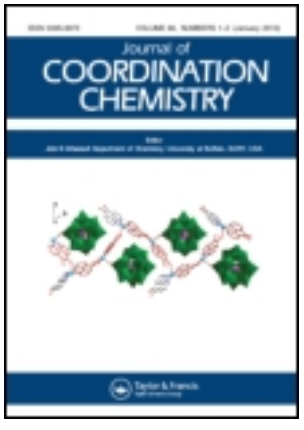

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Tuning the reaction products of ruthenium and ciprofloxacin for studies of DNA interactions

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Tuning the reaction products of ruthenium and ciprofloxacin for studies of DNA interactions

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This work presents two potential metallo-drugs, the ionic $(C_{17}H_{19}FN_3O_3)_3[RuCl_6]\cdot 3H_2O$ (**1**) and the coordination $[Ru(C_{17}H_{17}FN_3O_3)_3]\cdot 4H_2O$ (**2**) compounds, obtained by the combination of ruthenium(III) and ciprofloxacin in different synthetic conditions. The ESI MS spectrum of **1** displayed a main peak at $m/z = 994.6$, assigned to the gaseous phase adduct $(\text{ciprofloxacin})_3\cdot H^+$, while **2** featured peaks at m/z 1093.3 and 547.1 ascribed to $[Ru(C_{17}H_{17}FN_3O_3)_3\cdot H^+ - 4H_2O]^+$ and $[Ru(C_{17}H_{17}FN_3O_3)_3\cdot 2H^+ - 4H_2O]^{2+}$. Thermal analysis corroborated the proposed water content for both complexes. Absorption spectra of the compounds in aqueous medium are dominated by ciprofloxacin transitions in the UV region but displayed weak bands in the visible region, assigned to ligand field transitions. The cyclic voltammograms of **2** exhibited a quasi-reversible process ascribed to the Ru(II)/(III) redox pair at −0.25 V (*vs.* SHE) while **1** displayed this process at −0.11 V, showing that the central ruthenium ion is stabilized in the (III) oxidation state by the coordination to the hard oxygen atoms of ciprofloxacin. The solubility of **1** is pH dependent (as well as free ciprofloxacin) while **2** is fully water soluble and stable under physiological pH for at least 48 h. The compounds are also stable under incubation conditions (stomach pH and 37°C) without significant pH lowering. An interaction study of **2** with *ct*-DNA showed a value of $K_b = 2.47\ (\pm 0.89) \times 10^4\ mol^{-1}\ L$ for the intrinsic binding constant.

Keywords: Metallo-drugs; Ruthenium; Fluoroquinolones; Ciprofloxacin; Calf thymus DNA

1. Introduction

Quinolones and fluoroquinolones represent a large family of synthetic antibacterial agents which are widely used in human therapy as well as in veterinary medicine [1, 2]. The synthesis and characterization of fluoroquinolone complexes with metal ions such as Fe(III), Cu(II), Co(II), Zn(II), Al(III), Ba(II), Mg(II), and others have been described during the last two decades in different levels of detail [2–10]. In general, the

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reports focus on infrared (IR) and UV-Vis spectra as well as complexes' behavior as a function of pH. In some cases other spectroscopic techniques have been used, such as X-ray diffraction and NMR. Less frequent is the use of mass spectrometry and electrochemical experiments, such as cyclic voltammetry to characterize the fluoroquinolones and their coordination complexes [10].

Ruthenium complexes are versatile regarding their spectroscopic and electrochemical properties and reactivity toward different classes of ligands [11, 12]. With the discovery of ruthenium metallo-drugs such as NAMI-A and KP1019 (both in clinical trials), interest in ruthenium coordination complexes with potential biological activity has grown, due to their clinical application against metastatic cells and low systemic toxicity [13–17].

This work proposes the synthesis and characterization of two different ruthenium–ciprofloxacin complexes ($C_{17}H_{18}FN_3O_3$, cipro), aiming to obtain new potential metallo-drugs and focusing on the use of alternative techniques such as mass spectrometry in their characterization. It also addresses the interaction of compound **2** with calf thymus DNA (*ct*-DNA).

2. Experimental

2.1. *Materials and physical measurements*

All reactants were commercially available and used without purification. The mass spectrometer employed in the analyses was the ultrOTOF$_Q$-ESI-TOF Mass Spectrometer (Bruker Daltonics, Billerica, MA, USA), operating in the positive mode. The UV-Vis spectra were recorded on a Hitachi U-3501 spectrophotometer in different solvents, at a solution concentration of 10^{-5} mol L^{-1}. IR spectra were registered on an FTIR/Nicolet spectrometer, model Protegé 460, from KBr pellets. Thermal analysis was performed in SHIMADZU equipment, models TGA-50 and DTA-50. The samples were heated under a $N_{2(g)}$ flow of 10°C min^{-1}. Cyclic voltammetry was carried out with a potentiostat/galvanostat AUTOLAB®, model PGSTAT30 coupled to a computer. A conventional three-electrode cell was used, with a platinum disk and a platinum wire as working and auxiliary electrodes, respectively, and an Ag/AgCl reference electrode. The supporting electrolyte was tetrabutylammonium hexafluorophosphate and all the $E_{1/2}$ values presented here were converted to standard hydrogen electrode (SHE) by adding 0.197 V to the observed values. The pH values were recorded on a Labmeter pHmeter model pH 2. For pH 7.4 phosphate buffer was used; for other pH values the solutions were prepared with HCl or NaOH. In order to determine the p*K*a values of the compounds, a spectrophotometric titration was performed and the absorbance variation at a given wavelength was plotted as a function of pH at $T = 25$°C. The resulting curves were fit by a sigmoidal adjust, followed by application of the first differentiate tool of the Origin® 6.0 program.

2.2. *Synthesis*

$(C_{17}H_{19}FN_3O_3)_3[RuCl_6] \cdot 3H_2O$ ($(ciproH_2^+)_3[RuCl_6] \cdot 3H_2O$, **1**): Ciprofloxacin (480 mg, 1.50 mmol) and $RuCl_3 \cdot nH_2O$ (100 mg, 0.480 mmol) were dissolved in a mixture of

ethanol (25 mL) and HCl 0.1 mol L^{-1} (10 mL) and the resulting solution was kept under reflux for 2 h. The reaction mixture was cooled to room temperature and kept under refrigeration overnight. This procedure yielded a reddish solid which was isolated by filtration, washed with a dilute HCl solution and water, and dried under vacuum. Thin-layer chromatography (aluminum oxide as stationary phase and 1 acetonitrile : 1 methanol (*v* : *v*) solution as mobile phase) of **1** showed only one colored spot, which did not display the characteristic luminescence of ciprofloxacin under 254 and 365 nm irradiation. Yield: 360 mg (54%). $C_{51}H_{63}F_3N_9O_{12}Cl_6Ru$ (1364.90): Calcd – H 4.65, C 44.88, N 9.24; found H 4.42, C 44.51, N 8.75. IR (KBr, cm^{-1}): 1709, 1626, 1608, 1520, 1479, 1466, 1450, 1398, 1385, 1344, 1273.

$[Ru(C_{17}H_{17}FN_3O_3)_3]\cdot 4H_2O$ ($[Ru(cipro)_3]\cdot 4H_2O$, **2**): Ciprofloxacin (480 mg, 1.50 mmol) and $RuCl_3\cdot nH_2O$ (100 mg, 0.480 mmol) were suspended in water (15 mL) and heated under reflux for 3 h [4]. The reaction medium was allowed to cool and was then filtered. Acetone was added to the filtrate in a 25 : 1 proportion. The yellowish-brown precipitate was isolated by filtration and dried under vacuum. Compound **2** was purified by exclusion chromatography using Sephadex G-10 (Sigma-Aldrich). Yield: 355 mg (63%). $C_{51}H_{59}F_3N_9O_{13}Ru$ (1164.15): Calcd – H 5.11, C 52.62, N 10.80; Found – H 5.02, C 50.61, N 10.20. IR (KBr, cm^{-1}): 1630, 1578, 1554, 1516, 1488, 1456, 1404, 1382, 1339, 1295, 1269.

2.3. *ct-DNA interaction evaluated by UV-Visible spectroscopy*

5.0 mg of *ct*-DNA (Sigma-Aldrich) was added to 5.0 mL of phosphate buffer (pH 7.0), sodium chloride 0.15 mol L^{-1} and 0.015 mol L^{-1} sodium citrate. This mixture was stirred overnight at 2–8°C. The resulting solution was kept under refrigeration and used within a week [18]. The *ct*-DNA purity was verified by measuring the absorbance intensity ratio at 260 nm and 280 nm, which was found to be 1.88, compatible with the value reported [19]. Then, the *ct*-DNA concentration was determined from the value of $\varepsilon_{260\,nm} = 6600\,mol^{-1}\,L\,cm^{-1}$ as 1.47×10^{-3} mol L^{-1} [20, 21].

In order to evaluate the interaction between *ct*-DNA and **2** [10], *ct*-DNA solutions ranging from 4.9×10^{-5} to 4.9×10^{-4} mol L^{-1} were prepared by dilution of the initial solution with phosphate buffer (pH 7.0), sodium chloride 0.15 mol L^{-1}, and 0.015 mol L^{-1} sodium citrate. Aliquots of a $[Ru(cipro)_3]\cdot 4H_2O$ solution were added to the *ct*-DNA solutions to get a final concentration of 2.29×10^{-5} mol L^{-1} of the metal complex. A control solution in the absence of *ct*-DNA was also prepared. All mixtures were kept at 0°C for 1 h and then were left to equilibrate during 24 h, at room temperature and light protected. All UV-Visible measurements were made twice. The variation of absorbance was monitored at 353 nm and the data was analyzed following the Benesi–Hildebrand method [10, 22, 23], equation (1):

$$\frac{1}{\Delta A} = \frac{1}{[Ru]_T \Delta\varepsilon} + \frac{1}{[Ru]_T \Delta\varepsilon K[DNA]} \quad (1)$$

where ΔA is the absorbance variation monitored at 353 nm, $\Delta\varepsilon$ is the variation on the absorption coefficient from ruthenium compound to ruthenium · DNA adduct (intercept) and K is the binding constant (slope).

3. Results and discussion

3.1. *Synthesis and solubility of* $(ciproH_2^+)_3[RuCl_6]\cdot 3H_2O$ *(1) and* $[Ru(cipro)_3]\cdot 4H_2O$ *(2)*

Literature describes two main classes of metal-fluoroquinolone complexes. Synthetic approaches that employ low pH values lead to ionic complexes in which the fluoroquinolone appears in its fully protonated form, as the counter-ion of a simple coordination anion such as $BiCl_n$ [24]. When the synthesis is carried out in organic media, coordination compounds are isolated, with the metal center coordinated to one to three fluoroquinolate anions and solvent. The product is fine tuned by changing synthesis conditions such as solvent and pH, as observed in this work. Compound **1**, synthesized in an acid medium rich in Cl^-, is isolated as a salt of $[RuCl_6]^{3-}$, while **2**, synthesized in the absence of acid, is isolated as a "true" coordination compound. The suggested structures for **1** and **2** are depicted in figure 1.

Regarding the proposed structural differences between **1** and **2**, the most important feature is the difference in water solubility between these species. While **2** is soluble in aqueous medium (as is its iron analogue [10]), the ionic compound **1** is soluble under extreme pH conditions, just as free ciprofloxacin. This feature may be rationalized in terms of the p*K*a values of the fluoroquinolone and the total charge of the compounds.

For **1**, the p*K*a values determined are $pKa_1 = 3.4$ and $pKa_2 = 8.7$. For this compound, it is reasonable to assume that the p*K*a values should be similar to the values of free ciprofloxacin, since it is proposed that the fluoroquinolone is not coordinated to the metal center in **1**. Depending on the employed method, three protonation equilibria, one for the piperazine nitrogen (the most acidic center of the molecule), one for the carboxylate, and one for the peripheral amine on the piperazine ring, with p*K*a values ranging from $pKa_1 = 3.6$ to 4.1, $pKa_2 = 5.9$ to 6.4, and $pKa_3 = 8.2$ to 9.0 has been reported [25, 26]. By comparison, we can assign the first equilibrium observed for **1** (p*K*a = 3.4) to protonation of the piperazine nitrogen and the second one (p*K*a = 8.7) to the peripheral amine protonation. Presumably, protonation of the carboxylate was not observed due to the occurrence of two acid–base equilibria in a small pH interval (3–6) and to the simplicity of the method employed in this work.

Regarding **2**, it is proposed that the ciprofloxacin has its carboxylate coordinated to ruthenium, and should present only the protonation equilibrium of its amine group. The p*K*a observed in this work is 5.7. Literature has reported p*K*a values with significant fluctuations for the amine protonation equilibrium of free ciprofloxacin (8.2–9.0) [25], depending on the methodology employed. In our case, as expected, this p*K*a is much lower, due to the inductive effect of the Ru(III), that acts as a Lewis acid, removing electronic density of the ligand and increasing its overall acidity. This effect was corroborated by voltammetric results, which will be discussed below. This p*K*a value is also responsible for the differences observed in the electronic spectrum of **2** in distilled water (pH = 5.8) or physiological pH (pH = 7.4, phosphate buffer, see below); in water there is probably a major contribution of the species with three amines protonated, while deprotonated ciprofloxacin must prevail at physiological pH.

From the determined p*K*a values we conclude that, in distilled water (pH 5.8) and physiological pH, ciprofloxacin in **1** is present as zwitterions, which means, as a neutral molecule, probably responsible for the very low solubility of **1** in aqueous media. Actually, free ciprofloxacin is soluble under extreme pH conditions, since in low pH

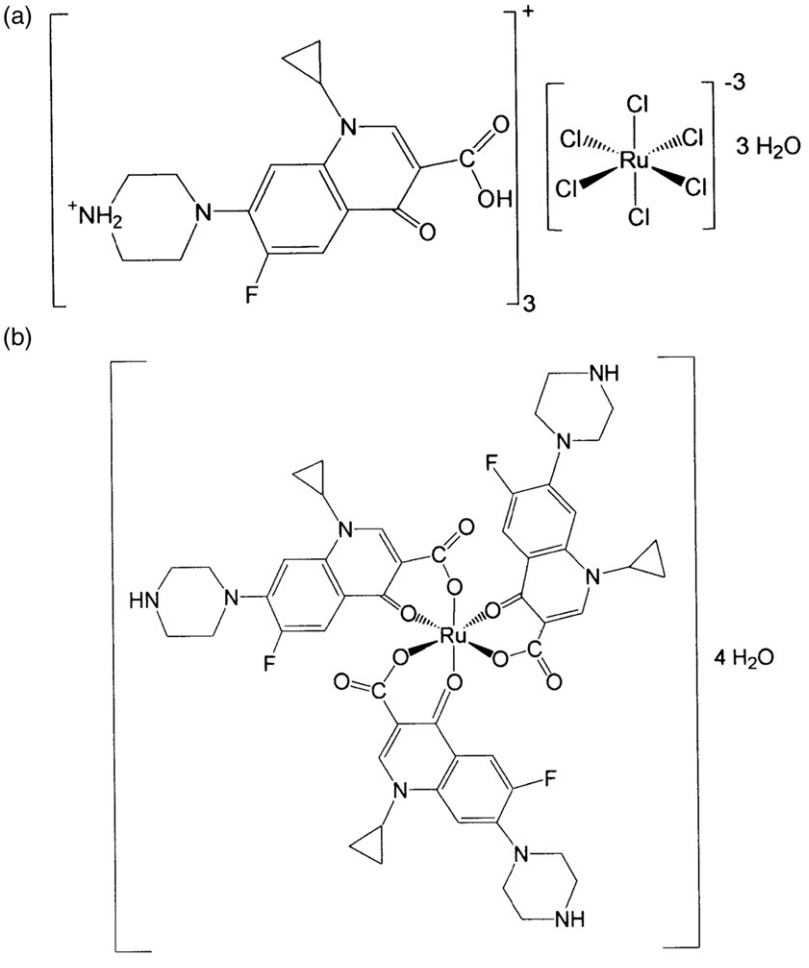

Figure 1. Pictorial view of the proposed structures for (a) $(\text{cipro}H_2^+)_3[RuCl_6]\cdot 3H_2O$ (**1**) and (b) $[Ru(\text{cipro})_3]\cdot 4H_2O$ (**2**).

values it presents +2 charge and in higher pH values, it presents −1 charge. Taking this feature into account, the charge of the whole molecule seems to determine the fluoroquinolone solubility, as it does in **1**.

3.2. *Structural characterization by ESI MS spectrometry and TG–DTA measurements*

For **1**, a low-intensity cluster of isotopolgue ions centered at m/z 1325.8 in the ESI MS/MS spectrum was ascribed to the adduct $\{[C_{17}H_{18}FN_3O_3{-}H^+]_3[RuCl_5]^{2-}\cdot H_2O\cdot CH_3OH\}^+$. However, the most intense peak is observed at m/z 994.6 and its isotopic distribution is compatible with the carbon isotopic profile [27, 28]. This observation leads to the conclusion that this fragment does not have ruthenium in its composition. This peak was assigned to $\{(C_{17}H_{18}FN_3O_3)_3\cdot H^+\}$ formed in gaseous phase by three

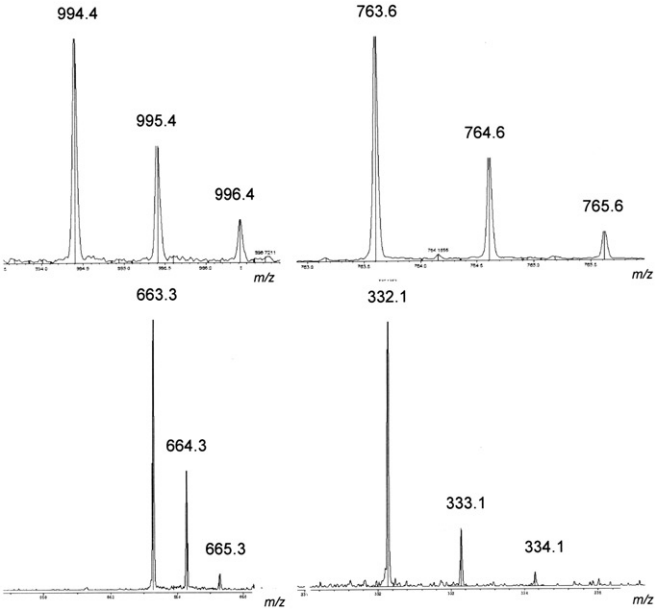

Figure 2. Expansion of the peaks observed in the ESI MS/MS spectrum of $(\text{ciproH}_2^+)_3[\text{RuCl}_6]\cdot 3\text{H}_2\text{O}$ (**1**), obtained from a methanolic solution (fragmentation of the ion at m/z 994.6), with emphasis on the carbon isotopic distribution.

ciprofloxacin molecules. In fact, in the ESI MS/MS spectrum of **1** (figure 2), a series of lower order adducts are assigned to the fragments $\{(C_{17}H_{18}FN_3O_3)_2\cdot H^+\}$ at m/z 663.3 and $\{(C_{17}H_{18}FN_3O_3)\cdot H^+\}$ at m/z 332.1. Another peak at $m/z = 763.6$ was ascribed to a ciprofloxacin fragment originated from its gaseous phase fragmentation. In recent work, Calza *et al.* [29] demonstrated that ciprofloxacin fragmentation in gas phase implies the loss of HF, CO_2, and a $-C_3H_5$ (cyclopropane). Considering the loss of the HF and CO_2 fragments from the three ciprofloxacin molecules of the adduct $\{(C_{17}H_{18}FN_3O_3)_3\cdot H^+\}$, and the loss of one alkyl group $-C_3H_5$ from one ciprofloxacin, a fragment of mass 763 is obtained, corresponding to the observed peak at $m/z = 763.6$. Therefore, these observations lead us to conclude that the ESI MS/MS spectrum of **1** is dominated by ciprofloxacin chemistry in the gaseous phase. This result strongly suggests that in **1** ciprofloxacin is not coordinated to the metal center, which is not detected because it occurs as an anion and the experiment was performed in the positive detection mode.

On the other hand, the ESI MS spectrum of **2** (figure 3) exhibits two clusters of isotopologue ions centered at m/z 1093.3 and 547.1, ascribed to the singly and doubly charged ions $[\text{Ru}(C_{17}H_{17}FN_3O_3)_3]\cdot H^+$ and $[\text{Ru}(C_{17}H_{17}FN_3O_3)_3]\cdot 2H^+$, respectively. The isotopic distribution observed is compatible with that of ruthenium [27, 28], corroborating the formulation proposed for **2**.

The compounds under investigation were analyzed by thermogravimetric (TG) measurements (Supplementary material); for comparison purposes the TG/DTA curves of free ciprofloxacin were also obtained in the same conditions used for the metallic compounds. TG results for free ciprofloxacin and ionic and coordination compounds of

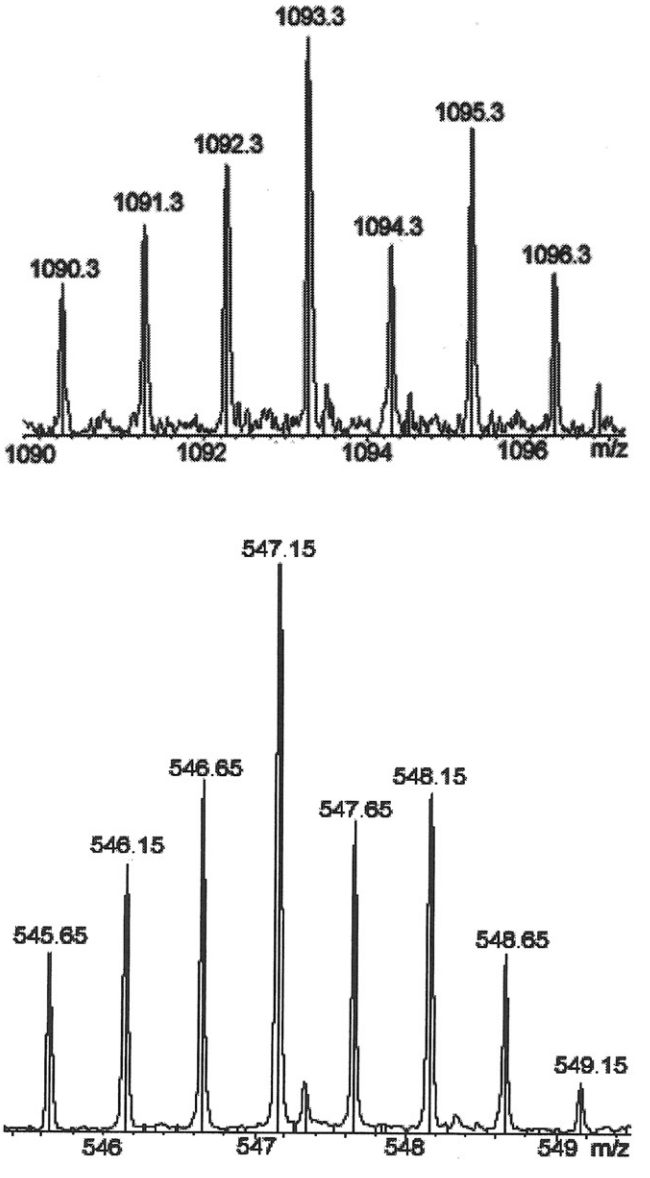

Figure 3. Expansion of the two peaks observed in the ESI MS spectrum of $[Ru(cipro)_3] \cdot 4H_2O$ (**2**), obtained from a methanolic solution, with emphasis on the ruthenium isotopic distribution. (a) $[\mathbf{2}.H^+–4H_2O]^+$ and (b) $[\mathbf{2}.2H^+–4H_2O]^{2+}$.

Cu, Zn, and Ru have been reported [30–33] and, although the literature data were collected in different conditions, the data reported in this work are compatible with the literature cases, except for the ruthenium complex reported earlier since its composition is rather different from that of **2** [33].

For free ciprofloxacin (in its zwitterionic form), there is no loss of hydration water in the beginning of the thermogram; the first mass loss (68%) is finished around 400°C

and is ascribed to a partial decomposition of the ciprofloxacin molecules with the loss of H_2O and CO_2. This first decomposition step was previously reported to occur at 277°C [30]. In our experiment an intense peak at 272°C is observed. However, a close inspection of the thermogram shows that at this temperature there is no mass loss, suggesting that this endothermic peak might be assigned to a phase change, e.g., to the ciprofloxacin fusion. Its complete decomposition occurs over 600°C (endothermic peak at 623°C); the whole profile of the thermogram is in accord with the literature [30].

The thermogram profiles of **1** and **2** are also in agreement with the data reported earlier [30–33] and, although they resemble each other, the TG data are fundamental to distinguish between the ionic and coordination compounds. In the case of the ionic **1**, a 4% mass loss at 60°C corresponding to the loss of three water molecules corroborates the elemental analysis results. Turel *et al.* [30, 31] reported this process to occur at 62.5°C for an analogous ionic compound of Cu, and it is followed by mass losses over 230°C, ascribed to the loss of CO_2, H_2O, and HCl (keeping in mind that the coordination anion – a copper compound in that case and a ruthenium one in our investigation – contains chlorides). The total decomposition of the Cu ionic compound was reported to be complete above 800°C. The behavior of **1** is fully compatible with the description.

For **2**, hydration water molecules are lost at 135°C (6% in mass, corresponding to four water molecules). For zinc(II) compound, this temperature was 140°C [32]. Apparently, the coordination promotes some thermal stability to ciprofloxacin, since its decomposition begins above 300°C, a value higher than observed for free ciprofloxacin and for **1**. Nevertheless, the most important feature that emerges from the thermal analysis regards the temperature of hydration water molecules lost. For **1** this occurs at 60°C, signaling weakly interacting water molecules; for **2**, the hydration water molecules are lost above 100°C, suggesting that in this case these water molecules are tightly bound to the compound. In accordance with the discussion presented by Turel *et al.* [30], water molecules play a role when the fluoroquinolones have sites for the formation of hydrogen bonds. In typical coordination compounds such as **2**, both the amine function on the piperazine ring and the C=O of the coordinated carboxylate participate in a hydrogen bond framework with water [9] and this structure is responsible for the increase in temperature for the loss of hydration water. In the case of ionic compounds such as **1**, these sites are already protonated, being unavailable to form a net of hydrogen bonds with solvent, in such a way that their hydration water molecules are loosely associated to the compound and lost more easily.

3.3. *Spectroscopic and electrochemical characterization*

The IR spectra of quinolones are rather complicated from 2000 to 1000 cm^{-1} due to different functional groups displayed by this family of drugs, and the picture is maintained in IR spectra of quinolone metal complexes [2, 10, 34]. Usually, only the most characteristic vibrations are assigned, e.g., the carboxylic acid stretch ν(COOH) (or $\nu(C{=}O)_c$, between 1730 and 1700 cm^{-1}), the pyridone stretch $\nu(C{=}O)_p$ (around 1630 cm^{-1}), and the symmetric and antisymmetric carboxylate stretch $\nu(O{-}C{-}O)_s$ (between 1400 and 1280 cm^{-1}) and $\nu(O{-}C{-}O)_{as}$ (between 1650 and 1510 cm^{-1}). These two last vibrations are very useful since the value of $\Delta\nu$(COO) permits an evaluation of the binding mode of the carboxylate ($\Delta\nu(COO) > 200\,cm^{-1}$ monodentate, $< 100\,cm^{-1}$

Table 1. Tentative assignment for the peaks observed in IR spectra of $[Ru(C_{17}H_{17}FN_3O_3)_3]\cdot 4H_2O$, free ciprofloxacin (chlorohydrate form) and $[C_{17}H_{19}FN_3O_3]_3[RuCl_6]\cdot 3H_2O$.

	$\nu(C=O)_p$ (cm^{-1})				
	$\nu(C=O)_p$	ν(COOH)	$\nu(O–C–O)_a$	$\nu(O–C–O)_s$	$\Delta\nu_{(COO)}$ (cm^{-1})
$[Ru(C_{17}H_{17}FN_3O_3)_3]\cdot 4H_2O$	1630	–	1608	1382	226
Ciprofloxacin	1620	1724	1542	1377	165
$[C_{17}H_{19}FN_3O_3]_3[RuCl_6]\cdot 3H_2O$	1626	1709	1608	1385	223

bidentate, and 150 cm^{-1} bridged or ionic) [10, 34]. However, most authors recognize that on the basis of IR measurements, it is not possible to unambiguously assign the coordination mode of such a complex ligand. Table 1 depicts the main IR data collected in this work for **1** and **2**, as well as a tentative assignment; it also presents data obtained for ciprofloxacin for comparison.

For free ciprofloxacin, a strong peak ascribed to the $\nu(C=O)_c$ should be observed. Actually, this band was observed at 1724 cm^{-1} but as a low intensity peak. The ciprofloxacin used in this work (Fluka) is in its zwitterionic form, which does not display the $\nu(C=O)_c$ vibration since the carboxylic acid function is deprotonated. Possibly, this band was still observed due to the presence of a residual amount of ciprofloxacin in its acidic form. The observed value of $\Delta\nu$ (165 cm^{-1}, near of 150 cm^{-1}) also corroborates the predominance of the drug in its ionic form. In the case of **1**, isolated from an acid medium, the IR spectrum displays the $\nu(C=O)_c$ as a strong peak at 1709 cm^{-1} and **2** does not display this vibration at all. In all cases studied in this work, the $\nu(C=O)_p$ appeared in its typical frequency range, between 1620 and 1630 cm^{-1}.

Regarding the coordination mode of ciprofloxacin in **2**, our data indicate that the carboxylate is coordinated monodentate ($\Delta\nu = 226\,cm^{-1}$), supported by other literature examples, in which it has been demonstrated (also based on X-ray results) [2] that the most common coordination mode for this class of metal-quinolone is the chelation of the central ion by one carboxylate oxygen and by the pyridone oxygen. The value found for **1**, $\Delta\nu = 223\,cm^{-1}$, also suggests monodentate coordination. As pointed above, unambiguous assignment is not possible, however, in **1** the carboxylate is protonated and the protonation might simulate coordination to a metal ion.

The electronic spectrum of **1** in DMSO displays the characteristic intense bands of ciprofloxacin in the UV region ($\lambda = 283$ nm, $\varepsilon = 144{,}978\,L\,cm^{-1}\,mol^{-1}$; $\lambda = 319$ nm, $\varepsilon = 39{,}393\,L\,cm^{-1}\,mol^{-1}$; and $\lambda = 332$ nm, $\varepsilon = 37{,}433\,L\,cm^{-1}\,mol^{-1}$), assigned to $\pi\rightarrow\pi^*$ transitions. It also presents low-intensity bands at $\lambda = 421$ nm ($\varepsilon = 3377\,L\,cm^{-1}\,mol^{-1}$) and $\lambda = 495$ nm ($\varepsilon = 472\,L\,cm^{-1}\,mol^{-1}$) that can be tentatively assigned to a ligand-to-metal charge-transfer and a d→d transition, respectively. The assignment was done based on the ruthenium oxidation state +3, the visible region of spectra where these bands occur, and on the molar absorptive coefficient values. The electronic spectrum of **2** in DMSO also displays the characteristic intense bands of the ciprofloxacin $\pi\rightarrow\pi^*$ transitions ($\lambda = 276$ nm, $\varepsilon = 119{,}087\,L\,cm^{-1}\,mol^{-1}$ and $\lambda = 314$ nm, $\varepsilon = 38{,}382\,L\,cm^{-1}\,mol^{-1}$), however, only a broad, low-intensity shoulder was observed between 400 and 500 nm.

The electrochemical profile of both compounds is rather similar (the cyclic voltammograms are available as "Supplementary material"), although they present important shifts on the $E_{1/2}$ values of the observed processes. Compound **1** presents a pair of quasi-reversible waves at −0.11 V (SHE), which was readily ascribed to $Ru^{+3/+2}$ since this wave is absent in the voltammogram of free ciprofloxacin [35]. This redox pair was observed at −0.25 V (SHE) for **2**. In fact, this negative shift is expected on going from the ionic to the coordination compound, since in the latter case the hard acid Ru(III) must be stabilized by coordination to the oxygen of ciprofloxacin, which are hard bases. Thus, the coordination should make the reduction of the central ruthenium ion more difficult.

Concerning the other two processes observed in a more negative region, little is known about the electrochemical behavior of ciprofloxacin and of fluoroquinolones in general. However, the two irreversible waves observed in both voltammograms in the negative region are consistent with the behavior previously presented by Saha *et al.* [35], which observed two ciprofloxacin reductions, the first assigned to the irreversible piperazine reduction and the other assigned to the reductive process centered at the pyridone ring. The interesting aspect is that for **2**, where ciprofloxacin molecules are coordinated to Ru(III), the reduction potential values ($E_{1/2} = -0.85$ V and −1.45 V) are smaller than the values observed for the ionic compound **1** ($E_{1/2} = -0.92$ V and −1.56 V). This fact is consistent with the removal of electron density of the ciprofloxacin ligands by the ruthenium ion in **2** (as already observed in the determination of the p*K*a values), facilitating the reduction processes of this coordinated ciprofloxacin, which is poor in electron density.

3.4. *Dependence of compounds behavior with variation of pH*

The electronic spectra of both compounds in water solution at different pH values: pH 1.5 (stomach), pH 7.4 (physiological), and pH 8.0 (intestine) were investigated (Supplementary material). The spectra profiles of **1** and **2** are pH dependent, due to the different degrees of protonation of ciprofloxacin in each situation. Under pH 1.5, ciprofloxacin is protonated and at pH 7.4 and 8, ciprofloxacin is not protonated at all in both compounds. Therefore, the spectra profiles present small differences due to the fact that the chemical species present in solution are pH dependent.

Compounds **1** and **2** were also incubated at stomach pH (1.5) at 37°C to probe their stability and eventual variations in the medium pH (figure 4) [36]. As one can see, the compounds are stable, since there are no significant variations of the pH values during the incubation period. Besides that, there was no color change or precipitation events [36], signaling to maintenance of their structures.

For **2**, which is very soluble in water, stability in water and at physiological pH (phosphate buffer) was also evaluated (Supplementary material), and the compound is stable for a period of at least 48 h.

3.5. *ct-DNA-binding evaluation*

Ciprofloxacin features antibacterial action by inhibiting DNA replication, especially for Gram-negative pathogens [1], and that is the reason of the fundamental interest to study the interaction of quinolone–metal complexes with DNA. Although both the carbonyl

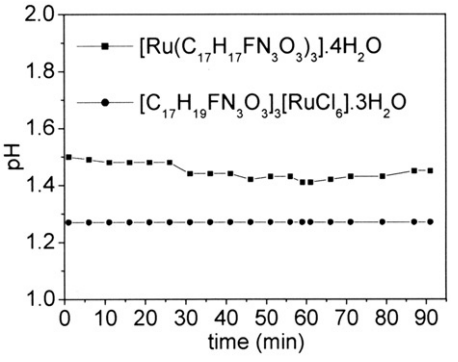

Figure 4. pH variation with time for $[Ru(C_{17}H_{17}FN_3O_3)_3]\cdot 4H_2O$ (8.0×10^{-6} mol L^{-1}) and $[C_{17}H_{19}FN_3O_3]_3$ $[RuCl_6]\cdot 3H_2O$ (2.5×10^{-4} mol L^{-1}) in HCl solution, pH = 1.5; 37°C.

and the carboxyl groups at positions C4 and C3, respectively, are involved with DNA interaction, in most cases coordination to a metal ion involving both groups do not preclude activity of the fluoroquinolone [2]. More recently, a theoretical study of ciprofloxacin using electron density methods showed that the positively charged amine in ciprofloxacin zwitterionic form is a strong hydrogen bond site, suggesting that it may also contribute to the interaction with the active sites of proteins or with DNA [37].

To probe if there is an interaction of **2** (which is the water soluble species) with *ct*-DNA and to evaluate the nature of this interaction, a spectrophotometric titration was performed (figure 5) at a fixed concentration of **2** (2.29×10^{-5} mol L^{-1}) and increasing amounts of *ct*-DNA (4.9×10^{-5} to 4.9×10^{-4} mol L^{-1}).

Figure 5(a) shows a broadening in the 276 nm band (ascribed to a transition of **2**) and an intensity increase in lower wavelengths due to the addition of *ct*-DNA, which has an absorption maximum in the UV region. In spite of the superposition of the complex and DNA absorptions bands, a bathochromic shift of the *ct*-DNA absorption is observed, leading to the shoulder between 290 and 300 nm. This observation allows the conclusion that there is a ground-state interaction between *ct*-DNA and **2**. Earlier literature reports [10, 18, 22, 38] point out that the observed red shifts are due to an intercalative interaction involving π-stacking of aromatic chromophores of the ligands and the DNA base pairs. The spectra presented in figure 5(a) are consistent with this behavior.

The binding strength of **2** to *ct*-DNA was evaluated by calculating the intrinsic binding constant K_b [10, 22, 23] which was obtained from the slope of the Benesi–Hildebrand plot (figure 5c). The observed value of $K_b = 2.5\ (\pm\ 0.9)\times10^4$ mol^{-1} L suggests a strong interaction, in the same range observed for other ciprofloxacinate compounds [10].

Even for ruthenium compounds having other ligands in their coordination sphere (such as modified phenanthrolines and imidazole derivatives [39, 40]), the binding constants to DNA as probed by spectroscopic methods typically fall in the range between 10^4 and 10^5 mol L^{-1}. In all cases, as for **2** (this work), the hydrophobicity of the ligands seems to be the leading aspect promoting intercalation (which is based on π-interactions), rather then their spatial configuration. Nevertheless, it is interesting to point out that, for a new copper–ciprofloxacin compound [41], the authors claim that

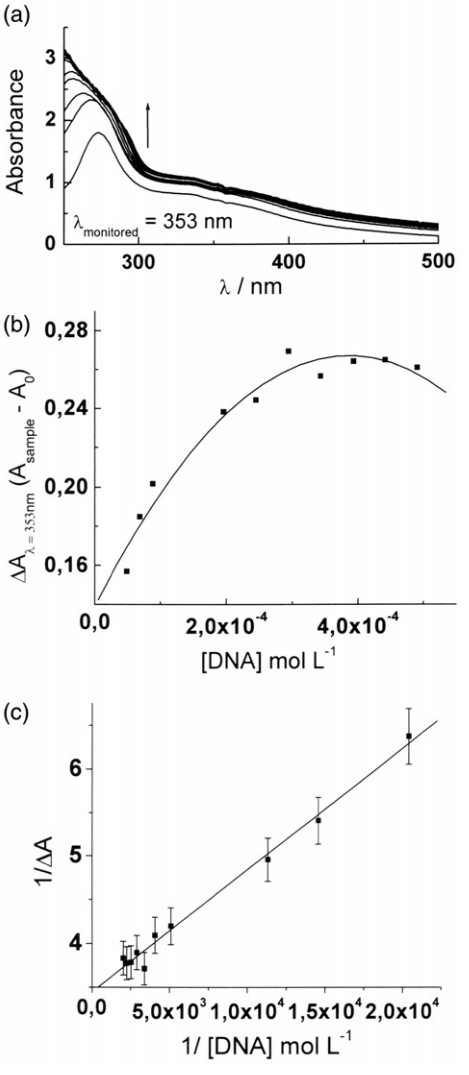

Figure 5. UV-Vis spectra of (a) $[Ru(cipro)_3] \cdot 4H_2O$ (2.29×10^{-5} mol L^{-1}) with increasing concentration of *ct*-DNA (from 4.9×10^{-5} to 4.9×10^{-4} mol L^{-1}) in phosphate buffer (0.15 mol L^{-1} sodium chloride and 0.015 mol L^{-1} sodium citrate, pH 7.0); (b) Absorbance variation (at 353 nm) with the addition of *ct*-DNA; (c) Double reciprocal Benesi–Hildebrand plot.

the positive charge of the coordination ion assists the drug-DNA interaction by means of electrostatic attraction to the phosphate backbone of DNA.

4. Conclusions

This work presents ruthenium–ciprofloxacin complexes obtained by two simple synthetic routes. The voltammetric characterization of the complexes shows ruthenium

ion is stabilized in its Ru(III) form by coordination with ciprofloxacin, having a low oxidation potential. Due to this stabilization, reduction of the ciprofloxacin is facilitated. A comparison of the complexes obtained from two different synthetic approaches shows that these products have two distinct structures that should have important implications for their potential application as metallo-drugs. The "true" coordination compound $[Ru(cipro)_3]\cdot 4H_2O$ is totally soluble in water, improving its bio-availability. Another important feature is that these compounds are stable under physiological and stomach pH. A preliminary investigation on the interaction of **2** and *ct*-DNA shows that the complex strongly binds to *ct*-DNA ($K_b \sim 10^4$), possibly by an intercalative interaction, as suggested by the UV-Visible spectra profiles. Therefore, in the next steps of this work, the biological activity of the compounds will be investigated in our laboratories.

Supplementary material

The TG and cyclic voltammetry measurements for compounds **1** and **2**, as well as electronic spectra of **2** in different pH values for a period of 48 hours are available as Supplementary Data.

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