Interaction Between Ciprofloxacin and Metal Cations: Its Influence on Physicochemical Characteristics and Antibacterial Activity

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INTRODUCTION

Interactions between metal cations and antimicrobial agents have been the focus of many previous studies (1–5). Metal cations were shown to affect the activity of tetracyclines, penicillins, macrolides, aminoglycosides, isoniazid and streptomycin (3). Many of these interactions can be accounted for by the formation of metal-antibiotic chelates. Recently, dramatic decreases in the oral bioavailability of quinolone antibiotics have been reported following concomitant administration of antacids containing aluminum, magnesium and calcium, and mineral supplements such as, iron and zinc (6–13). Formation of metal-quinolone chelates has been speculated to be the cause for this interaction ever since it was first reported. From a physicochemical perspective, metal chelate formation can potentially affect the antimicrobial activity of the quinolone antibiotics. However, information regarding this issue remains scanty in the literature.

Experiments here were conducted to examine possible alterations in the antimicrobial activity of ciprofloxacin at increasing concentrations of six different metal cations using standard strains of *Escherichia coli* and *Pseudomonas aeruginosa*. To better comprehend such interaction and its impacts on antimicrobial activity, studies were conducted to assess the potential effects of metal chelate formation on the physicochemical characteristics of ciprofloxacin. Specifically, attempts were made to define the stoichiometry of the Al^{3+}-ciprofloxacin chelate and to identify, by using data from both the literature and present study, if there is an association between the cation induced changes in ciprofloxacin's activity and the chelation potentials of different cations to various quinolone antibiotics.

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MATERIALS AND METHODS

Chemicals and solvents. Ciprofloxacin hydrochloride was a gift from Miles Pharmaceuticals, New Haven, CT. Unless otherwise specified, analytical grade inorganic salts of $CaCl_2$, $MgSO_4$, $ZnSO_4$, $MnCl_2$, $FeCl_3$, and $AlCl_3$ (J. T. Baker Chemicals Co., Phillipsburg, NJ) were used. These salts were soluble in water at all concentrations tested. Distilled and deionized water was used throughout the experiments. Two buffers, sodium acetate-acetic acid (0.1 m, pH 4.65) and morpholinopropane-sulfonic acid-KOH (0.1 M, pH 7.00), each in the presence of 0.1 M KCl, were employed.

Instruments. A variable wavelength spectrophotometer (Model 1201; Milton Roy Co., Rochester, NY) was utilized for measurements of light absorption either at fixed or variable wavelengths.

Microorganisms and culture medium. E. coli ATCC 25922 and *P. aeruginosa* ATCC 27853 (Difco Lab., Detroit, MI) were the test organisms employed in the study. Mueller Hinton broth (MHB) was procured from Difco Labs., Detroit, MI. The culture medium was prepared as suggested by the manufacturer and sterilized by autoclaving before use.

Checkerboard technique. The MICs of ciprofloxacin in MHB, either alone or in combination with various cations, were measured. The MIC is defined as the lowest antibiotic concentration to inhibit visible bacterial growth after overnight incubation at 35°C. A two-way full checkerboard design was achieved in duplicate on a 96 well microtiter plate using a two-fold dilution scheme by the Dynatech-2000 microdilutor (Dynatech Lab. Alexandria, VA). The starting concentration of ciprofloxacin was set at approximately 32–64 times the respective MIC of the test organisms. The concentration range for the six cations studied was from 7.8×10^{-4} to 0.1 M. The test organisms were inoculated onto the microtiter plate to yield an initial inoculum of $\sim 5 \times 10^5$ cfu/ml in each well. After 18 hr of incubation at 35°C, MIC measurements and growth pattern on each microtiter plate were visually determined and recorded.

Brief antibiotic exposure study. An overnight culture of *E. coli* was allowed to grow to the logarithmic phase in fresh MHB for 2–3 hrs. The actively growing culture was visually adjusted to achieve a 0.5 MacFarland standard. To initiate the time-kill studies, 0.1 ml of the adjusted culture was placed into 9.9 ml of MHB containing ciprofloxacin, Mg^{2+} or both in combination, to obtain an initial inoculum at a density of $\sim 10^6$ cfu/ml. For the antibiotic treated cultures, each contained ciprofloxacin at 3.125×10^{-2} μg/ml ($\sim 1 \times 10^{-7}$ M), with progressively increasing Mg^{2+} supplementation from 0 to 50, 250, and 500 mg per liter or from 0 to 2×10^{-3}, 1×10^{-2}, and 2×10^{-2} M, respectively. Two separate ciprofloxacin free controls in MHB were employed; one supplemented with 2×10^{-2} M of Mg^{2+} and the other with no Mg^{2+}. The cultures were maintained at 35°C and samples were taken at time zero and then hourly for a period of two hours for colony count assay via a pour plate technique.

Determination of stoichiometry for the Al^{3+}-ciprofloxacin chelate. Job's methods of continuous variation was employed; application of the method has been previously described (14). Briefly, ciprofloxacin and Al^{3+} cation at different molar concentrations were mixed such that the sum of

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the relative parts of the two components was equal to 1. The relative part of each component was increased or decreased at an increment of 0.0833 units. The total concentration of the two species was maintained at 2×10^{-5} M. For all combinations, absorbances were measured at the λ_{max} for ciprofloxacin or 276 nm. Absorbance measurements were also made for solutions at the respective ciprofloxacin concentrations containing no Al^{3+} cations. Differences between the two absorbance measurements, i.e., with or without Al^{3+}, were plotted against the relative parts of ciprofloxacin to Al^{3+} for all combinations. The stoichiometry of the chelate should theoretically be represented by the ratio of the relative parts for the two components which yielded the maximal difference in absorbance. For verification purpose, the same experiment was performed at two pHs, i.e., 4.65 and 7.00.

RESULTS

The MICs of ciprofloxacin against *E. coli* and *P. aeruginosa* in MHB were 0.0156 and 0.5 μg/ml, respectively. The degrees of antagonistic activity exhibited by the various cations against ciprofloxacin are shown in Table I for the two bacterial species. In general, the MIC of ciprofloxacin was higher (expressed as multiples of MIC for ciprofloxacin alone) as the cation concentrations were increased. For the two bacterial species, the order of antagonism demonstrated by the six cations was similar, i.e., $Al^{3+} > Fe^{3+} > Mn^{2+} > Zn^{2+} > Mg^{2+} > Ca^{2+}$ (the order for Zn^{2+} only pertained to *P. aeruginosa*). The above conclusion was based on the extent of shift in MIC at comparable cation concentrations. Interestingly, all cations except Mg^{2+} and Ca^{2+} also possessed inhibitory activity as reflected by the lack of bacterial growth at higher cation concentrations (Table I). Assessed from the lowest concentration for individual cations to produce an inhibitory effect against the test microorganisms in Table I, the following order was demonstrated for *E. coli;* $Fe^{3+} \approx Mn^{2+} < Al^{3+} < Zn^{2+}$. A somewhat different order was obtained for *P. aeruginosa;* $Zn^{2+} < Al^{3+} \approx Mn^{2+} \approx Fe^{3+}$.

Time-kill data for *E. coli* obtained after brief ciprofloxacin exposure at 2 × MIC showed a reduction in the bactericidal effect with increasing of Mg^{2+} supplementation (Fig. 1). Partial to almost complete reversal of bactericidal activity was observed at 2×10^{-3} and 1×10^{-2} M of Mg^{2+}. At the highest level of Mg^{2+} supplementation, i.e., 2×10^{-2} M, the bactericidal effect of ciprofloxacin was totally negated. For antibiotic free MHB in the presence of only Mg^{2+}, normal bacterial growth was evident at a Mg^{2+} concentration up to 2×10^{-2} M. However, based on the data obtained from the checkerboard experiments, bacterial growth could be maintained at Mg^{2+} concentration as high as 0.1 M for both bacterial species (Table I).

Data obtained from the two continuous variation experiments are shown in Fig. 2. At both pHs 4.65 and 7, a peak was identified with the relative part of ciprofloxacin to Al^{3+} at 0.5, i.e., equal portions of Al^{3+} and ciprofloxacin in combination. The absorbance differences measured at the lower pH were lower across all ratios.

DISCUSSION

It has been proposed that the carbonyl group and the adjacent ionized carboxylic acid functional group is the site for metal chelation on the ciprofloxacin molecule (Fig. 3). Estimates of apparent dissociation constants obtained in previous studies (15,16), between various metal cations and nalidixic acid and three other quinolone antibiotics (structurally similar to ciprofloxacin), have shown the following order of decreasing chelation potentials; $Cu^{2+} > Co^{2+} > Ni^{2+} > Zn^{2+} > Mg^{2+} > Ca^{2+} > Ba^{2+}$. Furthermore, two trivalent cations, Al^{3+} and Fe^{3+}, have been shown to possess the strongest chelation potentials (16). The present study revealed that the degrees of antagonism against ciprofloxacin by the various cations are dependent on the individual cations and their concentrations. Assuming that metal chelate formation is the direct cause for the observed antagonistic activity, metal cations that possess stronger affinity for ciprofloxacin should theoretically cause more reduction in antimicrobial activity. Indeed, for both bacterial species, the order of the antagonism exhibited by the six cations (Table I) corresponds nicely to the chelation potentials reported earlier for the different quinolone antibiotics (15,16).

Despite the fact that Al^{3+} is a trivalent cation, experiments employing the method of continuous variation showed that the typical stoichiometry for the Al^{3+}-ciprofloxacin chelate is 1:1. This conclusion is reinforced by the consistent findings that, at both pH 4.65 and 7.00, a peak absorbance differences was observed at equal relative parts of ciprofloxacin and Al^{3+} (Fig. 2). Steric hinderance by the first ciprofloxacin molecule may significantly affect chelation of subsequent molecules. Concurrent with the above interpretation, a 1:1 stoichiometry has also been reported for the metal

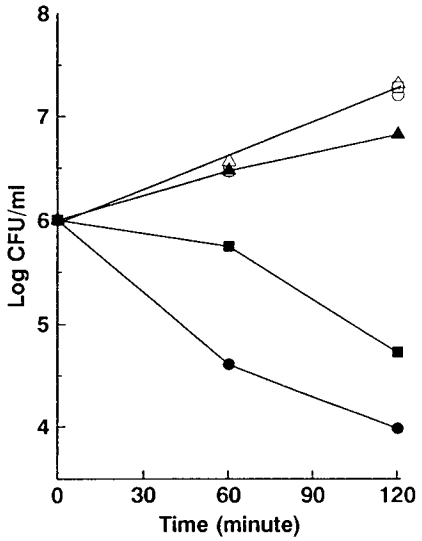

Fig. 1. Brief antibiotic exposure studies on ciprofloxacin at a concentration of 3.125×10^{-2} μg/ml (~1×10^{-7} M or 2 × MIC) against *E. coli* ATCC 25922 under the influence of increasing Mg^{2+} cation supplementation. (●) no Mg^{2+} added, (■) 2×10^{-3} M, (▲) 1×10^{-2} M and (○) 2×10^{-2} M. Data obtained at the highest level of Mg^{2+} supplementation in combination with ciprofloxacin (○) and the two controls without ciprofloxacin (in the presence (△) and absence (□) of 2×10^{-2} M of Mg^{2+} supplementation) essentially coincided with one another as demonstrated by the uppermost growth curve. Note: the usual level of Mg^{2+} supplementation in MHB for susceptibility testing is equivalent to ~0.5×10^{-3} M.

Table I. Changes in the MIC of ciprofloxacin in MHB against *E. coli* and *P. aeruginosa* with presence of various metal cations at different concentrations; metal cations are listed in the order of decreasing antagonistic activity.

Cation ($\times 10^{-4}$ M)	*E. coli* ATCC 25922						*P. aeruginosa* ATCC 27853					
	Al^{3+}	Fe^{3+}	Mn^{2+}	Zn^{2+}	Mg^{2+}	Ca^{2+}	Al^{3+}	Fe^{3+}	Mn^{2+}	Zn^{2+}	Mg^{2+}	Ca^{2+}
0	1x[a]	1x	1x	1x	1x	1x	1x	1x	1x	1x	1x	1x
7.8	4x	2x	2x	2x	1x	1x	1x	1x	1x	1x	1x	1x
15.6	8x	4x	2x	—	1x	1x	2x	1x	1x	1x	1x	1x
31.3	32x	32x	2x	—	2x	1x	8x	4x	2x	1x	1x	1x
62.5	—[b]	>64x	4x	—	4x	1x	—	—	—	2x	2x	1x
125	—	—	—	—	8x	2x	—	—	—	—	4x	2x
250	—	—	—	—	8x	4x	—	—	—	—	8x	2x
500	—	—	—	—	16x	8x	—	—	—	—	8x	4x
1000	—	—	—	—	32x	8x	—	—	—	—	16x	4x

[a]numbers are expressed as multiples of MIC; MIC for *E. coli* and *P. aeruginosa* are 0.0156 and 0.5 μg/ml, respectively.
[b]MIC could not be determined because of inhibitory activity produced by the metal cations.

chelates of nalidixic and oxolinic acids (15,17) including cations with lower chelation potentials than Al^{3+} (16).

The continuous variation experiments showed a reduction in the absorbance differences for all combinations at a lower pH (Fig. 2). In other words, the chelation potential of Al^{3+} for ciprofloxacin is lower at a higher H^+ concentration. This observation may reflect a reduction in the availability of chelation sites as the H^+ concentration increases. Such interpretation is in good agreement with the hypothesis that the negatively charged carboxylic group of the ciprofloxacin molecule, in conjunction with the carbonyl group, is the site for chelation (Fig. 3). Furthermore, an increase in the charge state of the ciprofloxacin molecule is to be anticipated following metal chelate formation; this is especially true for cations with a valency equal or higher than two. The increase in charge state should potentially translate to a higher polarity and dipole moment for the ciprofloxacin molecule. In fact, preliminary studies (data not shown) conducted in a 0.1 M acetate buffer (pH 4.65)/carbon tetrachloride system using UV absorption at the λ_{max} of 276 nm demonstrated an increase in the aqueous partitioning of ciprofloxacin at increasing aqueous concentrations of Al^{3+} or Mg^{2+}. Up to a 100- and 4-fold increase in the aqueous partitioning was recorded for Al^{3+} and Mg^{2+}, respectively. With the pK_a of the carboxylic functional group of ciprofloxacin at about 6 (18), the higher aqueous partitioning, when most of molecules exist in the unionized form (>90%) at pH 4.65, suggests a stronger affinity of Al^{3+} and Mg^{2+} for the ciprofloxacin molecule than its tendency to remain in the organic phase.

Several reasons may contribute to the cation induced reduction of ciprofloxacin's antimicrobial activity, namely; 1. a decrease in uptake of the antibiotic by the bacterial cells, 2. the metal chelates formed are not pharmacologically active, 3. alteration of bacterial cell physiology or induction of resistance by the metal cations at low concentrations. Although the present data do not allow exclusion of any of the three possibilities, recent studies have reported a decline in bacterial uptake of ciprofloxacin and fleroxacin in the presence of Mg^{2+} (19,20). The good correlation observed between the cation chelation potentials and the reduction in antimicrobial activity indicates that the formation of a metal-ciprofloxacin chelate is a probable cause for the antagonism.

In conclusion, the physicochemical characteristics of ciprofloxacin undergo some important changes following metal chelate formation. The formation of metal chelates may help explain the decrease in antimicrobial activity of

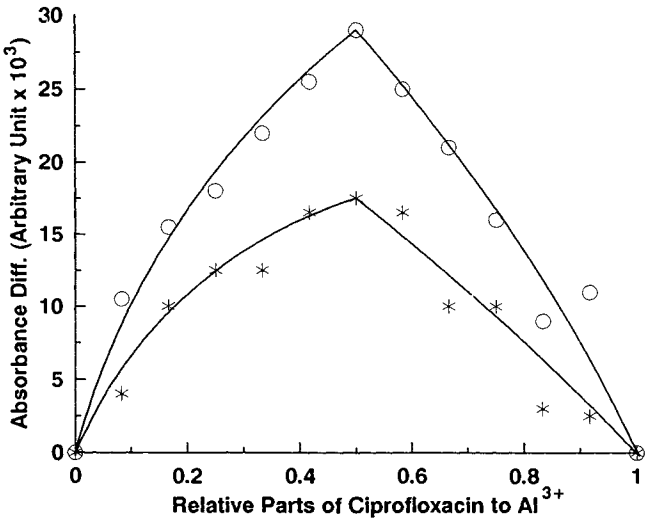

Fig. 2. The plot for the absorbance differences against the relative parts of ciprofloxacin to Al^{3+} in two different buffer systems according to the Job's method of continuous variation. Data obtained at pH 7 (○) and pH 4.65 (*) are represented by the upper and lower curves, respectively.

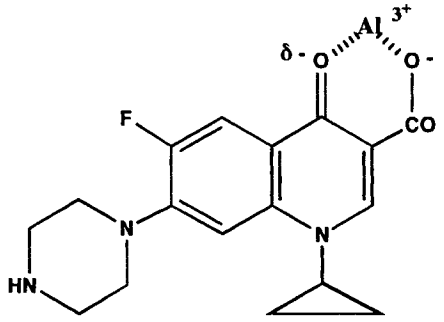

Fig. 3. The proposed molecular structure for the Al^{3+}-ciprofloxacin chelate.

ciprofloxacin in the presence of various cations. Under normal physiological conditions, presence of the two major cations, Mg^{2+} and Ca^{2+}, at the respective nominal concentrations of 1×10^{-3} and 3×10^{-3} M in serum and 2.5×10^{-3} and 5×10^{-3} M in urine (21), should have minimal effects on the antibacterial activity of ciprofloxacin (see Table I); however, the observed antagonism may be clinically relevant, especially for the treatment of urinary tract infections, when urinary levels of certain cations are elevated by regular ingestion of mineral supplements or antacids. Further studies on the pharmacological impacts of this interaction should be warranted.

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