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NMR investigation of pefloxacin/cation/DNA interactions. Mg^{2+} and Ca^{2+} Binding

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Abstract

Pefloxacin at low concentration (10^{-4} mol l^{-1}) and high $[cation]_0/[quinolone]_0$ ratios forms 1:1 and 1:2 (drug:cation) complexes with magnesium or calcium cation, at pH 7.4. The binding sites are, first, the carbonyl and carboxyl groups, then, the N-4′ piperazinyl atom, as shown by the investigation of the pefloxacin ethyl ester/magnesium interaction. As expected, the apparent affinity constants are larger for magnesium than for calcium. When the pefloxacin concentration increases to 10^{-3} mol l^{-1}, the affinity of this drug for magnesium becomes much larger. That is due to the formation of a 2:2 complex, what enhances the stacking of this fluoroquinolone.

Keywords: Fluoroquinolone; Cation binding; ^{13}C NMR; ^{19}F NMR

1. Introduction

Fluoroquinolones such as pefloxacin are antimicrobial agents with an excellent activity against various bacteria. However, complexation with metal ions such as aluminium, magnesium or calcium, commonly found in antacids and multivitamin preparations, reduce their bioavailability (Ross and Riley, 1993 and references therein). The antibacterial activity of these drugs depends mainly on two factors: their uptake by the cell and their activity against their target enzyme DNA gyrase. The interaction of fluoroquinolones with magnesium reduces, by at least 50%, their accumulation in bacteria such as *Escherichia coli* or *Staphylococcus aureus* (Lecomte et al., 1994). Furthermore, magnesium seems to be involved in the binding of these drugs to DNA (Tornaletti and Pedrini, 1988; Maxwell, 1992; Palu et al., 1992; Bazile-Pham Khac and Moreau, 1994). Therefore, for a better understanding of the different interactions between fluoroquinolones,

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DNA and magnesium, we have investigated them by NMR spectroscopy (Lecomte, 1995).

In a previous paper (Lecomte et al., 1994), we have reported the affinity constants of some fluoroquinolones for magnesium. In this first study, the $[cation]_0/[drug]_0$ ratio was limited to 100. However the magnesium concentration in cells can be up to 0.1 mol l^{-1} (Snavely, 1990). Therefore we have investigated again, by ^{19}F and ^{13}C NMR, the pefloxacin/magnesium interaction for high $[cation]_0/[drug]_0$ ratios and extended this study to the pefloxacin/calcium complexes.

Various stoichiometries for the quinolone/cation complexes have been reported in the literature. Their inconsistency comes partly from the different concentration ranges used. For nalidixic acid/magnesium, Timmers and Sternglanz (1978), and lately Ross and Riley (1993), reported a 1:1 quinolone/cation complex. However, for this same interaction, Cole et al. (1984) proposed both 1:1 and 2:1 stoichiometries. For norfloxacin and lomefloxacin, these same 1:1 and 2:1 stoichiometries were reported by Okabayashi et al. (1992), while Palu et al. (1992) proposed only a 1:1 complex for norfloxacin/magnesium. Finally, Ross and Riley (1993) reported a 2:2 stoichiometry for the magnesium (and calcium) complex of the 7-piperazinylfluoroquinolones involving coordination at the carboxylic acid, the adjacent 4-keto group and the 4′-nitrogen of the piperazine ring, despite the fact that the concentration of these quinolones were in the μmol l^{-1} ranges. For nalidixic acid/calcium, only a 1:1 complex was reported (Timmers and Sternglanz, 1978; Ross and Riley, 1993). Our study aimed to clarify these discrepancies. Two concentrations of pefloxacin were used, 10^{-4} mol l^{-1} (where pefloxacin is monomeric at pH 7.4 (Lecomte et al., 1996)) and 10^{-3} mol l^{-1}, and the $[cation]_0/[drug]_0$ ratios were up to 800.

2. Materials and methods

2.1. Materials

Pefloxacin mesylate and its ethyl ester were a gift from Rhone Poulenc Rorer (Vitry sur Seine, France) (Fig. 1)

Pefloxacin ethyl ester was first dissolved (10^{-2} mol l^{-1}) in ethylene glycol to make it soluble in aqueous solutions. Both compounds were dissolved in buffer containing 2×10^{-2} mol l^{-1} MOPS (pH 7.4) and 2×10^{-2} mol l^{-1} KCl. The aqueous solutions contained 20% v/v D_2O for the field-frequency lock of the NMR spectrometer. Therefore, the pH meter reading was adjusted to 7.32, using μl additions of HCl or KOH solutions, in order to take into account the change in the glass electrode potential induced by D_2O (Glasoe and Long, 1960). All buffer components, magnesium chloride and calcium chloride were purchased from Sigma Chemical Co. All solutions were prepared with deionized distilled water purified by a Milli-Q Water System (Millipore Corp., Bedford, MA).

2.2. NMR studies

The ^{13}C and ^{19}F spectra were recorded on a Bruker AM-300 spectrometer. The digital resolution for these ^{13}C and ^{19}F spectra was 0.012 and 0.001 ppm, respectively. Carbon shifts are referenced to DSS. The fluorine chemical shifts of pefloxacin and its ethyl ester (10^{-4} mol l^{-1} in buffer MOPS, pH 7.4, 283 K) are -48.700 and -47.371 ppm, respectively, from trifluoroacetic acid (external reference). To prevent any saturation of the fluorine signals, the recycle time was adjusted to 1.2 s for a flip angle ca. 52°. In order to slow down the exchange between the several species involved in the interaction between fluoroquinolones and magnesium (or calcium) the temperature was lowered to 283 K.

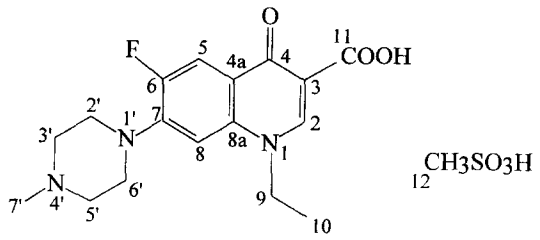

Fig. 1. Structure of pefloxacin mesylate.

3. Results and discussion

3.1. Pefloxacin (10^{-4} mol l^{-1})/cation interaction

In our previous study (Lecomte et al., 1994), the pefloxacin concentration was 10^{-4} mol l^{-1}, and the pH hold at 7.4. At these concentrations and pH, pefloxacin is monomeric (Lecomte et al., 1996). The percentages of the Bjerrum species are anionic 26%, zwitterionic 59%, neutral 4% and cationic 11% (Lecomte et al., 1996), taking into account the 20% v/v of D_2O added to the solution for locking the spectrometer.

For low $[Mg^{2+}]_0/[quinolone]_0$ ratio (< 25), the pefloxacin ^{19}F spectra obtained at 282 MHz and 283 K showed two broad lines assigned to the free and bound species in slow exchange. Their percentages, obtained first by integration, then by bandshape analysis (Stephenson and Binsch, 1978), were consistent with the formation of a 1:1 complex. However, when the $[cation]_0/[drug]_0$ ratio is increased from 100 to 700, the ^{19}F line assigned to the bound species narrows by 20 Hz, and shifts by 0.088 ppm. These variations do not depend on the ionic strength of the solutions since they have been observed whether this ionic strength is hold constant (0.2 mol l^{-1}) or not (maximum 0.35 mol l^{-1}).

These chemical shift and linewidth variations for high magnesium concentrations can be explained by the existence, at $[Mg^{2+}]_0/[drug]_0 = 100$, of several bound forms in fast exchange on the ^{19}F NMR time scale. Increasing the concentration of magnesium could favor the formation of only one complex characterized by a narrow line. This implies two exchange rates for the various species involved in the pefloxacin/magnesium interaction, a slow exchange between the free species and the bound ones, and a fast exchange between the free species on one hand, and the bound species on the other hand.

In order to check this assumption, we have investigated the interaction of pefloxacin with another cation. Calcium was chosen since it is involved, like magnesium, in some antiacids formulations, which reduce the bioavailabilty of fluoroquinolones (Ross and Riley, 1993 and references therein); furthermore, its interaction with

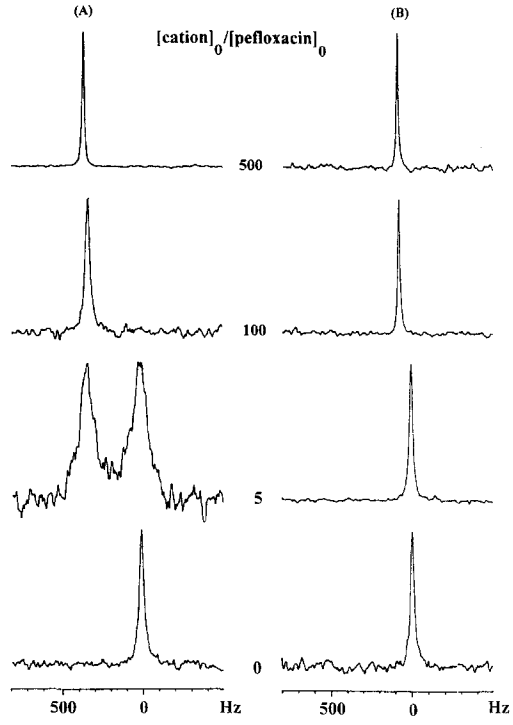

Fig. 2. ^{19}F spectra (282.38 MHz) of pefloxacin (10^{-4} mol l^{-1} in buffer MOPS) for several ratios of cation (A magnesium, B calcium) to drug, at pH 7.4 and temperature 283 K (line broadening 10 Hz).

the quinolones could explain some toxicity problems (Kato et al., 1995; Stahlmann et al., 1995). Finally, since the charge density is smaller for calcium than for magnesium (Demitras et al., 1972), the variations of the ^{19}F chemical shifts between the free and bound species were expected to be smaller, inducing a fast exchange rate between these species whatever the calcium concentration. As a matter of fact, the spectra ^{19}F obtained with calcium are different from those with magnesium, for the same concentration and temperature conditions (Fig. 2).

For calcium, only one line is observed, and its integration corresponds to the total pefloxacin concentration. This indicates that the exchange rates are, as expected, faster when calcium is involved instead of magnesium. When the $[Ca^{2+}]_0/[pefloxacin]_0$ ratio increases from 0 to 700, the ^{19}F chemical shift increases by 0.354 ppm and the

^{19}F linewidth decreases by 13 Hz (Fig. 3). For magnesium, the corresponding data are 1.281 ppm and 15 Hz, respectively. Since the exchange rate between all the free and bound species is fast for the whole calcium concentration range, that allows one to test, more easily than for magnesium solution data, several stoichiometries for the complexes.

The 1:1 stoichiometry which has seemed to fit our data for low $[magnesium]_0/[pefloxacin]_0$ ratios is not valid. As expected from the excess of cation (B) with respect to quinolone (A), the A_2B model did not fit the data, neither the $AB + A_2B$ nor $AB + A_2B_2$ models. In fact, only the $AB + AB_2$ model yields a good fit (Fig. 4) of the observed ^{19}F chemical shifts for the full range of calcium concentration studied (from $0–7 \times 10^{-2}$ mol l^{-1}).

When referenced to the chemical shift of the free species A, the observed fluroquinolone chemical shift $\Delta\delta_{obs}$ is given by:

$$\Delta\delta_{obs} = ([AB]\Delta\delta AB + [AB_2]\Delta\delta AB_2)/[A]_0 \quad (1)$$

$$\Delta\delta_{obs} = (K_1[B]\Delta\delta AB + K_1K_2[B]^2\Delta\delta AB_2) \times /(1 + K_1[B] + K_1K_2[B]^2) \quad (2)$$

with $K_1 = [AB]/[A][B]$ and $K_2 = [AB_2]/[AB][B]$. For calcium solutions, $[B]_0$ is always larger than $[A]_0$ and we have assumed $[B] = [B]_0$. However, this approximation is not valid for all the magnesium solutions and [B] has been calculated by

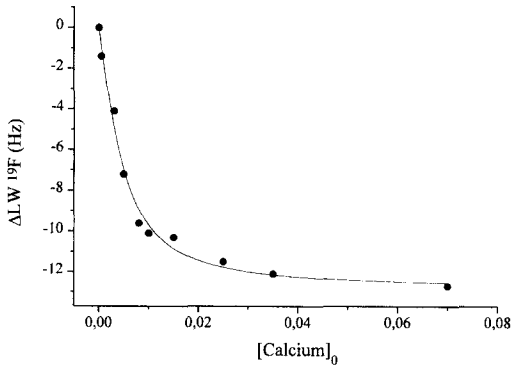

Fig. 3. Variations of the ^{19}F linewidth (282.38 MHz) of pefloxacin (10^{-4} mol l^{-1} in buffer MOPS) vs. calcium concentration at pH 7.4 and temperature 283 K. The linewidth is referenced to its value without calcium.

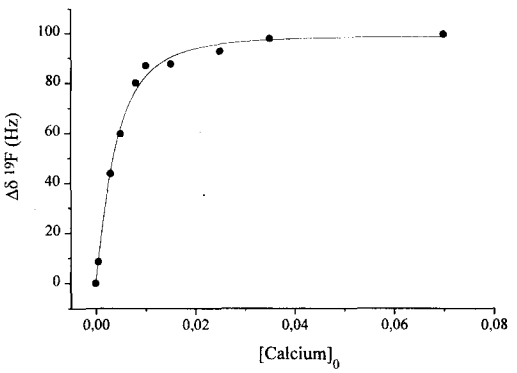

Fig. 4. Variations of the ^{19}F chemical shift (282.38 MHz) of pefloxacin (10^{-4} mol l^{-1} in buffer MOPS) vs. calcium concentration, at pH 7.4 and temperature 283 K. The chemical shift is referenced to its value without calcium. The solid curve represents the best least squares fit using Eq. (2).

$$K_1K_2[B]^3 + (K_1K_2(2[A]_0 - [B]_0) + K_1)[B]^2 + (1 + K_1([A]_0 - [B]_0))[B] - [B]_0 = 0 \quad (3)$$

$\Delta\delta AB$ and $\Delta\delta AB_2$ are the chemical shift of the 1:1 and 1:2 (drug:cation) complexes respectively, referenced to that of the free species A. A least squares fit of the curves $\Delta\delta$obs vs. $[B]_0$ allows to calculate K_1, K_2, $\Delta\delta AB$ and $\Delta\delta AB_2$.

Formation of an AB_2 complex is consistent with the fact that, at pH 7.4, pefloxacin has two protonation sites, the carboxylic and the N-4′ amine functions (Lecomte et al., 1996). Therefore, by analogy with protonation, the second site of binding of magnesium could be N-4′. In fact, Martin (1990) measured affinity constants of magnesium for amines. Furthermore, such a site was also proposed by Ross and Riley (1993) for 7-piperazinylfluoroquinolones for very high $[cation]_0/[drug]_0$ ratios (ca. 1000). In order to confirm without ambiguity this second site, we have studied the interaction of the ethyl ester of pefloxacin with magnesium. For increasing concentrations of magnesium, the ^{19}F chemical shift first clearly decreases, then increases (Fig. 5A). This decrease indicates that the first site of interaction with magnesium is N-4′ since protonation of this same atom induces a negative ^{19}F chemical shift (Lecomte et al., 1996). Afterwards, the formation of an AB_2 complex with both the carbonyl and the hindered carboxyl groups induces a posi-

tive ^{19}F chemical shift which overrides the effect of the first interaction. Cation binding involving first N-4′ then the carbonyl and carboxyl groups is confirmed by the variations of the ^{19}F linewidth vs. magnesium concentration. By analogy with protonation, the characteristic chemical shift of these AB and AB_2 complexes is expected to be small and large, respectively. As a matter of fact, the line broadens only when some AB_2 complexes begin to be formed (Fig. 5B). It is possible to fit the chemical shift variations on the basis of AB + AB_2 interactions. However, the values of the complexation parameters (K_i and $\Delta\delta AB_i$) thus obtained are meaningless since there are not enough complexes formed (Deranleau, 1969), as indicated by the absence of saturation of the curve for the high [magnesium]$_0$/[fluoroquinolone]$_0$ ratios.

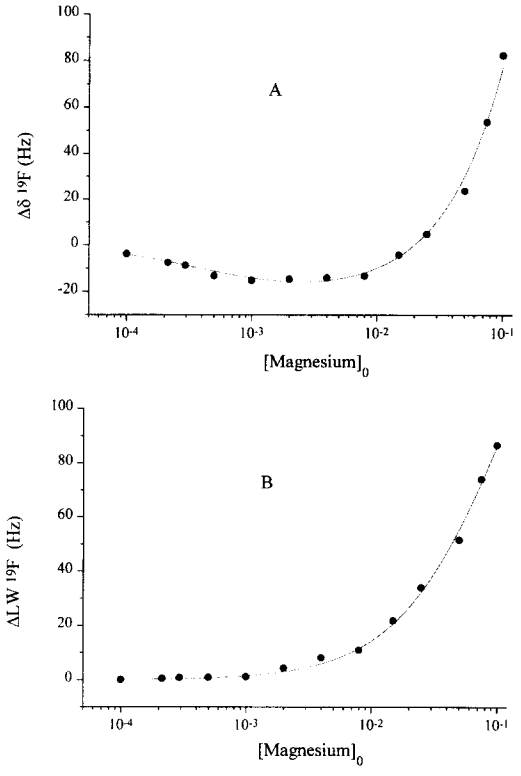

Fig. 5. Variations of the ^{19}F chemical shift (A) and linewidth (B) (282.38 MHz) of pefloxacin ethyl ester (10^{-4} mol l^{-1} in buffer MOPS) vs. magnesium concentration, at pH 7.4 and temperature 283 K. The chemical shift and linewidth are referenced to their values without magnesium.

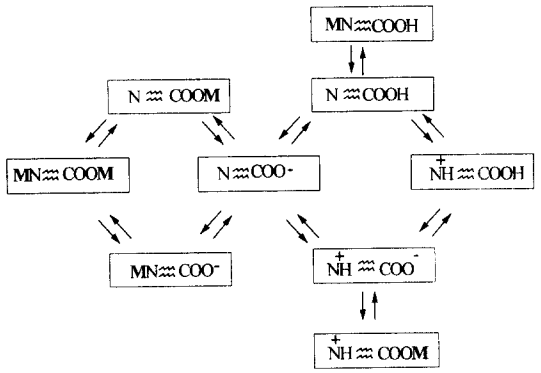

Fig. 6. Scheme of the equilibria involved in the interactions between pefloxacin (N=COOH) and magnesium (or calcium) (M).

It is important to note that this simple AB + AB_2 model yields only *apparent* complexation parameters. At pH 7.4, each Bjerrum species of free pefloxacin (see the above percentages) has its own affinity constants for a given cation. But the interaction equilibria that should be considered (Fig. 6) are too numerous to be taken into account in the fitting of our data, since we need to calculate not only the affinity constants but also the chemical shift characteristic of each complex.

The best fit of the variation of ^{19}F chemical shift vs. calcium total concentration (Fig. 4) is obtained with $K_1 = 180 \pm 20$ mol^{-1} l, $K_2 = 145 \pm 20$ mol^{-1} l, $\Delta\delta AB = 0.39 \pm 0.02$ ppm, and $\Delta\delta AB_2 = 0.350 \pm 0.007$ ppm. The close values for the chemical shift of the complexes explain why only one line is observed for the fluorine spectra whatever the calcium concentration.

The same model has been used to analyze magnesium solution data. Since at low magnesium concentrations the fluorine spectra have two lines (Fig. 2), the percentages of free [A] and bound ([AB] + [AB_2]) species have been determined by band shape analysis. A regression analysis of these percentages, using more data than in our previous paper (Lecomte et al., 1994), yields K_1 and K_2 values, 1600 ± 50 mol^{-1} l and 650 ± 50 mol^{-1} l, respectively. Then these values have been used to calculate the chemical shift of the complexes in the concentration range where the

amount of [A] is negligible, i.e. for $[Mg^{2+}]_0/[pefloxacin]_0 > 100$. These chemical shift values are $\Delta\delta AB = 1.07 \pm 0.05$ ppm, and $\Delta\delta AB_2 = 1.319 \pm 0.005$ ppm.

The $K_1(Mg^{2+})/K_1(Ca^{2+})$ ratio is 8.8. This value is larger than that obtained by Timmers and Sternglanz (1978) for oxolinic acid (7.9) and nalidixic acid (6.3), and by Okabayashi et al. (1992) for norfloxacin (5) and lomefloxacin (5). For both calcium and magnesium, the K_2 affinity constant is smaller that the K_1 constant, which reflects the constraints of binding two cations to the same pefloxacin molecule. The ratio of these constants is larger for calcium (0.8 ± 0.1) than for magnesium (0.41 ± 0.06). On the other hand, the ratio of the chemical shifts of the bound species, $\Delta\delta AB_2/\Delta\delta AB$, is smaller for calcium (0.9) than for magnesium (1.23). These discrepancies can be partially due to the simplicity of the model used. This model, as already emphasized, yields only *apparent* parameter values and does not take into account the several AB complexes (Fig. 6) which can have different percentages for calcium and magnesium. Furthermore, the chemical shifts of the bound species depend on both the electronic and steric effects of each cation; there is no reason that the result of these two effects be similar for calcium and magnesium.

3.2. Pefloxacin (10^{-3} mol l^{-1})/magnesium interaction

The increase of pefloxacin concentration from 10^{-4} to 10^{-3} mol l^{-1} induces large changes in its fluorine spectra obtained at the same pH and temperature (compare Figs. 2 and 7). Only one broad (≈ 350 Hz) line is observed with a shoulder first at high frequencies for low $[Mg^{2+}]_0/[drug]_0$ ratios, then at low frequencies for high $[Mg^{2+}]_0/[drug]_0$ ratios, corresponding to the bound and free species respectively. That can be explained by an exchange rate between these species slightly larger than for pefloxacin solutions ten times more diluted.

Furthermore, it is obvious that the affinity constants are larger. For the ratio $[Mg^{2+}]_0/[pefloxacin]_0 \approx 0.6$, there are approximately 50% of bound species while, for pefloxacin concentration which is ten times smaller, a ratio of 5 is needed to obtain this same percentage. The increase in affinity constants could reflect the formation of a new complex (A_2B_2 type) with magnesium, which will enhance the stacking of pefloxacin evaluated, in absence of magnesium, at only 2% at these 10^{-3} mol l^{-1} concentration and 7.4 pH (Lecomte et al., 1996).

In order to confirm this hypothesis, a 2.67×10^{-2} mol l^{-1} solution of pefloxacin, at pH 12, has been studied by carbon-13 NMR. At this concentration and pH, and without magnesium, the stacking of pefloxacin is ca. 20% (Lecomte et al., 1996). While it is not possible to increase the $[Mg^{2+}]_0/[pefloxacin]_0$ ratio above 0.56 without getting a precipitate, it is possible to assume from the spectra obtained for pefloxacin at 10^{-4} and 10^{-3} mol l^{-1} (Figs. 2 and 7) that, at this 2.67×10^{-2} mol l^{-1} drug concentration and 0.56

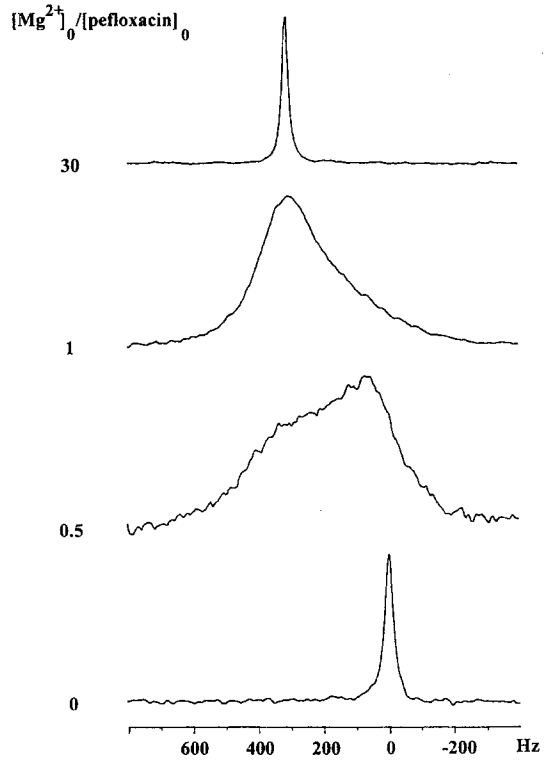

Fig. 7. ^{19}F spectra (282.38 MHz) of pefloxacin (10^{-3} mol l^{-1} in buffer MOPS) for several ratios of magnesium to drug, at pH 7.4 and temperature 283 K (line broadening 10 Hz).

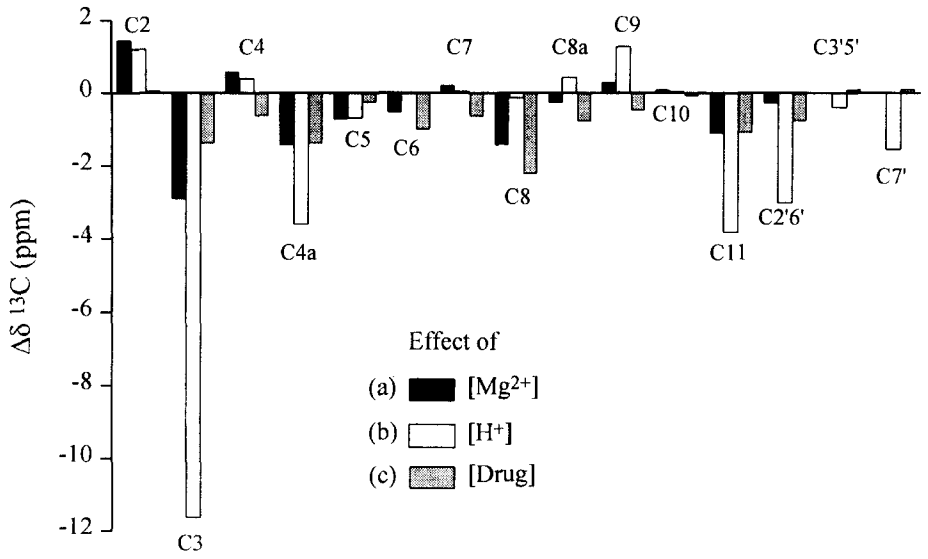

Fig. 8. Variations of ^{13}C chemical shifts vs. the effect of (a) magnesium concentration 1.52×10^{-2} mol l^{-1}, at pefloxacin concentration 2.67×10^{-2} mol l^{-1} and pH 12; (b) protonation (pH 2–13), at pefloxacin concentration $3\ 10^{-2}$ mol l^{-1}, without magnesium; (c) pefloxacin concentration ($0.7–3 \times 10^{-3}$ mol l^{-1}), at pH 13, without magnesium.

$[Mg^{2+}]_0/[drug]_0$ ratio, this quinolone is predominantly complexed.

The formation of an A_2B_2 complex can be inferred from the comparison of the effect of such a complexation with those of protonation of pefloxacin at 3×10^{-2} mol l^{-1}, and stacking (Fig. 8). The stacking effect induced by magnesium binding is clearly confirmed by the variations of C-6, C-8a, and particularly C-8, three carbons that have their chemical shift sensitive mainly to stacking and not to protonation. Furthermore, adding magnesium induces significant linewidth broadenings, the largest (10 Hz) being observed for C-3, some noticeable ones (5–4 Hz) for C-2, C-5, and C-8, and some smaller perturbations (3–2 Hz) for C-4, C-4a, C-11 and C-3′,5′. Their similarity with the line broadenings observed for the concentration effect of pefloxacin at pH 2 (pH where this stacking is maximum) indicates that they reflect the increase of the rotational correlation time of the compound due to the stacking rather than an exchange effect. However, this last effect is probably the major factor for C-3 line broadening. Its large chemical shift variation (-11.60 ppm) under protonation suggests that a significant decrease also happens in the complexation of pefloxacin with magnesium.

By infrared spectroscopy (Lecomte et al., 1994), we have observed that, at low $[Mg^{2+}]_0/[pefloxacin]_0$ ratio, magnesium is localized between the carbonyl and carboxyl groups. Therefore, it is not surprising that the C-3, C-4a and C-11 chemical shifts, which are very sensitive to protonation, are also the ones which are the more sensitive to complexation with magnesium. This complexation effect is smaller than the protonation one since binding of magnesium affects the electronic structure of the ring less than protonation that induces an intramolecular bonding C-11(O)OH–OC-4.

The variations observed for the chemical shifts of the piperazinyl carbons when magnesium is added confirm the formation of an A_2B_2 complex with N-4′ as second site of binding of magnesium since they are consistent with both a complexation effect smaller, as expected, than the protonation effect, and a stacking effect (Fig. 8).

4. Conclusion

Pefloxacin at low concentration (10^{-4} mol l^{-1}) and high $[cation]_0/[quinolone]_0$ ratios forms 1:1 and 1:2 (drug:cation) complexes with magnesium or calcium cation at pH 7.4. The binding sites are, first, the carbonyl and carboxyl groups, then, the N-4′ piperazinyl atom as shown by the investigation of the interaction between magnesium and pefloxacin ethyl ester. As expected, the affinity constants are larger for magnesium than for calcium.

When the pefloxacin concentration increases to 10^{-3} mol l^{-1}, the affinity of this drug for magnesium becomes much larger. That is due to the formation of a 2:2 complex that enhances the stacking of this fluoroquinolone.

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