses for all 180° pulses on f2 channel with decoupling during acquisition. Sensitivity improvement and gradients in back-inept were also used. Relaxation delays of 2s and 90° pulses of about 10 μ s were employed in all the experiments. Solvent suppression for 1D ^1H , 2D ^1H - ^1H TOCSY (total correlation spectroscopy) experiments were carried out by using excitation sculpting with gradients. The spin-lock mixing time of TOCSY experiments was obtained with MLEV17. ^1H - ^1H TOCSYs were performed by using mixing times of 60 ms. A combination of 1D, 2D TOCSY, HSQC experiments was used to assign the signals of both free and metal-bound ligands at different pH values. All NMR data were processed with TopSpin software (Bruker Instruments), and analysed by Sparky 3.11 and MestRe Nova 6.0.2 programs (Mestrelab Research S.L.).

2.6. ESI-MS

High-resolution mass spectra were obtained on a Bruker MicrO-TOF-Q spectrometer (Bruker Daltonik, Bremen, Germany), equipped with an Apollo II electrospray ionization source and an ion funnel. The spectra were recorded in the positive ion mode in the range 100 - 1500 m/z. The instrument parameters were dry gas–nitrogen, temperature 200 $^\circ\text{C}$, ion source voltage 4500 V, collision energy 10 eV. Analyte aqueous solutions (0.1 mM) were introduced at a flow rate of 3 $\mu\text{L}/\text{min}$. Stock solutions were prepared by using MeOH/ H_2O mixture (50:50 v/v) as a solvent. The metal to ligand molar ratio was 1:2. The instrument was calibrated using the Tunemix mixture (BrukerDaltonik, Germany) in the quadratic regression mode. The overall charge of the analyzed ion was calculated on the base of distance between isotopic peaks. Data were processed by Compass DataAnalysis 4.0 (Bruker Daltonik, Germany) program.[28]

2.7. Speciation.

All the calculations concerning the speciation were performed with HySS (Hyperquad Simulation and Speciation) program.[29] The details of the calculation procedure, the estimate of the concentration of zinc and copper in human plasma and of their interaction with albumin were described in depth in a previous paper.[30]

An analysis of the pharmacokinetic behaviour of Irbesartan and Losartan was necessary in order to correlate the amount of chelating agent available in plasma at a given time with the amount of drug administered. In the work by Marino et al.[31] Figure 1 reports the Irbesartan concentrations ($\mu\text{g}/\text{ml}$) in plasma for the 24 h following administration, for different administered doses. From this plot it was possible to evaluate that Irbesartan concentration reaches a peak at 12 μM just before the

2 hours, it is always greater than 5 μ M in the first 6 hours. In the following 18 hours before the next administration, the concentration slowly falls down to about 1 μ M.

3. Results and discussion

3.1. Ligand characterization

The acid–base behaviour of a drug is of great relevance in pharmacological sciences for its influence on bioavailability, activity and pharmacokinetics.[32] In particular, in the case of antihypertensive drugs, the knowledge of acid-base properties favours the comprehension of the physiological mechanisms in which their receptor antagonists are implied.[33] The charged protonated forms of lipophilic bases assume high relevance for the potential electrostatic interactions with anionic or zwitterionic lipids.[34] The value of the dissociation constants of habitually assumed cardiovascular drugs, increasingly formulated into controlled-release forms, is of great significance.[35] The solubility of Losartan and Irbesartan strongly depends on the protonation state of the molecule, and their water solubility becomes extremely low at acidic pH. The aqueous solubility of Losartan (5.72×10^{-5} M) and Irbesartan (4.64×10^{-4} M) was spectrophotometrically determined at 280 nm at pH 3. At the pH of stomach, Irbesartan is more soluble than Losartan, and this could be one of the reasons of the higher bioavailability of Irbesartan (60-80% [36]) than that of Losartan (33% [37]).

Both Losartan and Irbesartan contain an acidic centre on the tetrazole ring, and a basic centre on the imidazole in the case of Losartan and on the 5-oxo-imidazole in Irbesartan. A number of dissociation (or protonation) constants related to this centres can be found in literature, but they do not mention neither the experimental conditions in which were determined, nor to the used techniques (Losartan 2.36 and 5.55 [38], 4.9 [39], 5.6 [40] and Irbesartan 4.5 [41]). Tosco et al. [42] presented reliable pKa values of both groups, potentiometrically evaluated in co-solvents of variable composition. These values, reported as a function of the percent composition allowed the extrapolation of the values in water and to compare these values with the corresponding ones of some tetrazole molecules (tetrazole 4.89, [43] methyltetrazole 5.50, [44] and 5-phenyl-1H tetrazole 4.38 [45]). These results remarked that the acidity of the tetrazole ring increases with aromatic 5-substitution, which implies a larger delocalization of the negative charge on the aromatic moiety, and the influence of the electron withdrawing substituent. Hence, the first dissociation was attributed to the imidazole ring and the second to the tetrazole ring.[42]

3.1.1 Potentiometric results

Even if potentiometry and spectrophotometry represent the most suitable and handy techniques in the determination of equilibrium constants, the low water solubility of Irbesartan and Losartan at acidic pH prevented potentiometric studies in aqueous solution. [46, 47] Ligand dissociation studies were thus performed in methanol/water (MeOH/H₂O) mixtures 30:70 and 80:20, being the effects of methanol on pK_a values widely studied. [48]

Irbesartan and Losartan act as diprotic ligands LH₂⁺ and deprotonate in the same way. Actually, in the 30:70 MeOH/H₂O mixture, Irbesartan loses the imidazole proton with pK_{a1} 3.6, and Losartan with pK_{a1} 3.06 (Table 1). After the first deprotonation, both ligands exist in the zwitterionic form LH[±], being negatively charged on the tetrazole nitrogen. The tetrazole ring of Irbesartan deprotonates with pK_{a2} 5.0, and that of Losartan with pK_{a2} 4.38. According to Tosco et al. [42], the plots of pK_a's vs % of MeOH [42] show that the higher the % of methanol, the lower pK_{a1} and the higher pK_{a2}. Indeed, the results in Table 1 present the same behaviour, except pK_{a2} of Irbesartan that has almost the same value at the two solvent compositions.

Table 1. Dissociation constants determined potentiometrically at 25°C and 0.1 M NaCl ionic strength.

	Irbesartan		Losartan	
	30:70 MeOH/H ₂ O	80:20 MeOH/H ₂ O	30:70 MeOH/H ₂ O	80:20 MeOH/H ₂ O
pK _{a1}	3.6(1)	3.25(1)	3.06(7)	2.56(1)
pK _{a2}	5.0(1)	4.98(1)	4.38(4)	5.07(1)

3.1.2 Spectrophotometric results

Irbesartan and Losartan are characterized by complex molecular structures, which allow monitoring spectrophotometrically the two protonation equilibria with close dissociation constants. The two molecules exhibit similar absorption bands, and we selected the spectral range from 240 to 310 nm, with an isosbestic point at ~280 nm, as the most convenient for evaluating the dissociation constants.

The pK_a values calculated with HypSpec program [22] slightly differ from those potentiometrically determined (Table 1) and are less reliable. Actually, the low reliability depends on the small variations of the absorptivity spectra with pK_a (Figure S14).

3.1.3 Fluorescence results.

The presence of the biphenyl and the imidazole fluorescent groups (fluorophores) allowed to collect the fluorescence spectra of both the ligands from pH 1 to 7 [49],[50]. In the pH range 1 - 2 the fluorescence peak shifts towards higher energy both in 30:70 and 80:20 MeOH/H₂O solutions (Figures 1 and 2). In the pH range 2-7, upon excitation at 280 nm, both drugs show an emission band centered at 376 nm in 30:70 MeOH/H₂O solution (Fig. 1B and Fig. 2B), while this band is shifted to 370 nm in 80:20 MeOH/H₂O (Fig. 1E and Fig. 2D) due to the decreased polarity of the solvent.

Figure 1. Fluorescence spectra of Irbesartan in 30:70 MeOH/H₂O (A-C) and 80:20 MeOH/H₂O (D-F) solution in pH dependent titration. ($\lambda_{exc} = 280$ nm, [Irbesartan] = 0.032 mM).

Figure 2. Fluorescence spectra of Losartan in 30:70 MeOH/H₂O (A,B) and 80:20 MeOH/H₂O (C,D) solution in pH dependent titration. ($\lambda_{\text{exc}} = 280 \text{ nm}$, [Losartan] = 0.032 mM).

In Figure 3, the fluorescence intensities at fixed wavelength are superimposed to the speciation plots obtained from the potentiometric results (Fig. 3). The feature of these graphics indicates that the fluorescence emission is related to the deprotonation of imidazole ring. The mono-deprotonated $\text{LH}^{\pm}$ species of Losartan is almost non-emissive, while only when the second deprotonation process starts a fluorescence quenching is observed in the case of Irbesartan. The quenching process is completed when the anionic form L^{-} alone is present in solution. Figure 3 shows that the

fluorescence intensity of Irbesartan increases reaching a maximum at pH 3.6, then starts to decrease. On the contrary, the fluorescence intensity of Losartan, observable at acidic pH 2, progressively decreases vanishing at pH 5.

Figure 3. Speciation plots of Irbesartan (A, B) and Losartan (C, D), in 30:70 MeOH/H₂O (left plots A, C) and 80:20 MeOH/H₂O (right plots B, D). The normalized fluorescence intensity at 376 nm (A, C) and 370 nm (B, D) are superimposed as points. (λ_{exc} = 280 nm, [Losartan] = 0.032 mM).

A previous fluorescence study conducted in 5:95 MeOH/H₂O solution gave evidence of only one dissociation equilibrium for each molecule, related to the tetrazole group, characterized by a pK_a value 3.15 for Losartan and 4.70 for Irbesartan [33].

3.2. Cu²⁺ and Zn²⁺ complex formation equilibria.

The complex formation equilibria with Cu²⁺ and Zn²⁺ were studied potentiometrically in an 80:20 MeOH/H₂O solution due to the low solubility of the formed complexes both in water and 30:70 MeOH/H₂O solution. The studies were carried out at 0.5 mM ligand concentration in 1:2 and 1:4 metal/ligand molar ratios; at 1mM ligand concentration the precipitation of the complex occurred. The results are reported in Table 2.

In the following we will indicate with I⁻ and L⁻ the completely deprotonated forms of Irbesartan and Losartan.

Table 2. Logarithms of protonation and complex formation constants at 25.0 °C and 0.1 M NaCl ionic strength in 80:20 MeOH: H₂O solution. The relative pM values (pM defined as the negative logarithm of the concentration of the free metal ion in solution, when [Metal]_{tot} = 10⁻⁶M, [Ligand]_{tot} = 10⁻⁵M at pH 7.4) are also reported.

Species	Irbesartan	Species	Losartan
IH	4.98(1)	LH	5.07(1)
[IH ₂] ⁺	3.25(1)	[LH ₂] ⁺	2.56(1)
	-	[CuL] ⁺	4.12(7)
CuI ₂	6.89(3)	CuL ₂	7.1(3)
	-	[CuL ₂ H ₁] ⁻	2.40(9)
[CuI ₂ H ₂] ²⁻	-4.03(1)	[CuL ₂ H ₂] ²⁻	-3.6(4)
pCu ²⁺	6.7		7.4
	-	[ZnL]	3.7(1)
	-	ZnL ₂	6.2(1)
[ZnI ₂ H ₁] ⁻	0.42(8)	[ZnL ₂ H ₁] ⁻	0.4(2)
[ZnI ₂ H ₂] ²⁻	-6.51(4)	[ZnL ₂ H ₂] ²⁻	-6.69(8)
pZn ²⁺	6.0		6.0

Irbesartan and copper ions form a weak CuI_2 complex from pH 4, which loses two protons giving the $[\text{CuI}_2\text{H}_2]^{2-}$ complex from pH 5 (Fig. 4A). Zinc forms weaker complexes than copper, of the same stoichiometry (Fig. 4B, Table 2).

The copper complexes with Losartan are slightly stronger than the complexes with Irbesartan (Table 2). A 1:1 complex exists from pH 2.5, then replaced by variously protonated 1:2 complexes (Fig. 4C). As previously, zinc complexes with Losartan are weaker than copper complexes, with the same stoichiometry (Fig. 4D).

Copper complexes in 80:20 MeOH/ H_2O solution precipitate at $\text{pH} > 5$, while in the case of zinc complex precipitation occurs only at $\text{pH} > 7$. The formation of a neutral dimeric $[\text{Cu}(\text{Ligand})_2(\text{H}_2\text{O})_3]_2$ complex, previously synthesized and characterized, can explain the low solubility of copper complexes. FTIR and Raman studies gave evidence of copper coordination by tetrazole nitrogen atoms, and EPR measurements of the formation of a dimeric complex. The metal centers are in a tetragonal distorted environment. A monomeric complex was also found as a minor component [51].

Figure 4. Speciation plots relative of copper and zinc complexes calculated using the stability constants in Table 2. Ligand concentration was 0.5 mM and a 1:2 metal to ligand ratio was used.

Isothermal titration calorimetry showed that complexation in water occurs as a stepwise process, both enthalpy and entropy driven. 1:1 copper: Losartan complex forms at low molar ratio, and a 1:2 species is formed at molar ratios > 1 [52]. This last 1:2 complex is enthalpically favored, due to the metal-ligand bond strengthened by Ligand Field Stabilization Energy. The loss of water molecules during complexation and the loss of complex molecular structure, due to tetragonal distortion, account for the entropic contribution. In the solid state, the formation of copper-active Van der Waals interactions between the aliphatic chains of Losartan stabilizes the copper complexes.

3.3. ESI-MS characterization of Cu^{2+} and Zn^{2+} complexes

Mass spectrometry is a potent complementary technique to support speciation studies, which allows determining the metal-to-ligand stoichiometry directly from the m/z values. We successfully employed ESI-MS for this purpose in previous works.[53-57]

ESI-MS measurements of Irbesartan and Losartan complexes with the bivalent copper and zinc cations confirms the findings of Denadai et al. [52]. ESI-MS spectra of the pure ligands show the formation of dimers in solution: $[\text{I}_2\text{H}_2+\text{H}]^+$ (857.472 m/z , $\text{C}_{50}\text{H}_{57}\text{N}_{12}\text{O}_2$), $[\text{I}_2\text{H}_2+\text{Na}]^+$ (879.454 m/z , $\text{C}_{50}\text{H}_{56}\text{N}_{12}\text{O}_2\text{Na}$), $[\text{I}_2\text{H}+2\text{Na}]^+$ (901.436 m/z , $\text{C}_{50}\text{H}_{55}\text{N}_{12}\text{O}_2\text{Na}_2$), $[\text{L}_2\text{H}_2+\text{K}]^+$ (883.285 m/z , $\text{C}_{44}\text{H}_{46}\text{Cl}_2\text{KN}_{12}\text{O}_2$), $[\text{L}_2\text{H}+2\text{K}]^+$ (921.241 m/z , $\text{C}_{44}\text{H}_{45}\text{Cl}_2\text{K}_2\text{N}_{12}\text{O}_2$), $[\text{L}_2+3\text{K}]^+$ (959.199 m/z , $\text{C}_{44}\text{H}_{44}\text{Cl}_2\text{K}_3\text{N}_{12}\text{O}_2$) and $[\text{L}_2+3\text{K}+\text{Na}]^+$ (983.163 m/z , $\text{C}_{44}\text{H}_{44}\text{Cl}_2\text{K}_3\text{N}_{12}\text{O}_2\text{Na}$) (Fig. S1D-F and Fig. S7C-F). Van der Waals interactions between aliphatic chains account for the formation of the observed dimers.

ESI-MS signals of Irbesartan with Cu^{2+} ion can be assigned to the species $[\text{CuIH}]^+$ (491.162 m/z , $\text{C}_{25}\text{H}_{28}\text{N}_6\text{OCu}$, Fig. S2A) and $[\text{CuI}_2\text{H}_2]^+$ (919.394 m/z , $\text{C}_{50}\text{H}_{56}\text{N}_{12}\text{O}_2\text{Cu}$, Fig. S2B). The charge and the empirical formula of the complexes suggest a redox reaction. Electrospray ionization with tandem mass spectrometry (MS/MS) of the $[\text{CuIH}]^+$ complex show the four signals 463.16 m/z , 448.14 m/z , 352.09 m/z and 296.03 m/z . Their isotopic patterns (Fig.S3A-D) demonstrate that one copper atom is present in each collision induced dissociation (CID) product and they fit with Chemical formulas generated using the SmartFormula algorithm from Compass Data Analysis 4.0 software (Bruker), presented in Fig. 5. The lowest m/z signal suggests Cu^{2+} ion coordination by tetrazole ring.

Figure 5. Electrospray ionization with tandem mass spectrometry (MS/MS) spectrum of the $[\text{CuI}+\text{H}]^+$ complex (parent ion m/z 491). Chemical formulas of the fragment ions are presented.

The ESI-MS spectrum of Irbesartan with Zn^{2+} highlights the formation of a $[\text{ZnI}]^+$ complex ($\text{C}_{25}\text{H}_{27}\text{N}_6\text{OZn}$ 491.162 m/z , Fig. S4A) and of mono-hydrated $[\text{ZnI}+\text{H}_2\text{O}]^+$ and bis-hydrated $[\text{ZnI}+2\text{H}_2\text{O}]^+$ forms ($\text{C}_{25}\text{H}_{29}\text{N}_6\text{O}_2\text{Zn}$ 509.160 m/z , $\text{C}_{25}\text{H}_{31}\text{N}_6\text{O}_3\text{Zn}$ 527.131 m/z , Fig. S4B and S4C, respectively). The signal at 919.395 m/z is attributed to the $[\text{ZnI}_2\text{H}]^+$ complex ($\text{C}_{50}\text{H}_{55}\text{N}_{12}\text{O}_2\text{Zn}$, S4D9). MS/MS spectrum of $[\text{ZnI}]^+$ complex leads to the decomposition of the complex, even at low collision energy (15 eV). The MS/MS spectrum of the $[\text{ZnI}_2\text{H}]^+$ complex presents the signals at 491.16 m/z , 449.16 m/z and 429.25 m/z , attributed to the $[\text{ZnI}]^+$ complex, to the $[\text{C}_{22}\text{H}_{21}\text{N}_6\text{OZn}]^+$ fragment and to $[\text{IH}_2]^+$ (Fig. S6). ESI-MS spectra of pure Losartan and of its Cu^{2+} and Zn^{2+} complexes are reported in Figure 6.

Figure 6. ESI-MS spectra of Losartan A) pure, B) with copper at 1:1 molar ratio, and C) with zinc at 2:1 molar ratio in the 400-470 m/z range.

The signal at 461.124 m/z is attributed to the Losartan potassium salt $[LH+K]^+$ ($C_{22}H_{23}ClKN_6O$). In the presence of copper and zinc ions two additional signals at 423.169 and 405.158 m/z are present, attributable to $[LH_2]^+$ ($C_{22}H_{24}ClN_6O$) and $[LH_2-H_2O]^+$ ($C_{22}H_{22}ClN_6$) respectively. A possible explanation of this is that these two metal ions favor the reaction suggested in Scheme 2.

Scheme 2. Suggested formation of 3-butyl-1-chloro-8-[2-(1*H*-tetrazol-5-yl)phenyl]-5,10-dihydroimidazo[1,5-*b*]isoquinoline^{§§} by rearrangement of Losartan in presence of metal ions.

ESI-MS spectrum of Losartan in presence of Cu^{2+} (molar ratio 1:1) shows signals of low intensity attributed to $[\text{CuLH}]^+$ ($\text{C}_{22}\text{H}_{23}\text{ClN}_6\text{OCu}$ 485.092 m/z , Fig. S9A), $[\text{CuL}_2\text{H}_2]^+$ ($\text{C}_{44}\text{H}_{46}\text{Cl}_2\text{N}_{12}\text{O}_2\text{Cu}$ 907.252 m/z , Fig. S9C), $[\text{Cu}_3\text{L}_4]^{2+}$ ($\text{C}_{88}\text{H}_{88}\text{Cl}_4\text{N}_{24}\text{O}_4\text{Cu}_3$ 936.701 m/z , Fig. S9D), $[\text{CuL}_2\text{H}+\text{K}]^+$ ($\text{C}_{44}\text{H}_{45}\text{Cl}_2\text{N}_{12}\text{O}_2\text{CuK}$ 945.209 m/z , Fig. S9E) and $[\text{Cu}_3\text{L}_2]^+$ ($\text{C}_{44}\text{H}_{44}\text{Cl}_2\text{N}_{12}\text{O}_2\text{Cu}_3$ 1031.098 m/z , Fig. S9F). A redox reaction, similar to that observed with Irbesartan, can be hypothesized on the basis of charges and empirical formulae of $[\text{CuLH}]^+$, $[\text{CuL}_2\text{H}_2]^+$, $[\text{CuL}_2\text{H}+\text{K}]^+$ and $[\text{Cu}_3\text{L}_2]^+$ complexes. MS/MS of the $[\text{CuLH}]^+$ complex, even at low collision energy (10 eV), leads to its decomposition. MS/MS spectrum of $[\text{CuL}_2\text{H}_2]^+$ complex shows one signal at 461.134 m/z attributed to $[\text{L}+\text{K}]^+$ (Fig. S11).

The redox activity of copper-Losartan complexes was previously pointed out, showing a weak Super-oxide Dismutase activity of $[\text{CuL}_2(\text{H}_2\text{O})_3]_2$ complex at pH 10.2 [51].

ESI-MS spectrum of Losartan with Zn^{2+} ions at 1:2 metal:ligand ratio shows three signals at 485.083 m/z , 907.242 m/z and 937.701 m/z attributed to $[\text{ZnL}]^+$ ($\text{C}_{22}\text{H}_{22}\text{ClN}_6\text{OZn}$; Fig. S13A), $[\text{ZnL}_2\text{H}]^+$ ($\text{C}_{44}\text{H}_{45}\text{Cl}_2\text{N}_{12}\text{O}_2\text{Zn}$; Fig. S13B) and $[\text{Zn}_3\text{L}_4]^{2+}$ ($\text{C}_{88}\text{H}_{88}\text{Cl}_4\text{N}_{24}\text{O}_4\text{Zn}_3$; Fig. S13C), respectively.

3.4. NMR characterization of Zn^{2+} complexes

The free Losartan ligand was studied by NMR techniques in the different solvents D_2O at pH 7.0, MeOD- d_4 and DMSO- d_6 (Figure S15 and Table S1). The low solubility of Losartan in aqueous solution and the precipitation occurring after Zn^{2+} addition (Losartan concentration 5-7 mM and Zn:L 1:2, 1:1 ratios) prevented the NMR study in water. Therefore, the interaction between Losartan and Zn^{2+} was followed in MeOD and DMSO. ^1H 1D NMR spectra of pure Losartan and in presence of Zn^{2+} ions (1:1 and 1:2 Zn^{2+} : Losartan molar ratios in deuterated methanol) are presented in Figure 7.

Figure 7. ^1H 1D NMR spectra of Zn^{2+} : Losartan system in MeOD- d_4 solvent in the molar ratio of 0:1, 1:2 and 1:1 at 25°C and pH 7.

The spectra of Losartan in presence of Zn^{2+} at 1:2 metal:ligand molar ratio present clearly observable perturbations in the aromatic region. Changes in chemical shifts can be detected for H-22, the proton closest to tetrazole moiety, for H-19 and in a minor extent for H-13, H-17, H-14 and H-16 from biphenyl, which are further away from the tetrazole moiety. No evident changes were observed for the other protons in the molecule, except a slight broadening of the signals. These findings identified tetrazole as the anchoring site for Zn^{2+} ion, confirming the potentiometric and spectrophotometric results.

At a 1:1 Zn^{2+} : Losartan molar ratio the broadening of the signals is more evident, ascribable to a chemical exchange in an intermediate regime with respect to the NMR timescale. Also in DMSO- d_6 solution, a similar behaviour of Losartan in presence of Zn^{2+} ions was observed. ^1H 1D NMR spectra and 2D ^1H - ^{13}C HSQC spectra (aromatic region) of pure Losartan and of Losartan in presence of Zn^{2+} at a molar ratio 1:1 are reported in Figure 8 and 9, respectively. Indeed, the protons H-22, H-20, H-19 and H-21 were the most affected by Zn^{2+} ion addition, pointing out that the same coordination observed in MeOD is retained in DMSO- d_6 solvent.

Figure 8. Comparison ^1H 1D NMR spectra of free Losartan (red) and Zn^{2+} : Losartan system at a molar ratio of 1:1 (blue), in DMSO- d_6 solvent, at 25°C and pH 7.

Figure 9. Comparison of 2D ^1H - ^{13}C HSQC NMR spectra of free Losartan (red) and Losartan: Zn(II) system at a molar ratio of 1:1 (blue), in DMSO-d_6 solvent, at 25°C and pH 7.

Also in the case of Irbesartan, the spectra were acquired in MeOD solution, in which the ligand can be solved at the NMR used concentration. The ^1H 1D NMR spectrum and ^1H 2D COSY NMR spectra of aliphatic region were collected in MeOD solution (Figures S16, S17 and Table S2). Perturbation in NMR spectra were observed in the Irbesartan methanolic when adding Zn^{2+} ions. The ^1H 1D NMR spectra of free Irbesartan and in presence of Zn^{2+} at a molar ratio Zn^{2+} : Irbesartan 1:1 and 1:2 are presented in Fig. 10.

Figure 10. ^1H 1D NMR spectra of Zn^{2+} : Irbesartan system in MeOD-d_4 solvent at a molar ratio of 0:1 (red), 1:2 (green) and 1:1 (blue) at 25°C and pH 7.

The most apparent feature is the total disappearance of the signal of the protons in position 7 in the aliphatic chain, and a change of the multiplicity of the signal of the two protons in position 8, as highlighted in the inset. A possible tautomerism induced by Zn^{2+} ions, as reported in Scheme 3, can explain this behaviour.

Scheme 3. Possible tautomerism induced by Zn^{2+} ions.

The signal of methylene proton in position 7 can then shift towards lower field, under the water signal.

Since only minor changes in chemical shifts are presented, upon Zn^{2+} addition, by protons and carbons of tetrazole and biphenyl moieties, the coordination site of Zn^{2+} ions can be mostly located on the imidazole ring of Irbesartan ligand.

3.5. Speciation studies

Based on the calculated protonation and complex formation constants of Irbesartan and Losartan a speciation study has been performed. An estimate of the total chelatable Cu^{2+} and Zn^{2+} circulating in plasma, and of their interaction with albumin, was previously presented.[30] The estimate of available Irbesartan as a function of the time elapsed from its delivery is based on the work of Marino et al.[31] The same estimates were used for Losartan assuming a similar behaviour. The micro molar concentrations of the formed complex species and the total complexed metal in percent with respect to the total metal are reported in Table 3.

It is apparent that in correspondence of the drug concentration peak and in all the first six hours from delivery the drug is able to effectively scavenge non-negligible amounts of copper, accompanied by lower amounts of zinc. These results cannot be assumed as a quantitative evaluation of the real behaviour of the drug in vivo for the rough approximations necessarily assumed, as well as for the possible involvement of different copper pools accessible to the drug other than plasma. Nevertheless, they are indicative of a trend in the scavenging activity of the drugs. Furthermore, it must be remembered that the interaction between the drug and the metal ions is not only dictated by the thermodynamic properties, but that also the kinetic factors can play an important role in determining the behaviour of the system.

Table 3. Speciation of different simulated conditions in the presence of the two drugs Losartan and Irbesartan. Total concentrations of ligands, copper and zinc ($\mu\text{mol/L}$) are reported in columns 1 to 3, while the concentrations ($\mu\text{mol/L}$) of calculated complex species (disregarding the minor species) are reported in the remaining columns; in the last columns the percent bound metal ions are reported.

L	Cu _T	Zn _T	CuL	CuL ₂ H ₁	CuL ₂ H ₂	Cu _b %	ZnL	ZnL ₂ H ₁	ZnL ₂ H ₂	Zn _b %
12	5	12	0.005	0.025	0.618	13.0	0.164	0.021	0.044	1.9
12	2	12	0.002	0.011	0.276	14.5	0.174	0.024	0.050	2.1
5	5	12	0.002	0.005	0.135	2.8	0.073	0.004	0.009	0.7
5	2	12	0.001	0.002	0.058	3.1	0.076	0.004	0.009	0.7
2	5	12	0.001	0.001	0.025	0.5	0.031	<0.001	0.002	0.3
2	2	12	<0.001	<0.001	0.010	0.5	0.031	<0.001	0.002	0.3
1	5	12	<0.001	<0.001	0.006	0.1	0.015	<0.001	<0.001	0.1
1	2	12	<0.001	<0.001	0.002	0.1	0.015	<0.001	<0.001	0.1

I	Cu _T	Zn _T	CuL ₂ H ₂	Cu _b %	ZnL ₂ H ₁	ZnL ₂ H ₂	Zn _b %
12	5	12	0.288	5.8	0.026	0.079	0.9
12	2	12	0.122	6.1	0.028	0.084	0.9
5	5	12	0.057	1.1	0.005	0.015	0.2
5	2	12	0.023	1.2	0.005	0.015	0.2
2	5	12	0.010	0.2	0.001	0.002	<0.1
2	2	12	0.004	0.2	0.001	0.003	<0.1
1	5	12	0.002	<0.1	<0.001	<0.001	<0.1
1	2	12	0.001	<0.1	<0.001	<0.001	<0.1

Conclusions

The use of different complementary techniques has allowed both to give the protonation and complex formation constants of Irbesartan and Losartan with Cu²⁺ and Zn²⁺, and to attribute the protonation sequence and the coordination sites. The presence of variously protonated species also explains the fluorescence behaviour of both drugs.

In particular, the binding of both metal ions through the tetrazole moiety obtained clear evidence, except in the case of the Zn^{2+} /Irbesartan system. In this case, NMR measurements give evidence of a possible tautomerism involving the imidazole ring and the connected aliphatic chain. The possibility to form tautomers in the case of Irbesartan might also explain the differences observed in the luminescence properties of the two drugs. ESI-MS spectra remark that in the 1:1 Zn^{2+} /Irbesartan complex one or two water molecules are present. The speciation study, even in the remarked restraints of the made assumption, gives a realistic indication of a copper scavenging action of the two drugs, deserving further clinical attention.

Notes and references

§ Potentiometric equilibrium studies are normally conducted in the $10^{-4} - 10^{-3}$ M concentration range. Actually, the Irbesartan concentration in blood at the highest dosage can be approximately evaluated in 7.5×10^{-5} M, based on the data reported in the Irbesartan Data Sheet (http://www.sanofi.com.au/products/nzl_ds_karvea.pdf).

§§ Following oral administration, Losartan is rapidly absorbed, reaching maximum concentrations 1–2 hours post-administration. After oral administration approximately 14% of a Losartan dose is converted to the pharmacologically active E3174 carboxylic acid metabolite (2-n-butyl-4-chloro-1-[2- ϕ -(1H-tetrazol-5-yl) biphenyl-4-yl)methyl]imidazole-5-carboxyl acid).^[58] E3174 was used as a step-product in the synthesis of 3-butyl-1-chloro-8-[2-(1H-tetrazol-5-yl)phenyl]-5,10-dihydroimidazo[1,5-*b*]isoquinoline, which is completely devoid of activity in the Angiotensin II receptor.^[59]

[1] A.S. Go, D. Mozaffarian, V.L. Roger, E.J. Benjamin, J.D. Berry, W.B. Borden, D.M. Bravata, S. Dai, E.S. Ford, C.S. Fox, S. Franco, H.J. Fullerton, K. Gillespie, S.M. Hailpern, J.A. Heit, V.J. Howard, M.D. Huffman, B.M. Kissela, S.J. Kittner, D.T. Lackland, J.H. Lichtman, L.D. Lisabeth, D. Magid, G.M. Marcus, A. Marelli, D.B. Matchar, D.K. McGuire, E.R. Mohler, C.S. Moy, M.E. Mussolino, G. Nichol, N.P. Paynter, P.J. Schreiner, P.D. Sorlie, S. Stein, T.N. Turan, S.S. Virani, N.D. Wong, D. Woo, M.B. Turner, on behalf of the American Heart Association Statistics Committee and Stroke Statistics Subcommittee, *Circulation*, 127 (2013) e6-e245.

[2] G.J.S. Cooper, A.R.J. Phillips, S.Y. Choong, B.L. Leonard, D.J. Crossman, D.H. Brunton, E.L. Saafi, A.M. Dissanayake, B.R. Cowan, A.A. Young, C.J. Occleshaw, Y.K. Chan, F.E. Leahy, G.F. Keogh, G.D. Gamble, G.R. Allen, A.J. Pope, P.D. Boyd, S.D. Poppitt, T.K. Borg, R.N. Doughty, J.R. Baker, *Diabetes*, 53 (2004) 2501-2508.

- [3] G.J.S. Cooper, A.A. Young, G.D. Gamble, C.J. Occleshaw, A.M. Dissanayake, B.R. Cowan, D.H. Brunton, J.R. Baker, A.R. Phillips, C.M. Frampton, S.D. Poppitt, R.N. Doughty, *Diabetologia*, 52 (2009) 715-722.
- [4] G.J.S. Cooper, Y.K. Chan, A.M. Dissanayake, F.E. Leahy, G.F. Keogh, C.M. Frampton, G.D. Gamble, D.H. Brunton, J.R. Baker, S.D. Poppitt, *Diabetes*, 54 (2005) 1468-1476.
- [5] S. Zhang, H. Liu, G.V. Amarsingh, C.C.H. Cheung, S. Hogg, U. Narayanan, L. Zhang, S. McHarg, J. Xu, D. Gong, J. Kennedy, B. Barry, Y.S. Choong, A.R.J. Phillips, G.J.S. Cooper, *Cardiovasc Diabetol*, 13 (2014) 100.
- [6] J. Lu, D. Gong, S.Y. Choong, H. Xu, Y.K. Chan, X. Chen, S. Fitzpatrick, S. Glyn-Jones, S. Zhang, T. Nakamura, K. Ruggiero, V. Obolonkin, S.D. Poppitt, A.R. Phillips, G.J.S. Cooper, *Diabetologia*, 53 (2010) 1217-1226.
- [7] G.J.S. Cooper, *Curr Med Chem*, 19 (2012) 2828-2860.
- [8] W.B. Kannel, D.L. McGhee, *J Am Med Assoc*, 241 (1979) 2035-2038.
- [9] J. Karalliedde, A. Smith, L. DeAngelis, V. Mirenda, A. Kandra, J. Botha, P. Ferber, G. Viberti, *Hypertension*, 51 (2008) 1617-1623.
- [10] L. Zhang, M.-L. Ward, A.R.J. Phillips, S. Zhang, M.B. Cannell, G.J.S. Cooper, *Cardiovasc Diabetol*, 12 (2013) 123.
- [11] D. Gong, J. Lu, X. Chen, S.Y. Choong, S. Zhang, Y.K. Chan, S. Glyn-Jones, G.D. Gamble, A.R.J. Phillips, G.J.S. Cooper, *Mol Pharmacol*, 70 (2006) 2045-2051.
- [12] J. Lu, B. Pontr, S. Pickup, S.Y. Choong, M. Li, H. Xu, G.D. Gamble, A.R.J. Phillips, B.R. Cowan, A.A. Young, G.J.S. Cooper, *Cardiovasc Diabetol*, 12 (2013) 28.
- [13] G.J.S. Cooper, *Drugs*, 71 (2011) 1281-1320.
- [14] V.M. Nurchi, G. Crisponi, M. Crespo-Alonso, J.I. Lachowicz, Z. Szewczuk, G.J. Cooper, *Dalton Trans*, 42 (2013) 6161-6170.
- [15] J.J. Frasto da Silva, R.J. Williams, in: *The Biological Chemistry of the Elements: The Inorganic Chemistry of Life*, Clarendon Press, Oxford, U.K., 2001, pp. 418-435.
- [16] B.M. Massie, P.E. Carson, J.J.V. McMurray, M. Komajda, R. McKelvie, M.R. Zile, S. Anderson, M. Donovan, E. Iverson, C. Staiger, A. Ptaszynska, for the I-PRESERVE Investigators, *N Engl J Med*, 359 (2008) 2456-2467.
- [17] L.H. Lindholm, H. Ibsen, B. Dahlf, R.B. Devereux, G. Beevers, U. de Faire, F. Fhrquist, S. Julius, S.E. Kjeldsen, K. Kristiansson, O. Lederballe-Pedersen, M.S. Nieminen, P. Omvik, S. Oparil, H. Wedel, P. Aurup, J. Edelman, S. Snapinn, LIFE Study Group, *Lancet*, 359 (2002) 1004-1010.

- [18] M. Koren-Michowitz, V. Dishy, R. Zaidenstein, O. Yona, S. Berman, J. Weissgarten, A. Golik, *Am J Hypertens*, 18 (2005) 358-363.
- [19] G. Gran, *Analyst*, 77 (1952) 661-671.
- [20] P. Gans, A. Sabatini, A. Vacca, *Talanta*, 43 (1996) 1739-1753.
- [21] V.M. Nurchi, J.I. Lachowicz, G. Crisponi, S. Murgia, M. Arca, A. Pintus, P. Gans, J. Niclos-Gutierrez, A. Domínguez-Martín, A. Castineiras, M. Remelli, Z. Szewczuk, T. Lis, *Dalton Trans*, 40 (2011) 5984-5998.
- [22] P. Gans, A. Sabatini, A. Vacca, *Annali di Chimica*, 89 (1999) 45-49.
- [23] M. Peana, S. Medici, V.M. Nurchi, G. Crisponi, M.A. Zoroddu, *Coord Chem Rev*, 257 (2013) 2737-2751.
- [24] M.A. Zoroddu, M. Peana, S. Medici, *Dalton Trans*, (2007) 379-384.
- [25] M.A. Zoroddu, S. Medici, M. Peana, R. Anedda, *Dalton Trans*, 39 (2010) 1282-1294.
- [26] P.K. Glasoe, F. Long, *J Phys Chem*, 64 (1960) 188-190.
- [27] A.K. Covington, M. Paabo, R.A. Robinson, R.G. Bates, *Analytical Chemistry*, 40 (1968) 700-706.
- [28] Bruker, ESI Compass 1.3; DataAnalysis Version 4.0 (Build 234) Daltonik GmbH <http://www.bdal.com>.
- [29] L. Alderighi, P. Gans, A. Ienco, D. Peters, A. Sabatini, A. Vacca, *Coord Chem Rev*, 184 (1999) 311-318.
- [30] G. Crisponi, V.M. Nurchi, M. Crespo-Alonso, G. Sanna, M.A. Zoroddu, G. Alberti, R. Biesuz, *PLoS ONE*, 10 (2015) e0133050.
- [31] M.R. Marino, K. Langenbacher, N.F. Ford, H.D. Uderman, *J Clin Pharm*, 38 (1998) 246-255.
- [32] G.C. V.M. Nurchi, J. I. Lachowicz, M. A. Zoroddu, M. Peana, S. Medici, D. Veciani, M. Tolazzi, A. Melchior, *Eur J Pharm Sci*, 93 (2016) 380-391.
- [33] E. Cagigal, L. Gonzalez, R. Alonso, R. Jimenez, *J Pharm Biomed Anal*, 26 (2001) 477-486.
- [34] H.P. Limbacher, G.D. Blickenstaff, J.H. Bowen, H.H. Wang, *Biochim Biophys Acta (BBA)-Biomembranes*, 812 (1985) 268-276.
- [35] M. Meloun, T. Syrový, A. Vrána, *Anal Chim Acta*, 533 (2005) 97-110.
- [36] J.C. Gillis, A. Markham, *Drugs*, 54 (1997) 885-902.
- [37] K.L. Goa, A.J. Wagstaff, *Drugs*, 51 (1996) 820-845.
- [38] K.E. McCarthy, Q. Wang, E.W. Tsai, R.E. Gilbert, D.P. Ip, M.A. Brooks, *Journal of Pharmaceutical and Biomedical Analysis*, 17 (1998) 671-677.
- [39] R. Williams, M. Alasandro, V. Fasone, R. Boucher, J. Edwards, *J Pharm Biomed Anal*, 14 (1996) 1539-1546.

- [40] C.I. Furtek, M.-W. Lo, *J Chrom B: Biomedical Sciences and Applications*, 573 (1992) 295-301.
- [41] S.Y. Chang, D.B. Whigan, N.N. Vachharajani, R. Patel, *J Chrom B: Biomedical Sciences and Applications*, 702 (1997) 149-155.
- [42] P. Tosco, B. Rolando, R. Fruttero, Y. Henchoz, S. Martel, P.A. Carrupt, A. Gasco, *Helv Chim Acta*, 91 (2008) 468-482.
- [43] T. Eicher, S. Hauptmann, Thieme, New York, (1995).
- [44] V. Ostrovskii, G. Koldobskii, N. Shirokova, V. Poplavskii, *Chem Hetero Comp*, 17 (1981) 412-416.
- [45] L. Haussen, E. Baca, P. Scheiner, *J Heterocycl Chem*, 7 (1970) 991.
- [46] G. Crisponi, *Reactive and Functional Polymers*, 34 (1997) 121-126.
- [47] M.C. Aragoni, M. Arca, G. Crisponi, F. Cristiani, F. Isaia, V.M. Nurchi, *Talanta*, 43 (1996) 1357-1366.
- [48] A. Avdeef, J.E. Comer, S.J. Thomson, *Anal Chem*, 65 (1993) 42-49.
- [49] J. Bridges, P. Creaven, R. Williams, *Biochem J*, 96 (1965) 872-878.
- [50] J. Jayabharathi, V. Thanikachalam, N. Srinivasan, M.V. Perumal, *Spectrochim Acta Part A: Molecular and Biomolecular Spectroscopy*, 90 (2012) 125-130.
- [51] S.B. Etcheverry, E.G. Ferrer, L. Naso, D.A. Barrio, L. Lezama, T. Rojo, P.A. Williams, *Bioorg Med Chem*, 15 (2007) 6418-6424.
- [52] Â.M. Denadai, J.G. Da Silva, P.P. Guimarães, L.B.S. Gomes, A.S. Mangrich, E.I. de Rezende, I.M. Daniel, H. Beraldo, R.D. Sinisterra, *Mater Sci Eng: C*, 33 (2013) 3916-3922.
- [53] L. Toso, G. Crisponi, V.M. Nurchi, M. Crespo-Alonso, J.I. Lachowicz, M.A. Santos, S.M. Marques, J. Niclós-Gutiérrez, J.M. González-Pérez, A. Domínguez-Martín, D. Choquesillo-Lazarte, Z. Szewczuk, *J Inorg Biochem*, 127 (2013) 220-231.
- [54] L. Toso, G. Crisponi, V.M. Nurchi, M. Crespo-Alonso, J.I. Lachowicz, D. Mansoori, M. Arca, M.A. Santos, S.M. Marques, L. Gano, J. Niclós-Gutiérrez, J.M. González-Pérez, A. Domínguez-Martín, D. Choquesillo-Lazarte, Z. Szewczuk, *J Inorg Biochem*, 130 (2014) 112-121.
- [55] V.M. Nurchi, G. Crisponi, M. Arca, M. Crespo-Alonso, J.I. Lachowicz, M.A. Zoroddu, M. Peana, G. Pichiri, M.A. Santos, S.M. Marques, J. Niclos-Gutierrez, M.J. Gonzalez-Perez, A. Dominguez-Martin, D. Choquesillo-Lazarte, Z. Szewczuk, D. Mansoori, L. Toso, *J Inorg Biochem*, 141 (2014) 132-143.
- [56] Joanna Izabela Lachowicza, Valeria Marina Nurchi, Guido Crisponi, Maria de Guadalupe Jaraquemada-Pelaez, Małgorzata Ostrowska, Julia Jezierska, Elżbieta Gumienna – Kontecka,

Massimiliano Peana, Maria Antonietta Zoroddu, Duane Choquesillo-Lazarte, J. Niclós-Gutiérrez, J.M. González-Pérez, *J Inorg Biochem*, 151 (2015).

[57] J.I. Lachowicz, V.M. Nurchi, G. Crisponi, M.d.G.J. Pelaez, A. Rescigno, P. Stefanowicz, M. Cal, Z. Szewczuk, *J Inorg Biochem*, 151 (2015) 36-43.

[58] D.A. Sica, T.W. Gehr, S. Ghosh, *Clin Pharmacokin*, 44 (2005) 797-814.

[59] S.E. Yoo, Y.A. Shin, S.H. Lee, N.J. Kim, *Bioorg Med Chem*, 3 (1995) 289-295.

Graphical abstract