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Pharmaceuticals and Personal Care Products Toxicity to Japanese Medaka Fish (Oryzias latipes)

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We evaluated the acute toxicity of some pharmaceuticals and personal care products (PPCPs) to the survival of Japanese medaka (*Oryzias latipes*). Adult medaka were exposed to different concentrations of triclosan (TCS; 1, 2, 2.4 and 3 mg/L), diclofenac (DCF; 9, 12, 15 and 18 mg/L) or carbamazepine (CBMZ; 60, 65, 70, 90 and 100 mg/L) for 96 h in a semi–static water exposure system. In order of decreasing toxicity to medaka, the 96–h LC_{50} values were TCS, 1.7 mg/L; DCF, 10.1 mg/L; and CBMZ, 61.5 mg/L. We compared the no observed effect concentration (NOEC) determined from this study with the predicted environmental concentrations (PEC) and concluded that TCS and DCF pose potential risks to medaka but the risk from CBMZ is negligible

INTRODUCTION

Although toxicological interest in pharmaceuticals and personal care products (PPCPs) in the environment is fairly recent, the problem is not considered new. PPCPs have probably found their way into the environment for as long as they have been consumed. There, they directly affect aquatic organisms and can be incorporated into food chains (Daughton and Ternes, 1999). Little is known about the acute or chronic effects of these compounds in aquatic ecosystems. With a growing population and an increased demand for medicines, the amounts of PPCPs in the environment have been steadily increasing. Each year, large quantities of PPCPs are sold and consumed worldwide. From 1999 to 2002, pharmaceutical use increased worldwide by about 25% to 424 billion US dollars (German Association of Research–Based Pharmaceutical Companies, 2004). In addition to pharmaceutical compounds, large quantities of personal care products are produced and sold worldwide each year (Kang *et al.*, 2005). Among the most frequently detected PPCPs of greatest concern are triclosan (TCS), diclofenac (DCF) and carbamazepine (CBMZ).

TCS has been used for more than 35 years as an antimicrobial and antifungal agent. Its current widespread usage has raised concerns about possible effects on aquatic organisms (Dussault *et al.*, 2008). The highest environmental concentration of TCS (20 μg/L) was detected in water samples collected near the outfall of a waste water treatment plant (WWTP) in Rhode Island, USA (Lopez–Avila and Hites, 1980) and concentrations at other WWTPs ranged from 1,000 to 10,000 ng/L for influent and 40 to 2,000 ng/L for effluent (Singer *et al.*, 2002; Ying and Kookana, 2007).

DCF is a component of a widely applied antiphlogistic and antirheumatic drug, with an estimated 75 t/yr prescribed in Germany (Landsdrop *et al.*, 1990). The maximum concentrations of DCF detected in Germany have reached 15 μg/L in surface waters (Jux *et al.*, 2002) and 380 ng/L in groundwater in Berlin (Heberer *et al.*, 1998). DCF has been detected at a concentration of less than 10 ng/L in a water sample taken from a private Household tap in Berlin (Heberer, 2002b).

CBMZ is an antiepileptic drug, and its environmental persistence raises concerns about potential effects on non–target organisms. The highest aqueous concentration of CBMZ measured in surface water has been 2.85 mg/L (Oetken *et al.*, 2005). Heberer (2002) detected CBMZ at a concentration of 1,075 ng/L in surface water in Berlin. CBMZ has been detected in effluent from municipal sewage treatment plants (6.3 mg/L; Ternes, 1998), in groundwater (up to 1.1 mg/L), and in drinking water (30 ng/L) (Heberer, 2002).

Despite the recent attention received by PPCPs for their occurrence in the environment, there are substantial gaps regarding their potential ecological consequences (Kim *et al.*, 2007). There has been only limited risk–based analysis, and little is known about the ecotoxicological effects of PPCPs on aquatic and terrestrial organisms and wildlife. Although this information is necessary for estimation of the risk, a comprehensive review of ecotoxicological effects is lacking (Fent *et al.*, 2006). By studying the hazard potential of these three compounds to medaka, and comparing our results with those of previously published environmental risk assessments, we can classify the potential toxicity of these substances in the aquatic environment. Comparative toxicity studies are rarely performed, so comparisons of the toxicities of multiple PPCPs to one fish species are valuable

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Japanese medaka (*Oryzias latipes*) is an ideal aquatic model organism because it is small, easily maintained, and can be induced to propagate throughout the year in captivity (Holcombe *et al.*, 1995). Also, this species is appropriately sensitive to many toxic chemicals and serves as an excellent model fish for determining both acute and chronic toxicities (Zha and Wang, 2006).

The present study was conducted to evaluate the acute toxicities of three widely used PPCPs; TCS, DCF, and CBMZ in medaka (*Oryzias latipes*) and to predict their toxicities in the aquatic environment.

MATERIALS AND METHODS

Test chemicals

The chemicals used in toxicity tests were purchased from Wako Pure Chemical Co. (Tokyo, Japan): triclosan (TCS; 98% purity), diclofenac (DCF; 98% purity), carbamazepine (CMBZ; 97% purity), and dimethylsulfoxide (DMSO; 99% purity).

Stock and working solutions

Stock solutions of TCS and DCF were made up by first dissolving the pure chemicals in DMSO (TCS, 10 mg and DCF, 60 mg in 0.05 mL DMSO) and then adding artificial seawater (salinity, 0.035 psu) to a final volume of 1 L. Test media (TCS: 1, 2, 2.4, and 3 mg/L; DCF: 9, 12, 15, and 18 mg/L) were made from the stock solutions by a series of dilutions with the artificial seawater and additional DMSO to give equal concentrations in all solutions (DMSO, 0.05 mL/L). Stock solutions were wrapped in aluminum foil and stored at room temperature for no longer than 2 days before use.

A series of working solutions of CBMZ (60, 65, 70, 90, and 100 mg/L) were prepared by dissolving appropriate amount of CBMZ in equal volumes of DMSO (0.250 mL) and then adding artificial seawater (salinity, 0.035 psu) to a final volume of 1 L. Solvent controls consisted of 0.05 mL DMSO (TCS and DCF) or 0.250 mL DMSO (CBMZ) in 1 L artificial seawater. Controls and solvent controls were tested in parallel with all test solutions under the same conditions.

Test organisms

Adult Japanese medaka stock (Orange–Red strain) was purchased from a fish farm in Nagasu, Kumamoto Prefecture, Japan. The fish were acclimated for at least 2 weeks in a glass tank (60 cm long×30 cm wide×36 cm high) filled with the artificial seawater (salinity, 0.035 psu) at 25±1°C. The fish were kept under a consistent 16:8–h light: dark photoperiod and were fed freshly hatched (<24 h) *Artemia* nauplii twice a day. Half of the water in the holding tank was replaced daily.

Toxicity tests

Toxicity tests were carried out in triplicates using medaka with average body length of 3.25±0.34 cm and average body weight of 366±30 mg. For each exposure group, 10 fish were moved into glass aquaria (2 L; 18×12 ×15 cm) and exposed to TCS (1, 2, 2.4, and 3 mg/L), DCF (9, 12, 15, and 18 mg/L), or CMBZ (60, 65, 70, 90 and 100 mg/L) at 25±1°C for 96 h. The concentration of the solvent DMSO was never in excess of 0.005 (v/v) in the final exposure solutions, which is within acceptable levels according to the office of environmental compliance and documentation (OECD, USA) guidelines. Each test was performed in triplicate. Fish were not fed during acute exposure tests. Test waters were completely replaced after 48 h of exposure. Medaka in each treatment, control, and solvent control tank were checked every 24 h and considered dead when no response was detected.

Statistical Analysis

The lethal concentrations (96–h LC_{50}) of the tested chemicals were calculated by the Trimmed Spearman–Karber Method, version 1.5 (USEPA, Statistical Analysis for Biological Methods; http://www.epa.gov/nerleerd/stat2.htm).

RESULTS

In the TCS treatment group there was no fish death in the control groups. The mean 96–h survival rates of medaka exposed to TCS were 100% (1 mg/L), 16.7% (2 mg/L), 3.3% (2.4 mg/L), and 0% (3 mg/L) (**Fig. 1**). All fish were dead in the 3 mg/L treatment at 48 h post–exposure, and no mortality was observed in the 1 mg/L group after 96 h of exposure. Fish exposed to the higher TCS concentrations (2.4 and 3 mg/L) exhibited abnormal behaviors, such as erratic swimming and loss of equilibrium, immediately after exposure. No visible changes in behavior were observed in control groups.

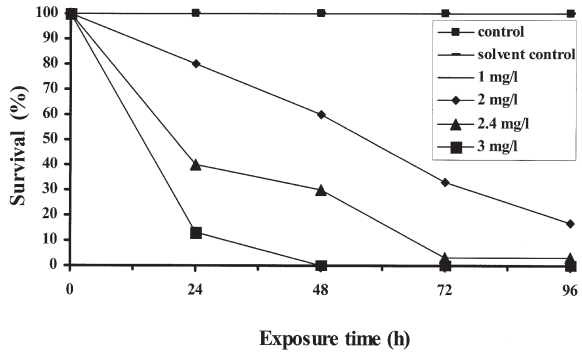

Fig. 1. Survival rate of adult medaka fish exposed to triclosan.

In DCF toxicity test, no fish died in the control or 9 mg/L exposure groups, whereas 100% mortality occurred at exposures of 15 or 18 mg/L after 96 h (**Fig. 2**). All fish were dead in the 18 mg/L group at 24 h post–exposure. Survival rates generally decreased with increases in both exposure duration and DCF concentration. Fish at the highest DCF exposure level (18 mg/L) attempted to swim to the water surface more often than fish in the control groups.

For the group exposed to CBMZ, the survival rate at 60 mg/L exposure was 100% over the exposure period

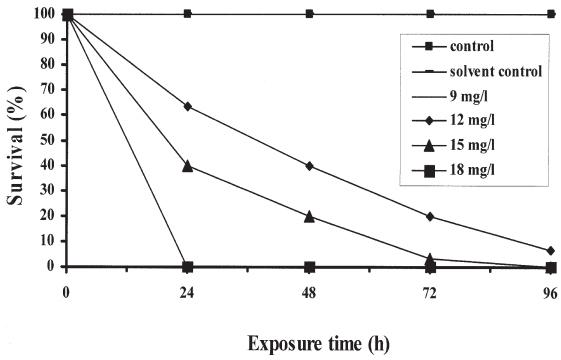

Fig. 2. Survival rate of adult medaka fish exposed to diclofenac.

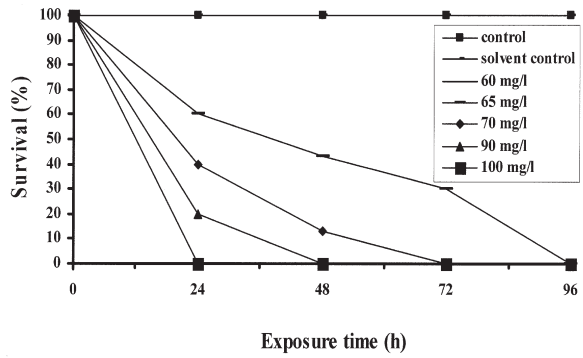

Fig. 3. Survival rate of adult medaka fish exposed to carbamazepine.

(**Fig. 3**), whereas survival was 0% at all other concentrations (65, 70, 90, and 100 mg/L) after 96 h of exposure. No animals survived to 24 h post–exposure in the 100 mg/L group. The survival rate in the control groups was 100%. At CBMZ concentrations of 90 and 100 mg/L, the medaka became lethargic, and after death their entire bodies were covered with a mucous substance.

Table 1 summarizes the mean of 24, 48, 72, and 96–h LC_{50} values of the tested PPCPs in adult medaka. Of the 3 PPCPs tested, TCS has the highest toxicity in medaka, followed by DCF and CBMZ in decreasing order, with 96–h LC_{50} values of 1.7, 10.1, and 61.5 mg/L, respectively.

DISCUSSION

Our experiment clearly showed that TCS is highly toxic and caused concentration– and time–dependent mortality in medaka. The 96–h LC_{50} value of TCS for medaka (1.7 mg/L; **Table 1**) is comparable with values from other studies. Ishibashi *et al.* (2004) reported acute TCS toxicity (96–h LC_{50}) values of 602 μg/L in 24 h–old medaka larvae and 399 μg/L in embryos less than 24 h post–fertilization. The differences in LC_{50} values between adult, larvae, and embryos might be due to the different sensitivities at different life stages, and to different exposure times in the experiments. The 96–h LC_{50} for fathead minnow (*Pimephales promelas*) and bluegill (*Lepomis macrochirus*) are 260 and 370 μg/L TCS, respectively (Orvos *et al.*, 2002). These differences may indicate a range of sensitivities among species.

We estimated the TCS no observed effect concentration (NOEC) for medaka at 1.7 μg/L by dividing the estimated 96–h LC_{50} by an assessment factor of 1,000 (European Commission, 2003). We used the environmentally highest measured value (20 μg/L) of TCS at the WWTP in Rhode Island, USA as a predicted environmental concentration (PEC) to determine the potential risk from TCS (Lopez–Avila and Hites, 1980) and determined that TCS poses a potentially high risk to medaka in the natural environment because the PEC (20 μg/L) was 12 times the NOEC (1.7 μg/L).

The high risk of TCS to medaka suggests harmful effects on other aquatic organisms, especially near large human populations, because of discharges of TCS from domestic detergents and cosmetic products (about 350 t/yr in European countries; Singer *et al.*, 2002). Ishibashi *et al.* (2004) concluded that TCS is highly toxic to the early life stages of medaka, and that TCS metabolites may include weak estrogenic compounds with the potential to induce vitellogenin production in male medaka. They observed significant decreases in hatchability and delays in the time to hatching in fertilized medaka embryos exposed to 313 μg/L TCS for 14 d. Even at TCS exposures of 20 and 100 μg/L, concentrations of hepatic vitellogenin in males increased significantly.

Our results showed DCF to be moderately toxic to medaka, with a 96–h LC_{50} of 10.1 mg/L. Our results are consistent with those of Han *et al.* (2007), who determined a DCF 96–h LC_{10} of 8 mg/L in juvenile medaka. Dietrich and Prietz (1999) exposed zebra fish embryos to DCF and determined 96–h LC_{50} and 96–h EC_{50} values of 480±50 and 90±20 μg/L, respectively. These differences in reported toxicities presumably reflect the range of sensitivities of different organisms.

Our toxicity results yielded a calculated NOEC

Table 1. Summary of the effects of each pharmaceuticals and personal care products tested to adult medaka fish. *: Data expressed in mean±SD, n= 3

PPCPs	LC_{50} (mg/l)*			
	24 h	48 h	72 h	96 h
Triclosan (TCS)	2.3±0.01	2±0.02	1.8±0.02	1.7±0.04
Diclofenac (DCF)	14±0.10	12.1±0.10	10.9±0.10	10.1±0.10
Carbamazepine (CBMZ)	72.15±0.00	65.3±0.04	63.2±0.03	61.5±0.00

(European Commission, 2003) for DCF in medaka of 10.1 μg/L. We estimated the PEC to be 15 μg/L on the basis of maximum DCF levels reported in surface water aquatic ecosystems in Germany (Jux *et al.*, 2002). A comparison of the NOEC for DCF (10.1 μg/L) with the PEC (15 μg/L) indicates that DCF poses little or moderate risk to medaka in the environment.

Recent studies have demonstrated physiological effects in DCF–exposed organisms. Han *et al.* (2007) found that, in medaka exposed to 1 μg/L DCF, expression of the p53 gene increased 31.6–fold in liver, 8.7–fold in gills, and 19.3–fold in intestines. In brown trout (Salmo trutta), haematocrit levels decreased after exposure to DCF for 7 d (Elliott *et al.*, 1995; Sanchez *et al.*, 2002). DCF might also have carcinogenic or apoptotic effects in medaka (Han *et al.*, 2007). These physiological effects could have deleterious impacts on fish health after long–term DCF exposure.

CBMZ is characterized by low acute toxicity to medaka, with a 96–h LC_{50} of 61.5 mg/L. Our results agree with those of Jones *et al.* (2002), who reported that CBMZ had a low toxicity for fish (EC_{50}>100 mg/L). Kim *et al.* (2007) found a 96–h EC_{50} for CBMZ of 35.4 mg/L in medaka. We compared the NOEC from our study (61.5 μg/L) with the PEC value (2.85 mg/L; Oetaken *et al.*, 2005) and concluded that CBMZ poses a negligible risk to medaka in the environment.

This conclusion agrees with that of Jos *et al.* (2003) who predicted no acute toxic effects on aquatic biota from CBMZ (EC_{50}, 4.5–383.5 mg/L). There are few reports available on the effects of CBMZ on aquatic organisms; this compound has no notable toxicity in crustaceans or fish (EC_{50}>100 mg/L) (Jones *et al.*, 2002). However, chronic effects or synergistic effects with other chemicals are possible and should be further investigated (Jos *et al.*, 2003).

None of the PPCPs tested seem to pose acute environmental risks by themselves, but combined exposures could produce more severe effects. There are many PPCPs that are consumed continuously and in large quantities, potentially resulting in chronic exposure of aquatic organisms to a mixture of these compounds (Schwaiger *et al.,* 2004). Chemicals in combination can have antagonistic or synergistic effects (Marking, 1977). Thus, to better assess the ecotoxicological potential of residual PPCPs in the aquatic environment, the effects of combined exposure should be determined.

Our results clearly demonstrated the differing degree of risk from PPCPs to aquatic ecosystems, in particular for fish species. In order of decreasing toxicity to medaka, the 96–h LC_{50} values for the tested PPCPs were TCS (1.7 mg/L) > DCF (10.1 mg/L) > CBMZ (61.5 mg/L). A comparison of the estimated NOEC and PEC values indicates that TCS and DCF have potential risk to fish in the environment, whereas CBMZ does not. There is a need for long–term exposure assessment of specific modes of action of residual pharmaceuticals in aquatic systems. This will allow more reliable environmental risk assessments. However, aquatic biota are exposed to mixtures of chemicals, including PPCPs, and the sub–acute effects of PPCPs, including behavioral effects, endocrine disruption, and developmental toxicity, are still unknown. Further study is needed into the toxicity of mixtures of these chemicals.

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