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Behavioral effects of citalopram, tramadol, and binary mixture in zebrafish (Danio rerio) larvae

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HIGHLIGHTS

- Citalopram and tramadol binary mixture effects studied in zebrafish (Danio rerio).
- Embryo-larvae were exposed from fertilization until six days post-fertilization.
- Heart rate, spontaneous tail coiling, and death/malformation incidence unaffected.
- Anxiolytic effects in citalopram (EC50: 471 µg L−1) and tramadol (EC50: 411 µg L−1).
- The mixture was less anxiolytic (EC50: 713 µg L−1), indicating interaction effects.

ABSTRACT

Pharmaceuticals are emerging as environmentally problematic compounds. As they are often not appropriately removed by sewage treatment plants, pharmaceutical compounds end up in surface water environments worldwide at concentrations in the ng to µg L−1 range. There is a need to further explore single compound and mixture effects using e.g. in vivo test model systems. We have investigated, for the first time, behavioral effects in larval zebrafish (Danio rerio) exposed to a binary mixture of an antidepressant drug (citalopram) and a synthetic opioid (tramadol). Citalopram and tramadol have a similar mode of action (serotonin reuptake inhibition) and are known to produce drug-drug interactional effects resulting in serotonin syndrome (SS) in humans. Zebrafish embryo-larvae were exposed to citalopram, tramadol and 1:1 binary mixture from fertilization until 144 h post-fertilization. No effects on heart rate, spontaneous tail coiling, or death/malformations were observed in any treatment at tested concentrations. Behavior (hypoactivity in dark periods) was on the other hand affected, with lowest observed effect concentrations (LOECs) of 373 µg L−1 for citalopram, 320 µg L−1 for tramadol, and 473 µg L−1 for the 1:1 mixture. Behavioral EC50 was calculated to be 471 µg L−1 for citalopram, 411 µg L−1 for tramadol, and 713 µg L−1 for the 1:1 mixture. The results of this study conclude that tramadol and citalopram produce hypoactivity in 144 hpf zebrafish larvae. Further, a 1:1 binary mixture of the two caused the same response, albeit at a higher concentration, possibly due to SS.

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1. Introduction

Pharmaceutical drug residues are regularly detected in the surface water environment in the ng to µg L−1 concentration range (Fick et al., 2009; Grabicova et al., 2017). Pharmaceutical compounds end up in surface water mainly due to inadequate removal in sewage treatment plants (Lindberg et al., 2014; Luo et al., 2014). Although most drugs are fully metabolized to inactive metabolites, some compounds are preserved in their active form and eliminated through the kidneys (Bergheim et al., 2012). As a consequence, minute concentrations of drugs are constantly released into the aquatic environment (Lindberg et al., 2014). Numerous pharmaceuticals may bioaccumulate in aquatic and semi-aquatic invertebrates, thereby exposing predators (including fish) to levels exceeding surface water concentrations (Richmond et al., 2018). The exposure to pharmaceuticals may result in ecological effects influencing aquatic lifeforms since drug target sites are evolutionarily conserved. For example, the zebrafish (Danio rerio) has been...
proposed to have about 86% orthologs to human drug targets (Gunnarsson et al., 2008). Water-dwelling organisms continuously exposed to anti-depressant and anti-anxiety drugs might be affected by e.g. increased predation pressure (Klaminder et al., 2019). There is thus a need for research establishing drug-drug interaction properties of pharmaceuticals mixtures in non-target species (Backhaus, 2014; Gunnarsson et al., 2019).

One pharmaceutical drug frequently found in surface waters is citalopram (Fick et al., 2009; Vasskog et al., 2006). Citalopram is a selective serotonin reuptake inhibitor (SSRI) type drug and is one of the most prescribed and used antidepressants in the world. The drug has two racemic enantiomers, S- (+) and R- (−) which are the active enantiomer and R- (−) is inactive (Hytte et al., 1992), although some studies have shown that the R-enantiomer may have an inhibiting effect on the S-enantiomer (Zhong et al., 2009). The antidepressant effect arises through a mechanism inhibiting synaptic serotonin (5-hydroxytryptamine (5-HT)) reuptake, giving rise to increased 5-HT concentrations in the central nervous system pre-synaptic clefts (Bechzhiiby-Butler et al., 2006). When citalopram is consumed by humans, about 12% of the parent compound leaves the body unmetabolized (Milhe and Goa, 1991). The remainder is metabolized into citalopram-N-oxide, citalopram propionic acid and the active metabolite desmethylcitalopram (Hytte et al., 1992). Desmethylocitalopram is then further degraded to didesmethylcitalopram (Sangkuhl et al., 2011). Relatively high citalopram concentrations (~8 µg L−1) have been detected downstream from pharmaceutical production sites in India (Fick et al., 2009).

Tramadol is a commonly used synthetic opioid medication for pain treatment (Rougemon-Bücking et al., 2017). The effect causes analgesia (pain relief) when tramadol is converted into the active metabolite O-desmethyltramadol which acts as an agonist to the µ-opioid receptor (Vazzana et al., 2015). However, it is reported that tramadol may also be used for depression and anxiety treatment via its 5-HT and norephedrine reuptake inhibition properties (Miotto et al., 2017). Tramadol consists of two chiral centers with two enantiomers, the S,S-enantiomer and the R,R-enantiomer. The S,S-enantiomer has a strong effect on the norephedrine and 5-HT reuptake by inhibition while the R,R-enantiomer has 5-HT reuptake properties also derived from inhibition (Miotto et al., 2017). The unchanged excretion of the tramadol parent compound in urine is about 15–35% (Kasprzyk-Hordern et al., 2009). Tramadol is relatively stable in the aquatic environment, with concentrations of up to 50 µg L−1 measured in Cameroon river sediments (Kusari et al., 2016).

When citalopram and tramadol are co-administered in humans, a drug-drug interaction termed serotonin syndrome (SS) may occur (Park et al., 2014). SS is manifested when a single reuptake inhibitor (tacrine or tramadol) is co-administered with a drug that increases 5-HT concentrations in the synaptic cleft, causing an imbalance in the binding of 5-HT to the 5-HT1A- and 5-HT2A receptor (Beakley et al., 2015). The exhibited symptoms of SS are related to neuromuscular activity, tachycardia (increased resting heart rate), tremors, and mental conditions including confusion and agitation (Boyer and Shannon, 2005). Co-administering these compounds should be avoided as the resulting SS syndrome may ultimately be fatal (Rougemon-Bücking et al., 2017). Drug-drug interactions leading to clinical SS are reproduced in mammal studies have been reproduced in fish studies, suggesting fish could be sensitive to 5-HT-linked adverse effects from co-exposure of several SSRI and mechanistically related compounds (Stewart et al., 2013). Zebrafish embryo models are increasingly used within the framework of biomedical science, including research on behavioral toxicity (Noyes et al., 2018). Zebrafish are genetically similar to mammals and possess a highly developed serotonergic system both in the central nervous system and peripheral system (Winberg et al., 1997). Zebrafish larvae develop the 5-HT system around 96 h post-fertilization (hpf; Brustein et al. (2003)). The zebrafish larvae also possess a distinct behavioral repertoire in response to stress stimuli (e.g. rapid changes in illumination; Padilla et al. (2011)).

The present study aimed to identify behavioral and embroyotoxic effects in embryo-larval zebrafish exposed to citalopram, tramadol, and a 1:1 binary mixture. To our knowledge, this study is the first to combine an SSRI (citalopram) and opioid (tramadol) in a zebrafish embryo-larvae behavioral test. The rationale for studying the combination of the compounds stemmed from the hypothesis that tramadol co-exposure would potentiate citalopram embryo-larval behavioral effects, in accordance with studies showing such outcome in rodents (e.g. Fox et al. (2009)). In order to capture SS-like effects, we recorded endpoints including the incidence of lethality and malformations, spontaneous tail coiling, heart rate, and swimming activity in response to alternating dark and light periods in a 6d static zebrafish embryo-larvae exposure study.

2. Materials & methods

2.1. Chemicals and test solutions

The chemicals used in the present study were citalopram hydrobromide (CAS number: 59729–32–7, purity ≥98%) and tramadol hydrochloride (CAS number: 36282-47-0, purity ≥99%), and isotope-labeled internal standards of tramadol (13C, D3) and citalopram (D4). All chemicals were purchased from Sigma Aldrich (Sweden). Test solutions of citalopram, tramadol and 1:1 citalopram:tramadol mixture were prepared by dissolving the respective compound in carbon-filtered tap water (used as control treatment) by stirring and vortexing at room temperature (22 °C). The pharmaceutical concentrations used for the exposure studies were determined by prior concentration-response range screenings. Citalopram was serially diluted 1:4 corresponding to five nominal test concentrations (8000, 2000, 500, 125 and 31 µg L−1). Tramadol was likewise serially diluted 1:4 corresponding to five nominal test concentrations (6250, 1563, 391, 98 and 24 µg L−1). A binary mixture solution with a ratio of 1:1 citalopram:tramadol was prepared with at a nominal maximum concentration of 10000 µg L−1 (combination of 5000 µg L−1 citalopram and 5000 µg L−1 tramadol) and serially diluted 1:4, yielding in total four nominal test concentrations (10000, 2500, 625, 156 and 39 µg L−1). Water quality parameters were monitored for each test solution before and after each test, and did not differ significantly from the control treatment (pH: 8.36 ± 0.04, conductivity: 450 ± 30 µS/cm, dissolved O2: 90 ± 5%).

2.2. Chemical analysis

The measured concentrations at the start of the embryo exposure (t = 0 h) are represented by a mean of triplicate samples and the measured concentrations at the end of the embryo exposure (t = 144 h) are based on single samples. No samples from t = 144 were analyzed in the mixture study. The chemical analysis method was based on a previously published study (Rostvall et al., 2018). For chemical analysis, the samples were filtered using a regenerated cellulose syringe filter (0.22 µm pores). One milliliter of the filtered extract was placed in an autosampler vial with 10 ng of the isotope-labeled internal standards of tramadol (13C, D3) and citalopram (D4) per aliquot of sample. The samples were analyzed using liquid chromatography tandem-mass spectrometry (LC-MS/MS) with an LC system from Thermo Fisher Scientific and a triple-stage quadrupole MS/MS TSQ Quantiva (Thermo Fisher Scientific,
2.3. Embryotoxicity- and behavioral tests

The zebrafish embryotoxicity test (ZFET) assay used in this study was based on the methodology described in Pohl et al. (2019). Adult zebrafish were kept under 12 h dark and light interval conditions at 26 °C. Groups (n = 10) of adult zebrafish were moved to aquaria equipped with stainless steel net cages with carbon-filtered tap water. During the next morning (09:00–09:30 a.m.), the stainless-steel net cages containing zebrafish were moved to aquaria filled with fresh carbon-filtered tap water where they could spawn. Eggs were collected from the aquarium with the highest proportion of fertilized eggs for each test. Embryos (n = 32 per treatment group, distributed on two plates) were exposed to each test solution at –3 hpf and placed in two 96-well round bottom plates with a 200 μL volume until 144 hpf. The plates were covered with Parafilm M (Bemis Company, United States) throughout the six d exposure (12:12 h light cycle, 26 ± 1 °C air temperature). The eggs were exposed statically (i.e. test solutions were not renewed over time) from fertilization until 144 hpf.

Incidences of lethal and sublethal effects were observed by stereo microscopy at 24, 48 and 144 hpf. For instance, coagulation from fertilization until 144 hpf.

2.4. Statistical analysis

The R software (R Core team, 2019) with the RStudio version 1.1.463 interface (RStudio Team, 2019) was used for statistical computing and plotting. Statistical analysis of the endpoints heart rate, spontaneous movements and behavior were done by ANOVA and Dunnett’s post hoc test. Fisher’s test was used to analyze lethal (coagulation) and sublethal (malformations) effects which were categorized into one group (affected). The R package ggpplot2 was used to produce plots (Wickham, 2016). Swimming activity in response to dark conditions was normalized against control mean and modeled in a 4 parameter logistic regression (the Hill equation), before plotting and estimation of effect concentrations using the drc R package (Ritz et al., 2015). The significance level was set at 0.05 for all parametric- and nonparametric tests.

3. Results & discussion

The present study was performed to investigate embryotoxicity- and neurobehavioral endpoints in zebrafish exposed to citalopram, tramadol and a 1:1 binary mixture thereof. The endpoints studied included spontaneous tail coiling frequency, heart rate, the incidence of lethality/malformations, and behavioral effects (swimming activity during alternating dark and light conditions). Measured citalopram and tramadol concentrations are shown in Table 1.

Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Nominal concentration (μg L⁻¹)</th>
<th>Measured concentration at t = 0 h (mean, μg L⁻¹)</th>
<th>Measured in % of nominal concentration</th>
<th>Measured concentration at t = 144 h (μg L⁻¹)</th>
<th>LOQ (μg L⁻¹)</th>
</tr>
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3.1. Embryotoxicity of citalopram, tramadol, and mixture

There were no significant effects in any recorded embryotoxicity endpoint (i.e., heart rate, spontaneous tail coiling, and incidence of death/malformations) in zebrafish embryo-larvae exposed to citalopram, tramadol, or the mixture (Supplementary Data Fig. S1). Early spontaneous tail-coilings appear before 24 hpf as the axons start to proliferate in the embryonic somites, and the tail-coiling frequency at 24 hpf can be used for neurotoxicity assessment (Basnet et al., 2017; Saint-Amant and Drapeau, 1998). As observed in this study, embryo spontaneous tail movements at 24 hpf were not affected (Supplementary Data Fig. S1). This may indicate a low sensitivity when studying the endpoint in zebrafish embryos exposed to the compounds citalopram, tramadol, and the mixture at concentrations about 20–5000 μg L⁻¹. The functionality of the 5-HT system in zebrafish embryo is initiated around 96 hpf (Brustein et al., 2003). Hence, serotonergic compounds would not directly affect the undeveloped brain, or other organs, at 24 hpf.

At 48 hpf the heart rate was visually measured for all drugs compositions at different concentrations. The results indicated that there were no significant effects on embryo heart rate compared to the controls (Supplementary Data Fig. S1). Earlier studies have shown the effects of 5-HT on zebrafish heart rate via the occurrence of 5-HT in the heart cells (Stoyek et al., 2017). Exposure to other SSRI pharmaceuticals such as fluoxetine has been shown to cause a heart rate reduction, meaning the SSRI also affected the 5-HT re-uptake in the heart (Stoyek et al., 2017). On the other hand, increasing levels of 5-HT due to deprenyl exposure has been associated with increased heart rate in nine d post-fertilization zebrafish larvae in a previous study (Sallinen et al., 2009). The present study could however not detect heart rate alterations caused by citalopram, tramadol, or the mixture 48 hpf zebrafish embryos. This may be due to the fact that the 5-HT system is not active until 96 hpf in zebrafish embryos (Brustein et al., 2003).

At 144 hpf the proportion affected (dead and malformed) embryos were summarized. The results showed no significant effect on the embryos exposed to citalopram, tramadol or the mixture compared to the controls (Supplementary Data Fig. S1). Citalopram LC₅₀-values of 38.4 mg L⁻¹ in zebrafish embryo-larvae (Steele et al., 2018) and 9.1 mg L⁻¹ in Japanese medaka (Oryzias latipes) larvae.

![Figure 1](image-url)
have been reported previously. –2 to 7 fold higher than the highest concentration used in the present study. Zebrafish embryos have been exposed to tramadol in earlier studies with concentrations between 10 and 200 µg L⁻¹ resulting in delayed hatching, but no significant embryo mortality (Sehonova et al., 2016).

3.2. Behavioral effects at 144 hpf

Larval zebrafish behavior (swimming activity) in response to alternating light- and dark periods was assessed at 144 hpf. It is known that zebrafish larvae display a higher activity during dark conditions compared to light conditions, presumably due to a stress response triggered by the sudden shift from light to total darkness (Burgess and Granato, 2007; MacPhead et al., 2009). Several psychoactive compounds can cause decreased swimming activity (hypoactivity) in response to stress cues, which may affect fish species adversely since hypoactivity may result in increased predation risk (Saaristo et al., 2018). In the present study, hypoactivity during dark conditions was recorded in larvae exposed to citalopram, tramadol and the 1:1 binary mixture in comparison with unexposed control, demonstrating anxiolytic properties of these treatments with LOECs between ~300 and 400 µg L⁻¹ (Fig. 1). When comparing the treatments in concentration-response curves (4 parameter logistic regression) it was revealed that the binary mixture was less potent (~50% higher EC₅₀) in terms of hypoactivity during dark conditions than citalopram and tramadol respectively (Fig. 2).

3.2.1. Citalopram

The swimming activity during dark conditions in larvae exposed to citalopram significantly decreased and the lowest observed effect concentration (LOEC) was 373 µg L⁻¹ (Fig. 1). Citalopram has the pharmacological effect of antagonizing serotonin reuptake transporters (SERT) in studied fish and thereby provoking a multitude of behavioral effects (Gould et al., 2007; Sehonova et al., 2018). Previous studies on zebrafish larval behavior have reported effects at 20 µg L⁻¹ (Steeie et al., 2018). Japanese medaka larvae are reportedly affected at concentrations of 10 µg citalopram L⁻¹ cit- alopram (Chiffre et al., 2016). Crayfish exposed to citalopram (1 µg L⁻¹), exhibit anxiolytic behaviors (Burt et al., 2018). Citalopram has also been shown to exert behavioral effects in adult three-spine stickleback (Gasterosteus aculeatus; Kellner et al. (2015)), guppy (Poecilia wingei; Olsén et al. (2014)) and zebrafish (Nielsen et al., 2018), at concentrations well below the LC₅₀ reported for each respective species. Anxiolytic responses in wild-caught three-spine stickleback exposed to citalopram alone at concentrations of up to 15 µg L⁻¹ have also been reported (Kellner et al., 2016). Furthermore, citalopram has been shown to interfere with fish embryonic development concerning behavior (Kellner et al., 2018) and bone development (Fraher et al., 2016).

According to the literature, citalopram has yet not been found in the environment at concentrations approximating the effect concentration observed in the present study (Fick et al., 2009; Grabicova et al., 2017). However, under real-life conditions, cit- alopram typically occurs in combination with a host of other SSRI and their metabolites, some of which retain SSRI activity, and the total SSRI concentration can be much higher than single compound measurements will show (Vasskog et al., 2008).

3.2.2. Tramadol

The results of the present study indicated a significant anxiolytic effect on zebrafish embryos exposed to tramadol and a LOEC of 320 µg L⁻¹ (Fig. 1). Tramadol has previously been investigated in the context of aquatic behavioral toxicology, with examples including studies on crayfish displaying increased shelter dwelling after treatment with environmental relevant (1 µg L⁻¹) concentrations (Burt et al., 2018; Lozé et al., 2019). Clinical side-effects of tramadol exposure include drowsiness and confusion, possibly explaining anxiolytic outcomes in non-target organisms (Langley et al., 2010). However, it may also be suspected that other mechanisms, e.g. mitochondrial dysfunction leading to disturbed energy metabolism, could also be involved in decreasing swimming activity (Zhuo et al., 2012).

3.2.3. Mixture

A decrease in swimming activity was observed in zebrafish embryos exposed to the 1:1 binary mixture with a LOEC of 473 µg L⁻¹ (Fig. 1). By modelling the concentration-response curves of the swimming activity in darkness in % relative control of the three tests we could observe that the 1:1 mixture was ~50% less potent (EC₅₀: 411 µg L⁻¹ ± 118 SE) than tramadol (EC₅₀: 713 µg L⁻¹ ± 251 SE) or citalopram (EC₅₀: 471 µg L⁻¹ ± 177 SE) individually (Fig. 2).

To our knowledge, the present work is the first where a mixture of an SSRI drug and tramadol were investigated to identify behavioral effects in larval zebrafish possibly linked to SS. Adult zebrafish has earlier been used to demonstrate possible signs of SS (top dwelling) after exposure to a fluoxetine (SSRI) and l-tryptophan (SSRI) combination (Stewart et al., 2013). In the present study, we could not detect any indications of synergism following citalopram and tramadol co-exposure. On the other hand, the decreased potency of the 1:1 binary mixture could perhaps
illustrate signs of SS mental symptoms including e.g. agitation or increased anxiety (Maximino et al., 2013). However, this interpretation is speculative and more mechanistic research is needed to provide evidence.

4. Conclusions

Uncovering mixture effects of SSRIs and opioids on zebrafish models can inform about the risk of polluting the environment with human pharmaceuticals affecting the 5-HT system. This study was therefore performed to understand whether citalopram, tramadol, and a mixture thereof would affect developing zebrafish embryo spontaneous tail coiling, heart rate, death/malformation incidence, and larval behavior (swimming activity in light and dark conditions). The recorded endpoints tail coiling, heart rate and incidence of death/malformations were not affected by any treatment. Larval behavior at 144 hpf was however affected by citralopram (LOEC: 373 μg L⁻¹), tramadol (LOEC: 320 μg L⁻¹), and mixture (LOEC: 473 μg L⁻¹). The mixture effect was seemingly antagonistic, as the EC₅₀ of larval hypoactivity in dark conditions was higher in 1:1 binary mixture (713 μg L⁻¹ ± 177 SE) than citralopram (EC₅₀: 471 μg L⁻¹ ± 177 SE) and tramadol (EC₅₀: 411 μg L⁻¹ ± 118 SE), respectively. Since both compounds exert similar behavioral responses in zebrafish larvae, the combined concentrations of citalopram and tramadol (and other SSRIs) found in the surface water environment may be of concern. Further studies combining drugs with different pharmacological effects to evaluate potential adverse effects in aquatic organisms are needed.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2019.124587.

References


