Ecotoxic effects of loratadine and its metabolic and light-induced derivatives

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Abstract

Loratadine and desloratadine are second-generation antihistaminic drugs. Because of human administration, they are continuously released ***via excreta*** into wastewater treatment plants and occur in surface waters as residues and transformation products (TPs).

Loratadine and desloratadine residues have been found at very low concentrations (ng/L) in the aquatic environment but their toxic effects are still not well known. Both drugs are light-sensitive even under environmentally simulated conditions and some of the photoproducts have been isolated and characterized. The aim of the present study was to investigate the acute and chronic ecotoxicity of loratadine, desloratadine and their light-induced transformation products in organisms of the aquatic trophic chain. Bioassays were performed in the alga *Pseudokirchneriella subcapitata*, the rotifer *Brachionus calyciflorus* and in two crustaceans, *Thamnocephalus platyurus* and *Ceriodaphnia dubia*. Loratadine exerted its acute and chronic toxicity especially on *Ceriodaphnia dubia* (LC50: **600 µg** /L, EC50: 28.14 µg/L) while desloratadine showed similar acute toxicity among the organisms tested and it was more chronically effective compound in *Ceriodaphnia dubia* and *Pseudokirchneriella subcapitata*. Generally, transformation products were less active both in acute and chronic assays.

Keywords: antihistaminic drug; loratadine; desloratadine; acute toxicity; chronic toxicity; photoproducts.

Highlights

- Loratadine was irradiated by UVB and sunlight and its photoproducts were isolated and characterized.
- Loratadine, desloratadine and light-induced TPs were tested in aquatic organisms.
-Generally, transformation products were less active both in acute and chronic assays.
-Desloratadine was the most chronically effective compound in *C. dubia* and *P. subcapitata*.

1. Introduction

Drugs are continuously released as mixtures of parent compounds and metabolites and enter the aquatic environment through hospital and municipal wastewaters. Here, these mixtures may undergo transformations due to redox or light-induced reactions, hydrolysis, and other reactions leading to transformation products, in some cases more harmful than parent compounds (DellaGreca et al., 2014; Passananti et al., 2015; Isidori et al., 2016). The importance of these events in the breakdown of drugs has stimulated a large number of researches concerning kinetics, degradation mechanism, isolation and toxicity of the transformation products (Lambropoulou and Nollet, 2014). Generally, the most commonly occurring drugs in the aquatic systems are the most administered. However, some classes of drugs highly utilized by patients are not detected in the waters because rapidly degraded, while in some cases drugs less utilized are detected at high concentrations because resistant to biodegradation. Among the most administered drugs, antihistamines are detected in surface waters because of their poor removal by conventional wastewater treatments (Kosonen and Kronberg, 2009; Radjenovic' et al., 2009; Valcarcel et al., 2011) and due to their low polarity and scarce volatility, they may represent a hazard for the aquatic ecosystem (Berninger and Brooks, 2010; Kristofco and Brooks, 2017).

Among antihistamines, ranitidine, difenidramine, cimetidine and loratadine are the most detected in the effluents of sewage treatment plants and the detection of loratadine in surface waters has exceeded therapeutic hazard values (THVs) showing the need of understanding the aquatic toxicology, hazards and risks associated with this drug (Kristofco and Brooks, 2017). Loratadine is a second-generation antihistaminic drug so called because it causes less sedation and drowsiness than the first-generation antihistamines used to treat allergic reactions, approved by US Food and Drug Administration in 1993. Loratadine is a selective inverse agonist of peripheral H_1-receptors (Witiak, 1970, Peyrovi and Hadjmohammadi, 2015). It is mainly metabolized through the hepatic system to desloratadine, which is a pharmacologically active compound, deriving from the loss of carbamate moiety (Yumibe et al., 1996). **Forty percent and 42% of the ingested loratadine dose is excreted unchanged in urine and the feces, respectively** (Ramanathan et al., 2007). It has been detected in surface waters in Europe (in some Spanish river samples) in the low concentration range of 3.96-17.1 ng/L (Lopez-Serna et al., 2012) but also in wastewater effluents in Europe, North-America and Asia-Pacific with a maximum concentration of 58.5 ng/L (Kristofco and Brooks, 2017). Desloratadine has been detected in Europe with a maximum concentration of 81 ng/L (Kristofco and Brooks, 2017). Both drugs have also been recovered in lower amounts in marine water of Mediterranean coasts (Moreno-Gonzalez et al., 2015). Based on the antihistamines consumption data, loratadine and desloratadine should occur in wastewater at higher concentrations.

However, loratadine has low affinity for suspended matter (octanol/water partition coefficient log P equal to 5 for loratadine and 3.2 for desloratadine; El-Awady et al., 2013) and therefore does not accumulate appreciably in sediments and remains in the water column (Moreno-Gonzales et al., 2015). Loratadine is known as photolabile, in fact it is stated that the drug should be stored protected from light (Parfitt, 1999). Its UV spectrum shows an absorption band at λ 280 nm with a tail up to 300 nm, hence the drug is able to adsorb sunlight at ground level and to undergo light-induced transformations in the aquatic compartment. While its photostability has been investigated, no data on photoproducts identification are reported (Abounassif et al., 2005). In this context, we have examined the photochemical behavior of loratadine and desloratadine in aqueous medium under UVB and sunlight irradiation in order to isolate and fully characterized the photoproducts. For this purpose, concentrated solutions, far from environmental concentrations, were used (DellaGreca et al., 2014). The ecotoxicological effects of the parent drug, its metabolite and its transformation products were evaluated in producers and primary consumers.

2. Materials and methods

2.1 Chemicals

Loratadine (99.4%, CAS Number: 79794-75-5) and desloratadine (99.6%, CAS Number: 100643-71-8) were purchased by Kemprotec. All chemicals were used without further purification unless otherwise indicated. Solvents (acetonitrile, methanol and diethylether) were of HPLC grade and were purchased from Sigma Aldrich. Water was of Milli-Q quality and was obtained from a Milli-Q gradient system (Millipore).

2.2 Apparatus

2.2.1 Spectroscopic techniques

Nuclear magnetic resonance (NMR) spectra were recorded on a Varian Inova-500 instrument operating at 499.6 and 125.6 MHz for ^{1}H and ^{13}C, respectively, and referenced with $CDCl_3$. The carbon multiplicity was evidenced by DEPT experiments. The proton couplings were evidenced by ^{1}H-^{1}H COSY experiments. The heteronuclear chemical shift correlations were determined by HMQC and HMBC pulse sequences.

IR spectra were recorded on a Jasco FT/IR-430 instrument equipped with single reflection ATR using $CHCl_3$ as solvent.

UV–Vis spectra were recorded with a Varian Cary 300 UV–Vis spectrophotometer or on a PerkinElmer Lambda 7 spectrophotometer.

2.2.2 Chromatographic analysis

HPLC experiments were carried out on an Agilent 1100 HPLC system, equipped with an UV detector set at 254 nm, using a RP-18 column (Gemini, 5 µm, 110 A, 250 mm × 4.6 mm). at a flow rate of 0.8 mL min^{-1}.

The analysis of the solutions used for determining the kinetic constant of loratadine photodegradation was carried out using the gradient elution as follows: at initial time 30 % acetonitrile and 70 % water for 7 min, followed by an increase of acetonitrile up to 70% in two minutes. Then, the same ratio was maintained constant for 24 minutes; finally, the initial ratio (30 % acetonitrile and 70 % water) was reached in two minutes.

In other cases HPLC analysis was performed under isocratic conditions and H_2O /CH_3CN 4:6 v/v was used as eluent.

GC-MS analyses were performed on a 6890 MSD quadrupole mass spectrometer (Agilent technologies) equipped with a gas chromatograph by using a Zebron ZB-5HT Inferno (5%-Phenyl-95%-Dimethylpolysiloxane) fused silica capillary column (Column 30 m x 0.32 mm x 0.10 µm) from Phenomenex. The injection temperature was 250°C, the oven temperature was held at 50°C for 3 min and then increased to 150°C at 12°C/min, increasing to 230°C at 18°C/min, to 280°C at 10°C/min and finally to 300°C at 30°C/min and held for 3 min. Electron Ionization mass spectra were recorded by continuous quadrupole scanning at 70eV ionization energy, in the mass range of m/z 30-600.

2.2.3 Irradiation apparatus

The photoreactor (Multirays, Helios Italquartz) was equipped with six 15W lamps with a maximum at 310 nm (UV-B). Open quartz tubes (1 cm optical path) and open and closed pyrex tubes (20 cm x 1 cm, 25 mL) were used.

2.3 Chromatographic separation materials

Analytical and preparative Thin Layer Chromatography (TLC) was made on Kieselgel 60 F_{254} plates with 0.2 mm, 0.5 or 1 mm layer thickness, respectively (Merck).

2.4 Experiments

2.4.1 Stability in aqueous solution in the dark

Loratadine (**1**) solutions (1 x 10^{-4} M) in H_2O/CH_3CN (9:1, v/v) at pH 4, 7 and 9 were prepared. The acid and alkaline solutions were made using NaOH 2M and HCl 2M to adjust pH level. All solutions were kept in the dark and analyzed by HPLC (isocratic conditions) at 12 h and 48 h.

2.4.2 Kinetic constant and quantum yield determination

Kinetics data were obtained by irradiating the drug (1x10^{-4} M solution in H_2O/CH_3CN 9:1, v/v) in open quartz tubes and monitoring the solution at fixed time intervals by HPLC using the proposed gradient elution. The time evolution was fitted with a pseudo-first order equation $C_0 = C_t \times e^{-Kt}$

where C_0 is the initial drug concentration, C_t the concentration at time t and k the pseudo-first order degradation rate constant.

The incident photon flux (4.98 x 10^{21} photons m^{-2} s^{-1}) in solution, used to calculate the quantum yield of loratadine, was calculated using *p*-nitroanisole/pyridine actinometer (Dulin and Mill, 1982).

2.4.3 Irradiation experiments. Two solutions of loratadine in H_2O/CH_3CN (9:1, v/v, 1 x 10^{-4} M) and H_2O/CH_3CN (75:25, v/v, 1 x 10^{-4} M) were irradiated in open quartz tube and analysed by HPLC at selected times. The photoproducts were identified by HPLC comparing their R_t values with those of standard compounds which were isolated and characterized by performing preparative photochemical experiments (see below). An aliquot of the H_2O/CH_3CN (75:25, v/v, 1 x 10^{-4} M) was analyzed by GC-MS after 6 min irradiation.

2.4.4 Preparative experiments for photoproducts isolation

The photoproducts were isolated by means of preparative TLCs of irradiation mixtures obtained by appropriate experiments. Their structures were determined by spectroscopic analyses (Hesse et al., 2008). The presence of functional groups was deduced by IR spectra and identification of all different protons and carbons was obtained by NMR spectra.

*2.4.4.1. Isolation of isoloratadine **2***

Loratadine (35 mg) was dissolved in 92 mL of H_2O/CH_3CN (75:25 v/v, 1 x 10^{-3} M) and divided in four closed quartz tubes. Each solution was saturated with argon and irradiated by UV-B lamps. After 20 min of irradiation the solvents were evaporated under vacuum and the residue was analysed by ^{1}H NMR and separated by preparative TLC. Elution with Et_2O gave a fraction consisting of **3** and **4** (2 mg), isoloratadine **2** (6 mg), loratadine **1** (3 mg) and an intractable polar material (11 mg).

Ethyl 4-(8-chloro-6,11-dihydro-5H-benzo[5,6]cyclohepta[1,2-b]pyridin-11-yl)-5,6-dihydropyridine-1(2H)-carboxylate (**2**): EI-MS *m/z* 382/384 ; UVλ_{max} (CH_3OH) nm 266 (log ε 3.8); IRν_{max} ($CHCl_3$) 1690 (-N-CO-O-), 1606 (stretching vibrations of aromatic rings), 1371 (C-O stretching) cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.39 (1H, d, *J* = 4.0 Hz, H-2), 7.42 (1H, d, *J* = 7.3 Hz, H-4), 7.20-7.12 (4H, m, H-3, H-7, H-9 and H-10), 4.84-4.80 (2H, m, H-11 e H-3'), 4.10 (2H, q, *J* = 7.0 Hz, CH_2O), 3.88 (2H, m, H-2'), 3.50-3.45 (4H, m,), 2.88-2.74 (2H, m), 1.94-1.71 (2H, m,), 1.23 (3H, t, *J* = 7.0 Hz, CH_3). ^{13}C NMR (126 MHz, $CDCl_3$) δ 157.0 (C-1a), 155.5 (CO), 146.8 (C-2), 141.8 (C-6a), 138.5 (C-4), 135.9 (C-10a), 135.0 (C-4a), 133.1 (C-7), 133.0 (C-8), 131.1 (C-4'), 129.8 (C-10), 126.3 (C-9), 122.4 (C-3), 121.1 (C-3'), 62.3 (C-11), 61.2 (CH_2O), 43.4 (C-2'), 40.5 (C-6'), 31.3 (x2, C-5 e C-6), 28.0 (C-5'), 14.9 (CH_3).

*2.4.4.2 Isolation of compounds **3** and **4***

Loratadine (50 mg) was dissolved in 130 mL of H_2O/CH_3CN (75:25, v/v, 1 x 10^{-3} M) and irradiated by UV-B lamps. The irradiation mixture was analyzed at different time by HPLC. After 40 min of irradiation the solvents were evaporated under vacuum, and the residue was analysed by ^{1}H NMR and separated by preparative TLC. Elution with Et_2O gave a fraction consisting of **3** and **4** in ca. 3:1 molar ratio (11 mg), tricycle **3** (2 mg), isoloratadine **2** (2 mg), loratadine **1** (5 mg) and an intractable polar residue (8 mg).

8-Chloro-6,11-dihydro-5H-benzo[5,6]cyclohepta[1,2-b]pyridine (**3**): EI-MS *m/z* 229/231; UVλ_{max} (CH_3OH) nm: 279 (log ε 3.1); IR ν_{max} ($CHCl_3$) 1580 (stretching vibrations of aromatic ring), 1070 (aryl C-halogen stretching) cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.35 (1H, d, *J* = 4.9 Hz, H-2), 7.40 (1H, dd, *J* = 7.5, 1.4 Hz, H-4), 7.20 (1H, d, *J* = 8.1 Hz, H-10), 7.16 (3H, m, H-4, H-7 and H-9), 4.35 (2H, s, H-11), 3.33 (4H, brs, H-5 and H-6);^{13}C NMR (50 MHz, $CDCl_3$) δ 156.9 (C-1a), 146.5 (C-2), 140.8 (C-6a), 137.7 (C-4), 136.0 (C-10a), 133.8 (C-4a), 132.3 (C-8), 130.7 (C-10), 128.9 (C-7), 126.3 (C-9), 122.0 (C-3), 35.7 (C-11), 31.3 (C-5 and C-6).

Spectral data of piperidinone **4** were deduced by those of the mixture of **3** and **4** after the signals of tricycle **3** were subtracted; it was identified by comparison of its signals with those reported in literature (Hirsch and Havinga, 1976).

Ethyl 4-oxopiperidine-1-carboxylate (**4**) (in mixture with **3** in ca. 1:3 molar ratio**)**: EI-MS *m/z* 171; ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.19 (2H, q, *J* = 7.1 Hz, CH_2O), 3.76 (4H, t, *J* = 6.1 Hz, H-2 and H-6), 2.45 (4H, t, *J* = 6.1 Hz, H-3 e H-5), 1.29 (3H, t, *J* = 7.1 Hz, CH_3). ^{13}C NMR (125 MHz, $CDCl_3$) δ 207.1 (C-4), 155.0 (CO), 61.8 (CH_2O), 43.0 (C-2 and C-6), 41.1, (C-3 and C-5), 14.6 (CH_3).

2.4.4.3*.UV-B irradiation experiments for mechanistic purposes*

Two 1 x 10^{-3} M solutions of loratadine in pure CH_3CN were prepared by dissolving 5 mg in 13 mL. A solution was irradiated in open quartz tubes and the other one in closed quartz tubes after saturating with argon. After 15 min the solvent was evaporated and each residue analysed by ^{1}H NMR.

A similar procedure was used for two 1 x 10^{-3} M solutions of loratadine in methanol and for two solutions of loratadine in H_2O/CH_3CN (7:3 v/v).

2.4.4.4 *Irradiation of isolaratadine* **2**

A 1 x 10^{-4} M solution of compound **2** in H_2O/CH_3CN (9:1 v/v) was irradiated in open quartz tubes with UV-B lamps and analysed by HPLC and ^{1}H-NMR.

2.4.4.5 *Irradiation of desloratadine* **5**

1 x 10^{-3} M solution of desloratadine **5** was prepared by dissolving 5 mg in 16 mL of H_2O/CH_3CN 7:3 v/v. The solution was irradiated by UV-B lamps and analysed by ^{1}H-NMR.

2.5 Toxicity testing

Samples were dissolved in dimethylsulphoxide (DMSO, **3% v/v**), stored in the dark at 4 °C, further diluted in deionized water (Elix 10, Millipore, Milan, Italy) and sonicated for 30 minutes to obtain stock solutions. The test solutions were prepared by mixing the appropriate volumes of the stock solutions and ISO test media. Toxicity assays were performed in the following organisms: the green alga *Pseudokirchneriella subcapitata*, the planktonic rotifer *Brachionus calyciflorus* abundant in freshwaters, the anostracan crustacean *Thamnocephalus platyurus*, highly sensitive in acute toxicity testing and the cladoceran crustacean *Ceriodaphnia dubia*, worldwide distributed and often employed in acute and chronic toxicity testing.

2.5.1 Determination of drugs concentration in test samples

The concentrations of drugs were **measured (n=1)** using the solid phase extraction (SPE) coupled with HPLC. Each test solution containing drugs at the beginning of each toxicity test and after 24 h, 48 h and 72h passed through a C18 Sep-Pak® light column (Waters) used as a solid phase extraction cartridge, previously conditioned with 5.0 mL methanol followed by 5.0 mL water. The cartridge was then eluted with 5 mL methanol. The eluate was evaporated to dryness under reduced pressure and the residue was suspended in 1.0 mL acetonitrile. Portions of 200 μL volume were then injected into the HPLC system.

2.5.2 Acute toxicity tests

B. calyciflorus organisms were hatched from cysts (MicroBioTest Inc., Nazareth, Belgium) in synthetic moderately hard freshwater (80-100mg/L $CaCO_3$, pH7.5 ± 0.3) at 25 ± 1 °C under continuous illumination (3000-4000 lux) for 16-18 h prior to test initiation, as reported in the ASTM E 1440-91 guidelines. Six replicates with five animals/well, less than 2h old, were performed for each concentrations (0.3mL of test solution for each test well in 36-well plates, MicroBioTest Inc., Nazareth, Belgium) of each compound.

The *T. platyurus* test was performed in according to ISO 14380 (2011) using larvae hatched from cysts (Thamnotoxkit F, MicroBioTest Inc., Nazareth, Belgium) in 20-22 h before the assay in the standard freshwater (dilution 1:8 with deionized water) at 25°C under continuous illumination (3000-4000 lux). Tests were performed in 24-well plates with 10 crustaceans/well (1.0 mL of test solution), in three replicates.

The *C. dubia* test was performed over 24 h of exposure using young organisms less than 24 h old following test conditions reported in EPA-600-4-90 (US EPA 1993) with slight modifications. Neonates of at least third generation coming from a healthy mass culture (starting organisms were purchased from Aquatic Research Organisms, Inc., Hampton, NH, USA) were maintained at 25 ± 1 °C in synthetic medium (hardness 250 mg/L expressed as $CaCO_3$) with a 16:8 h light:dark cycle

(600 lux) Tests were performed in 24-well plates with 10 crustaceans per well (1.0 mL of test solution), in three replicates.

For each test considered above, both a negative control (only test-medium) and a solvent control (DMSO 1% v/v related to the maximum concentration of compounds tested equal to 100 mg/L) were performed. The plates were incubated in darkness at 25 °C for 24 h.

The end-point considered was mortality, and the concentration resulting in a 50% effect in 24 h-exposure was indicated as Median Lethal Concentration (LC50).

In acute assays, compounds were tested for a maximum of eight dilutions depending on the respective sensitivity of the organisms (100-31.25-9.76-3.15-0.98-0.31-0.09 mg/L) starting from the highest concentration of 100 mg/L with a geometric progression of 3.2.

2.5.3 Chronic toxicity tests

The *B. calyciflorus* chronic test was based on the offspring reduction over 48 h exposure (ISO, 20666, 2008) and was performed on young organisms less than 2 h old. Cysts were hatched as previously described for the acute test. Tests were performed in 48-well plates with one rotifer/well (0.9 mL of test solution prepared in moderately hard dilution water, ASTM E1440-91), in six replicates. The organisms were fed with a fresh suspension (0.1 mL) of 10^7 cells/mL of the unicellular alga *P. subcapitata*. Plates were incubated in darkness at 25 °C.

The chronic test in *C. dubia* was performed with female neonates < 24 h old from at least the third generation of a stock culture maintained in synthetic water with ISO medium were individually exposed to 25 mL of test solution in beakers over 7 days (ISO, 20665, 2008). Tests were conducted in semi-static conditions (all test media were exchanged five times per week) and, from the fourth day-exposure onward, the offspring produced by each parent organism were counted and removed daily. The organisms were fed daily with 200 μL of a combination of yeast *Saccharomyces cerevisiae*, alfalfa and flake food in addition to the unicellular green alga *P. subcapitata* (10^8 cells/mL). Ten replicates per concentration were incubated at 25 ± 1 °C with a 16:8 h light:dark cycle (600 lux).

The *P. subcapitata* growth inhibition test was performed according to OECD 201, 2011 with slight modifications reported by Paixao et al., 2008. The single samples were incubated with 10^4 cells/mL of algal suspension in 96-well microplates in six replicates under continuous illumination at 25 ± 1 °C on a microplate shaker (450 rpm). The plates were read at 450 nm (SpectraFluor, Tecan, Switzerland) immediately before the test and after 24 h, 48 h and 72 h.

For all chronic tests, a negative control (test-medium control) was used to the test series. Only for *P. subcapitata* growth inhibition test, the % DMSO exceeded the maximum % recommended in toxicity testing (0.01%). Thus, for this kind of assay, a solvent control (DMSO 0.1% v/v related to

the maximum concentration of compounds tested equal to 10000 µg/L) was performed. The number of the offspring or the algal growth outputs were compared to the values obtained for the negative control in order to determine the chronic effective percentages and to evaluate the chronic Effective Concentrations (ECx).

In *B. calyciflorus* and *C. dubia* chronic assays, compounds were tested for a maximum of nine dilutions (1000-312.5-97.66-30.52-9.54-2.98-0.93-0.29-0.09 µg/L) starting from the highest concentrations of 1000 µg/L with a geometric progression of 3.2. For the *P. subcapitata* growth inhibition test, compounds were tested for a maximum of ten dilutions (10000-3125-976.56-305.17-95.37-29.80-9.31-2.91-0.91-0.28 µg/L) starting from the highest concentration of 10000 µg/L with a geometric progression of 3.2.

2.5.4 Ecotoxicological data analysis

For each kind of assay, three independent experiments were performed. For each independent experiment, the effect percentages were calculated comparing each specific negative control. For each assay, the effect percentages coming from three independent experiments were pooled using Prism5 software (Graphpad Inc., CA, USA) to estimate the concentrations giving x% effect (L(E)Cx) by non-linear regression (log agonist vs. normalized response-variable slope). The LC50 value, corresponding to the 50% of mortality for each test-organism, was the test parameter for acute tests, whereas EC50, EC20, and EC10 were the concentrations giving 50%, 20% or 10% of the effect used in chronic tests to evaluate the inhibition of reproduction or the inhibition of the algal growth.

3. Results

The SPE coupled with HPLC analysis revealed a non-appreciable difference between nominal and actual concentrations: the actual concentrations of tested chemicals **diverged from the nominal concentrations by 5% after 24h, around 10% after 48h, and around 15% after 72h**. According to Li (2012), when the actual concentrations are at least 80% of the nominal concentrations, the measured and the expected concentrations are considered to be very close and no significantly different, so that in the present study the effective concentrations are reported as nominal concentrations.

3.1 Photochemical behaviour of loratadine

Loratadine **1** is slightly soluble in water, hence acetonitrile was chosen as co-solvent to obtain clear solutions (Figure 1). Preliminary experiments were carried out in the dark using 1 x 10^{-4} M solutions in H_2O/CH_3CN 9:1 v/v. The drug was stable after 48 h even when tested in acidic (pH 4)

and alkaline (pH 9) solutions. These pH ranges are usually considered in environmental analysis (Valenti et al., 2009).

Loratadine solution was then irradiated in a photoreactor with UV-B lamps. HPLC analysis showed the formation of photoproducts already after 2 min (Figure 2): compound **2** at R_t16.2 min and compound **3** at R_t10.2 min (Figure 1). The photoproducts were identified comparing their R_t values with those of standard compounds which were isolated and characterized by performing preparative photochemical experiments.

Kinetic experiment under these conditions showed that loratadine has a half-life of 137.4 seconds and a polychromatic quantum yield of 5.89×10^{-4} (Table S1).

Preparative experiments to isolate and characterize the photoproducts were carried out by UV-B irradiation of 1×10^{-3} M solutions of the drug in H_2O/CH_3CN 75:25 v/v. HPLC analysis confirmed the trend observed in dilute conditions and revealed the presence of the peaks of products **2** and **3** together with other minor products. After 40 min of irradiation, TLC on silica gel afforded three photoproducts: compounds **2** and **3** and a new product **4**. Structures **2-4** (Figure 1) were assigned on the basis of spectral data. In particular, 1D and 2D NMR spectroscopy was used because it is a powerful technique for identification and structure elucidation of small organic molecules (Elyashberg, 2015; Fuloria and Fuloria, 2013).

The mass spectrum of photoproduct **2** (R_t 16.5 min) shows a molecular peak at 382/384 *m*/*z* corresponding to the molecular formula $C_{22}H_{23}ClN_2O_2$, hence suggesting that it is a loratadine isomer. The mass spectrum evidences a peak at *m*/*z* 154, absent in the mass spectrum of loratadine, attributable to the tetrapyridine fragment $C_8H_{12}NO_2$.The ^{1}H-NMR spectrum shows significant differences only in the aliphatic proton region. In particular, two overlapping signals due to protons H-11 and H-3' (singlet + multiplet, respectively) are observed at δ 4.80 as expected due to the shift of the double bond. The shift of the double bond produces, in the ^{13}C-NMR spectrum, the disappearance of the singlet carbon signal at δ 133.3 (C-11 of **1**) and the appearance of a doublet carbon signal at δ 120.8 (C-3' of **2**).

The structure of photoproduct **3** (R_t 10.2 min) was confirmed by the presence in the mass spectrum of molecular peak at 229/231 *m*/*z* corresponding to the molecular formula $C_{14}H_{12}ClN_2$. The ^{1}H-NMR spectrum shows the presence of six aromatic protons in the δ range 8.40-7.10, of signals at δ 4.35 and at δ 3.33 due to di-benzylic methylene proton H-11 and to benzylic methylene protons H-5 and H-6, respectively.

Piperidinone **4** was obtained by TLC in mixture with compound **3** (ca. 1:3 molar ratio) and its data were deduced by comparison with those reported in literature (Hirsch and Havinga, 1976). It was not observed by HPLC analysis since it is transparent at the selected wavelength (254 nm) of the

detector. Its presence in the irradiation mixture was confirmed by GC-MS (Figure 2) and ^{1}H-NMR analysis of the crude irradiation mixture.

NMR analysis was particularly useful to examine the irradiation mixtures since loratadine and its photoproducts **2**-**4** have characteristic identifiable signals.

Aqueous solutions of the drug were also exposed to sunlight in Naples (Italy) in July 2017, under environmental-like conditions. As expected, degradation was slower. HPLC analysis showed a decrease of drug concentration to approximately 50% after 2 days and a complex mixture of photoproducts. The chromatographic and spectroscopic analysis of the irradiation mixture showed the presence of photoproducts **2**-**4**.

3.2 Mechanistic interpretation

In order to gain more mechanistic information on photoproducts formation, UV-B irradiation experiments were performed under various conditions (in different solvents such as acetonitrile, methanol, water/acetonitrile; in the presence and absence of oxygen) and the reactions were monitored by HPLC and ^{1}H NMR. The experimental conditions and the results are reported in Table S2.

Accordingly with previous data (Abounassif et al., 2005), loratadine degraded faster in solutions containing water (after 15 min of irradiation only 5% photodegradation in methanol or acetonitrile vs. 55% in H_2O/CH_3CN 7:3 v/v, Table S2 runs **c**, **e**, **g**). In all irradiation conditions, especially under argon, photoproduct **2** was present while the formation of compounds **3** and **4** was observed only in aqueous solution. Control experiments showed that isoloratadine **2** was photolabile and converted to unidentified material after 20 min of UV-B irradiation.

On the basis of literature data, a plausible mechanistic interpretation is reported in Figure 3. Compound **2** should derive from a 1,3-hydrogen shift, probably via a radical pair, from an excited triplet state of loratadine **1**, as suggested by the quenching with oxygen. The radical recombination can give loratadine **1** or its isomer **2** (Turro et al., 2010a). Addition of water to give intermediate **6** and β-cleavage of the alkoxy radical intermediate **7** should give products **3** and **4**. β-Cleavage of alkoxy radicals to give ketones and stable radicals is well known (Turro et al., 2010b).

In all the experimental irradiation conditions, desloratadine **5** was not observed. This result is not surprising considering that the carbamate function is quite photostable and it does not absorb light in the UV-C and UV-B regions (Iesce et al., 2006). However, control experiments showed that when a 1 x 10^{-3} M solution of desloratadine **5** in H_2O/CH_3CN 7:3 v/v was irradiated by UV-B lamps as reported above for loratadine, it was photodegraded within 60 min and gave a complex mixture of products among which the sole identifiable product was tricycle **3**.

3.3 Ecotoxicological experiments

Tests were performed with loratadine **1,** and its photoisomer **2** and the mixture of compounds **3** and **4** (in ca. 3: 1 molar ratio) obtained by preparative experiments (see 2.4.4). We also examined desloratadine **5** and its photodegradation mixture (DPM) obtained as reported in 2.4.4.5.

3.4 Acute toxicity results

In order to verify that the acute effects were not DMSO-dependent, a solvent control was performed for each kind of assay, at the highest tested percentage (1% v/v), and referred to the highest tested concentration of 100 mg/L, observing no significant difference with the negative control, with a survival higher than the 90% both in negative and in solvent controls (Table S3) as recommended by test validity criteria. The parent compound loratadine, its metabolite desloratadine, the transformation products and the degradation mixture of desloratadine were found to cause mortality in both crustaceans and rotifers and the LC50 values, obtained after 24 h exposure (coming from three independent experiments pooled using Prism5) are reported in Table 1. In addition, LC50 values espressed as mean ± SD of the indipendent experiments are reported in Table S4. Loratadine was able to cause the 50% of mortality in *C. dubia* at hundreds of µg/L, differently from the effects found at dozens of mg/L in the rotifers and in the anostracan crustacean. On the other hand, all the aquatic organisms showed the same sensitivity to the metabolite, with LC50 values found at units of mg/L, while DPM was more active in the rotifer *B. calyciflorus* (LC50 equal to 2.02 mg/L) than in crustaceans. Transformation products such as isoloratadine **2** and the mixture of tricycle **3** and piperidinone **4** showed different acute effects. In fact, albeit isoloratadine was more effective in *C. dubia* (LC50= 1.19 mg/L), the mixture of tricycle **3** and piperidinone **4** caused 50% mortality at units of mg/L not only in the cladoceran crustacean but also in the rotifer and it is more lethal than parent loratadine 1 for *B. calyciforus* (Table 1). To the best of our knowledge, scientific data on the aquatic toxicity of the compounds here tested is rather scarce. **Nevertheless, there are several data on diphenhydramine (DPH), the same histamine H1-receptor antagonist as loratadine.** In 2013 Goolsby and collaborators found that the diphenhydramine (DPH) was acutely toxic in *C. dubia* with an LC50 value equal to 3.94 mg/L, while in 2015 Kristofco at al., found that DPH caused a 50% immobilisation in the cladoceran crustacean *Daphnia magna* at 374 µg/L after 48h-exposure and the 50% mortality in the fish *Danio rerio* at 45.5 mg/L after 72h exposure. In the 2011, Berninger et al. found a median acute effect in *D. magna* at 0.37 mg/L after DPH exposure and from units to dozens of mg/L in the fish *Pimephales promelas*. Differently from vertebrates which are known to possess some degree of genetic homology for DPH targets like histamine-H1 receptors, with a similarity from 40 to 70% (Gunnarsson et al., 2008, Berninger et al., 2011), the effects of the antihistamines in invertebrates may likely be related to other mechanisms of actions

affecting histamine ion channel transporters, as suggested by Haas et al., 2008 and Berninger et al., 2011. Regarding transformation products, they were found to be slightly toxic for all aquatic organisms tested excepted isoloratadine 2 in *C. dubia*. **Although acute toxicity data are generally very far from those of environmental concern and from the water solubility of chemicals, they are still relevant regarding the assessment of environmental risk since chronic data are often lacking.**

3.5 Chronic toxicity results

The chronic toxicity data for the five samples, reported as EC50, EC20, and EC10 values (coming from three independent experiments pooled using Prism5) and expressed in µg/L, are shown in Table 2. In addition, EC50 values espressed as mean ± SD of the indipendent experiments are reported in Table S5. In order to verify that the chronic effects were not DMSO-dependent, a solvent control was performed only for *P. subcapitata* growth inhibition assay, at the highest tested percentage (0.1% v/v), and referred to the highest tested concentration of 10000 µg/L, as explained above. No significant difference with the negative control was found, with a growth higher than the 90% both in negative and in solvent controls (Table S3) as recommended by test validity criteria. Loratadine **1** was the most chronically active compound in the rotifer (EC50= 51.32 mg/L), while its metabolite desloratadine **5** was the most active both in *C. dubia*, with a median effective concentration at few units of µg/L, and in the green alga *P. subcapitata* (EC50 = 220.20 µg/L), as also depicted in Figure 4, where the concentration/effect curves of samples are reported for each aquatic organism. As shown in Figure 4, the parent compound was less active in *C. dubia* than its metabolite but more active than the transformation products. Furthermore, DPM determined a median chronic toxic effect at hundreds or thousands µg/L, in the case of aquatic consumers and producers, respectively (Table 2). In fact, as also reported in Figure 4, differently from consumers (*C. dubia* and *B. calyciflorus*), in producers (*P. subcapitata*) there is a slow increase in concentration/effect relationship with an evident response only at the highest concentrations.

In 2015 Watanabe and collaborators tested the diphenhydramine histamine H1-receptor antagonist in the alga *P. subcapitata* finding an EC50 value equal to 1240 µg/L, the same order of magnitude of the EC50 obtained in this study.

In the 2009, Isidori and collaborators tested the ranitidine, a histamine H2-receptor antagonist in the consumers *C. dubia* and *B. calyciflorus* finding a median offspring reduction in the order of thousands of µg/L, therefore underlining a higher sensitivity of these aquatic organisms to histamine H1- than to histamine H2-receptor antagonists. The environmental chronic toxicity of the histamine H1-receptor antagonist such as loratadine and its derivatives towards the aquatic tested organisms is observable already at few units-dozens µg/L (EC20 values, Table 2), however these

residues occur in surface waters at ng/L levels which are too low to cause an immediate threat to exposed organisms but can pose delayed long term effects interfering with organism metabolic pathways.

The EC10 and EC20 values of loratadine and EC10, EC20 and EC50 values of desloratadine (Table 2) are lower than their respective water solubility equal to 11 µg/L and 3950 µg/L, making the results of this study interesting to understand the behaviour of these drugs in real water samples. At the best of our knowledge, no data of photoproducts water solubility is available.

4. Conclusion

Loratadine **1** is transformed either under UVB or by sunlight exposure. The reactive site is the double bond while the carbamate moiety is unreactive. Transformation products derive by photoisomerization and water photoaddition followed by a cleavage reaction. Photolability is also observed in desloratadine **5** but this drug leads to a complex photodegradation mixture.

The toxic effects of loratadine **1** and desloratadine **5** occur in both acute and chronic assays at concentrations higher than their environmental occurring concentrations differently affecting the organisms selected from two trophic levels. However, the environmental transformations of the parent compounds, here simulated by the UV-irradiation treatments, lead to the formation of a bioactive mixture of residues and transformation products, which could represent a harmful combination to some of the organisms tested**. In order to define water quality criteria protective for all aquatic species, further toxicity studies towards other aquatic species are needed especially to increase species sensitivity diagrams used in EU and North American approach to anti-histamine management to derive water quality benchmark, and to** broaden knowledges of mechanisms involved in the different biological responses of the organisms.

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