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Sildenafil and tadalafil in simulated chlorination conditions: Ecotoxicity of drugs and their derivatives

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HIGHLIGHTS

- Simulated disinfection process of pharmaceuticals was performed.
- Toxicity and genotoxicity of sildenafil, tadalafil and their derivatives were evaluated.
- Chlorine derivatives caused chronic toxicity on rotifers and crustaceans.
- A mutagenic potential was found for all the compounds investigated.

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ABSTRACT

Chlorination experiments on two drugs (sildenafil and tadalafil) were performed mimicking the conditions of a typical wastewater treatment process. The main transformation products were isolated by chromatographic techniques (Thin Layer Chromatography (TLC), Column Chromatography (CC), High Performance Liquid Chromatography (HPLC)) and fully characterized employing Nuclear Magnetic Resonance (NMR) and Mass Spectrometry (MS) analyses. The environmental effects of the parent compounds and transformation products were evaluated using an overall toxicity approach that considered aquatic acute and chronic toxicity on *Brachionus calyciflorus* and *Ceriodaphnia dubia* as well as mutagenesis and genotoxicity on bacterial strains. The results revealed that both parent drugs did not show high acute and chronic toxicity for the organisms utilized in the bioassays while, chronic exposure to chlorine derivatives caused inhibition of growth population on rotifers and crustaceans. A mutagenic potential was found for all the compounds investigated.

1. Introduction

The consumption of stimulating drugs is increasing and usually has much greater quantities than legally prescribed occur in sewage treatment plants. Among the drugs illegally purchased there are the phosphodiesterase type V inhibitors such as sildenafil and tadalafil, active agents of Viagra®, and Cialis® used for erectile dysfunction treatment. Phosphodiesterase type V inhibitors are responsible for the breakdown of cGMP which relaxes the smooth muscle and increases blood flow to the corpus cavernosum. The presence of uncontrolled quantities of so-called lifestyle drugs in waters could be of great interest especially for the evaluation of the environmental risk considering that often, it is based on the calculation of the Predicted Environmental Concentrations (PEC) obtained from consumption data. Data on the occurrence of these drugs were reported by Nieto et al. (2010) in sewage treatment plant influents, effluents and sludge in Spain, where they were found at ng/L concentrations. Comparable amounts of these drugs were also found in Germany by Schroeder et al. (2010). The potential risk posed by these drugs could be increased by their susceptibility to produce phototransformation products as reported by Eichhorn et al. (2012) and Temussi et al. (2010), also considering that the removal efficiency in sewage treatment plants of the parent compounds was 68% and 69% for sildenafil and tadalafil, respectively. The percentage of removal of a certain drug from wastewater not only depends on its mineralization, but also on its capacity to be transformed into a different chemical substance during sewage treatments, in particular during disinfection step. Chlorination is by far the most used process in the disinfection step in wastewater treatment plants (WWTPs). Chlorine is often added to wastewater as a sodium hypochlorite solution and the mixture $HOCl/OCl^-$, known as free available chlorine (FAC), is a powerful

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non-specific oxidant, capable of inducing transformation of different micro-pollutants. Products obtained are usually chlorinated and/or oxidized, and could be more toxic than parent compounds (Escher and Fenner, 2011). In this work the selection of sildenafil and tadalafil was based on the presence in the molecular structure of potentially reactive groups as well as their detection in wastewater and sewage sludge (Nieto et al., 2010; Schroeder et al., 2010). Furthermore, their PECs in waters are difficult to estimate due to their illegal use worldwide.

A photochemical investigation was performed on these two compounds very recently. In our laboratories irradiation of tadalafil in aqueous solutions led to 6-epimer and/or water adducts depending on concentration and pH (Temussi et al., 2010). In a recent paper sildenafil phototransformation was reported under simulated sunlight (Eichhorn et al., 2012), the authors observed the formation of different photoproducts using ultra-performance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight mass spectrometry (UPLC-ESI-QToF-MS).

In this work, drug chlorination experiments were performed mimicking conditions of a typical wastewater treatment process. The main transformation products were isolated and fully characterized. Furthermore, in order to evaluate the environmental effects of parent compounds and transformation products, aquatic acute and chronic toxicity testing on *Brachionus calyciflorus* and *Ceriodaphnia dubia* as well as mutagenesis and genotoxicity on bacterial strains were carried out. The use of the freshwater rotifer *B. calyciflorus* and of the microcrustacean *C. dubia* (Cladocera, Crustacea) as representative aquatic organisms in such tests was justified because they have a widespread geographic distribution and a strong impact on several important ecological processes in waters. The mutagenesis and genotoxicity were performed using the Ames test on *Salmonella typhimurium* and the SOS Chromotest on *Escherichia coli* PQ37, respectively to detect point mutations and the induction of SOS DNA repair system.

2. Materials and methods

2.1. Test substances

Sildenafil citrate and tadalafil were purchased from Kemprotec Limited. Sodium hypochlorite solution NaOCl (10% available chlorine) was purchased from Fluka. Water used in all experiments and in the preparation of aqueous buffers was purified by a MilliQ filtration system (Millipore). Sodium thiosulfate, analytical reagent grade, was obtained from Carlo Erba. All organic solvents were purchased from Fluka and were of HPLC grade.

For toxicity and genotoxicity tests, parental drugs and their derivatives were dissolved in dimethylsulfoxide (Sigma-Aldrich Chemicals) because of their low solubility in water, sonicated and further diluted in double-deionized water to make stock solutions. The test solutions were prepared by mixing the appropriate volume of the stock solutions to be tested and the test medium. The dimethylsulfoxide (DMSO) concentration in the test solutions for ecotoxicological assays did not exceed 0.01% as recommended by international standard guidelines even though a solvent control was included in each test. Preliminary range finding tests (dilution factor = 10) were performed in order to identify the concentration range to be used in definitive tests. Final compound concentrations followed a geometric progression (dilution factor = 2) as recommended by standard guidelines and the concentration range was chosen to define an ECx with an appropriate level of confidence. According to guidelines the nominal concentrations of drug test solutions were checked using HPLC-analysis to evaluate the actual concentrations. HPLC experiments were carried out on a System Waters 1525 Binary HPLC Pumps equipped with a Waters 2996 Photodiode Array Detector using a Synergy Polar-RP 80A column (4 m, 250 × 4.6 mm, Phenomenex). The eluents used were MeOH–CH_3CN: 1 v/v, A) and H_2O Milli-Q (B) with A:B 6:4 isocratic run 0.6 mL min^{-1}.

2.2. Chlorination (general information)

The concentration of commercial NaOCl solutions was spectrophotometrically determined (λ_{max}292 nm; $\varepsilon = 350\ dm^3\ mol^{-1}\ cm^{-1}$). All chlorination experiments were performed at room temperature.

Sodium hypochlorite solutions were obtained for dilution of Fluka solution and the available chlorine in the solution was measured by iodometric titration method (Jackson et al., 2006).

Sodium thiosulfate solution (0.1 mM, $K_2Cr_2O_7$ iodometric titration) was used to quench residual free available chlorine in experiments conducted with excess of hypochlorite.

2.3. Procedures for the spectral characterization of transformation products

All products were identified by ^1H NMR and ^{13}C NMR spectra. NMR is an excellent method for quality control and quality assurance since it provides the most detailed information concerning the molecular structure.

NMR spectra were recorded under following conditions: NMR spectra were recorded at 500 MHz for [^1H] and 125 MHz for [^{13}C] on a Fourier transform NMR Varian 500 Unity Inova spectrometer; operating temperature was 25 °C; samples 0.6 mL, 0.02 M concentration in CD_3OD.

Carbon multiplicity was evidenced by Distortionless Enhancement by Polarization Transfer (DEPT) experiments. The proton couplings were evidenced by ^1H–^1H Correlation Spectroscopy (COSY) experiments. The heteronuclear chemical shift correlations were determined by Heteronuclear Single-Quantum Correlation (HSQC) and Heteronuclear Multiple-Bond Correlation (HMBC) pulse sequences.

Accurate mass measurements of the purified compounds were obtained by electrospray hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer (Q-TOF) fitted with a Z-spray electrospray ion source (Waters S.p.A.).

2.4. Sildenafil chlorination

A solution of sildenafil citrate in 1:18 molar ratio was prepared by dissolving 44 mg of the drug in 500 mL of a 1.67 mM sodium hypochlorite solution ([sildenafil]/[NaOCl] = 1:18). The solution was stirred and kept in the dark, after 1 h the experiment was quenched and the solution concentrated in vacuum and then extracted three times with ethyl acetate using separatory funnel. The organic phase was concentrated in vacuum and submitted to Thin Layer Chromatography (TLC) [dichloromethane/acetone (CH_2Cl_2/CH_3COCH_3) = 9/1] yielding compounds **S1** (50%), **S2** (25%) and **S3** (25%).

2.4.1. Spectral data

Compound **S1**: ^1H NMR (CD_3OD): δ 8.19 (1H, s, H-15), 7.90 (1H, dd, J = 9.0, 2.0 Hz, H-17), 7.40 (1H, d, J = 9.0 Hz, H-18), 4.31 (2H, q, J = 7.0 Hz, H-20), 4.24 (3H, s, H-10), 3.31 (4H, bs, H-23 e H-27), 3.22 (4H, bs, H-24 e H-26), 2.89 (2H, t, J = 7.5 Hz, H-11), 1.82 (2H, m, J = 7.5 Hz, H-12), 1.47 (3H, t, J = 7.0 Hz, H-21), 1.00 (3H, t, J = 7.5 Hz, H-13); ^{13}C NMR (CD_3OD): δ 161.7 (C-19), 157.1 (C-7), 150.0 (C-5), 147.5 (C-3), 140.0 (C-9), 133.2 (C-17), 131.7 (C-15), 129.1 (C-16), 124.4 (C-8), 114.3 (C-18), 66.7 (C-20), 61.6 (C-24 e C-26), 47.1 (C-23 e C-27), 38.4 (C-10), 28.4 (C-11), 23.4 (C-12), 14.7 (C-21), 14.2 (C-13); ESI-MS: m/z 495.0 $[M + H]^+$, 497.0 $[M + 2 + H]^+$ (33%), 517.0 $[M + Na]^+$, 533.1 $[M + K]^+$. Q-TOF HRMS (ESI^+): m/z 517.1410 (calcd. 517.1401 for $C_{21}H_{27}ClN_6O_4S$ Na).

Compound **S2**: ^1H NMR ($CDCl_3$): δ 10.8 (1H, bs, H-6), 8.84 (1H, d, J = 2.0 Hz, H-15), 7.88 (1H, dd, J = 9.0, 2.0 Hz, H-17), 7.16 (1H, d, J = 9.0 Hz, H-18), 4.39 (2H, q, J = 6.5 Hz, H-20), 4.28 (3H, s, H-10),