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Electron beam irradiation of pharmaceuticals aiming at toxicity reduction: a binary mixture of fluoxetine and propranolol

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Abstract

Significant evidence is available in the literature justifying the search for treatment technologies or process combinations to improve the decomposition of dozens of pharmaceuticals in wastewater. Conventional processing techniques are insufficient in removal of the pharmaceuticals, for having resistant waste and low biodegradability. Electron beam irradiation (EBI) may play an important role in this context, and relatively low doses have been reported for such purposes. The objective of this study was to apply the process of irradiation with electron beam in order to reduce the toxic effects of fluoxetine, propranolol, and a binary mixture of these pharmaceuticals in aqueous solution. Ecotoxicological tests conducted in two model organisms, *Daphnia similis* microcrustacean, and *Vibrio fischeri* bacterium. It was observed that *D. similis* was more sensitive to the pharmaceuticals and binary mixture, when compared to *V. fischeri.* When EBI was applied, all doses showed significant reduction of toxicity for *D. similis*, and the opposite for *V. fischeri*, when only 5.0 kGy showed a significant reduced of toxicity for the pharmaceuticals and binary mixture. 5.0 kGy was the best removal efficiency for toxicity, approximately 80% for *D. similis* and 20% for *V. fischeri*.

Keywords**:** Daphnids, Electron Beam Irradiation; Fluoxetine; Mixture; Propranolol.

INTRODUCTION

Water pollution is a current issue around the world. In this context, pharmaceuticals from various therapeutic classes have been detected in surface waters, wastewater and groundwater worldwide, including in marine ecosystems. This indicates that further attention is required in this regard to avoid the discharge of these biologically active substances into waterways.

Fluoxetine (commercial name: Prozac®), prescribed for the treatment of depressive and anxiety disorders, has been reported as remaining in water bodies after biological treatment, for example at 5.85 μg L^{-1} in influents and 0.112 μg L^{-1} in effluents (Deblonde *et al.,* 2011). Lower values, however, have also been reported (Kolpin *et al.,* 2002; Metcalfe *et al.,* 2003). Fluoxetine toxicity to aquatic organisms is relatively high. Despite determinations of acute levels of this compound in water bodies, other important findings have also reported, such as ocular lateralization during the aggressive behavior of *Betta splendens* males exposed to Prozac® and changes in *Pimephales promelas* feeding and reproduction behavior (Weinberger & Klaper, 2014; Hedayatirad *et al*., 2017).

Another class of widely evidenced pharmaceuticals in many countries includes β-blockers, such as Propranolol®. Several compounds belonging to this class have also been detected in superficial waters, like propranolol, bisoprolol, and metroprolol, in concentrations of 0.59, 2.9 and 2.2 μg L^{-1} respectively. An average concentration of propranolol in a sewage plant in the United States has been reported as 76 μg L^{-1} by several authors (Sacher *et al.,* 2001; Ternes *et al.,* 2003). The highest environmental concentrations of propranolol reported are 1.9 μg L^{-1} in effluents and 0.59 μg L^{-1} in surface water, which may pose a risk for the most sensitive freshwater species (Godoy *et al.* 2015).

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When developing treatment technology improvements, acute effects are the first basic information related to ecotoxicity measurements. Nevertheless, emerging contaminants are now spread in the environment and account for several chronic effects on living organisms. However, lack of information reporting the effects of contaminant mixtures, as well their interactions, is noted. The mechanisms of action of pharmaceuticals in living organisms, thus, requires further study, and the fact that many pharmaceuticals may be decomposed or transformed into less toxic byproducts when discharged into the aquatic environment should also be taken into account. The increasing use of pharmaceuticals is also a risk factor to the biota (Geiger *et al*., 2016), and risk assessment studies concerning pharmaceutical and healthcare products have been recently reported by Arnold *et al.* (2018). Considerable acute toxicity, for example, has been reported for a mixture of diclofenac, ibuprofen, naproxen and aspirin (1 – 320 mg L^{-1}) (Cleuvers, 2004), while a significant reduction in embryo survival was observed in female *Danio rerio* exposed to a pharmaceutical mixture of acetaminophen, carbazepine, gemfibrozil, and venlafaxine (Galus *et al*., 2013).

Advanced Oxidation Processes (POA) are reported as a complementary technology for wastewater treatment, required for several types of effluents. This technique may contribute to the removal of organic contaminants in water bodies, aiding in the improvement and biodegradability of industrial wastewaters (Rizzo, 2011). This includes the application of ionizing radiation aiming at pollutant degradation, which may be applied through the use of electron beam accelerator (EB) or gamma source irradiators. This technology is based on the action of oxidative molecules produced during water radiolysis and chemical degradation. Some examples in this regard include studies on diclofenac (Homlok e*t al,* 2011; Yu *et al*., 2013; Tominaga *et al*., 2018) fluoroquinolone antibiotics (Tegze *et al.,* 2018); sulfonamide antibiotics (Sági *et al.,* 2018) and chloramphenicol (Csay *et al.,* 2012); paracetamol (Szabo *et al.,* 2012) degradation and detoxification, as well as fluoxetine in municipal sewage (Silva *et al.,* 2016), among others. However, most studies are carried out with pure substances, not mixtures. Thus, advanced treatment-processing studies considering pharmaceutical mixtures are scarce in the literature, indicating a knowledge gap in this regard. Previous studies demonstrated that relatively low doses were enough to reduce toxicity and other paramethers in different effluents (Silva *et al*, 2016; Tegze *et al.,* 2018; Tominaga *et al,* 2018).

In respect to industrial scale, one Electron Beam Facility was installed in China in 2018, with a low energy accelerator, for the enhancement of biodegradability of real textile and dyeing wastewater - combined system as activated sludge and EB irradiation (He *et al.*, 2016). Brazil has been studied different type of effluents also irradiated by EB. (Madureira *et al.,* 2018).

The aim of the present study was to assess the toxicity of a binary mixture of fluoxetine and propranolol and apply EBI in order to reduce their toxicity in an aqueous solution. These pharmaceuticals were elected because they are widely used by Brazilian population and have also been detected in different countries.

MATERIAL AND METHODS

The acute toxicity of the assessed pharmaceuticals, commercial trade as fluoxetine hydrochloride and propranolol, was determined in a binary mixture. *Daphnia similis* and *Vibrio fischeri* were exposed to both pharmaceuticals, individually and in a binary mixture. Electron beam irradiation was applied to aqueous solutions containing both pharmaceuticals, aiming at their decomposition and decreased ecotoxicity, assessed as acute biological effects.

Propranolol β-blocker consists in (RS)-1-(isopropylamino)-3-(naphthalen-1-yloxy)-propan-2-ol; CAS number 318-98-9, molecular formula $C_{16}H_{21}NO_2$, molecular weight 259.34 g mol^{-1}, commercial name Propranolol® (PRP). Fluoxetine hydrochloride consists in -N-methyl-3-phenyl-3 - [(α, α, α-trifluoro-p-tolyl) oxy] propylamine hydrochloride; CAS number 56296-78-7, molecular formula $C_{17}H_{18}F_3NO$, molecular weight 345.79 g mol^{-1}, commercial name Prozac®, (FLX). A 1:1 ratio mixture of both pharmaceuticals was assessed.

Irradiation procedure

A Dynamitron Electron Beam Accelerator was used for sample irradiations and they were irradiated in aqueous solution. Fixed energy machine was 1.4 MeV and conveyor speed of 6.72 m min^{-1} during all the experiments. Fluoxetine hydrochloride (10 mg L^{-1}) and propranolol (80 mg L^{-1}) mixture was considered as an effluent sample, these concentrations were based in the commercial formulation of the pills. The samples were irradiated using a batch system in borosilicate containers (Pyrex). Samples were covered with a plastic wrap during irradiation, for protection. The applied doses were 2.5 kGy, 5.0 kGy, 7.5 kGy, and 10 kGy. Previous experiments and public data were important for electing the applied doses of radiation.

Toxicity assays

The toxicity assays were carried out regarding acute exposure to the assessed pharmaceuticals in the crustacean *Daphnia similis* and the bacteria *Vibrio fischeri.* The test organisms were exposed to the samples immediately after the irradiation of the pharmaceuticals aqueous solutions.

The acute *D. similis* toxicity tests were performed according to the standard Brazilian method NBR 12713/2009. Results were based on the EC50 for the species, which represents the average effective concentration for immobilization of 50% of the exposed organisms, using a 95% confidence interval, calculated using the Trimmed Spearman Karber statistical method (Hamilton *et al.,* 1977). Cultures of the organisms were kept in an incubator (Brand: Quimis) at a temperature of 20º C ± 1ºC, with photoperiod 16 hours light, in crystallizers with a capacity of 2 L, containing between 30 and 40 organisms kept in suitable standard water.

The acute *V. fischeri* toxicity tests were carried out based on the Brazilian standard method NBR 15411-3/2012. The EC_{50} was obtained by a loss of luminescence data analysis, based on gamma values (ratio between the lost and remaining luminescence) and the related concentration of the pharmaceuticals. *V. fischeri* bacteria were purchased in the freeze-dried form of the Biolux® brand, which were kept at -20º C ± 2ºC in a freezer. Reactivation of bacterial solution was the first step before running samples - hydrated with 1000 µ L of reactivation buffer solution. M500 Microbics® Toxicity Analyzer was used for measuring the light in all set of samples. The statistical analysis have been used to calculate EC_{50}, it was based on the gamma values (relation between the light lost and the remaining light from each pair of cuvettes – samples concentration) and the concentration of the sample. Linear regression analysis was performed based on the luminescence decreasing and sample concentration. Thus, the values of $EC_{50/15min}$, and the respective confidence interval were obtained.

The efficiency of the irradiation treatment was calculated by:

$$TU = \frac{100}{EC_{50}} \quad (01)$$

$$TR\ \% = \frac{(TU_0 - TU_{irrad})}{TU_0} \times 100$$

Where: TU_0= Toxic Units before irradiation; TU_{irrad} = Toxic Units after irradiation.

RESULTS AND DISCUSSION

Water radiolysis is the main and initial starting factor for organic degradation (Wojnarovits & Takacs, 2008) as the following equation:

$$H_2O \rightarrow 2.6\ e_{aq}^- + 0.6\ H^\bullet + 2.7\ OH^\bullet + 0.7\ H_2O_2 + 2.6\ H_3O^+ + 0.45\ H_2 \quad (02)$$

Concerning oxidative processes, the measurements of whole toxicity are desirable. A comparison between the toxicity values of non-irradiated solutions for both exposed organisms indicates that the effects were more significant for *D. similis*, at a 4-fold higher toxicity when compared to *V. fischeri.* Fluoxetine was less toxic than propranolol and mixture for the organism *D. similis,* and more toxic for *V. fischeri.*

EC50 values increased after 2.5 and 5.0 kGy for both pharmaceuticals and for binary mixture (exposure for *D. similis*). Before irradiation, propranolol was relatively more toxic than the mixture for *D. similis* and the opposite for *V. fischeri*.

The results of pharmaceuticals aqueous solutions after EBI are at Figures 1, 2 and 3.

Considering that EC_{50} is inversely proportional to toxicity, the data was transformed into Toxic Units (TU). The toxicity removal efficiency by radiation is presented in Table 1, Figure 3, indicating that 2.5 kGy and 5.0 kGy were more effective than higher doses (7.5 and 10 kGy) for the reduction of

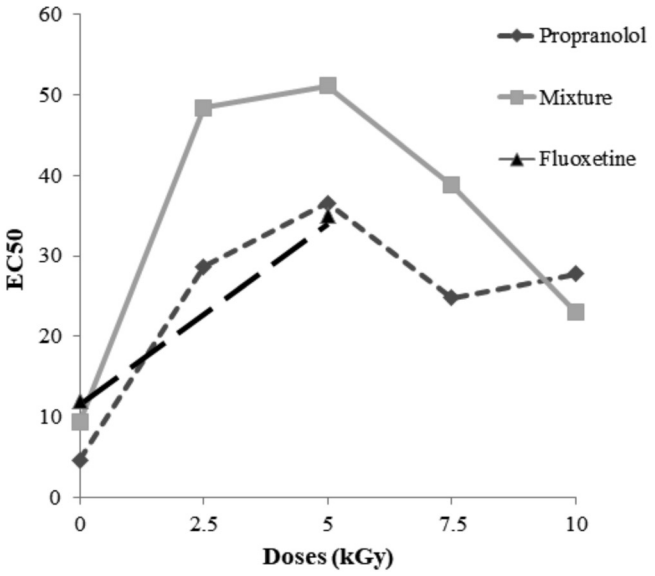

Figure 1 – Effective average concentration (EC50) versus dose. *D. similis* exposed to propranolol (80 mg L^{-1}) and fluoxetine hydrochloride (10 mg L^{-1}) isolated, and a mixture of fluoxetine hydrochloride (10 mg L^{-1}) + propranolol (80 mg L^{-1}) for 48 h.

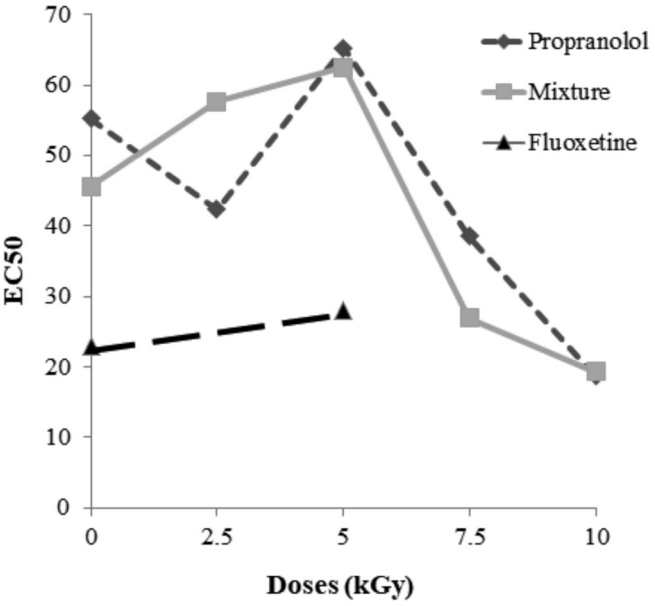

Figure 2 Effective average concentration (EC50) versus dose. *V. fischeri* exposed to propranolol (80 mg L^{-1}) and fluoxetine hydrochloride (10 mg L^{-1}) isolated, and a mixture of fluoxetine hydrochloride (10 mg L^{-1}) + propranolol (80 mg L^{-1}) for 15 min.

acute effects in the studied conditions. Approximately 80% of the toxicity removal was achieved at 5.0 kGy for both pharmaceuticals and binary mixture in *D. similis,* and 20% in *V. fischeri* experiments. On the other hand, 7.5 and 10 kGy enhanced toxic effects compared to non-irradiated samples.

The application of ionizing radiation is a feasible technology, and it will probably be included in the combination of treatment technologies for wastewater. When the applied irradiation is able to decrease toxicity loads, activated sludge process will be able to guarantee proper combined treatment.

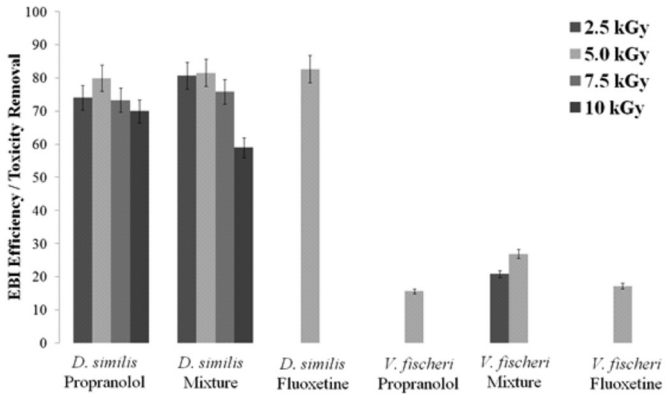

Figure 3 – Toxicity removal efficiency (%) of fluoxetine hydrochloride (10 mg L^{-1}); propranolol (80 mg L^{-1}) isolated; and mixture fluoxetine hydrochloride (10 mg L^{-1}) + propranolol (80 mg L^{-1}), versus dose (*D. similis* and *V. fischeri*).

Comparing the EBI efficacy regarding toxicity removal, the 5.0 kGy dose reduced 81.6% toxicity in *D. similis* and 27% in *V. fischeri*. This is interesting, since *D. similis* was more sensitive to each compound and the mixture than *V. fischeri.* Several recommendations are available for environment protection actions indicating that it is necessary to expose more sensitive species during ecotoxicological assessment.

Several studies have shown 5.0 kGy as a suitable dose for the reduction of toxicity (Borrely *et al*., 2004; Santos *et al.,* 2011; Silva *et al.,* 2016, Tominaga *et al.,* 2018). Borrely *et al.* (2004) analyzed acute toxicity with *D. similis* and *V. fischeri*, in order to evaluate the EBI treatment for sewage effluent, demonstrating the reduction of acute toxicity above 85%, in response to the increased dose. In the same study, effluents from textile industries, doses of 0.5 kGy and 3.0 kGy showed significant toxicity removal. 5.0 kGy presented the highest removal efficiency for the fluoxetine hydrochloride, with reduction values: 91.95% for *Hyalella azteca*, 82.97% for *D. similis* and 79.21% for *V. fischeri* (Santos *et al.*, 2011). Similar results were obtained when fluoxetine hydrochloride was irradiated in the presence of dodecyl sodium sulfate (surfactant), toxicity removals of 91.89%, 87.57% and 89.10%, for *H. azteca, D. similis* and *V. fischeri*, respectively. Removal efficiency of 100% and 79.32% were reported for domestic sewage and its mixture with pharmaceutical fluoxetine hydrochloride, at 5.0 kGy (Silva *et al.,* 2016). Complete diclofenac degradation has been achieved at 5.0 kGy, by EBI (Tominaga *et al.,* 2018).

Multiple factors influenced irradiation pharmaceutical degradation, such as product concentration, pH and absorbed dose. Here, pH values also decreased after irradiation (Figure 4).

A decrease in the pH solution from 7.4 to about 4.0 was associated with the formation of acidic transformation products; HCl elimination following HO radical addition to chlorine bearing carbon atoms in the DCF molecules; and to the formation of recalcitrant carboxylic acids through hydroxylated aromatic rings opening, as a consequence of hydroxyl radical attack. These trends are also associated with the increase in the oxygen-to-carbon ratio in the degradation products, as well as the conversion of carbon bound fluorine and nitrogen to inorganic ions (Homlok *et al.,* 2011). Opening of the hydroxylated aromatic ring formed upon the addition of the HO· radical and formation of lower molecular weight organic compounds, including organic acidic species, are common steps during degradation of contaminants containing aromatic groups (Silva *et al.,* 2016). The decrease in pH with increasing dose has been previously reported for treating dye-contaminated wastewater exposed to electron beam (Vahdat *et al.,* 2010).

Nine transformation products (Table 2) were elucidated during EBI-driven fluoxetine degradation using direct injection mass spectrometry. The degradation pathway includes the

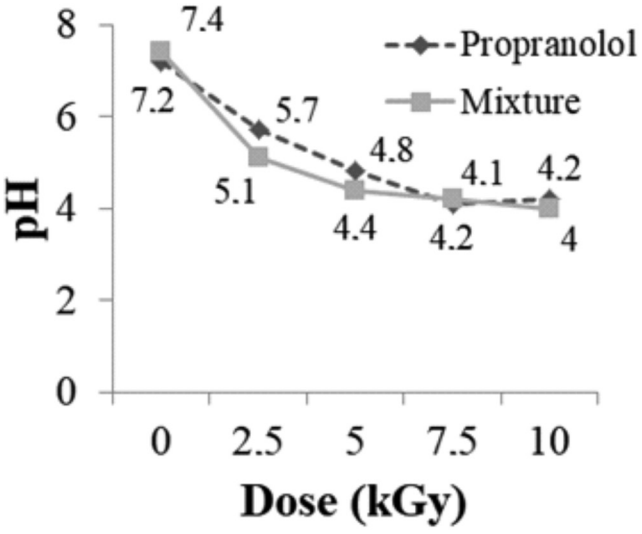

Figure 4 - pH values obtained in the trials for propranolol and for their mixture with fluoxetine hydrichloride, before and after irradiation.

Table 1 – Toxicity removal efficiency before electron beam irradiation.

Doses (kGy)	EBI Efficiency%					
	Propranolol		Fluoxetine		Mixture	
	D. similis	*V. fischeri*	*D. similis*	*V. fischeri*	*D. similis*	*V. fischeri*
2.5	74.14	*	-	-	80.65	20.84
5.0	79.94	15.65	82.67	26.93	81.59	17.26
7.5	73.30	*	-	-	75.85	*
10	69.97	*	-	-	58.96	*

Legend: (*) There was no removal efficiency. (-) No data.

electrophilic addition of hydroxyl radicals generated from water radiolysis under EBI to the aromatic groups, further hydroxylation of ring systems, and also release of fluoride anions. About one third of the carbon bound fluorine atoms originally present in fluoxetine molecules were released to the solution as F− ions for doses higher than 1 kGy.

The electrophilic addition of hydroxyl radicals to aromatics ring systems constitutes an important route of HO· radical attack on organic pollutants (Batista *et al.,* 2014). FLX molecules ($[M+H]^+$ at *m/z* 310.1587, $C_{17}H_{18}F_3NO$) undergo electrophilic addition of hydroxyl radicals to the aromatic rings (Lam *et al.,* 2005).

Table 2 - Identification of organic by-products from fluoxetine degradation, performed by direct injection experiments using UHR-QqTOF mass spectrometry.

By-products		
Compound P1	$[M+H]^+$ at *m/z* 326.1547	$C_{17}H_{18}F_3NO_2$
Compound P2	$[M+H]^+$ at *m/z* 166.1315	$C_{10}H_{15}NO$
Compound P3	$[M+H]^+$ at m/z 143.0090	$C_7H_4F_2O$
Compound P4	$[M+H]^+$ at m/z 342.1507	$C_{17}H_{18}F_3NO_3$
Compound P5	$[M+H]^+$ at m/z 358.1467	$C_{17}H_{18}F_3NO_4$
Compound P6	$[M+H]^+$ at m/z 376.1583	$C_{17}H_{20}F_3NO_5$
Compound P7	$[M+H]^+$ at m/z 392.1543	$C_{17}H_{20}F_3NO_6$
Compound P8	$[M+H]^+$ at m/z 182.1273	$C_{10}H_{15}NO_2$
Compound P9	$[M+H]^+$ at m/z 198.1233	$C_{10}H_{15}NO_3$

CONCLUSIONS

Many studies and detection techniques have been developed to demonstrate the presence and risks of several pharmaceuticals discharged into the aquatic environment. However, these compounds still contaminate aquatic systems and are reported as emerging pollutants. In the present study, fluoxetine was more toxic than propranolol and than a binary mixture for *V. fischeri* and the opposite for *D. similis* under the studied conditions. Regarding the EBI application, 5.0 kGy was effective for reducing toxicity of pharmaceuticals. Statistical analyses concerning the EC_{50} values obtained in the ecotoxicological assays comparing irradiated and non-irradiated samples showed that 5.0 kGy leads to a significant decrease in whole toxicity.

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