Mechanistic Investigation of the Reduction in Antimicrobial Activity of Ciprofloxacin by Metal Cations

Henry H. M. Ma,[1] Francis C. K. Chiu,[1] and Ronald C. Li[1,2]

Received October 8, 1996; accepted December 24, 1996

KEY WORDS: ciprofloxacin; chelate; metal cation; cellular uptake; time-kill study.

INTRODUCTION

In the presence of metal cations, the quinolone class of antibiotics is known to exhibit extensive bioavailability problem (1–5). This drug-drug interaction is believed to be induced by the formation of antibiotic-metal chelates. Because of this, concurrent administration of cation-containing agents such as sucralfate, antacids and mineral supplements is not advisable for patients on oral quinolone therapy. If both therapies are necessary, it is recommended that adequate time be spaced between the two agents. From a pharmacokinetic point of view, such interaction leads to reduced quinolone absorption; however, only a few studies have been conducted to examine how this interaction affects the antimicrobial activity of quinolone antibiotics (6–8).

In our previous study (8), a number of cations was found to cause a significant reduction in the antimicrobial activity of ciprofloxacin. Since the formation of metal chelates is believed to be the primary reason, a close association between the degrees of activity reduction and the dissociation constants (K_D) of various ciprofloxacin-metal chelates should exist. To better understand the impact of metal chelate formation on the antimicrobial activity of ciprofloxacin, it is, therefore, essential that the K_D values for different ciprofloxacin-metal chelates are simultaneously assessed in conjunction with the alterations in activity.

Pharmacologically, there are two possible mechanisms that would explain the reduction in ciprofloxacin's activity by metal cations. One possible reason is a decrease in permeation of the antibiotic into bacterial cells. This is because, in comparison to the parent compound, a higher ionic charge state, polarity, and hydrophilicity of the metal chelates are anticipated. This is especially true when the stoichiometry of the quinolone-metal chelates is known to be 1:1 (8–10) and the chelating cations are multivalent. For example, the formation of Al^{3+}-ciprofloxacin chelate renders a gain of two cationic charges above neutrality for the ciprofloxacin molecule. Indeed, these physicochemical changes imposed on the molecule may hinder permeation of the antibiotic across the lipophilic bacterial cell membrane. The second possible reason is the formation of a chelate that may lack of any antibacterial activity, i.e., an inactive chelate. Hence, without performing an antibiotic uptake study, conventional methods of susceptibility testing and bacterial kinetic studies cannot differentiate the two possibilities.

In this report, we describe the determination of various K_D values of ciprofloxacin with Al^{3+}, Mn^{2+}, Zn^{2+}, Fe^{3+} and Mg^{2+} using fluorescence spectroscopy. In parallel with these K_D measurements, changes in the antibacterial activity of ciprofloxacin in the presence of Al^{3+}, Mn^{2+}, and Mg^{2+} were assessed and quantitated via time-kill studies using *Escherichia coli* as the test organism. Association between the two study variables, i.e., bactericidal activity and K_D, was examined. Furthermore, bacterial uptake of ciprofloxacin in the absence and presence of the three corresponding cations was investigated. The relationship between extent of cellular ciprofloxacin uptake and shifts in antimicrobial activity was also studied.

MATERIALS AND METHODS

Chemicals and Solvents

Ciprofloxacin hydrochloride was a gift from Bayer AG, Germany. Analytical grade $Al_2(SO_4)_3 \cdot 18H_2O$, $MnCl_2 \cdot 4H_2O$, $MgCl_2 \cdot 6H_2O$, $Fe(NO_3)_3 \cdot 9H_2O$, $MnCl_2 \cdot 4H_2O$, and NaCl were purchased from BDH, UK. Normal saline (0.9% w/v of NaCl) was employed as a physiological diluent for bacterial cultures. Distilled and deionized water was used for the preparation of various salt solutions and culture media throughout the experiments.

Apparent Dissociation Constant (K_D) Measurements for Ciprofloxacin-Metal Chelates

Measurements of K_D with respect to ciprofloxacin and the five different cations, i.e., Mg^{2+}, Mn^{2+}, Zn^{2+}, Fe^{3+}, and Al^{3+}, were performed using fluorescence detection. Different methods of detection including ultraviolet absorption and potentiometric titration have also been employed for other quinolones (9–11). Fluorescence measurements were recorded using a Hitachi F-2000 fluorescence spectrophotometer (Hitachi, Japan). Stock solutions of ciprofloxacin (1.5 mM) and various metal cations (1 M) were prepared in 200 mM triethylamine-acetic acid buffer at a pH of 4.5 and were kept at 4°C. The working solutions were freshly prepared by dilution before use. Spectral shifts induced by each metal cation on the fluorescence emission of ciprofloxacin was first studied in the presence of excessive amount of that particular cation for the determination of emission maximum of the metal chelate. For the K_D measurements, metal cation titration experiments were carried out. An aliquot (1 ml, 20 mM) of ciprofloxacin solution was mixed with 0.1 ml of metal cation solution at increasing concentrations. Concentration ranges employed for the individual cations studied were dependent on their respective affinities to the antibiotic. After equilibration for 90 seconds, the fluorescence intensity of the mixture was measured at the emission maximum for that metal chelate. A series of measurements was taken until the change in fluorescence intensity leveled off, i.e. 100% chelation, at high cation concentrations. The principle of K_D determination employed in this study was analogous to the conventional assessment of pK_a value by acid or base titration; likewise,

[1] Department of Pharmacy, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, Hong Kong.

[2] To whom correspondence should be addressed. (e-mail:ronli@cuhk.edu.hk)

0724-8741/97/0300-0366$12.50/0

plotting of the resultant fluorescence intensity measurements against the logarithm of cation concentrations would yield a curve which is sigmoidal in shape. The fluorescence of the Al^{3+}-ciprofloxacin chelate was monitored over the concentration range of 0.0018 to 0.909 mM at the excitation and emission wavelengths of 271 nm and 432 nm, respectively. Similarly, Fe^{3+}, Zn^{2+}, Mn^{2+}, and Mg^{2+} chelates were studied over 0.0045 to 0.36 mM at 278 nm and 444 nm, 0.909 to 45.5 mM at 271 nm and 428 nm, 0.909 to 81.8 mM at 271 nm and 444 nm, and 12.7 to 181.8 mM at 271 nm and 438 nm, respectively.

The plateau observed following the initial increase in fluorescence intensity was suggestive of 100% formation of the 1:1 metal-ciprofloxacin chelates. The fraction of ciprofloxacin chelated (F_b) at each cation concentration was calculated as the difference between the fluorescence intensity at that concentration (I_c) and at zero cation concentration (I_0) relative to the maximum intensity difference, i.e. maximum fluorescence intensity (I_{100}) minus I_0. The equation describing F_b can be written as $F_b = (I_c - I_0)/(I_{100} - I_0)$. Therefore, the fraction of free (non-chelated) ciprofloxacin (F_f) could be computed as $F_f = 1 - F_b$. The ratio of F_f/F_b at each cation concentration was then calculated. Values of log (molar cation concentration) were plotted against log (F_f/F_b). Data obtained at the middle linear portion of the double log plot were analyzed further by linear regression to determine the y-intercept, i.e., log K_D (12). This mathematical operation allowed linearization of the sigmoidal cation titration curve and thus simple K_D estimation for individual metal-ciprofloxacin chelates.

Organism and Culture Media

Escherichia coli ATCC 25922 (Difco Lab., Detroit, MI) was employed as the test organism. Mueller Hinton Broth (MHB) and nutrient agar (Difco Lab., Detroit, MI) were used as the liquid and solid media in the study. Both culture media were prepared as suggested by the manufacturer and sterilized by autoclaving before use. Following initial isolation, bacteria from a single colony were maintained on agar slants and were stored at 4°C. The MIC of ciprofloxacin for the test organism was determined per macrodilution method (13).

Preparation of Bacterial Culture

Prior to the short-term time-kill and ciprofloxacin uptake studies, an overnight culture of *E. coli* was allowed to grow to the logarithmic phase in fresh MHB for 2–3 hours. The actively growing culture was visually adjusted to achieve a 0.5 MacFarland standard. This adjusted culture containing a bacterial density of 10^8 organisms (CFU)/ml was employed as the stock culture for both studies described below.

Short-Term Time-Kill Study Following Antibiotic Exposure

To initiate the time-kill studies, 0.1 ml of the adjusted culture was added to 9.9 ml of MHB containing either ciprofloxacin, metal cations, or both in combination, to yield an initial inoculum of approximately 10^6 organisms/ml. The concentration of ciprofloxacin employed was maintained at 2 × MIC, i.e., 3.125×10^{-2} μg/ml or 1×10^{-7} M. The concentrations of metal cations tested in combination with the antibiotic were 0, 0.1, 0.25, 0.5, 0.75, 1.0, and 2.0 mM for Al^{3+}, 0, 0.5, 1.0, 2.0, 3.0, and 6.0 mM for Mn^{2+}, and 0, 1.0, 1.5, 2.0, 5.0, 10.0, and 20.0 mM for Mg^{2+}, respectively. In addition to the inclusion of an antibiotic- and cation-free control, controls employing cation only were also utilized to account for any possible antibacterial effects exhibited by the cations. For the cation only controls, cation concentrations employed were 0, 2.0 mM for Al^{3+}, 0, 0.5, 1.0, 2.0, 3.0, and 6.0 mM for Mn^{2+}, and 0, 20.0 mM for Mg^{2+}, respectively. All cultures were maintained at 37°C. Samples were withdrawn at 15 min intervals for a period up to 2 hrs and, after dilution with normal saline, were submitted to the pour plate and colony count assay using 10 ml of molten agar maintained at 45–50°C.

Under each condition tested, the initial bactericidal rate constant was determined from the bacterial count data collected in the log-linear bactericidal phase using the equation, $K' = K - K_{app}$ (14,15), where K is the rate constant describing the growth of the control culture without any cation or antibiotic treatment. At each cation concentration and in the presence of ciprofloxacin, K′ is the initial bactericidal rate constant and K_{app} is the rate constant describing the apparent rate of decline in bacterial counts during the log-linear bactericidal phase which was determined by linear least square regression analysis. In the case when the cation under studied was determined to possess bactericidal effect, such additional effect at the cation concentration tested was subtracted from the total effect measured for the antibiotic-cation combination at the same cation concentration. To generate a concentration-response curve, the K′ estimates were plotted against the log cation concentrations studied. For the purpose of graphic depiction, individual curves were generated by an exponential smoothing function which is built-in to the Lotus Freelance graphics program, ver. 4.0. From these individual curves, the strength of various cations to reduce the antimicrobial activity of ciprofloxacin was expressed as their respective concentrations causing a 50% reduction of the bactericidal effect (IC_{50}) measured at 2 × MIC of ciprofloxacin. The IC_{50} estimates for the three cations tested were interpolated directly from the corresponding concentration-response curves.

Ciprofloxacin Uptake Studies

To assess the effect of various metal cations on the total uptake of ciprofloxacin by bacterial cells, a 1 ml aliquot of the adjusted culture was mixed with 0.9% NaCl solution containing the cation to yield a final volume of 10 ml. Ciprofloxacin concentration was maintained at 3.125×10^{-2} μg/ml (1×10^{-7} M) in these studies and the resultant concentrations of Al^{3+}, Mn^{2+}, and Mg^{2+} were chosen to be 0.427 mM, 1.4 mM, and 4.39 mM, respectively. These concentrations corresponded to the IC_{50} values of individual metal cations and were chosen for their equivalent effect to reduce the bactericidal activity of ciprofloxacin. Two controls were employed for each of the cation tested; one was free of ciprofloxacin and cations and the second carried ciprofloxacin with no cations. The first control would determine if there were any substances from within the bacterial cells and/or the diluted culture medium in normal saline that could interfere with the HPLC assay (described below) and the second control would serve as a reference to the bacterial cell preparation containing both the antibiotic and the cation. The bacteria were then incubated at 37°C for 15 minutes and were subsequently centrifuged at 4500 rpm for 5

minutes. The cell pellets were washed twice with 10 ml of 0.9% NaCl solution, and lysed by sonication in 200 μl of HPLC mobile phase for 10 minutes. The cell debris was removed again by centrifugation, and 100 μl of the supernatant was analyzed by the HPLC assay.

Due to the high affinity of ciprofloxacin for Al^{3+}, ciprofloxacin permeation might be influenced by the presence of intracellular Al^{3+}. To investigate any possible 'siphoning' effect caused by intracellular Al^{3+}, additional uptake experiments were performed with bacterial cells pre-incubated in the diluted medium with Al^{3+} at 0.427 mM for 15 minutes. Following pre-incubation with Al^{3+}, antibiotic uptake was initiated by adding ciprofloxacin solution to the bacterial cell preparation at a final ciprofloxacin concentration of 1×10^{-7} M. After 15 minutes of antibiotic exposure, the cells were centrifuged and washed with 0.9% NaCl (2x). The cells were lysed by sonication and the lysate (100 μl) was prepared for subsequent HPLC assay. Data obtained from this experiment were used to contrast the cell preparation when Al^{3+} was added together with ciprofloxacin.

High Performance Liquid Chromatography (HPLC) Assay for Ciprofloxacin

The HPLC assay previously reported by Weber *et al.* (16) was adapted in the present study with minor modifications. In brief, the assay was carried out using a Hewlett-Packard series 1050 HPLC system equipped with a HP 1046A programmable fluorescence detector and ChemStation™ software package (Hewlett-Packard Company, Wilmington, DE). This assay utilized a mobile phase consisted of 30% v/v methanol in a triethylamine-phosphate buffer (0.5% w/v TEA adjusted to pH 3.0 with phosphoric acid). Separation was achieved using a Waters Associate Novapak C_{18} radial compression cartridge column (5 × 100 mm) at a flow rate of 1.4 ml/min. The eluent was monitored by fluorescence detection at the optimal wavelengths of 280 nm (excitation) and 449 nm (emission).

RESULTS

The degrees of chelation were reflected by the changes in the fluorescence intensity exhibited by ciprofloxacin at varying concentrations of different metal cations. The apparent K_D estimates for the five metal-ciprofloxacin chelates show a decreasing order of $Mg^{2+} > Mn^{2+} > Zn^{2+} >> Fe^{3+} > Al^{3+}$ (Table 1). A smaller K_D value indicates a stronger affinity of the cation to the ciprofloxacin molecule.

The minimum inhibitory concentration (MIC) of ciprofloxacin against *E. coli* in MHB was determined to be 0.0156 μg/ml. Among the three cations studied, i.e., Al^{3+}, Mn^{2+}, and Mg^{2+}, in the short-term time-kill study, only Mn^{2+} showed a low degree of bactericidal activity in the concentration range tested. This intrinsic bactericidal activity for Mn^{2+} was corrected as previously described prior to the presentation of bactericidal activity observed. Time-kill data for *E. coli* obtained during ciprofloxacin exposure at 2 × MIC or 1×10^{-7} M showed that the three cations at increasing concentrations caused a progressive reduction in the bactericidal effect of the antibiotic (Fig. 1). The extent of decrease was most significant for Al^{3+} which was followed by Mn^{2+} and Mg^{2+}. The magnitude of shift on the profile of the bactericidal effect vs. cation concentration at the various cation concentrations was well described by the IC_{50} estimates (Table 1, Fig. 2). The direction of shifts demonstrated by the three cations were similar to that observed for the K_D values measured for the three metal chelates (Table 1). Interestingly, uptake of ciprofloxacin by *E. coli* assessed at IC_{50} was enhanced in the presence of metal cations with the most significant increase by Al^{3+}; as compared to the control with no cations added, cellular drug uptake increased by 14.3-, 2.7- and 2.0-fold for Al^{3+}, Mn^{2+} and Mg^{2+}, respectively. Furthermore, this pattern of increased antibiotic uptake corresponded with the observed decrease in bactericidal activity, i.e., IC_{50}. Nevertheless, the degrees of antibiotic uptake were found to inversely relate to the K_D values. Moreover, cellular uptake of ciprofloxacin was 34% higher when Al^{3+} and ciprofloxacin were added simultaneously to the bacterial cell preparation than that following a period of pre-incubation with Al^{3+}.

DISCUSSION

Ciprofloxacin shows a characteristic fluorescence spectrum with the excitation wavelength at 271 nm. The carbonyl group and the adjacent carboxylate functional group of the ciprofloxacin molecule have been proposed to be the site for metal chelate formation (Fig. 3) (8–10). The chelation potentials of the metal cations to the antibiotic followed the increasing order of $Mg^{2+} < Mn^{2+} < Zn^{2+} << Fe^{3+} < Al^{3+}$ (Table 1). Coincidentally, the degrees of attenuation of bactericidal activity by the three metal cations showed a similar order of $Mg^{2+} < Mn^{2+} < Al^{3+}$ (Table 1). These observations suggest a significant contribution of metal chelate formation to the activity reduction of the antibiotic. Nevertheless, differences in the K_D estimates among Al^{3+}, Mn^{2+} and Mg^{2+} obtained in a buffer system were not in the same order of magnitude to those observed for the IC_{50} values in a culture system (Table 1). For instance, K_D of the magnesium-ciprofloxacin chelates was estimated to be approximately 1,000 fold lower than that of the aluminum chelates; however, the difference in the ability to attenuate bactericidal activity was only 10 fold between the two cations as indicated by the IC_{50} values. This discrepancy is probably related to the higher potential for Al^{3+} to precipitate in MHB during the time-kill studies as compared to the buffer used for K_D determination. However, the shifts observed for the IC_{50} values with regard to Mn^{2+}, Mg^{2+} and Al^{3+} appear to correspond well with the increase in cellular ciprofloxacin uptake when the same biological system (bacterial cells) is concerned.

Time-kill data obtained in the present study demonstrate a reduction in the bactericidal activity of ciprofloxacin in the presence of various metal cations. However, based on this direct observation alone, a firm conclusion cannot be reached with respect to which of the two contending mechanisms, i.e., reduced antibiotic uptake or formation of inactive metal chelates, is responsible for the reduction in activity. Presumably, the increase in polarity due to the higher ionic charge state as a result of chelate formation may render poor permeation of the metal-chelates through the phospholipid bilayer of the bacterial cell membrane. On the contrary, data show that the presence of Al^{3+}, Mn^{2+} or Mg^{2+} resulted in higher ciprofloxacin uptake by bacterial cells when compared to that of the drug alone. At a similar level of activity reduction for the three cations at their respective IC_{50} values, the degrees of cellular antibiotic uptake contrasted in a reversed manner. The uptake data strongly suggest that ciprofloxacin loses its activity when the cation chelates

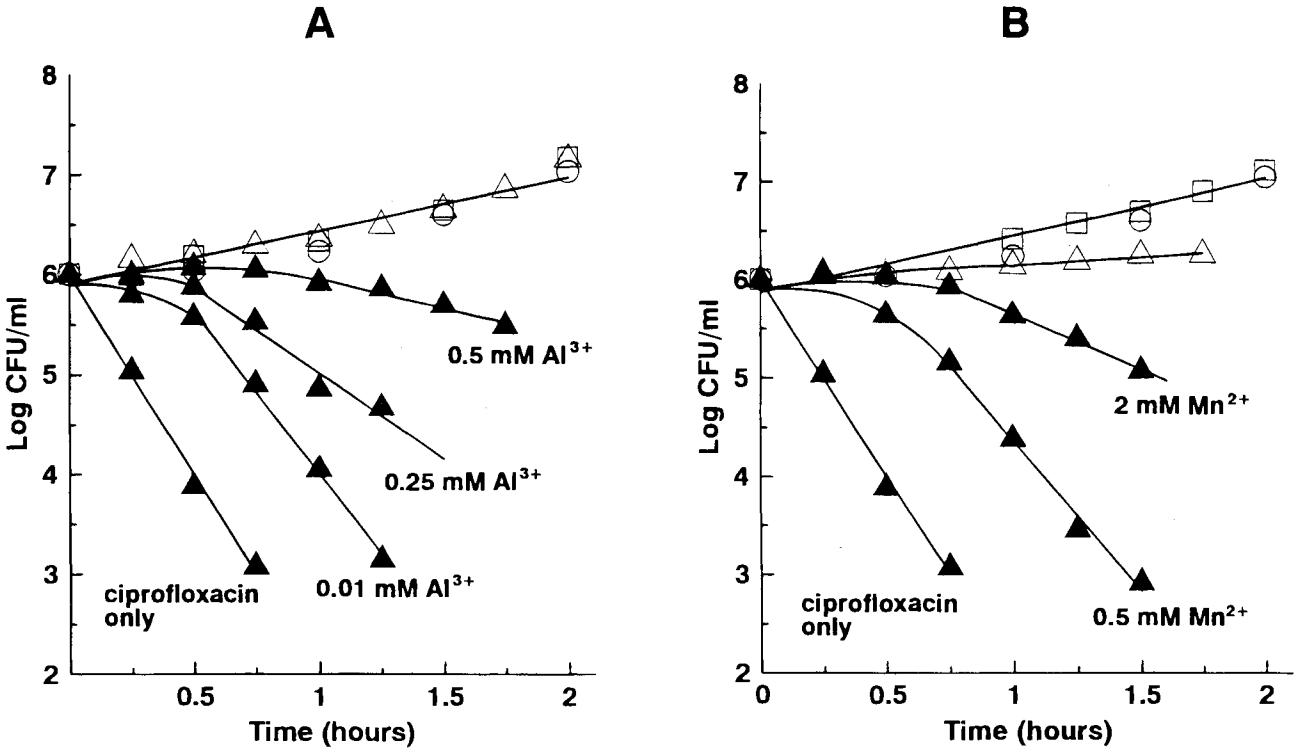

Fig. 1. Cation and concentration dependent reduction of ciprofloxacin's bactericidal activity. Fig. 1A shows the effects of increasing Al^{3+} concentrations on the bactericidal activity of ciprofloxacin at 2 × MIC (1×10^{-7} M); open circle, open square, and open triangle represent control with no antibiotic and no Al^{3+}, 2 mM Al^{3+} (no ciprofloxacin) and 2 mM Al^{3+} with ciprofloxacin, respectively. For all closed triangles, cation concentrations tested in the presence of ciprofloxacin are given next to the individual time-kill curves. The highest concentration of Al^{3+} tested, i.e., 2 mM, did not produce any measurable bactericidal effect. Fig. 1B demonstrates the impact of increasing Mn^{2+} concentrations on the bactericidal activity of ciprofloxacin at 2 × MIC. Open circle, open square, and open triangle represent control with no antibiotic and no Mn^{2+}, 2 mM Mn^{2+} and 6 mM Mn^{2+} antibiotic free controls, respectively. At higher concentrations of Mn^{2+} tested, e.g., > 2 to 6 mM, low levels of bactericidal activity were measured; such activity was subtracted from the bactericidal rate data measured for the corresponding Mn^{2+}-ciprofloxacin combination. Similarly, cation concentrations tested in the presence of ciprofloxacin are given next to the individual time-kill curves depicted by solid triangles. Note: Due to the large number of time-kill curves generated, not all available data are presented. Regression lines describing the log-linear bactericidal phase under individual study conditions are shown; no data treatment was applied to the lag phases.

Table 1. Physicochemical Characteristics of Various Metal-Ciprofloxacin Chelates and Antimicrobial Activity of Ciprofloxacin in the Presence of Different Metal Cations

Metal cation	K_D (mM)	IC_{50} (mM)	Increase in MIC[a]
Al^{3+}	0.05	0.43	8x
Fe^{3+}	0.07	—[b]	4x
Zn^{2+}	19.4	—	—
Mn^{2+}	37.7	1.40	2x
Mg^{2+}	60.9	4.39	1x

[a] Increase in MIC (expressed as multiples of MIC determined without addition of cations) for ciprofloxacin against *E. coli* ATCC 25922 at a cation concentration of 1.56 mM; data adapted from reference 8.
[b] —not determined.

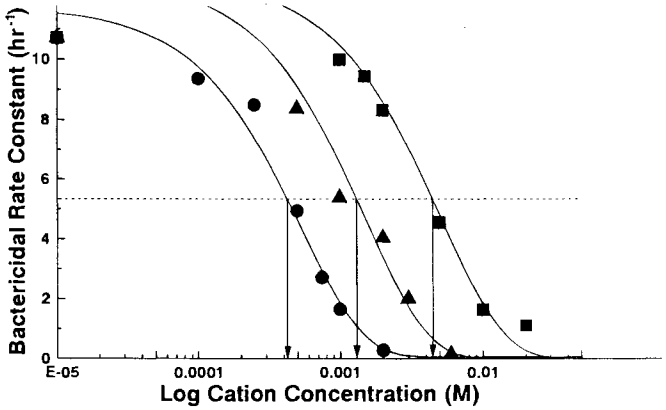

Fig. 2. Parallel shift of the bactericidal rate constants measured at increasing concentrations of Al^{3+} (solid circle), Mn^{2+} (solid triangle) and Mg^{2+} (solid square). The IC_{50} estimates for the cations tested are located at the interceptions of the dotted horizontal line and the individual concentration-response curves as indicated by the arrows.

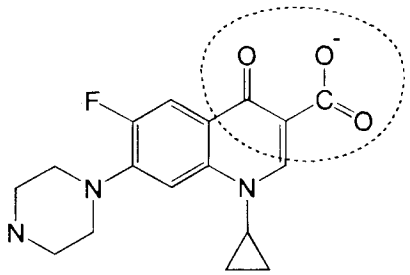

Fig. 3. The proposed site of metal chelate formation for ciprofloxacin.

the two functional groups of the molecule (Fig. 3) even though antibiotic permeation is enhanced in the form of a metal chelate. Furthermore, uptake of ciprofloxacin by *E. coli* was found to be higher when ciprofloxacin and Al^{3+} were introduced simultaneously to the bacterial cells than that following a period of pre-incubation with Al^{3+}. This finding excludes the possibility of any 'siphoning' effect of intracellular Al^{3+} on ciprofloxacin uptake. In fact, this observation is consistent with the argument that the metal chelates formed extracellularly have a higher propensity to permeate into the bacterial cells. It has been shown in this study that metal cations can reduce the antimicrobial activity of ciprofloxacin and the degree of reduction is in agreement with the affinity of the metal cations to the antibiotic. Since ciprofloxacin uptake by bacterial cells was enhanced in the presence of metal cations, the reduction in activity is most likely a result of drug inactivation. The limited data obtained for ciprofloxacin in the present study may help explain the similar phenomenon observed for other quinolone antibiotics (17).

REFERENCES

1. J. Sahai, K. Gallicano, L. Oliveras, S. Khaliq, N. Hawley-Foss, and G. Garber. *Clin. Pharmcol. Ther.* **53**:292–297 (1993).
2. D. E. Nix, W. A. Watson, M. E. Lener, R. W. Frost, G. Krol, H. Goldstein, J. Lettieri, and J. J. Schentag. *Clin. Pharmacol. Ther.* **46**:700–705 (1989).
3. S. H. Parpia, D. E. Nix, L. G. Hejmanowski, H. R. Goldstein, J. H. Wilton, and J. J. Schentag. *Antimicrob. Agents Chemother.* **33**:99–102 (1989).
4. D. E. Nix, W. A. Watson, L. Handy, R. W. Frost, D. L. Rescott, and H. R. Goldstein. *Pharmacotherapy* **9**:377–380 (1989).
5. E. Rubinstein and S. Segev. *Am. J. Med.* **82**:119–123 (1987).
6. N. T. Ratcliffe and J. T. Smith. *J. Pharm. Pharmacol.* **35**:61 (1983).
7. J. Blaser and R. Luthy. *J. Antimicrob. Chemother.* **22**:15–22 (1988).
8. R. C. Li, D. E. Nix, and J. J. Schentag. *Pharmaceut. Res.* **11**:917–920 (1994).
9. K. Timmers and R. Sternglanz. *Bioinorg. Chem.* **9**:145–155 (1978).
10. A. J. G. Bailey, A. Cole, J. Goodfield, P. M. May, M. E. Dreyfuss, J. M. Midgley, and D. R. Williams. *Int. J. Pharm.* **22**:283–290 (1984).
11. R. Kelly and T. Schultz. *Pharm. Res.* **6**:S139 (1989).
12. F. J. C. Rossotti and H. Rossotti. *The Determination of Stability Constants and other Equilibrium Constants in Solution*, McGraw-Hill Book Company, Inc., New York, (1961).
13. National Committee for Clinical Laboratory Standards. NCCLS document M7-A3, Villanova, PA (1993).
14. R. C. Li, D. E. Nix, and J. J. Schentag. *J. Pharm. Sci.* **83**:970–975 (1994).
15. R. C. Li, J. J. Schentag, and D. E. Nix. *Antimicrob. Agents Chemother.* **37**:523–531 (1993).
16. A. Weber, D. Chaffin, A. Smith, and K. E. Opheim. *Antimicrob. Agents Chemother.* **27**:531–534 (1985).
17. B. M. Lomaestro and G. R. Bailie. *DICP, Ann. Pharmacother.* **25**:1249–1258 (1991).