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Solution studies on binary and ternary complexes of copper(II) with some fluoroquinolones and 1,10-phenanthroline: Antimicrobial activity of ternary metalloantibiotics

Paula Gameiro [a,*], Catarina Rodrigues [b], Teresa Baptista [b], Isabel Sousa [a], Baltazar de Castro [a]

[a] *Requimte, Departamento de Química, Faculdade de Ciências, 4169-007 Porto, Portugal*
[b] *Requimte, Instituto de Ciências de Saúde do Norte, 4585-116 Gandra, Portugal*

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Abstract

Interaction of norfloxacin and ofloxacin with copper(II) and copper(II)/phenanthroline has been studied in aqueous solution and the stability constants of the binary complexes Cu(II)/fluoroquinolone and of the ternary complexes Cu(II)/phenanthroline/fluoroquinolone have been determined by potentiometry and UV–vis spectrophotometry. The stability constants for the binary and ternary complexes of norfloxacin were always higher than those found for ofloxacin and comparing the values obtained for the binary and ternary species ($\Delta \log K$) it is possible to conclude that the ternary complexes are more stable than the binary ones, suggesting that an interaction occurs between the ligands in the ternary complexes. From the distribution diagrams it is possible to state that at physiological pH 7.4, the copper ternary complexes, are the main species in solution not only at the concentration used to determined the stability constants but also at the minimum inhibitory concentration. The antibacterial activity of these complexes, in different bacterial strains, was determined, at physiological pH, and the results obtain show that these ternary complexes may be good candidates as metalloantibiotics.

Keywords: Fluoroquinolones; Ternary complexes; Distribution diagrams; Antibacterial activity

1. Introduction

Fluoroquinolones are a group of antibacterial agents used currently against a wide variety of infections. Currently, these drugs are known to have two prime enzyme targets in the bacterial cell, DNA gyrase and topoisomerase IV (Levine et al., 1998; Anderson et al., 1998; Khodursky and Cozzarelli, 1998; Marians and Hiasa, 1997) and that they inhibit these enzymes by stabilizing the DNA–DNA gyrase complex and/or the DNA–topoisomerase IV complex (Shen and Pernet, 1985; Shen et al., 1989a,b,c). Although, to some extent, all fluoroquinolones are active against these enzymes, exposure to the drugs induces mutations in both proteins promoting higher levels of bacterial resistance and modifications to the fluoroquinolones based on structure–activity-relationships (SARs) have been made to achieve lower level of resistance (Yun-Liang et al., 2004; Weigel et al., 2002; Piddock, 2002; Hegde et al., 2005; Piddock et al., 2003).

Synthesis of fluoroquinolone metal complexes has been carried out as an attempt to clarify their physico-chemical properties and some antibacterial activity studies show that these complexes allowed the alteration of the potency and specificity of fluoroquinolones (Ross and Riley, 1994a; Wallis et al., 1996; Macias et al., 2001, 2002; Turel, 2002; Lopez-Gresa et al., 2002; Wu et al., 2003). Moreover, the studies of a few ternary complexes metal/fluoroquinolone/phenanthroline have also been carried out (Bivian-Castro et al., 2004; Alvarez et al., 1997; Robles et al., 2000; Ramirez-Ramirez et al., 1998) on the basis that DNAase activity of copper phenanthroline has been widely documented (Sigman et al., 1993; Macias et al., 2003; Garcia-Raso et al., 2003). Nevertheless, aqueous speciation studies of these binary and ternary systems are scarce and

* Corresponding author. Tel.: +351 226082889; fax: +351 226082959.
E-mail address: agsantos@fc.up.pt (P. Gameiro).

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the study of the bacterial activity of possible ternary complexes, which potentially combines the antibacterial activity of fluoroquinolones and the DNAase activity of copper/phenanthroline, is poorly investigated (Ramirez-Ramirez et al., 1998).

In this work, we determined the complex behaviour of norfloxacin and ofloxacin with copper(II) ion in the presence and absence of phenanthroline and the bacterial activity of the binary and ternary species present at physiological pH in a standard ATCC strain and two *E. coli*B^E host strains, one of which was devoid of major outer membrane proteins.

The values obtained for the stability constant of the binary and ternary copper(II) complexes are very high and clearly show that the ternary complexes are more stable than the binary ones, suggesting that an interaction occurs between the ligands in the ternary complexes. The distribution diagrams indicate that fluoroquinolones binary or ternary species are very stable at physiological pH and the antibacterial studies, performed at this pH, confirm the existence of a strong antibacterial activity of ternary species. These results suggest that the ternary copper complexes of fluoroquinolone can be seen as an approach to develop new antibacterial drugs, very stable at physiological pH, with a probable lower level of resistance against bacteria possible related with a high nuclease activity (Ramirez-Ramirez et al., 1998).

2. Experimental

2.1. Reagents and solutions

All compounds were used as received: norfloxacin (Nor), ofloxacin (Ofl) and 1,10-phenanthroline (Phe) from Sigma, HCl (Titrisol) and all other chemicals were from Merck (grade pro analysis). Solutions were prepared with double deionised water (conductivity less than 0.1 $\mu S\,cm^{-1}$). Iso-Sensitest broth and Mueller–Hinton broth were obtained from Oxoid, Basingstoke, UK.

2.2. Bacterial strains

Susceptibility of antibiotics was determined for National Committee for Clinical Laboratory Standards (NCCLS) reference strain: *Escherichia coli* ATCC 25922 and for a series of *E. coli*B^E host strains devoid of major outer membrane proteins BL21(DE3)omp1 to BL21(DE3)omp8 that were a gift from the Department of Microbiology of the University of Basel (Prilipov et al., 1998). Studies were peformed in BL21(DE3) and the mutant, without porins, BL21(DE3)omp8 → BL21(DE3), Δ*lamB, ompF::Tn5,* Δ*ompA,* Δ*ompC.*

2.3. Potentiometric pH titration

All potentiometric measurements were carried out with a Crison 2002 pH meter and a Crison 2031 burette controlled by a microcomputer. The electrode assembly consisted of an Orion 900029 double-junction AgCl/Ag reference electrode, and a Russell SWL07 glass electrode as indicator. System calibration was performed by the Gran method in terms of hydrogen ion concentration (Gran, 1952), by titrating solutions of strong acid with strong base. A calibration was performed before each run used to determine stability constants; this calibration also provided a check to the electrode behaviour. All titrations were carried out under argon atmosphere in a thermostat-controlled double-walled glass cell; the temperature was set at 25.0 (0.1 °C, and the ionic strength was adjusted to 0.10 M with sodium chloride.

2.4. Potentiometric determination of stability constants

Stock solutions of fluoroquinolones and 1,10-phenanthroline (1.0×10^{-2} M) were prepared in water ($I = 0.1$ M NaCl). The concentration of fluoroquinolones was measured by checking the compliance of the absorbance of the isosbestic points with the Beer–Lambert law. Aqueous copper(II) nitrate trihydrate solution (0.01 M) was standardized with standard solution of EDTA 0.1 M (Titriplex).

For the determination of the acid dissociation constants of the ligands (Nor, Ofl and Phe) an aqueous solution (1–4 mM) of the protonated ligand was titrated with NaOH (ca. 0.02 M; $I = 0.1$ M NaCl; 25 °C) under argon. For the determination of the association constants between, each of the fluoroquinolones and Phen, an aqueous solution of HCl (1–2 mM; $I = 0.1$ M NaCl; 25 °C), in the presence of both ligands (1–2 mM) was titrated with ∼0.03 M NaOH, under an argon atmosphere. The stability constants of the binary and ternary complexes were determined by titrating aqueous HCl (1–2.5 mM; $I = 0.1$ M NaCl; 25 °C), in the presence of $Cu(NO_3)_2$ (1–2.5 mM), and of the ligands (1–8 mM), with ∼0.03 NaOH, under an argon atmosphere. Each titration was repeated four times in order to check the reproducibility of the data.

The equilibrium constants defined by Eqs. (1) and (2):

$$pM + qHL + rH + sA \Leftrightarrow M_pL_qH_rA_s \quad (1)$$

$$\beta_{pqrs} = \frac{[M_p(HL)_qH_rA_s]}{[M]^p[HL]^q[H]^r[A]^s} \quad (2)$$

(where M is metal, HL the fluoroquinolones, in the zwitterionic form, H the proton and A is 1,10-phenanthroline) were refined by least-squares calculation using the computer program Hyperquad (Gans et al., 1996) taking into account the presence of the hydroxide species of copper and the autoprotolysis of water.

2.5. Spectrophotometric measurements

Absorption spectra were recorded in a UNICAM UV-300 spectrophotometer equipped with a constant-temperature cell holder. Spectra were recorded at 25.0 °C in 1 cm quartz cuvettes with a slit width of 2 nm in the range 230–400 nm. Spectrophotometric pH titrations were performed in stock solutions of the same metal/ligand molar ratio as previously used in potentiometry—1:1 and 1:2 (1.25×10^{-5}–2.5×10^{-5} M) for the binary species and 1:1:1 (2.5×10^{-5} M) for the ternary species, and aliquots of strong acid or base were added to adjust pH to the desired value. pH measurements and system calibration were performed by potentiometry as described previously.

2.6. Antibiotic susceptibility testing—inoculation procedures

Minimal inhibitory concentrations (MICs) were determined in Iso-Sensitest broth by a standard microdilution technique (NCCLS, 2000). In this method, the final desired inoculum concentration was 5×10^5 CFU/ml. A suspension of 10^8 CFU/ml (turbidity of 0.5 McFarland standard) made from colonies grown in Iso-Sensitest agar medium overnight, was initially prepared. For the microbroth dilution procedure used it was required an inoculum volume of 0.05 ml to inoculate 0.05 ml of broth. So, it was necessary to do a 1:100 dilution of the 0.5 McFarland suspension. When the inoculum was added to the wells, the 1:2 dilution of the 10^6 CFU/ml inoculum results in a final inoculum concentration of 5×10^5 CFU/ml and so halves the antibiotic concentration in each well.

Stock solutions of the fluoroquinolones and derivatives were prepared in Hepes buffer, sterilized by membrane filtration and then diluted in Iso-Sensitest broth as proposed by National Committee for Clinical Laboratory Standards (NCCLS, 2000).

Microbroth dilution trays were inoculated within 15 and 20 min of inoculum preparation.

A total of 11 concentrations of each sample were prepared. A positive control (growth) consisting of organisms in broth and a negative control (sterility) consisting of uninoculated broth were included for each bacterial stain tested. After inoculation, each tray was covered with plastic tape and sealed in a plastic box to prevent evaporation during incubation.

Plates were incubated at 37 °C and read after 18–24 h.

Aliquots of the bacterial suspensions were subcultured to check the purity of the isolates, and colony counts were performed to check the accuracy of the inoculum concentration.

Viable cell counts were performed in duplicate by diluting cell samples in sterile distilled water, and spreading 100 μl of each dilution over the surface of Mueller–Hinton agar plates. Plates were incubated at 37 °C and the colonies were counted after 18–24 h. Results were expressed as CFU/ml.

Each assay was repeated six times with each antimicrobial agent formulation and six additional times on a different day with all formulations to ensure reproducibility of results.

3. Results and discussion

3.1. Potentiometric studies

The logarithm of the protonation constants of the fluoroquinolones, the log β values of the association constants between the fluoroquinolones and Phe, and the log β values of the formation constants of the complexes found in the binary and ternary systems are shown in Table 1. The Phe acidity constant determined in this work was 4.95 ± 0.02, a pK_a value that is in agreement with values already reported (Quentel et al., 1978; Erim et al., 1994). The values of the binary copper(II)/Phe (CuA) used were: $\log \beta_{1001} = 9.25$ and $\log \beta_{1002} = 16.00$ (Quentel et al., 1978).

Fluoroquinolones have two relevant ionizable functional groups, a basic piperazinyl group in the 7-position and a carboxylic acid group in the 3-position (Scheme 1). At low pH, both groups are protonated (H_2L) and at higher pH, none is protonated (L) (Ross and Riley, 1994b; Kawai et al., 1996).

Table 1
Logarithm of protonation and association constants of the ligands and stability constants ($\log \beta_{pqrs}$) of Cu(II) complexes at 25 °C and $I = 0.1$ M (NaCl)

		Norfloxacin	Ofloxacin
H_2L	$\log \beta_{0110}$	6.25 ± 0.01	6.10 ± 0.02
L	$\log \beta_{01-10}$	−8.44 ± 0.01	−8.60 ± 0.01
CuHL	$\log \beta_{1100}$	6.95 ± 0.03	6.24 ± 0.04
$Cu(HL)_2$	$\log \beta_{1200}$	12.70 ± 0.04	11.20 ± 0.04
HLHA	$\log \beta_{0111}$	9.61 ± 0.06	9.41 ± 0.08
HLA	$\log \beta_{0101}$	3.81 ± 0.08	3.92 ± 0.07
LA	$\log \beta_{01-11}$	−4.50 ± 0.06	−3.35 ± 0.07
Cu(HL)A	$\log \beta_{1101}$	17.56 ± 0.05	16.69 ± 0.03
CuLA	$\log \beta_{11-11}$	9.55 ± 0.04	9.21 ± 0.01

Charges are omitted, as usual, for simplicity. For Cu/Phe the stability constants used, for the binary system were $\log \beta_{1001} = 9.24$ and $\log \beta_{1002} = 16.00$.

The carboxylic group is normally a stronger acid than the ammonium group, therefore the neutral form is rearranged spontaneously to the zwitter ion and there is extensive literature showing that the values for pK_{a1} and pK_{a2} are approximately 5.7–6.2 and 7.9–8.9, respectively. The pK_{a1} value for the fluoroquinolones is higher than is generally observed for carboxylic acids which are normally associated with an intramolecular hydrogen-bond formation between the carboxylic acid and the neighboring keto function which results in the stabilization of the protonated species (Turel, 2002). The value of pK_{a2} is normally sensitive to substitution on the piperazine ring. The values of the acidity constants obtained in this study are similar to some of those already found in literature, regarding the experimental conditions used (Quentel et al., 1978; Erim et al., 1994). Furthermore, the pK_{a2} values herein described, are in agreement with what is expected from the differences in the piperazine ring: Ofl pK_{a2} is higher than Nor pK_{a2}, since the $-CH_3$ group in Ofl increases its basicity.

3.2. Binary systems

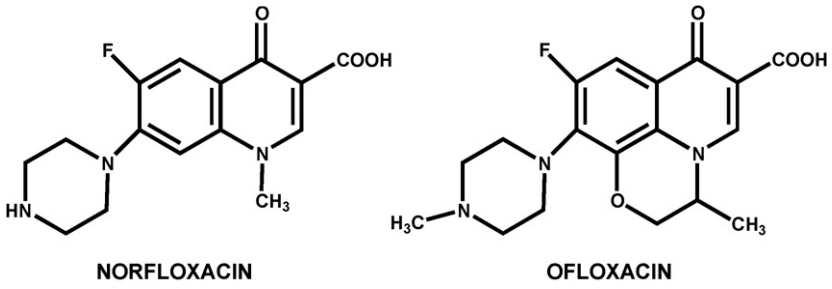
The data obtained from the titration curves of the Cu(II)-containing binary system, for fluoroquinolones, in all the Cu/L molar ratio was treated with the program Hyperquad assuming the model (charge omitted for simplicity):

$$Cu^{2+} + HL \Leftrightarrow CuHL \quad (3)$$

$$Cu^{2+} + 2HL \Leftrightarrow Cu(HL)_2 \quad (4)$$

$$Cu^{2+} + HL \Leftrightarrow CuL + H^+ \quad (5)$$

The results obtained show that only the equilibria described by Eqs. (3) and (4) exist, and that the values obtained are similar to those found in the literature for the stability constants of copper(II) with other fluoroquinolones (Lopez-Gresa et al., 2002; Turel, 2002; Kawai et al., 1996). The speciation as a function of pH (Fig. 1) shows the predominance of the binary species, $Cu(HL)_2$ in a wide pH range (~4.5–9.5) for Cu/L molar ratios ≥1:2.



Scheme 1.

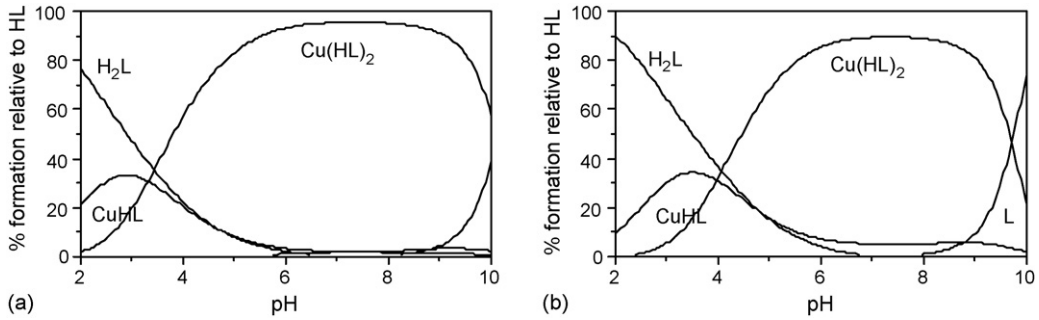


Fig. 1. Species distribution as a function of pH of 1:2 Cu:Nor(a) and Cu:Ofl (b), calculated from the stability constants listed in Table 1. Concentrations: Cu(II), 2 mM; fluoroquinolone, 4 mM.

For the system Phe/Nor and Phe/Ofl, the data obtained were treated assuming the model

$$A + HL \Leftrightarrow HLA \quad (6)$$

$$A + HL + H^+ \Leftrightarrow HLHA \quad (7)$$

$$A + HL \Leftrightarrow LA + H \quad (8)$$

The results obtained account for the formation of the adduct HLA, with $\log \beta_{0101}$, between Phe and the fluoroquinolones and the acidity equilibria, of this adduct, described by Eqs. (7) and (8), $\log \beta_{0111}$ and $\log \beta_{01-11}$. These latter equilibria can be transformed in the adduct protonated constants, as:

$$HLHA \Leftrightarrow HLA + H^+ \quad pK_{a1}^{HLA}$$

$$HLA \Leftrightarrow LA + H^+ \quad pK_{a2}^{HLA}$$

and the values obtained are summarized in Table 2. The fluoroquinolones/Phe adduct can occur by charge transfer, aromatic ring stacking and/or hydrogen bond formation (Fernandez-Botello et al., 2004; Luth et al., 1999; Sigel, 1975).

According to the speciation as a function of pH, it is possible to conclude that the adduct is the species that prevails (∼70%) at a molar ratio of 1:1 Phe/Nor or Phe/Ofl (Fig. 2) and pH 7.4. At pH below 6 the acid species HLHA forms (∼50%) and it can be associated to the interaction H_2L/Phe where the fluoroquinolones are in their cationic form and Phe in its neutral form (pK_{a1}^{HLA}).

At pH above 8 the predominant species is LA, where the fluoroquinolones are in their anionic form and Phe in its neutral form (pK_{a2}^{HLA}). The values obtained for these adducts acidity constants can thus be related to the acidity functions of the fluoroquinolones alone and the results show that in the adduct form, the acidity increase can be a consequence of the aromatic ring stacking that occurs between the Phe aromatic ring and the fluoroquinole aromatic ring, that withdraw electronic density from the carboxylic group in the 3-position and the piperazinyl group in the 7-position making these groups more acidic. The values obtained for the association constants of the Ofl and Nor adduct are in the same order of magnitude and agree well with what was expected from this kind of adduct interaction (Fernandez-Botello et al., 2004; Luth et al., 1999).

Table 2
pK_a values of the species in the fluoroquinolone, fluoroquinolone/phenanthroline and Cu(II)/fluoroquinolone/phenanthroline systems

		Norfloxacin	Ofloxacin
H_2L	pK_{a1}	6.25 ± 0.01	6.10 ± 0.02
L	pK_{a2}	8.44 ± 0.01	8.60 ± 0.01
HLHA	pK_{a1}^{HLA}	5.60 ± 0.07	5.49 ± 0.04
LA	pK_{a2}^{HLA}	8.31 ± 0.07	7.27 ± 0.07
CuLA	pK_{a1}^{CuHLA}	8.01 ± 0.04	7.48 ± 0.02

pK_{a1} of 1,10-phenanthroline (A) is 4.95 ± 0.02.

3.3. Ternary systems

The stability constants for the ternary systems, Cu(II)/Phe/fluoroquinolone were obtained by the program Hyperquad assuming the model:

$$Cu^{2+} + HL + A \Leftrightarrow CuHLA \quad (9)$$

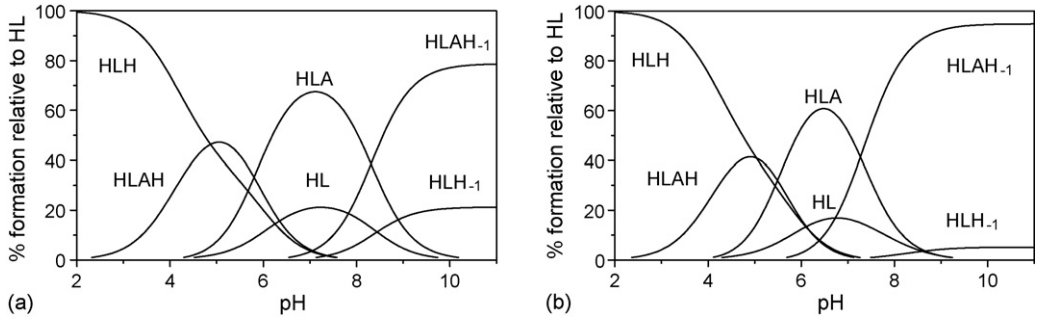


Fig. 2. Species distribution as a function of pH of 1:1 Phe:Nor (a) and Phe:Ofl (b), calculated from the stability constants listed in Table 1. Concentrations: Phe, 2 mM; fluoroquinolone, 2 mM.

$$Cu^{2+} + HL + A \Leftrightarrow CuLA + H^{+} \quad (10)$$

and, all the equilibria already determined for the binary systems, the acidity constant of the ligands and the protolysis of copper(II). The speciation as a function of pH (Fig. 3) shows the predominance of the ternary species described by Eqs. (9) and (10) in all pH range for Cu/HL/A molar ratios 1:1:1 and, an equimolar mixture of the CuHLA (∼50%) and CuLA is observed at pH 7.5.

The values obtained for these constants (Table 1) are very high and an acidity constant of the ternary complex can be associated to these equilibria (Table 2):

$$CuHLA \Leftrightarrow CuLA + H^{+} \quad pK_{a1}^{CuHLA}$$

These values show, as already observed for the binary system, that the acidity of the ternary complex can be related to the acidity functions of the fluoroquinolones alone and the results show that in the ternary complex the acidity also increases. This enhancement must be related to the synergy that copper(II) enforces on the possible stacking interaction between the Phe aromatic ring and the fluoroquinolones aromatic ring, since they are identical to those obtained for pK_{a2}^{HLA}.

In order to verify the tendency of formation of ternary complexes with intramolecular interactions the difference between the stability constants of the ternary and the binary complexes was calculated by

$$\Delta logK = \log K_{CuAB}^{CuA} - \log K_{CuB}^{Cu} \quad (11)$$

The values obtained for our ternary complexes are very high (∼1) which can only be justified by the presence of interactions between the ligands. This much higher stability of the ternary complexes can be expected since they join the existence of five- and six-membered ring, formation of stacking interactions and possibly formation of π back bonding a synergy that will favored considerable the formation of these complexes (Fernandez-Botello et al., 2004; Luth et al., 1999; Sigel, 1975).

3.4. Spectrophotometric analysis

Independent support for the values of the stability constants was provided by the good agreement with the values obtained by analyzing the spectrophotometric pH titration data with program Hyperquad, using the models already described for potentiometric studies. Spectral changes with isosbestic points were observed in all binary and ternary systems denoting the presence of new species as the pH changes, as was expected from the potentiometric studies (Connors, 1987).

The values obtained for the stability constants of the binary and ternary systems were identical, within experimental error to those obtained by potentiometry.

The spectra of the complex solution was followed during a month and it is safe to assert that, under the experimental conditions used, no changes were observed on the absorption spectra of the binary and ternary species in Hepes buffer, pH 7.4, during this period.

3.5. Antibiotic susceptibility testing

Minimal inhibitory concentrations (MICs), were obtained in a standard reference stain *E. coli* ATCC 25922 to validate the

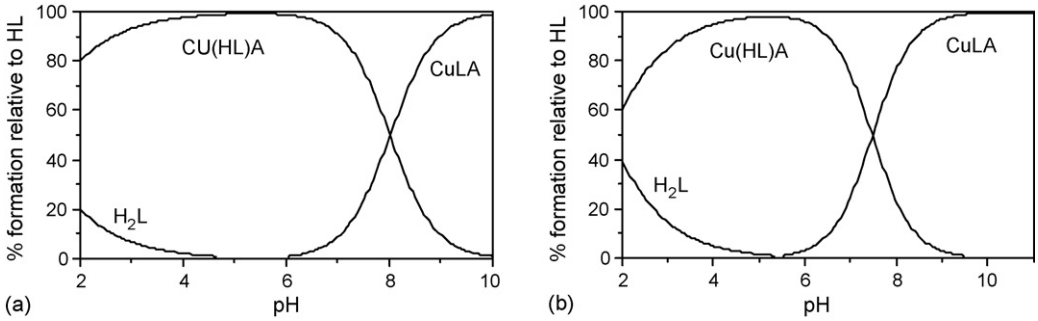


Fig. 3. Species distribution as a function of pH of 1:1:1 Cu:Phe:Nor (a) and Cu:Phe:Ofl (b), calculated from the stability constants listed in Table 1. Concentrations: Cu, 2 mM, Phe, 2 mM; fluoroquinolone, 2 mM.

Table 3
Minimal inhibitory concentration (MIC, μg/ml) of the drugs and related binary and ternary species

Microorganism	Nor	Ofl	Cu:Nor	Cu:Ofl	Cu:Nor:Phe	Cu:Ofl:Phe
ATCC 25922	0.03	0.015	0.12	0.015	0.06	0.03
E. coliB^E;BL21(DE3)	0.0075	0.00375	0.03	0.00375	0.015	0.0075
BL21(DE3)omp8	0.06	0.015	0.12	0.015	0.12	0.03

data and in a strain that we normally use to express the outer-membrane proteins (*E. coli*B^E; BL21(DE3) (Neves et al., 2005). MICs were also determined in a mutant devoid of porins to understand whether the cellular uptake mechanism of fluoroquinolone derivatives was porin dependent.

Table 3 shows the MICs of Nor, Ofl and of the binary and ternary species formed between these ligands and copper(II), against the Gram-negative bacteria tested.

The MICs of ofloxacin and norfloxacin obtained by the NCCLS standard method were similar and within the NCCLS quality control range for *E. coli* ATCC 25922 (Kawai et al., 1996). In general, the activity of the ofloxacin derivatives is higher than the norfloxacin derivatives, a trend that is in agreement with other results obtained for ofloxacin and norfloxacin in several different strains (Takei et al., 2001; Bolmstrom and Karlsson, 2002). For ofloxacin, the activity of the binary species is very similar to that found for the fluoroquinolone alone, but the activity of the ternary specie is lower (if only by a factor of 2). For norfloxacin, the ternary species are only less active than the fluoroquinolone alone by a factor of 2 but are more active than the binary ones. It is also interesting to note that all the species exhibit an increase in activity in the presence of porins and, in general, norfloxacin derivatives have a higher porin-dependence than ofloxacin derivatives, results that can be related to the lower hydrophobicity of norfloxacin causing a lower penetration without the porins (Chevalier et al., 2000).

Combining these MIC results with those obtained for the stability constants, it is possible to determine the concentration of the several species that are present in solution at pH 7.4 and that can contribute, or not, to the activity of the fluoroquinolones or derivatives. For free fluoroquinolones, the species that can contribute mostly to the activity are L and HL with a main contribution of HL for Nor and Ofl as expected from the pK_a values. When fluoroquinolones are coordinated to copper the species that contribute to the activity are HL and CuHL, since at MIC concentration there is a dissociation of the $Cu(HL)_2$ complexes for both systems. These results suggest that the activity of the binary copper complexes must be very small and that the main species that contributes to activity is HL. For the ternary complexes, the main species that contribute to the activity are HL, Cu(HL)A and CuLA with the two copper complexes being present in higher quantity. As free concentrations of HL is low it cannot account for the small difference obtained for the activity of the complexes compared to the activity of fluoroquinolones alone, consequently the copper ternary complexes must account for the bactericidal activity, as the copper free concentration is below the limit of its toxicity (Fraústo and Williams, 1994).

From these speciation results determined at MIC, for all the species studied (results not show) it is possible to conclude that copper binary complexes do not have or have a very small antimicrobial activity, but the copper ternary species must have a good antimicrobial activity. It is also possible to stress that for these latter complexes the activity is higher in the presence of porins, hence, these proteins are important for the entry of these complexes into the bacterial cell.

4. Concluding remarks

The pK_a values of the two fluoroquinolones studied in this work are similar to those previously reported for several fluoroquinolones and as was expected from their chelating properties they form very stable complexes with copper(II). The values of the stability constants determined for binary copper(II) complexes are very high and similar to those found in literature for other fluoroquinolones (Turel, 2002; Lopez-Gresa et al., 2002; Wu et al., 2003); an analysis of the distribution diagrams shows that they are the main species at physiological pH. For the ternary system, copper(II)/Phe/Flu, the values of the stability constants determined are even higher and from the distribution diagrams it is evident that they are the predominant species at physiological pH. This higher stability of the ternary complexes, a result already observed for similar ligands in ternary complexes (Fernandez-Botello et al., 2004; Luth et al., 1999; Sigel, 1975), can be attributed to: the existence of two ligands with five and/or six member rings and the π–π stacking between the aromatic rings with an interaction mediated by copper(II).

Due to the stability of the complexes at physiological pH they can be suitable metalloantibiotic candidates. The complexation seems to cause profound chemical and biochemical changes to the antibiotic, which may not significantly affect the structure of the drugs (Ming, 2003), but can lead to a lower level of bacterial resistance, a most probable property for the ternary complexes with their possible DNAase activity.

The values of the MICs obtained by the susceptibility tests performed for Nor and Ofl are identical, within experimental error, to those previously reported for the ATCC standard strain (NCCLS, 2000), and the values obtained for the metalloantibiotics are similar, although, always a little smaller. For the other stain, BL21(BE), the MICs are always smaller but the values obtained for the fluoroquinolones and for the metalloantibiotics have the same profile of those obtained for the ATCC strain. Comparing these results with the MICs obtained for the mutant without porins it is evident that, for all the chemical species, fluoroquinolones and metallofluoroquinolones, the presence of porins is important for their activity. In view of these results, the uptake of metalloantibiotics seems to be performed in a simi-

lar way as that described for fluoroquinolones: the higher the hydrophobicity of the species the higher the need for porins for their uptake (Chevalier et al., 2000).

Speciation studies were very important as they allowed us to conclude that: (i) for binary systems, at MIC concentration, the copper complexes have almost no microbial activity as the complexes partially dissociate in solution, and (ii) for the ternary systems, the active species are definitely the metalloantibiotics and not the fluoroquinolones alone as at MICs, the concentration of ternary species are much higher than those of the fluoroquinolones alone.

As a final conclusion this work shows that copper(II)/phenanthroline complexes with fluoroquinolones are very stable, at physiological pH and they seem to be a good approach for the development of drugs with similar activity against bacteria but with the possibility of lowering their level of resistance.

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