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A multispecies study to assess the toxic and genotoxic effect of pharmaceuticals: Furosemide and its photoproduct

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Abstract

Pharmaceutical products for humans and animals, as well as their related metabolites end up in the aquatic environment after use. Recent investigations show that concentrations of pharmaceuticals are detectable in the order of ng/l–μg/l in municipal wastewater, groundwater and also drinking water. Little is known about the effects, and the hazard of long-term exposure to low concentrations of pharmaceuticals for non-target aquatic organisms.

This study was designed to assess the ecotoxicity of furosemide, a potent diuretic agent, and its photoproduct in the aquatic environment. Bioassays were performed on bacteria, algae, rotifers and microcrustaceans to assess acute and chronic toxicity, while the SOS Chromotest and the Ames test were utilized to detect the genotoxic potential of the investigated compounds.

A first approach to risk characterization was to calculate the environmental impact of furosemide by measured environmental concentration and predicted no effect concentration ratio (MEC/PNEC). To do so we used occurrence data reported in the literature and our toxicity results. The results showed that acute toxicity was in the order of mg/l for the crustaceans and absent for bacteria and rotifers. Chronic exposure to these compounds caused inhibition of growth population on the consumers, while the algae did not seem to be affected. A mutagenic potential was found for the photoproduct compared to the parental compound suggesting that byproducts ought to be considered in the environmental assessment of drugs. The risk calculated for furosemide suggested its harmlessness on the aquatic compartment.

Keywords: Furosemide; Photoproduct; Toxicity testing; Genotoxicity; Mutagenesis

1. Introduction

In recent years the growing and uncontrolled use of drugs in farming, aquaculture and human health, in addition to improper disposal of expired medicines, has led to concern about their occurrence in the environment. Not surprisingly, many monitoring studies have detected low concentrations of a wide range of pharmacologically

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active substances, including hormones, steroids, antibiotics and diuretics in soils, surface waters and ground waters (Christen, 1998; Hirsch et al., 1999; Zuccato et al., 2000; Jones et al., 2002; Kolpin et al., 2002). Pharmaceuticals and their metabolic products reach the sewage systems through wastewaters, but often are not completely removed by Sewage Treatment Plants (Ternes, 1998; Stumpf et al., 1999). The end result is a contamination of the aquatic environment (Zuccato et al., 2000; Boyd et al., 2003; Calamari et al., 2003; Derksen et al., 2004). Here, pharmaceutical substances may also be degraded by biotic (Winkler et al., 2001) and/or abiotic processes such as hydrolysis and photolysis (Boree et al., 2003). However, the literature rarely provides extensive information about the environmental fate of these compounds and their possible interactions with aquatic organisms (Tixier et al., 2003). Generally, degradative processes reduce the potency of medicines, but some byproducts have similar toxicity (Halling-Sørensen et al., 2002) or, many times, are more persistent and toxic than their parent compounds (Andreozzi et al., 2003; DellaGreca et al., 2003; Doll and Frimmel, 2003). The occurrence data and the complex behaviour of drugs have raised questions about how the mixture of veterinary and human medicines and their transformation products impact the resident organisms and finally, human health. In fact, pharmaceuticals, that have been designed to cause beneficial effects on target organisms, could potentially determine changes at low concentrations in other animals exposed, from microbial communities to the higher organisms in the food chain (Halling-Sørensen et al., 1998; Daughton and Ternes, 1999; Calamari et al., 2003).

Diuretics are an important group of drugs used for heart and kidney failure, nefrotic syndrome, edema, cirrhosis and hypertension (Gotardo et al., 2004).

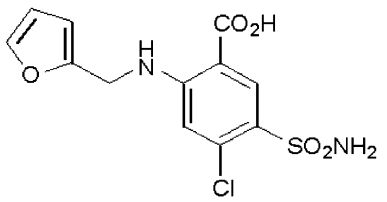
Furosemide (4-chloro-*N*-furfuryl-5-sulphamoyl anthranilic acid) is a potent diuretic inducing a powerful diuresis, followed by sodium, potassium and chloride wasting. The drug acts on the thick ascending limb of the loop of Henle (Agygralides et al., 2004). It is one of the most widely prescribed pharmaceutical (Stuer-Lauridsen et al., 2000). About 90% of the drug is excreted as a parent compound and its finding in Northern Italian Rivers such as the Po and Lambro is in the order of ng/l. This has recently been reported by Calamari et al. (2003). In this work the ratio between predicted environmental concentration (PEC) and measured environmental concentration (MEC) for furosemide was about 0.3 in both rivers, thus indicating that the ratio was probably affected by the behaviour of the drug and level of its degradation in the environment, as stated by authors when this ratio ranged from 0.01 to 0.3.

Photochemical studies on furosemide supported its high photodegradability (reduction, oxygenation, dechlorination) depending on the reaction conditions (Bundgaard et al., 1988; Zanocco et al., 1998) so that DellaGreca et al. (2004b) tested this pharmaceutical in environmental conditions (solar light, water, presence of humic acids) to obtain possible photoproducts. They found that furosemide was largely transformed (45% in three days) into a dimer (indicated as compound **2** in Fig. 1), stable and completely soluble in water. This behaviour could justify the low MEC/PEC value found by Calamari et al. (2003).

In this study, we examined the environmental effects of furosemide and its recently isolated photoproduct. The environmental impact was performed through ecotoxicological studies detecting the potential negative acute as well as chronic effects on different organisms of the aquatic chain: bacteria (*Vibrio fischeri*), algae (*Pseudokirchneriella subcapitata*), rotifers (*Brachionus calyciflorus*) and crustaceans (*Thamnocephalus platyurus*, *Daphnia magna* and *Ceriodaphnia dubia*).

The toxicity results obtained in this study and the available occurrence data from the literature were used to calculate the MEC/PNEC (predicted no-effect concentration) ratio. This value provided a first approach for the aquatic environmental risk estimation as reported by EMEA (2003).

In view of the increasing concern on biological effects induced by drugs, we also evaluated the potential mutagenic and/or genotoxic activity of furosemide and its photoderivative. Not much information is available in the literature on the latter aspect of pharmaceuticals although we recently reported the mutagenic and/or



Furosemide (**1**)

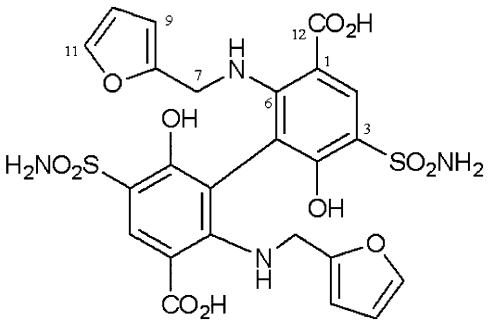


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Fig. 1. Furosemide and its photoproduct.

genotoxic activity of some compounds on antibacterial agents (Isidori et al., 2005a).

The genotoxic activity was determined using the SOS Chromotest, a quantitative bacterial colorimetric assay on *Escherichia coli* and the Ames test on *Salmonella typhimurium*, the widely used short-term test for monitoring water samples (APHA, 1998). The former is currently used to detect DNA-damaging agents (Dayan et al., 1987; Kümmerer et al., 2000; Jolibois et al., 2003) and the latter to reveal point mutations.

2. Materials and methods

2.1. Test substances

Furosemide (**1**) (purity > 99%) was purchased from Sigma Chemical, St Louis, MO, USA. Its photoproduct (**2**) (purity > 99%) was obtained in more than 45% yield from the solar simulator irradiation of furosemide in aqueous medium.

2.2. Phototransformation of furosemide

A solution of furosemide (**1**) (0.6 mM) in distilled water was irradiated at room temperature for 36 h by a 150-W solar simulator equipped with a Xenon lamp with a spectral output 200–2400 nm and an irradiance at 0.5 m higher than 10 $mW/m^2/nm$, using a filter to simulate irradiation at the earth surface (Oriel Instruments). The water was concentrated and the residue was filtered on HV13 Millex filter (Millipore Co.). The mixture (100 mg) separated by silica gel flash column chromatography eluting with ($CHCl_3$–acetone–CH_3OH 3:1:1), yields two fractions: A (60 mg) and B (40 mg). Fraction A mostly contained furosemide. Fraction B was further purified by reverse phase C-18 (Synergy Hydro) HPLC performed on an Agilent 1100 apparatus equipped with a UV-detector. The column was equilibrated with a mixture of A (H_2O containing 1% acetic acid)–B (MeOH containing 1% acetic acid) 10:0 (v/v) and the run was with the following program: an increase of B up to 5% in 5 min and then an increase of B up to 100% in 10 min, finally, an isocratic run for 5 min. The detector was set at 325 nm. From HPLC compound **2** (30 mg) was obtained.

2.3. Acute toxicity testing

Acute toxicity evaluation was performed on aquatic reducers and primary consumers: *V. fischeri* (luminescent bacterium), *B. calyciflorus* (rotifer), *T. platyurus* (crustacean anostraca), *D. magna* Straus and *C. dubia* (crustacean cladocera).

Furosemide and its photoderivative were initially dissolved in dimethylsulphoxide (DMSO) and then diluted further in double-deionized water to make the final stock solutions. The highest DMSO concentration, including controls, in the test samples never exceeded 0.01% (v/v). Range-finding tests were previously performed both for acute and chronic evaluation to identify the appropriate toxic concentration to expose each organism. The test solutions were prepared by mixing appropriate volumes of stock solutions with synthetic freshwater. All bioassays were conducted under static conditions and included a negative, a solvent and a reference toxicant control which was potassium dichromate (Aldrich) for all the organisms test (concentrations starting from 30 mg/l for *B. calyciflorus* and from 4 mg/l for the others) except *C. dubia* and *P. subcapitata* for which pentahydrate copper sulphate (Aldrich) was used (concentrations starting from 1 mg/l as Cu).

Toxicity testing on bacteria was conducted using the Microtox® system (Microbics, Carlsbad, CA, USA) according to the procedure described in the Microtox Manual (1995). Microtox test measures the decrease in bioluminescence of the marine bacterium *V. fischeri*, induced by depletion in the cell metabolism due to toxicants. The measurements were made after 5, 15, and 30 min of exposure and compared to the control (Bulich, 1979).

Toxic effects were expressed as median effective concentrations (EC50s) for *V. fischeri* at the time when the most toxic response was obtained (30 min).

The bioassay on the rotifer *B. calyciflorus* was performed according to the ASTM (American Society for Testing and Materials) E1440-91 procedure (ASTM, 1991). Juveniles (age, 0–2 h) of the rotifer were hatched from cysts provided by MicroBioTest, Nazareth, Belgium, after 16–18 h of incubation at 25 °C in synthetic freshwater (moderately hard medium according to EPA-600/4-85-013) under continuous illumination (3000–4000 lux) and then exposed to five test dilutions prepared in a 50% dilution series for each sample with six replicates of five animals. After 24 h in a 25 °C incubator in the dark the dead rotifers were counted and the concentration found to kill 50% rotifers in 24 h was indicated as the LC50.

The test on the anostracan crustacean *T. platyurus* was conducted using second- and third-instar fairy shrimp larvae hatched from cysts provided by MicroBioTest, Nazareth, Belgium, after 20–22 h of incubation at 25 °C in synthetic reconstituted freshwater (the same moderately hard EPA medium as rotifers) under continuous illumination (light source 3000–4000 lux) and then exposed to five dilutions with three replicates of ten animals. After 24 h in a 25 °C incubator in the dark, the number of dead crustaceans was recorded and the concentration found to kill 50% animals in 24 h was indicated as LC50 (Thamnotoxkit, 1995).

D. magna was obtained from our laboratory cultures and the test was performed according to the ISO

(International Organization for Standardization) 6341 (ISO, 1996). The bioassay was performed with neonates <24 h. Five daphnids per vessel, four replicates for each of five concentrations were exposed to the samples at temperature of 20 °C in the dark. The number of immobile daphnids was recorded after 24 h to determine the concentration able to achieve 50% immobilization (EC50).

The test on *C. dubia* during 48 h of exposure was performed on young organisms, less than 24 h old, obtained from our laboratory cultures. The test was performed according to USEPA (1993) utilizing ten crustaceans for each dilution, three replicates and five concentrations; the test parameter considered was mobility and the concentration found to immobilize 50% crustaceans in 48 h was indicated as EC50.

2.4. Chronic toxicity testing

Chronic effects of the pharmaceutical investigated and its derivative were assessed using the standard methods for the alga *P. subcapitata*, the rotifer *B. calyciflorus* and the crustacean *C. dubia*.

The algal test was based on the measurement of growth inhibition of the *P. subcapitata* during 72 h of exposure according to the International Standard Organization procedure 8692 (ISO, 1989). The algal inoculum was taken from our laboratory culture. Each compound was tested on five concentrations and three replicates. Flasks were placed in a growth chamber at 25 °C under continuous illumination (fluorescent lamps at 8000 lux). In addition to the controls, above described, a control constituted only by medium and furosemide was added in this bioassay to exclude its transformation in the photoproduct. The cell density of test solutions was measured at 0 time and every 24 h for three days by an electronic particle dual threshold counter (Coulter Counter Z2, 100 μm capillary, Instrumentation Laboratory, Miami, FL, USA).

The test on *B. calyciflorus* was based on the population growth inhibition after 48 h of exposure. It was conducted according to the standard ISO/CD 20666 (ISO, 2001b). The test organisms were hatched from cysts provided by MicroBioTests after 18 h of incubation. Each compound was tested in four replicates per concentration and the test samples were incubated at 25 ± 1 °C in the dark for 48 h. At the end of the test, the total number of living rotifers was counted and, by comparison with the control, the population growth inhibition (in %) was determined for each concentration.

The test on *C. dubia* was based on a population growth inhibition in seven days of exposure to samples and was performed according to the standard ISO/CD 20665 procedure (ISO, 2001a). Test animals were obtained by acyclical parthenogenesis of individual adult females (coming from our laboratory mass culture). The young organisms, less than 24 h old, were individually exposed to seven concentrations in ten replicates for seven days. Organisms were monitored for survival, and released neonates were counted every day prior to renewals and then discharged. By comparison between the number of offspring at the end of the test in the sample batch and the control, it was possible to calculate the concentration which gave rise to 50% population growth inhibition, indicated as EC50.

2.5. Mutagenesis/genotoxicity testing

Mutagenicity was assessed by the Ames test, using two different bacterial strains of *S. typhimurium*: TA98 for frame-shift mutations and TA100 for base-pair substitution. The plate incorporation assay was conducted without metabolic activation (S9) to detect direct mutagenic compounds (Maron and Ames, 1983). Six increasing concentrations of the compounds were analyzed in triplicate (from 3.125 to 100 mg/l). Sodium azide (2, 1 and 0.5 μg/plate) and 2-nitrofluoren (1, 0.5 and 0.25 μg/plate) were used as positive controls for TA100 and TA98, respectively. Results obtained showed that these positive controls behaved as expected and that consequently all tests could be considered valid. The plates were incubated at 37 °C for 72 h in the dark. The number of His^+ revertants was counted with the colony counter Cardinal, Perceptive Instruments, Suffolk, UK. A compound was considered mutagenic when the ratio between the number of revertant colonies of the plates containing the test substance and the number of revertants of the plates containing the negative control (mutagenic ratio) was more than double and showed a clear dose–response relationship.

Genotoxicity was assessed by the SOS Chromotest (Quillardet and Hofnung, 1985, 1993), a simple bacterial colorimetric assay. This is based on the measure of the induction of *sfiA*, a gene controlled by the general repressor of the SOS system in *E. coli*. Expression of *sfiA*, induced after DNA damage, is monitored by assaying β-galactosidase activity in a *sfiA::lacZ* operon fusion tester strain. In this study we utilized *E. coli* PQ37 (provided by Prof. Quillardet, Institute Pasteur, Paris, France) which is constitutive for alkaline phosphatase synthesis. The assay is quantitative and allows the association with each compound that actively induces a single parameter, the SOS-inducing potency (SOSIP), which reflects the ability of the sample to induce gene *sfiA* expression (Quillardet and Hofnung, 1985, 1993; Hofnung and Quillardet, 1986).

The β-galactosidase and alkaline phosphatase activity were measured as the o-nitrophenol and 4-nitrophenyl concentration, respectively, by photometric measurement at 420 nm. The test was performed at the same compound concentrations used for the Ames test.

2.6. Statistic treatment of data

Compounds **1** and **2** were examined three times (three independent assays). All results were expressed as nominal concentrations and, except algal test, were analyzed using the Toxcalc™ (1996). For acute toxicity tests, the LC50s and EC50s were calculated by concentration/response regression using probit or trimmed Spearman–Karber method, as appropriate (Peltier and Weber, 1985). For the chronic test with *C. dubia*, the value of the concentration that gave 50% population growth inhibition was calculated using maximum likelihood-logit method. Raw test data from algae were analyzed by a Microsoft Excel 5.0 program (Phoenix, AZ, USA) tailored for this test. From these data the algal growth inhibition in percentage was calculated by integrating the mean values from t_0 to t_{72} h (area under the curve). Inhibition (%) values were tabulated against log-transformed data of concentrations to evaluate the test concentration corresponding to 50% algal growth inhibition. For the chronic tests the lowest observed effect concentration (LOEC) and no observed effect concentration (NOEC) were calculated. If unequal numbers of replicates occurred for the concentrations tested, *T*-test with a Bonferroni adjustment was used.

The genotoxic activity was expressed in the ratio $R = \beta/p$ where β represents β-galactosidase activity and p phosphatase alkaline activity. A compound was considered as an SOS repair system inducer in *E. coli* if the induction factor, defined as $IF = R/R_0$ in which R_0 is the spontaneous ratio measured in the blank test (solvent control), was higher than 2, the β-galactosidase activity was significantly increased compared to the negative control and the induction factor versus concentration showed a clear dose-effect relationship.

The Ames test results were expressed not only as mutagenic ratio but also as revertants/μg of compound by analyzing linear regression of the dose–response curves of the samples found to be mutagenic since pure substances were used (APHA, 1998). The mutagenic potency was determined as the slope of the linear portion of the dose–response curve.

Experimental data allowed us to perform a first approach of risk for the aquatic environment. This evaluates whether the concentrations of pollutants in the environment exceed their PNEC and represents the environmental level at which no adverse effect on the ecosystem is expected. The calculation of PNEC may be based on NOEC, estimated in standard bioassays or on an effect concentration ECx, where x is a chosen effect level on the concentration-response curve. The NOEC is the most frequently used measure for toxicity in the low effect region, but it is not a measure of precision as it is based on operator decisions for the chosen concentrations and their spacing. In our preliminary estimation we chose EC50 values as ECx, because it is more accurate, since it is found in the linear region of the dose–response curve. Furthermore, the EC50 values are always determined by all the researchers to know the toxic potential of substances so that the comparison of toxicities is easier. The estimation of the risk considers the ratio calculated between the highest PEC or the MEC and the PNEC both for acute and chronic toxicity. If the MEC/PNEC ratio is $\geqslant 1$ then an ecological risk is suspected.

In the present study we considered only the chronic PNEC by dividing the MEC by the worst chronic EC50, obtained by tests on different aquatic organisms and assuming the assessment factor = 1000. The assessment factor reduces the degree of uncertainty in the extrapolation from the test data on a limited number of species compared to the real environment. Actually, the European Agency for the Evaluation of Medicinal Products (EMEA) suggests an assessment factor equal to 50 in case of species representing two trophic levels even if the Committee does not exclude different factors justifying their use from the applicants.

3. Results

The photoproduct from furosemide in water was identified as dimer 2 on the basis of its spectroscopic features as reported by DellaGreca et al. (2004b).

The results of acute toxicity for furosemide and its photoproduct are reported in Table 1. Data showed that both substances had a limited acute toxicity on the tested organisms. The crustacean *D. magna* was the most sensitive species for furosemide (EC50 = 60.62 mg/l) while no effects were observed towards the rotifer *B. calyciflorus* and the bacterium *V. fischeri* at the highest tested concentrations, 100 and 200 mg/l, respectively. No effect was found for the photoderivative on *D. magna* up to 100 mg/l with a similar trend for *V. fischeri* and *B. calyciflorus* (up to 120 mg/l).

Table 1
L(E)C50 values in mg/l for acute toxicity tests with confidence limits (95% probability)

Compounds	*V. fischeri*	*B. calyciflorus*	*T. platyurus*	*D. magna*	*C. dubia*
1	NE 200	NE 100	70.57 (61.12–76.82)	60.62 (44.82–81.98)	84.09 (70.11–91.01)
2	NE 120	NE 120	81.02 (75.98–86.40)	NE 100	75.79 (64.31–79.12)

NE = No effect at.

Table 2
NOEC, LOEC and EC50 values in mg/l for chronic toxicity tests with confidence limits (95% probability)

Compounds		*P. subcapitata*	*B. calyciflorus*	*C. dubia*
1	EC50	NE 70	2.493 (2.097–3.103)	2.354 (1.383–6.492)
	NOEC	–	0.625	0.156
	LOEC	–	1.250	0.312
2	EC50	NE 50	1.037 (0.761–1.385)	0.566 (0.275–3.018)
	NOEC	–	0.156	0.008
	LOEC	–	0.312	0.015

NE = No effect at.

Chronic data about the inhibition of the reproduction for *P. subcapitata*, *B. calyciflorus* and *C. dubia* are reported in Table 2 together with NOEC and LOEC values. By 72 h, algae showed no effect at the highest concentration tested both for the parental compound and its photoproduct at 70 and 50 mg/l, respectively. Instead, an EC50 value was found for rotifers and crustaceans at concentrations ranging from 0.56 to 2.49 mg/l. The two organisms demonstrated an overlapping sensitivity to the toxic potential of the tested compounds but always at concentrations far from environmental concern.

The results on mutagenesis and genotoxicity are summarized in Table 3. The two compounds were not able to determine a significant SOS system induction, while a variability among the mutagenic responses was observed in the Salmonella mutagenicity assay. Here, only the photoderivative was mutagenic showing the maximum mutagenic ratio (MR = 94.38) and a good dose–response relationship, but only on the strain TA98. This suggests that the compound is a direct and frame-shift mutagen (Mortelmans and Zeiger, 2000). No mutagenic effect was found for the parental compound.

Using the occurrence data for surface-waters in Italy (up to 254.70 ng/l, Calamari et al., 2003) and our toxicity results, we estimated the aquatic risk only for furosemide because no occurrence data were available for its photoproduct. As already reported in Data Analysis, we only considered the chronic PNEC assuming the assessment factor = 1000. The choice of 1000 was based on the concentration of test substance which resulted in 50% of effect for the organisms exposed (EC50) instead of NOEC. An acceptable risk (<1) was found for the drug investigated ($R = 0.256$).

4. Discussion

Pharmaceuticals, as other compounds such as endocrine disruptors, differ from the typical pollutants because of their bioactivity and continuous release to surface waters with exposure of wildlife organisms. Nevertheless, our results indicate that high, environmentally unrealistic concentrations of furosemide and its photoproduct are needed to cause acute effects. For the medical substances, detected in the environment at low concentrations, life cycle tests on organisms from different trophic layers are needed to identify the hazard of these compounds. In our investigation on furosemide, we found no effects on algae with compounds **1** and **2**, and EC50s ranging from 0.56 to 2.49 mg/l for the other organisms. These values are far from the environmental concentrations detected for this drug that are usually below parts per billion (Heberer, 2002; Calamari et al., 2003; Castiglioni et al., 2004).

Our data are confirmed by another investigation on acute and chronic exposure of the cnidarian *Hydra vulgaris* to furosemide indicating a lack of toxic risk at concentrations up to 1 mg/l (Pascoe et al., 2003). Many other widely prescribed pharmaceuticals, however, showed long-term effects on the aquatic fauna, particularly the invertebrates which play an important role in the functioning of freshwater systems, as recently reported in studies on carbamazepine (Ferrari et al., 2003; Jos et al., 2003), clofibric acid, diclofenac (Ferrari et al., 2003) and antibiotics (Isidori et al., 2005a). In this latter, we found considerable differences between acute and chronic data for the antibiotics investigated. This is well clarified by the acute/chronic toxicity ratios that suggested not to be excluded sublethal effects at environmentally relevant concentrations.

For the assessment of the ecotoxicological effects of drugs on aquatic organisms it is important to consider transformation products that are often more toxic than parent compounds (DellaGreca et al., 2003, 2004a; Brigante et al., 2004; Isidori et al., 2005b). For the furosemide derivative, comparable acute and chronic toxicity were found for all the organisms tested.

The behaviour of the photoproduct was peculiar in the Ames test where a significant mutagenic activity (maximum MR = 94.38) was found for the TA98 strain in contrast with the parent compound, already found negative at mutagenicity in Salmonella in other studies (Zeiger et al., 1988; Gold et al., 1993; Benigni et al., 1995; Waters et al., 1998; Rosenkranz and Karol, 1999). The photoproduct was, however, tested at concentrations (6.25–100 mg/l) far exceeding those found in the aquatic environment. This result, however,

Table 3
Mutagenicity/genotoxicity results (±SD) of the furosemide (**1**) and its photoproduct (**2**)

Compounds	Mutagenicity (Ames test)								Genotoxicity (SOS Chromotest)		
	TA98				TA100						
	Range of mutagenic concentration (μg/ml)	Revertants (μg)	R^2	Maximum mutagenic ratio[a]	Range of mutagenic concentration (μg/ml)	Revertants (μg)	R^2	Maximum mutagenic ratio[a]	Range of genotoxic concentration (μg/ml)	Maximum IF[b]	R^2
1	nm	ns	0.85	1.52 ± 0.35	nm	ns	0.99	1.17 ± 0.63	ng	1.40 ± 0.11	0.20
2	6.25–100	8.28 ± 1.12	0.99	94.38 ± 0.65	nm	0.087 ± 0.24	0.62	1.00 ± 0.98	ng	1.11 ± 0.14	0.62

nm = No mutagenic effect detected on *S. typhimurium* strains TA98 or TA100.
ns = No significative number of revertants compared to the control.
ng = No genotoxic effect detected on *E. coli* PQ37.
[a] Higher number of revertants/plate compared to the control.
[b] IF (induction factor) = R/R_0.

strongly indicates the importance of investigating not only the parent compounds but also the potential byproducts when assessing the environmental impact of active agents. Both furosemide and its derivative were not able to induce SOS activation response. This work provides a preliminary investigation in the field of mutagenesis/genotoxicity utilizing only two tests. More tests, based on different mechanisms, would give a better indication of genotoxicity.

A tolerable risk (<1) was found for furosemide ($R = 0.256$) even if, in terms of real ecological impact of pharmaceuticals on aquatic environment, the risk calculation for drugs should differ from the environmental assessment of industrial chemicals generally based on the use of a limited number of standard tests. In fact, medical substances are, by nature, biologically active and have a variety of specific modes of action. For this reason it would be suitable to investigate other relevant endpoints, such as mutagenic effects and include them in the environmental risk assessment since compounds that did not show any toxicity, such as the furosemide photoproduct, proved to be mutagenic.

5. Conclusions

The most prescribed pharmaceuticals have already been detected (generally at ng/l or low μg/l concentrations) in different environmental aquatic samples (Stuer-Lauridsen et al., 2000; Andreozzi et al., 2003; Calamari et al., 2003; Castiglioni et al., 2004). It is unlikely that non target organisms would be exposed to concentrations causing acute toxicity, but often at these concentrations, chronic, sublethal effects were found. Results from this study demonstrate that both acute and chronic exposure to furosemide and its byproduct do not cause a toxic risk at the order of magnitude of effective concentrations found. On the other hand, the harmfulness of the bioactive agent investigated cannot be excluded if the DNA damage found for its derivative is considered. To date, however, no further information on the occurrence in the environment, is available for this compound.

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