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Study of the photolytic and photocatalytic transformation of amiloride in water

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Abstract

The diffusion of drug residues in wastewaters and surface waters as rivers and streams may constitute a problem for the environment, with consequences on the ecosystem and also on the human health. This paper deals with the study of the photo-induced transformation of amiloride, an orally administered diuretic agent, under simulated solar light. Direct photolysis and photocatalyzed degradation processes, using titanium dioxide as a photocatalyst, were investigated. The study involved the monitoring of the drug decomposition, the identification of intermediate compounds of the decomposition, the assessment of mineralization, as well as the evaluation of the toxicity associated to the degradation products.

Amiloride underwent complete degradation within 30 min of irradiation (heterogeneous photocatalysis) or 4 h (homogeneous photolysis). HPLC coupled to HRMS, via ESI interface, demonstrated to be a powerful tool to identify and measure degradation products of the studied drug. By considering the photocatalytic process, the identified intermediates are formed through: (1) dechlorination and hydroxylation of the heteroaromatic ring; (2) the detachment of the guanidinic moiety; (3) cleavage of the heteroaromatic ring. The drug photomineralization was a rather slow process and after 4 h of irradiation 25% of the total organic carbon (TOC) was still present. Chlorine was stoichiometrically released as chloride ions within the considered irradiation times (4 h), while nitrogen was only partially converted into ammonium ions. This was due to the formation of guanidine, known to be hardly mineralized photocatalytically, and some other small molecules still containing the nitrogen. Acute toxicity, measured with a Vibrio fischery assay, showed that amiloride transformation proceeded through the formation of toxic compounds.

Keywords: Amiloride; Drug; Photolysis; HPLC/HRMS; Toxicity; Mineralization; Guanidine

1. Introduction

The presence in the environment of new xenobiotic compounds as a consequence of the massive use of chemicals in different productive fields constitutes a ticklish and complex emerging subject-matter. Many of these substances are now considered "persistent organic pollutants" (POPs) and their effects on the ecosystem and on the living organisms are becoming target of various studies [1–3].

In recent years, pharmaceutical residues have appeared as a new class of pollutants, for which public and scientific concern has progressively increased, due to their potential impact on the human health and on the environment [4–8]. Human and veterinary drugs can be released in the environment mainly as a consequence of manufacturing processes, disposal of unused or expired products and accidental spills during manufacturing and distribution or excreta by humans and animals. These substances and their metabolites may accumulate in soils and sediments and contaminate groundwater, or be discharged into sewers through urine and faeces and then enter sewage-treatment plants (STPs), prior to entering rivers and streams, lakes and sea [4,9–12]. Sometimes, not only the parent compound may arouse adverse effects on ecosystem and human health but also its metabolites. For most medical substances and their metabolites the transformation pathways in the aquatic system are largely unknown and investigations into their occurrence in environmental compartments are still rare [13,14]. Pharmaceuticals can undergo both abiotic and biotic processes of transformation. Abiotic reactions in surface waters may occur via hydrolysis or direct and indirect photolysis [15–17].

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Some compounds may be unaffected by sewage-treatment and remain in the water effluents or be transformed into breakdown products. The presence of pharmaceuticals in the environment was considered a consequence of a combination of a partial removal in STP and of refractoriness in natural (biotic or abiotic) transformations. Heterogeneous photocatalysis represents an example of advanced oxidation processes able to achieve a complete oxidation of organic and inorganic species, including also pharmaceutical substances [18–22]. It takes advantage of some semiconductor solids, which can be used as photocatalysts suspended in the water effluent to be treated, or immobilised on various types of supports. Among them TiO_2 is widely used because it is non-toxic, inexpensive, as well as biologically and chemically inert. The electron/hole pair (e^-/h^+) generated under light illumination of energy greater than 3.2 eV reacts with the molecules, objects of degradation, or water molecules oxidized by the photoholes (h^+) and gives rise to the generation of hydroxyl radicals, responsible for the complete decomposition of the chemical substances. Moreover, intermediates coming from an artificial photocatalytic process can be identical to those found in the metabolic system of living organisms [23] and in the environment, as a consequence of naturally occurring reactions [24]. The target drug of this study is amiloride (3,5-diamino-*N*-diaminomethylene)-6-chloropyrazine-carboxamide monohydrochloride), an orally administered diuretic agent, which acts as a sodium channel inhibitor and is excreted 50% in urine and 40% in faeces [25]. Amiloride showed photosensitization properties, related to the adverse clinical photobiological responses observed in patients exposed to sunlight [26].

The main objectives of this research were to assess the degradation of the pollutant through the identification of possible intermediate products as well as the determination of the final products by employing liquid chromatography coupled to mass spectrometry and ion chromatography.

Identification of intermediate compounds formed during the degradation is necessary to verify the real ability of the oxidation technology in reducing toxicity of water and to inspect the possible formation of dangerous substances. Toxic effects of drugs have been tested on microorganisms [3,27,28], on phytoplankton [29] and on insects [30]. In recent years, the attention has been focused also on genotoxicological effects connected to the spreading of some types of drugs in the aquatic environment, in particular in drinking water, wastewater and sludge [31,32]. Toxicity was also demonstrated at concentrations at which pharmaceuticals are normally found in the aquatic environment in the range of μg/l to ng/l [4,33–35]. Some drugs are suspected to be able to affect endocrine system of living organisms such as fish [2] and, even if in trace quantities, they may cause endocrine disruption also in humans, with consequent alterations in reproduction or development [9,36]. In the present study, the acute toxicity of the irradiated solutions was also evaluated; a bacterial assay based on the bioluminescence reduction of the marine bacterium *Vibrio fischeri* was carried out.

2. Experimental

2.1. Materials and reagents

Amiloride was purchased from Aldrich. The photocatalytic experiments were carried out using TiO_2 Degussa P25 as a photocatalyst (surface area $50\,m^2\,g^{-1}$). In order to avoid possible interference from ions adsorbed on the photocatalyst, the TiO_2 powder was irradiated and washed with distilled water until no signal due to chloride, sulphate or sodium ions could be detected by ion chromatography. HPLC grade methanol (BDH, Milan, Italy) was filtered through a 0.45-μm filter before use.

2.2. Irradiation procedures

Irradiations were performed using a xenon lamp ($30\,W/m^2$) simulating AM1 solar light and equipped with a 340-nm cut-off filter. Irradiation experiments were carried out in pyrex glass cells (Ø = 4 cm) containing 5 ml of amiloride ($15\,mg\,l^{-1}$) and TiO_2 ($200\,mg\,l^{-1}$) or 5 ml of amiloride ($15\,mg\,l^{-1}$) solution. The distance of the cells from the lamp was 10 cm, the photon flux was 6.4×10^{-7} Einstein s^{-1} and the temperature measured during the experiments was about 35 °C. The entire content of the cells was filtered through a 0.45-μm filter and then analyzed with the appropriate techniques.

2.3. Analytical procedures

2.3.1. Liquid chromatography

The chromatographic separations followed by a MS analysis were run on a C18 column Phenomenex luna (Phenomenex, Bologna, Italy) 150 mm × 2.0 mm. Injection volume was 10 μl and flow rate 200 μl/min. The gradient program was as follows: methanol/formic acid 0.05% in water 5/95 to 100/0 in 20 min.

2.3.2. Mass spectrometry

A LTQ Orbitrap mass spectrometer (ThermoFisher) equipped with an atmospheric pressure interface and an ESI ion source was used. The LC column effluent was delivered into the ion source using nitrogen as sheath and auxiliary gas. The source voltage was set at 4.1 kV. The heated capillary value was maintained at 275 °C. The acquisition method used was previously optimized in the tuning sections for the parent compound (capillary, magnetic lenses and collimating octapoles voltages) in order to achieve maximum sensitivity. The tuning parameters adopted for ESI source were the following: capillary voltage 13.00 V, tube lens 70 V; for ions optics, multipole 00 offset −1.25 V, lens 0 voltage −4.00 V, multipole 0 offset −4.75 V, lens 1 voltage −13.00 V, gate lens voltage −52.00 V, multipole 1 offset −15.00 V, front lens voltage −5.00 V. Mass accuracy of recorded ions (vs. calculated) was ±15 ppm (without internal calibration).

2.3.3. Ion chromatography

A Dionex instrument was employed, equipped with a conductometric detector. Ammonium ions were determined using

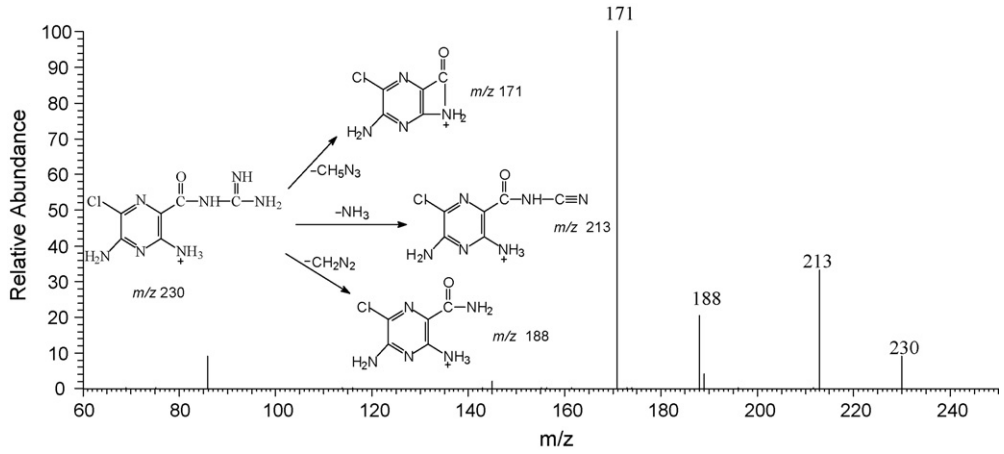


Fig. 1. Amiloride MS/MS spectrum and proposed fragmentation pathways.

a CS12A column with 25 mM methanesulphonic acid as eluent; flow rate 1 ml min^{-1}. The anions were analyzed using an AS9HC anionic column and a mixture of $NaHCO_3$ 12 mM and K_2CO_3 5 mM at a flow rate 1 ml min^{-1}.

2.3.4. Total organic carbon analyzer

Total organic carbon (TOC) was measured on filtered suspensions using a Shimadzu TOC-5000 analyzer (catalytic oxidation on Pt at 680 °C). Calibration was achieved by injecting standards of potassium phthalate.

2.3.5. Toxicity measurements

The toxicity of amiloride solution and of its aqueous samples at different irradiation times was examined by Microtox Model 500 Toxicity Analyzer. Acute toxicity was measured by a bioluminescence inhibition assay using the marine bacterium *V. fischery*, monitoring changes in the natural light emission of the luminescent bacteria when challenged with toxic compounds [37]. Freeze-dried bacteria, reconstitution solution, diluent (2% NaCl) and an adjustment solution (non-toxic 22% sodium chloride) were obtained from Strategic Diagnostic Inc. (SDI). Samples were tested in a medium containing 2% sodium chloride, in five dilutions and luminescence was recorded after 5 min of incubation at 15 °C. The inhibition of the luminescence, compared with a toxic-free control to give the percentage of inhibition, was calculated following the established protocol using the Microtox calculation program.

3. Results and discussion

Amiloride was irradiated alone or in the presence of TiO_2 as a photocatalyst. HPLC coupled to high-resolution mass spectrometer with an ESI interface in positive ions mode was performed to recognize the unknown intermediates. Table 1 summarizes the *m/z* ratios, empirical formulae, main MS/MS fragments and possible structures for amiloride and the detected intermediates.

3.1. Amiloride MS/MS study

MS/MS spectrum of amiloride was investigated, as its fragmentation is helpful in identifying the unknown compounds formed in the course of the photo-induced degradation. The proposed fragmentation pathway is shown in Fig. 1. The ion at *m/z* 171 (loss of guanidine) was the base peak. As other product ions the fragment at *m/z* 213 (loss of ammonia) and at *m/z* 188 (loss of methyldiamine) were formed.

3.2. Amiloride degradation under homogeneous photolysis

Amiloride was irradiated in pure water, in order to verify the extent of the photolysis process. After 30 min of irradiation, approximately 50% of amiloride was degraded and 4 h of irradiation were required to achieve its total disappearance (Fig. 2).

Along with the amiloride photolysis, two intermediate compounds at *m/z* 212.0869 and 213.0709 were identified. Full mass spectra for these species did not present the typical chlorine

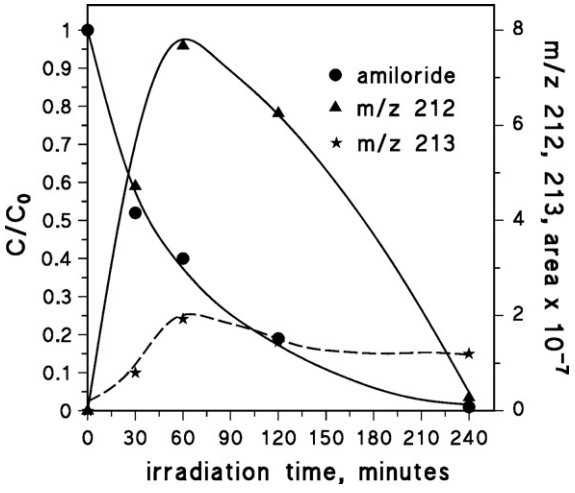
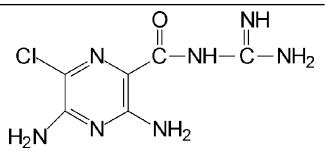
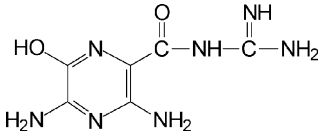
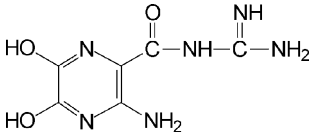
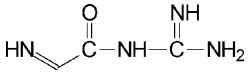
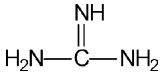


Fig. 2. Amiloride disappearance under homogeneous photolysis and formation of intermediate compounds as a function of the irradiation time.

Table 1
Amiloride (first line [M+H$^+$] 230.0513) and intermediate compounds originating from amiloride photodegradation, empirical formula, retention times, MS/MS fragment ions with relative abundances and proposed structures

[M+H]$^+$	Formula	RT (min)	MS/MS	Structure
230.0513	$C_6H_9N_6O_3$	9.9	171 (100), 213 (30), 188 (20)	
212.0869	$C_6H_9N_6O_2$	7.7	195 (100), 170 (50), 141 (15)	
213.0709	$C_6H_7N_5O_3$	5.9	–	
115.0597	$C_3H_7N_4O$	1.7	–	
60.0542	CH_6N_3	1.6	–	

isotopic pattern thus proving a dechlorination process. The structures for these intermediate compounds were suggested on the basis of their exact masses.

The main intermediate at *m/z* 212.0869 was quickly formed, reached the maximum amount after 1 h of irradiation and then was completely degraded after 4 h of irradiation (Fig. 2). Its MS/MS spectrum still presented the typical losses observed for amiloride (ammonia and methyldiamine), thus confirming the presence of unaltered guanidinic moiety and of one amino group on the heteroaromatic ring. This intermediate was also recognized by Li et al. [38] as an amiloride photolysis product and corresponds to the structure in Table 1. It was formed through the photo-induced cleavage of the C–Cl bond with the substitution of a Cl group with an OH group.

The formation of the species at *m/z* 213.0709 was slow and it did not disappear within the considered times. High-resolution mass spectrum suggested an empirical formula of $C_6H_7N_6O_3$, that permitted to attribute it to the structure shown in Table 1. Its formation involves the replacement of one (of the two) amino group by a hydroxyl group and dechlorination/hydroxylation of the heterocyclic ring.

3.3. Photocatalytic transformation of amiloride on TiO_2

The rate of the disappearance of amiloride was enhanced by the addition of TiO_2; $t_{1/2}$ decreased from 30 to 3 min of irradiation and it was completely degraded within 30 min (see Fig. 3).

Amiloride underwent dechlorination and hydroxylation of the heteroaromatic ring with the production of the compound at *m/z* 212, both by direct photolysis and under photocatalytic treatment. While for direct photolysis it was the main transformation products, by photocatalyzed process it represented only a secondary product (maximum amount was 20 times lower).

Interestingly, when considering the species at *m/z* 213, while its formation by direct photolysis occurred in a small amount (*m/z* 213:*m/z* 212 ratio 1:4), its formation was greatly increased through the photocatalytic process (*m/z* 213:*m/z* 212 ratio 10:1); it became the more important initial intermediate.

In addition to these shared intermediate compounds, the presence of titanium dioxide initiated additional transforma-

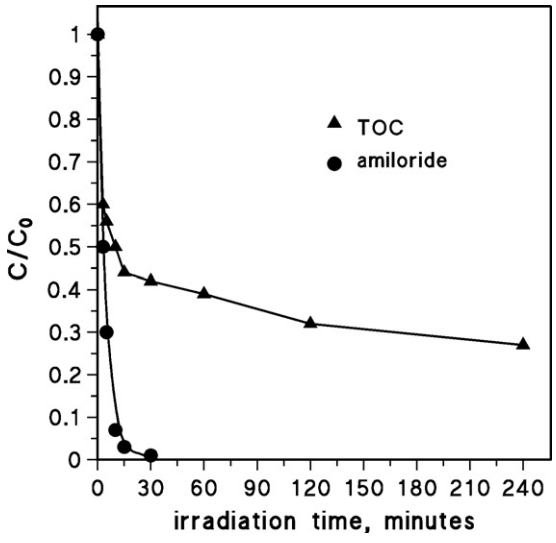


Fig. 3. Heterogeneous photocatalyzed disappearance of amiloride on TiO_2 200 mg l^{-1} and total organic carbon (TOC) profile as a function of the irradiation time.

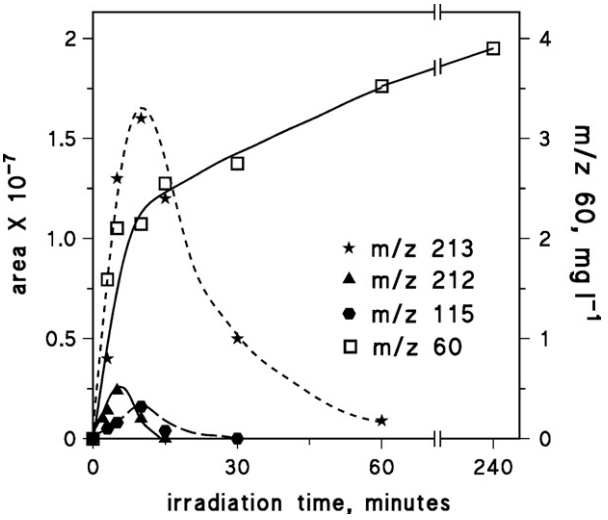


Fig. 4. Intermediates formed during amiloride heterogeneous photocatalyzed degradation on TiO_2 200 mg l^{-1} as a function of the irradiation time.

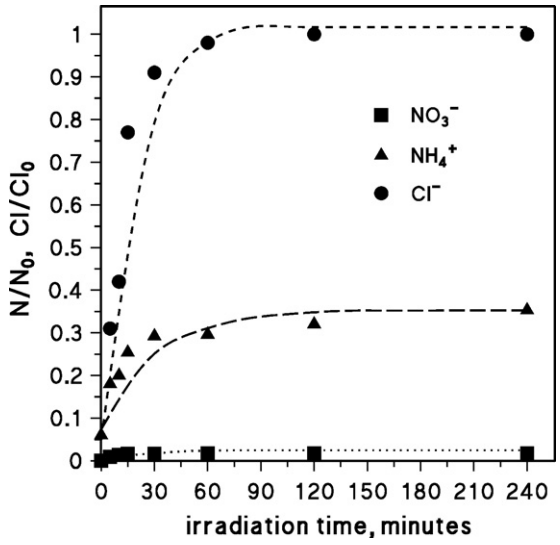


Fig. 5. Evolution of nitrate, ammonium and chloride ions formed from amiloride heterogeneous photocatalyzed degradation as a function of the irradiation time.

tion pathways. Other two intermediates at *m/z* 115.0597 and 60.0542 were detected. Their main MS/MS fragments and possible structures are reported in Table 1, while their time profiles are plotted in Fig. 4. A transient species, with *m/z* 115 and an empirical formula $C_3H_7N_4O$ (see Table 1), was supposed to be formed through the opening of the heterocyclic ring. It was easily formed and transformed into smaller molecules not detectable under our experimental conditions. Another species, at *m/z* 60 (formula CH_5N_3) was formed at high amount and recognized as guanidine. Its identity was also confirmed by the injection of a standard solution. It seemed to be the main intermediate; it was rapidly formed in the course of 1 h irradiation and its concentration then slowly increased until 4 h to reach 3.9 mg l^{-1}, that corresponds to the stoichiometric conversion of the guanidinic moiety into guanidine. Guanidine was recognized as a highly stable compound and it required 45 h of irradiation to be completely degraded [39].

3.4. Final products

The photocatalytic conversion of chlorine and nitrogen atoms into inorganic ions was plotted in Fig. 5 as a function of the irradiation time. While chlorine was recovered as chloride ions, nitrogen moieties can be photocatalytically transformed to either N_2, NH_3/NH_4^+ and/or nitrite and nitrate ions, whose ratio depends on the different features of the N-containing structure [40]. Chlorine was easily transformed into chloride ions and reached the stoichiometric concentration within 1 h of irradiation, in agreement with the identified chlorine-less intermediates. By contrast, nitrogen was only partially mineralized. After 4 h of irradiation, 30% of the initial nitrogen was mineralized into ammonium ions, while nitrate was formed at trace amounts. When the oxidation state of the bound nitrogen was −3, ammonium ions were formed directly from the two amino groups by the release of nitrogen as ammonia. The smaller extent of nitrogen mineralization was surely due to the formation of guanidine as the main intermediate, a very stable compound that can be photocatalytically mineralized only after prolonged times (up to 70 h of irradiation) [39,41]. It accounted for 43% of the total nitrogen, while the 20% lacking was probably caused by the formation of other small molecules still containing nitrogen.

Total organic carbon was also measured (Fig. 3). A rapid initial decrease is followed by a much slower decrease. After 1 h of irradiation, when amiloride was totally degraded, the residual organic carbon was 40% and it decreased to 25% after 4 h of irradiation. The lack in carbon mineralization accounted for the guanidine (17%) and other non-identified small molecules (8%).

3.5. Toxicity assessment

Acute toxicity was evaluated by monitoring changes in the natural light emission of the luminescent bacteria *V. fischeri* when challenged with toxic compounds and was expressed as percentage of inhibition of the bacteria luminescence. The toxicity of amiloride solutions after different irradiation times was examined and is plotted in Fig. 6. No toxicity was associated to amiloride solution (percentage of inhibition zero). In the course

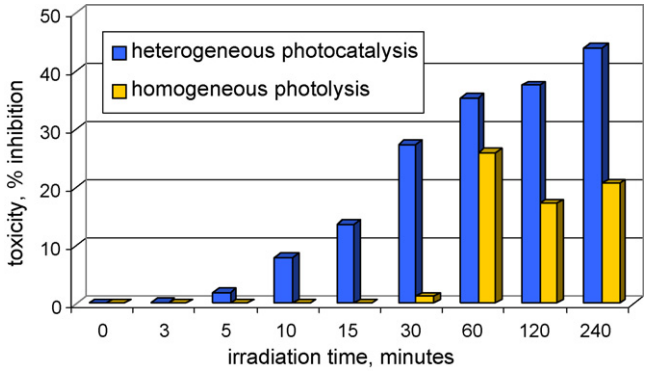


Fig. 6. Inhibition (%) of the luminescence of bacteria *Vibrio fischeri* as a function of the irradiation time by homogeneous photolysis and heterogeneous photocatalysis.

of homogeneous photolysis the inhibition reached a maximum after 1 h (20% of inhibition) and then slowly decreased. It has to be underlined that this toxicity curve presents a shape similar to that observed with the identified intermediates (see Fig. 2).

In the heterogeneous photocatalysis experiments the percentage of inhibition gradually increased until 4 h of irradiation to reach about 40%. Thus, even if initial transformation compounds still showed toxicity (in agreement with photolysis experiments), the more toxic compounds seemed to be the ultimate organic intermediates, i.e. guanidine and other not identified nitrogenous compounds.

4. Conclusions

The study of amiloride transformations through direct photolysis and photocatalyzed degradation process, using titanium dioxide as a photocatalyst, has been investigated. Amiloride underwent complete degradation under both homogeneous and heterogeneous photocatalytic treatments. The main products formed in the course of photocatalyzed transformation were mainly hydroxylated and hydrolysis products. The amiloride transformation proceeded through: (1) dechlorination and hydroxylation of the heteroaromatic ring; (2) the detachment of the guanidinic moiety; (3) cleavage of the heteroaromatic ring.

Complete mineralization was not reached in the investigated time, due to the formation of guanidine, known to be hardly mineralized and some other unidentified (and toxic) compounds. The evolution of inorganic ions confirmed that a part of the nitrogen was still present as organic nitrogen. Acute toxicity measurements showed that, even if amiloride is a non-toxic drug, its photo-induced transformation produced toxic compounds.

References

[1] R. Andreozzi, V. Caprio, R. Marotta, D. Vogna, Water Res. 37 (2003) 993–1004.
[2] G. Rodgers, S. Jobling, S. Morris, C. Kelly, S. Kirby, A. Janbakhsh, J.E. Harries, M.J. Waldock, J.P. Sumpter, C.R. Tyler, Environ. Sci. Technol. 34 (2000) 1521–1528.
[3] B. Halling-Sorensen, S.N. Nielsen, P.F. Lanzky, F. Ingerslev, H.C. Lutzhorft, S.E. Jorgensen, Chemosphere 36 (1998) 357–393.
[4] M.D. Hernando, M. Mezcua, A.R. Fernández-Alba, D. Barceló, Talanta 69 (2006) 334–342.
[5] P. Calza, C. Medana, C. Baiocchi, E. Pelizzetti, Curr. Anal. Chem. 1 (2005) 267–287.
[6] T. Heberer, Toxicol. Lett. 131 (2002) 5–17.
[7] J. Tolls, Environ. Sci. Technol. 35 (2001) 3397–3406.
[8] B. Quintero, M.A. Miranda, Ars Pharm. 41 (2000) 27–46.
[9] S.K. Khetan, T.J. Collins, Chem. Rev. 107 (2007) 2319–2364.
[10] T.A. Ternes, Water Res. 32 (1998) 3245–3260.
[11] E. Zuccato, R. Bagnati, F. Fioretti, M. Natangelo, D. Calamari, R. Fanelli, in: K. Kummerer (Ed.), Pharmaceuticals in the Environment. Sources, Fate, Effect and Risks, Springer, Heidelberg, 2001.
[12] T. Poiger, H.R. Buser, M.D. Müller, Environ. Toxicol. Chem. 20 (2001) 256–263.
[13] M.S. Díaz-Cruz, M.J. López de Alda, D. Barceló, Trends Anal. Chem. 22 (2003) 340–351.
[14] S. Pérez, D. Barceló, Trends Anal. Chem. 26 (2007) 494–514.
[15] R. Andreozzi, M. Raffaele, P. Nicklas, Chemosphere 50 (2003) 1319–1330.
[16] R.G. Zepp, D.M. Cline, Environ. Sci. Technol. 11 (1977) 359–366.
[17] R.G. Zepp, G.L. Baughman, P.F. Schlotzhauer, Chemosphere 10 (1981) 109–117.
[18] C. Medana, P. Calza, F. Carbone, E. Pelizzetti, H. Hidaka, C. Baiocchi, Rapid Commun. Mass Spectrom. 22 (2008) 301–313.
[19] C. Zwiener, F.H. Frimmel, Water Res. 34 (2000) 1881–1885.
[20] P. Calza, V.A. Sakkas, C. Medana, C. Baiocchi, A. Dimou, E. Pelizzetti, T. Albanis, Appl. Catal. B: Environ. 67 (2006) 197–205.
[21] T.E. Doll, F.H. Frimmel, Catal. Today 101 (2005) 195–202.
[22] R. Molinari, F. Pirillo, V. Loddo, L. Palmisano, Catal. Today 118 (2006) 205–213.
[23] P. Calza, E. Pelizzetti, M. Brussino, C. Baiocchi, J. Am. Soc. Mass. Spectrom. 12 (2001) 1286–1295.
[24] S.Y. Panshin, D.S. Carter, E.R. Bayless, Environ. Sci. Technol. 34 (2000) 2131–2137.
[25] H. Spahn, K. Reuter, E. Mutschler, W. Gerok, H. Knauf, Eur. J. Clin. Pharmacol. 33 (1987) 493–498.
[26] I.A. Magnus, Dermatological Photobiology, Blackwell, London, 1977, pp. 211-235.
[27] K.T. Elvers, S.J.L. Wright, Lett. Appl. Microbiol. 20 (1995) 82–84.
[28] A.K. Sanyal, D. Roy, B. Chowdhury, A.B. Baneuec, Lett. Appl. Microbiol. 17 (1993) 109–111.
[29] M.C. Harrass, A.C. Kinding, F.B. Taub, Aquat. Toxicol. 6 (1985) 1–11.
[30] C. Sommer, B. Steffansen, B. Overgaard Nielsen, J. Gronvold, K.M. Vagn Jensen, J. Brochner Jespersen, L. Springborg, P. Nansen, Bull. Entomol. Res. 82 (1992) 257–264.
[31] J.J. Espinosa-Aquirre, R.A. de la Torre, I. Lares-Asseff, J. Rubio, V. Dorado, M. Wong, J.M. Hernandez, Mutat. Res. 359 (1996) 133–140.
[32] F. Giuliana, T. Koller, F.E. Würgler, R.M. Widmer, Mutat. Res. 368 (1996) 49–57.
[33] H.R. Buser, T. Poiger, M.D. Muller, Environ. Sci. Technol. 32 (1998) 3449–3456.
[34] H.R. Buser, M.D. Muller, T. Norbert, Environ. Sci. Technol. 32 (1998) 188–192.
[35] E. Zuccato, D. Calamari, M. Natangelo, R. Fanelli, Lancet 355 (2000) 1789–1790.
[36] B.E. Erickson, Environ. Sci. Technol. 36 (2002) 140–145.
[37] International Organization for Standardization (ISO/DIS) 11348-2, Determination of the Inhibitory Effect of Water Samples on the Light Emission of *Vibrio fisheri* (Luminescent Bacteria Test), ISO, Geneva, Switzerland, 1998.
[38] Y.N.B. Li, D.E. Moore, B.N. Tattam, Int. J. Pharm. 183 (1999) 109–116.
[39] P. Calza, C. Medana, C. Baiocchi, E. Pelizzetti, Appl. Catal. B: Environ. 52 (2004) 267–274.
[40] P. Calza, E. Pelizzetti, C. Minero, J. Appl. Electrochem. 35 (2005) 665–673.
[41] P. Calza, C. Medana, C. Baiocchi, E. Pelizzetti, Appl. Catal. B: Environ. 63 (2006) 124–130.