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ORIGINAL ARTICLE

Acute toxicity of acetylsalicylic acid to juvenile and embryonic stages of *Danio rerio*

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Abstract

OBJECTIVES: The aim of this study was to compare the acute toxicity of acetylsalicylic acid to embryonic and juvenile stages of aquarium fish – zebrafish (*Danio rerio*), oxidative stress parameters and detoxifying enzyme.
METHODS: Tests were performed according to OECD No. 203 (Fish, acute toxicity test) and OECD No. 212 (Fish, short-term toxicity test on embryo and sac-fry stages) methodology.
RESULTS: The results showed the mean acetylsalicylic acid LC50 value to be 567.7 mg/L in juvenile zebrafish. The acute toxicity of acetylsalicylic acid for zebrafish embryos was 274.6 mg/L. Statistically significantly higher activity of GST was found in concentrations 340, 380 and 420 mg/L of acetylsalicylic acid. TBARS, GPx and GST didn't show statistically significant activity in tested concentrations of acetylsalicylic acid.
CONCLUSIONS: The results revealed a statistically significantly higher degree of sensitivity in the embryonic stages of zebrafish compared to its juveniles. Acetylsalicylic acid did not cause statistically significantly higher antioxidative defence in zebrafish.

Abbreviations:

$ANC_{4.5}$	- acid neutralizing capacity
CAS	- chemical abstracts service registry number
COD_{Mn}	- chemical oxygen demand
COX	- cyclooxygenase
GPx	- glutathione peroxidase
GR	- glutathione reductase
GST	- glutathione S-transferase
HPLC	- high performance liquid chromatography
NADPH	- nicotinamide adenine dinucleotide phosphate
NSAIDs	- non-steroidal anti-inflammatory drugs
OECD	- Organisation for Economic Cooperation and Development
STP	- sewage treatment plant
TBARS	- Thiobarbituric Acid Reactive Substances
96h EC50	- median effective concentration (50% mortality or immobility after a 96 h interval)
96h LC50	- median lethal concentration (50% mortality after a 96 h interval)

INTRODUCTION

Pharmaceuticals are a class of environmental contaminants that are extensively and increasingly being used in both human and veterinary medicine. These chemicals are designed to have a specific mode of action, and many of them have some persistence in the body. In contrast to the broad knowledge of their pharmacokinetic and pharmacodynamic properties in target organisms, only very little is known about the ecotoxicological effects of pharmaceuticals on aquatic and terrestrial organisms. Current knowledge indicates that residues of pharmaceuticals in trace quantities are widespread in aquatic systems (Fent *et al.* 2006; Corcoran *et al.* 2010). Aquatic organisms are thus particularly important targets, as they are exposed to drug residues over their whole life via wastewater released into their natural environment. Standard acute ecotoxicity data have been reported for a number of pharmaceuticals. However, such data alone may not be suitable for specifically addressing the question of environmental effects, and subsequently the question of hazard and risk assessment. In spite of the sizeable quantities of human drugs released into the environment, concise regulations for ecological risk assessment are largely missing.

The consumption of pharmaceuticals is substantial in both human and veterinary medicine; antibiotics and anti-inflammatory drugs are among the most widely prescribed and widely used medicines. Some drugs are regularly documented within the most frequently applied range: the class of non-steroidal anti-inflammatory drugs (NSAIDs) including acetylsalicylic acid (e.g. 836 tons in Germany in 2001) (Tixier *et al.* 2003; Fent *et al.* 2006; Corcoran *et al.* 2010).

Pharmaceuticals are excreted in their native form or as metabolites and enter aquatic systems in different ways. Municipal wastewater is the main route bringing human pharmaceuticals into the environment, after their normal use or the disposal of unused medicines.

Hospital wastewater, wastewater from industrial processes, and landfill leachates may contain significant concentrations of pharmaceuticals (Holm *et al.* 1995). Pharmaceuticals not readily degraded in the sewage treatment plant (STP) are discharged into treated effluents resulting in the contamination of rivers, lakes, estuaries and, rarely, groundwater and drinking water. Where sewage sludge is applied to agricultural fields, the contamination of soil, runoff into surface water, and also drainage may occur. In addition, veterinary pharmaceuticals may enter aquatic systems via manure application to fields and subsequent runoff, but also via direct application in aquaculture (fish farming) (Tixier *et al.* 2003; Corcoran *et al.* 2010).

A high production volume of a certain pharmaceutical does not necessarily have to cause environmental concern, but there are properties of drugs which are environmentally persistent and effect critical biological activity (e.g. high toxicity and the high potency of effects on key biological functions such as reproduction) (Fent *et al.* 2006).

NSAIDs inhibit the synthesis and release of prostaglandins via COX inhibition, and such compounds are the most consumed category of drugs (Vane and Botting 1998). NSAIDs are also commonly found in the aquatic environment; mostly chronic data are reported. Despite an average elimination rate in STPs of up to 81% for acetylsalicylic acid, and 99% for salicylic acid (Ternes 1998; Heberer 2002; Ternes *et al.* 2002). Acetylsalicylic acid has been found in many municipal wastewaters at levels up to 4.1 µg/L (Ternes 1998), 13 µg/L (Farre *et al.* 2001; Heberer 2002) or even 59.6 µg/L with median levels of 3.6 µg/L (Metcalfe *et al.* 2003).

Acetylsalicylic acid was selected for the experiment, because of both the frequency with which the particular maternal substance is prescribed and the frequency with which the residues of its metabolites are found in the aquatic environment, and because of its potential biological effects on non-target animal species.

The aim of this study was to determine the acute toxicity of acetylsalicylic acid to embryonic and juvenile stages of zebrafish (*Danio rerio*), activity of detoxifying enzyme, selected parameters of oxidative stress and to extend present knowledge of its effects on aquatic organisms.

MATERIALS AND METHODS

Acute toxicity tests

Acute toxicity tests with acetylsalicylic acid (Sigma-Aldrich, CAS 50-78-2) were performed on juvenile stages of the aquarium fish *D. rerio* in accordance with OECD No. 203 guidelines (Fish, acute toxicity test).

The zebrafish were 2–3 months old, weighed 0.4±0.1 g and their total length was 30±5 mm. The fish were obtained from a commercial dealer. The zebrafish were divided into 7 groups (including one control group) each containing 10 fish and were kept in 3 L full glass tanks. The tests were conducted using a semi-static method with solution replacement after 48 hours. During the tests, records of the temperature, pH, the concentration of oxygen dissolved in test tanks, and fish behaviour and mortality rate were noted. The temperature of the experimental bath was 22.5±0.6 °C with a 12-h/12-h light/dark cycle, the dissolved oxygen concentrations did not fall below 60% (89–100%), and the pH was between 7.5 and 8.2. The tested concentrations ranged from 100 to 600 mg/L in the preliminary test. In the subsequent toxicity tests the tested concentrations were 550, 560, 570, 580, 590 and 600 mg/L. The tests were performed in 4 repetitions. No fish died in the control tanks during the experiments. Due to the low solubility of acetylsalicylic acid in water, dissolution of the substance was achieved using an ultrasound device.

Embryonic toxicity tests

Embryonic toxicity tests were performed on embryos of *D. rerio*. These tests were conducted according to OECD No. 212 (Fish, short-term toxicity test on embryo and sac-fry stages) guidelines. A series of 7 ascending concentrations of tested substance were used in the test. Petri dishes each containing 10 fertilized eggs were tested at each concentration; one dish was used as control. The eggs were placed in each Petri dish within 5 hours of fertilization at the latest. The tests were terminated after hatching and the absorption of the yolk sack in all individuals in the control dish (96 h after placement into the dish). The baths were replaced at 24 h intervals. Early life stage parameters such as egg and embryo mortality, gastrulation, somite formation, movement and tail detachment, pigmentation, pulse, and hatching success were noted. During the test, the number of dead embryos in individual concentrations was recorded. The mortality rate of the control embryos did not exceed 20%. Test bath temperatures were 27.4±0.5 °C. The tested concentrations of acetylsalicylic acid were 100, 200, 300, 400, 500, 600 and 700 mg/L. The tests were performed in 4 repetitions.

Determination of detoxifying enzyme and oxidative stress parameters

At the end of all tests, juvenile fish, which were alive, were killed by carbon dioxide. Whole body samples were used for analysis. The total catalytic concentration of GST was determined by measuring the conjugation of 1-chloro-2,4-dinitrobenzene with reduced glutathione at 340 nm (Habig *et al.* 1974). The specific activity was expressed as the nmol of the formed product per min per mg of protein. The catalytic concentration of GR was determined spectrophotometrically by measuring NADPH oxidation at 340 nm (Carlberg and Mannervik 1975). The catalytic concentration of GPx was calculated from the rate of NADPH oxidation by the reaction with GR at 340 nm (Flohe & Gunzler 1984). The specific activity of GR and GPx was expressed as the nmol of NADPH consumption per min per mg of protein. Protein concentrations were determined by a Bicinchoninic Acid Protein Essay Kit (Sigma-Aldrich, St. Louis, MO, USA) using bovine serum albumin as the standard (Smith *et al.* 1985). To check lipid peroxidation, malondialdehyde was measured by the TBARS method as described by Lushchak *et al.* (2005) at 535 nm. The concentration is expressed as nmol per gram wet weight of tissue.

Water quality parameters

The basic physical and chemical parameters of the dilution water used in the toxicity tests on embryonic and juvenile stages were: $ANC_{4.5}$ 4.6 mmol/L; COD_{Mn} 3.1 mg/L; total ammonia below the limit of determination; NO_3^- 24.20 mg/L; NO_2^- below the limit of determination; Cl^- 19.16 mg/L; $\Sigma\ Ca^{2+} + Mg^{2+}$ 3.18 mmol/L.

Determination of acetylsalicylic acid

Acetylsalicylic acid determination in water samples was performed by high performance liquid chromatography (HPLC) with photometric detection. Samples were filtered through a 0.45-µm nylon filter (Millipore, Billerica, MA) and used for analysis. The sample volume injected into the HPLC system was 10 µL. The acetylsalicylic acid was separated by an isocratic elution method with acetonitrile/water 50/50 (v/v) on a 150×4.6 mm, 5-µm Zorbax Eclipse XBD-C18 column (Agilent Technologies, Santa Clara, CA). The mobile phase flow rate was 1 mL/min, the column temperature was 35 °C, and UV detection was performed at 235 nm. Chromatographic analysis was accomplished by means of an Alliance 2695 chromatographic system (Waters, Milford, MA) with a PDA 2996 photodiode array detector (Waters, Milford, MA). Acetylsalicylic acid was purchased from Sigma-Aldrich (St. Louis, MO). All solvents were HPLC-grade purity (Chromservis, s.r.o., CZ). The detection limit for acetylsalicylic acid was 50 ng/mL. The limit of quantification for acetylsalicylic acid was 64 ng/mL. The coefficient of variation was 4.5%. In all toxicity tests, the concentration of acetylsalicylic acid after 48 h was above 80% of the dosed initial concentration.

Statistical analysis

The results (mortality of fish and embryos in individual test concentrations) were subjected to a probit analysis (EKO-TOX 5.2 programme) to determine the LC50 (lethal concentration for 50% of tested organisms) acetylsalicylic acid values. The statistical significance of the difference between the LC50 values for the juvenile and for the embryonic stages of *D. rerio* was calculated using the non-parametric Mann-Whitney test and Unistat 5.1 (Unistat Ltd., GB) software.

Values of GST, GR, GPx, and TBARS were tested for normal distribution using the Shapiro–Wilk test and data were log-transformed to improve the homogeneity of variance. A one-way analysis of variance (ANOVA) was applied to the differences in determined parameters between tested groups. Individual differences between the means were tested successively using Dunnett test and $p<0.05$ was chosen as the level of significance. Statistical analysis of the data was performed using the program Unistat 5.6.

RESULTS AND DISCUSSION

Comparison of the toxicities of acetylsalicylic acid

The results showed the mean acetylsalicylic acid LC50 value to be 567.7 mg/L in juvenile zebrafish and 274.6 mg/L in zebrafish embryos.

The acute 48h LC50 toxicity of acetylsalicylic acid determined for the water flea *Daphnia magna* is between 168 mg/L and 1468 mg/L; the LOEC of acetylsalicylic acid is 37 mg/L (Cleuvers 2004). Henschel *et al.* (1997) state that the effective concentration of acetyl-

salicylic acid causing mortality or immobility in 50% of the tested organisms (EC50) is >100 mg/L for algae and 37 mg/L for zebrafish embryos.

The results of our acute toxicity tests on zebrafish embryos and juveniles did not confirm these observations.

A comparison of acetylsalicylic acid toxicity to the embryonic and juvenile life stages of *D. rerio* revealed a statistically significantly higher degree of sensitivity in the embryonic stage ($p<0.01$) compared to the juvenile life stage. It is generally stated that early developmental stages of fish (embryos and larvae) are more sensitive to different stimuli compared to juveniles and adults, which has also been proven by many studies with chemicals (Nagel 2002; Plhalova *et al.* 2011; Praskova *et al.* 2011). Kovriznych and Urbancikova (2001) compared the LC50 values of 8 different chemical agents assessed in acute tests of toxicity performed in *D. rerio* and ascertained different sensitivities to them in these two tested life stages. This difference between the sensitivities of fish embryos and of juvenile/adult individuals of the same species may arise because of the underdeveloped enzymatic system in embryos, differences in metabolism pathways, and different ways in which the substance is absorbed into the organism (Van Leeuwen *et al.* 1985).

Studies by Marques *et al.* (2004a,b) showed that chronic influence is much more toxic than short influence. In these studies, acute (1293.1 mg/L) and chronic (1.4 mg/L) toxicity of acetylsalicylic acid for the water flea *Ceriodaphnia dubia* were compared. Marques *et al.* (2004a,b) also discovered that acetylsalicylic acid affected reproduction in *D. magna* and *D. longispina* at a concentration of 1.8 mg/L.

Tab. 1. Results of detoxifying enzyme and oxidative stress parameters

Conc. of ASA	GPx	GST	TBARS	GR
0	45.17±2.54	57.84±2.99	118.35±20.55	12.19±1.02
300	30.14±2.20	55.99±1.94	280.71±64.74	10.55±1.14
340	39.00±2.52	77.15±7.68*	144.61±42.92	15.31±2.11
380	59.15±8.81	90.01±6.90**	78.75±10.70	21.76±1.61
420	63.54±18.14	77.11±2.48**	143.85±42.20	30.26±10.87
460	48.98±5.79	68.48±4.59	102.77±27.18	12.52±1.36
560	44.39±7.01	64.28±5.67	165.41±49.29	11.39±0.90
570	44.51±2.55	59.22±2.32	59.80±17.76	9.81±0.91
580	44.11±4.96	56.45±3.64	50.19±25.62	8.95±0.86

*$p<0.05$, **$p<0.01$; Values are given as means ± SE;
ASA - acetylsalicylic acid (mg/L); GPx - glutathione peroxidase (nmol/min.mg/protein); GR - glutathione reductase (nmol/min.mg/protein); GST - glutathione S-transferase (nmol/min.mg/protein); TBARS - Thiobarbituric Acid Reactive Substances (nmol/g of w/w)

Determination of detoxifying enzyme and oxidative stress parameters

Results of detoxifying enzyme and oxidative stress parameters are given in Table 1. Statistically significantly higher activity of GST was found in concentrations 340 ($p<0.05$), 380 and 420 ($p<0.01$) mg/L of acetylsalicylic acid. We have not found statistically significantly higher activity in other tested parameters (GPx, GR and TBARS).

Acetylsalicylic acid did not cause statistically significantly higher antioxidative defence in juvenile zebrafish, only activation of detoxification of metabolism was observed. The effect of xenobiotic exposure is described as an increase in levels of antioxidant enzymes (van der Oost et al., 2003). The results of our analysis of selected parameters of oxidative stress appear to show an upward trend of GST activity in concentrations of acetylsalicylic acid 340, 380, and 420 mg/L, which may be connected with the effort of the enzymatic system to balance the activity of free oxygen radicals caused by acetylsalicylic acid. At the higher concentrations of acetylsalicylic acid (460–580 mg/L) we observed a decrease in enzyme activity, which may be explained by the collapse of the enzymatic systems of the tested organisms.

Pharmaceuticals such as acetylsalicylic acid which display LC50 values higher than 100 mg/L are, according to EU-Directive 93/67/EEC (Commission of the European Communities, 1993), classified as not being harmful to aquatic organisms. In our experiment, mean LC50 values of acetylsalicylic acid agreed with this classification.

CONCLUSION

The determined LC50 values (274.6 mg/L for embryos and 567.7 mg/L for juvenile zebrafish) are higher than environmental concentrations of this substance, which are ordinarily in µg/L. It is suggested that pharmaceuticals in the environment pose only a low risk with respect to acute toxicity. For chronic effects, the situation may be different, but there is a considerable lack of information. Thorough investigation of multigenerational life-cycle effects at various life stages is yet to be carried out, although many aquatic organisms are exposed to pharmaceuticals for their entire life. There is also a need to focus on the assessment of long-term exposure with respect to specific modes of action of pharmaceuticals in order to better assess the implications for aquatic systems contaminated by pharmaceutical residues. Only after these knowledge gaps are filled can more reliable environmental risk assessments with much lower degrees of uncertainty be performed.

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