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R. Masmoudi

Universite de Batna: Universite Hadj Lakhdar Batna

S. Khettaf

Universite Hadj Lakhdar Batna

A. Soltani

Universite Hadj Lakhdar Batna

A. Dibi

Universite de Batna: Universite Hadj Lakhdar Batna

L. Messaadia

Universite de Jijel

Messaoud BENAMIRA (✉ benamira18@yahoo.fr)

Universite de Jijel <https://orcid.org/0000-0002-2460-9457>

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Cephalexin degradation initiated by OH radicals: Theoretical prediction of the mechanisms and the toxicity of byproducts

R. Masmoudi¹, S. Khettaf¹, A. Soltani¹, A. Dibi¹, L. Messaadia² [0000-0003-2542-0098]

, M. Benamira^{3,*} [0000-0002-2460-9457]

¹Laboratory of Chemistry and Environmental Chemistry LCEE, Department of Chemistry, Faculty of Material Sciences, University of Batna 1, Batna, 05000, Algeria

²Laboratory of Applied Energy and Materials (LEAM), University of Jijel, BP. 98, Ouled Aissa 18000, Jijel, Algeria

³Laboratory of Interaction Materials and Environment (LIME), University of Jijel, BP. 98, Ouled Aissa 18000, Jijel, Algeria

*Corresponding author: redhachem@yahoo.fr, m_benamira@univ-jijel.dz.

Abstract: In this work, density functional theory is used to study the local reactivity of cephalexin (CLX) to radical attack and explain the mechanism of the reaction between CLX and hydroxyl radical attack leading to degradation byproducts. The reaction between •OH and CLX is supposed to lead to either an addition of a hydroxyl radical or an abstraction of a hydrogen. The results showed that the affinity of cephalexin for addition reactions increases as it passes from the gas to the aqueous phase and decreases as it passes from the neutral to the ionized form. Thermodynamic data confirmed that OH addition radicals (R_{add}) are thermodynamically favored over H abstraction radicals (R_{abs}). The ecotoxicity assessments of CLX and its byproducts are estimated from the acute toxicities toward green algae, Daphnia and fish. The formation of byproducts is safe for aquatic organisms, and only one byproduct is harmful to Daphnia.

Keywords: Quantum chemical calculations; DFT; Advanced oxidation processes; Cephalexin; Hydroxyl radical; Wastewater treatment.

1. Introduction

Growing concentrations of antibiotics and their metabolites in aqueous environments have caused great concerns to human health and different ecological systems. Cephalexin, (6*R*,7*R*)-7-[(2*R*)-2-amino-2-phenylacetyl]amino]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (CLX), is a first generation cephalosporin used in human and veterinary medicine in the treatment of bacterial infections [1]. Cephalexin has a biotransformation rate

of only 10%, and the remaining 90% is excreted unchanged in the urine [2]. Cephalexin is frequently detected in the effluents of wastewater treatment plants (WWTPs) and exhibits very low biodegradability [3]. Consequently, the World Health Organization (WHO) warned about the presence of pharmaceuticals in drinking water and the potential implications on human health and the environment and classified cephalosporin antibiotics as emergent environmental contaminants [4]. WWTPs use advanced oxidation processes (AOPs) to remove contaminants from wastewater. This technique is based on the in situ generation of reactive oxygen species (ROS) and hydroxyl radicals ($\bullet\text{OH}$) through chemical processes that employ ozone and hydrogen peroxide combined with irradiation of UV light, electromagnetic radiation, and ultrasound [5-7]. The advantage of $\bullet\text{OH}$ is that it is highly reactive and nonselective with a large range of organic compounds [8]. There are numerous methods for the determination of local reactivity descriptors. The study of He et al. [2] located the reactive sites of cephalexin on the basis of frontier electron densities FED_{HOMO} and FED_{LUMO} for each site. The research established by Cao et al. [9] compared the different methods used for local reactivity determination, and these methods differed in their accuracy. The most accurate approach is the one proposed by Yang and Mortier [10] using the atom condensed Fukui function. The byproduct structures of CLX hydroxyl-mediated degradation have been reported by some authors [11-15], while the thermodynamic data and a mechanism of the degradation process are still uncovered. Previous studies indicate that the most common degradation pathways include hydroxylation, decarboxylation, demethylation and dealkylation. Another study claimed that the majority of oxidation intermediates result from OH attack on the CLX cephalosporin ring, where the intermediates undergo opening of the active site of the molecule responsible for the antibacterial activity. Another intermediate formation pathway involves the electrophilic addition of OH to the aromatic moiety [16]. Terminal degradation products such as lactic, acetic and propionic acids are rapidly further oxidized (mineralization processes). However, fumaric, oxalic and oxamic acids are hard to further oxidize. More recently, there has been a growing interest in predicting the photodegradation mechanism, kinetics, and ecotoxicological assessment of water emerging contaminants (EOCs) with the assistance of theoretical calculations. Several experimental studies have been conducted on cephalexin hydroxyl-mediated degradation, sonochemical processes [17], electrochemical advanced oxidation processes [16], photo-Fenton processes [18] and photocatalytic nanowire oxidation processes [2], but little is known about its degradation mechanism. Gathering information on the degradation mechanisms and pathways of cephalexin and the ecotoxicological impact of its byproducts has become an issue of great concern for the treatment of CLX and other

pharmaceutical pollutants.

2. Materials and methods

2.1. Quantum chemical calculations

All calculations were performed with the Gaussian 16 program package. Geometry optimization was conducted using the DFT/B3LYP/6-31+G(d) basis set [19]. Stationary points were characterized by frequency analysis where the free energy and thermal enthalpy were obtained at 298 K. Polarizable continuum model (CPCM) was used to study the effect of the solvent environment, i.e., water ($\epsilon = 78.36$) [20]. The pKa of cephalexin was calculated in this work using the ChemAxon Marvin Sketch software (ChemAxon Kft, Budapest, Hungary). Its value indicated in the work of some authors was 2.56 for the carboxylic function and 6.88 for the primary amine function [21]. Since degradation takes place in a medium at pH = 7 (for water), the degradation of CLX was modeled into neutral and anionic forms in both gas and aqueous phases. From the values of the charges of neutral cephalexin $q(N)$, the cation and the corresponding anion $q(N - 1)$, $q(N + 1)$, respectively, the Fukui indices for electrophilic, nucleophilic and radical attacks, i.e., f_k^+ , f_k^- and f_k^0 , respectively, are calculated as follows [22]:

$$f_k^+ = q(N + 1) - q(N) \text{ for nucleophilic attack} \quad (1)$$

$$f_k^- = q(N) - q(N - 1) \text{ for electrophilic attack} \quad (2)$$

$$f_k^0 = \frac{1}{2} [q(N + 1) - q(N - 1)] \text{ for radical attack} \quad (3)$$

Eq. (5) and Eq. (7) were used to calculate the stability of the generated radicals of OH addition and H abstraction, respectively [23,24]:

$$\Delta G_{rxn} = G(R_{add}\bullet) - G(\bullet\text{OH}) - G(\text{CLX}) \quad (5)$$

$$\Delta G_{rxn} = G(\text{H}_2\text{O}) + G(R_{abs}\bullet) - G(\bullet\text{OH}) - G(\text{CLX}) \quad (7)$$

In addition to the above measures, the standard radical stabilization energies ($RSE = \Delta H_{298}^0$) of the produced radicals were calculated at 298 K using Eq. (9), where $R\bullet$ is the concerned radical.

$$\Delta H_{298}^0 = \Delta_f H_{298}^0(CH_3\bullet) + \Delta_f H_{298}^0(R-H) - \Delta_f H_{298}^0(CH_4) - \Delta_f H_{298}^0(R\bullet) \quad (9)$$

ΔH is the enthalpy change of the reaction. In the case where it corresponds to a positive value, $R\bullet$ is expected to be more stable than $CH_3\bullet$; otherwise, $R\bullet$ is expected to be less stable than $CH_3\bullet$.

The Gibbs free energies of fragmentation help to identify the most suitable fragmentation pathways of CLX/CLX^- , which are calculated using Eq. (10):

$$\Delta G_{rxn} = (G(\text{fragment 1}) + G(\text{fragment 2})) - G(CLX) \quad (10)$$

2.2. Ecotoxicity assessment

The ecotoxicological impact of cephalexin and its degradation byproducts were estimated for three tropical levels (fish, green algae and *Daphnia*) in terms of both efficient and lethal concentrations. Ecological Structure Activity Relationship software (ECOSAR) was used in this study [25]. ECOSAR Class Program is a computerized predictive system used to predict the aquatic toxicity. The program estimates a chemical's acute toxicity and chronic toxicity to aquatic organisms, such as fish, aquatic invertebrates, and aquatic plants, by using computerized Structure Activity Relationships (SARs). EC_{50} refers to the pollutant level of the tested compound that could inhibit 50% growth of green algae after 96 h of exposure time. LC_{50} represents the concentration of the tested pollutant for 50% death of fish and *Daphnia* after 96 and 48 h of contact times, respectively.

3. Results and discussion

3.1. Initial reaction of CLX with $\bullet OH$

In the aqueous phase, cephalexin can exist in six forms depending on the pH of the solution (Fig. 1). The ChemAxon Marvin Sketch software was used to calculate the pK_a and to predict which structure of acid or base will dominate in the pH range of 0–14. In this diagram of the

species repartition presented in terms of molar fractions (%), six forms were observed. In the first region (pH range of 0-4), the predominant species is C, with molar fractions of 99.91%. The first deprotonation takes place at the OH of carboxylic acid, with a relative pKa value of 3.26 (Fig. 2). The second region is described by the B species from pH 7 to 12 with molar fractions of 99.09%. In this case, deprotonation took place at the primary amine NH₂ functional group (pKa = 7.23), and these results are in good agreement with the literature [26,27]. In the third region, from pH=4 to 7, the most important species is D in a zwitterion form with a molar fraction of 97.95%. The other species (E, F and G) existed in the pH region of 10-14 with molar fractions of 53.43, 12.40 and 94.97%, respectively.

Cephalexin has two functions mainly responsible for the acid-base behavior in the molecule, a carboxylic acid function and primary amine, and the degradation of cephalexin takes place in a medium at pH = 7 (for water). This allows us to model the molecule in both neutral and anionic forms. The solvent intervenes in the geometric parameters by influencing, mainly, the bonds of the aliphatic chain and, by a less remarkable effect, the bonds of the cephem ring, while the lengths of the bonds of benzene are not practically affected. The most stable conformers of the neutral and anionic forms are depicted in Fig. 3.

Advanced oxidation products (AOPs) produce powerful nonselective oxidant agents, mainly •OH radicals, which interact with contaminant molecules to initiate the degradation process [7]. Information on the behavior of cephalexin in the presence of •OH radicals is first required to determine the most likely sites in view of radical attack. The reactivity of cephalexin toward •OH is supposed to be either by hydrogen abstraction or hydroxyl addition. The concerned sites on cephalexin molecules are either hydrogen atoms, in which the outcome of their interaction with hydroxyl radicals is an abstraction reaction, or unsaturated carbon atoms, in which the result of their interaction with hydroxyl radicals is an addition reaction [28]. The reactivity of the candidate sites is studied through atom condensed Fukui index analysis. The Fukui indices for neutral and ionized cephalexin in gas and aqueous phases are calculated with equations 1, 2 and 3; the results are summarized in Tables 1 to 4, respectively.

The Fukui indices were determined to predict the initial attack position during the photocatalytic degradation by reactive oxygen species (ROS). According to the Table1 (in gas phase), the most positive atoms with higher values of f_k^+ function is localized at C25 (0.46), C31 (0.04) and C40 (0.01). However, in aqueous phases (Table 2), C14 (0.07), H24 (0.17) and H21 (0.12) atoms showed the more positive values of f_k^+ function (atom abstraction by •OH). Consequently, the addition of •OH maybe took place on these atoms (Nucleophilic

attack). Nevertheless, the most negative part of f_k^- function in gas phase is localized in C25 (-0.14), C26 (-0.31) and C40 (-0.30). Whereas C14 (-0.12), C25 (-0.39), C26 (-0.29) and C40 (-0.15) are regarded as the most favorable sites for electrophilic attack in aqueous phases. In the case of ionized cephalixin in gas phases (Table 3), the atoms with more positive values of f_k^+ function are localized in C26 (0.13), C39 (0.03). However, C25 (0.23) and C26 (0.10) atoms in aqueous phases (Table 4) showed the most positive values and the best negative part of f_k^- function are localized in C26 (-0.68) and C31 (-0.10).

The reaction sites extracted from the analysis of atom condensed Fukui indices are circled (in red) and labeled, as shown in Figs. 4 and 5. To facilitate the recognition of the reaction site in future studies, abstraction sites are assigned and labeled with numbers (from 1 to 11), and additional sites are labeled with letters (from *a* to *e*), as shown in Figs. 6 and 7.

The free energies, enthalpies and radical stabilization energies of the abstraction and addition radicals are calculated with equations 7, 9 and 10, and the results are displayed in Tables 5-7, respectively. The most stable abstraction radical corresponds to R_{abs5} (-H38) with the highest stabilization energy (-0.08 kcal/mol). The R_{abs3} (-H9) and R_{abs2} (-H8/H10) radicals have almost the same stability, although the generation of the R_{abs3} (-H9) radical is more energetically favorable (para position) than that of H8 and H10 (meta position). The abstraction of an H proton from positions 10 (-H34) and 4 (-H13) is found to be more favorable than the abstraction of an H proton from the benzene ring; this is explained on the basis of the stabilization generated by the conjugation of the carboxyl radical and the benzyl radical in the second and third cases. The generation of these three radicals, R_{abs5} , R_{abs3} , and R_{abs2} , is exergonic and thermodynamically favorable compared to other abstraction reactions.

The abstraction radical R_{abs9} (-H28/29/30) is the most stable radical after the three preceding radicals; its formation is accompanied by energy absorption (endergonic), and its stability is also explained by conjugation with the double bond (allylic radical).

The R_{abs11} (-H20), R_{abs7} (-H21) and R_{abs8} (-H23/H24) radicals are the least stable. Their formations are the most endergonic, and their low stability is explained by the absence of stabilizing effects (conjugation and hyperconjugation) next to the radical.

In general, in the gas phase, abstraction radicals are more stable than those produced in the aqueous phase. The most stable addition radical is that corresponding to R_{add}^e (C31) with a

stabilization energy of 0.02 kcal/mol. This radical seems to be more unstable by moving from the neutral form *CLX* to the anionic form *CLX*⁻ (-0.94 kcal/mol).

The R_{add}^a (C25) radical is the least stable. Its formation is the most endergonic (0.08 kcal/mol), and its low stability is explained by the fact that the center of the radical is adjacent to the carboxyl group which exerts an inductive attracting effect on it (destabilizing effect). On the other hand, the addition of the OH radical to the C25 carbon (R_{add}^a (C25)) generates a more stable tertiary radical (because of the inductive donor effect of the three groups that surround it).

The addition of the OH radical to C14 (R_{add}^c (C14)) and C40 (R_{add}^d (C40)) ends with the breaking of the amide bond (hydrolysis reaction) which is cyclic in the first case and aliphatic in the second case [16,29]. The resistance of the cyclic amide to the 4-chain link is weaker than that of the aliphatic amide, resulting in a high tendency to react, i.e., -27.73 kcal/mol compared to 9.85 kcal/mol.

3.2. Fragmentation *CLX/CLX*⁻

The fragmentation of *CLX/CLX*⁻ is modeled to predict the possible fates of the generated radical intermediates and to predict the most likely degradation pathways. All fragmentation pathways are shown in Fig. 8. The reaction energies of all optimized fragments are calculated using Eq. (10) and presented in Table 8 in ascending order. Cleavage of the benzylamine moiety (entry 1) appears to be easier than the other fragmentation pathways. In the gas phase, deamination (entry 2) and demethylation (entry 3) have the same difficulty. Moreover, the losses of (OH) (entry 8) in neutral form and (O⁻) in anionic form are more endergonic than those of the others. The decarboxylation is less endergonic for *CLX*⁻ (71.84 (64.5) kcal/mol) than for *CLX* (76.74 (76.56) kcal/mol). The reactions in the aqueous phase are, in general, less endergonic than those in the gas phase (solvent effect). In the ionized form of cephalexin, demethylation (entry 3) and the loss of benzylamine carbamide (entry 5) are the most exergonic and appear to have almost the same spontaneity. The eliminations of benzylamine carbonyl (entry 7) and benzylamine carbamide (entry 5) and decarboxylation (entry 6) are very close in energy. According to Hammond's postulate [30], the structure of the transition state is similar for these three reactions. The negative values of Gibbs free energy ($\Delta G < 0$) mean that the pathway was exergonic processes (the release of energy from the reaction), indicating that the hydroxyl radical, •OH adding to this reaction site P3 (Fig.9) may be more favored by thermodynamics, in addition, the Cephalexin molecule was ruptured (Entry 5) and then formed a series of stable products P4, P5 and P6.

3.3. Proposed mechanism of degradation

Some authors have contributed to the study of the degradation byproducts of cephalexin, including theoretical studies, by using FED_{HOMO} and FED_{LUMO} to identify the reaction sites involved in radical attacks [2]. In this part, we are concerned with supporting our thermodynamic study by mechanistic research taking into account the abstraction, addition and $CLX/CLX^{\cdot-}$ fragmentation radicals previously indicated. Fig. 9 shows the mechanisms suggested in the generation of degradation byproducts (P1-P7).

The degradation byproduct P1 is formed by opening the cyclic amide after the addition of the OH radical to give the intermediate R_{add}^c , and the degradation reaction progresses to fragmentation (removal of OH) or decarboxylation to form P3 from P2 [2,16,29]. The byproduct P3 reacts with the OH radical at C14 (hydroxylation reaction) passing to the R_{add}^d radical followed by removing NH_2 (deamination) to generate P7. The other route is fragmentation (removal of benzylamine carbamide), which ends in dehydration to form P6. The theoretical results are well correlated with the previous experimental findings.

3.4. Toxicity estimation

The ecotoxicity of CLX and its byproducts to aquatic organisms was estimated at three trophic levels (fish, Daphnia and green algae) based on the European Union evaluation criteria for new chemical substances. The octanol-water partition coefficient ($\log K_{ow}$) was predicted to be 0.4 for CLX, and this value is in good correlation with that reported in the literature obtained experimentally (0.65) [28,31].

The toxicities of degradation byproducts were classified according to the Globally Harmonized System of Classification and Labeling of Chemicals (GHS) [28], as shown in Table 9. The toxicity was measured for the degradation byproducts (P1-P7) as well as the starting molecule (cephalexin); the results are presented in Table 10.

The toxicity of degradation byproducts was examined for the three trophic levels (fish, daphnia and green algae). For green algae, 4 degradation byproducts (P1, P3, P4, and P5) are less toxic than CLX, and P2, P6, and P7 are more toxic. In the case of Daphnia, 5 degradation byproducts (P1, P2, P3, P4, and P5) are less toxic than CLX, and P6 and P7 are more toxic. For fish, 4 degradation byproducts (P1, P3, P4, and P5) are less harmful than CLX, and P2, P6, and P7 are more toxic.

As degradation continues, the toxicity worsens; furthermore, the degradation byproducts P1, P3, P4 and P5 are less toxic than cephalexin to all three organisms, while the byproducts P2, P6 and P7 are always more toxic than cephalexin, except for Daphnia, where P2 is less toxic.

For green algae, all byproducts have an $LC50/EC50 > 100$ mg/l and are classified as nonharmful, with the exception of P7, which has an $EC50$ of 42.3 and is classified as harmful. CLX and most of the byproducts were found to be safe to aquatic organisms, and P7 is the only byproduct that is harmful to *Daphnia*. As a consequence, ZnO nanowire photocatalytic oxidation is a safe and clean process and is encouraged in the treatment of wastewater contaminated by cephalexin.

4. Conclusion

In this study, theoretical scale degradation of cephalexin was investigated in terms of reactivity, degradation thermodynamics, mechanism and ecotoxicological impact of degradation byproducts. It was revealed that the affinity of cephalexin toward addition reactions increases as it passes from the gas phase to aqueous phase and from the neutral to ionized form and decreases in the same way for the abstraction reactions. Thermodynamic data showed that the most favorable abstraction and addition radicals formed are those in which the reactant site is weakened by the inductive withdrawing effect, and the most stable radicals formed are those possessing larger mesomeric forms. The degradation byproducts (P1-P7) identified experimentally in the photocatalytic degradation of CLX by ZnO nanowires under simulated sunlight were verified in this computational work. The ecotoxicological impact of the degradation byproducts was clarified with ECOSAR software, where toxicity analysis revealed that CLX/ CLX^- was not toxic. Only its degradation product P7 was harmful, and the other oxidation byproducts (P1, P3, P4, and P5) were less toxic to fish and green algae. At the same time, P1, P2, P3, P4, and P5 were not toxic to *Daphnia*, and the rest of the byproducts were more toxic than cephalexin but still can be classified as nontoxic to the three organisms. Based on the collected information about the byproduct toxicities, we conclude that the photocatalytic degradation of cephalexin is a safe process for ecological organism studies. This study is the first to assess the safety of a photocatalytic process for the degradation of cephalexin based on a DFT approach toward the intermediate and byproduct structures of the degradation process.

Statements & Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose

Author Contributions

R. Masmoudi: Investigation, Methodology, Writing- Original draft. Writing - reviewing and editing. **S. Khettaf:** Methodology, Validation. **A. Soltani:** Formal analysis, Validation. **A. Dibi:** Conceptualization, Validation. **L. Messaadia:** Software, Formal analysis. **M. Benamira:** Supervision, Validation, Writing - reviewing and editing.

Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Code availability

Gaussian 16 program package: N° Licence G32214419065828W-4627N, G16W Full Version, 32-Bit, Rev: A.03 Front: 1.1. Version: Multiprocessor. GaussView6 for Windows, Rev: 6.0.16, 32-bit. V32108462166828W-4627N. Ecosar Class Program (Free Ware).

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Figures

Figure 1

a) Species distribution diagram of cephalexin as a function of solution pH. b) Molar fractions (%) of the main different species of cephalexin.

Figure 2

The 2D structure of CLX

Figure 3

The optimized structure of CLX/CLX using DFT/B3LYP/6-31+G(d)

Figure 4

The sites concerned with radical attack on neutral cephalixin in gas (1) and aqueous phase (2)

Figure 5

The sites concerned with radical attack on ionized cephalixin in gas (3) and aqueous phase (4)

Figure 6

H-abstraction by $\cdot\text{OH}$ from CLX, generating radical intermediate (R_{abs})

Figure 7

OH addition by $\cdot\text{OH}$ from CLX, generating radical intermediate (R_{add})

Figure 8

Fragmentation pathways of CLX/CLX

Figure 9

Proposed mechanism of *cephalexin* degradation.

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