Application of XCMS online and toxicity bioassays to the study of transformation products of levofloxacin

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The final version of this paper was published in *Water Science and Technology:*

Segura P. A., Saadi K., Clair A., Lecours M.-A., Yargeau V. (2015) Application of XCMS online and toxicity bioassays to the study of transformation products of levofloxacin. *Water Science and Technology* 72(9):1578-1587. doi:10.2166/wst.2015.369

Abstract

We studied the nature and antimicrobial activity of ozonolysis transformation products (OTPs) of levofloxacin (LEV), a frequently detected fluoroquinolone antimicrobial in environmental waters. Two bioassays, the Kirby-Bauer test and the broth microdilution assay were used to measure changes in the antimicrobial activity of solutions at low LEV to O_3 molar ratios (2:1, 2:3 and 1:3) compared to solutions without added O_3 (LEV:O_3 1:0). The Kirby-Bauer test was not sensitive enough to detect significant differences in the growth inhibition zones in samples LEV:O_3 2:1 and LEV:O_3 1:0, however the broth microdilution assay showed that bacterial growth inhibition was significantly lower ($p < 0.001$) in the solutions exposed to O_3. Loss of antimicrobial activity in LEV:O_3 2:1 solutions (48 ± 16)% was in agreement with the concentration decrease of LEV (36 ± 3)% in those same samples. A method of identification of OTPs using XCMS online was applied to LEV:O_3 2:1 and 1:0 samples and indicated the presence of an OTP of LEV of formula $C_{18}H_{20}O_5N_3F$, which was identified as LEV-*N*-oxide. The molecular structure of this compound was partially confirmed by tandem mass spectrometry experiments. This study showed that even at sub-optimal ozone doses, OTPs of higher antimicrobial activity than LEV were not formed.

Keywords: transformation products; ozonation; emerging contaminants; bioassays; high-resolution mass spectrometry; toxicity

Introduction

After almost two decades of research on the occurrence of pharmaceuticals active compounds (PhACs) in the environment, it has become clear that these substances originate mainly from excreta of individuals taking medication. Consequently, they are almost ubiquitous at nanogram-per-liter concentrations in urban wastewaters (Monteiro and Boxall 2010). Detrimental effects on aquatic biota have been observed at concentrations between 5-100 ng L^{-1} for a few compounds, *e.g.* ibuprofen and fluoxetine (De Lange et al. 2006) and 17α-ethinylestradiol (Kidd et al. 2007).

Elimination of PhACs using more effective treatment processes in wastewater treatment plants is one of the most investigated approaches to mitigate this problem. The removal of PhACs by ozone (O_3) has been proposed as viable wastewater treatment since elimination rates are > 90% for many PhACs using O_3 doses around to 2-10 mg L^{-1} (Joss et al. 2006). However, oxidation of PhACs using those O_3 doses not result in the complete mineralization of these compounds (Huber et al. 2003). To date, ozonolysis transformation products (OTPs) of many PhACs have been identified (Ikehata et al. 2006) however, there are far less studies on their residual toxicity (Yargeau et al. 2008; Radjenović et al. 2009; Larcher et al. 2012).

Among PhACs, anti-infectives (more commonly known as antibiotics), have received a lot of attention from the scientific community since their occurrence in surface waters and sewage is of concern because of their potential role in the dissemination of antibiotic resistance in bacteria and other harmful effects on non-target species such as algae and shellfish (Binelli et al. 2009; Segura et al. 2009). OTPs of antibiotics could conserve their antimicrobial activity if their pharmacophore, *i.e.* key functional groups to the biological activity of the substance, are not affected by ozonolysis (Boxall et al. 2004). A study showed that for several types of antibiotics (except some β-lactams) residual antimicrobial activity decreases with increasing doses of $O_{3(aq)}$, which suggested that the formation of toxic OTPs of antibiotics are rarely formed (Dodd et al. 2009). However, a more recent paper showed that OTPs of levofloxacin were toxic to the bioluminescent bacterium *Vibrio fischeri* (El Najjar et al. 2013).

The objective of this study was to identify OTPs formed at low LEV to O_3 molar ratios (2:1, 2:3 and 1:3) equivalent respectively to 0.4, 1.4 and 2.8 mg L^{-1} of ozone, and measure their antimicrobial activity. These conditions of ozonolysis represent situations that could be observed in treatment of water containing high amounts of dissolved organic matter, which would decrease the effective concentration of O_3 available to react with antibiotics or other PhACs. Ozonolysis experiments with illicit drugs performed by Rodayan et al. (2013) have shown that the rate of removal of organic micropollutants is affected by the composition of the matrix. Therefore, under conditions of low dose of O_3, the formation of biologically active OTPs is more likely since the pharmacophore of levofloxacin could be preserved due to the limited availability of ozone to further react with the transformation products formed.

Methods

Ozonolysis of levofloxacin-containing water

An Ozomax OZO4VTT O_3 generator (Granby, QC, Canada) was used to produce gaseous O_3 in-situ from pure (99.5%) compressed $O_{2(g)}$. An in-house 2-L acrylic reactor (height: 60 cm; diameter: 7 cm) was used to prepare an aqueous O_3 stock solution (7.1 mg L^{-1}). Levofloxacin (LEV) with a purity of 99.4 % was purchased from Sigma-Aldrich Canada (Oakville, ON, Canada). Deionized water (di-H_2O) was produced by a Milli-Q system from Millipore (Billerica, MA, USA). A stock solution of 29.7 mg L^{-1} of LEV was prepared in di-H_2O (initial pH value of 7.91 ± 0.05). Complete dissolution of the antibiotic was achieved by placing the solution in an Branson Ultrasonics model 2510 ultrasonic bath (frequency: 40kHz, power:80 W) for 30 min. In these conditions, sonochemical composition of LEV was not observed (Fig. S1, supplementary material). Working solutions of 15.4 mg L^{-1} of LEV in di-H_2O were prepared before each experiment from the LEV stock solution. While the concentration used for the ozonolysis experiments is several orders of magnitude higher than environmental concentrations, it was necessary to use such concentration in order to facilitate the identification of OTPs without a preconcentration step prior to analysis. Volumes of 15 mL of the working solution were placed in 40-mL amber vials with a silicone septum to allow for addition of $O_{3(aq)}$. A fresh $O_{3(aq)}$ stock solution was prepared before each experiment by introducing $O_{3(g)}$ from the O_3 generator into the reactor at a flow rate of 15 standard cubic feet per minute. The concentration of $O_{3\ (aq)}$ was determined by measuring the absorbance at 260 nm and a molar absorptivity value of 3300 $M^{-1}cm^{-1}$. Test solutions of LEV were prepared by adding different volumes of the $O_{3\ (aq)}$ stock solution (2, 7 and 14 mL) using gas tight syringes to obtain approximately molar ratios of LEV to O_3 of 2:1, 2:3 and 1:3, respectively. Therefore, applied O_3 doses were in the range 0.4 to 2.8 mg L^{-1}. O_3 blanks (molar ratio of LEV to O_3 of 1:0) were also prepared during each experiment. After addition of $O_{3(aq)}$, samples were agitated with a vortex mixer for a few seconds and their volume was brought to 35 mL by addition of di-H_2O. This last step was necessary to obtain the same volume for all samples. Samples were then uncapped and stirred for 5 min to remove all residual O_3. All ozonolysis experiments were done in triplicate and were carried out at 22 °C.

Quantification of levofloxacin

The residual concentration of LEV in the test solutions was determined on the same day that the ozonolysis experiments were done by liquid chromatography-high resolution mass spectrometry. This system was composed of an Accela ultra-high performance liquid chromatograph (UHPLC) coupled with a linear ion trap-orbital trap mass spectrometer (LIT-OrbitrapMS) model LTQ Orbitrap XL, both manufactured by Thermo Fisher Scientific (Waltham, MA, USA). The protocol of analysis is described in detail elsewhere (Rodayan et al. 2013). A five-point calibration curve injected in triplicate was used for quantification of levofloxacin.

Kirby-Bauer disk diffusion susceptibility test

The Kirby-Bauer assay was performed to monitor antimicrobial activity of LEV test solutions. The protocol used was based on Hudzicki (2012) with two minor modifications. *Escherichia coli* strain ATCC 11303 was employed as a replacement to *E. coli* strain ATCC 25922 and a hole-puncher was used to make paper disks (diameter: 6.0 ± 0.5 mm) using Express Plus filter paper

of a pore size of 0.22 μm (Millipore). Aseptic techniques (sterile work area, sterile handling, etc.) were used throughout all bioassays experiments to ensure that bacterial cultures were free of contamination. The disks were soaked in the LEV test solutions described previously using tweezers. Three disks were placed per Mueller-Hinton (MH) agar plate previously inoculated with *E. coli*. The plates were then incubated at 37 °C and the growth inhibition zone diameters were measured to the nearest millimeter after 24 h of incubation. The magnitude of the diameter of the growth inhibition zone provides a semi-quantitative method of analyzing the residual antibacterial activity after exposure of antibiotics to ozone (Nasuhoglu et al. 2012). In order to determine which diameters of the growth inhibition zones were significantly different, an analysis of variance (ANOVA) test followed by Tamhane's T2 post hoc test using SPSS statistics 22 (IBM, Armonk, NY, USA) was performed.

Broth microdilution assay

The protocol described by Dodd et al. (2009) was used to measure the antimicrobial activity of LEV test solutions. Minor modifications to the original protocol were applied to adapt it to the material and instruments available in the laboratory: *i) Escherichia coli* strain ATCC 11303 was employed as a replacement to *E. coli* strain ATCC 25922; *ii)* the incubation time of the plates was extended to 20 h instead of 8 h, to obtain sufficient growth in the agar and *iii)* antimicrobial activity was evaluated measuring the absorbance of the microplate wells at 625 nm, and the data obtained was normalized to the maximum absorbance value and plotted as a function of the LEV concentration. The obtained dose-response curve was fitted using the logistic non-linear fit of Origin 7.0 (OriginLab Corp., Northampton, MA, USA). Optimization of the growth medium is described in the Supplementary material.

This assay was used to obtain a more quantitative measure of the subtle differences in the antimicrobial activity of LEV test solutions. For this reason, data from two different ozonolysis experiments performed on two different days (total of 6 samples and 120 dilution points per test solution) were pooled for analysis. To facilitate the comparison of the broth microdilution assay experiments, normalized growth inhibition was plotted as a function of the dilution factor, instead of LEV concentration, of each solution. Therefore the median effective dilution factor (EDF_{50}) was used as a parameter for comparison. The EDF_{50} represents the dilution factor necessary to inhibit bacterial growth by 50%. Therefore, substances with low antimicrobial activity have higher EDF_{50} values than substances with high antimicrobial activity, since the former have to be less diluted than the latter to produce the same inhibitory effect. A Student's t-test and an F-test were used to evaluate differences between samples at the 95 % confidence level.

Method validation: Identification of transformation products

In order to identify the main OTPs of LEV generated after addition low doses of O_3, a method using differential of analysis of chromatograms obtained by high resolution mass spectrometry was developed and validated using carbamazepine (CBZ), an anticonvulsant and mood regulator frequently detected in the environment. CBZ was used to validate the identification method since

its OTPs are known and have been studied extensively (Hübner et al. 2014). Triplicate samples with a molar ratio CBZ: O_3 1:0 (2.3 mg L^{-1} of CBZ and no ozone added) and 1:4 (same CBZ concentration and 1.8 mg L^{-1} of O_3) were analysed in a UHPLC-QqTOFMS system composed of a Nexera ultra-high performance liquid chromatograph manufactured by Shimadzu (Kyoto, Japan) coupled to a Maxis quadrupole-time-of-flight mass spectrometer made by Bruker (Billerica, MA, USA). Ionization was performed by ESI+ and the mass analyzer was used in the full scan mode (*m/z* 50-800) with a resolution power, measured at full width half-maximum, of 19725 at *m/z* 237.1025. Chromatographic separation was done with an Acquity UPLC HSS-T3 column (2.1×50mm, 1.8µm) at a flow rate of 0.5 mL min^{-1} and a column temperature of 30°C. Solvent A (0.1% formic acid in H_2O) and solvent B (0.1% formic acid in acetonitrile) were used with the following mobile phase gradient: 0 min (10% B), 1 min (10% B), 7 min (35% B), 9 min (100% B), 12min (100% B), 13 min (10% B), 16min (10% B). The ionization conditions were: capillary voltage of 3000 V, dry gas at 10 L min^{-1}, dry temperature of 200°C. Nitrogen (99%) was used in the source to assist nebulization and in the collision cell as collision gas.

UHPLC-QqTOFMS data files were uploaded to the web-based metabolomics data processing platform XCMS online, freely accessible at https://xcmsonline.scripps.edu. XCMS online compares by differential analysis two sets of samples and identifies peaks that increase or decrease significantly by calculating *p*-values and fold changes (Tautenhahn et al. 2012). Dataset#1 (control) contained triplicate samples of CBZ:O_3 1:0 and dataset #2 contained triplicate samples of CBZ:O_3 1:4. The parameters used to perform the differential analysis in XCMS online are indicated in the Supplementary material, Table S1. In order to classify as potential OTP, a peak must: *i)* be present in all ozonated samples and absent in non-ozonated samples; *ii)* have *p*-values < 0.05; ii) have fold changes > 10; *iii)* have a mean intensity >1% of the mean intensity of the parent compound signal in the non-ozonated samples and *iv)* elute at a percentage of organic solvent in the mobile phase <85%. This set of rules ensured that only peaks that appeared in ozonated samples with sufficient intensity and not present in the non-ozonated (control) samples were discriminated from the rest of the peaks detected in the samples. Also, our previous study (Segura et al. 2013) showed that peaks eluted at high percentages of organic solvent in the mobile phase are identified by differential analysis as potential OTPs but in fact they are not related to the parent compound.

To confirm the identification of OTPs of CBZ, the most intense peaks were further studied by tandem mass spectrometry. In order to find the best energy able to completely fragment the precursor ion, collision-induced dissociation (CID) experiments were performed in specific retention time ranges with increasing collision energies in the cell, from 10 to 30 eV.

Identification of ozonolysis transformation products of levofloxacin

The validated method was applied to the identification of OTPs of LEV. Samples were grouped in two different datasets: dataset#1 (control) contained triplicate samples of LEV:O_3 1:0 while dataset #2 contained triplicate samples of LEV:O_3 2:1. Data files generated after UHPLC-LIT-OrbitrapMS analysis in the .raw format of Thermo Scientific were converted to the .mzXML format using MSConvert application of the ProteoWizard toolkit (Chambers et al. 2012) and

then uploaded to XCMS online. In order to identify potential OTPs of LEV from the list of results, the set of rules described in the previous section was used to filter the data and reduce the number of false positives. Elemental formulas were assigned to the retained peaks using a mass tolerance ≤ 10 mmu, ring and double bond equivalents (RDBE) within ± 4 units of the value for LEV and the nitrogen rule for even-electron ions. For element limits, only H and O atoms were allowed to be higher than the parent compound. For N and C, the maximum number of atoms was the same as LEV. In order to assist the structural elucidation of the potential OTPs, the most intense precursor ions were studied by tandem mass spectrometry experiments in the UHPLC-QqTOFMS system or by multi-stage tandem mass spectrometry (MS^n) experiments performed by flow-injection analysis in a linear ion trap mass spectrometer; model LTQ-XL from Thermo Scientific.

Results and discussion

Ozonolysis of levofloxacin and antimicrobial activity measured with the Kirby-Bauer disk diffusion susceptibility test

Analysis of the test solutions by UHPLC-LIT-OrbitrapMS showed that the residual concentration of LEV rapidly decreased as a function of O_3 dose (Fig. 1). This was expected as LEV has a reaction rate constant of $6.0 \pm 0.2 \times 10^4$ M^{-1} s^{-1} with O_3 at pH 7.2 and 20°C (El Najjar et al. 2013). Using a concentration of 0.4 mg L^{-1} of O_3 (LEV:O_3 molar ratio of 2:1), LEV initial concentration decreased by (36 ± 3)% while in the samples with the highest applied ozone dose (2.8 mg L^{-1}, equivalent to a LEV:O_3 molar ratio of 1:3), about (96 ± 2)% of the initial LEV concentration was transformed. Next, the antimicrobial activity of the test solutions was measured with the Kirby-Bauer test. In this bioassay, the ability of a compound to inhibit microbial growth is determined by the presence or absence of bacteria around the paper disks impregnated with the test substance, identified by a circular zone around the disk without bacterial growth. The magnitude of the activity is correlated to the diameter of the zone of growth inhibition.

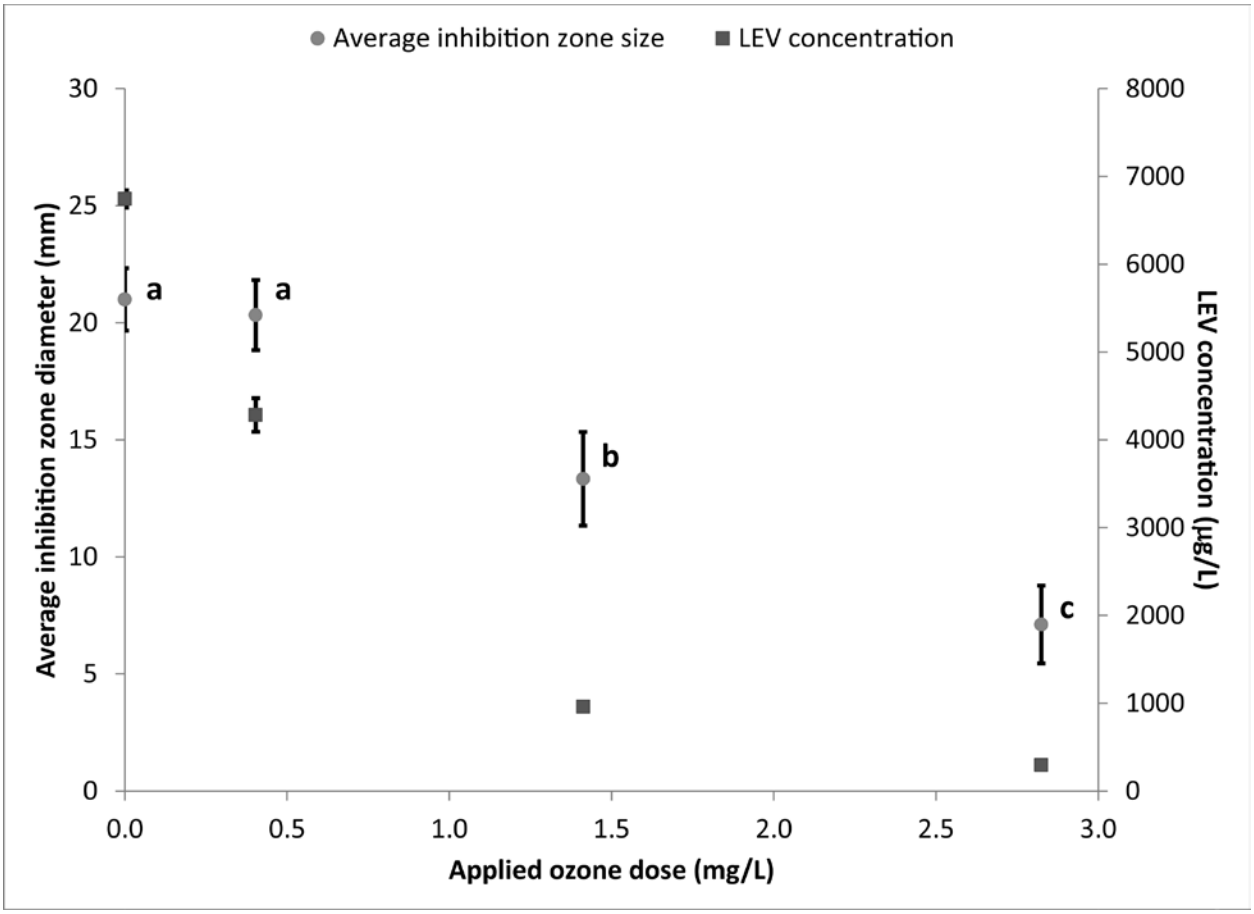

Figure 1. Average inhibition zone diameters (n=9) and residual levofloxacin concentration of the ozonated solutions. Letters that are different indicate a significant difference ($p < 0.05$) between the inhibition zones of the respective applied ozone doses according to Tamhane's T2 post hoc test. Length of error bars indicates two standard deviations.

Results of the Kirby-Bauer test (Fig. 1) showed that ozonolysis of LEV was also accompanied by a diminution of its antimicrobial activity, as demonstrated by the lower average inhibition zone diameter, and reached a point where it was equal to a blank (about 7-8 mm). However, the trend did not correspond completely to the one observed for the residual LEV concentration. An analysis of variance followed by Tamhane's T2 post hoc test indicated that the difference between the average inhibition zone diameter for the test solutions LEV:O_3 1:0 (no O_3) and 2:1 (0.4 mg L^{-1} of O_3) was not significantly different (p=0.997). This result might be explained by either: *i)* OTPs created in the LEV:O_3 2:1 sample had an antimicrobial activity similar to that of LEV or *ii)* the sensitivity of the Kirby-Bauer is insufficient to detect differences in the antimicrobial activity of those two test solutions. We decided to test the validity of those two hypotheses by using a more sensitive antimicrobial activity assay.

Determination of antimicrobial activity with the Broth microdilution assay

Since the Kirby-Bauer test was not able to detect differences between the LEV test solutions with no O_3 added and the solution in which 0.4 $mgL^{-1}$of O_3 was added (LEV:O_3 1:0 and 2:1, respectively), experiments were conducted with a more sensitive and quantitative bioassay, the broth microdilution assay. In this bioassay, antimicrobial activity is measured as a result of the diminution of the absorbance at 625 nm of a series of growth media solutions containing a microorganism and several dilutions of the substance to be tested for antimicrobial activity. If the test substance has an antimicrobial activity, bacterial growth will be increasingly inhibited in the wells and absorbance will decrease as the concentration of the test substance increases. Therefore, a dose-response (sigmoidal) curve will be observed when plotting normalized growth inhibition as a function of the test substance concentration or dilution factor. Results of the optimization of the growth medium for the broth microdilution assay are shown in the Supplementary material (Fig. S2, S3 and S4, supplementary material). Based on the results obtained, the Mueller-Hinton (MH) was identified as the growth medium providing the best testing conditions for this work. Logistic fit to the data obtained with the broth microdilution assay experiments with the LEV:O_3 1:0 and LEV 2:1 solutions (Fig. 2) showed that their median dilution factors (EDF_{50}) were 0.0058 ± 0.0006 and 0.0086 ± 0.0006, respectively. The EDF_{50} values of both regressions indicated that ozonolysis reduced the antimicrobial activity of LEV:O_3 2:1 solution since it had to be less diluted than the LEV:O_3 1:0 solution to have the same effect (50% growth inhibition). Comparison of those two EDF_{50} values with an unpaired Student's t test (degrees of freedom=232) indicated that they were significantly different with a *p* value < 0.001 (Motulsky and Chrsitopoulos 2004). Also we performed an F test to determine if the LEV:O_3 1:0 and LEV 2:1 datasets are significantly different. The calculated test value (F=4.013) with degrees of freedom 4 and 232 for numerator and denominator, respectively had a *p*-value of 0.0035. These results confirmed that the second hypothesis proposed earlier, *i.e.* that the Kirby-Bauer test is not sufficiently sensitive to detect differences in the antimicrobial activity of the two test solutions, was correct. Additionally, the percent loss of antimicrobial activity of the LEV:O_3 2:1 solution is (48±16)% which is in agreement with the diminution of the concentration of LEV after addition of 0.4 mg L^{-1} of O_3 (36±3)%. A disagreement between loss of antimicrobial activity and LEV concentration after ozonolysis would indicate the presence of OTPs active against microorganisms (higher activity than accounted for by the concentration of LEV) or capable of inhibiting antimicrobial activity (lower activity). The broth microdilution assay results demonstrated that ozonolysis of LEV, even at very low molar ratios or doses, forms OTPs without either property. However, the emergence of a different type of toxicity after ozonolysis of LEV is not ruled out and these results do not contradict the increased toxicity using bioluminescent bacteria previously reported by El Najjar et al. (2013).

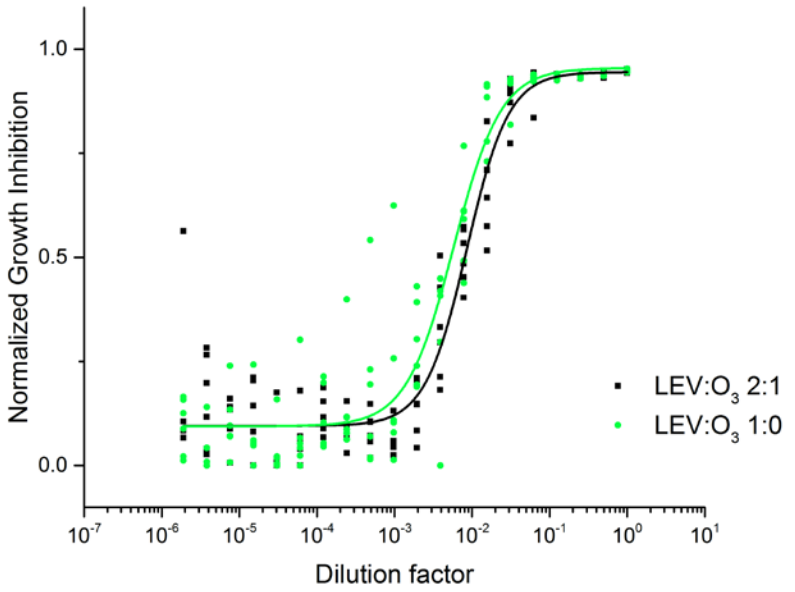

Figure 2. Comparison of dose-response curves for LEV before ozonation (LEV:O_3 1:0) and after ozonation (LEV:O_3 2:1). Median effective dilution factors (EDF_{50}) were 0.0058 ± 0.0006 and 0.0086 ± 0.0006, respectively.

Results of the validation of the identification method using carbamazepine

XCMS online was originally developed to process untargeted metabolomic data, but its unique features such as free access, ease of use and wide range of statistical analyses make it an interesting software for the identification of transformation products of emerging contaminants. Results of the differential analysis performed with CBZ to validate our XCMS online differential analysis method are shown in Table 1. The table shows that out of 620 unregulated features that the software originally found only 24 followed the rules specified in section *Method validation: Identification of transformation products*.

In XCMS online an "upregulated" feature is a peak that is significantly more intense in the test than in the control samples (Tautenhahn et al. 2012). Similarly a "downregulated" feature is a peak that has higher intensity in the control samples than in the test samples. The ion with the highest normalized intensity of the list was *m/z* 251.0821 which corresponds to 1-(2-benzaldehyde)-4-hydro-(1H,3H)-quinazoline-2-one also known as BQM ($C_{15}H_{10}N_2O_2$, $\Delta m/z$=0.596 mmu). The presence of BQM was confirmed by the presence in Table 1 of several related ions: *m/z* 252.0852 (M+1 isotopic peak, theoretical abundance= 17%, experimental abundance=16%), *m/z* 273.0641 (sodium adduct, $\Delta m/z$ =0.651 mmu) and *m/z* 274.0673 (M+1 isotopic peak of ion BQM+Na^+, theoretical abundance= 0.17, experimental abundance=0.16). Two other OTPs of CBZ identified by XCMS online were *m/z* 269.0928 that corresponds to dihydroxy-carbamazepine ($C_{15}H_{12}N_2O_3$, $\Delta m/z$ =0.731 mmu) and *m/z* 267.0771 that could

correspond to either 1-(2-benzaldehyde)-(1H,3H)-quinazoline-2,4-one (BQD) or to 1-(2-benzoic acid)-4-hydro-(1H,3H)-quinazoline-2-one also known as BaQM. These two OTPs are isomers ($C_{15}H_{10}N_2O_3$, $\Delta m/z$=0.681 mmu) but BQD is the result of the attack one O_3 molecule on CBZ while BaQM results from the attack of two O_3 molecules (Hübner et al. 2014).

TABLE 1. Results of the XCMS online differential analysis

***m/z* value** [a]	**t_R** [b] **(min)**	**Fold change**	***p*-value**	**Identity**	**Normalized Intensity** [c] **(%)**
273.0641	4.78	597	0.001	$[BQM+Na]^+$	48.1
251.0821	4.78	592	0.002	$[BQM+H]^+$	84.0
165.5318	4.78	273	0.001	N.I.	15.4
274.0673	4.78	163	0.002	(M+1) peak of $[BQM+Na]^+$	7.5
291.0748	4.78	87	0.002	$[2OH\text{-}CBZ+Na]^+$	13.0
292.0780	4.78	62	0.002	(M+1) peak of $[2OH\text{-}CBZ+Na]^+$	2.1
174.5370	4.78	48	0.002	N.I.	2.6
269.0928	4.78	33	0.0008	$[2OH\text{-}CBZ+H]^+$	3.2
289.0380	4.78	31	0.001	N.I.	1.5
252.0852	4.78	29	0.002	(M+1) peak of $[BQM+H]^+$	13.1
335.0352	4.78	27	0.001	N.I.	1.5
145.5198	4.78	23	0.002	N.I.	1.4
166.0333	4.78	20	0.002	N.I.	2.8
154.0234	4.78	19	0.002	N.I.	1.8
173.5182	4.78	17	0.003	N.I.	0.8
193.0125	4.78	15	0.002	N.I.	1.0
157.5429	4.78	13	0.003	N.I.	0.9
289.0591	5.38	17	0.04	$[BQD+Na]^+$	1.2
173.5292	5.40	41	0.03	N.I.	2.4
267.0771	5.40	40	0.0	$[BQD+H]^+$	3.9
237.1025	6.61	-264	0.006	$[CBZ+H]^+$	0.4
236.1544	8.03	20	0.00001	N.I.	4.4
236.6560	8.03	21	0.001	N.I.	1.2
248.0686	8.42	700	0.006	N.I.	29.7

[a] Median value; [b] Median retention time; [b] Percentage of CBZ median peak height in CBZ:O_3 1:0 samples. N.I.: ion not identified. Values for CBZ are only included to indicate differences in retention time, fold change and *p*-value.

A major OTP of CBZ that was not present in the samples was 1-(2-benzoic acid)-(1H,3H)-quinazoline-2,4-one (BaQD), which could be explained by the low amount of O_3 used in the experiments compared to previous studies (Hübner et al. 2014). The absence of BaQD suggests that *m/z* 267.0771 is BQD rather than BaQM since both BaQD and BaQM are the result of two O_3 attacks on the CBZ molecule. This was proven by high resolution-tandem mass spectrometry

experiments (Table 2) which showed that a product ion of *m/z* 196.0755, generated after CID of *m/z* 267.0771, was observed by Hübner et al. (2014) only in the tandem mass spectrum of BQD. Additionally, neutral losses of H_2O or CO_2, typically observed when carboxylic acid groups are present as is the case of BaQM, were not observed in the mass spectrum of *m/z* 267.0771.

TABLE 2. High resolution-tandem mass spectrometry experiments performed on the precursor ions of CBZ and its OTPs

Precursor ion (*m/z*)	**Neutral formula (Name)**	**Product ions [a] (*m/z*)**
237.1027	$C_{15}H_{12}N_2O$ (CBZ)	194.0970 (100%)
		192.0814 (19%)
		179.0735 (1%)
251.0810	$C_{15}H_{10}N_2O_2$ (BQM)	223.0863 (4%)
		208.0752 (53%)
		195.0674 (12%)
		180.0805 (100%)
267.1208	$C_{15}H_{10}N_20_3$ (BQD)	239.0823 (5%)
		224.0709 (6%)
		206.0589 (3%)
		196.0755 (100%)

[a] Values in parenthesis represent the relative abundance. Collision energy in the collision cell was 30 eV.

While the developed identification method of OTPs still suffers from the presence of false positives, *i.e.* only 41.7% of the filtered ions were related to known OTPs of CBZ; the results represent an improvement from our previous method (Segura et al. 2013) in which only 13% of the potential OTPs were good candidates. In summary, the method of identification of OTPs by high-resolution mass spectrometry and differential analysis with XCMS online presented here is a simple and efficient way to identify potential transformation products of organic micropollutants. The method identified three of the major OTPs of CBZ: dihydroxy-carbamazepine, BQM and BQD. Other OTPs, reported elsewhere (Hübner et al. 2014), such as BaQD and BaQM were not identified and could be the result of different ozonolysis setups as well as low intensity.

Identification of ozonolysis transformation products of levofloxacin

The validated method of identification of OTPs was applied to the analysis of triplicate LEV:O_3 1:0 (control) and LEV:O_3 2:1 test solutions. Results obtained with XCMS online showed the presence of 277 upregulated features. As expected, LEV was the main downregulated feature, with a fold change of -1.6 and *p*-value of 0.002. Application of the selection rules specified in

section *Method validation: Identification of transformation products*, reduced the number of upregulated features to only three (Table 3). Among those potential OTPs of LEV, one appears to be an isotopic peak: *m/z* 379.1481, M+1 isotopic peak of *m/z* 378.1461 (theoretical abundance= 21%, experimental abundance=20%). The most probable elemental formulas presented in Table 3 were then assigned to the 2 potential OTPs according to mass accuracy and RDBE and the nitrogen rule for even electron ions.

TABLE 3. List of potential OTPs of LEV found using XCMS online

Peaks [a] **(*m/z*)**	**t_R** [b] **(min)**	**Fold change**	***p*-value**	**Normalized Intensity** [c] **(%)**	**Possible neutral formulas**	**Formula difference with LEV**	**RDBE**	**Δ*m/z* (mmu)**
360.1366	6.71	20	0.001	1.5	$C_{18}H_{18}O_4N_3F$	-2 H	10.5	1.189
362.1509	6.71	-1.6	0.002	61.1	$C_{18}H_{20}O_4N_3F$ (LEV)	N.A.	9.5	-0.211
378.1461	6.79	198	0.013	23.7	$C_{18}H_{20}O_5N_3F$	+1 O	9.5	0.125
379.1481	6.79	237	0.014	4.8	M+1 peak of *m/z* 378.14606	N.A.	N.A.	0.955

[a] Median values; [b] Median retention time; [c] Percentage of LEV median peak height in LEV:O_3 1:0 samples. Values for LEV are shown to indicate differences in mass and composition. N. A.: not applicable.

Out of these two potential OTPs, $C_{18}H_{20}O_5N_3F$ has been previously reported and was identified as LEV-*N*-oxide (Fig. 3) and is one of the major OTPs of LEV identified so far (De Witte et al. 2009). The other potential OTP of LEV, $C_{18}H_{18}O_4N_3F$ is puzzling since the observed formula difference with LEV, 2 H atoms, is difficult to interpret according to known O_3 chemistry and further analysis was performed and discussed in the Supplementary material.

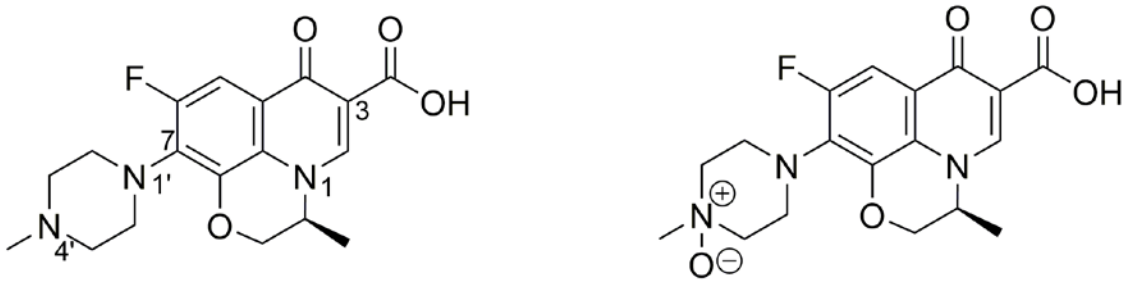

Figure 3. Molecular structure of LEV (left) and LEV-*N*-oxide (right) the major OTP identified in the LEV:O_3 2:1 samples.

In order to confirm the structure of LEV-*N*-oxide (neutral formula $C_{18}H_{20}O_5N_3F$), tandem mass spectrometry experiments were performed in the UHPLC-QqTOFMS system. The product ion scan of *m/z* 378 showed three major ions (Table 4). For product ion *m/z* 361.1445, only one formula within 10 mmu of mass tolerance atom number equal or inferior to the number elements

in LEV-*N*-oxide is possible: $C_{18}H_{20}O_4N_3F^{\bullet+}$ ($\Delta m/z$=1.264 mmu). This indicates a loss of $^{\bullet}OH$. This type of loss has been previously observed with *N*-oxides in ESI+ (Ma et al. 2005) and hints the presence of an O atom bonded to a N atom in the structure of this OTP. Molecular orbital calculations performed by De Witte et al. (2009) showed that the N′$_4$-atom in piperazinyl functional group of LEV (Fig. 3) is the most likely site of reaction towards an electrophilic attack by O_3, therefore the O atom added after ozonolysis must be bonded to this nitrogen atom. As for the second product ion, *m/z* 317.1544, two possible formulas within the limits specified previously are possible: $C_{17}H_{20}O_2N_3F^{\bullet+}$ or $C_{17}H_{21}O_4N_2^{+}$. They indicate losses of $CHO_3^{\bullet}$ or CONF, respectively. Since the F atom in fluoroquinolones is usually lost after collision-induced dissociation as HF (Volmer et al. 1997), the first loss is more likely and is probably produced by the combined loss of $^{\bullet}OH$ followed by CO_2. A loss of CO_2 indicates also the presence of a carboxylic acid functional group (Volmer et al. 1997). Finally, among the four formulas possible for the product ion *m/z* 254.1058, none are characteristic of a functional group of LEV-*N*-oxide. This product ion is probably formed after gas phase bond cleavages and rearrangement reactions that we did not try elucidate, since it was not in the scope of the present work.

TABLE 4. Analysis of the tandem mass spectrum of the precursor ion of LEV-*N*-oxide, *m/z* 378.

Product ion (*m/z*)	**Possible ion formulas and mass accuracy in mmu**	**Neutral loss**	**Functional groups characteristic of the loss**
361.1445	$C_{18}H_{20}O_4N_3F^{\bullet+}$ ($\Delta m/z$ =1.264 mmu)	$^{\bullet}OH$	*N*-oxide
317.1544	$C_{17}H_{20}O_2N_3F^{\bullet+}$ ($\Delta m/z$ =1.264 mmu)	$CHO_3^{\bullet}$ (CO_2+$^{\bullet}OH$)	Carboxylic acid, N-oxide
	$C_{17}H_{21}O_4N_2^{+}$ ($\Delta m/z$ =1.264 mmu)	CONF	?
254.1058	$C_{15}H_{14}O_2N_2^{\bullet+}$ ($\Delta m/z$ =1.264 mmu)	$C_3H_7FNO_3$	?
	$C_{15}H_{13}N_3F^{+}$ ($\Delta m/z$ =1.264 mmu)	$C_3H_8O_5$	?
	$C_{17}H_{15}OF^{\bullet+}$ ($\Delta m/z$ =1.264 mmu)	$CH_6O_4N_3$	?
	$C_{16}H_{13}ONF^{+}$ ($\Delta m/z$ =1.264 mmu)	$C_2H_8O_4N_2$	?

The structure of LEV-*N*-oxide can be used to predict its antimicrobial activity based on the known relationship between the structure and the antimicrobial activity of quinolones. The mode of action of quinolones is based on the selective inhibition of bacterial DNA synthesis (Mitscher 2005). Inside the bacterial cell, quinolones induce the formation of a molecular ternary complex, composed of DNA, a type II topoisomerase (an enzyme essential for DNA functioning) and two quinolone molecules. This ternary complex which disturbs basic bacterial DNA functions such as transcription, replication and reparation, is held together by multiple interaction sites between

the quinolones, the enzyme and DNA (Mitscher 2005; Drlica et al. 2009). Therefore, slight modifications of the structure of LEV such as the formation of a new bond between oxygen and the N′$_4$-atom, as in LEV-*N*-oxide, can disrupt the formation of the ternary complex and thus decrease antimicrobial activity, as observed with the bioassays.

Conclusion

This study demonstrated that LEV-*N*-oxide, the major OTP generated after addition of a low dose of O_3 (molar ratio LEV:O_3 2:1, equivalent to 6.7 mg L^{-1} of LEV and 0.4 mg L^{-1} of O_3) does not possess the same antimicrobial activity as LEV. After optimization of the growth medium, the broth microdilution bioassay showed that ozonolysis of LEV with that amount of O_3 resulted in a significantly lower (p <0.001) antimicrobial activity compared to LEV samples not exposed to ozone (LEV:O_3 1:0) as measured by the EDF_{50}. According to the broth microdilution assay, percent loss of antimicrobial activity of the LEV:O_3 2:1 solution was (48 ± 16)%, which is in agreement, considering experimental error, with the diminution of the concentration of LEV after ozonolysis (36 ± 3)%, measured by UHPLC-LIT-OrbitrapMS.

The presence of LEV-*N*-oxide in LEV:O_3 2:1 samples was detected using a validated and improved method of identification of transformation products based on the differential analysis of liquid chromatography-high resolution mass spectrometry data with XCMS online. Tandem mass spectrometry experiments with the precursor ion of LEV-*N*-oxide revealed the presence of two functional groups, *N*-oxide and carboxylic acid, in its molecular structure that partially confirm the structure proposed by De Witte et al. (2009).

Ozonolysis of other quinolones even at low doses of O_3 may give similar results in terms of reduction of antimicrobial activity given the effect that small changes in their molecular structure have on their antimicrobial activity. More studies are necessary to confirm the absence of biological activity of LEV-*N*-oxide and other OTPs, since a new mode of toxic action could arise as a consequence of the changes caused in the molecular structure of the parent compounds after reaction with O_3.

Acknowledgments

This study was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) through a Discovery Grant awarded to Prof. Viviane Yargeau and a start-up fund attributed to Prof. Pedro A. Segura by the Faculty of Sciences of Université de Sherbrooke.

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