New rhenium complexes with ciprofloxacin as useful models for understanding the properties of [^{99m}Tc]-ciprofloxacin radiopharmaceutical

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Abstract

Rhenium complexes with the antibiotic ciprofloxacin have been prepared to be studied as models of technetium radiopharmaceuticals. With this aim, the new rhenium complexes **1** {$[ReO(Cpf)_2]Cl$}, **2** {$[ReO(CpfH)_2]Cl_3$} and **3** {*fac*-$[Re(CO)_3(H_2O)(Cpf)]$} with ciprofloxacin (CpfH = ciprofloxacin; Cpf = conjugated base of ciprofloxacin) have been synthesised and characterised by elemental analyses, IR, NMR (^1H, ^{19}F and ^{13}C CP-MAS) spectroscopy, as well as MS measurements. All spectroscopic data are consistent with the coordination of ciprofloxacin in all these complexes through the carbonyl and the carboxylate oxygen atoms with the formation of a six member chelate ring.

The study of a Tc-ciprofloxacin solution by ESI-MS reveals the presence of $[TcO(Cpf)_2]^+$ cations, which agrees with the hypothesis that complexes **1** and **2** can be seen as model rhenium complexes of this radiopharmaceutical.

Antimicrobial and DNA Gyrase inhibition studies performed with complexes **2** and **3** have shown a very similar behaviour between complex **2** and the free antibiotic, whereas complex **3** exhibit a lower antimicrobial activity.

Based on a joint analysis of the data reported in the literature and the chemical and biological results obtained in this study, a tentative proposal to explain some aspects of the behaviour of Tc-ciprofloxacin radiopharmaceutical has been made

Keywords

Rhenium, Technetium, Ciprofloxacin, Metal Carbonyl, Radiopharmaceutical, Infection

1. Introduction

Molecular imaging techniques are very powerful tools in medicine because they allow visualising biochemical process in living organisms by non-invasive methods. Images can be obtained either by measuring the absorption of externally applied radiation (NMR, CT), or the radiation escaping from the body. In the second case, a diagnostic radiopharmaceutical, a molecule that contains an unstable radionuclide which decomposes with the emission of gamma rays, should be administered to the patient. The radionuclide Tc-99m is widely used in radiopharmacy for this purpose due to its availability through the ^{99}Mo/^{99m}Tc generator and its near ideal nuclear properties (half-life of 6h and γ-ray energy of 141 keV).[1,2] The high penetration ability of photons allows gamma radiation to escape from the body and to be detected externally. The analysis of the *in vivo* behaviour of the radiopharmaceutical makes it possible to differentiate normal from abnormal anatomical structures or biochemical functions. Current research with technetium radiopharmaceuticals is focused on the development of bioactive substances with specific biological properties linked to the technetium metal.[3,4]

Despite significant advances in medicine, infections remain a major health problem and a cause of patient mortality worldwide. The use of specific radiopharmaceuticals for infection imaging is an attracting approach, as it is a sensitive methodology that allows a site of an occult infection to be located earlier.[5,6] Radiolabelled leucocytes is currently one of the most commonly used radiopharmaceuticals in hospitals for this purpose, but radiolabelled antimicrobial peptides and radiolabelled antibiotics are also being studied as potential alternatives.[5,6] Ciprofloxacin is one of the most common and widely used antibacterial agents (Scheme

1). It is a synthetic antibiotic belonging to fluoroquinolone group with a broad-spectrum bacteria-localising capability.[7,8] Fluoroquinolones suppress the growth of bacteria by inhibiting the activity of DNA gyrase, an essential bacterial enzyme that relieves strain while double-stranded DNA is being unwound.[7,9,10] Technetium-99m labelled ciprofloxacin (^{99m}Tc-ciprofloxacin or Infecton) was first introduced in the 1990s and has been extensively studied as an infection-specific radiopharmaceutical.[5,11-16] However, the global analysis of numerous published studies (including clinical trials) shows contradictory data about the specificity of ^{99m}Tc-ciprofloxacin. Thus, different authors have claimed that this radiopharmaceutical can not properly differentiate a bacterial infection from an aseptic inflammation.[5,15,16] One of the main problems to elucidate the behaviour of this radiopharmaceutical is that, although it has been studied for two decades, there is no information about its molecular structure. This situation is due to attempts to prepare a model rhenium complex of ^{99m}Tc-ciprofloxacin have been unsuccessful,[17] and the structural analysis of the Tc-99m complex is not possible as it is prepared at very low concentration (10^{-7}-10^{-8} M).[1,2] If a model rhenium complex was available, the influence of metal coordination in the antibacterial properties of ciprofloxacin could be analyzed, and this is a key point to understand how this compound works as diagnostic radiopharmaceutical. We must realise that, in a first approximation, it seems very strange that the coordination of ciprofloxacin to the metal does not significantly alter its antibacterial properties. Particularly, if we take into account that the atoms that probably interact with the metal (4-oxo carbonyl and carboxylate oxygen atoms) are the same that have been proposed to interact with DNA gyrase.[7,9] In contrast to this view, other authors have proposed that metal atoms can be involved in the interaction between ciprofloxacin and DNA-gyrase ,[7, 18-20] which could

explain the antibacterial properties of Tc-ciprofloxacin.

(Scheme 1)

An interesting aspect is that the metal complexes of quinolones have been extensively studied. Thus, complexes with different metals such as, Mg,[20, 21] Al,[22] V,[23] Mn,[21, 24] Fe,[24, 25] Co,[21, 24, 26] Ni,[24, 26] Cu,[26, 27] Zn,[21, 26, 28] Y,[29] Zr,[29] Mo,[24] Ag,[30] Cd,[21, 26] Pt,[31] U[32] and Eu[33] have been reported, and there is even a review of these metal complexes.[19] As mentioned above, the most common coordination mode of ciprofloxacin is through the oxygen atoms of the 4-oxo carbonyl and the carboxylate groups, forming a chelating ring of six atoms. Only a few examples have been reported, in which the coordination through the nitrogen atoms of the piperazine ring of ciprofloxacin is shown or proposed.[30, 31]

As far as we know, only one rhenium complex with a derivative of ciprofloxacin has been reported,[34] but in this case the studied ligand is obtained by a coupling reaction between a tridentate chelator and the piperazinyl nitrogen of ciprofloxacin. This structural modification changes the coordination ability of the molecule and the metal is coordinated to the tridentate chelator, while the carbonyl and the carboxylate groups of ciprofloxacin remain free. The objective of this modification is to obtain a new molecule that preserves the capability of the antibiotic to bind bacteria, and that can also form stable bonds with technetium. Following the same approach, three works with Tc-99m have been published that contain other structural modifications of ciprofloxacin.[35-37] In recent years, and in some cases in conjunction with structural modifications of ciprofloxacin, the substitution of the hypothetical Tc(V) fragment $\{TcO\}^{3+}$ by the technetium tricarbonyl $\{fac\text{-}Tc(CO)_3\}$ has been explored in order to improve the potential radiopharmaceutical properties of technetium complexes with ciprofloxacin.[34,

[37, 38] The use of technetium carbonyls in radiopharmacy has been greatly stimulated after the development of *fac*-[^{99m}Tc$(H_2O)_3(CO)_3]^+$ because this cation contains three labile water molecules that can be easily substituted by other ligands yielding a wide range of stable complexes useful for radiopharmaceutical purposes.[3, 39, 40]

The aim of this paper is to prepare rhenium complexes with ciprofloxacin that can be useful as model compounds of ^{99m}Tc-ciprofloxacin to study their antibacterial properties, and to provide valuable data for understanding the behaviour of this radiopharmaceutical. This work will be complemented with a study in which the Re(V) will be replaced by the rhenium tricarbonyl fragment. This approach will allow us to compare the interaction of the two different fragments with ciprofloxacin and to supply comparative data for their antibacterial properties, which can shed some light on the understanding of the radiopharmaceutical properties of technetium ciprofloxacin derivatives. Part of this work was previously communicated.[41]

2. Materials and methods

2.1. Starting materials and methods

All reactions were performed under nitrogen using standard Schlenk tube techniques. Infrared spectra were recorded with a Perkin-Elmer 2000 FT spectrometer. The NMR spectra were recorded in the *Servei de Ressonància Magnètica Nuclear de la Universitat Autònoma de Barcelona* on Bruker DPX-250, DPX-360 and AV400 instruments. Microanalyses were performed by the *Servei d'Anàlisi Química del Departament de Química de la Universitat Autònoma de Barcelona*. Mass spectra and exact mass measurements were respectively obtained on an Esquire 3000 with electrospray ionization and an ion trap Bruker Daltonics and on a Bruker microTOFQ with electrospray ionization Apollo by *Servei d'Anàlisi Química del Departament de Química de la Universitat Autònoma de Barcelona*.

Rhenium complexes $[ReO_2(py)_4]Cl{\cdot}2H_2O$,[42] $[ReO_2(py)_4][PF_6]$,[43] $[NBu_4][ReOCl_4]$[44] and $[Re(CO)_5(O_3SCF_3)]$[45] were prepared by published procedures.

The preparation method for ^{99}Tc-ciprofloxacin was identical to that previously reported[46] but the technetium concentration was increased to 1.4 nmol/mL (the mass amount of technetium was calculated using algorithms derived from the radioactive equilibriums in the generator[47]) to improve performance in ESI-MS. This was achieved by radioactive decay of solutions with high activity. The concentration of technetium under these conditions was 10-100 times higher than in the no-carrier added conditions. The HPLC-MS (ESI) system used for this study consisted of a Liquid Chromatograph (Agillent 1100 HPLC) equipped with a RP C18 column (XTerra MS C18 Waters; 50 ×

2.1 mm × 3.5 μm) eluted at a flow rate of 0.3 mL/min. The column was eluted with gradient mixtures of acetonitrile (0': 5 %, 6': 5 %, 12': 60 %, 13': 60 %, 15': 5 %, 22': 5 %) and formic acid 0.25 %. The column eluate was directed to the spectrometer (Esquire 3000 with electrospray ionization and an ion trap Bruker Daltonics). Although the study was performed in the positive and negative mode, only a significant signal in the positive mode with the appropriate isotopic pattern was observed in the positive mode at 774.9 m/z (R_t = 3.2, 10.4 min).

2.2. Chemical synthesis

2.2.1 Synthesis of **1**

The precursor $[ReO_2(py)_4]Cl{\cdot}2H_2O$ (130 mg, 0.21 mmol) was dissolved in MeOH (5 mL) and added to a suspension of ciprofloxacin (150 mg, 0.45 mmol) in the same solvent (45 mL). The mixture was heated at the reflux temperature for 2 h. The obtained dark brown solution was concentrated under vacuum to 5 mL and the resulting solution was cooled to 4 ºC to crystallize the complex. The solid was washed with cold MeOH and Et_2O to give a brown solid **1** (120 mg). **IR** (ATR, cm^{-1}, relevant peaks): $1730_{(m)}$ (CO, free ciprofloxacin), $1630_{(s)}$, $1454_{(s)}$ (CO, OCO), $896_{(s)}$ (Re=O). **^{1}H-NMR** (DMSO-d_6, δ): 8.82 (s, 1H, H-2), 8.02 (d, *J* = 12.8 Hz, 1H, H-5), 7.68 (b, 1H, H-8), 3.81 (b, 1H, H-9), 3.64 (b, 4H, H-11), 3.50 (b, 4H, H-12), 1.30 (m, 4H, H-10). **^{19}F{^{1}H}-NMR** (DMSO-d_6, δ): -123.6. **^{13}C CP-MAS**: 174.5 (C-4), 165.7 (carboxylate), 154.8, 152.4 (C-6), 148.6 (C-2), 144.9 (C-7), 139.3 (C-4a), 117.0 (C-8a), 109.7 (C-5), 106.3 (C-3), 105.0

(C-8), 46.2 (C-11/ C-12), 36.1 (C-9), 8.2 (C-10). **HRMS (ESI)** (positive mode, m/z): Calcd. for $C_{34}H_{34}F_2N_6O_7Re$ ($M\text{-}Cl^-$) 863.2011, found 863.1977.

2.2.2 Synthesis of **2**

A solution of $[NBu_4][ReOCl_4]$ (70 mg, 0.12 mmol) in dry methanol (10 mL) was added dropwise to a suspension of ciprofloxacin (86 mg, 0.26 mmol) in the same solvent (10 mL) at 0°C. Next, the mixture was stirred for 1 h at the same temperature. The resulting dark-brown solution was reduced to 5 mL under vacuum and cooled to -30 ºC for 12 h. The dark brown precipitated was filtered and washed with 5 mL of dry acetone, 5 mL of dry Et_2O and dried under vacuum to obtain the complex **2** (63 mg, 53 %). **IR** (ATR, cm^{-1}, relevant peaks): $1626_{(s)}$, $1451_{(s)}$ (CO, OCO), $899_{(m)}$ (Re=O). **^{1}H-NMR** (MeOD, δ): 8.82 (s, 1H, H-2), 8.02 (d, *J* = 12.8 Hz, 1H, H-5), 7.68 (b, 1H, H-8), 3.81 (b, 1H, H-9), 3.64 (b, 4H, H-11), 3.50 (b, 4H, H-12), 1.30 (m, 4H: H-10).**^{19}F{^{1}H}-NMR** (MeOD, δ): -123.6. **^{13}C CP-MAS**(δ): 175.4 (C-4), 167.9 (carboxilate), 153.6, 151.0 (C-6), 146.5 (C-2), 143.9 (C-7), 139.2 (C-4a), 117.8 (C-8a), 112.2 (C-5), 106.9 (C-3), 104.8 (C-8), 44.6 (C-11/ C-12), 36.8 (C-9), 8.5 (C-10). Anal. Calcd. for $C_{34}H_{36}Cl_3F_2N_6O_7Re{\cdot}H_2O$: C, 41.28 H, 3.87 N, 8.50 %. Found: C, 41.08 H, 3.98 N, 8.32 %. **HRMS (ESI)** (positive mode, m/z): Calcd. for $C_{34}H_{34}F_2N_6O_7Re$ ($M\text{-}3Cl^-\text{-}2H^+$) 863.2011, found 863.2015.

2.2.3 Synthesis of **3**

The complex $Re(CO)_5O_3SCF_3$ (154 mg, 0.60 mmol) was dissolved in water (10 mL) and heated to reflux temperature for 1 h. Then, a water solution of ciprofloxacin (200

mg, 0.60 mmol) at pH = 3 was added and the heterogeneous mixture was heated to reflux temperature for 2 h with vigorous stirring. Next, the mixture was cooled in an ice bath and a suspension of a pale-yellow solid was formed. The solid was filtered and washed with 10 mL of cold water, 10 mL of methanol, 10 mL of Et_2O and dried under vacuum to afford **3** (231 mg, 62%). **IR** (ATR, cm^{-1}, relevant peaks): $2018_{(s)}$, $1871_{(s)}$ (C≡O), $1614_{(s)}$, $1480_{(s)}$ (CO, OCO). **^{1}H-NMR** (DMSO-d_6, δ): 8.85 (m, 1H, H-2), 7.85 (m, 1H, H-5), 7.45 (m, 1H, H-8), 3.35 (m, 9H, H-9/ H-11/ H-12), 1.20 (m, 4H, H-10). **^{19}F{^{1}H}-NMR** (DMSO-d_6, δ): -120.9. **^{13}C CP-MAS**: δ 198.3 (C≡O), 175.9 (C-4), 168 (carboxylate), 148.6 (C-2, C-6, C-7), 138.9 (C-4a) 121.2 (C-8a), 111.5 (C-3/ C-5/ C-8), 44.9 (C-11/ C-12), 36.8 (C-9), 8.1 (C-10). **ESI MS** (positive mode, m/z): 602.2 (M-H_2O+H^+). **Anal. Calcd.** for $C_{20}H_{19}FN_3O_7Re$: C, 38.83 H, 3.10 N, 6.79 %. Found: C, 38.44 H, 3.25 N, 6.67 %.

2.3. Antimicrobial susceptibility test

Antimicrobial activities of the complexes **2** and **3** were evaluated using a methodology similar as previously described.[48, 49] Briefly, 24h cultures of tested microorganisms: *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923) and *Enterococcus faecalis* (ATCC 29212) were stirred with NaCl 0.9 % solution and adapted to the concentration of 2x 10^8 cfu/ml. A sterile cotton swab was dipped into bacterial suspension and streaked over Mueller-Hinton agar plates (Oxoid). Filter paper discs Ø 6 mm (Whatman) were drenched with test complexes (5 µg of product). Plates were incubated at 37ºC for 18h.

The diameters of inhibition zones were measured. Ciprofloxacin (5 µg; Oxoid) was used as reference substance.

2.4. DNA gyrase cleavage assay

The solutions of complexes were prepared as follow: compound **2** and ciprofloxacin hydrochloride (used as control) were dissolved in sterile MilliQ grade water. Complex **3** was dissolved in a small volume of DMSO and the solution was diluted with sterile MilliQ grade water to the desired assay concentrations (from 1 to 250 µM). In all cases, the DMSO final concentration was less than 2.5 %.

DNA gyrase cleavage assay was carried out following the recommendations of the manufacturer (Inspiralis). Briefly, 0.5 µl (1U) of DNA gyrase was incubated with 0.5 µg of supercoiled pBR322 in a reaction volume of 30µl at 37ºC for 1 hour in a 1X assay buffer (Inspiralis). Ciprofloxacin or the assay complexes (**2**, **3**) were added at the desired concentrations (from 1 to 250 µM). Following, sodium dodecyl sulfate and proteinase K were added to final concentrations of 0.2 % and 0.1 mg/ml, respectively, and the incubation was continued for 30 min at 37 ºC. The reactions were stopped by adding 30 µl Stop buffer (40 % sucrose, 100 mM Tris.HCl (pH 7.5), 100 mM EDTA, 0.5 µg/mL bromophenol blue), followed by extraction with 1 volume of chloroform/isoamyl alcohol (24:1). Then 20 µl of aqueous phase of each sample was analysed on 1% agarose gels in 1 % TAE (40 mM Tris-Acetate, 2 mM EDTA) and visualized after staining with ethidium bromide in a ChemiDoc XRS apparatus (Bio Rad Laboratories).

3. Results and discussion

3.1. MS-ESI spectrum of ^{99}Tc-Ciprofloxacin

As has been stated in the introduction, is not possible to obtain structural information from very dilute solutions of Tc-99m compounds. However, in recent years has been demonstrated that it is possible to obtain MS-ESI spectra of solutions of technetium complexes, particularly if the technetium concentration is increased.[50, 51] Using this approach, an aqueous solution of ^{99}Tc-ciprofloxacin (prepared using previously reported method[46]) was analysed by MS-ESI spectrometry. It should be emphasized that the concentration of different species (free ciprofloxacin, tartrate, Cl^-, Na^+, Sn^{2+}) in the analyzed solution is several orders of magnitude higher than the concentration of technetium, which represents a major drawback to detect the ionization of the technetium species. However, using an HPLC column previously to the injection it was possible to observe a peak in the positive mode (Figure 1) corresponding to the ion $[TcO(Cpf)_2]^+$ (Cpf = conjugate base of ciprofloxacin). Although, due to of the experimental conditions it can not be excluded the formation of other species, this stoichiometry is reasonable and agrees with the expected reactivity of ciprofloxacin because it is consistent with the formation of a bis-chelate complex as it is shown in Scheme 2. As mentioned in the introduction, the coordination of ciprofloxacin through the carbonyl and the carboxylate oxygen atoms with formation of a six members chelate ring is the most frequently observed in ciprofloxacin metal complexes.[19] Regarding the metal, Tc(V) complexes are commonly obtained after the reaction between $[TcO_4]^-$ and

Sn^{2+}, and mono-oxo $[Tc^{V}O]^{3+}$ complexes are formed if Tc(V) is coordinated to anionic ligands which contain carboxylate groups that can act as (O,O)-chelate ligands.[2, 52] (Figure 1)

Therefore, the stoichiometry $[TcO(Cpf)_2]^+$ obtained from MS-ESI study with ^{99}Tc-ciprofloxacin is consistent with a plausible structure for this complex, and it was chosen as a reference to prepare a model rhenium complex. (Scheme 2)

3.2. Synthesis and characterisation of rhenium ciprofloxacin complexes

3.2.1. Rhenium (V) complexes

The first approach to prepare a $[ReO(Cpf)_2]^+$ complex was based on the substitution reaction of labile pyridine ligands in the *trans*-dioxo rhenium precursor *trans*-$[ReO_2(py)_4]^+$ by ciprofloxacin, as shown in Scheme 3. This reaction was studied using a wide range of experimental conditions, such as: different precursors ($[ReO_2(py)_4]Cl$, $[ReO_2(py)_4][PF_6]$), solvents (acetone, ethanol, methanol), reaction times and temperatures. The hydrothermal reaction was also studied using similar reaction conditions to those reported in the preparation of some ciprofloxacin metal complexes.[19, 21, 28] The worst results were obtained in those tests carried out in hydrothermal conditions, or in acetone as a reaction medium, which led to slightly coloured solutions and the recovery of ciprofloxacin as the main product. In contrast, reactions in methanol gave rise to dark brown solutions, and no significant quantities of

free ciprofloxacin were observed. Experiments in ethanol showed better results than in acetone, but appreciably lower than tests performed in methanol. Hence, after choosing methanol as the most appropriate reaction medium, the influence of temperature and reaction time was tested in this solvent, which showed that the best results are obtained if the reaction is performed at reflux temperature, and with a reaction time of nearly 2 hours (if reaction time is considerably increased above this value, the product was contaminated by substantial amounts of free ciprofloxacin). The concentration of the methanol solution under reduced pressure and subsequent cooling at 4ºC led to the formation of a dark brown precipitate (**1**) that was characterised by the usual spectroscopic and spectrometric methods.

(Scheme 3)

The ESI-MS of a methanolic solution of **1** in the positive region shows a sole main signal with the characteristic isotopic pattern of ions that contain rhenium and the high-resolution mass spectrum (HRMS) evidence that this signal at 863.1977 (m/z) fully agrees with the stoichiometry $[ReO(Cpf)_2]^+$.

The ^{1}H NMR spectrum of **1** is very similar to the spectrum of free ciprofloxacin, although show some small changes that agree more with the coordination of ciprofloxacin through the oxygen atoms of carboxylate and carbonyl groups than with the coordination through the piperazine ring. Thus, no significant changes are observed in the methylene hydrogens of piperazine ring,[31] whereas slight down field shifts are noticed in the H2, H5 and H8 hydrogen atoms of the quinolone ring.[53] In order to obtain information about the binding mode of ciprofloxacin, the ^{13}C spectrum is more useful because carbon atoms are directly linked to the heteroatoms susceptible of bonding to the metal. However, attempts to obtain a ^{13}C NMR spectrum of **1** in solution were

unsuccessful because the solubility of this compound is low, which force too long acquisition times in order to observe the signals of atoms of quinolone ring. This long time involves some decomposition of **1**, and a slight precipitate of free ciprofloxacin was observed in the NMR tube. Attempts to isolate a monocrystal of complex **1** for X-ray analysis were unsuccessful for this same reason. This behaviour forced us to study this complex by solid-state NMR methods using cross-polarization and magic angle spinning experiments (CP-MAS). On comparing the ^{13}C CP-MAS spectra of complex **1** and ciprofloxacin, small changes in the 0-50 ppm region can be observed (Figure 2, numbering of carbon atoms is shown in Scheme 1), whereas the most relevant modifications are detected in the 160-180 ppm region. Thus, in free ciprofloxacin a sole signal is observed that has been assigned to the carboxylate and C4 carbon atoms.[22] The coordination of the metal through the oxygen atoms of carbonyl and carboxylate groups exerts a strong influence on these carbon atoms directly bonded to these oxygen and these resonances are observed in the complex **1** as two different peaks. This assignment is consistent with the reported study with complex $[Al(CpfH)_3]Cl_3$ (CpfH = ciprofloxacin).[22]

(Figure 2)

The IR spectrum also suggests ciprofloxacin coordination through the oxygen atoms of carboxylate and carbonyl groups, in accordance with the NMR spectroscopy**.** Thus, two intense signals are observed at 1630 and 1454 cm^{-1}, which have been assigned to the vibrations of carboxylate and ketone groups in previously reported ciprofloxacin metal complexes with the proposed coordination of ciprofloxacin.[19, 29, 54] The presence of an oxorhenium metal fragment in **1** is also consistent with this IR spectrum, since an intense band at 896 cm^{-1} is observed, which can be assigned to the

ν(Re=O) vibration.[55] The contamination of **1** by small quantities of free ciprofloxacin was revealed by a signal of medium intensity observed at 1730 cm^{-1}. This fact agrees with the results observed in the different synthetic assays and with the observed tendency of the solutions of **1** to decompose, yielding free ciprofloxacin. In order to obtain the complex **1** free of ciprofloxacin or another complex with a similar structure, a new synthetic method was designed, based on a substitution reaction with a rhenium precursor that can be performed at low temperature. Hence, the reaction between the very reactive rhenium precursor $[ReOCl_4]^-$ and ciprofloxacin leads to complex **2**, as shown in Scheme 4. The ESI-MS of methanol solutions of **1** and **2** in the positive region are identical, and no significant differences are observed in the ^{13}C CP-MAS spectra of both complexes. The main difference is observed in the intensity of signal at C11-C12 atoms, which is more intense for complex **2** and it is slightly shifted. These data suggest that the structure of the two complexes should be very similar. However, the elemental analysis shows values significantly different for **2**, which agree with the stoichiometry $[ReO(CpfH)_2]Cl_3$ for this compound. This result agrees with the above spectroscopic data and provides evidence that the structures of the two complexes are nearly identical but the secondary nitrogen atom of the piperazine ring is protonated in complex **2** or, in other words, the ciprofloxacin ligand is coordinated in its zwitterionic form. It is consistent with the synthetic method used to prepare this complex, since the $[ReOCl_4]^-$ anion reacts vigorously with acidic hydrogen atoms to form hydrogen chloride. Therefore, we can suppose that the acid generated by the reaction with the carboxylic hydrogen atom is responsible for the protonation of the piperazine nitrogens. The absence of free ciprofloxacin in this complex is evidenced by the IR spectrum (no signal

is observed at 1730 cm^{-1}), and this fact agrees with the idea that the reaction at a lower temperature minimises the presence of free ciprofloxacin in the final product.

(Scheme 4)

3.2.2. Rhenium (I) complex

A rhenium carbonyl complex with ciprofloxacin was prepared by direct reaction between aqueous solutions of *fac*-$[Re(H_2O)_3(CO)_3]^+$ [56] and ciprofloxacin, as shown in Scheme 5. The pH is a key factor in this process, because the complex *fac*-$[Re(H_2O)_3(CO)_3]^+$ can undergo a deprotonation in water, leading to dimeric or trimeric species as $[Re_2(\mu\text{-}OH)_3(CO)_6]^-$,[57] so it is necessary to maintain this solution at pH neutral or slightly acidic. Furthermore, the acidic medium avoids contaminating the final product by free ciprofloxacin, since the solubility of ciprofloxacin in water is very low at neutral pH, but it is soluble at the pH of the reaction. Complex **3** was obtained as a yellow solid that showed very low solubility in most of the solvents, but it was possible to obtain the ESI-MS spectrum. It shows a peak at 602.2 (m/z) with the characteristic isotopic pattern of rhenium compounds that is assigned to the $M+H^+\text{-}H_2O$ signal of complex **3**. As in the previous rhenium ciprofloxacin complexes, the ^1H NMR spectrum gives very little information but shows slight down field shifts of hydrogen atoms of the quinolone ring, which agrees with the coordination of ciprofloxacin through the oxygen atoms of carboxylate and carbonyl groups. This hypothesis agrees with the ^{13}C CP-MAS spectrum of complex **3**, which is very similar to the spectrum of **1** and **2** in the 160-180 ppm region, showing two signals that are assigned to the carbon atoms directly bonded to the oxygen atoms linked to rhenium.[22] The IR spectra also agrees with the proposed

coordination for ciprofloxacin, showing two intense signals at 1614 and 1480 cm^{-1}, in concordance with the observed values for complexes **1**-**2**, and with reported data for other complexes with this coordination.[19, 29, 54] No signal was observed at 1730 cm^{-1}, showing that this complex is free of contamination with ciprofloxacin and two intense peaks, assigned to the characteristic υ(C≡O) absorptions of the *fac*-{$Re(CO)_3$} fragment, are shown at 2018 and 1871 cm^{-1}. All these data agree with the structure proposed in Scheme 5 for complex **3**, in which the ciprofloxacin coordination is identical to that of complexes **1**-**2**, but in this case the metal fragment is *fac*-{$Re(CO)_3$} instead of {ReO^{3+}}.

(Scheme 5)

3.3. Antimicrobial activity

Antimicrobial activities of the rhenium complexes **2** and **3** were evaluated using the agar disk-diffusion method against Gram-positive and Gram-negative bacteria, and the results are presented in Table 1. The low solubility of rhenium complexes forces dimethylsulfoxide to be used as solvent and, for that reason, the activity against this molecule is shown. The results reveal that the activity of rhenium complex **2** against all studied strains is nearly identical to that found for the ciprofloxacin, which is used as a reference compound. Alternatively, the activity of the rhenium complex **3** is slightly lower than the activity of the free ciprofloxacin. These results agree with previous reported data about the antimicrobial activity of quinolone metal complexes. Thus, in most of these studies, the reported antimicrobial activities are very similar to, or slightly lower than, those of free quinolones.[19, 23, 54, 58]

(Table 1)

The fact that the rhenium metal complexes of ciprofloxacin exhibits antimicrobial activity can explain the properties of Tc-ciprofloxacin radiopharmaceutical, since the similarities between technetium and rhenium chemistry and between the radiuses of both elements are well known.[1] In other words, if the coordination of the antibiotic to the metal does not significantly alter their antimicrobial properties, it could explain the high concentration of radionuclide in infected regions. Another interesting result, is to observe that the coordination of ciprofloxacin to the metal fragment *fac*-{$Re(CO)_3$} has more influence on the antimicrobial activity than if it is coordinated to the {ReO^{3+}} fragment. We will discuss this result further in the next section.

3.4. Inhibition of DNA Gyrase

As previously stated in the introduction, DNA Gyrase is a type II topoisomerase that relaxes supercoiled DNA and also introduces negative supercoils into the double stranded DNA in bacterial replication. The antibacterial properties of quinolones are due to their capability of acting as inhibitors of this enzyme.[7, 10, 59] Different proposals have been made to understand the mechanism of Gyrase inhibition but the molecular details are unknown.[7, 9, 10, 59] It should be mentioned that some authors support the idea that metal atoms are involved in the inhibitions mechanism of Gyrase by quinolones. For this reason, quinolones are also known as metallo-antibiotics,[60] and different studies on Gyrase inhibition by quinolone metal complexes have been reported.[19, 58, 61, 62]

Since in ^{99m}Tc-ciprofloxacin radiopharmaceutical the ability to act as a diagnosis agent is a consequence of their antibiotic properties and the presence of the technetium radionuclide, we have performed a study of the Gyrase inhibition by the model rhenium ciprofloxacin complexes in order to obtain experimental data on the inhibition capabilities of the metal complexes.

The study of *Escherichia coli* topoisomerase II (named DNA Gyrase) inhibition by complexes **2** and **3** was performed by agarose gel electrophoresis using *E coli* DNA Gyrase cleavage assay kit (Inspiralis) and supercoiled pBR322 plasmid DNA (the study of **1** was omitted due to the contamination of this complex by free ciprofloxacin). The plasmid was incubated with a unit of DNA Gyrase in the presence of different concentrations (1 to 250 μM) of the free antibiotic (ciprofloxacin chlorhydrate) or the metal complexes **2** and **3**, and following the manufacturer's methodology. The results obtained in the assay control with ciprofloxacin chlorhydrate are shown in Figure 3. Thus, when Gyrase was added to the supercoiled DNA (lane 1), a series of topoisomers are formed due to the presence of various degrees of cleavage by the action of the enzyme (these topoisomers are less compacted and move at different speeds in agar) and the track is observed as a ladder. Lanes 2-7 show the influence of different concentrations of tested compounds on the activity of Gyrase. The top image corresponds to results obtained with ciprofloxacin chlorhydrate and show that, even at very low antibiotic concentrations, the intensity of the lower band assigned to supercoiled DNA increases, which is caused by the inhibitory action of the antibiotic. On comparing these results with data obtained for complex **2** (Figure 3, central image), we can observe a very similar behaviour between this compound and free ciprofloxacin, which points to a inhibition capacity of complex **2** very similar to that of the free

antibiotic. In contrast, the analysis of the results obtained with complex **3** (Figure 3, bottom image) shows that, although this complex also inhibits DNA Gyrase, the behaviour is slightly different than in the other two compounds. Thus, in this case a higher concentration of the tested compound is necessary (≈ 10 μM) to minimise the formation of the different topoisomers that are observed as a ladder in the agar gel. Consequently, these results are consistent with the observed antimicrobial activity, and confirm that the antibacterial properties of complex **2** are nearly identical to those of the free ciprofloxacin, while complex **3** shows a slightly different behaviour. Hence, although this complex also shows antibacterial properties, exhibits a lower activity than free ciprofloxacin.

(Figure 3)

On comparing the results obtained in the biological assays with complexes **2** and **3** with the chemical behaviour observed with these compounds, we can tentatively suggest a plausible explanation for the radiopharmaceutical properties of the homologous technetium complexes. Thus, the fact that the antibacterial properties of complex **2** are nearly identical to those of free ciprofloxacin, together with the observation that complex **2** has a manifest tendency to decompose to free ciprofloxacin, suggest a probable dissociation of ^{99m}Tc-ciprofloxacin during the interaction between the radiopharmaceutical and the bacteria. This hypothesis agrees with the observed labile properties of ^{99m}Tc-ciprofloxacin[46] and with the reported study on its accumulation in *Staphylococcus aureus* and *Pseudomonas aeruginosa*[63] that showed that ^{99m}Tc-ciprofloxacin was accumulated intracellularly in the ciprofloxacin-resistant strains. This result was associated with a possible dissociation of ^{99m}Tc-ciprofloxacin after entering the cell.[63] The labile character of O-donor complexes with Tc(V) as ^{99m}Tc-

Gluconate,[64] as well as the higher labile character of technetium complexes as regards their homologous rhenium complexes,[65] also points in this direction. According to this hypothesis, the behaviour of ^{99m}Tc-ciprofloxacin could be seen as similar to that of the radiopharmaceutical ^{111}In-oxine, which is known to dissociate after entering the cell.[66]

4. Conclusions

Three new rhenium complexes with ciprofloxacin have been prepared, and in all cases the spectroscopic measurements agree with a coordination of the ciprofloxacin molecule to the metal thorough the carbonyl and the carboxylate oxygen atoms with formation of a six member chelate ring. These rhenium complexes can be used as model compounds of technetium radiopharmaceuticals. The MS analysis of ^{99}Tc-ciprofloxacin is consistent with this hypothesis, since it shows the formation of homologous $[MO(Cpf)_2]^+$ (M = Tc, Re) ions with technetium and rhenium.

The biological studies with rhenium ciprofloxacin complexes have shown that, when the antibiotic is coordinated to the $\{ReO\}^{3+}$ fragment, the antibacterial activity is nearly identical to that of free ciprofloxacin, whereas if it is linked to the *fac*-$\{Re(CO)_3\}$ fragment it is significantly lower. From these results and previously reported data, we propose that the behaviour of Tc-ciprofloxacin radiopharmaceutical (Infecton) can be

associated with the dissociation of the labile $[TcO(Cpf)_2]^+$ complex after entering the cell.

Acknowledgements

This research was supported by *Dirección General de Investigación Científica y Técnica, Ministerio de Economía y Competitividad* (Projects CTQ2007-63913, BIO2009-12513-C02-02 and BIO2012-39682-C02-02) and by grant SGR 2009/1106 from *Generalitat de Catalunya*.

We are deeply indebted to Dr. Carmen Ardanuy and Fe Tubau from *Hospital Universitari de Bellvitge* (Barcelona, Spain) for providing us with ATCC strains. Finally, we also agree to *Servei de Ressonància Magnètica Nuclear de la Universitat Autònoma de Barcelona* for valuable helping in the NMR experiments.

Supplementary data

Supplementary data associated with this article (IR, ^{1}H NMR, ^{13}C NMR and HRMS spectra of complexes **1**, **2** and **3)** can be found in the online version at

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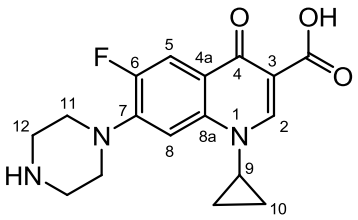
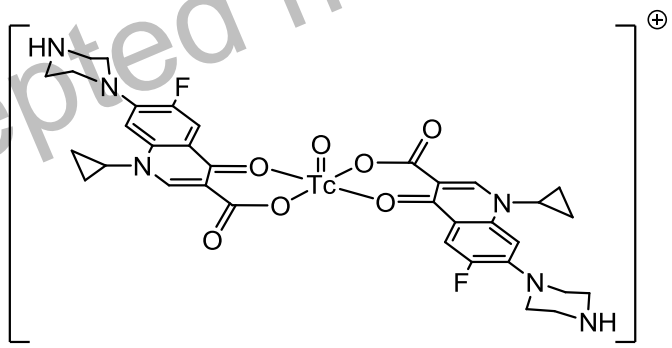
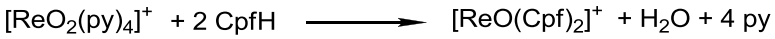
Scheme 1



Scheme 2



Scheme 3



Scheme 4

Scheme 5

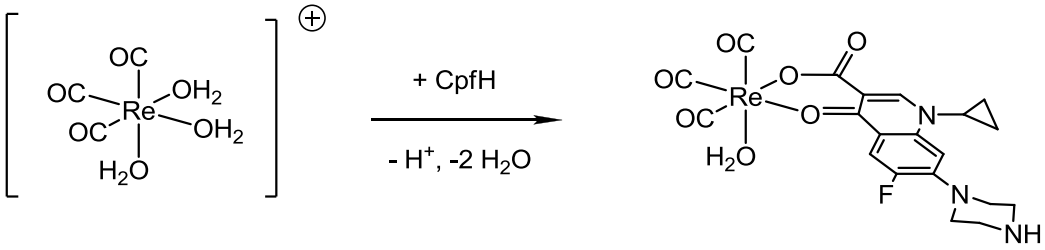


Figure 1

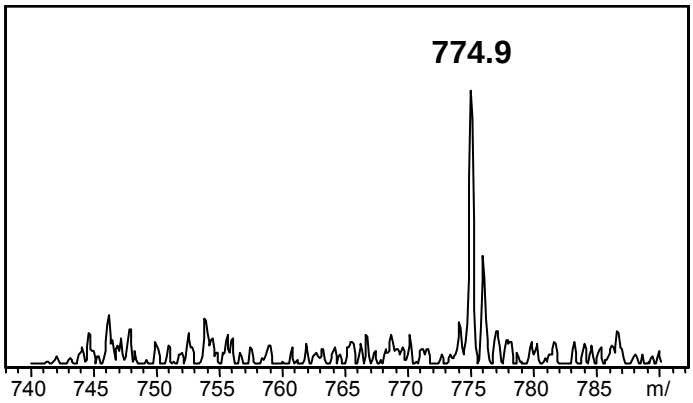


Figure 2

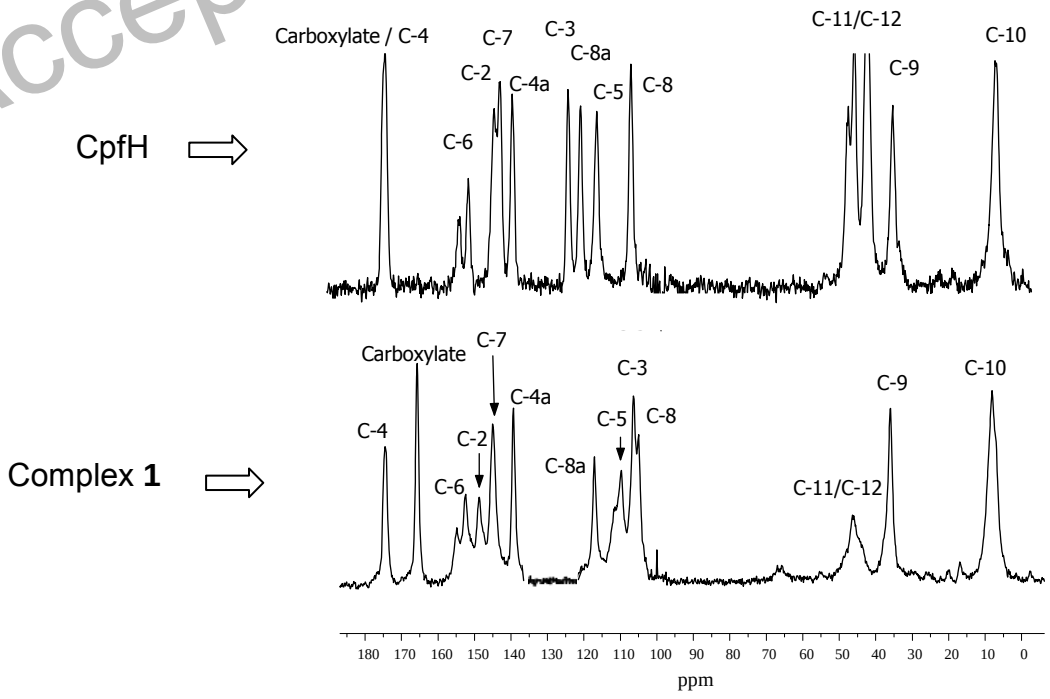


Figure 3

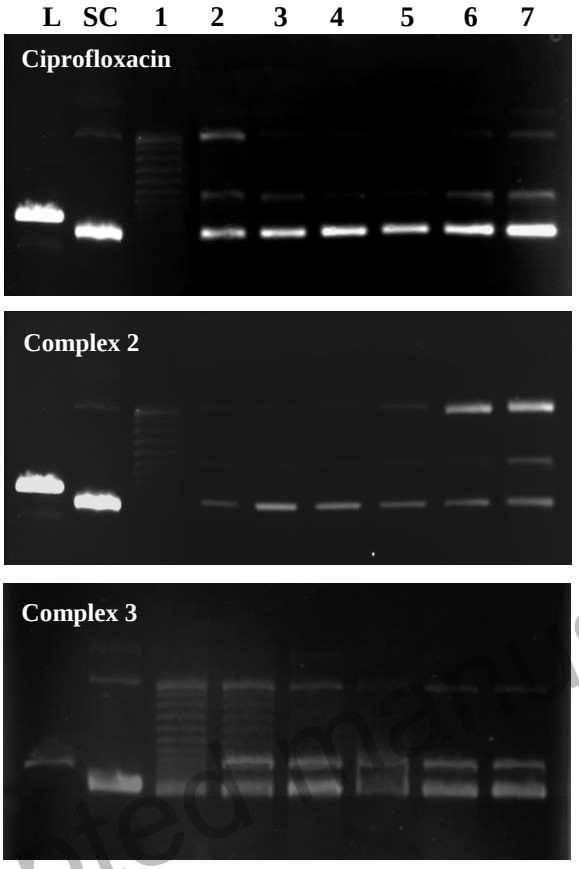


Table 1

Antimicrobial activities for complexes **2** and **3** measured by disk-diffusion method following the Clinical and Laboratory Standards Institute guidelines for ciprofloxacin: +(≤15mm); ++(16-20mm); +++(≥21mm) [49].

	Products			
Microorganisms	**CpfH**	**2**	**3**	**DMSO**
Staphylococcus aureus	+++	+++	++	-
Enterococcus faecalis	+++	+++	++	-
Escherichia coli	+++	+++	+++	-
Pseudomonas aeruginosa	+++	+++	+++	-

FIGURE CAPTIONS

Figure 1: ESI-MS spectrum (positive mode) of a ^{99}Tc-ciprofloxacin aqueous solution

Figure 2: ^{13}C CP-MAS spectra of free ciprofloxacin (top) and complex **1** (bottom).

Figure 3: Agarose gel electrophoresis of pBR322 plasmid DNA showing the effect of ciprofloxacin and metal complexes **2** and **3** on *E. coli* Gyrase cleavage assay. Lane **L**, Linear conformation; Lane **SC**, supercoiled pBR322; Lane **1**, pBR322+Gyrase; Lane **2**, pBR322 + Gyrase + 1 µM tested compound; Lane **3**, pBR322 + Gyrase + 5 µM tested compound; Lane **4**, pBR322 + Gyrase + 10 µM tested compound; Lane **5**, pBR322 + Gyrase + 50 µM tested compound; Lane **6**, pBR322 + Gyrase + 100 µM tested compound; Lane **7**, pBR322 + Gyrase + 250 µM tested compound.

GRAPHICAL ABSTRACT