

4-hydroxy-3,5-pyridinedicarboxylic acids: synthesis, complexation properties towards Fe(III), Al(III), Cu(II), Zn(II), human serum albumin, and cellular toxicity

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Abstract

4-hydroxy-3,5-pyridinedicarboxylic acid (DQ58) and 4-hydroxy-1-methyl-3,5-pyridinedicarboxylic acid (DQ71508) have been synthesized, and their Fe(III), Al(III), Cu(II), and Zn(II) coordination properties have been studied by potentiometry, UV/Vis (in the case of Fe(III), Al(III), Cu(II)), ¹H-NMR (for Al(III)), and EPR (for Cu(II)). Thermodynamic results were used to model the extent of the toxic metal ions decorporation (Fe(III) or Al(III)) in the presence of the essential metal ion (Cu(II) or Zn(II)). DQ58 and DQ71508 were demonstrated to interact with human serum albumin (HSA), which is assumed to be the main serum transporter of the chelators, and binding constants have been obtained by ultrafiltration. IC₅₀ values of 5.185 mM and 1.033 mM were collected after 24 and 48 h of treatment with DQ71508 towards human embryonic kidney HEK-293 cells, demonstrating the relatively low cytotoxicity of this compound. According to these results, both DQ58 and DQ71508 seem to be potential candidates for Fe chelation therapy, and DQ58 is a better Fe(III) chelator than DQ71508.

Keywords

iron - aluminium - chelation therapy - potentiometry - hydroxypyridinedicarboxylic acids - cytotoxicity

Introduction

Chelation therapy is the most efficient therapeutic approach for pathologies due to iron (Fe) and aluminium (Al) overload. The presently available chelators are Desferal (DFO), Deferiprone (L1), and Deferasirox (ICL670). DFO and L1 have several drawbacks, and also the recently developed Fe chelator ICL670 has controversial efficiency and side effects. Therefore, the search for alternative molecules is strongly and continuously requested [1-3].

3,4-Hydroxypyridinecarboxylic acids (HPCs) are being proposed as possible chelating agents for Fe and Al because they display a number of favourable properties [4], which include strong complexation strength towards metal ions under overload conditions (Fe(III) and Al(III) in Fe and Al chelation therapy), low stability of non-overloaded essential metal ions complexes, low cellular toxicity, no redox activity, and a low molecular weight (prerequisite for oral activity [5]). Several methyl HPC derivatives have been studied so far, which demonstrate that the methyl group, especially the N-methyl substitution, has the positive effect to increase the stability of the Fe(III)-HPC complexes [4,6], thus reasonably leading to a more efficient toxic metal ion decorporation in vivo.

Ortho-carboxylic HPC derivatives, i.e., hydroxypyridinedicarboxylic acids (HPDCs), can be investigated as alternative HPC for a possible application as Fe(III) and Al(III) chelating agents, because the second carboxylate can in principle enhance the complexation affinity towards hard metal ions with respect to hydroxypyridine*monocarboxylic* acids. The compounds considered in this work are the unsubstituted 4-hydroxy-3,5-pyridinedicarboxylic acid (DQ58, Figure 1), and the N-methylated derivative of DQ58 (4-hydroxy-1-methyl-3,5-pyridinedicarboxylic acid, DQ71508, Figure 1). DQ71508 was chosen to check the effects (in particular towards the Fe(III)-ligand complexation strength) of a simultaneous N-methyl and *o*-carboxylic substitution on the pyridinic ring. It is worth noting that HPDCs have been hitherto completely ignored in the literature.

DQ58 and DQ71508 have been synthesized as they are not commercially available. The Fe(III) and Al(III) coordination properties of these HPDCs were studied in order to obtain the thermodynamic speciation properties of the toxic metal ions in the presence of each chelator. Cu(II) and Zn(II) complexes have been investigated as well, with the aim to highlight the chelator affinity towards these essential metal ions and to evaluate the competition between toxic and essential metal ions towards the chelator. The binding constants of DQ58 and DQ71508 with human serum albumin (HSA, which is assumed to be the main serum transporter of the chelators) have been obtained in order to estimate if these HPDCs interact with this important plasma protein. The in vitro cytotoxicity of DQ71508 has been studied in order to assess the effect induced by the insertion of a second carboxylic in HPC molecule on cell viability.

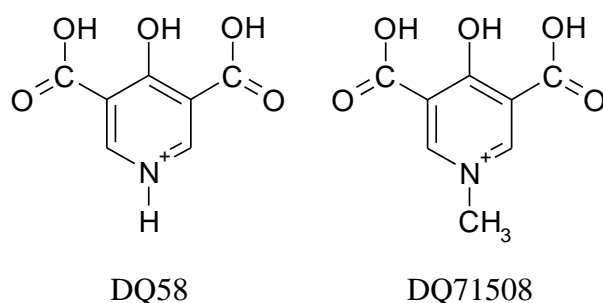


Figure 1. 4-hydroxy-3,5-pyridinedicarboxylic acid (DQ58), and 4-hydroxy-1-methyl-3,5-pyridinedicarboxylic acid (DQ71508). Both ligands are shown in their charged (most protonated) form.

Experimental

Synthesis of 4-hydroxy-3,5-pyridinedicarboxylic acid (DQ58), and 4-hydroxy-1-methyl-3,5-pyridinedicarboxylic acid (DQ71508).

All chemicals and solvents were obtained from Aldrich Chimica, Lancaster or Acros. The identity and purity of the intermediates and of the final compounds were checked by $^1\text{H-NMR}$ spectra (Bruker 400 MHz spectrometer) with the solvents indicated. Chemical shifts are reported in δ (ppm) values downfield from tetramethylsilane which is taken as internal reference. Elemental analyses were performed in the Microanalytical Laboratory, Department of Pharmaceutical Sciences, University of Padova, on a Perkin-Elmer C, H, N elemental analyzer model 240B. Mass spectra were obtained on a Mat 112 Varian Mat Bremen (70 eV) mass spectrometer and Applied Biosystems Mariner System 5220 LC/MS (nozzle potential 250.00). Chemical reactions were monitored by analytical thin-layer chromatography (TLC) on Merck silica gel 60 F-254 glass plates. Solutions were concentrated on a rotary evaporator under reduced pressure.

The synthetic procedure leading to DQ58 e DQ71058 is reviewed in the Scheme of Figure 2 and it is described in the following subsections. Both compounds were synthesized on the basis of the preparation of similar compounds described by Balogh et al. [7]. A similar synthetic path was followed for the synthesis of 4-hydroxy-5-methyl-3-pyridinedicarboxylic acid and 4-hydroxy-1,5-dimethyl-3-pyridinedicarboxylic acid [8].

DQ58

Equimolar amounts of 1,3,5 triazine (3.15 g, 38.5 mmol) and dimethyl-1,3-acetonedicarboxylate (6.70 g, 38.5 mmol) were slowly added under magnetic stirring and at room temperature to a freshly prepared sodium ethoxide solution in anhydrous ethanol. The solution was always maintained under magnetic stirring and nitrogen gas flow. During the addition the solution became yellow and a white precipitate was formed. After the addition, the solution was allowed to react under heating (reflux) for

2 hours. The resulting orange solution, after cooling, was slowly acidified to ca. pH 4 with HCl solution.

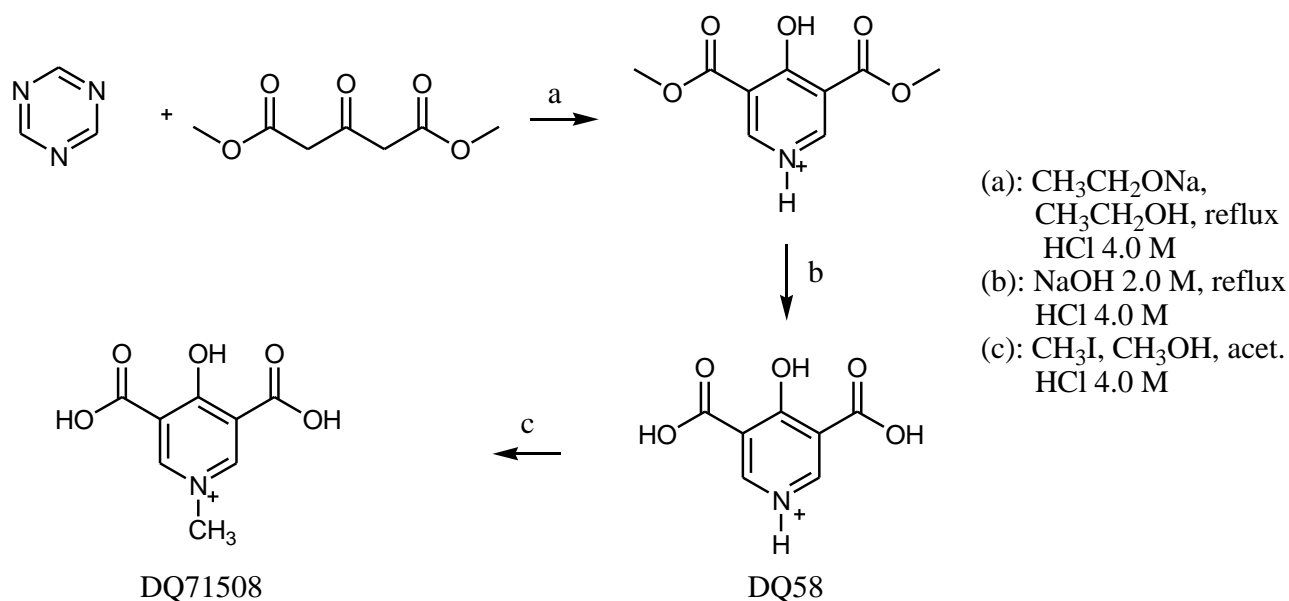


Figure 2. Scheme of the synthesis of DQ58 and DQ71508.

The prepared diethyl ester was recovered by filtration through gooch G3 and treated with 90 mL of 2.0 M NaOH solution. Under heating for 4 hours (reflux) the ester dissolved and the resulting yellow solution was allowed to stir overnight at room temperature. Then the solution was acidified to pH 2.5 with 4.0 M HCl solution. A white solid was formed, that was filtrated and purified by hot-cold water treatment. Yellow crystals of DQ58 were obtained with a 98.2 % purity determined from potentiometric titrations. The final yield was 85 %. Elemental analysis: calcd. C 46.2, H 2.2, N 7.7; found C 45.1, H 2.1, N 7.2. $^1\text{H-NMR}$ (D_2O , pD = 3): δ 8.80 (s, 2 H, -H). HR-MS: m/z = 184.12 ($[\text{MH}]^+$; MM of $\text{C}_7\text{H}_5\text{NO}_5$ = 183.12).

DQ71508

DQ58 (1 g, 54.6 mmol) was dissolved in a 100 mL flask with a little amount of NaOH (5 M, 3 mL). After complete dissolution, methanol (10 mL), acetone (40 mL) and a large excess of methyl iodide (6 mL, 96.3 mmol) were added. The mixture was heated to reflux for 4 h, and then it was allowed to react at room temperature overnight. After removal of the solvent, the residue was dissolved in 30 mL HCl 2.0 M and stirred in the presence of 2-3 drops of H_2O_2 to remove iodine. After concentration in a rotary evaporator, the formed solid product was collected by filtration. In order to remove NaCl present as an impurity, the residue was treated with a small volume of absolute ethanol (15 mL), the suspension was filtered, and the filtrate was added with an excess of diethyl ether, obtaining the precipitate of DQ71508. This was collected and dried under vacuum (yield 60 %). A

95.6% purity was obtained from potentiometric titrations, which agrees with the elemental analysis results (below), so that the copresence of DQ71508 and DQ71508·HCl can be supposed in the product. Elemental analysis: calcd. C 48.7, H 3.6, N 7.1; found C 46.2, H 3.1, N 6.7. $^1\text{H-NMR}$ (D_2O , $\text{pD} = 3$): δ 8.75 (s, 2 H, -H), 4.02 (s, 3H, $-\text{CH}_3$). HR-MS: $m/z = 198.19$ ($[\text{MH}]^+$; MM of $\text{C}_8\text{H}_7\text{NO}_5 = 197.11$).

Complexometric study

Compounds

The pH-potentiometric titrations were performed with 0.1 m ($m = \text{mol/kg}$) NaOH or 0.2 M ($M = \text{mol/L}$) KOH prepared from NaOH (Fluka) and KOH pellets (Merck). The base was standardised by 0.1 m or 0.2 M HCl solutions prepared from 37% HCl (Merck). The 0.2 m Fe^{3+} and Al^{3+} solutions were prepared from electrolytic dissolution of pure Fe granules and Al wire, respectively (Aldrich), added with distilled HCl (which was diluted to ca. 0.05 m in the final solution), and standardized as described previously [9,10]. The 0.1 M Cu^{2+} and Zn^{2+} solutions were prepared from CuCl_2 and ZnCl_2 (Aldrich), and their exact concentrations was determined gravimetrically via the oxinates. DQ58 and DQ71508 were obtained by synthesis as described above, and their 10^{-3} M solutions were standardised by base. Solutions for UV-Vis were the same as for potentiometric titrations. Solutions for $^1\text{H-NMR}$ measurements were prepared by dissolving weighed amounts of ligand (DQ58 or DQ71508) and AlCl_3 (Carlo Erba) in D_2O (Aldrich, 99.9% atom D). The internal reference was $\text{Me}_3\text{SiCH}_2\text{CH}_2\text{COOH}$ (TSP, Aldrich).

Equilibrium measurements

The stability constants of the proton and metal complexes of the ligands were determined by pH-potentiometric titrations. A Metrohm 715 Dosimat burette was used. The samples were in all cases thermostatted at 25.0 ± 0.1 °C, and completely deoxygenated by bubbling purified nitrogen or argon for ca. 15 min. before the measurements. The bubbling gas was also passed over the solutions during the titrations. The electrode system was calibrated according to Irving et al. [11] and the pH-metric readings could therefore be converted into hydrogen ion concentration. About 200 titration points were used for each system. The accepted fitting of the titration curves was always less than 0.01 cm^3 . Duplicate titrations were performed. The reproducibility of the titrations was within 0.005 pH unit. The pH-metric titrations were performed in the pH range 2.0–11.0 or until precipitation occurred in the samples. The ligand concentration in the potentiometric cell varied in the range $3.00 \cdot 10^{-4}$ – $1.00 \cdot 10^{-3}$ (M or m). The metal ion to ligand ratios was in the range 1:1–1:4. After the mixing of Al(III) and DQ71508 in an acidic solution (pH below ca. 3.5), the measured pH drifted and reached a constant value only after ca. 1 hour, suggesting a low complex formation rate. The kinetics of the Al(III) + DQ71508 reaction became “normal” (equilibration after ca. 1 min) at pH values above 3.5. These

findings are in agreement with previous results with other HPCs ([8] and references therein). Experimental details regarding the handling of the slow kinetics during the titrations are reported [12].

For Fe(III) and Al(III)-related titrations, the ionic strength of the solutions was adjusted to 0.6 m (Na)Cl aqueous solution, and the titrant was a 0.1 m carbonate-free NaOH solution. For Cu(II) and Zn(II)-related titrations, medium was a 0.2 M KCl, and a 0.2 M carbonate-free KOH solution was the titrant. These ionic strengths and concentration units have been used to allow a rigorous comparison among metal-HPCs data which have been always obtained in these conditions ([4,6] and references in [4]). The water ionization constant, pK_w , is 13.69 ± 0.01 in (Na)Cl 0.6 m and 13.76 ± 0.01 in KCl 0.2 M.

The general equilibrium reaction is: $pM + qL + rH = M_pL_qH_r$, where M denotes the metal ion and L the non-protonated ligand molecule (see caption of Table 2). Charges are omitted for simplicity. The corresponding concentration stability constants $\beta_{pqr} = [M_pL_qH_r]/[M]^p[L]^q[H]^r$ were calculated using the PITMAP [13] or the PSEQUAD [14] computer programmes. Calculations were always made on the experimental results obtained before precipitation. Stability constants for iron - and aluminium/hydroxo complexes have been taken from the literature: $\log\beta_{FeOH} = -2.87$, $\log\beta_{Fe(OH)_2} = -6.16$ [10], $\log\beta_{Fe(OH)_3} = -12.16$, $\log\beta_{Fe(OH)_4} = -22.16$, $\log\beta_{Fe_2(OH)_2} = -2.9$, $\log\beta_{Fe_3(OH)_4} = -6.3$, $\log\beta_{Fe_{12}(OH)_{34}} = -48.9$ [15], $\log\beta_{AlOH} = -5.52$, $\log\beta_{Al(OH)_2} = -11.3$, $\log\beta_{Al(OH)_3} = -17.3$, $\log\beta_{Al(OH)_4} = -23.46$, $\log\beta_{Al_3(OH)_4} = -13.57$, $\log\beta_{Al_{13}(OH)_{32}} = -109.2$ [16]. pK_a values were calculated from the protonation constants obtained by the same programmes and refer to the general reaction: $H_hL = H_{h-1}L + H$.

UV-Vis spectra were recorded at 25.0 ± 0.1 °C, in 0.5 or 1 cm quartz cuvettes, using a Perkin-Elmer Lambda 25 and a Hewlett Packard 8452A diode array spectrophotometer, for solutions containing each ligand alone (DQ58 or DQ71508), and for solutions containing the ligand and the metal ion (Fe(III), Al(III), or Cu(II)), at various pH values. If the pH was below 2, the pH was computed from the stoichiometric concentration of HCl, because the $[H^+]$ modifications produced by the other species were negligible under these conditions. Otherwise, the pH was measured with the same electrodes and procedures as for potentiometric titrations. Calculations of the stability constants (values are reported in Tables 1 and 2) were performed at the wavelength displaying the maximum absorbance variation with pH.

1H -NMR spectra were obtained at 25 °C using a Bruker DRX-400 spectrometer operating at 400.13 MHz. Chemical shift values are given in δ units with reference to internal TSP. Suitable integral values for the proton signals were obtained by a pre-scan delay of 10 s. The assignment of the proton resonances was performed by standard chemical shift correlations and NOESY measurements when necessary. Spectra were collected in D₂O solutions containing each free ligand, and Al(III) + ligand. The pH was measured with a Crison 5014 combined glass electrode previously calibrated in buffered

aqueous solutions at pH = 4 and pH = 7. The values of pD were computed by adding 0.41 pH units to the pH meter readings, in order to correct for isotopic and solvent effects due to the use of D₂O instead of H₂O [17].

Continuous wave anisotropic EPR spectra (9.7 GHz) were recorded with a Bruker EleXsys E500 spectrometer at room temperature in circulating aqueous solutions during a titration. Three titrations were performed with KOH in solutions containing ligand (either DQ58 or DQ71508) $1.3 \cdot 10^{-3}$ M, Cu(II) $4 \cdot 10^{-4}$ M, and KCl 0.2 M, under nitrogen flow, in the pH range 1.8-3.8, by using the same procedure as for potentiometric measurements. At various pH values, samples of 100 μ L were taken from each solution and frozen in liquid nitrogen to perform isotropic spectra. Isotropic EPR parameters for some of the complexes formed are reported in Table 1S (online resource).

Ultrafiltration measurements

Samples were separated by ultrafiltration through 10-kDa membrane filters (Microcon YM-10 centrifugal filter unit, Amicon, Millipore) according to a standard procedure [18]. In the protein-ligand interaction studies, all 1-mL samples contained 20 μ M HSA and the carrier ligand in 0.10 M HEPES buffer at pH 7.4. The ligand concentrations were varied from 20 μ M to 1 mM. Time-dependent measurements were carried out to determine the time needed to reach equilibrium between the reacting partners: an incubation time of 10 min was found to be enough. The samples were then transferred to filter tubes and centrifuged for 10 min at 10,000 rpm (25 °C). After the separation, the low molecular mass (LMM) fractions were obtained. The non-bound ligand was determined by UV spectrophotometry. Control samples containing the carrier ligand and HEPES at the same concentrations but without the protein were used to determine the analytical concentration of the ligand. No adsorption of the drug on the filter was observed. The binding constants were calculated with the computer program PSEQUAD from the total and free concentration data pairs obtained at different protein-ligand ratios.

Cytotoxicity assay (MTT assay).

Human Embryonic Kidney 293 (HEK-293) cell line was obtained by ATCC, Rockville, MD. Cells were maintained in the logarithmic phase at 37 °C in a 5 % carbon dioxide atmosphere using the DMEM medium (Euroclone) containing 10% fetal calf serum (Euroclone, Milan, Italy), antibiotics (50 units mL⁻¹ penicillin and 50 μ g mL⁻¹ streptomycin) and 2 mM L-glutamine.

The growth inhibitory effect towards human HEK-293 cell line was evaluated by means of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; tetrazolium salt reduction) assay [19]. Briefly, $3 \cdot 10^3$ cells/well were seeded in 96-well microplates in growth medium (100 μ L) and then incubated at 37 °C in 5% CO₂. After 24 h, the medium was removed and replaced with a fresh one

containing the compound to be studied at the appropriate concentration. Triplicate cultures were established for each treatment. After 24 and 48 h, each well was treated with 10 μL of a 5 $\text{mg}\cdot\text{mL}^{-1}$ MTT saline solution. After 5 h additional incubation, 100 μL of a sodium dodecylsulfate (SDS) solution in 0.01 M HCl were added. Following overnight incubation, the inhibition of cell growth was detected by measuring the absorbance of each well at 570 nm using a Bio-Rad 680 microplate reader (Bio-Rad, Hercules, CA, USA). Mean absorbance for each drug dose was expressed as a percentage of the control untreated well absorbance and plotted *vs.* drug concentration. IC_{50} values represent the drug concentrations that reduced the mean absorbance at 570 nm to 50% of those in the untreated control wells.

Results and discussion

Acidity constants of the ligands

The $\text{p}K_{\text{a}}$ values for each ligand are reported in Table 1. The assignment of the $\text{p}K_{\text{a}}$ values is not straightforward, because 4-hydroxypyridines can in principle exist in two tautomeric forms, the aromatic and the quinoid one. However, previous results indicate that for HPCs no significant quinoid form should exist in aqueous solutions [4], and the same is expected to occur for HPDCs, too. Therefore, for both ligands $\text{p}K_{\text{a}1}$ and $\text{p}K_{\text{a}2}$ are reasonably assigned to the two $-\text{COOH}$, the $\text{p}K_{\text{a}3}$ of DQ58 is due to the pyridinic $-\text{NH}$, and the $\text{p}K_{\text{a}3}$ of DQ71508 and the $\text{p}K_{\text{a}4}$ of DQ58 are assigned to $-\text{OH}$. The $\text{p}K_{\text{a}}$ values increase by ca. 0.2 log units when passing from (Na)Cl 0.6 m to KCl 0.2 M ionic strength. The low $\text{p}K_{\text{a}}$ values for the phenolic-OH, based on the comparison with salicylic acid derivatives, was justified [20], and it agrees with values obtained for the other HPCs [4].

Table 1. $\text{p}K_{\text{a}}$ values of DQ58 and DQ71508, at 25.0 $^{\circ}\text{C}$ in aqueous (Na)Cl 0.6 m or KCl 0.2 M. Standard deviations are given in brackets.

$\text{p}K_{\text{a}}$	DQ58 (Na)Cl 0.6 m	DQ58 KCl 0.2 M	DQ71508 (Na)Cl 0.6 m	DQ71508 KCl 0.2 M
$\text{p}K_{\text{a}1}$	0.3 (0.5) ^[a]	< 2	< 0 ^[a]	< 2
$\text{p}K_{\text{a}2}$	3.05 (0.01)	3.22 (0.02) 3.24 (0.01) ^[a]	3.101 (0.005)	3.16 (0.06) 3.14 (0.01) ^[a]
$\text{p}K_{\text{a}3}$	6.20 (0.01)	6.37 (0.01) 6.56 (0.01) ^[a]	6.530 (0.005)	6.83 (0.02) 6.78 (0.01) ^[a]
$\text{p}K_{\text{a}4}$	10.85 (0.01)	11.20 (0.01) 11.16 (0.01) ^[a]	–	–

^[a] values obtained by UV/Vis.

All $\text{p}K_{\text{a}}$ values were determined by potentiometric titrations, except $\text{p}K_{\text{a}1}$, which is very low (< 2) and therefore cannot be obtained by this technique because the potential measurements at very acidic pH have a high intrinsic uncertainty. The $\text{p}K_{\text{a}1}$ values in aqueous (Na)Cl 0.6 m were measured by

UV/Vis, but only for DQ58 a sufficiently high (> 0) value was determined. UV/Vis spectra in KCl 0.2 M allowed also to obtain the other pK_a values, which were in agreement with the potentiometric ones (Table 1). The UV/Vis spectra of both ligands at various pH values are shown in the Online Resource (Figures 1S and 2S).

Metal/ligand complexes

Potentiometric data for Fe(III) and Al(III)-ligand solutions were elaborated in a sequential manner, as described previously [21], to avoid the difficulties produced by the strong correlation between parameters to be optimised in the presence of a large number of metal/ligand species. Less complicated speciations were obtained for Cu(II) and especially for Zn(II). Stoichiometry and stability constants of metal/ligand complexes identified in solution are reported in Table 2. Distribution diagrams for Fe(III)/ligand and Al(III)/ligand solutions, computed at $5 \cdot 10^{-4}$ m metal ion and $2 \cdot 10^{-3}$ m ligand, are shown in Figure 3. Zn(II)/ligand and Cu(II)/ligand distribution diagrams are reported in the Online Resource (Figure 3S).

The observed Fe(III) and Al(III) speciations resemble the general characteristics observed for the metal/HPCs solutions studied so far ([4] and references therein), so that mono, bis and tris-complexes are formed with variable protonation states. The tris-complexes of HPDCs (MA_3H_x , MB_3H_x) are less stable than the tris-complexes of the HPCs, as their fraction in solution is always lower than 1. The presence of the second carboxylate group in HPDCs, which has an intrinsic low pK_a (between 0 and 3, see Table 1), causes the ligand and thus the tris-complexes to be highly negatively charged (Table 2): this charge likely destabilizes the HPDCs tris-complexes when compared to the analogous, less charged, HPCs tris-complexes.

pFe and pAl values ($pM = -\log[M^{3+}]$) can be calculated [22] from speciation data at $pH = 7.4$ to indicate the effective complexation strength of different ligands towards the given metal ion. These data are reported in Table 2, and they indicate that DQ58 is a stronger chelator than DQ71508, especially as regards Fe(III). It can be concluded that the simultaneous insertion of a 5-carboxylate and a 1-methyl group in the pyridinic ring of HPCs is less beneficial than the insertion of the carboxylate only, as regards the Fe chelation efficiency, whereas it has little effects as regards the Al chelation efficiency. The pFe and pAl values of DQ58 are lower than those of the presently used drugs (e.g. pFe for deferiprone = 21), and are comparable to those of 1,6-dimethyl-4-hydroxy-3-pyridinecarboxylic acid ($pFe = 18.7$, $pAl = 12.6$) [4], which up to now has displayed the strongest Fe(III) and Al(III) affinity among the 4-hydroxy-3-pyridinecarboxylic acid compounds.

Significant differences were observed among the speciation of Cu(II)/HPDCs and Cu(II)/HPCs solutions, as in the presence of HPDCs the formation of dinuclear species (Cu_2L_2 -type) was detected (Cu(II)/HPCs complexes studied up to now are only mononuclear [4,23]). A possible structure of the

dinuclear complexes is shown in Figure 4S; all coordinating groups and the two metal ions are in the same plane. The same structures were published earlier for the complexes formed by 2,6-carboxyphenolate or derivatives with Cu(II) (e.g. [24-26]). Fe(III) and Al(III) cannot form such species probably because of the high charge repulsion among the two metal ions. For Zn(II) only weak mononuclear complexes were detected.

Table 2. Stoichiometry and stability constants ($\log\beta$) for metal/ligand complexes, at 25.0 °C and in the reported ionic strength. Standard deviations are given in brackets. A is the completely deprotonated form of DQ58, charged -3 , B is the completely deprotonated form of DQ71508, charged -2 (Figure 1 displays the H_4A^+ and H_3B^+ forms). pFe and pAl represents $-\log[M^{3+}]$ and have been computed at $5 \cdot 10^{-5}$ m of ligand and 10^{-6} m of metal ion, respectively [22], and at pH = 7.4.

metal/ligand	DQ58		DQ71508	
Fe(III), (Na)Cl 0.6 m	FeAH ⁺	19.67 (0.03) ^[a]	FeBH ²⁺	12.35 (0.05) ^[a]
	FeA	16.80 (0.01)	FeB ⁺	9.16 (0.03)
	FeAH ₋₁ ⁻	13.05 (0.01)	FeB ₂ H	21.0 (0.2)
	FeA ₂ H ₂ ⁻	37.33 (0.08)	FeB ₂ ⁻	17.9 (0.2)
	FeA ₂ H ²⁻	31.62 (0.08)	FeB ₂ H ₋₁ ²⁻	12.8 (0.2)
	FeA ₃ H ₃ ³⁻	53.40 (0.03)	FeB ₃ H ₋₁ ⁴⁻	15.4 (0.3)
	FeA ₃ H ₂ ⁴⁻	47.84 (0.02)	pFe = 17.6	
	FeA ₃ H ⁵⁻	40.88 (0.06)		
	FeA ₃ ⁶⁻	34.29 (0.08)		
	pFe = 18.6			
Al(III), (Na)Cl 0.6 m	AlAH ⁺	18.14 (0.05)	AlB ⁺	7.74 (0.01)
	AlA ₂ H ₂ ⁻	35.20 (0.03)	AlBH ₋₁	2.08 (0.08)
	AlA ₂ H ²⁻	28.98 (0.02)	AlB ₂ H	18.24 (0.02)
	AlA ₂ ³⁻	21.68 (0.01)	AlB ₂ ⁻	14.13 (0.05)
	AlA ₃ H ₃ ³⁻	50.56 (0.01)	AlB ₃ H ²⁻	23.89 (0.03)
	AlA ₃ ⁶⁻	27.81 (0.01)	AlB ₃ ³⁻	18.22 (0.02)
		pAl = 12.6	pAl = 12.3	
Cu(II), KCl 0.2 M	CuAH ₂ ⁺	20.82 (0.03)	CuBH ⁺	10.43 (0.02)
	CuA ₂ ⁴⁻	17.2 (0.2) ^[a]	CuB ₂ ²⁻	10.9 (0.2) ^[a]
	Cu ₂ A ₂ H ₂	39.12 (0.08)	Cu ₂ B ₂	18.06 (0.02)
	Cu ₂ A ₂ H	34.3 (0.3) ^[a]		
	Cu ₂ A ₂	28.0 (0.3) ^[a]		
Zn(II), KCl 0.2 M	ZnAH	15.09 (0.03)	ZnBH ⁺	9.25 (0.06)
			ZnB	4.59 (0.06)

^[a] values obtained by UV/Vis.

UV/Vis spectra were collected for Fe(III)/ligand, Al(III)/ligand, and Cu(II)/ligand solutions. Spectra display the typical absorption of substituted pyridinic rings. One weak charge-transfer peak appears between 350 nm and 400 nm when Fe(III) coordinates to each ligand. Al(III) and Cu(II) complexation produces changes of the absorption coefficients in the wavelengths range 200-340 nm. Selected UV/Vis spectra of metal/ligand solutions are reported in the Online Resource (Figures 5S-7S).

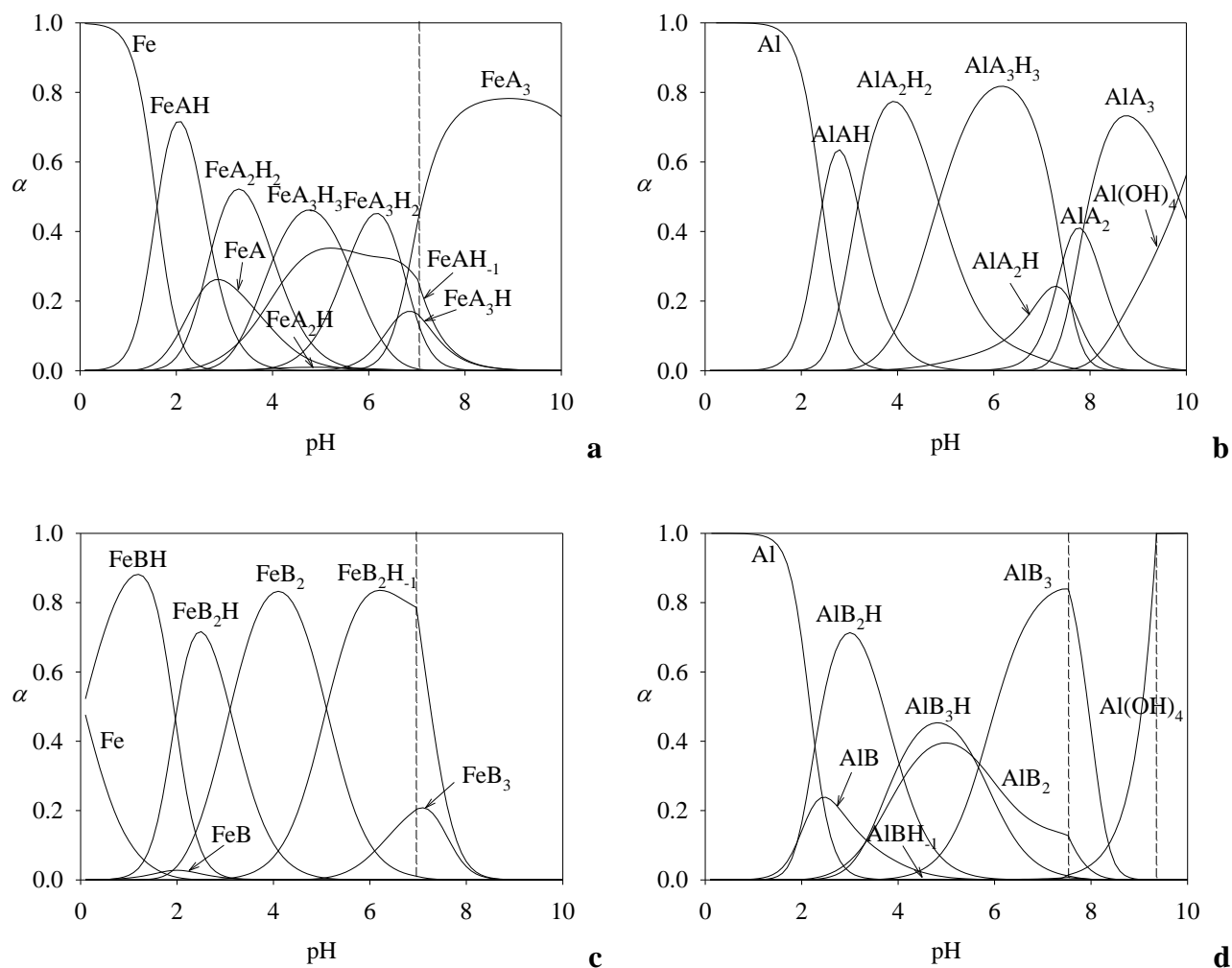


Figure 3. Distribution diagrams of the most important Fe(III) (a, c) and Al(III) (b, d) species in the presence of DQ58 (a, b) and DQ71508 (c, d) in aqueous (Na)Cl 0.6 m, $T = 25.0\text{ }^{\circ}\text{C}$; $C_{\text{metal}} = 5 \cdot 10^{-4}\text{ m}$, $C_{\text{ligand}} = 2 \cdot 10^{-3}\text{ m}$. For the definitions of A and B see caption of Table 2. Charges of the species are omitted for simplicity. The dashed lines show the theoretical starting pH for metal hydroxide precipitation (if occurring).

Fe(III)-ligand UV/Vis spectra were used to determine the stability constants of the FeAH^+ and FeBH^{2+} complexes. These species are already formed at very acidic pH values (see e.g. their distribution in Figures 3a and 3c), and therefore their formation constant cannot be measured by potentiometry. The obtained values are reported in Table 2. For Cu(II), UV-Vis allowed to investigate neutral and basic pH values, which could not be reached by potentiometric titrations. These had to be stopped at pH below ca. 4 due to the precipitation of a solid, which, according to the Cu(II)/ligand distribution diagrams, should be the neutral species CuA_2H_2 formed by DQ58 and Cu_2B_2 formed by DQ71508. By UV-Vis it was possible to work at lower metal and ligand concentrations, which avoided precipitation, so that additional species were detected in solution, and their stability constants were obtained (Table 2).

UV/Vis measurements were performed also for Al(III)/ligand and Cu(II)/ligand solutions to check some potentiometric values. For AlAH^+ , AlB^+ , AlB_2H , the $\log\beta$ values of 18.4 (0.2), 7.9 (0.1), and 18.21 (0.03), respectively, were obtained. As well, for CuAH_2^+ , $\text{Cu}_2\text{A}_2\text{H}_2$, CuBH^+ , and Cu_2B_2 , the $\log\beta$ values of 21.4 (0.2), 40.2 (0.2) 9.3 (0.2) and 17.84 (0.07), were computed. These results agree with the corresponding potentiometric values (Table 2), thus suggesting their accuracy.

Also EPR spectra (Figures 8S and 9S, Online Resource) allowed to calculate some stability constants of the Cu(II) complexes. For DQ58 the CuAH_2^+ complex was detected ($\log\beta = 20.9 \pm 0.1$), and for DQ71508 the CuBH^+ and Cu_2B_2 species were detected ($\log\beta = 9.8 \pm 0.1$ and 17.2 ± 0.1). As well, stability constants values agree with the potentiometric results.

$^1\text{H-NMR}$ spectra were obtained in deuterated water for solutions containing each ligand alone, and for solutions containing the ligand and Al(III). Some spectra are shown in the Online Resource (Figure 10S). Both DQ58 and DQ71508 are symmetric molecules, so that the aromatic protons at the ring positions 2 and 6 are chemically identical and give rise to one signal at 8.8 ppm (at pD 2.7). This signal moves upfield at higher pD values (e.g. for DQ58 it resonates at 8.7 ppm if pD = 3.9, and at 8.55 ppm if pD = 6.4) due to the carboxylate deprotonation. DQ58 and DQ71508 aromatic protons resonate at very similar ppm values. Upon complexation, the symmetry is lost, and two new aromatic signals appear at 8.4 and 8.9 ppm, which maintain their chemical shifts both at pD = 2.7 and 3.9. These results indicate that the complex is unsymmetric (i.e. the metal ion is bound by only one of the carboxylate groups), and that no deprotonation occurs in the Al(III) complexes between pD 2.7 and 3.9. The latter evidence is coherent with the potentiometric data, according to which only the free ligands can deprotonate in this pD range. At larger pD values, more complicated and broad signals appear, as observed for other HPCs (e.g. see [20]). Broadening can be attributed [20] to the formation of several isomers (up to 4 and 2 diastereoisomers can exist in solution for the bis- and tris-complexes, respectively) and to a relatively slow kinetics of interconversion between them.

Decorporation of Fe(III) or Al(III) in the presence of Cu(II) or Zn(II)

An ideal Fe and Al chelator should be able to massively decorporate Fe(III) and Al(III), and only negligibly the other essential ions. Although no definitive data exist, Zn appears to be an essential metal ion which can be more strongly complexed at in vivo conditions by chelating agents [5], probably due to the low thermodynamic stability and to the marked kinetic lability of its physiological complexes. Among other essential metal ions, Cu(II) is most often considered as possible target of Fe and Al chelating agents [27].

In a previous work a model was proposed to estimate the extent of metal interference during a possible chelation therapy treatment of Fe or Al [28], which can be applied if the speciation of both the toxic and the essential metal ions is known. By "interference" it is meant that the ligand may coordinate

the essential instead of the toxic metal. This not only would cause toxic side effects because of the removal of the essential ion, but it would also lower the efficiency of the chelation therapy. The proposed model is a ternary system [28], which contains the essential metal (either Zn(II) or Cu(II)), the toxic metal (either Al(III) or Fe(III)), and the chelator (either DQ58 or DQ71508). The essential metal interference was evaluated at various pH values, calculating the $\log[M']$ values as a function of pH (where $[M']$ represents the sum of the concentrations of all metal species which are not bound to the ligand). Low $\log[M']$ values of the toxic metals indicate a high efficiency of the chelating agent, and high $\log[M']$ values of the essential metals indicate a low perturbation of their metabolism. The ligand and the toxic metal (Fe or Al) concentrations were chosen to be $5 \cdot 10^{-5}$ m and 10^{-6} m, respectively [22]. The Zn and Cu concentrations were set to 10^{-4} m, which represents (or slightly overcomes) the highest limit of the typical concentration levels found in human blood [29]. The thermodynamic values of Tables 1 and 2 allowed the set-up of eight ternary model systems (Fe(III)–Zn(II)–DQ58, Fe(III)–Zn(II)–DQ71508, etc.). Figures 4 and 5 report the results obtained for the ternary systems which include Fe(III). The corresponding results for Al(III) are shown in the Online Resource (Figures 11S and 12S).

According to this model calculation, DQ58 appears to be a better Fe chelator than DQ71508 in connection with Zn and Cu interference, because DQ58 is able to more efficiently chelate the toxic metal ion in the presence of the essential one. In particular, DQ58 is able to chelate 99.7% of Fe at pH = 7.4 ($\log[Fe'] = -8.49$, Figure 4) in the presence of $Zn^{2+} 10^{-4}$ m, whereas DQ71508 chelates Fe around thirty times less effectively ($\log[Fe'] = -7.00$, Figure 4). The interference of $Cu^{2+} 10^{-4}$ m is more pronounced, but again DQ58 is a better chelator than DQ71508, because the former chelates 60% of Fe at pH = 7.4 ($\log[Fe'] = -6.40$, Figure 5), and the latter chelates only a negligible amount of the toxic metal ion ($\log[Fe'] \approx -6.00$, Figure 5). The same graphs obtained for the ternary systems including Al (Figures 11S and 12S) demonstrate that DQ58 and DQ71508 have very similar chelation efficiencies towards this toxic metal ion, and especially that the Cu and Zn interference on Al chelation is significant.

Binding constants with human serum albumin

Centrifugal devices with ultrafiltration coupled with UV spectrophotometry was performed to find the binding constants of DQ58 and DQ71508 with HSA at pH = 7.40. The formation of 1:1 L:HSA complexes were found, and the corresponding constants were: $\log\beta(DQ58:HSA) = 3.7$, $\log\beta(DQ71508:HSA) = 3.1$. These values agree with previous results obtained in the interaction study of HSA with other HPC ligands [23]. If the concentrations are 600 μ M for HSA (average blood level) and 50 μ M for the ligand (general blood concentration in chelation therapy regiment), the fraction of

ligand bound to HSA can be computed to be 42% for DQ58 and 74% for DQ71508. These values indicate that HSA can have a significant role in the transport of these HPDCs in the blood stream.

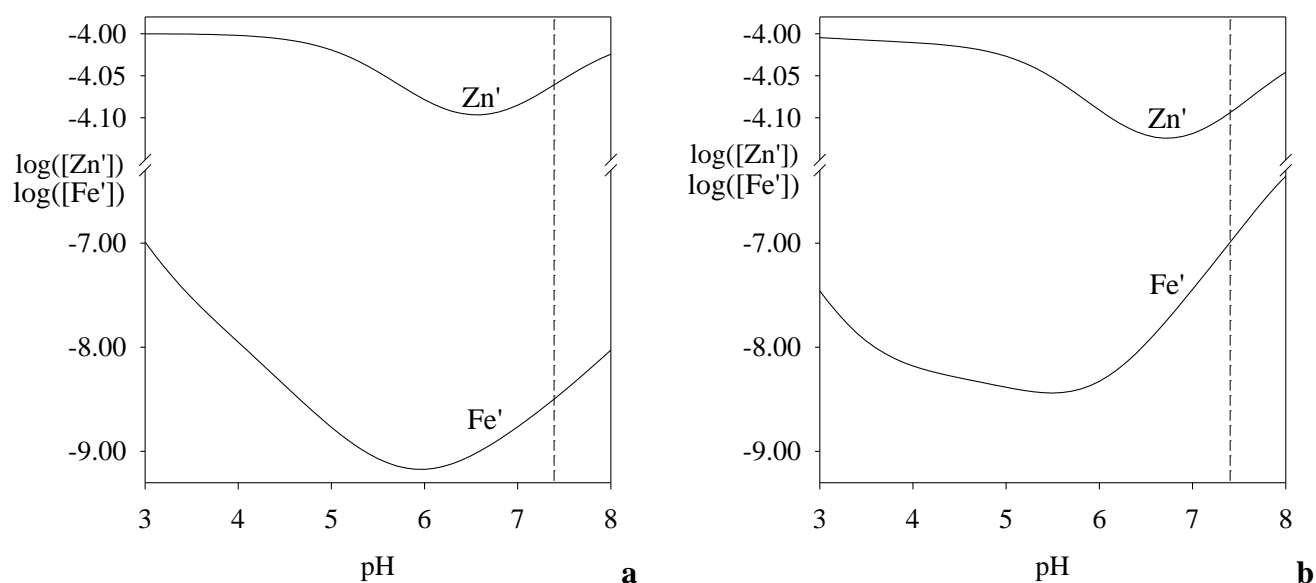


Figure 4. $\log[M']$ graphs (see text) for the ternary systems Fe(III)–Zn(II)–DQ58 (a) and Fe(III)–Zn(II)–DQ71508 (b); $C_{Fe} = 10^{-6}$ m, $C_{Zn} = 10^{-4}$ m, $C_{ligand} = 5 \cdot 10^{-5}$ m. Dashed lines indicate pH = 7.4.

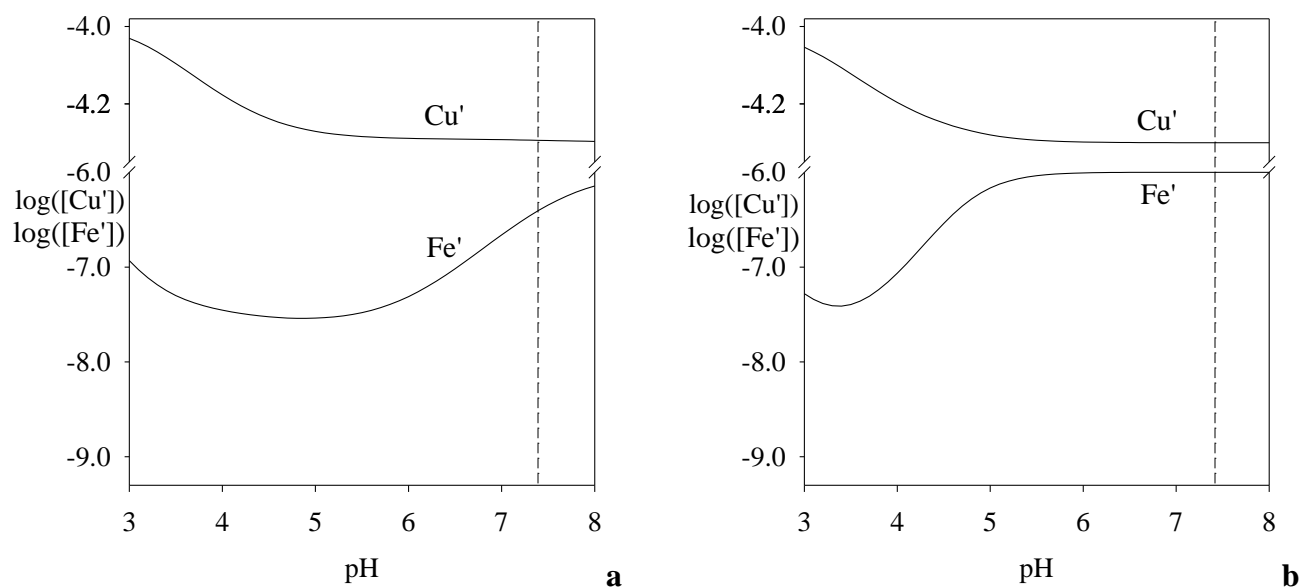


Figure 5. $\log[M']$ graphs (see text) for the ternary systems Fe(III)–Cu(II)–DQ58 (a) and Fe(III)–Cu(II)–DQ71508 (b); $C_{Fe} = 10^{-6}$ m, $C_{Cu} = 10^{-4}$ m, $C_{ligand} = 5 \cdot 10^{-5}$ m. Dashed lines indicate pH = 7.4.

Cytotoxicity data.

As a preliminary toxicity assessment of the compounds, the effect of DQ71508 on cell growth was monitored on HEK-293 human embryonic kidney cells. Cells were treated for 24 and 48 h with increasing concentrations of DQ71508, and the cell viability was determined by using the MTT test.

Dose-survival curves and IC_{50} values, calculated from the curves by a four parameter logistic model ($p < 0.05$) are reported in Figure 6. The cytotoxicity profile of DQ71508 was concentration- and time-dependent, with IC_{50} values of 5.185 mM and 1.033 mM after 24 and 48 h of treatment, respectively. Cytotoxicity is somewhat higher than that obtained for other HPC, as e.g. 1,5-dimethyl-4-hydroxy-3-pyridinecarboxylic acid [8], for which the calculated IC_{50} values were 1.4 mM and 0.8 mM after 24 h and 48 h of treatment, respectively.

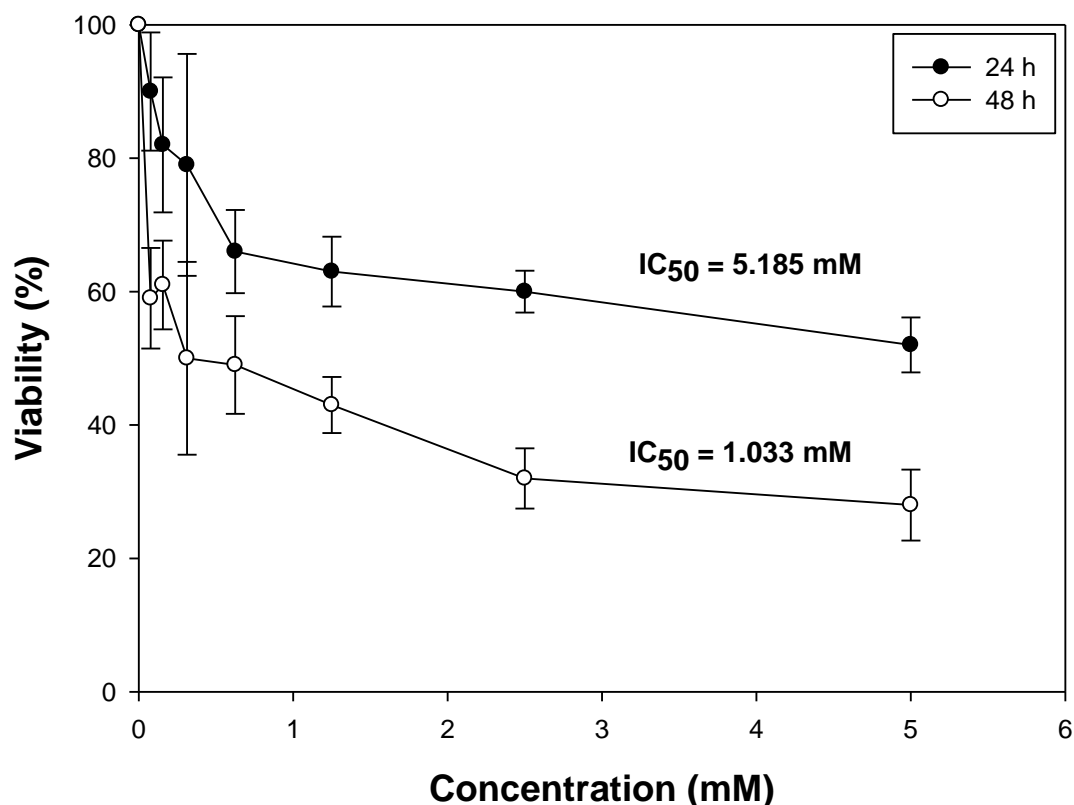


Figure 6. IC_{50} values calculated through MTT test (four parameter logistic model, $p < 0.05$) by treatment of HEK-293 cells for 24 or 48 h with increasing concentrations of DQ71508.

Conclusions

4-hydroxy-3,5-pyridinedicarboxylic acid (DQ58) and 4-hydroxy-1-methyl-3,5-pyridinedicarboxylic acid (DQ71508) have been synthesized, and their Fe(III), Al(III), Cu(II), and Zn(II) coordination properties were studied by potentiometry, UV-Vis, EPR, and 1H -NMR. The relatively high stability of DQ58 and DQ71508 complexes with Fe(III) and Al(III) suggest that both ligands can in principle be proposed as Fe and Al chelators. When the thermodynamic results were used to model the extent of the toxic metal ion decorporation in the presence of an excess of essential metal ion (Cu(II) or Zn(II)), it resulted that the Al(III) complexation is significantly affected by Cu(II)

and Zn(II). Therefore, neither DQ58 nor DQ71508 appear to be promising Al chelators. As regards Fe, better results were obtained, suggesting that these compounds can be employed rather in Fe chelation therapy. DQ58 is able to complex practically all Fe(III) in the presence of Zn(II), and most of it in the presence of Cu(II), whereas DQ71508 has lower efficiencies. This agrees also with the higher pFe obtained for DQ58 than for DQ71508. For complex stability point of view, it follows that the simultaneous insertion of a 5-carboxylate and a 1-methyl group in the pyridinic ring of HPCs is less beneficial than the insertion of a single carboxylate only. DQ58 is thus a more promising Fe chelator than DQ71508.

The in vitro cytotoxicity of DQ71508 on human embryonic kidney HEK-293 cells, proved to be reasonably low. Ultrafiltration measurements allowed us to obtain the binding constants of DQ58 and DQ71508 with HSA: From this interaction it can be modelled that a significant amount of the ligands can be bound and transported by HSA in a chelation therapy regimen.

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