Sonochemical degradation of antibiotics from representative classes- Considerations on structural effects, initial transformation products, antimicrobial activity and matrix

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Abstract

In this work, the sonochemical treatment (at 354 kHz and 88 W L^{-1}) of six relevant antibiotics belonging to fluoroquinolones (ciprofloxacin and norfloxacin), penicillins (oxacillin and cloxacillin) and cephalosporins (cephalexin and cephadroxyl) classes was evaluated. Firstly, the ability of the process to eliminate them was tested, showing that sonodegradation of these antibiotics is strongly chemical structure-dependent. Thus, correlations among initial degradation rate of pollutants (Rd), solubility in water (Sw), water-octanol partition coefficient (Log P) and topological polar surface area (TPSA) were tested. Rd exhibited a good correlation with Log P (i.e., the hydrophobicity degree of antibiotics). The considered penicillins had the fastest elimination and from the constitutional analysis using Lemke method was clear that the functional groups arrangement on these antibiotics made them highly hydrophobics. The penicillins were degraded closer at cavitation bubble than the fluoroquinolones or cephalosporins. The investigation of degradation products showed that sonogenerated hydroxyl radical primary attacked the β-lactam ring of cloxacillin and cephalexin, whereas on norfloxacin induced a decarboxylation. On the other hand, the evolution of antimicrobial activity was also followed. It was evidenced the process capacity to remove antimicrobial activity from treated solutions, which was associated to the transformations of functional groups on antibiotics with important role for interaction with bacteria. Additionally, degradation of antibiotics having the highest (the most hydrophobic, i.e., cloxacillin) and lowest (the most hydrophilic, i.e., cephadroxyl) Rd, was performed in synthetic matrices (hospital wastewater and seawater). Ultrasound degraded both antibiotics; for

cloxacillin in such waters higher eliminations than in distilled water were observed (probably due to a salting-out effect exerted by matrix components). Meanwhile, for cephadroxyl a moderate inhibition of degradation in hospital wastewater and seawater respect to distilled water was found, this was related to competition by hydroxyl radical of the other substances in the matrices. These results show the quite selectivity of high frequency ultrasound to eliminate antibiotics form different classes even in complex matrices.

Keywords: Antibiotic structure differences; Advanced oxidation process, Matrix effects, Water treatment; Pollutants degradation; Ultrasound.

1. Introduction

Antibiotics are commonly utilized for treatment and prevention of deadly infections in humans and animals. The antibiotics over-prescription in medical centers and increasing uses in agriculture and livestock are pushing the development of antibiotic resistant microorganisms, which represents a global environmental and health concern [1,2]. Nowadays, effluents from municipal wastewater treatment plants (MWTP) and hospital wastewaters (HWW) are considered among the main sources of antibiotics discharge into the environment [3,4]. This is because most of these substances are recalcitrant to conventional water treatments [5]. Thus, the application of complementary processes able to efficiently eliminate antibiotics from water is urgently required.

The advanced oxidation processes (AOP) are alternatives, which take advantage of short lived and highly reactive radical species, that have been successfully utilized for organic pollutants degradation [6]. Among AOP, the sonochemical treatment has shown a high proficiency in remediation of water contaminated with pharmaceuticals [7–10]. Sonochemistry is based on the acoustic cavitation process: bubbles formation and growth until reaching a critical size at which they violently collapse generating small hot spots with singular conditions of pressure (~1000 atm) and temperature (~5000 K) [11]. As a consequence, hydroxyl radicals are generated from water molecules and oxygen rupture (Eq. 1-4). Also, hydrogen peroxide can be formed by recombination of hydroxyl radicals (Eq. 5).

$H_2O +))) \rightarrow \bullet H + \bullet OH$ (Eq. 1)

$O_2 +))) \rightarrow 2 \bullet O$ (Eq. 2)

$H_2O + \bullet O \rightarrow 2 \bullet OH$ (Eq. 3)

$O_2 + \bullet H \rightarrow \bullet O + \bullet OH$ (Eq. 4)

$2 \bullet OH \rightarrow H_2O_2$ (Eq. 5)

On the other hand, fluoroquinolones and β-lactams (cephalosporin and penicillins) are in the top of antibiotics consumption [7,12,13]. In fact, they are frequently found in the MWTP effluents [3,14] and HWW (which is one of the main contributors of antibiotic to municipal wastewater, [3,15]). Considering the structural diversity of such pollutants, the present work was focused on the evaluation of the chemical structure effect of representative fluoroquinolones, penicillins and cephalosporins antibiotics during their elimination by sonochemistry. Initially, rates of antibiotics degradation and hydrogen peroxide accumulation were established. Besides, the closeness of antibiotics to cavitation bubble was determined by the degradation inhibition degree in 2-propanol presence. Then, correlations among initial degradation rate of pollutants and diverse molecular properties of antibiotics were studied. Also, the primary transformation products were identified and the structural control on sonochemical degradation was discussed under the constitutional approach (Lemke method). After that, the evolution of antimicrobial activity during treatment was tested and related to antibiotic modifications. Finally, degradations of both the most hydrophobic and hydrophilic antibiotics in complex matrixes were assessed.

2. Experimental

2.1 Reagents and reaction system

Fluoroquinolones: ciprofloxacin (CIP) and norfloxacin (NOR) were provided by Laproff laboratories (Medellín, Colombia). Cephalosporins: cephalexin (CPX) and cefadroxil (CDX) were provided by Syntopharma laboratories (Bogotá, Colombia). Penicillins: oxacillin (OXA) and cloxacillin (CLX) were purchased from Syntopharma laboratories and Sigma-Aldrich (St Louis, USA), respectively. Sodium chloride, calcium chloride dihydrate, potassium chloride, ammonium chloride, sodium sulfate, potassium dihydrogen phosphate, urea, acetonitrile, 2-propanol and nutrient agar were provided by Merck (Darmstadt, Germany). Peptone, meat extract and potato dextrose agar were purchased from Oxoid (Basingstoke, England). Formic acid was provided by Carlo-Erba (Val de Reuil, France). All chemicals were used as received. The solutions were prepared using distilled water and the experiments were carried out at least by duplicate.

For UHPLC-QTOF MS analysis, HPLC-grade water was obtained by purifying demineralized water using a Milli-Q system from Millipore (Bedford, MA, USA). HPLC-grade methanol (MeOH), HPLC-grade acetonitrile (ACN), formic acid (HCOOH), acetone, and sodium hydroxide (NaOH) were acquired from Scharlau. Leucine enkephalin was purchased from Sigma-Aldrich (St. Louis, MO, USA).

A Meinhardt ultrasound reactor with capacity of 500 mL was used. The reactor was operated at 88 W L^{-1} of actual ultrasonic power density (measured by calorimetric

118 method [16]). Reactor temperature was controlled at 20°C using a Huber
119 Minichiller. It should be mentioned that the energy consumption by the ultrasound
120 reactor plus the cooling system (minichiller) is 0.514 kWh. On the other hand, the
121 antibiotics were individually treated (300 mL, at 40 µM and pH 6.5). These
122 experimental conditions were selected based on previous works (details about it
123 are indicated in Text SM1).

124 **2.2 Analyses**

125 Antibiotics degradations were followed using a UHPLC Thermoscientific Dionex
126 UltiMate 3000 instrument equipped with an Acclaim™ 120 RP C18 column (5 µm,
127 4.6 x150 mm) and a diode array detector as previously reported [17]. Accumulation
128 of hydrogen peroxide was estimated by iodometry method [18]. The antimicrobial
129 activity (AA) was determined by analyzing the inhibition zone in the agar diffusion
130 test [19].

131 The primary transformation products were elucidated by liquid chromatography
132 coupled with quadrupole-time of flight mass spectrometry (UHPLC-QTOF MS).
133 Briefly, a Waters Acquity UHPLC system (Waters, Milford, MA, USA) coupled to a
134 hybrid quadrupole-orthogonal acceleration-TOF mass spectrometer (XEVO G2
135 QTOF, Waters Micromass, Manchester, UK), using an orthogonal Z-spray-ESI
136 interface was used, operating in both positive and negative ionization modes. For
137 further details about the conditions employed, see Supplementary Material (Text
138 SM2).

For MS^E experiments, two acquisition functions with different collision energies were selected. The low energy function (LE), selecting a collision energy of 4 eV, and the high energy (HE) function, with a collision energy ramp ranging from 15 to 40 eV, were used in order to obtain a greater range of fragment ions. Additional MS/MS experiments at different collision energies (10, 20, 30, 40 and 50 eV) were also performed to helping the elucidation process. Mass data was acquired with MassLynx v 4.1 (Waters).

3. Results and discussion

3.1 Rate of antibiotics degradation and hydrogen peroxide accumulation

As previously reported by several authors, the highest sonochemical production of hydroxyl radical is obtained at frequencies around 300 kHz [20]. Therefore, the ultrasonic reactor was operated at 354 kHz (88 W L^{-1}) for the pollutants treatment. **Fig. 1A** presents the comparison of initial degradation rate (Rd) for the six antibiotics. It can be observed that the compounds belonging to the penicillin class (CLX and OXA) are faster degraded than fluoroquinolones (NOR and CIP) or cephalosporins (CPX and CDX) by the sonochemical system.

In addition to Rd, the rate of accumulation of hydrogen peroxide (Ra), which is an indirect indicator of •OH formation, in presence and absence of antibiotics (BK) was also analyzed (**Fig. 1B**). In all of cases, the Ra value in antibiotics presence is lower than in the blank (BK). Moreover, it can be noted that the antibiotics with the lowest accumulation of H_2O_2 (CLX and OXA), presented the highest rate of

degradation. Interestingly, the Rd and Ra are correlated showing that a higher antibiotic degradation implies a decreasing accumulation of H_2O_2. This suggests that the reaction of antibiotics (An) with the sonogenerated hydroxyl radical (Eq. 6) reduces the recombination of radicals to produce hydrogen peroxide (Eq. 5) [21].

$$\bullet OH + An \rightarrow Products \qquad \text{(Eq. 6)}$$

At this point, it is clear the ability of the ultrasound to degrade antibiotics from three different classes. However, as previously indicated, the six antibiotics showed a differential response to the sonochemical treatment. The explanation of such fact should take into account the structural and chemical characteristics, which will be discussed in the next section.

3.2 Structural effects interpretation

The sonochemical system can be divided in three degradation zones: 1) the bulk of solution, 2) the bubble-solution interface and 3) the inner part of cavitation bubble [11]. As the evaluated antibiotics are not volatile molecules, the degradation depends on their proximity to the cavitation bubble. Thus, to study the antibiotics closeness to cavitation bubble, the inhibition degree of sonochemical degradation (IDS) by the addition of the well-known radical scavenger 2-propanol was evaluated [22]. A representative molecule from each antibiotic class was taken (Table 1). It was found that for NOR the inhibition was total, for CPX was 80% and for CLX was 55%. These results indicate that the penicillin is closer to cavitation bubbles (because its degradation is the least inhibited), whereas the

183 fluoroquinolone and cephalosporin are farther (in the bulk of solution). Therefore,
184 due to the higher proximity to the bubbles (where •OH is at high concentration),
185 CLX experiments a faster the degradation, which is coherent with the observed Ra
186 values in **Fig. 1A**.

187 To better understand the differences among the pollutants, possible relationships
188 among the initial degradation rate and the physico-chemical characteristics were
189 examined. Solubility in water (expressed as Log Sw), water-octanol partition
190 coefficient (Log P), and topological polar surface area (TPSA), which reflect
191 interaction of antibiotics with water and cavitation bubble, were considered. From
192 Fig. 2B can be noted the high relationship between initial degradation rate and Log
193 P of antibiotics. In fact, the correlation coefficient is R = 0.911. Meanwhile, Sw and
194 TPSA showed a low interrelation with Rd (Fig. 2A and 2B), having correlation
195 coefficient values of R = 0.588 and 0.129, respectively.

196 The octanol-water partition coefficient is a physico-chemical property related to
197 hydrophobicity and it is recognized that hydrophobic compounds tend to
198 accumulate at the cavitation bubble interface, in which they can have a high
199 interaction with hydroxyl radicals [23]. Thus, as hydrophobicity increases the rate of
200 degradation is higher. Consequently, the antibiotics with the highest Log P values
201 (i.e., CLX and OXA) are faster degraded than the others (CIP, NOR, CPX and
202 CDX).

203 The Log P of a compound can be estimated by addition of all contributions of
204 atom/fragment present in its chemical structure [24]. Although Log P is influenced

by geometrical, constitutional, electronic and electrostatic characteristics of molecules [25], the constitutional analysis (Lemke method, based on hydrophilic-lipophilic fragments computation) has been a useful tool to easy and fastly estimate hydrophilic-hydrophobic properties of organic substances [26]. Thus, to better interpreting the structural and Log P differences of the tested antibiotics, the Lemke methodology was applied. As Table 2 shown, the hydrophobic and hydrophilic contribution of constitutional fragments on each antibiotic are pondered. Subsequently, the total hydrophobicity is estimated by subtraction of the hydrophilic contribution from the hydrophobic.

It can be noted that CLX and OXA have a predominance of hydrophobic parts (e.g., chlorine, phenyl-isoxazolyl and aliphatic moieties); this can explain their highest Log P values and sonochemical degradation. Meanwhile, NOR, CIP, CPX and CDX have other functional groups (e.g., hydroxyl, amines, or ketones) on their structures, which make these molecules less hydrophobics (i.e., more hydrophilics). At this point, it is important to clarify that the Lemke approach is a basic guide for interpretation of structural effect. However, if exact numbers are required, methodologies including geometrical, electronic and electrostatic criteria must be applied

3.3 Primary antibiotics transformation, removal of the antimicrobial activity and prediction of toxicity changes

226 The primary transformations of antibiotics during the sonochemical treatment were
227 studied through establishing the initial degradation products by UHPLC-HRMS. For
228 such purpose, CLO, CPX and NOR (a representative antibiotic of each class) were
229 selected. Table 3 contains the elucidated transformation products (TPs). In the
230 case of CLO, three initial products were found, whereas for NOR and CPX a by-
231 product from each one was identified. This result is coherent with the indicated in
232 section 3.2, as CLO is closer to the cavitation bubble than the other antibiotics, and
233 therefore it can react more efficiently with sonogenerated hydroxyl radical and
234 consequently more initial degradation products are observed. MS/MS data for both
235 parent compounds and identified TPs are shown in Supplementary Material (SM2-
236 SM15).

237 According to the identified TPs (Table 3), the primary attacks of •OH to CLO seem
238 to occur on two sites: 1) the β-lactam moiety (DP1 and DP2, two isomers), this ring
239 is highly reactive due to its strained linkages, which does labile the carbonyl-
240 nitrogen bond [32]; 2) the central secondary amide (DP3), where the radical attack
241 is favored by inductive and resonant effects generated by the oxazolyl substituent
242 [35], such effects leave the electrons on the nitrogen more available to react (see
243 Figure SM1, in Supplementary Material). Interestingly, previous works on
244 sonochemical elimination of oxacillin have shown degradation products analogous
245 to those obtained for CLX [19,27]. Besides, the primary transformation product of
246 CPX (DP5) comes from the opening of β-lactam ring, similar to that observed for
247 CLX (see DP1 and DP2). These results evidence the high susceptibility of the β-
248 lactam moiety to transformations by sonogenerated hydroxyl radical.

DP4 indicates that the initial NOR degradation proceeds via decarboxylation. Although in typical AOPs, such as TiO_2-photocatalysis and photo-Fenton, the primary •OH attacks are on the piperazyl ring of NOR (or CIP) [28,29], here we observed the rupture of carboxylic group. This difference could be associated to the nature of sonochemical process (having three differential degradation zones), which may favor other routes. In fact, in the sonochemical degradation of the pharmaceuticals ciprofloxacin and ibuprofen, the decarboxylation joined to hydroxylation, dehalogenation, demethylation were also reported as major elimination pathways [30].

Many works on the degradation of organic pollutants have shown that ultrasound induces oxidative pathways on substances (as observed for the antibiotics (Table 3)), but this process has low mineralizing ability [8,9,27,30]. In fact, a previous paper about the sonochemical treatment of Oxacillin (OXA, one of the antibiotics with highest degradation rate here) demonstrated that even after 6h of process application there was no mineralization [27]. Although ultrasound alone is not mineralizing, its combination with others AOP such as Fenton, TiO_2-photocalaysis or ozonation may lead to pollutants transformation into carbon dioxide, water and inorganic ions [30–32]. Thus, considering the limitation of sonochemistry in the mineralization other parameters to verify the process efficiency must be evaluated.

In the case of antibiotics degradation, it is important to determine the residual antimicrobial activity (AA) because in some cases the initial molecule may be degraded, but the transformation products continue generating AA [17]. Therefore, the evolution of AA for the three representative antibiotics was followed (**Fig. 3**).

For both CPX and NOR, the antimicrobial activity was removed at short treatment time (75 min). In contrast CLX, which experienced a faster degradation, required 120 min of ultrasound application to remove its associated AA. As the evolution of AA can be related to the antibiotics transformations, the faster antimicrobial activity elimination suggests that the degradation products of CPX or NOR are less active than the by-products from CLX.

From DP4 is noted that the sonochemical process initially removes the carboxylic group on NOR. Such moiety is essential for the antibiotic binding to DNA-gyrase. It is well-known that fluoroquinolones inhibit the bacterial DNA synthesis by blocking the DNA-gyrase (Topoisomerase II) and Topoisomerase IV enzymes [33,34]; thus, the removal of carboxylic group on NOR leads to the decreasing of AA. Although here is presented the primary transformation product, during the whole period of process application, it may occur the formation of other substances, in which the quinolone core is modified, contributing to the AA elimination.

Meanwhile, DP1, DP2, DP3 and DP5 show the elimination of β-lactam ring from the penicillin and cephalosporin. This part of such antibiotics plays the main active role against bacteria [35,36]; consequently, its elimination produces the reduction of antimicrobial activity. In fact, different works on the treatment of penicillins and cephalosporins have also evidenced that the AA of treated solutions toward bacteria is removed by the β-lactam ring breakdown [37,38]. It can be pointed out that the removal of antimicrobial activity evidenced the ability of sonochemistry to diminish the development of antibiotic resistance.

Other important factor to be considered during treatment of water pollutants is toxicity changes. Recently, it was demonstrated the direct relationship between Log P and aquatic toxicity [39]. Indeed, widely used toxicity prediction tools such as ECOSAR, ADMET, CADRE-AT, KATE or TEST rely on octanol-water partition coefficient [40]. Thereby, Log P could be assumed as an indirect indicator of aquatic toxicity. Then, to obtain a rough idea on the toxicity changes, it was calculated the variation of octanol-water partition coefficient (ΔLog P) for the identified primary transformations products (Table 3).

The ΔLog P of the initial transformation products from CLX (DP1, DP2 and DP3) and CPX (DP5) were negative values, which indicates that these new substances are less lipophilic than the parent antibiotics. In contrast, for the product DP4 is positive indicating that it has higher lipophilicity than NOR. The negative ΔLog P values for degradation products can be associated to the generation of polar functional groups such carboxylic acid (DP1, DP2 and DP5) or primary amide (DP3) by the sonochemical action. Whereas a positive ΔLog P is related to removal of polar functional groups (e.g., the decarboxylation of NOR, DP4) [40].

The lipophilicity is determinant for the toxicity and bioaccumulation of compounds [41]. Thus, a low lipophilicity points out an inferior affinity of substances for the lipid bilayer of cells [42]. Therefore, in the case of the DP1, DP2, DP3 and DP5, it could be expected an aquatic toxicity lower than for the parent antibiotics. A contrary situation is expected for DP4, which is more lipophilic than NOR. Here, it should be indicated that this information on toxicity is a reasonable prediction based on the primary structural modifications of antibiotics. However, as final solutions of longer

317 treatment times may contain other products in addition to the initially identified,
318 experimental analyses of aquatic toxicity using biological models (e.g., *Vibrio*
319 *fishery* or fishes) must be applied to guarantee a beneficial impact on treated water
320 on the environment (topic to be studied in further works).

321

322 **3.4 Treatment of complex matrices**

323 To study the process application to complex matrices, simulated seawater and
324 hospital wastewater (Table SM1) were considered. These matrices were selected
325 taking into account that hospital wastewater is recognized as a constant source of
326 antibiotics [15,43] and historically in many coastal cities the wastewater has been
327 directly disposed on sea [44,45]. Furthermore, two antibiotics were used in this
328 part: the fastest (CLX) and the slowest (CDX) degraded by the sonochemical
329 process. **Fig. 4** presents the ratio between degradation rate in the matrices and in
330 distilled water (**ρ:** *Rd in Matrix / Rd in distilled water*). Interestingly, the elimination
331 of cloxacillin in the matrices was higher than in distilled water. On the contrary,
332 cephadroxyl degradation was inhibited by the matrix components.

333 A careful revision of matrices composition (Table SM1) indicates that the
334 substances present in such waters are inorganic ions and urea, which have mainly
335 hydrophilic nature. Thus, due to the high hydrophobic character of CLX, the matrix
336 components did not affect the antibiotic elimination. Indeed, because of such
337 chemical differences a *salting-out* effect can be induced on CLX. This means that
338 in presence of inorganic anions or urea, which are at much higher concentrations

than the antibiotic (Table SM1), the water molecules surround more effectively these species, thereby pushing more molecules of the target compound towards the interfacial zone of the cavitation bubbles [46]. As a result, the degradation of CLX increased (i.e., **ρ** >1).

For cephadroxyl, the matrix components would compete by hydroxyl radicals. Because of its hydrophilic nature, CDX is far to the cavitation bubble and at concentration much lower than such components. Hence, the pollutant degradation in seawater and hospital wastewater was found to be lower than in distilled water. However, it can be remarked a moderate competition effect (i.e., 0.7 < **ρ** < 1) indicating that even at such matrix conditions the sonochemical process is applicable.

At this point, it should be indicated that ultrasound has some advantages and limitations for the elimination of pollutants. Sonochemistry can selectively degrade antibiotics, which is an important advantage over others AOP [19]; in contrast, ultrasound operation demands higher electrical consumption. Processes such as UVC photolysis or UVC/persulfate are more affected than ultrasound by matrix components for antibiotics degradation [17]. On the other hand, anodic oxidation using Ti/IrO_2 as anode and sodium chloride as supporting electrolyte induces the formation of chlorinated degradation products in the treatment of fluoroquinolones [47], which may be toxic substances. Meanwhile, during the treatment of these antibiotics with high frequency ultrasound such chlorinated transformation products were not found here; which is other advantage of the sonochemical process. Additionally, HWW matrix strongly retards the electrochemical elimination of

362 antibiotics, whereas using ultrasound, in cases as CLX; this matrix can even
363 enhance the pollutant degradation. Furthermore, it should be also mentioned that
364 seawater matrix accelerates the antibiotic elimination by anodic oxidation [48],
365 while in sonochemistry this matrix exerts a dual effect (i.e., acceleration or
366 inhibition) depending on the antibiotic nature (Figure 4). All these comparative
367 aspects highlight the interesting potentialities of high frequency ultrasound toward
368 future applications in the removal of antibiotics in complex matrices.

369

370 **4. Conclusions**

371 This study shows that the application of ultrasonic waves leads to degradation of
372 antibiotics from diverse classes. The rate of pollutants elimination (Rd) was
373 strongly dependent of pollutant closeness at the cavitation bubbles, and as the
374 considered antibiotics are non-volatile molecules, the closeness to the bubbles was
375 determined by their hydrophobicity. In fact, the Rd value correlated well with the
376 Log P value of the antibiotics. Moreover, the utilization of Lemke methodology
377 allowed estimate the contribution of constitutional functional groups to antibiotics
378 hydrophobicity, which explained the structural differences reflected in both Log P
379 values and sonochemical degradation rate.

380 The elucidation of primary transformation products of representative pollutants
381 (cloxacillin, norfloxacin and cephalexin) indicated that sonogenerated hydroxyl
382 radical modified the penicillin and cephalosporin cores from the β-lactam
383 antibiotics, whereas the fluoroquinolone was decarboxylated. Additionally, the

antimicrobial activity removal depended on the structural changes of antibiotics under treatment. Finally, the degradation in seawater and hospital wastewater evidenced that highly hydrophobic antibiotics could be selectively eliminated in complex matrices, whereas elimination of hydrophilic pollutants can experiment moderate inhibition. These facts illustrate the potential of sonochemical process for the treatment of real-world waters.

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