Effect of pH on ciprofloxacin ozonation in hospital WWTP effluent

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Abstract
A bubble column was used for ozonation of the quinolone antibiotic ciprofloxacin and the effect of pH was tested. Degradation at pH 7 increased the ciprofloxacin half life time to 29.1 min compared to pH 3 (26.8 min) and pH 10 (18.7 min), possibly due to increased sorption at neutral pH. Degradation product identification revealed strongest degradation at the piperazinyl substituent at pH 10 while degradation at the quinolone moiety seems promising at pH 7. For *P. fluorescens* and *E. coli*, reduction in antibacterial activity, monitored by agar diffusion tests, was in correlation with the ciprofloxacin degradation rate. For *B. coagulans*, however, no differences in residual antibacterial activity were found in function of pH, indicating that degradation products also affect antibacterial activity of ozonated samples.

Keywords
Ciprofloxacin, agar diffusion tests, degradation products, sorption

INTRODUCTION

Antibiotics belong to the so-called emerging contaminants in the aqueous environment. One of the major concerns with respect to their occurrence in the environment is the introduction of bacterial resistance. Ciprofloxacin (Fig.1a) is the most widely prescribed quinolone antibiotic in Europe (Ferech et al., 2006). An increase in resistance against ciprofloxacin from 5% to more than 80% of the bacterial population was shown in fish ponds when fluoroquinolones were used as medicated feed (Petersen et al., 2002). Because of its limited biodegradability (Kümmerer et al., 2000), ciprofloxacin is not well eliminated in conventional wastewater treatment systems. As a result, ciprofloxacin can be found at concentration levels up to 5.6 µg/l in wastewater treatment plant (WWTP) effluents originating from household waters (Batt et al., 2006) and even up to 31 $mg.l^{-1}$ in effluent of WWTPs, treating wastewaters of pharmaceutical manufacturers (Larsson et al., 2007). For efficient removal of ciprofloxacin, physical-chemical removal technologies such as advanced oxidation processes (AOPs) are indispensable. AOPs are characterized by the generation of hydroxyl radicals at ambient conditions. Ozonation can also be seen as an AOP because ozone decomposes into hydroxyl radicals at higher pH.
In this work, the effect of pH on the ozonation of ciprofloxacin, spiked in hospital WWTP effluent, is studied in detail. Ozone experiments at pH 3, 7 and 10 were performed, degradation products were identified and reduction of antibacterial activity in function of pH was studied by agar diffusion tests.

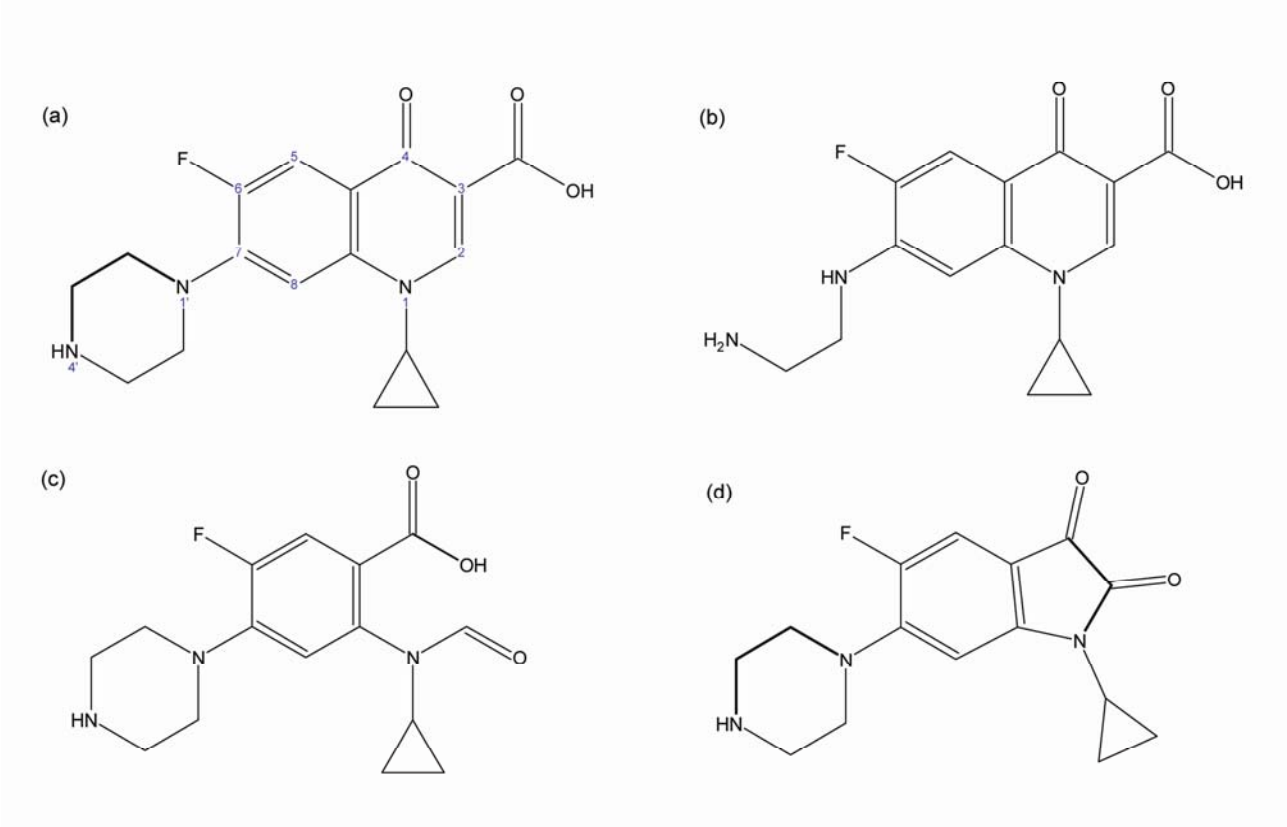

Figure 1. Molecular structure of (a) ciprofloxacin, (b) desethylene ciprofloxacin, (c) an anthranilic acid analogue and (d) an isatin analogue.

METHODS

Chemicals

Ciprofloxacin.HCl (≥ 98%) was purchased from MP Biomedicals Inc (USA). Chemicals used for solutions were of reagents grade and were used without further purification.

Experimental setup

A temperature controlled bubble column (27.5 ± 0.1 °C) with a height of 41.8 cm and an inner and outer diameter of 10.3 and 14.1 cm, respectively, was used for ozonation experiments. Ozone (2500 ppm_v) was generated in dry air by a LAB2B ozone generator (Ozonia, Switzerland). After adjusting the flow to 120 $ml.min^{-1}$, the O_3/air stream was introduced in the reactor through a porous glass plate positioned at the bottom. The reactor contained 2.4 l of hospital WWTP effluent, spiked with a ciprofloxacin concentration of 45.3 µM (15 $mg.l^{-1}$). Hospital WWTP effluent was sampled at the Maria Middelares hospital (Ghent, Belgium). The effluent was stored for at maximum 2 weeks at 4 °C and pH < 2. Before each ozone experiment, the effluent was pH adjusted by NaOH and HCl, buffered with 10.12 mM phosphate buffer (pH 3 and 7) or 2.53 mM borax buffer (pH 10) and temperature controlled at 27.5 °C for 24 h in order to reach ciprofloxacin sorption equilibrium at the start of each experiment. Ozonated liquid samples were taken by a tap at 6 cm water height. Immediately after sampling, the samples were flushed with nitrogen for 3 minutes at 15 mL min^{-1} in order to remove residual ozone.

Analytical procedures

For ozone measurements in the water phase, 10 ml AccuVac ampuls (ozone LR, 0.004 – 0.25 mg L^{-1}; ozone MR, 0.005 – 0.75 mg L^{-1}) were immersed in 40 ml of aqueous sample and analyzed with a Pocket Colorimeter (Hach, USA) at 600 nm.

For identification of ciprofloxacin degradation products, 25 ml samples were preconcentrated by a factor of 125 by solid phase extraction on OASIS MAX cartridges (30 µm; 150 mg; 6 ml, Waters, USA), followed by LC-MS analysis. The concentration method was modified from the Atlantis columns applications notebook (Atlantis columns, 2004). For LC-MS analysis, a Luna C18(2) column was used with a mobile phase containing water (0.1% formic acid) as eluent A and methanol as eluent B. Eluent A/eluent B ratios changed from 90/10 (0 min) over 60/40 (23 min) and 80/20 (33 min) to a ratio of 10/90 (34 min) which was kept constant for a final 11 minutes. MS spectra (m/z = 149.5 – 450.5) were recorded on a high resolution (HR) multi dimension MAT 95XP-Trap mass spectrometer (Thermo Finnigan) equipped with a TSQ/SSQ 7000 atmospheric pressure ionisation source. Analyses were done in positive ionisation mode by electrospray ionisation (ESI).
For quantification of ciprofloxacin and its degradation products, 6 ml sample was concentrated by a factor 12 by the same SPE method. For ciprofloxacin and desethylene ciprofloxacin determination, 5 µL concentrated sample was injected and separated on LC by an isocratic mobile phase containing 87.5% water (0.1% formic acid) and 12.5% acetonitrile. Other degradation products were separated by the gradient method described above after injection of 25 µL of concentrated sample. Quantification of ciprofloxacin and degradation products took place at the UV-absorbance maximum ± 4.5 nm on a photodiode array detector (Thermo Finnigan, Germany), coupled to the LC.

Sorption experiments
In additional batch experiments, ciprofloxacin sorption on suspended solids (SS) in hospital WWTP effluent was investigated as a function of pH (3, 7 and 10; 27.5 °C). Therefore, 100 mL of samples, identical to the initial solutions for ozonation, were stored in closed amber colored glass bottles of 118 mL and aqueous ciprofloxacin concentrations were determined after 24, 48, 120 and 168 h by sampling through a 0.45 µM filter (GHP membrane Acrodisc syringe filter with glass fiber prefilter, Pall, USA) followed by LC-UV analysis, described above.

Antibacterial activity tests
Pour plates of *Escherichia coli*, *Pseudomonas fluorescens* and *Bacillus coagulans* were prepared in 2% nutrient agar (Biokar Diagnostics, France) for agar diffusion tests. Three wells of 0.64 cm diameter each were cut manually into the agar for application of 20 µL of the ciprofloxacin ozonation samples at pH 3, 7 and 10. Samples taken after 0, 20, 40 and 60 minutes of ozonation were applied and diffusion tests were performed in triplicate. Samples for *P. fluorescens* and *B. coagulans* were diluted five times. Plates were incubated for 48h at 30 °C (*B. coagulans, P. fluorescens*) or 37 °C (*E. coli*) before measuring inhibition zone diameters as semi-quantitative indication for residual antibacterial activity.

RESULTS AND DISCUSSION

Effect of pH on ciprofloxacin degradation rate
Ciprofloxacin ozonation in hospital WWTP effluent is presented in Fig. 2. Half life times are 26.8, 29.1 and 18.7 min at pH 3, 7 and 10, respectively. Fast ozonation at pH 10 can be explained by fast direct ozonation at the deprotonated N_4, atom of the piperazinyl substituent (Fig. 1a) since deprotonated amines are known to react fast with ozone (Muñoz and von Sonntag, 2000).
At pH 3, ciprofloxacin degradation is faster as compared to pH 7 although direct ozonation is expected to be slow at acidic pH due to protonation of amine groups. Moreover, less reactive hydroxyl radicals are expected at pH 3. A sorption coefficient (log K_d) of 4.7 $l.kg^{-1}$ SS was found

for pH 7 compared to 4.3 $l.kg^{-1}$ SS for pH 3. Possibly, ciprofloxacin reactivity is mediated by sorption on organic material. Ozonation of deionized water at pH 7 without ciprofloxacin leads to steady state aqueous ozone concentrations of 16.0 µM while ciprofloxacin addition (45.27 µM) reduces aqueous ozone concentrations to ≤ 0.8 µM in deionized water and beneath limit of detection (< 0.1 µM) in hospital WWTP effluent. This indicates strong reactivity between ozone and ciprofloxacin and suggests reactions at the gas-liquid interface. By consequence, diffusion from sorbed ciprofloxacin into the aqueous phase may become rate limiting.

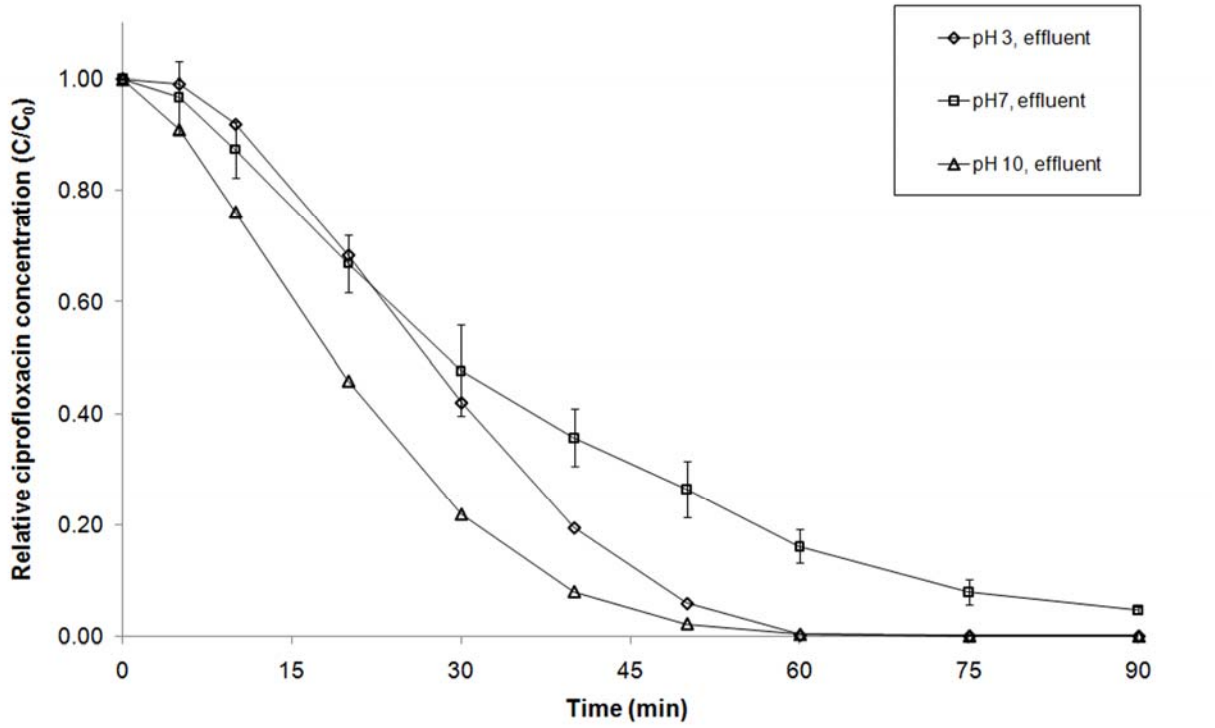

Figure 2. Relative ciprofloxacin concentration during ozonation in hospital WWTP effluent at pH 3, 7 (n = 3) and 10. ($[ciprofloxacin]_0$ = 45.3 µM, $O_{3,inlet}$ = 2500 ppm_v, T = 27.5 ± 0.1 °C)

Effect of pH on degradation product formation

Ciprofloxacin ozonation products were detected, based on LC-MS analysis. Degradation shows to occur (1) at the piperazinyl substituent, (2) at the quinolone moiety with formation of anthranilic acid analogues and (3) at the quinolone moiety with formation of isatin analogues. Identified degradation products were identical to degradation products, formed during ciprofloxacin ozonation in deionized water (De Witte et al., 2008). This indicates that the effluent composition does not affect the reaction mechanism, in contrast to earlier research on carbamazepine ozonation (Gebhardt and Schröder, 2007). Fig. 1 presents molecular structures of some degradation products. Formation proved strongly pH dependent with maximum concentrations of the anthranilic acid (Fig. 1c) and isatin analogue (Fig. 1d) being respectively at least 7 and 14 times higher at pH 7 compared to pH 3 and 10. Strongest degradation at the piperazinyl substituent occurred at pH 10 with maximum desethylene ciprofloxacin (Fig. 1b) concentrations of 5.3 µM compared to 3.3 (pH 3) and 1.9 µM (pH 7).

Antibacterial activity tests

Since the carboxyl and keto group at the quinolonic moiety are reported to be essential for antibacterial activity (Chu and Fernandes, 1989), degradation at pH 7, with formation of isatin and anthranilic acid analogues, seems promising. At pH 3 and 10, however, ciprofloxacin concentrations were reduced faster than at pH 7. The residual antibacterial activity of ozonated ciprofloxacin solutions was tested by means of agar disk diffusion tests.

For each bacterial species tested, residual antibacterial activity reduced during ozonation at pH 3, 7 and 10. For the Gram-negative *P. fluorescens* and *E. Coli*, strongest reduction of antibacterial activity occurred at pH 10, related to the parent compound degradation rate. For the Gram-positive *B. Coagulans*, however, inhibition zone diameters at 40 and 60 minutes of ozonation were not significantly different from each other, independent of pH (Fig. 3). The lack of differences indicate that not only ciprofloxacin degradation rate but also differences in reaction pathway, dependent on the ozonation pH, affect antibacterial activity against this species.

Figure 3. Agar diffusion tests for *B. coagulans* after addition of 5 times diluted ozonated hospital WWTP effluent spiked with 45.27 µM ciprofloxacin at pH 3, 7 and 10. Left side: 0 min of ozonation (repetition 1). Right side: 40 minutes of ozonation (repetition 1).

CONCLUSIONS

Ozonation of ciprofloxacin in hospital WWTP effluent as a function of pH showed fastest ciprofloxacin degradation at pH 10, explained by the direct ozonation at deprotonated amines of the piperazinyl substituent. At pH 7, ciprofloxacin degradation was slowest which may be affected by the higher degree of sorption on SS compared to pH 3 and 10. Identified ciprofloxacin ozonation products revealed strongest degradation at the piperazinyl substituent at pH 10 and strongest degradation at the quinolone moiety at pH 7. For *P. fluorescens* and *E. coli*, residual antibacterial activity indicated to be mainly determined by the parent compound degradation rate while degradation products seem to be more important for residual antibacterial activity against *B. coagulans*.

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