

Impact of excipients in the chronic toxicity of fluoxetine on the alga *Chlorella vulgaris*

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Fluoxetine is a selective serotonin reuptake inhibitor (SSRI) widely used in the treatment of major depression. It has been detected in surface and wastewaters, being able to negatively affect aquatic organisms. Most of the ecotoxicity studies focused only in pharmaceuticals, though excipients can also pose a risk to non-target organisms. In this work the ecotoxicity of five medicines (three generic formulations and two brand labels) containing the same active substance (fluoxetine hydrochloride) was tested on the alga *Chlorella vulgaris*, in order to evaluate if excipients can influence their ecotoxicity. Effective concentrations that cause 50% of inhibition (EC_{50}) ranging from 0.25 to 15 mg L⁻¹ were obtained in the growth inhibition test performed for the different medicines. The corresponding values for fluoxetine concentration are 10 times lower. Higher EC_{50} values had been published for the same alga considering only the toxicity of fluoxetine. Therefore, this increase in toxicity may be attributed to the presence of excipients. Thus more studies on ecotoxicological effects of excipients are required in order to assess the environmental risk they may pose to aquatic organisms.

Keywords: Antidepressant, *Chlorella vulgaris*, ecotoxicity, excipients, fluoxetine

Introduction

Over the last decade, the consumption of antidepressants has considerably been growing in Europe [1] and Iceland, Denmark and Portugal head the ranking of the European countries with the highest levels of consumption.[2]

Selective serotonin reuptake inhibitors (SSRIs) are amongst the most widely used antidepressants, fluoxetine being one of the most prescribed.[3] It stands out in the treatment of major depression, anxiety disorders and obsessive-compulsive disorders [4] and acts by inhibiting the reuptake of the neurotransmitter serotonin from the synaptic cleavage, enhancing serotonergic neurotransmission.[4]

Fluoxetine has been detected in surface waters [5–9] and wastewaters [10–13] within the ng L⁻¹ range. In Canada, it was detected in surface waters adjacent to discharges of wastewater treatment plants (WWTPs) effluents reaching maximum concentrations of 46 ng L⁻¹. [14]

Discharge of WWTPs was identified as one of the most important sources of entrance of pharmaceuticals into the environment. However, direct disposal of pharmaceuticals into the sink/toilet or via waste collection can also be pointed out as sources of environmental contamination by pharmaceuticals.[15,16] Therefore, besides the ecotoxicological risk caused by pharmaceuticals, environmental risk assessment should also be extended to excipients, since they are an important part of the final medicine. However, in literature, environmental risk assessment of excipients is rarely focused.[17,18]

Serotonin is also an important neurotransmitter in hormonal and neuronal mechanisms, participating in regulatory and endocrine functions in lower vertebrates and invertebrates.[15] Thus, the presence of fluoxetine in the environment, even at low concentrations (few ng L⁻¹), can adversely affect aquatic organisms. Christensen et al. [19] studied five SSRIs toxicity towards algae and crustaceans, showing that fluoxetine was the most toxic for the alga *Pseudokirchneriella subcapitata* with an effective concentration that causes 50% of inhibition (EC_{50}), for a 48 h period, of 27 µg L⁻¹; while Brooks et al. [20] reported a decrease in *P. subcapitata* growth when exposed to 14 µg L⁻¹ of fluoxetine. The same authors also described a decrease in *Ceriodaphnia dubia* fecundity for concentrations of 223 µg L⁻¹. [21] Fish species are also affected by the exposure to fluoxetine, which is able to significantly reduce their growth [22] and also affect their reproduction, being reported an increase in developmental abnormalities in fish embryos as well as in the plasmatic concentration of oestradiol in females.[23] On the other hand, the locomotion of the epibenthic crab *Carcinus maenas* is significantly increased for concentrations of fluoxetine equal or above 120 µg L⁻¹. [24]

In the present work, the toxicity of five medicines (three generic and two brand labels) used in the treatment of depression, which are available in the Portuguese market, was tested on the alga *Chlorella vulgaris*. All the medicines have the same active substance – fluoxetine. Their effect in

Table 1. Excipients of the studied medicines according to the producers.

Digassim	Prozac	Fluoxetina Actavis	Fluoxetina Ratiopharm	Fluoxetina Sandoz
Cornstarch	Cornstarch	Pre-gelatinized cornstarch	Lactose monohydrate	Microcrystalline cellulose
Dimethicone	Dimethicone	Anhydrous colloidal silica	Microcrystalline cellulose	Pre-gelatinized starch
Gelatine ^a	Patent blue V (E131) ^a	Magnesium stearate	Magnesium stearate	Talc
Titanium oxide (E171) ^a	Yellow iron oxide (E172) ^a	Talc	Anhydrous colloidal silica	Gelatine ^a
Indigo carmine (E132) ^a	Titanium oxide (E171) ^a	Quinoline yellow (E104) ^a	Gelatine ^a	Yellow iron oxide (E172) ^a
Red iron oxide (E172) ^a	Gelatine ^a	Erythrosine (E127) ^a	Titanium dioxide (E171) ^a	Titanium oxide (E171) ^a
	Shellac ^a	Indigo carmine (E132) ^a	Yellow iron oxide (E172) ^a	Shellac ^a
	Black iron oxide (E172) ^a	Titanium oxide (E171) ^a	Quinoline yellow (E104) ^a	Soy lecithin
		Gelatine ^a	Indigo carmine (E132) ^a	Antifoam dc150
		Shellac glaze ^a		Black iron oxide (E172) ^a
		Black iron oxide (E172) ^a		

^aCapsule constituents (not tested).

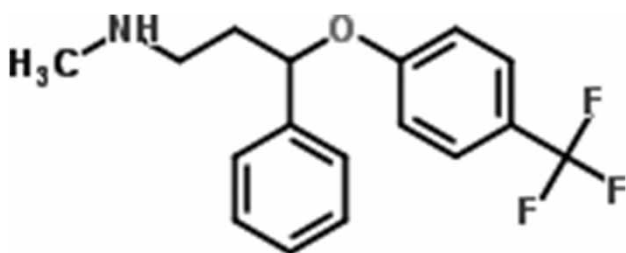


Figure 1. Chemical structure of fluoxetine.

the growth rate of the alga was evaluated over a 72-h period. The aim of the work was to evaluate the chronic toxicity of fluoxetine in this alga as well as to assess the contribution of excipients for the observed toxicity. Algae were chosen as test organisms because any disturbance in their dynamics might affect the higher levels of trophic chain. The unicellular green alga *C. vulgaris* was selected as test organism due to its good sensitivity to toxicants,[25,26] it is easily cultured in laboratory [27,28] and is representative of the algae present in nature, being found in freshwater, saltwater and soil.[29]

Materials and methods

Test substances

Five medicines containing fluoxetine hydrochloride as active substance were studied. Three of them are generic (Fluoxetina Actavis®, Fluoxetina Ratiopharm® and Fluoxetina Sandoz®); and Digassim and Prozac are two brand labels from Vitória and Lilly laboratories, respectively. All the medicines are commercialized as capsules and were obtained in the local market. Each capsule contains 20 mg of fluoxetine hydrochloride, which corresponds to approximately 10% of the capsule content, and different excipients that are enumerated in Table 1 by decreasing order of weight percentage. Only the capsule content was used in tests. The chemical structure of fluoxetine is presented in Figure 1.

Test organism

The ecotoxicity tests were carried out with the freshwater unicellular green alga *C. vulgaris*. The test organism was cultured in laboratory under aseptic conditions. A new culture was started weekly by aseptically transferring 1–2 mL of stock culture to a 50–100 mL of new culture medium in order to adapt the alga to the test conditions and ensure that the alga is in exponential growth phase when it was used to inoculate the test solutions. The stock cultures were kept at $21 \pm 2^\circ\text{C}$, under cool white fluorescent lighting, for four days. Agitation was performed by filtered air bubbling. Each stock culture was examined with an optical microscope (Nikon Alphaphot-2 YS2) to ensure that there were no contaminating microorganisms. The medium for the algal growth inhibition test was prepared in accordance with the OECD Test Guideline 201,[30] using deionized water with conductivity lower than $5 \mu\text{S cm}^{-1}$ and suitable nutrients. The final pH of the nutrient medium solution was approximately 8.

Bioassays

The tests were carried out based on the OECD 201 Guidelines and the US EPA suggested procedure.[30,31] The algae population was exposed in a static non-renewal system, to series of different concentrations of medicine between 0.25 and 18.5 mg L^{-1} , for $72 \pm 2 \text{ h}$, in order to evaluate its chronic toxicity. The test temperature was $21\text{--}24 \pm 2^\circ\text{C}$ (maximum variation = 3°C), using continuous illumination ('Cool white' fluorescent lighting, 6000 to 10,000 lux) and a test solution volume of 100 mL. Three replicates were tested.

The inoculum of the green alga, *C. vulgaris*, provided a concentration around 10^6 cells/mL in each test flask. Initial biomass did not exceed 0.5 mg L^{-1} as dry weight, allowing exponential growth through the incubation period, without risk of nutrient depletion. Aseptic techniques were used in the algal cultures, handling and extreme

care were exercised to avoid contamination. A laminar air flow chamber (FASTER, model two-30) and a sterilization chamber (AJC, model Uniclave 88) were used.

A set of four different concentrations and a control were used for each sample tested, except for Fluoxetina Sandoz for which an additional lower concentration was tested. The diluted water was the culture medium, in order to avoid nutrient limitation.

The test flasks position was randomized and changed daily, being agitated twice by hand. The agitation by air bubbling was not used in order to prevent changes in the concentration and pH.[30,31]

The growth of the algae population was measured in terms of changes in cell density, which was evaluated by optical density (Shimadzu UV-2101 PC spectrophotometer) at 440 nm.[28,32] A linear relationship was verified between cell counts and biomass versus optical density. The optical microscope was used for cell counts. The biomass was determined by filtration over a 0.45 μm membrane (GN-6 Metrical Grid, Pall Corporation) followed by drying until constant weight.

The pH (Crison® CWL/s7 combined glass electrode connected to a decimilivoltammeter Crison®, pH meter, GLP 22) was evaluated in the beginning and at the end of the test, its variation should not exceed 1.5.

The test conditions were validated through the use of a reference toxicant, the potassium dichromate, tested in the same conditions.[31]

Data analysis

The optical density values obtained at 440 nm [32] were transformed in cell density (cells/mL) using the linear experimental relation previously determined. The acceptability criterion considered was variability less than 20% among replicates.

The statistical analysis of results was done as suggested by EPA.[31] The EC_{50} was calculated using a point estimation technique, the linear interpolation method. Due to the use of a linear interpolation technique to calculate an estimate of the EC_{50} , standard statistical methods for calculating confidence intervals are not applicable.

Results and discussion

The chronic toxicity of five medicines (three generic and two brand labels) with the same active substance (fluoxetine) was tested on the alga *C. vulgaris*. Considering that medicines were tested as a whole (pharmaceutical plus excipients) and subjected to a very rigorous quality control (concentrations and stability) by pharmaceutical industry, it was assumed that the real concentrations of fluoxetine are the nominal ones. According to literature [30,31] it was considered that there was no significant change in the nominal concentration during the test period (less than 20%). Moreover fluoxetine has low volatility

Table 2. Toxicity of the five studied medicines containing fluoxetine to *Chlorella vulgaris*.

Medicine	72 h EC_{50} (mg L^{-1})
Fluoxetina Actavis	0.57
Fluoxetina Ratiopharm	15
Fluoxetina Sandoz	0.25
Digassim	3.8
Prozac	8.1

and biodegradability, being described as one of the most persistent pharmaceuticals in the environment.[33]

The obtained average inhibition rates, and also the maximum and minimum values, are presented in Figure 2. It was noticed that there was an increasing growth inhibition of algae to increasing concentrations of the tested medicine.

EC_{50} ranging from 0.25 to 15 mg L^{-1} were obtained in the growth inhibition test performed on *C. vulgaris* for the different medicines (Table 2). The corresponding values for fluoxetine content are 10 times lower. Nevertheless, a higher value ($\text{EC}_{50} = 4.3 \text{ mg L}^{-1}$) was previously published for *C. vulgaris*, when exposed to fluoxetine for a period of 96 h,[34] albeit the incubation time used was lower (72 h).

Although similar EC_{50} values were reported in literature for the algae *Scenedesmus acutus* and *S. quadricauda*, with EC_{50} of 91 and 213 $\mu\text{g L}^{-1}$, respectively,[34] and also for *P. subcapitata* (EC_{50} ranging from 24 to 90 $\mu\text{g L}^{-1}$).[3,19,20,34] The latter also showed smaller cells with deformities for 13.6 $\mu\text{g L}^{-1}$ of fluoxetine.[20] On the other hand, this SSRI did not show any effect on the efficiency of photosynthesis of *P. subcapitata*. [3]

In the present study the medicines were tested as a whole (active substance plus excipients), since the quantitative composition of the medicine is not of public domain and excipients represent 90% of their total weight. They share the same active substance (fluoxetine), so differences in their toxicity might be attributed to the excipients attending that: all test conditions were the same, being controlled and kept constant; experiments were made in triplicate, the results being reproducible among replicates; the only parameter that changed was the supplier of the medicine, which implies different composition in terms of excipients and their quantities.

As it was mentioned, EC_{50} values are lower than data for fluoxetine toxicity in *C. vulgaris*, which could be attributed to the presence of excipients. In accordance with literature, most of the excipients present in the studied medicines (Table 1), namely starch, cellulose and lactose, are considered environmentally safe.[17] The lowest EC_{50} values were obtained with two generic formulations (Fluoxetina Sandoz and Fluoxetina Actavis). This could be due to the presence of talc in their formulations (Table 1), which might have caused some turbidity in the test solution decreasing the penetration of light and, consequently, the growth of the alga.

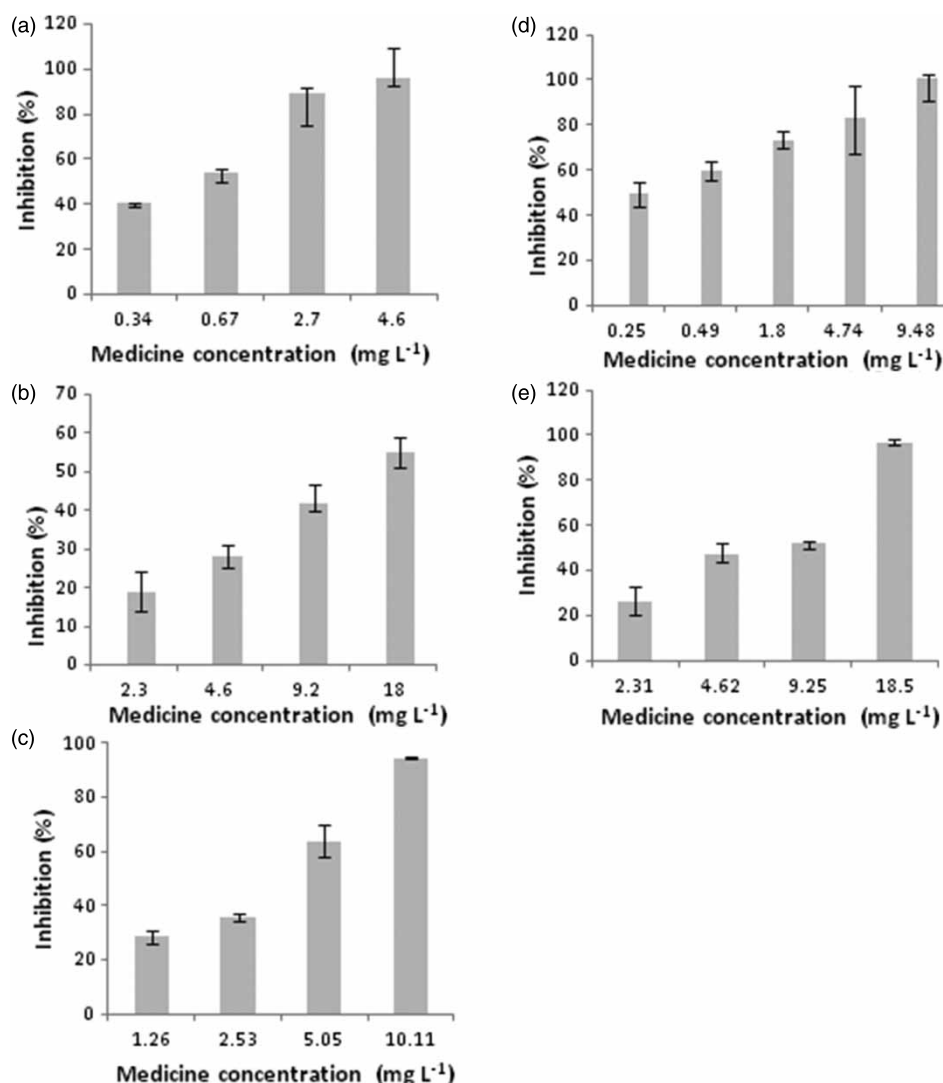


Figure 2. Inhibition rates for: (a) Fluoxetina Actavis; (b) Fluoxetina Rathiopharm; (c) Fluoxetina Sandoz; (d) Digassim; (e) Prozac.

Fluoxetine has been detected in surface waters at concentrations of few ng L^{-1} . For example, 66.1 ng L^{-1} were detected in the Henares–Jarama–Tajo river system (Spain),[9] in USA surface waters levels can go upto 43.2 ng L^{-1} ,[7] while in St Lawrence river (Canada) concentrations ranging from 0.42 to 1.3 ng L^{-1} . [5] Attending to the EC_{50} values obtained in the present study (Table 2), environmental concentrations of fluoxetine may not pose a risk for algae. However, it was proved that different SSRIs can have additive effects,[3,19] therefore as pharmaceuticals contaminate the environment as a mixture and not as single compounds, should be taken into account that fluoxetine might have toxicity for non-target organisms even at lower concentrations. On the other hand, the chronic exposure of fish to low levels of fluoxetine may cause negative effects in reproduction or behavioural functions.[20] For instance, a decrease in swimming velocity was observed when the Arabian killifish was exposed to concentrations of fluoxetine as lower as $0.03 \mu\text{g L}^{-1}$, [35] while a delay in the

development of external sexual morphology in both male and female western mosquitofish was reported for the levels of $71 \mu\text{g L}^{-1}$. [36]

In fact, fluoxetine was included in a shortlist of 10 pharmaceuticals potentially dangerous for aquatic organisms due to environmental exposure (by WWTP effluents) that should be further monitored.[37]

Conclusions

The evaluation of chronic toxicity of five medicines (three generic and two brand labels) with the same active substance (fluoxetine) to the alga *C. vulgaris* was performed on a 72 h growth inhibition test. EC_{50} ranging from 0.25 to 15 mg L^{-1} were obtained for the studied medicines, corresponding to concentrations 10 times lower than that of fluoxetine. Higher EC_{50} values were reported in literature for the same alga, taking into account only the toxicity of fluoxetine. Therefore, the observed increase in

toxicity may be attributed to the presence of excipients. However, nowadays there is a scarce knowledge in ecotoxicity of excipients present in pharmaceutical products. Although they are present in small amounts, environmental risk assessment should consider the whole medicine (active substance(s) plus excipients). Therefore more studies on ecotoxicological effects of excipients are required in order to assess the environmental risk they may pose to aquatic organisms, since they show a more realistic impact of medicines than testing only their individual constituents.

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